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IJSM is published 4 issues per year (March, June, September, December), and accepting manuscripts related to secondary metabolites of plant and allied organisms (algae, fungi, and lichens). Research areas covered in the journal are phytochemistry, biochemistry, biotechnology, ethnopharmacology, biological and pharmacological activities (antimicrobial activity, antioxidant activity, antiulcer activity, anti-convulsant activity, anti-anxiety activity, antidiabetic activity, anti-gout activity, antiprotozoal activity, anti-inflammatory activity, antispasmodic activity, antiparasitic activity, anti-mutagenic activity, anticholinesterase activity, antidepressant activity, hepatoprotective activity, anti-anxiety activity, anti-convulsant activity, anti-spasmolytic activity, anticancer activity). IJSM welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Authors are required to frame their research questions and discuss their results in terms of major questions in plant biology. Contribution is open to researchers of all nationalities. The following types of articles will be considered:

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2. Research notes: These include articles such as preliminary notes on a study or manuscripts new records on secondary metabolites.

3. Reviews: Reviews of recent developments, improvements, discoveries, and ideas in various fields of plant and allied organisms (algae, fungi, and lichens) will be requested by the editor or advisory board.

4. Letters to the editor: These include opinions, comments relating to the publishing policy of the International Journal of Secondary Metabolite, news, and suggestions. Letters are not to exceed one journal page.

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Research Article

In-silico investigation of alpha-bisabolol and derivatives as inhibitors of bcl-2 family proteins for targeting glioblastoma

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Abstract: Glioblastoma is one of the most common and destructive types of tumors, with an increasing number around the world. Alpha-bisabolol is a plant secondary metabolite with discovered anticancer activity, which can also be considered a potential treatment for glioblastoma. *In silico* investigations can provide adequate information for understanding the roles of alpha-bisabolol compounds in glioblastoma. For this purpose, computational drug design procedures were applied to investigate the anti-glioblastoma biotherapeutic potential of alpha-bisabolol compounds. In this study, bcl-2 family proteins' inhibitory activity of alpha-bisabolol compounds and their toxicity properties were investigated by molecular docking studies. Toxicity properties were evaluated by the prediction tools as, CarcinoPred for carcinogenicity and LAZAR for mutagenicity, pkCSM, and SwissADME for absorption, distribution, metabolism, excretion and toxicity (ADMET) analysis and BOILED-Egg model, PASS prediction to analyze biological functions and druggability, DruLiTo program to compute the drug likeness property and QSAR Toolbox for QSAR modeling. The results reveal the potential of alpha-bisabolol oxide B, a plant secondary metabolite and an alpha bisabolol derivative, in glioblastoma for the inhibitory mechanisms of bcl-2 family proteins, being non-toxic and non-mutagenic.

1. INTRODUCTION

Brain tumors have recently become a trending topic for researchers because of their negative impact on neurological functions and physiological behaviors with poor diagnosis and treatment. Approximately 60% of all brain tumors are gliomas, which are rapidly progressive malignant brain tumors driven by glial activity (Hanif *et al*., 2017). Today, treatment strategies are limited in this tumor group, as in other types of cancer, and investigation of high-efficiency chemotherapeutic drug molecules for glioma is essential for increasing the quality of life of patients and their life span with accurate treatment strategies (Davis, 2016). When radiotherapy, chemotherapy, and surgical treatment strategies are combined with late diagnosis and awareness processes, the applicability and effectiveness of these treatments decrease (Al-Azri, 2016). Therefore, the discovery of direct-to-target innovative drug agents is an essential preliminary step in controlled drug delivery studies. There are numerous natural substances

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originating from plants that have anticancer properties (Gielecińska *et al*., 2023). One of the most common anticancer drug compounds is paclitaxel, also known as taxol, derived from *Taxus brevifolia* Nutt. (Lim *et al*., 2022). This compound is actively used in first-line treatments for various other cancers, including ovarian carcinoma and breast cancer (Mosca *et al*., 2021). Another phytochemical alkaloid compound is oncovin, also known as vincristine, which is derived from *Vinca rosea* L. and has great potential for treating acute leukemia and malignant lymphoma (Škubník *et al*., 2021). Terpenes and their derivatives are large, diverse phytochemical compounds with great potential for biotherapeutic effects such as antimicrobial, anti-inflammatory, antioxidant, and anticancer (Kamran *et al*., 2022). The impact of terpenes on different cancer types also has been studied both in vitro and in vivo (Tomko *et al*., 2020). One of the sesquiterpenoid, alpha bisabolol, is mostly found in aromatic floral plants with anti-inflammatory, skin-soothing, wound healing, nephroprotective, and anticancer biotherapeutic features (Eddin *et al*., 2022; Ramazani *et al*., 2022). Alpha bisabolol tends to oxidize; therefore, the most common derivatives are alpha bisabolol oxides A and B (Detoni *et al*., 2009). Although many researchers have focused on the anticancer activities (such as pancreatic, endometrial and lung cancer) of bisabolol and its derivatives, *in silico* drug design against glioma based on these compounds has not been investigated (Murata *et al*., 2017; Fang *et al*., 2019). The main step in the *in silico* anticancer drug design was to determine how the phytochemical agent acts. Specification of cancer and related signaling pathways is important for the specification of targeted biological structures. Evading apoptosis and uncontrolled proliferation are characteristic features of cancer cells; hence, anticancer drug development strategies mostly focus on the apoptotic pathway and related protein structures (Pfeffer & Singh, 2018). For glioma, bcl-2 family proteins play key roles with upregulation and downregulation during the apoptosis and influencing the degree (Kale *et al*., 2018). This family proteins include bcl-xl, bcl-2, bcl-w, and mcl-1. In this study, the aim was to reveal the biotherapeutic potential of bioactive compounds that may exhibit anti-apoptotic effects against glioblastoma and to evaluate them within the context of glioblastoma. For this purpose, alpha bisabolol and its oxide derivatives were analyzed using *in silico* methods. Carcinogenicity and mutagenicity were predicted. Then, drug similarity and PASS properties were computationally observed using web-based tools. QSAR models for alpha bisabolol and oxides A and B were created, and their ligand structures were analyzed in detail. Subsequently, molecular docking experiments for bcl-2 family target proteins were performed, and chemical bond interactions were observed at the molecular level. The ADMET properties of the alpha bisabolol, oxide A, and oxide B structures were estimated separately, and their drug features were predicted.

The results obtained after the analysis indicate that the bcl-2 family proteins can be inhibited by alpha-bisabolol and its derivatives, showing high binding affinity. Specifically, it has been demonstrated that alpha-bisabolol oxide B holds great potential against glioblastoma in this context.

2. MATERIAL and METHODS

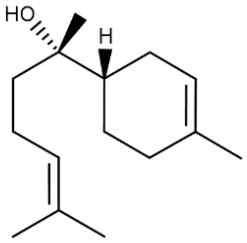
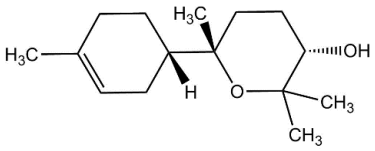
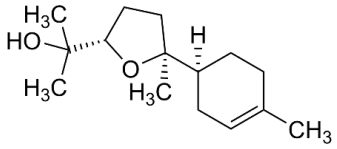
2.1. Dataset Sources

The main source of alpha-bisabolol and its derivatives as secondary metabolites is plants. Some of these plants and the research studies from which they were obtained are presented in Table 1.

2.2. Glioblastoma Target Protein-Protein Interaction (PPI) Analysis

The interactions among bcl-2 family proteins responsible for modulating apoptosis in glioblastoma were analyzed using the STRING online web tool (Szklarczyk *et al*., 2015). Initially, the protein names were entered sequentially into the multiple protein tab, and suitable structures were selected. Then the interaction score was set to high confidence (0.7) and all active interaction sources were examined.

Table 1. Aromatic plants containing alpha bisabolol and its derivative as an active ingredient.

Compound	Chemical Structure	Plant Sources
Alpha bisabolol		• *Lavandula angustifolia* (Mantovani *et al*., 2013) • *Hypericum perforatum* (Morshedloo *et al*., 2015) • *Rosmarinus officinalis* (Tawfeeq *et al*., 2016) • *Artemisia absinthium* (Rizvi *et al*., 2018)
Alpha bisabolol oxide A		• *Thymus vulgaris,* and *Salvia officinalis* (Kowalski *et al*., 2009) • *Achillea millefolium* (Costescu *et al*., 2014)
Alpha bisabolol oxide B		• *Mentha piperita*, *Thymus vulgaris* and *Salvia officinalis* (Kowalski *et al*., 2009) • *Achillea millefolium* (Costescu *et al*., 2014)

2.3. Molecular Docking

Structures of bcl-2 (PDB:2W3L), bcl-xl (PDB: 3ZK6), bcl-w (PDB: 2Y6W), and mcl-1 (PCB: 6OQC) were retrieved from the Protein Data Bank (PDB). Each protein structure was used as a receptor and remained rigid. For the docking preparation procedure of proteins, the following steps were applied: (i) energy minimization was performed with 100 steepest descent steps with a 0.02 Å step size and an update interval of 10, (ii) water molecules were removed, (iii) solvent and non-complex ions were deleted, and (iv) polar hydrogen atom and AM1-BCC charges were added (Jakalian *et al*., 2002). For ligand structure retrieval and preparation, alpha bisabolol, oxide A, and B structures were downloaded from the PubChem database in .sdf format. These three phytochemicals were prepared similarly to the protein preparation steps, but charge addition was based on the Gasteiger charge model (Gasteiger & Marsili, 1978). To determine phytochemicals and apoptotic proteins target interactions at the molecular level, semi-flexible molecular docking simulations were performed by using AutoDock Vina. First, the blind docking method was implemented to investigate the binding sites of the protein, and the results were compared with those reported in the literature (Poustforoosh *et al*., 2022). Then, oriented docking was performed with specified coordinates, and the top 2 minimum binding energy poses of each protein were visualized with Discovery Studio Visualizer (Biovia, 2021).

2.4. Mutagenicity Prediction

The AMES test, also known as the bacterial reverse mutation assay, is a common method used to determine the mutagenic properties of various chemical substances. Lazar, a web-based computational tool helps in the prediction of complicated toxicological outcomes such as toxicity, carcinogenicity, and blood brain barrier (BBB) permeation (Helma *et al*., 2017). Lazar employs data mining algorithms to input experimental data and generate predictions for unknown chemical ligands.

2.5. Carcinogenicity Prediction

The potential of any chemical to induce carcinogenicity in humans and animals can be predicted computationally using Carcinogenicity Prediction using Ensembled Learning Methods (CarcinoPred-EL). This prediction algorithm is created by combining different programs (RF, SVM and XGBoost), resulting in values with high sensitivity, accuracy, and specificity rates

(Zhang *et al*., 2017). The relevant prediction program compares the data entered in SMILES format with the chemicals in its database and provides an average carcinogenicity result. The program's algorithm conducts this by analyzing the functional groups and main skeleton similarities of the chemicals.

2.6. PASS Prediction

The PASS (Prediction of Activity Spectra for Substances) online web tool enables prediction of the expected biological function profile of a chemical compound with similarities to a drug. Computational predictions can be obtained by inserting chemical SMILES code of the chemical structures. The PASS tool prediction results 2 category labels of "probability to be active" (Pa) or "probability to be inactive" (Pi) as biological activity (Filimonov *et al*., 2014)

2.7. Drug-likeness Prediction

Drug-likeness prediction of alpha-bisabolol and its derivatives was predicted using the drug-likeness tool (DruLiTo) software. The chemical structure of the bioactive compounds was inputted into the software in the form of Structure -Data Files (.sdf) file format. To investigate druggability properties, three filters were applied which are Lipinski's rule, Veber filter, and Ghose filter (Bickerton *et al*., 2012).

2.8. Toxicity Prediction (Pimephales promelas) by QSAR Modeling

QSAR activity analyses were performed using the QSAR Activity Toolbox Version 4.6 package program. After the relevant parameters were estimated for the phytochemicals, the standard QSAR activity calculation path was followed which included selection of the true chemical structures, categorization, and gathering the data for model building (Mombelli *et al*., 2021). Adhering to the standard algorithm of the program, "Fish, LC50 (EC50) at 96h for *P. promelas* effect Mortality" was implemented via an automated workflow (Yordanova *et al*., 2019). This program is a system that performs predictions through a mathematical model based on processed data. The QSAR model for the processed data is trained to predict the 96-hour LC50 (EC50) values for *P. promelas* and defines the relationship between chemical structural properties and mortality. Subsequently, the study standardizes the physicochemical and topological properties of the entered chemicals and makes predictions by defining a mathematical equation related to the actual values.

2.9. ADME/T Analysis

ADME/T (absorption, distribution, metabolism, excretion, and toxicity) analysis was performed as the last step of the *in silico* experiments. The SwissADME (Daina *et al*., 2017) and pkCSM (Pires *et al*., 2015) web tools and literature data were used for these predictions. For analysis, the SMILES chemical data format of all three bioactive compounds was retrieved from the PubChem database.

2.10. Predicting Gastrointestinal Absorption and Brain Penetration

The two primary pharmacokinetic parameters crucial for assessing the bioactive substance's absorption in the gastrointestinal tract and its permeability into the brain can be estimated through the Brain or Intestinal Estimated Permeation (BOILED-Egg) computational *in silico* prediction model. This model evaluates the lipophilicity and polarity of the molecule entered, producing results accompanied by easily understandable graphical representations (Daina & Zoete, 2016).

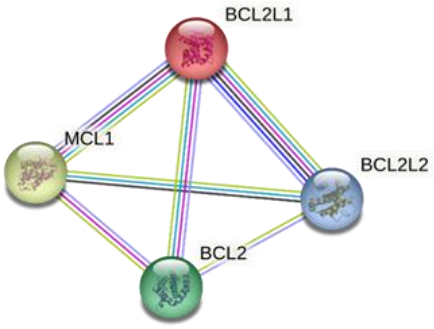
3. RESULTS and DISCUSSION

3.1. PPI Analysis of bcl-2 Family Proteins

The interactions among the proteins of the bcl-2 family are presented in Figure 1. The colors of interactions between proteins are represented in different colors: purple indicates experimentally determined interactions, blue indicates interactions from curated databases,

green indicates gene neighborhood, red indicates gene fusion, navy blue indicates gene co-occurrence, yellow indicates data mining, and gray indicates protein homology.

Figure 1. bcl-2 family proteins PPI (BCL2L1: bcl-xl and BCL2L2: bcl-w).



The consistency of the conducted PPI analysis with literature data has been determined (Calis *et al*., 2022; He *et al*., 2022). As seen in the Figure, proteins interact with each other in multiple ways, suggesting their involvement together in the regulation of disrupted apoptotic processes during cancer.

3.2. Molecular Docking

The analyses were conducted using AutoDock Vina, with UCSF Chimera utilized as the interface program. The binding affinity values obtained from the ligand-protein binding analyses were selected from RMSD 0 groups and ranked accordingly. Binding affinities are calculated by the AutoDock Vina algorithms based on Gibbs free energy (ΔG); the more negative the energy, the stronger affinity and binding. The molecular docking results are given in Table 2.

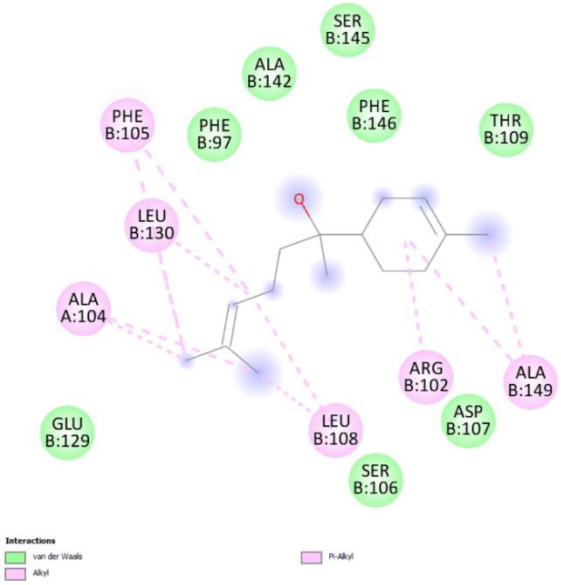
Table 2. Molecular docking scores and hydrogen bond interactions.

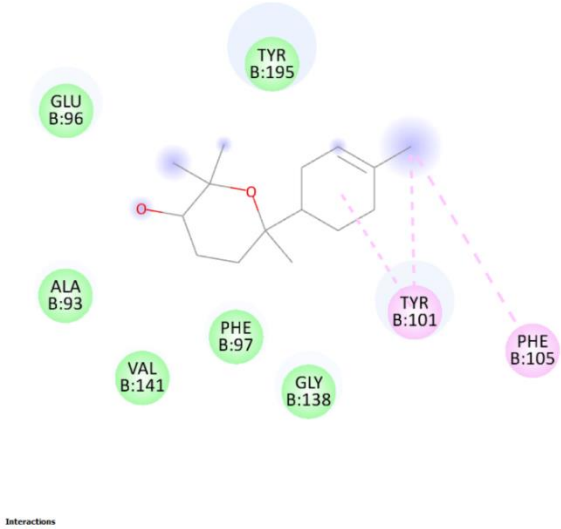
	Binding Affinities (kcal/mol)				Hydrogen Bond Residues			
Compounds	bcl-2	bcl-xl	bcl-w	mcl-1	bcl-2	bcl-xl	bcl-w	mcl-1
Alpha Bisabolol (AB)	-5.7	-8.1	-7	-7.5	-	-	-	-
AB Oxide A	-6.6	-7.9	-7.2	-7.9	SER75	-	ALA98 PHE 102	-
AB Oxide B	-6.5	-8.5	-7.2	-7.3	ARG26	ARG100 ALA104	PHE57	-

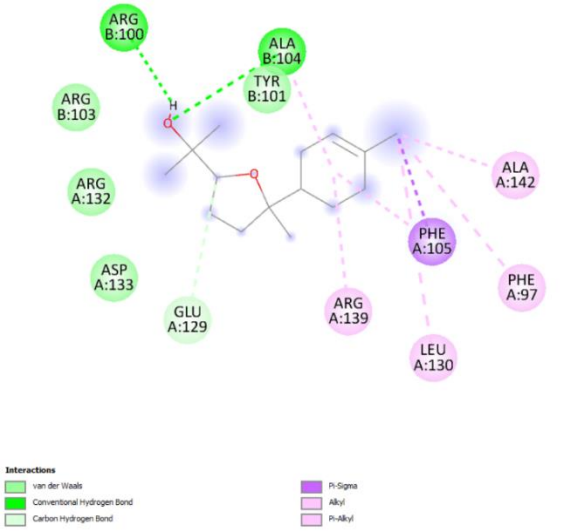
As noticed in Table 2, the highest interaction scores were determined on the bcl-xl protein, while the lowest interaction was observed against the bcl-2 protein. According to a glioblastoma study with the same method, the bcl-xl target was determined as the most effective and potential target, as found in our results (Poustforoosh *et al*., 2022). The 2D illustration of molecular docking targeting the bcl-xl receptor is provided in Figure 2.

Alpha bisabolol oxide B exhibited the highest activity and was determined to be the compound most tended to create hydrogen bonding. This indicates the inhibitor behavior strength of the bioactive component towards the target and its anticancer potential as well. Alpha bisabolol demonstrated a tendency to create alkyl bond interactions, forming a high number of alkyl bonds with all members of the bcl-2 family proteins. However, the binding score of alpha-bisabolol oxide A remained lower compared to the other two bioactive compounds, resulting in a relatively lower number of Van der Waals interactions and alkyl bonds. Common alkyl bonds were found in the residues PHE105 and LEU130, while a Van der Waals interaction was observed in the PHE97 residue.

Figure 2. Interaction of (a) alpha bisabolol, (b) alpha bisabolol oxide A, (c) alpha bisabolol oxide B with bcl-xl.







The reference study of the protein identified a hydrophobic pocket, which includes the PHE105 residue, where alkyl bonds are formed within hydrophobic regions (Lessene *et al*., 2013). The blue circular shapes surrounding some ligand points represent the intensity of interaction attraction from a pharmacophore perspective (Biovia, 2021). There is a significant intensity of interaction attraction at different binding sites of the ligand, especially for alpha bisabolol oxide B. The inhibition of the bcl-xl protein in the reference study also focused on anticancer strategies (Lessene *et al*., 2013), and when the molecular docking strategy employed in our study was applied, the obtained binding affinity value was found to be -12.3 kcal/mol. Comparing our determined binding affinity value of -8.5 kcal/mol with the reference value, reveals a considerably high inhibitor interaction.

Our bcl-2 family protein inhibitor results are also consistent with studies conducted in the literature. In an *in vivo* anti-apoptotic study conducted against testicular toxicity in rats, alpha-bisabolol provided overall anti-inflammatory activity by activating Nrf2 antioxidant activity and reducing NF-κB / MAPK signal activation (Arunachalam *et al*., 2022). In another study, it was determined that oral consumption of alpha-bisabolol reduced neurodegeneration in Parkinson's disease by decreasing oxidative stress, neuroinflammation, and apoptosis (Javed *et al*., 2020)

3.3. Mutagenicity and Carcinogenicity Predictions

The computational analysis results regarding the probability of alpha bisabolol and its derivatives being mutagenic and carcinogenic are presented in Table 3. The average values calculated using the RF method for highly carcinogenic compounds are close to 1, whereas the values obtained for alpha-bisabolol, and its derivatives remain below the average carcinogenicity (0.5) (Helma, 2006). These bioactive compounds, currently utilized in the pharmaceutical and cosmetic industries, as evident from the results, do not possess carcinogenic or mutagenic effects at moderate concentrations (Eddin *et al*., 2022).

Table 3. Carcinogenicity and mutagenicity prediction results.

Compound Name	CarcinoPred -EL Method	Average	Predicted Result	Mutagenicity Prediction
Alpha Bisabolol (AB)	RF	0.31	Non-carcinogen	Non-mutagenic
AB Oxide A	RF	0.29	Non-carcinogen	Non-mutagenic
AB Oxide B	RF	0.34	Non-carcinogen	Non-mutagenic

3.4. PASS Prediction Results

In this study, analysis of the possibilities of alpha-bisabolol and derivatives activity through the PASS online tool revealed that all 3 molecules were drug-like with CYP2J substrate (Pa>0.7 and Pi>0.01) and antimetastatic (Pa>0.6 and Pi:0.04) and other activities are given in Table 4. It is known that Pa values above 60% indicate strong similarity, as known from previous studies. Consistently with our other *in silico* analyses, the highest antimetastatic effect was found in alpha-bisabolol oxide B. Surprisingly, alpha bisabolol exhibited a significantly higher apoptosis agonist effect compared to the other two compounds. The high antitumor property of alpha-bisabolol oxide B is further supported by its antineoplastic (lung cancer) drug similarity, which was also the highest with a Pa of 0.798. Considering that the PASS analysis values reported in the literature for flavonoid trimethoxyflavone are Pa > 70% (Pires *et al*., 2015) and for apigenin are Pa > 90% (Divya Rajaselvi *et al*., 2023), it can be inferred from the obtained drug similarity values that alpha bisabolol has high druggability potential.

In molecular docking studies, alpha bisabolol oxide B shows the highest affinity; however, the higher potential of alpha bisabolol against various diseases, as indicated by PASS analysis, can be attributed to its specificity. Alpha bisabolol oxide B is a derivative bioactive compound that has shown activity against apoptosis markers studied in this work, while alpha bisabolol

presents a broad spectrum of biotherapeutic potential. Additionally, literature indicates that alpha-bisabolol exhibits antiviral, anti-inflammatory, and immunosuppressant effects, as demonstrated through *in vitro* and *in vivo* studies (Sun *et al*., 2017; Al-Ghanim *et al*., 2023).

Table 4. PASS predictions.

	Alpha Bisabolol		AB Oxide A		AB Oxide B	
Activity	Pa	Pi	Pa	Pi	Pa	Pi
Apoptosis agonist	0.847	0.005	0.434	0.058	0.342	0.102
Anti-eczematic	0.830	0.013	0.749	0.031	0.709	0.043
CYP2J substrate	0.807	0.019	0.818	0.017	0.719	0.044
Retinol dehydrogenase inhibitor	0.763	0.002	0.399	0.012	0.359	0.016
Immunosuppressant	0.736	0.013	0.347	0.087	0.690	0.018
BRAF expression inhibitor	0.731	0.003	0.198	0.040	0.266	0.026
Antithrombotic	0.727	0.006	0.230	0.167	0.270	0.124
Antiviral (Rhinovirus)	0.664	0.004	0.412	0.079	0.414	0.077
Chemo-preventive	0.660	0.008	0.302	0.036	0.270	0.043
Carminative	0.678	0.008	0.556	0.014	0.316	0.042
Antineoplastic	0.657	0.034	0.374	0.027	0.798	0.012
Anti-inflammatory	0.652	0.022	0.241	0.199	0.388	0.102
Antimetastatic	0.638	0.004	0.617	0.004	0.639	0.004

Lipinski's Rule of Five is specifically designed to illustrate a compound's oral bioavailability and assists in evaluating its absorption and penetration into lipid bilayers. According to this rule, a compound should not exceed certain thresholds: a molecular weight limit of 500 Da, no more than five hydrogen bond donors, a log P value of no more than five, and fewer than five hydrogen bond acceptors (Lipinski *et al*., 1997). Table 5 obtained from DruLiTo software indicates that alpha-bisabolol and its derivatives satisfy the Lipinski, Veber, and Ghosh rules. It has been predicted that the molecular weights (MW) of all three molecules are greater than 220 g/mol, with log P values of 4.085 for alpha-bisabolol and 3.176 for both alpha-bisabolol oxide A and B with at least 1 H donor and acceptor atoms. The total polar surface area (TPSA) refers to the cumulative surface area occupied by all polar atoms, including nitrogen, hydrogen, and oxygen, within a molecule. This measurement indicates a molecule's ability to penetrate biological barriers such as the blood-brain barrier (BBB). The TPSA values obtained from the analysis, around 20 $Å^2$, indicate blood-brain barrier (BBB) permeability (Prasanna & Doerksen, 2009). Although the TPSA values of secondary metabolites vary depending on the molecular structure, values obtained for standard bioactive compounds such as limonin, quercetin, and kaempferol derived from different plant species are typically around 100 $Å^2$, as reported in the literature (Oner *et al*., 2023).

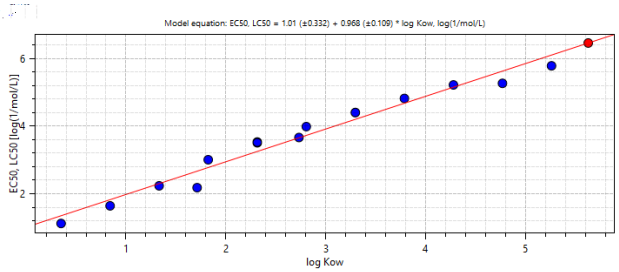
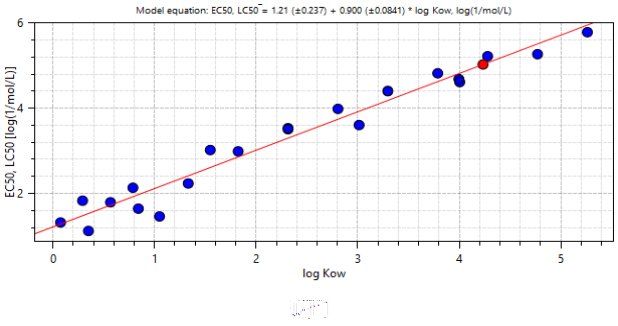
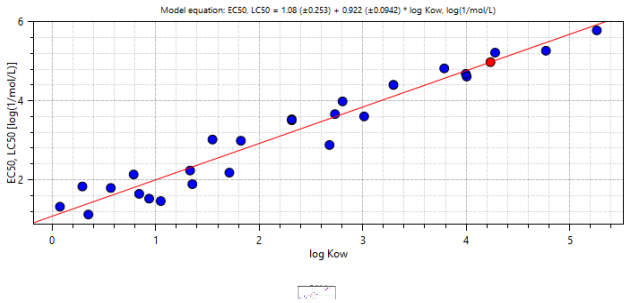
Table 5. Druglikeness assessment with DruLiTo program.

Compound	MW (g/mol)	logP	HBA	HBD	TPSA	Lipinski's rule	Ghose filter	Verber filter
Alpha Bisabolol (AB)	222.2	4.085	1	1	20.23	-	-	-
AB Oxide A	238.19	3.176	2	1	29.46	-	-	-
AB Oxide B	238.19	3.176	2	1	29.46	-	-	-

3.5. QSAR Analysis

In Table 6, the correlation between the LogLC50 value and the log-Kow value of alpha-bisabolol and its derivatives, which affect the organism used *in silico* experiments, is shown. As observed, there is a positive correlation between the log-Kow value and the LogLC50 value, with a simultaneous increase observed. An increase in the LogLC50 value indicates a decrease in toxicity, and this phenomenon resulted in similar observations on the organism for all three compounds (Ha *et al.*, 2019). On the other hand, when comparing the obtained LC50 values with the toxicity report of bisabolol, it can be said that the values are close to the average toxicity limit (Andersen, 1999). Considering that the toxicity value for ibuprofen, commonly used in comparative toxicity studies, is 3 mg/l, the values obtained for alpha bisabolol oxide A and B, which are 2.24 and 2.5 mg/l respectively are in the average toxicity range (Wade *et al.*, 1997). Other important QSAR result values are R^2 and Q^2, which means the optimistic evaluation of model fit and consistency lacks a proper standard of comparison. R^2 value generally gives a model fit to the original data measurement, and Q^2 gives a measurement of consistency between the original and predicted data (Worley & Powers, 2013). Both values can be a maximum of 1, in which case the obtained measurement values from QSAR analysis are quite high.

Table 6. Computational QSAR models for alpha bisabolol and derivatives.

No	Name	Model	LC_{50}	R^2	Q^2
1	Alpha bisabolol		0.0773 mg/l	0.978	0.971
2	Alpha bisabolol oxide A		2.24 mg/l	0.953	0.943
3	Alpha bisabolol oxide B		2.5 mg/l	0.942	0.940

3.6. ADMET Prediction

Estimation of various ADMET properties of alpha-bisabolol and its derivatives are given in Table 7. For an average ADMET property, the molecular weight of the phytochemicals should be in the range of 180–480 Da (see Table 5). Because heavy molecules will take longer to be absorbed and their mechanism of action is unclear, it is mostly expected that drug molecules will be as light as possible. Similarly, because of the prediction that heavy atoms will be difficult to pass through membranes and the toxicity profile will increase, it is expected that drug molecules will not contain as many heavy atoms as possible (<36) (Zhong *et al*., 2013). The Csp3 fraction affects the metabolic stability of the drug formula and molecules with a high sp3 hybridization value are more stable than molecules of the sp2 hybridization fraction. The Csp3 value is mostly expected to be around 0.6 (Cervelli & Russ, 2010). On the other hand, the lipophilicity of the drug directly affects its distribution in the body, drug metabolism, and biocompatibility. In drug development studies conducted for a lipid-rich tissue target, drug lipophilicity is expected to be quite high. Because the target mechanism mentioned in this study is the brain and surroundings, it is expected that the phytochemicals examined by the presence of adipose tissue will have high lipophilicity (1-3). Like lipophilicity, solubility should be high for better distribution, bioavailability, and absorption. For the glioma study, alpha-bisabolol and its derivatives are expected to be soluble (Chandrasekaran *et al*., 2018).

Most cancer drugs are administered orally for patient comfort and ease of use. Therefore, GI absorption is critical for determining the bioavailability of these drugs. Enough of the drug must reach the systemic circulation to achieve effective absorption. Therefore, for effective glioma-targeted drug development, drug molecules should also pass through the blood-brain barrier (BBB) (Chandrasekaran *et al*., 2018).

Table 7. ADMET prediction.

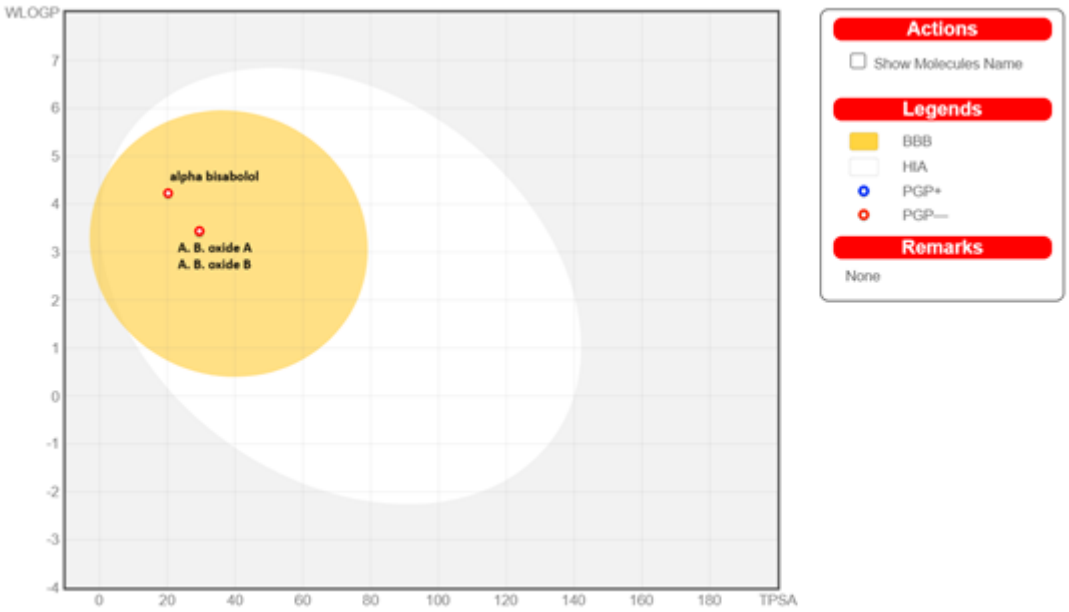
Predicted Model	Alpha Bisabolol	AB Oxide A	AB Oxide B
Water solubility (Log mol/L)	-4.379	-3.41	-3.396
Fraction Csp3	0.321	0.45	0.481
Caco2 permeability (Log Papp in 10^{-6} cm/s)	1.505	1.607	1.605
Intestinal (GI) absorption (%)	93.014	93.989	93.958
Skin permeability (log Kp)	-1.761	-2.919	-2.912
P-glycoprotein substrate	No	No	No
P-glycoprotein inhibitor	No	No	No
BBB permeability (log)	0.605	0.514	0.533
Total clearance (log ml/min/kg)	1.363	1.115	1.117
CYP3A4 inhibitor	No	No	No
CYP2C19 inhibitor	Yes	No	No
Hepatotoxicity	No	No	No

3.7. BOILED-EGG Model

The BOILED-Egg model predictions for alpha-bisabolol and its derivatives are presented in Figure 3. Due to their structural similarities, alpha-bisabolol oxide A and B are localized in the same region of the model. Additionally, also all three compounds are situated in the yolk region of the egg. From these observations, it can be inferred that all three compounds exhibit hydrophilic properties, have BBB permeability, and may have relatively slow cellular permeability due to P-glycoprotein (PGP-) substrates inhibition.All three bioactive components investigated in the study have shown BBB permeability, which indicates the potential for direct access to brain tissue and more effective intervention with tumor cells, reflecting high efficacy and bioavailability. Another advantage of these components crossing the BBB is the possibility

of using them in combination with other treatment methods (e.g., radiotherapy or surgery). Figure 3 shows that alpha bisabolol exhibits higher permeability compared to the other two components, which is quite normal considering their molecular weights and sizes.

Figure 3. Prediction of GI absorption and BBB permeation by BOILED-EGG model.



4. CONCLUSION

The immediate need to screen, discover, and develop novel potential anticancer drugs with optimal therapeutic efficacy and minimal adverse effects is crucial in today's pharmacological topic. Apart from conventional in vitro models, computer-assisted algorithms or *in silico* modeling and prediction tools emerge as highly useful resources in the available array of alternative approaches. The toxicological attributes of a compound can be promptly and efficiently assessed through computerized methodologies. This study, based on various *in silico* prediction methods, demonstrates that alpha bisabol and oxidized products (alpha bisabolol oxide A and B) exhibit favorable drug-like properties without any evidence of carcinogenicity or mutagenicity. The research evaluates the anticancer activity of the alpha-bisabolol and derivatives targeting bcl-2 family proteins. The results indicate that alpha bisabolol oxide B exhibits high inhibitory activity at the molecular level with a binding affinity score of -8.5 kcal/mol. According to QSAR analysis results, the components do not show toxic effects and have high BBB permeability. They not only have anti-apoptotic effects against glioblastoma but also possess high biotherapeutic potential against various diseases, as evidenced by PASS analysis results. Further *in vitro* and *in vivo* validations are necessary to investigate their safety profile and potential interactions with other drugs.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Nilüfer Vural: Methodology, Supervision, Writing and Validation. **Sibel Kaymak**: Investigation, Visualization, Software, Formal Analysis, and Writing-original draft.

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Research Article

Bioactivity of secondary metabolite of endophytic fungi extract isolated from root of Jambu Mawar (*Syzygium jambos* (L.) Alston)

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Abstract: This research aims to isolate endophytic fungi from *Syzygium jambos* and identify their active compounds. Endophytic fungi were isolated from the roots of *S. jambos* and cultured on Potato Dextrose Agar media. Antibacterial activity using the Kirby–Bauer method was tested on four Gram-positive and Gram-negative bacteria. Molecular identification was carried out on selected isolates to determine the species of endophytic fungi and isolate their active compounds. Column chromatography was used for compound isolation. The pure compounds were then analyzed spectroscopically using ^{1}H-NMR, ^{13}C-NMR, DEPT 135, HMQC, HMBC, COSY. The results of the isolation of endophytic fungi found four isolates SJR1 – 4, which show antibacterial activity. The strongest antibacterial activity was demonstrated by isolate SJR1, so it was continued with molecular identification. Molecular identification of SJR1 indicates that it is *Lasiodiplodia iranensis*. The pure compound *L. iranensis* was isolated and found to be 3-butyl-3,4-dihydroxy-6-((2-hydroxy-5-oxocyclopentyl) methyl)tetrahydro-pyran-2-one, which belongs to the phenolic group and has potential as an antibacterial. This compound can be used as an alternative medicinal ingredient.

1. INTRODUCTION

Endophytic fungi are known to produce many new metabolites that have the potential as natural agents in the pharmaceutical, agricultural, and environmental fields (Selim *et al*., 2012). Secondary metabolites produced by endophytic fungi are not needed for the growth of microorganisms, but play an important role in the health, nutritional needs, and economy of society (Abdel-Aziz *et al*., 2017). Secondary metabolites of endophytic fungi have various chemical compounds, such as terpenoids, polyketides, non-ribosomal peptides, or a

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combination of both. These secondary metabolites function as antibiotics or medical drugs, but there are also mycotoxins which are dangerous to health (Avalos & Limón, 2021). Secondary metabolites are natural products in the form of molecules with low molecular weight, having diverse chemical structures and biological activities produced by plants, bacteria, and fungi. Secondary metabolites (SM) are byproducts whose function is to defend an organism from environmental stress (Mosunova *et al*., 2021).

Endophytic fungi are organisms that live in all healthy plant tissues without showing signs of disease or morphological changes in either part or all of the plant's life cycle. The term endophyte was introduced by De Bary, namely organisms that reside in plant tissue (Khiralla *et al.,* 2016). Based on studies, endophytic fungi isolated from medicinal plants are known to be able to help their hosts in synthesizing secondary metabolite compounds whose effects are the same as natural medicines synthesized directly from plant tissue (Zheng *et al*., 2021).

There have been many studies on the discovery of new secondary metabolites produced by endophytic fungi and their bioactivity which has the same properties as compounds produced by their hosts. *Penicillium griseofulvum* isolated from the medicinal plant *Mentha pulegium* has activity against *Escherichia coli* bacteria (Amina *et al*., 2018). *Curvularia papendorii i*solated from *Vernonia amygdalina* contains kheiric acid which has activity against Methicillin-resistant *Staphylococcus aureus* (Khiralla *et al*., 2020). The endophytic fungi *Nigrospora, Diaporthe* and *Epicoccum* isolated from *Leucas martinicensis* have activity against *E. coli*, *S. aureus* (Ruth *et al*., 2020). Drug-resistant bacteria, such as *Staphylococcus aureus* resistant to Methicillin, *Streptococcus pneumonia* resistant to penicillin (Deshmukh *et al*., 2015).

Plants from the genus *Syzygium* Gaertn. (Myrtaceae) are known to have medicinal properties. *Syzygium* is known for having very strong aromatics because it is rich in essential oils, flavonoids, flavonols, anthocyanins, tannins, and phenolic acids (Sobeh *et al*., 2018). Jambu mawar (*Syzygium jambos* (L.) Alston) is one of the genera *Syzygium* which has medicinal properties. *S. jambos* leaves contain phenolic compounds which have antidiabetic potential (Gavillán-Suárez *et al*., 2015), alkaloid, flavonoid, and steroid compounds that have antioxidant and cytotoxic activity (Devakumar & Sudha, 2017). According to Wamba *et al*. (2018) the leaves and bark of *S. jambos* contain saponin, triterpenoid, anthraquinone, and tannin compounds which have antibacterial activity. However, this species is difficult to find, especially in South Sumatra, as an alternative, endophytic fungi have been isolated to overcome the need for compounds needed as medicinal ingredients. Previously, research had been carried out on the diversity and activity of endophytic fungal compounds from *S. jambos*.

A diversity of endophytic fungi isolated from *S. jambos* was found on the skin (Roux *et al*., 2020), as well as on leaves and root bark (Aini *et al.*, 2022b). Besides that, several pure compounds that have biological activity were also found in the endophyte function of *S. jambos*, namely the compound 3-hydroxy-4-(hydroxy(4hydroxyphenyl)methyl)dihydrofuran-2-on which was isolated from *Fusarium verticillioides* (Aini *et al*., 2022), the compound 3,5-dihydroxy-4-(4-hydroxyphenyl) tetrahydro-2H-pyran-2-one which was isolated from *Lasiodiplodia theobromae* (Aini *et al.*, 2022a) and 5-acetyl-6-hydroxy-3-methyl-2H-pyran-2-one which was isolated from *Botryosphaeria mamane* (Aini *et al*., 2023). These compounds have antibacterial and antioxidant activity.

A variety of endophytic fungi have also been isolated from other *Syzygium* genera. Endophytic fungi isolated from *S. zeylanicum* (L.) DC. have antioxidant and antibacterial activity (Syarifah *et al*., 2022). Endophytic fungi isolated from *S. aqueum* (Burm.f.) Alston have antibacterial and antifungal activity (Habisukan *et al*., 2021). Endophytic fungi isolated from *S. malaccense* (L.) Merr. & L.M.Perry have antibacterial potential (Hapida *et al*., 2021). Therefore, this research aims to identify the diversity of endophytic fungi isolated from the roots of the *S. jambos* plant and discover new compounds synthesized by endophytic fungi and their activities.

2. MATERIAL and METHODS

2.1. Isolation and Identification of Endophytic Fungi

Syzygium jambos root samples were taken in March 2021 at the multipurpose field, Jalan Sakura 3, Kencana Damai housing complex, Sako, Palembang 2°54'49.8"S - 104°45'43.9"E (-2.913824, 104.762194).

Isolation of endophytic fungi using a modified method from Elfita *et al*. (2014). *S. jambos* root samples were washed with sterile water 5-10 times, then used 70% ethanol for 3 minutes, rinsed again with sterile water, and finally washed in sodium hypochlorite (NaOCl) solution for 5 minutes. Sterilized root samples were cut approximately 1 - 2 cm and placed on Potato Dextrose Agar (PDA) media for 1-2 weeks in a dark room at room temperature 27 ± 2ºC. The hyphae that have grown are transferred to new PDA media to obtain pure isolates.

The growing endophytic fungal isolates were identified morphologically macroscopically and microscopically. Macroscopic characterization through observing the color of the top and bottom surface colonies, colony shape, texture, and topography. Microscopic characterization through observation under a microscope (Hirox MXB-2500REZ), namely the type and shape of spores, hyphae, and other distinctive characteristics. The results of these observations were then compared with fungal identification books and relevant articles.

2.2. Cultivation and Extraction

The pure isolate of the root endophytic fungus of *S. jambos* was cultivated in 5 x 300 mL in potato dextrose broth (PDB) media by placing 6 blocks of pure culture agar in a bottle. Then the culture was incubated for four weeks (±28 days) at room temperature under static conditions. After the incubation period, the endophytic fungal mycelia were separated from the liquid culture and partitioned in ethyl acetate at a ratio of 1:1 three times. The ethyl acetate extract was separated from the liquid culture, then evaporated using a rotary evaporator (R300+ V-300 with interface I-300 Pro+ F305) until a thick extract was obtained (Supaphon & Preedanon, 2019)

2.3. Antibacterial Activity of Fungal Endophytes

The endophytic fungal isolates obtained were screened for antibacterial activity using the paper disk diffusion method with an ethanol extract concentration of 400 µg/mL, positive control using 30 µg/mL tetracycline antibiotics. The test bacteria were represented by Gram-negative – *Escherichia coli* and *Salmonella typhi*, while Gram-positive – *Staphylococcus aureus* and *Bacillus subtilis*. Antibacterial activity was shown by the clear zone around the paper disc. Criteria for antibacterial activity are determined by comparing the clear zone of endophytic fungal extract (A) and the clear zone of tetracycline antibiotics (B). For the criteria, $weak < 50\%$; $50\% < moderat < 70\%$; $strong > 70\%$. Calculations based on the following equation (Elfita *et al*., 2019):

$$Clear\ zone\ percentage\ (\%) = \frac{A}{B} x100\%$$

2.4. Molecular Identification

Endophytic fungal isolates that have strong antibacterial activity are subjected to molecular identification to determine the species of the endophytic fungus. Molecular testing of endophytic fungi was carried out at the Genetic Science Indonesia laboratory, Banten, Indonesia using the Genomic DNA extraction method with Quick-DNA Fungal Miniprep Kit (Zymo Research, D6005), PCR amplifcation with My Taq HS Red Mix (Bioline, BIO-25048) at twice. Standard PCR primers use ITS1 and ITS4 (Singha et al., 2016). Analysis of DNA structure using Molecular Evolution Genetics Analysis Versi 11 (Tamura *et al*., 2021).

2.5. Isolation of Chemical Compounds of Endophytic Fungi

Endophytic fungi that have strong antibacterial activity will have their active compounds isolated. Filtrate containing active secondary metabolites by liquid-liquid fractionation

(partition) with the organic solvent ethyl acetate. The fraction filtrate was then concentrated using a rotary evaporator until a thick ethyl acetate fraction was obtained. The concentrated extract was separated through column chromatography using silica gel 60 (70–230 mesh) as the stationary phase (1:30) and eluent. The eluent was chosen based on previous determination through thin-layer chromatography using silica gel 60 F254. The selected eluent with increased polarity was *n*-hexane:EtOAc in a ratio ranging from 10:0 to 0:10 (v/v). An eluate was collected and then combined via thin-layer chromatography into column fractions. Each fraction was evaporated and purified through chromatography to isolate the pure compound. The chemical structure of the compound was determined through various spectroscopy methods: Spectrum of ^{1}H-NMR, ^{13}C-NMR, DEPT 135, HMQC, HMBC and COSY (Elfita *et al*., 2012, 2016).

3. FINDINGS

3.1. Isolation of Endophytic Fungi

The results of the isolation of endophytic fungi from the roots of *S. jambos* found four isolates, namely SJR1 - 4 (Figure 1). This endophytic fungal isolate was then subjected to macroscopic (Figure 1a, Table 1) and microscopic morphological characterization (Figure 1b, Table 2). The results of macroscopic characterization identified four genera, namely *Lasiodiplodia*, *Rhizopus*, *Mortierella*, and *Cylindrocladium*. These four genera have the same type and shape of spores, namely conidia and globose. However, the hyphae of the genus *Rhizopus* are rhizoid, and the other three genera are septate.

Figure 1. Macroscopic (a); Microscopic (b) morphological characterization of endophytic fungi.

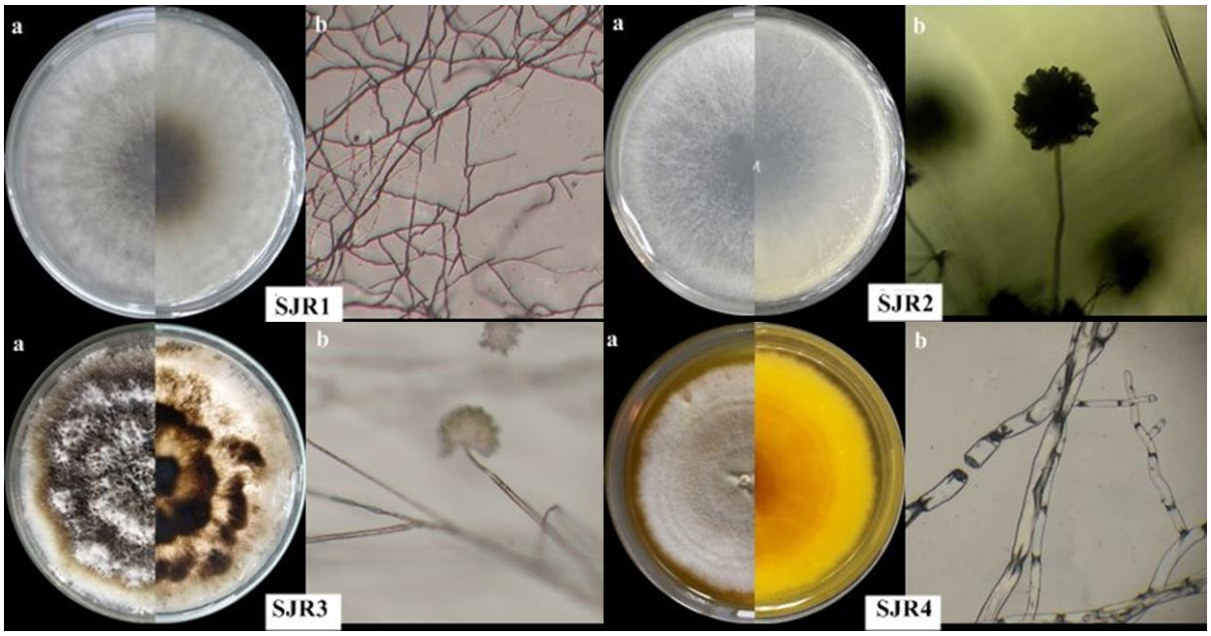


Table 1. Macroscopic characteristics of endophytic fungal isolation from *S. jambos* roots.

Isolate	Colony color	Reverse colony color	Texture	Topography	Pattern	Exudate drops	Radial line	Concentric circle
SJR1	White	Black and White	cottony	Raised	Zonate	-	√	√
SJR2	White	White	cottony	Raised	Zonate	-	-	-
SJR3	Black and White	Black and White	cottony	Raised	Flowery	-	-	√
SJR4	White	White to yellow pigmented	velvety	Umbonate	Radiated	-	√	√

Table 2. Microscopic characteristics of endophytic fungal isolation from *S. jambos* roots.

Isolate	Type of spore	Shape of spore	Hyphae	Specific characteristic	Genus / species
SJR1	Conidia	Globose	Septate	Hyaline conidia, usually two	*Lasiodiplodia* sp.
SJR2	Conidia	Globose	Rhizoids	Numerous stolon run among the mycelia	*Rhizopus* sp.
SJR3	Conidia	Globose	Septate	Sporagiosphores branched, long	*Mortierella* sp.
SJR4	Conidia	Globose	Septate	Hyaline conidia, conidiophores tapering towards the apex, branched, phialides terminal	*Cylindrocladium* sp.

3.2. Bioactivity of Endophytic Fungi

The results of antibacterial activity screening against the bacteria *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Salmonella typhi*, showed that the extract of endophytic fungi isolated from *S. jambos* roots had antibacterial activity (see Table 3). SJR1 isolate has strong activity against *B. subtilis* and *S. aureus* with percentages of 71.8% and 70.6%, and moderate activity against *S. typhi* (69.2%) and *E. coli* (69.4%). The isolates SJR3 and SJ4 have moderate activity toward four testing bacteria. The isolate SJR2 has moderate activity against *S. typhi*, *E. coli*, and *B. subtilis*, weak toward *S. aureus* (49.8%). Of the four fungi found, SJR 1 had the highest percentage of antibacterial activity, so it was chosen to continue with molecular identification.

Table 3. Antibacterial activity of endophytic fungal extracts.

Isolate	Ethyl acetate extract (gr)	Antibacterial Activity (%)			
		S. typhi	*E. coli*	*B. subtilis*	*S. aureus*
Tetracycline	-	100	100	100	100
SJR1	4.1	69.2**	69.4**	71.8***	70.6***
SJR2	3.8	54.8**	56.6**	51.7**	49.8*
SJR3	3.2	52.8**	55.8**	52.2**	53.8**
SJR4	3.2	52.9**	53.5**	51.4**	51.8**

Notet: < 50% = weak*; 50 – 70% = moderate**; > 70% = strong***

3.3. Molecular Identification

Molecular identification of SJR1 isolates using PCR amplification with universal primers of the ITS1 - ITS4 rDNA region varied by ± 500 - 600bp the result of assembling the PCR amplification sequence for SJR1 is 540bp (GenBank OM746696) (Figure 2). The results of phylogenetic analysis (Figure 3) SJR1 has a very close distance with a value of 99, this shows that these three isolates are closely related. Isolate SJR1 has a bootstrap value of 1000 and is in the genus *Lasiodiplodia*.

Figure 2. Electrophoresis of SJR1 isolates. M (Marker 100 bp DNA ladder).

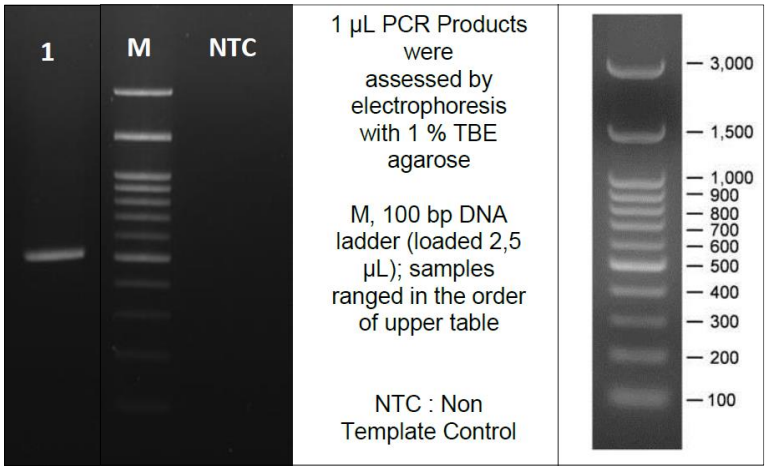
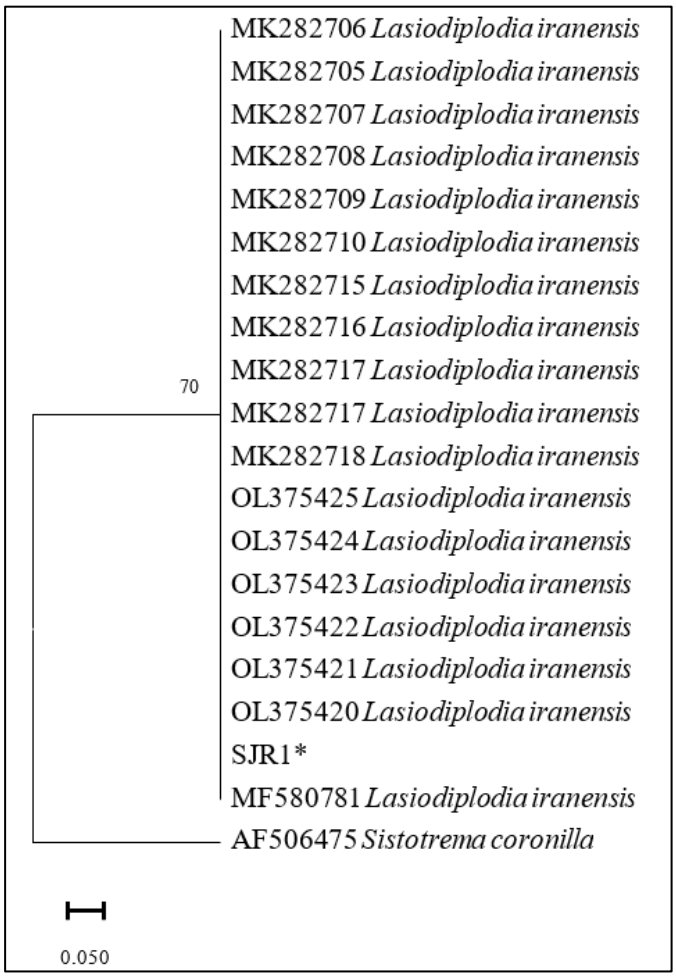


Figure 3. Phylogenetic tree of SJR1 endophytic fungal isolates.

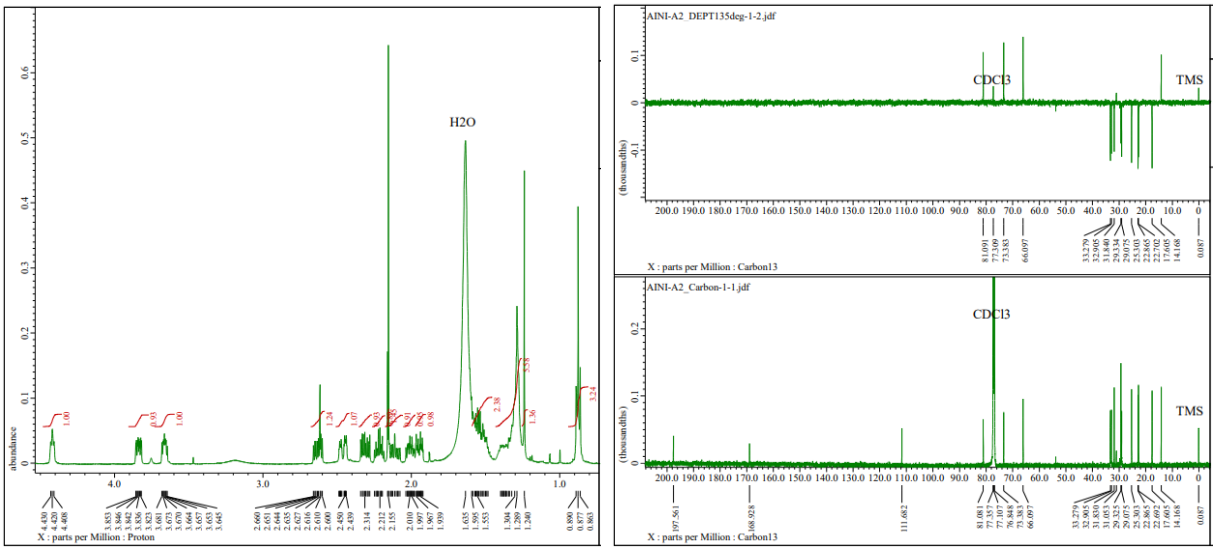


3.4. Isolation of Chemical Compounds of Endophytic Fungi

The chemical structure of compound **1** was determined based on ^{1}H-NMR, ^{13}C-NMR, DEPT 135, HMQC, HMBC, and COSY spectrum analysis. The spectrum of ^{1}H-NMR of compound **1** (Figure 4A) showed there are 17 proton signals. All proton signals are at δ_H < 4.5 ppm which indicates that all protons of compound **1** are sp^3. Proton at δ_H 3.5 – 4.5 ppm are sp^3 protons in oxygenated carbon. In the spectrum it can be seen that there are two triplet signals, namely at δ_H 0.88 (3H, J= 6.5 Hz); 4.42 ppm (1H, J= 5 Hz), and the other is a multiplet signal. Based on ^{1}H-NMR, spectrum analysis, compound **1** is non-aromatic which has an oxygenated carbon atom in the form of either a hydroxyl group or a cyclic ester.

The ^{13}C-NMR spectrum of compound **1** (Figure 4B) shows the presence of 15 signals. There are three carbon signals at δ_C > 100 ppm, three carbon signals at δ_C 65-85 ppm, and another at δ_C < 33.0 ppm. The spectrum of DEPT 135 compound **1** (Figure 4B) shows that all three signals at δ_C > 100 ppm are quaternary carbon. Two carbon signals at low fields, namely at δ_C 197.6 and 168.9 ppm indicate the presence of carbonyl ketone and carbonyl ester groups. The three oxygenated carbon signals at δ_C 65-85 are methine carbons, and all carbons at δ_C < 33.0 ppm are methylene carbons, except for one carbon at δ_C 29.3 ppm which is methine carbon and 14.2 ppm which is methyl carbon.

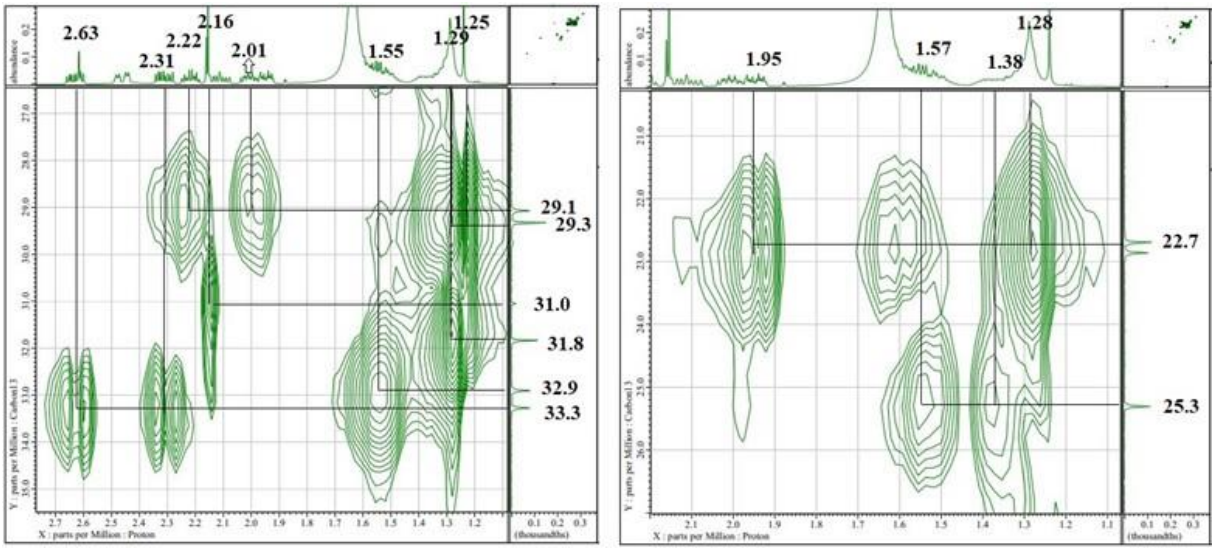
Figure 4.[1]H-NMR (A), [13]C-NMR and DEPT 135 (B) spectrum compound **1** ([1]H-500 MHz; [13]C-125 MHz in $CDCl_3$).



A B

Analysis of the NMR spectrum of protons and carbon is strengthened by data on the HMQC spectrum shown in Figure 5 and Table 4, namely the correlation of [1]H-[13]C through one bond.

Figure 5. The spectrum of HMQC of compound **1**.



The HMQC spectrum shows 17 correlations, there are 10 proton signals that correlate with five methylene carbon atoms and two other proton signals correlate with two methylene carbon atoms. Furthermore, three proton signals at δ_H 3.5 – 4.5 ppm correlate with three oxygenated methine carbons. Apart from that, there is also a correlation of a proton signal with methine carbon and a proton signal with methyl carbon.

The HMBC spectrum (see Figure 6) shows the correlation of [1]H-[13]C through two or three bonds. There is a methylene group that correlates with the ketone carbonyl carbon and two methylene groups that correlate with the ester carbonyl carbon. Methylene protons at δ_H 2.63 (1H, m) and 2.31 (1H, m) correlate two and three bonds with carbon at δ_C 197.6; 66.1; 29.1 ppm. Methylene protons at δ_H 2.22 (1H, m) and 2.01 (1H, m) correlate with carbon at δ_C 168.9; 197.6; 66.1; 33.3 ppm. Methylene protons at δ_H 2.46 (1H, m) and 2.11 (1H, m) correlate with carbon at δ_C 168.9; 81.1; 111.7; 22.7 ppm. This correlation indicates that there is a methylene group that is three bonds away from the ester carbonyl carbon and two methylene groups that are two and three

bonds away from the ketone carbonyl carbon. Furthermore, there is a correlation of the methyl proton with two methylene carbons at δ_C 22.7; 31.8 ppm, methylene proton correlation δ_H 1.25 (2H, m) with carbon at δ_C 29.1 ppm, and methylene proton correlation δ_H 1.95; 1.28 ppm with carbon at δ_C 111.7; 17.6; 31.8 ppm. This correlation indicates the presence of a straight carbon chain group in compound **1**.

Figure 6. The Spectrum of HMBC of compound **1**.

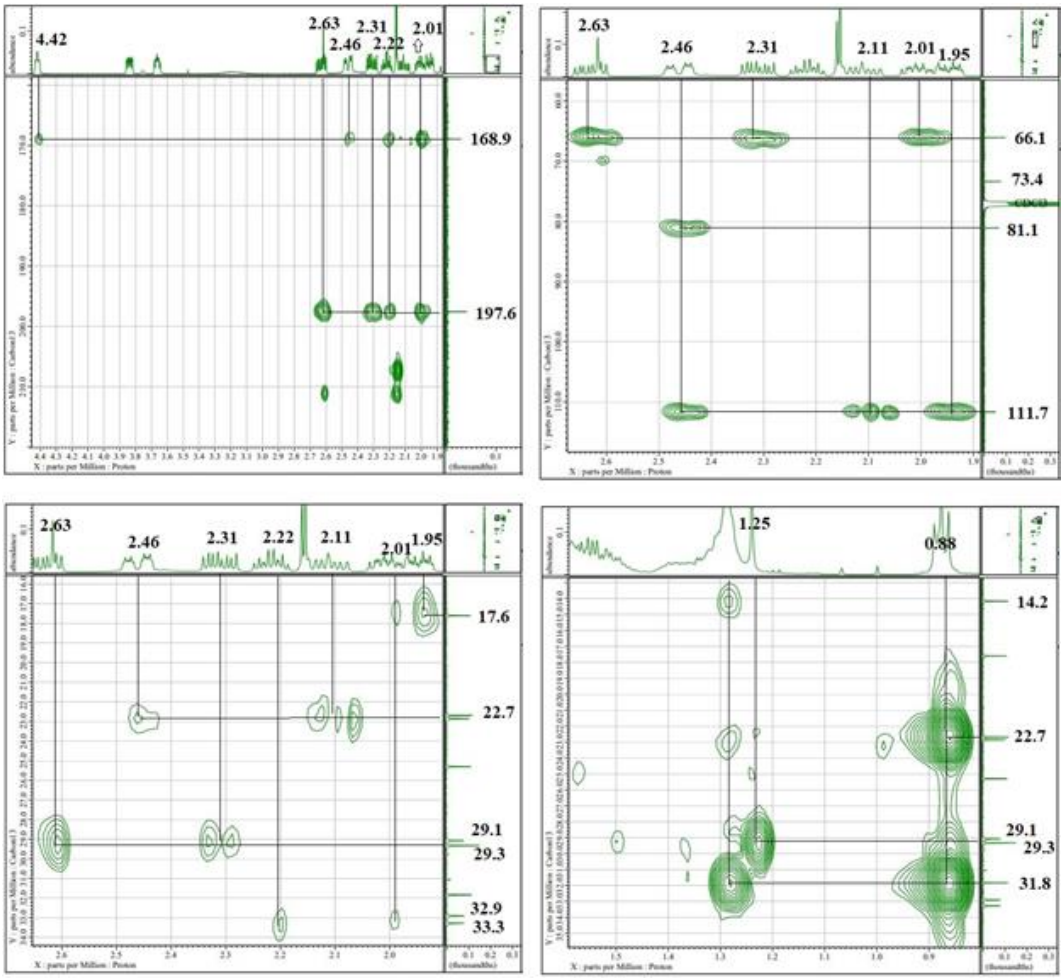
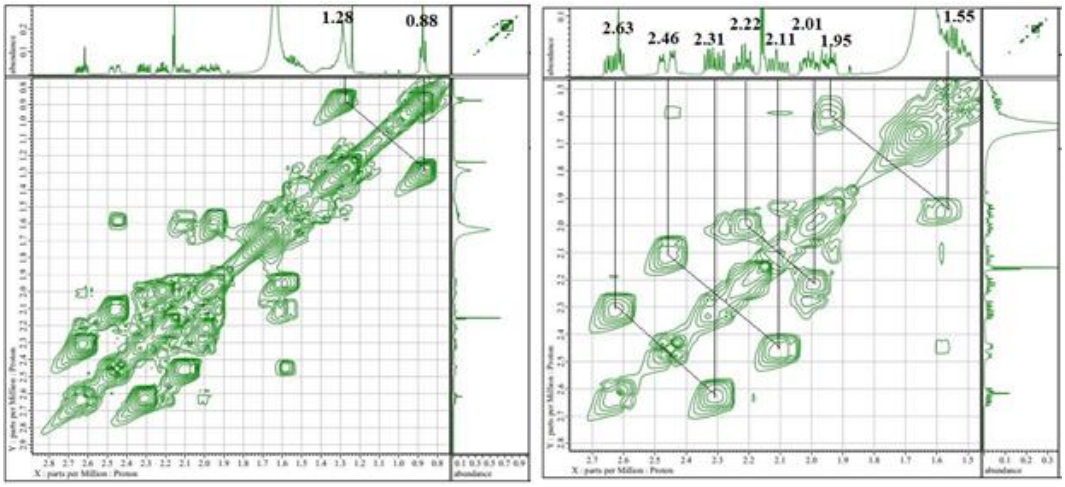


Figure 7. COSY spectrum of compound **1**.

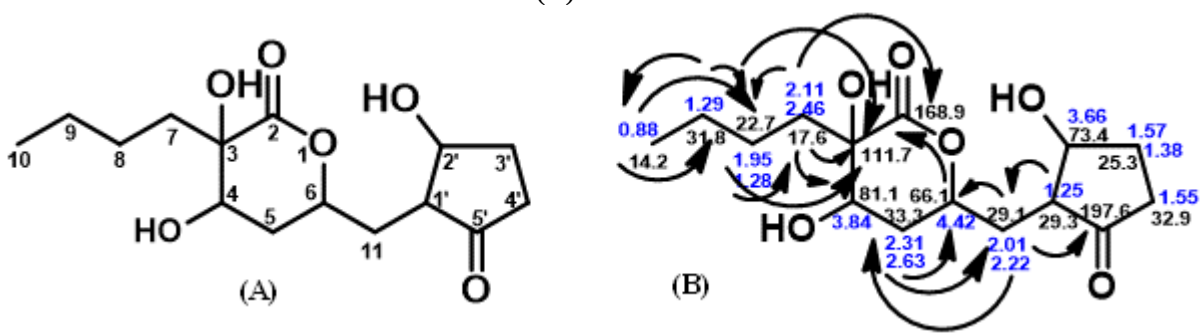


The COSY spectrum (see Figure 7) shows a ^{1}H-^{1}H correlation through two or three bonds. There are three ^{1}H-^{1}H correlations through two bonds, namely the correlation between methylene protons bound to the same carbon but with different chemical shifts. Apart from that, there is also a correlation between methyl protons and methylene protons through three bonds. The 1D and 2D NMR spectrum data for compound **1** are listed in Table 4. Based on the ^{1}H-NMR, ^{13}C-NMR, DEPT 135, HMQC, HMBC and COSY spectrum analysis, it can be explained that compound **1** has a straight chain consisting of four carbons (n-butyl), three hydroxyl groups, a carbonyl ketone group, a carbonyl ester, a methyl group, seven methylene groups (two methylene groups are on the butyl cyclo). Furthermore, there are four methine groups, including three oxygenated methine groups, and a quaternary carbon. The molecular formula of compound **1** is $C_{15}H_{24}O_6$ with the equivalent double bond being 4. Thus, the proposed chemical structure of compound **1** is 3-butyl-3,4-dihydroxy-6-((2-hydroxy-5-oxocyclopentyl) methyl)tetrahydro-pyran-2-one as shown in Figure 8.

Table 4. NMR data for compound **1**, recorded at ^{1}H-500 MHz; ^{13}C-125 MHz in $CDCl_3$

No. C	δ_C ppm	Type of C	δ_H ppm (ΣH. multiplicity. *J* (Hz))	HMBC	COSY
2	168.9	C			
3	111.7	C			
4	81.1	CH	3.84 (1H, m)		
5	33.3	CH_2	2.63 (1H, m)	66.1; 29.1	2.31
			2.31 (1H, m)	66.1; 29.1	2.63
6	66.1	CH	4.42 (1H, t, J= 5 Hz)	168.9	
7	17.6	CH_2	2.46 (1H, m)	168.9; 81.1; 111.7; 22.7	2.11
			2.11 (1H, m)	111.7; 22.7	2.46
8	22.7	CH_2	1.95 (1H, m)	111.7; 17.6; 31.8	
			1.28 (1H, m)		
9	31.8	CH_2	1.29 (2H, m)	14.2; 22.7	0.88
10	14.2	CH_3	0.88 (3H, t, J= 6.5 Hz)	22.7; 31.8	1.29
11	29.1	CH_2	2.22 (1H, m)	168.9; 197.6; 33.3	2.01
			2.01 (1H, m)	168.9; 197.6; 66.1; 33.3	2.22
1’	29.3	CH	1.25 (1H, m)	29.1	
2’	73.4	CH	3.66 (1H, m)		
3’	25.3	CH_2	1.57 (1H, m)		
			1.38 (1H, m)		
4’	32.9	CH_2	1.55 (2H, m)		
5’	197.6	C			

Figure 8. Structure of compound **1**: 3-butyl-3,4-dihydroxy-6-((2-hydroxy-5-oxocyclopentyl) methyl)tetrahydro-pyran-2-one with carbon atom numbering (A) and placement of proton and carbon chemical shifts as well as HMBC correlation (B).



4. DISCUSSION and CONCLUSION

The results of the isolation of endophytic fungi found four isolates, namely SJR1 – SJR4, based on microscopic and macroscopic characterization identified as the genus *Lasiodiplodia* (SJR1), *Rhizopus* (SJR2), *Mortierella* (SJR3), and *Cylindrocladium* (SJR4). These four isolates have antibacterial activity against the bacteria *B. subtilis*, *S. aureus*, *E. coli* and *S. typhi* with the strongest activity being SJR1 (*Lasiodiplodia*). The antibacterial activity of these endophytic fungi is due to the secondary metabolite content they produce. It is known that endophytic fungi are an alternative source of plant-derived bioactive compounds (Wen *et al*., 2022). Secondary metabolites produced by endophytic fungi act as antimicrobial, insecticides, anticancer, cytotoxic, and antioxidant (Bano *et al*., 2016). Apart from that, it also has the ability to act as an immunosuppressive, antidiabetic, antimalarial, antituberculosis and antiviral agent (Singh & Kumar, 2023).

Isolate SJR2 is characterized by white top colonies and white bottom colonies. Microscopically, the hyphae appear like roots or rhizoids, long sporangiopores and at the tip there is a round, dark colored sporangium. Based on these characteristics SJR2 was identified as *Rhizopus* (Walsh *et al*., 2018). The *Rhizopus* genus plays a role in increasing the nutrition of food ingredients through the fermentation process (Endrawati & Kusumaningtyas, 2018). *Rhizopus* can also be a pathogen that causes rot in plants (Hartanti *et al*., 2020). *Rhizopus oryzae* has antioxidant properties (Kang *et al*., 2016; Massarolo *et al*., 2017), antibacterial against *Salmonella typhi* (Jannah *et al*., 2020). *Rhizopus oligosporus* as an antioxidant (Dulf *et al*., 2018; Starzyńska-Janiszewska *et al*., 2020; de Lima *et al*., 2021), produces linoleic acid, α-linoeat acid and monolinolenins as antibacterial (Kusumah *et al*., 2020). Monohexosylceramides compounds inhibit the growth of *Bacillus terrae*, *Micrococcus luteus*, *Pseudomonas stutzeri* and antibiofilm from MRSA bacteria (Vieira *et al*., 2018).

The endophytic fungus SJR3 was identified as *Mortierella*, belonging to the order Mortierellales. Members of Mortierellales have a very high ecological and physiological diversity that allows them to be distributed throughout the world (Voigt & Kirk, 2014). *Mortierella parvispora* as antiparasitic activities, *Mortierella* has herbicidal activity (Vaca & Chávez, 2019), as bioremediation (Cui *et al*., 2017). *Mortierella alpine* as an antioxidant and antimicrobial (Goyzueta *et al*., 2020), synthesizes indoleacetic acid, gibberellic acid and ACC-deaminase (Ozimek *et al*., 2018). *Mortierella isabellina* is a type of fungi that produces compounds that have been found to have lower inhibitory effects than those detected in the dexamethasone-treated group. However, one of the compounds 15-ene steviol showed better effects than dexamethasone in reducing the release of monocyte chemoattractant protein (MCP)-1, 2, and 3, which LPS induces. Furthermore, three specialized products similarly showed better effects than dexamethasone in inhibiting the secretion of regulated on activation, normal T cell expressed and secreted (RANTES) in response to LPS. None of the tested compounds showed any cytotoxicity or triggered cell apoptosis, and none affected the protein integrity of toll-like receptor 4 (TLR4) or MyD88. This suggests that these compounds may exert the anti-inflammatory activity downstream of membrane-associated TLR4 and MyD88 molecules (Chang *et al*., 2021).

The SJR1 isolate has strong antibacterial activity, which was then identified molecularly to determine the species. Phylogenetic tree construction analysis using the Neighbor-Joining method (Saitou & Nei, 1987) with bootstrap 1000x repetitions (Felsenstein, 1985). This analysis involved 20 nucleotide sequences. Evolutionary analysis was performed in MEGA11 software (Tamura *et al*., 2021). Trees are drawn to scale and the units of branch length are the same as the evolutionary distances used to derive the phylogenetic tree. Evolutionary distances were calculated using the number-of-differences method (Nei & Kumar, 2000) and is measured in the number of basic differences per sequence. The analysis included 42 nucleotide sequences. From the SJR1 phylogenetic tree, the isolate sequence with a bootstrap value of 1000 shows 98–100% similarity to the species sequence, namely *Lasiodiplodia iranensis* (Figure 3).

Figure 3 shows that almost all samples have Bootstrap values in phylogenetic tree construction ranging >80. A bootstrap value of >80 means that the species are identical or have almost the same nucleotide base sequence and have a high level of similarity. According to that a branch of a phylogenetic tree is declared stable if the Bootstrap value is >80 and if it is <50 it is declared unstable. The similarity percentage can also influence the closeness of positions in the phylogenetic tree construction.

L. iranensis is found on *Arachis hypogeae* (Isalar *et al*., 2021), but is pathogenic on *Dioscorea* spp. causes rot in plant (Jibrin *et al*., 2022), causes death in *Coffea canephora* (Ramos *et al*., 2023). Based on research by Gagana & Shivanna (2020), *L. iranensis* found in *Memecylon umbellatum* has antibacterial activity, but the compounds contained in it have not been reported. Shen *et al*. (2022) reported that *L. iranensis* can produce jasmonic acid, a plant hormone that controls development, growth, photosynthesis, protects plants against insects and is applied in agriculture, industry and other fields. *Lasiodiplodia* sp. isolated from flower of *Viscum coloratum* produces essential oils in the form of cyclo-(Trp-Ala), indole-3-carboxylic acid (ICA), indole-3-carbaldehyde, mellein and 2-phenylethanol (Qian *et al*., 2014). *L. iranensis* isolated from the mangrove of *Avicenna ger-minans* was found to contain the compounds 11,12-didehydro-7-iso-jasmonic acid, 4,5-didehydro-7-iso-jasmonic acid, cyclo-(L-Leu-L-Pro), jasmonate-threonine, and abscisic acid (Delgado Gómez *et al*., 2023). In research Li *et al*. (2023), *L. iranensis* synthesizes 1,3,6,8-tetrahydroxynaphthalene, dimethylcoprogen, and (R)-melanin.

Based on this research, it is known that the four isolates found had antibacterial activity. The endophytic fungus *L. iranensis* produces the compound 3-butyl-3,4-dihydroxy-6-(3-(2-hydroxycyclobutyl)-3-oxopropyl) tetrahydro-2H-pyran-2-one which is included in the phenolic group. Phenolic compounds form a broad group of compounds derived from the secondary metabolism of plants found in various natural sources such as fruits, vegetables, tea, wine and honey (Lima *et al*., 2019). The antibacterial activity of phenolic compounds is related to the hydroxyl groups located in the cytoplasmic membrane of bacterial cells (Gyawali & Ibrahim, 2014). According to Griffin *et al*. (2005) and Figueiredo *et al*. (2008) said that the number and position of hydroxyl groups play a role in antimicrobial activity. The main antibacterial mechanism of phenolics such as eugenol is to disrupt the cytoplasmic membrane of bacteria, which increases their non-specific permeability (Li *et al*., 2015). In this study, compound **1** was found to have a hydroxyl group. It is suspected that this hydroxyl group is what disrupts the bacterial cytoplasmic membrane.

The presence of hydrophobic phenolic groups in the lipid bilayer disturbs interactions between lipids and proteins and leads to an increase in membrane permeability. This causes alterations in the structure of the membrane and speeds up the release of intracellular contents, ultimately leading to the disruption of membrane integrity. As a result, this process allows for the entry of substantial quantities of antibacterial agents (Char *et al*., 2010). Carvacrol and thymol, which are phenolic isomers, are non-polar compounds that insert themselves into the bacterial cell membrane, disrupting its normal function. The hydroxyl groups and the presence of a double bond in carvacrol and thymol enable them to function as proton exchangers, leading to a reduction in the gradient across the cytoplasmic membrane. This ultimately leads to the breakdown of the proton motive force and a decrease in the ATP pool, resulting in the death of the cell (Kachur & Suntres, 2020).

The start of disruption to the bacterial cell is suggested by shifts in cell membrane potential, which manifest as hyperpolarization of the membrane. This hyperpolarization is a result of pH changes or heightened movement of K^+ ions, leading to outward diffusion in order to maintain membrane potential balance (Whiteaker *et al*., 2001; Wu *et al*., 2016). Ensuring the balance of ions is crucial for the growth of cells and is involved in essential cellular functions like transporting substances, regulating metabolism, managing turgor pressure, and facilitating movement (Cox *et al*., 2001). The permeability of bacterial membranes is indicated by relative

electrical conductivity to explain how antimicrobial action works. Small ions like K^+, Na^+, and H^+ are crucial for supporting cell membrane function, enzyme activity, and normal metabolism, but the bacterial cytoplasmic membrane blocks their access. When electrical conductivity increases, it leads to cytolysis and bacterial death (Diao *et al*., 2014).

Maintaining the internal conditions of metabolism and energy transduction of cells relies on the integrity of the cell membrane, which is an important factor. (Sánchez *et al*., 2010). The functioning of the cell metabolism can be impacted by damage to the integrity of the cell membrane, leading to the inhibition of cell growth and potentially causing cell death. (Cox *et al*., 2001). The interactions between chemicals and membrane lipids can impact the fluidity of the membrane by affecting the order, shape, packing, and curvature of the lipids (Mykytczuk *et al*., 2007). Certain types of essential oils have the potential to harm bacterial cells and enhance the fluidity of the core of the lipid bilayer membrane. Changes in fluidity can lead to an increase in cell membrane permeability and result in harm to cellular contents, impacting cellular processes (Cherrat *et al*., 2016).

Compound **1** produced by *L. iranensis* in this research is expected to be an alternative source of medicinal ingredients, due to the need in the health sector. The limitation of this research is that it has not been tested *in vivo*, so further research needs to be carried out by applying it to test animals

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Kurratul 'Aini: Investigation, Resources, Software, Writing, original draft preparation, editing. **Elfita Elfita**: Methodology, Supervision, Validation, Analysis of new compound, Writing, original draft preparation, editing. **Hary Widjajanti**: Methodology, Validation and Analysis of Endophytic fungi. **Arum Setiawan**: Methodology, Validation and Analysis of Endophytic fungi. **Rian Oktiansyah**: Methodology, Analysis of Endophytic Fungi and Discussion.

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Research Article

Comprehensive analysis of antibacterial and antioxidant properties in *Calotropis gigantean* (Apocynaceae) leaf extracts

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Abstract: *Calotropis gigantea* (L.) W.T.Aiton., commonly known as arka, is a wild tropical plant from the Apocynaceae family with various medicinal properties. Previous studies have stated its antioxidant and antibacterial properties. For this purpose, leaf extracts of *C. gigantea* were prepared using a variety of solvents: ethanol, methanol, and n-hexane. For phytochemical bioactive compounds identification LC-MS analysis was performed, and to evaluate antibacterial activity against different gram positive (*Bacillus subtilis*, *Bacillus muralis*) and gram negative (*E. coli, Acetobactor rhizospherensis*) bacterial strains well diffusion method was applied. Antioxidant activity was observed using ABTS (2,2′-azino-bis-(3-ethylenebenzothiazoline)-6-sulfonic acid assay) and DPPH (2,2-diphenylpicrylhydrazyl assa) assays. The LC-MS (liquid chromatography-mass spectrometry) analysis revealed that ethanol leaf extract contained the highest number of compounds (14), while a 50% ethanol-methanol mixture had the fewest. In antimicrobial activity tests, methanol leaf extract exhibited the greatest inhibition zone against Bacillus subtilis (8 mm), and n-hexane the smallest (2 mm). Ethanol leaf extract had the highest inhibition against E. coli (9.5 mm), with methanol showing the lowest (4 mm). For *Acetobacter rhizopherensis*, ethanol extract demonstrated the largest inhibition zone (7.75 mm), while n-hexane showed the smallest (6 mm). Against *Bacillus muralis*, n-hexane showed the highest inhibition (5.75 mm), and methanol the lowest (3.5 mm). The ABTS assay indicated that ethanol extract had the highest inhibition activity (9.73%), and n-hexane the lowest (5.64%). The DPPH assay revealed that methanol extract had the highest radical remaining activity (99.68%), with n-hexane having the lowest (97.19%). *C. gigantea* is a prospective source of antioxidant and antibacterial agent.

1. INTRODUCTION

C. gigantea is a prevalent plant species found in barren and uncultivated areas, and it is usually referred to as gigantic milkweed (see Figure 1). This plant is indigenous to Bangladesh, Burma, China, India, Indonesia, Malaysia, Pakistan, Philippines, Thailand, and Sri Lanka. The plant

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has ovate, pale green foliage, a latex-filled stem, and clusters of glossy blooms that may be either white or lavender in hue. *C. gigantea* is readily accessible in India and is used for several therapeutic reasons in traditional medicine (Kumar *et al.*, 2012). Leaf of the *C. gigantea* are significantly known to have antibacterial and antioxidants activities. *C. gigantea* and *Calotropis procera* (Aiton) W.T.Aiton are the two species of *Calotropis* that are now in existence. In the past, these plants were mostly employed as ayurvedic medicines, and they were known by the common names "Sweet Arka" and "Raktha Arka," respectively. The botanical characteristics and pharmacological actions of these plants are identical (Bhatia *et al.*, 2022). The taxonomical classification of the plant *Calotropis gigantea* is described in the (Table 1).

Figure 1. General view photos of *Calotropis gigantea.*

Table 1. Taxonomical classification of *C. gigantea.*

Kingdom	Plantae
Phylum	Tracheophyte
Sub-phylum	Euphyllophyte
Class	Magnoliopsida
Sub-class	Asteridae
Order	Gentianales
Family	Apocynaceae
Subfamily	Asclepiadoideae
Tribe	Asclepiadeae
Genus	*Calotropis* R.Br.
Species	*Calotropis gigantea* (L.) W.T.Aiton

1.1. Vernacular Names

In many nations throughout the world, *Calotropis gigantea* is known by the following common names (Mahajan & Badgujar, 2008; Tiwari *et al.*, 2014). That was displayed in Table 2. *C. gigantea* can reach heights of 8–10 feet (Choudhary *et al.*, 2013). This plant contains clusters of waxy white or lavender blooms, oval light green leaves, and a milky latex stem. In India, *C. gigantea* is frequently found and used as medicine for a variety of illnesses (Alam *et al.*, 2008). The *Calotropis* plant has different names in different countries, as shown in Table 2 (Sharma & Tripathi, 2009).

Madagascar, Asia (including India, Pakistan, and Afghanistan), and tropical and subtropical Africa all have it (Somalia, Egypt, Libya, south Algeria, Morocco, Mauritania, and Senegal). Jordan, India, Iran, Arabia, Southern Asia, and Indochina (Rajamohan *et al.*, 2014; Sharma *et al.*, 2016). The plant has naturally grown on islands in Australia, including those in Indonesia and the Pacific, the Caribbean, and South and Central America (Rajamohan *et al.*, 2014).

Table 2. Vernacular names of the *C. gigantea* in the world.

Names of countries	Common name of *Calotropis gigantea* .
India	In Sanskrit it is called as Arka, Vasuki, Alarka, pratapass. Safed aak, Aak, Alarkh, Madar, Sveta Arka, Akanda, and Bara Akand are all used in Hindi.
Malaysia	In Malaysia, it is called Rembega, Remiges, and kemengu.
English countries	In almost all English countries it is called Giant milkweed, a Crown flower.
Indonesia	In Indonesia it is called as Sidaguri in Javanese, Rubik in Aceh.
Thailand	Paan-theun and Po-theun are commonly used.
Laos	Dok kap, Dok hak and Kok are mostly used.
French	Faux Arbre and Mercure vegetal are common names in French countries.

Calotropis may grow wild up to 900 meters across the nation and is very tolerant to salt and drought (Rani *et al.,* 2019). When dried, the milky latex found in the stem of *C. gigantea* serves as an antinode for the venom (snake poison). Its dried leaves are anti-inflammatory and are used to make cough medication and to cure paralysis (Al-Maskri *et al.,* 2011; Gyawali *et al.,* 2020; Muhammad *et al.,* 2011; Shahzadi *et al.,* 2017). *Calotropis* is regarded as an aromatic herb with therapeutic properties. The latex of the *Calotropis* plant contains cardiac toxins (Shahzadi *et al.,* 2017). The *Calotropis* plant's stems and leaves also contain essential oil, which is discovered through GC-MS (gas chromatography-mass spectrometry) analysis. (Merzaia *et al.,* 2017; Shahzadi *et al.,* 2017; Tiwari *et al.,* 2014). Many parts of the *Calotropis* plant, including the leaves, stem flowers, and root barks, are used in medicine to treat illnesses that are frequently found in people, including fever, arthritis, digestion issues, cough, loose stools, and nausea (Patel *et al.,* 2014).

The aim of the current research was to evaluate the antioxidant and antibacterial activities *of C. gigantea* leaf extract. It was meticulously examined for a variety of therapeutic uses, including the utilization of the flowers for cytotoxic, analgesic, and antibacterial properties (Choudhary *et al.,* 2013; Pathak & Argal, 2007). Plant leaves and other internal components have been employed for their antibacterial, antifungal, and antidiarrheal properties (Chitme *et al.,* 2004; Habib & Karim, 2009; Kumar *et al.,* 2010a). *Calotropis* roots have been utilized for CNS activity, wound healing, cytotoxicity, antibacterial, and antipyretic effects (Alam *et al.,* 2009; Alam *et al.,* 2008; Argal & Pathak, 2006; Chitme *et al.,* 2005; Deshmukh *et al.,* 2009; Namrata *et al.,* 2010; Wang *et al.,* 2008). This plant's latex was employed for its procoagulant, antibacterial, and wound-healing properties (Kumar *et al.,* 2010b; Lodhi *et al.,* 2009; Nalwaya *et al.,* 2009; Rajesh *et al.,* 2005). The stem of the plant was employed as a hepatoprotective agent(Sivapalan *et al.,* 2023).

2. MATERIAL and METHODS

2.1. Extraction of the Plant Material

Calotropis gigantea fully matured leaves of 10 biological replicates and three technical replicates were collected from the botanical garden, Government College University Lahore in May 2022, washed with distilled water and allowed to dry (in shade) for a week. Leaves were ground to fine powder by using a pistil and motor. Extraction was done by dissolving 5 g of leaf powder in 10 mL of various types of solvents like ethanol, methanol, 50% (ethanol and methanol), and n-hexane. 5g of powdered leaves were dissolved in 10 mL of aforementioned solvents and allowed to stand for 36 hrs. Each type of extract was filtered out by using simple filtration method. And then, these solutions were examined for antibacterial and antioxidant activities (Yesmin *et al.,* 2008).

2.2. Antibacterial Activity

Fully characterized bacterial strains *Bacillus subtilis*, *Bacillus muralis*, *E. coli*, *Acetobacter*

rhizospherensis were purchased from the microbiology lab of COMSTS University Islamabad, Abbottabad Campus. Antimicrobial agents are biological and chemical substances that inhibit the development of microorganisms and aid in their eradication. It is necessary to conduct an antimicrobial analysis on the pure separated ingredient from a natural source or the crude extract of the plant to ascertain their effectiveness against various pathogenic organism types. The well-diffusion method was employed to evaluate the anti-bacterial effect of *C. gigantea* leaf extract. Gram-positive (*Bacillus subtilis*, *Bacillus muralis*) and gram-negative (*E. coli*, *Acetobacter rhizospherensis*) bacterial strains were utilized as test organisms (Mandal *et al.*, 2022).

2.3. Well-Diffusion Method

Agar solution was prepared: 1.25g yeast, 2.5g NaCl, 2.5g tryptone, and 3.75g Agar were added in 250 mL of distilled water. Maintained the pH of the solution at 7 by adding a few drops of NaOH. A clear solution was obtained accompanied by stirring and covered with aluminum foil. Petri plates, Agar solution, micro-tips (blue and yellow), inoculating loops, glass spreaders and solvents (distilled water and DMSO) were autoclaved for sterilization. The agar media was spread on petri plates, and the plates were then allowed to dry. On the solidified agar media freshly grown bacterial strains were applied using a glass spreader. Yellow microtips were used to create wells in the agar diffused plates. Then, using a micropipette, a 1 mg/mL solution of the crude extract of *C. gigantea* leaves in DMSO was injected into the wells. For positive control, 1 mg/1mL ampicillin solution in distilled water was utilized. DMSO was used as a negative control. To test the samples' antibacterial activity, all the samples were injected through micropipettes into the wells of the petri plates, covered the plates, and left for 24 hours (Julius *et al.*, 2021).

2.4. Antioxidant Activity

2.4.1. *ABTS assay*

7 mM ABTS and 2.45 mM potassium persulfate solutions were prepared in distilled water. 7.5 mL of ABTS solution was added to 2.5 mL of potassium persulfate solution in a test tube and left for 24 hours in the dark. this radical cation ABTS+ was used to check the antioxidant activity of the sample. The absorbance of stock solution was maintained between (0.709 - 0.693) at 734 nm. 2.5 mL of ABTS solution was added to different test tubes, and then 10 µL of each sample was added to determine the antioxidant activity of *C. gigantea* leaves extract. Test tubes were placed in the dark for 8 minutes. UV/Vis spectroscopy was used to observe(Srivastava *et al.*, 2020).

2.4.2. *DPPH assay*

DPPH solution was prepared using 25 mg/L in methanol, and methanol was used to dilute it until absorbance was maintained at 0.963 (λ_{max} =763nm). After adding 2.5 mL of the stock solution to several test tubes, 10µL of samples were mixed in it. Test tubes were placed in dark for 30 minutes, and the change in absorbance was measured(Sangeetha *et al.*, 2020).

3. FINDINGS

3.1 LC-MS Analysis

3.1.1. *Phytochemical screening of leaves of C. gigantea*

Using a Bruker Dionex Ultimate 3000 quadrupole time-of-flight mass spectrometer, the chemicals in all of the *C. gigantea* samples were identified. Water containing 0.1% formic acid (A) and acetonitrile served as the eluents. A flow rate of 0.4 mL/min was used to complete the gradient programme over the course of 30 minutes. The injection volume for the sample was 2 L. The Bruker Dionex ultimate 3000 qTOF mass spectrometer, which has electrospray ionization in both positive and negative modes, was used to conduct the qualitative analysis. As a nebulizing, collision, and drying gas, nitrogen was utilized.

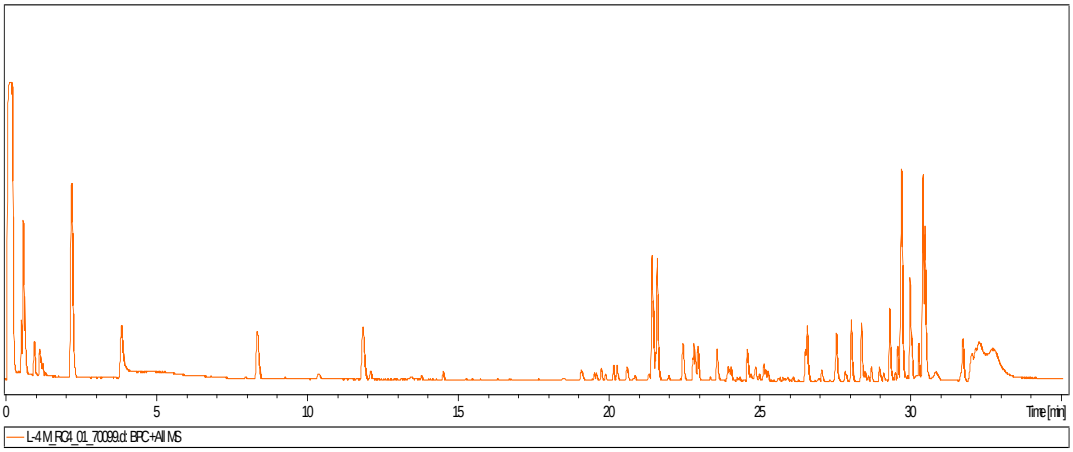
3.1.2. *LC-MS analysis of methanolic extract*

The studies on the active principle of LC-MS analysis of methanol extract of leaves of the plant clearly showed the presence of different compounds. The active principles with their Retention time (RT), Compound names, Molecular formula (MF), and Parent ion that showed the molecular weight of that compound are shown in the Table 3, Figure 2.

Table 3. Different types of compounds and their retention time in MeOH leaves extract.

Sr. No	Retention Time	Compound	Molecular weight
1	2.2	4-Heptylobenzoic acid	237.145
2	3.9	Oxypeucedanin	287.092
3	11.9	2,4,6-Triphenyl-1-hexene	313.194
4	21.4	Hexadecasphinganine	274.274
5	23.0	Phytosphingosine	318.299
6	26.6	Monoethylhexyl phthalic acid	301.140
7	27.5	Acetyl tributyl citrate	425.213
8	28.4	Oleamide	282.279

Figure 2. Graphical representation of different compounds in methanol leaf extract.



3.1.3. *LC-MS analysis of ethanolic extract*

The studies on the active principle of LC-MS analysis of ethanol extract of leaves of the plant clearly showed the presence of different compounds. The active principles with their Retention time (RT), Compound names, Molecular formula (MF), and Parent ion that showed the molecular weight of that compound are shown in Table 4, Figure 3.

Figure 3. Graphical representation of different compounds in ethanol leaves extract.

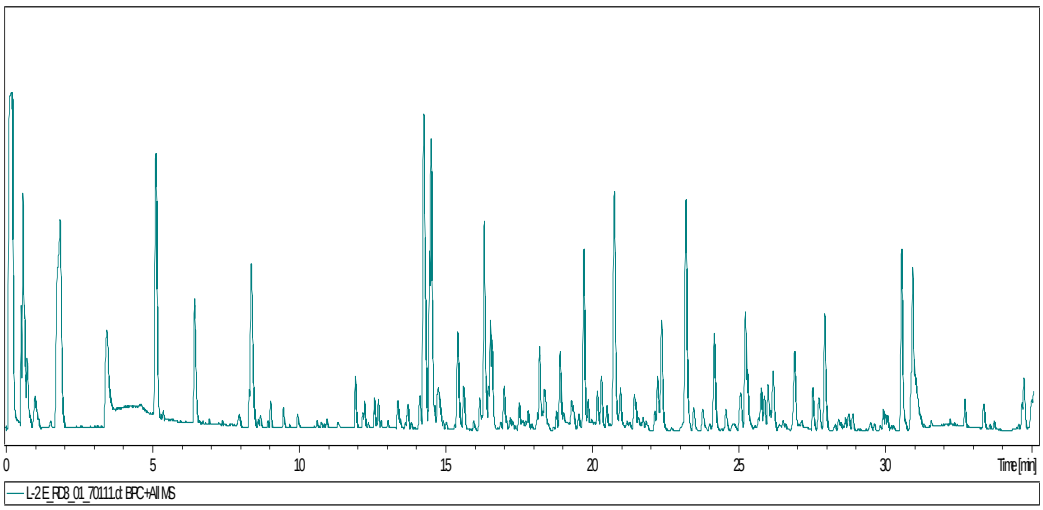


Table 4. Different types of compounds and their retention time in EtOH leaves extract.

Sr. No	Retention Time	Compound	Molecular weight
1	5.2	Ethofumesate	287.092
2	11.9	Calactin	533.273
3	12.6	4,4-Difluoropregn-5ene-3,20-dione	351.212
4	13.4	Stearidonic acid	277.215
5	13.8	6-Sgofaol	277.179
6	14.3	Hexadecasphinganine	274.275
7	17.0	Resolvin E2	357.202
8	18.9	13-cis-Retinoic acid	301.216
9	19.7	All-trans-4-Oxoretinoic acid	315.195
10	20.8	Resolvin E2	357.202
11	23.2	13-cis-Retinoic acid	323.197
12	25.2	Icosapentaemoic acid	303.230
13	25.8	Metholone.	327.228
14	26.9	Arachidonic acid	305.247
15	27.9	Octadecanamide	284.294
16	30.9	Bis(2-ethylhexyl) phthalate.	413.264

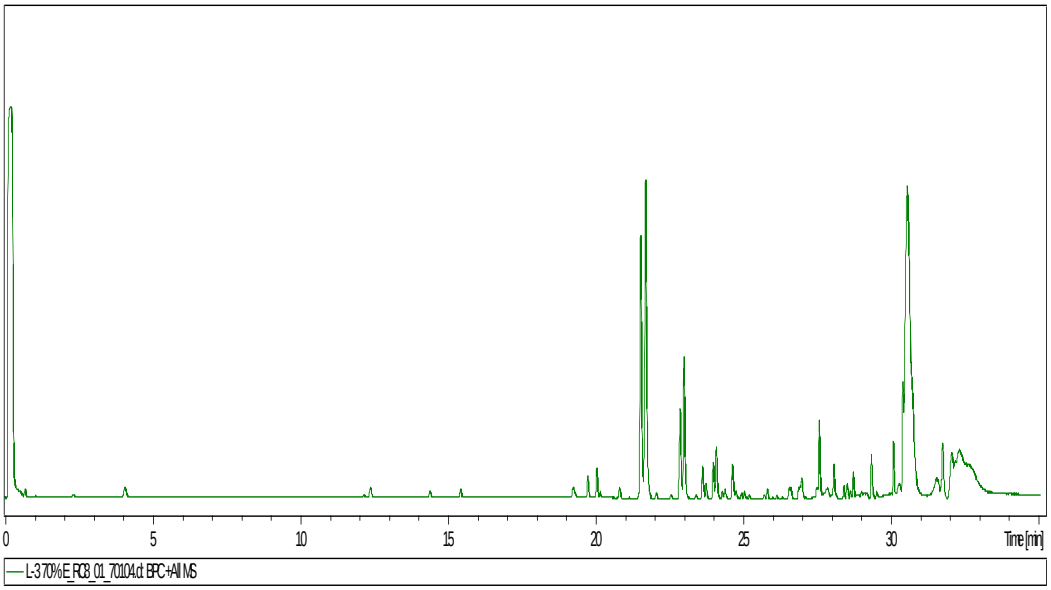
3.1.4. *LC-MS analysis of n-hexane extract*

The studies on the active principle of LC-MS analysis of n-hexane extract of leaves of the plant clearly showed the presence of different compounds. The active principles with their Retention time (RT), Compound names, Molecular formula (MF), and Parent ion that showed the molecular weight of that compound are shown in Table 5, Figure 4.

Table 5. Different types of compounds and their retention time in n-hexane leaf extract.

Sr. No	Retention Time	Compound	Molecular weight
1	21.5	Hexadecasphinganine	274.274
2	22.8	Sphinganine	302.305
3	23.0	Phytosphingosine	318.300
4	27.5	Acetyl tributyl citrate	425.213
5	30.1	Cepharanthine	607.291

Figure 4. Graphical representation of different compounds in n-hexane leaf extract.



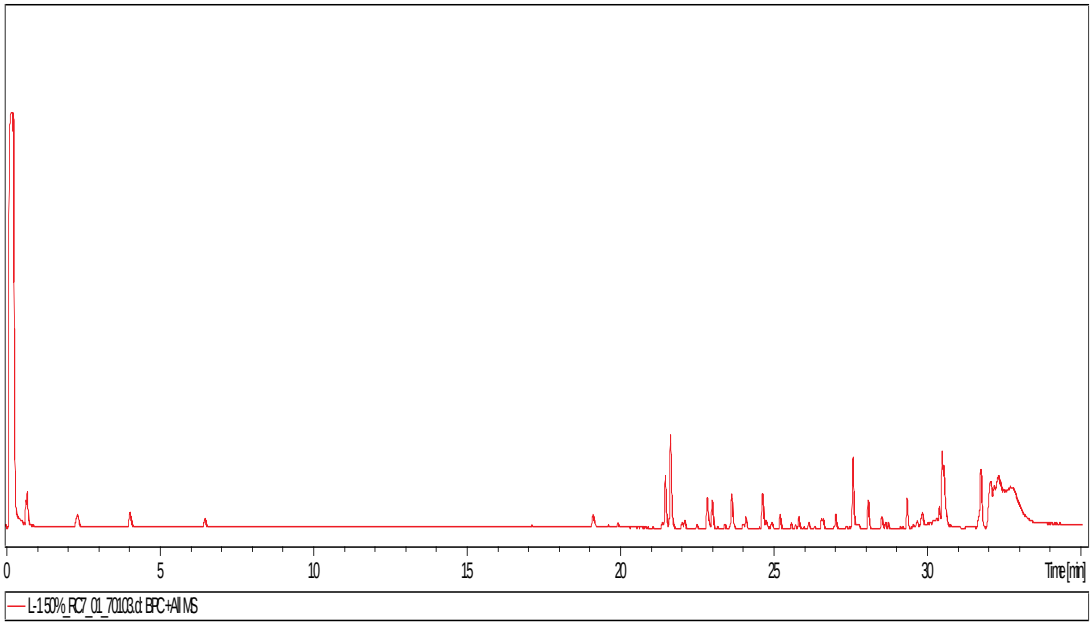
3.1.5. *LC-MS analysis of 50% (Methanol + Ethanol) extract*

The studies on the active principle of LC-MS analysis of 50%(methanol-ethanol) extract of leaves of the plant clearly showed the presence of different compounds. The active principles with their Retention time (RT), Compound names, Molecular formula (MF), and Parent ion that showed the molecular weight of that compound are shown in Table 6, Figure 5.

Table 6. Different compoaunds and their retention time in 50% (MeOH-EtOH) leaf extract.

Sr. No	Retention Time	Compound	Molecular weight
1	25.2	All-trans-4-Oxoretinoic acid	315.194
2	25.8	Prostaglandin J2	357.202
3	27.0	4,5-Leukotriene A4	341.207

Figure 5. Graphical representation of different compounds in 50% (MeOH-EtOH) leaf extract.



3.2. Antibacterial Activity

After 24 hours, the antibacterial activity of the *C. gigantea* leaf extract against the bacterial strains is observed in each well of the agar-diffused medium.

3.2.1. *Zone of inhibition of antibacterial activity*

The zone of inhibition was calculated for each sample, and the extract of *C. gigantea* leaves showed a different zone of inhibition for each type of bacteria that we utilized, which are displayed in the Table 7, Figure 6, Figure 7. Sample representations applied on the petridishes is shown in Table 8.

Table 7. Petri plates various signs.

Signs	Extracts
1	Ethanol leaves extract
2	Methanol leaves extract
3	n-hexane leaves extract
(+)	positive control
(-)	negative control

Table 8. Calculated zone of inhibition against various bacterial strains.

Bacteria	Sample	Zone of inhibition
Bacillus-muralis	Ethanol leaves extract	4.75 mm
Bacillus-muralis	Methanol leaves extract	3.5 mm
Bacillus-muralis	n-hexane leaves extract	5.75 mm
Bacillus-muralis	+ve control	6.5 mm
Bacillus-muralis	-ve control	3.5 mm
Acetobacter-rhizospheres	Ethanol leaves extract	7.75mm
Acetobacter-rhizospheres	Methanol leaves extract	4.25 mm
Acetobacter-rhizospheres	n-hexane leaves extract	6 mm
Acetobacter-rhizospheres	+ve control	6.5mm
Acetobacter-rhizospheres	-ve control	2 mm
E. coli	Ethanol leaves extract	9.5mm
E. coli	Methanol leaves extract	4mm
E. coli	n-hexane leaves extract	8mm
E. coli	+ve control	1.5mm
E. coli	-ve control	4.25mm
Bacillus-subtilis	Ethanol leaves extract	3.75 mm
Bacillus-subtilis	Methanol leaves extract	8.5 mm
Bacillus-subtilis	n-hexane leaves extract	2 mm
Bacillus-subtilis	+ve control	12 mm
Bacillus-subtilis	-ve control	No activity

Figure 6. Zone of inhibition shown against *B. subtilis* and *A. rhizospheres.*

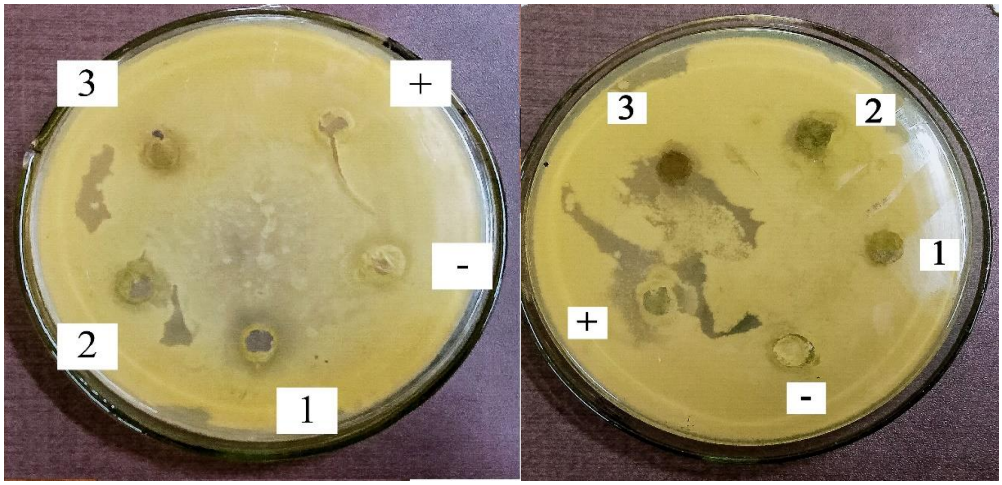
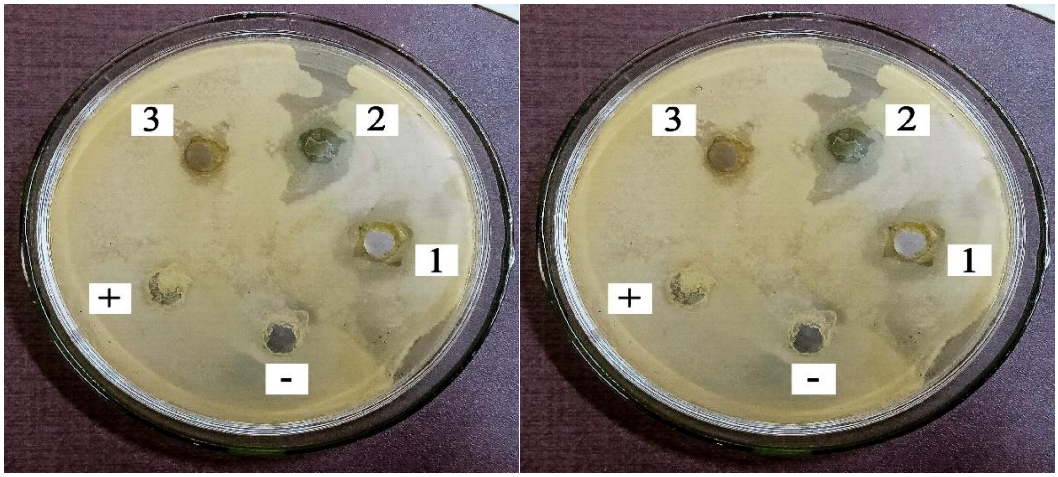


Figure 7. Zone of inhibition shown against *E. coli* and *B. muralis.*



3.3. Antioxidant Activity

3.3.1. *ABTS+ assay*

Reduction in the absorbance was observed and then % inhibition of each sample was calculated by using the following formula, all the values of each sample are written in Table 9.

$$\% \text{ Inhibition (734nm)} = (1-A_s/A^0) \times 100$$

A_s is the absorbance of the sample and A^0 is the absorbance of ABTS+. Here $\mathbf{A^0} = 0.709$

Table 9. % Inhibition of ABTS and absorbance of the sample are shown.

Sample	The absorbance of the sample (As)	% Inhibition of ABTS
n-hexane leaves extract	0.669	5.64
Ethanol leaves extract	0.640	9.73
Methanol leaves extract	0.654	7.75

The addition of the crude leaf extract caused the absorbance to decrease, demonstrating that it has antioxidant properties and the potential to stop oxidative damage brought on by free radical systems. MeOH, EtOH, and N-hexane leaf extract samples were compared in a comparative analysis. Better antioxidant activity was demonstrated by ethanolic leaf extract. As seen below, each extract exhibits distinct antioxidant activity when compared to one another.

Ethanol leaves extract > Methanol leaves extract > N- hexane leaves extract

3.3.2. *DPPH assay*

The change in the absorbance was measured and then the % of DPPH radical remaining was calculated using the following formula and all the data is written in the Table 10.

$$\% \text{ DPPH radical remaining} = A_S/A_o \times 100$$

A_s is the absorbance of the sample, A^0 is the absorbance of DPPH. Here $A_o = 0.963$

Table 10. %DPPH radical remaining and absorbance are shown.

Sample	The absorbance of the sample (As)	% DPPH radical remaining
n-hexane leaves extract	0.960	97.19
Ethanol leaves extract	0.959	99.68
Methanol leaves extract	0.936	99.58

When *C. gigantea* leaves extract was added to test tubes containing DPPH solution, the absorbance was found to decrease, indicating the extract's antioxidant properties. Comparative analysis between different extracts revealed that methanol leaf extract of the leaves had higher antioxidant properties. The antioxidant activity of each extract varies when compared to one another, as can be seen below.

Methanol leaves extract > Ethanol leaves extract > n-hexane leaves extract

4. DISCUSSION and CONCLUSION

The LC-MS assessment of the different solvent leaf extracts revealed substantial variation in chemical composition. The ethanol leaf extract exhibited the maximum number of compounds (14), while the 50% ethanol-methanol combination had the lowest number. The difference in the composition of compounds may directly impact the biological activities of the extracts, emphasizing the need to choose the appropriate solvent in phytochemical research.

The methanol leaf extract had the highest level of antimicrobial activity, as shown by the largest inhibition zone (8.5 mm) against *Bacillus subtilis*. This suggests that methanol is more efficient

in extracting antibacterial chemicals that specifically target this bacterium. In contrast, the n-hexane extract exhibited the lowest inhibitory zone measuring 2 mm, suggesting a lesser level of effectiveness. This discovery aligns with prior research that has shown methanol's superior efficacy as a solvent for extracting polar molecules with antibacterial characteristics (Altemimi *et al.*, 2017).

The ethanol leaf extract exhibited the greatest inhibition (9.5 mm) against *Escherichia coli*, whilst methanol showed the lowest inhibition (4 mm). This result highlights the varying effectiveness of solvents in extracting chemicals that have activity against Gram-negative bacteria such as *E. coli.* According to (Klūga *et al.*, 2021) , ethanol, due to its moderate polarity, has the ability to extract a wider variety of bioactive chemicals compared to methanol or n-hexane.

The investigation further revealed that the ethanol extract exhibited the most significant inhibitory zone against *Acetobacter rhizopherensis*, measuring 7.75 mm. Conversely, the n-hexane extract had the smallest zone, measuring 6 mm. This indicates that the chemicals that are capable of effectively combating *A. rhizopherensis* have a higher solubility in ethanol compared to n-hexane, which is a less polar solvent. This is consistent with the widely accepted notion that polar solvents are more efficient in extracting antimicrobial drugs (Borah *et al.*, 2020).

N-hexane exhibited the greatest inhibition (5.75 mm) for *Bacillus muralis*, whilst methanol had the lowest inhibition (3.5 mm). This suggests that the bacteria in question is more susceptible to non-polar chemicals, which have a higher affinity for extraction by n-hexane. This result emphasizes the intricacy of microbial resistance and the need for a wide variety of solvents in antimicrobial research (Osungunna, 2021).

The extracts showed different levels of efficacy in terms of antioxidant activity, as determined by the ABTS and DPPH tests. According to the ABTS test, the ethanol extract exhibited the maximum level of inhibitory activity at 9.73%, while the n-hexane extract showed the lowest level at 5.64%. According to the DPPH test, the methanol extract exhibited the maximum level of residual radical activity (99.68%), while the n-hexane extract had the lowest level (97.19%). The findings emphasize that various assays might provide distinct perspectives on antioxidant capabilities, and suggest that methanol may be more efficient in extracting molecules with potent abilities to scavenge free radicals (Sadowska-Bartosz & Bartosz, 2022).

It is concluded that ethanolic leaves' extract of *C. gigantea* had more phytochemical compounds as compared to others. Anti-bacterial and anti-oxidant activities showed promising results. Ethanolic extract revealed significant outcome against gram-negative bacteria. Methanolic and n-hexane leaf extract divulged convincing antibacterial activities against *Bacillus subtilis* and *Bacillus muralis* respectively. DPPH and ABTS assays demonstrated that the antioxidant activity of ethanolic and methanolic leaves extract is prominent. It can be employed in green synthesis in the future to boost medical and pharmaceutical applications.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Hazqail Umar Khan: Main Concept, Writing -original draft, Investigation, **Muhammad Shahid Cholistani**: Resources, Visualization, Software, Formal Analysis, **Eliza Iqbal**: Visualization, Software, Formal Analysis, **Kashif Kareem**: Validation, Writing draft, Formatting, Grammar and Structure. **Hafiz Muhammad Kashif Zahoor**: Methodology, Formal Analysis, **Muhammad Farhan**: Investigation and Visualization, **Hafiz Shozab Ahmad Khan**: Visualization, **Muhammad Pervaiz Bhatti**: Supervision, and Validation. **Jallat Khan**: Supervision, Formal Analysis

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Research Article

Some biological activities of *Thalictrum minus* (Ranunculaceae)

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Abstract: Plants are vital natural resources that are used in a wide range of applications. Throughout history, these tools have proven to be valuable assets for individuals. We conducted a study to assess the biological activity of the aerial portions of *Thalictrum minus* L. In this particular situation, the plant's ethanol and methanol extracts were obtained using Soxhlet equipment. The Rel Assay kits were used to measure the total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI). The antiproliferative effectiveness against the A549 lung cancer cell line was assessed using the MTT test. The anticholinesterase activity was assessed by measuring the activities of acetylcholinesterase and butyrylcholinesterase. The plant's ethanol and methanol extracts were analyzed, and their TAS values were determined to be 4.371±0.083 and 4.027±0.081, respectively. The TOS values were determined to be 11.816±0.121 and 13.580±0.176, respectively, whereas the OSI values were determined to be 0.271±0.007 and 0.337±0.009. The antiproliferative activity of the methanol extract of the plant was found to be greater than that of the ethanol extract. The ethanol extract had an anti-AChE value of 58.90±1.41, while the methanol extract had an anti-AChE value of 65.11±1.01. Similarly, the ethanol extract had an anti-BChE value of 72.25±0.79, while the methanol extract had an anti-BChE value of 85.79±0.68. Consequently, it was established that the plant has antioxidant, anticancer, and anticholinesterase properties.

1. INTRODUCTION

Throughout human history, individuals have utilized a variety of natural substances for various objectives. Individuals have employed natural substances for many functions, including generating warmth, constructing dwellings, procuring sustenance, manufacturing tools, and combating illnesses (Eraslan *et al.*, 2021; Mohammed *et al.*, 2023). Plants hold a significant

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position among natural commodities. Plants play a crucial role in the human diet because of their nutritional and aromatic characteristics (El-Chaghaby *et al.,* 2024). Plants are utilized by individuals not only for their nutritional attributes but also for their role in illness prevention and treatment. Several studies have shown that plants have many biological activities, such as antioxidant, antiproliferative, antiaging, anti-inflammatory, antimicrobial, anticancer, antitumor, DNA protective, and hepatoprotective properties (Selvi *et al.,* 2022; Aladı *et al.,* 2023; Kalkan *et al.,* 2023; El-Chaghaby *et al.,* 2024; Özcandır *et al.,* 2024; Seğmenoğlu and Sevindik, 2024; Yagi *et al.,* 2024; Zengin *et al.,* 2024). We conducted a study to assess the biological activities of *Thalictrum minus* L.

T. minus (Ranunculaceae) is commonly referred to as lesser meadow-rue. This plant species is widely distributed in various locations of the world and is known for its cosmopolitan nature. It thrives in several habitats including, gravel areas, coastal rocks, calcareous meadows, and rocky valleys. The plant reaches a maximum height of 30 cm, with upright stems and 3-4 sets of triple, hairy, and highly split leaves measuring 1 cm each. It is commonly employed for the management of diarrhea, elevated body temperature, and headaches (Popović *et al.,* 1992; Mushtaq *et al.,* 2016; Singh *et al.,* 2023).

2. MATERIAL and METHODS

We collected specimens from Kahramanmaraş, a city in Türkiye. The plant's aerial components were utilized for the process of extraction. The identification of the plant was made using Flora of Turkey and the East Aegean Islands, Volume 1 (Davis, 1965). The samples were desiccated in a controlled laboratory setting, shielded from direct sunlight. Weighed 30 grams of dry samples. Subsequently, it was extracted using 250 mL of ethanol in a Soxhlet apparatus, keeping at a temperature of 50 °C for a duration of 6 hours. The aforementioned procedure was replicated for the methanol extract. The solvents from the extracted substances were removed by evaporating them using a Buchi R100 Rotary Evaporator at a temperature of 40 °C. The crude extracts were refrigerated at +4 °C until the experiment was conducted.

2.1. Total Antioxidant and Oxidant Tests

The extracts' total antioxidant levels were quantified using the TAS kit. The TOS kit was used to measure the overall amounts of oxidants. Trolox served as a calibrator in the TAS test. The values were denoted in terms of molar concentration (mmol/L). Hydrogen peroxide served as a calibrator in the TOS test. The values were denoted in micromoles per liter (μmol/L). The kit manufacturer's methodology (Erel, 2004; Erel, 2005) guided the conduct of the tests. The OSI value was determined by comparing the unit of the TOS value with the unit of the TAS value, scaling the TOS value to match the TAS value, and then calculating the percentage (Sevindik, 2019).

2.2. Anticholinesterase Activity Tests

The anticholinesterase activity of the extracts was assessed using the Ellman method, as described by Ellman *et al.* in 1961. The extracts were evaluated for their ability to inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) within the context of anticholinesterase activity. Galantamine served as the study's benchmark. The extracts were diluted to generate stock solutions with concentrations ranging from 200 to 3.125 μg/mL. Next, we introduced 130 microliters of a 0.1 molar phosphate buffer with a pH of 8, 10 microliters of a stock solution, and 20 microliters of either AChE or BChE enzyme solution into the microplate. The sample then underwent a 10-minute incubation period at a temperature of 25 °C. Next, 20 μL of DTNB (5.5''-dithiobis-(2-nitrobenzoic acid)) solution and 20 μL of either acetylcholine iodide or butyrylcholine iodide substrate were added. The measurement was then taken at a wavelength of 412 nm. The extracts were analyzed in three repetitions. The IC50 values, representing the percentage inhibition of the samples, were given in units of micrograms per milliliter (μg/mL).

2.3. Antiproliferative Activity Test

The antiproliferative properties of plant extracts were assessed against the A549 lung cancer cell line using the MTT test. Stock solutions were generated by diluting the extracts to concentrations of 25, 50, 100, and 200 µg/mL. Subsequently, the cells reached a state of confluence, with 70-80% coverage. Subsequently, a 3.0 mL solution of Trypsin-EDTA (Sigma-Aldrich, MO, USA) was employed for the procedure of separation. Subsequently, the specimens were placed on a plate and subjected to incubation for a duration of 24 hours. Subsequently, stock solutions were introduced. Subsequently, the specimens were subjected to a 24-hour incubation period. Next, the liquid portion of the samples was mixed with the culture medium and substituted with a solution containing 1 mg/mL MTT. Subsequently, the samples were subjected to incubation at a temperature of 37 °C until the formation of a purple precipitate occurred. At this point, dimethyl sulfoxide (DMSO) from Sigma-Aldrich, MO, USA was introduced to the MTT solution. Ultimately, the plates were analyzed at a wavelength of 570 nm using an Epoch spectrophotometer (BioTek Instruments, Winooska, VT) (Bal *et al.*, 2017).

3. RESULTS and DISCUSSION

3.1. Antioxidant and Oxidant Status

Free radicals are reactive molecules formed during normal metabolic processes. Although low concentrations of these substances are not toxic, their amounts can lead to significant cellular damage as they increase (Krupodorova and Sevindik, 2020). The antioxidant defense system operates to mitigate the impact of free radicals. Occasionally, the antioxidant defense system may be inadequate in its ability to control the activity of free radicals (Bal *et al.*, 2019; Akkaya *et al.*, 2024). In this scenario, oxidative stress is present. Oxidative stress can lead to the development of various major diseases in humans, including diabetes, multiple sclerosis, Alzheimer's, Parkinson's, cardiological disorders, and cancer. Supplemental antioxidants can mitigate the impact of oxidative stress (Sevindik *et al.*, 2017; Korkmaz *et al.*, 2018; Baba *et al.*, 2020; Sudirman *et al.*, 2024). Plants possess significant natural antioxidant properties, making them highly valuable. In our work, we assessed the antioxidant capacity of *T. minus*. The Table 1 displays the TAS, TOS, and OSI values.

Table 1. TAS, TOS and OSI values of *Thalictrum minus.*

Extract	TAS (mmol/L)	TOS (µmol/L)	OSI (TOS/(TASx10))
Ethanol	4.371±0.083	11.816±0.121	0.271±0.007
Methanol	4.027±0.081	13.580±0.176	0.337±0.009

Values are presented as mean ± SD

Previous studies have documented the antioxidant capacity of *T. minus* using various techniques (Karyagina et al., 2011; Malik et al., 2017; Mishra et al., 2021). In our study, the TAS, TOS, and OSI values of *T. minus* were determined for the first time. The literature contains recorded TAS, TOS, and OSI values for several plant species. The TAS values of *Ferulago platycarpa, Helianthemum salicifolium, Silybum marianum, Asparagus acutifolius, Galium aparine, Glycyrrhiza glabra*, and *Alcea kurdica* were reported as 5.688, 9.490, 5.767, 6.238, 5.147, 8.770, and 3.298 mmol/L, respectively, in these studies. Furthermore, these studies recorded the TOS values as 15.552, 14.839, 12.144, 13.892, 18.679, 14.590, and 8.312 mmol/L, respectively. The OSI values were recorded as 0.273, 0.157, 0.211, 0.221, 0.346, 0.167, and 0.252, respectively (Mohammed *et al.*, 2019; Mohammed *et al.*, 2020; Korkmaz *et al.*, 2021; Mohammed *et al.*, 2021a; Mohammed *et al.*, 2021b; Mohammed *et al.*, 2021c; Mohammed *et al.*, 2022). *T. minus* had lower TAS values in our study than *F. platycarpa, H. salicifolium, S. marianum, A. acutifolius, G. aparine*, and *G. glabra*. This was true for both ethanol and methanol extracts of the plant. However, the TAS values of *T. minus* extracts were higher than those of *A. kurdica*. The TAS value serves as a comprehensive measure of the collective antioxidant molecules generated in natural goods (Ahmad *et al.*, 2023). In our study, we

observed that the ethanol extract of *T. minus* exhibited a greater TAS value. Furthermore, our study established the plant's antioxidant capabilities. The total oxidant status (TOS) is a quantitative measure of the collective amount of oxidant chemicals generated in natural products (Ahmad *et al.*, 2023). Our study assessed the total oxidant status (TOS) of the ethanol extract of *T. minus* and found it to be lower than that of *F. platycarpa, H. salicifolium, S. marianum, A. acutifolius, G. aparine*, and *G. glabra*. However, it was greater than *A. kurdica*'s TOS. The total oxidant status of the methanol extract was found to be lower than that of *F. platycarpa, H. salicifolium, A. acutifolius, G. aparine*, and *G. glabra*, while it was higher than that of *S. marianum* and *A. kurdica*. Upon analysis, it was shown that the OSI value of the ethanol extract of *T. minus* was lower than that of *F. platycarpa* and *G. aparine* but greater than that of *H. salicifolium, S. marianum, A. acutifolius, G. glabra*, and *A. kurdica*. Furthermore, the OSI value of the methanol extract was found to be greater than that of *F. platycarpa, H. salicifolium, S. marianum, A. acutifolius, G. glabra*, and *A. kurdica*, but lower than *G. aparine*. The OSI value quantifies the degree of reduction in oxidant chemicals present in natural goods due to the presence of endogenous antioxidant molecules (Ahmad *et al.*, 2023). The investigation revealed that *T. minus*, exhibited the capability to inhibit oxidant chemicals. Within this particular framework, it was determined that the plant possesses antioxidant capabilities.

3.2. Anticholinesterase Activity

Alzheimer's disease is a prevalent neurodegenerative illness in contemporary times. It is particularly prevalent among individuals aged 60 and above. Experts predict that the next few years will see the identification of over 80 million cases globally. One of the therapy techniques for this disease is the inhibition of cholinesterase enzymes (Sevindik *et al.*, 2024). In our investigation, we evaluated the anticholinesterase properties of *T. minus* ethanol and methanol extracts. Table 2 displays the results.

Table 2. Anti-AChE and anti-BChE values of *Thalictrum minus.*

Extract	AChE	BChE
Ethanol	58.90±1.41	72.25±0.79
Methanol	65.11±1.01	85.79±0.68
Galantamine	9.84±0.15	16.39±0.20

Values are presented as mean ± SD

The existing literature lacks any research on the acetylcholinesterase and butyrylcholinesterase activities of *T. minus*. We conducted this study for the first time. According to the literature, some plant species have anticholinesterase activity, as documented by Adewusi *et al.* (2010) and Mohammed *et al.* (2024). Our investigation found that the ethanol extract of *T. minus* exhibited higher levels of both anti-AChE and anti-BChE activities compared to the methanol extract. Furthermore, we used galantamine as a benchmark and found that the extracts exhibited reduced effectiveness. The existence of enzymes that contribute to the development of illnesses, as well as the inhibition of these enzymes, are critical for therapy approaches (Sevindik et al., 2024). Our investigation suggests that *T. minus* possesses both acetylcholinesterase and butyrylcholinesterase activity, making it a potential natural source in this setting.

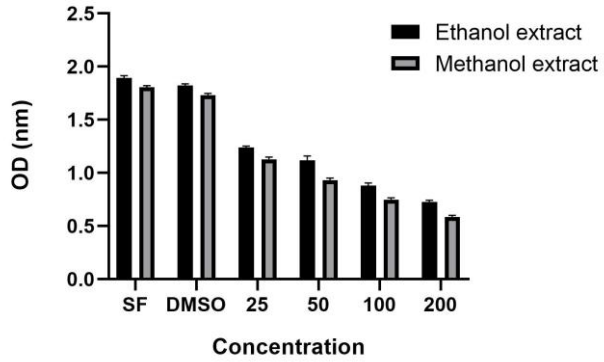
3.3. Antiproliferative Activity

Currently, a wide array of cancer kinds is observed. Novel therapeutic approaches have been devised to combat these specific forms of cancer. Currently, researchers have developed a multitude of therapy modalities to combat various forms of cancer. Furthermore, the utilization of supplements may play a crucial role in achieving victory in the battle against cancer (Karaltı et al., 2021; Esparza et al., 2024). Plants serve as valuable resources for other natural products.

We conducted a study to assess *T. minus*'s inhibitory effect on the growth of A549 lung cancer cells. Figure 1 displays the results.

The literature does not contain any reports on the antiproliferative activity of T. *minus*. According to a paper by Li *et al.* (2016), the species *T. foliolosum* has been found to have cytotoxic effects on various cancer cell lines, including MCF-7 (human breast cancer), PC-3 (human prostate cancer), HL-60 (human leukemia), and U937 (pro-monocytic model).

Figure 1. Antiproliferative activity of *Thalictrum minus*



We conducted a study to examine the impact of ethanol and methanol extracts from *T. minus* on A549 lung cancer cells. The study concluded that the methanol extract had a greater impact than the ethanol extract. Furthermore, it was established that the extracts displayed potent cytotoxic properties that were directly proportional to the concentration rise. Consequently, our investigation determined that *T. minus* may possess natural anticancer properties.

4. CONCLUSION

This study determined the antioxidant, anticholinesterase, and antiproliferative activities of ethanol and methanol extracts of *T. minus*. The obtained results indicated the plant's potential as a natural antioxidant agent. Additionally, the plant showed potential for use in pharmacological designs to combat Alzheimer's disease. The plant also demonstrated strong cytotoxic effects on A549 lung cancer cells. Consequently, the biological activities of *T. minus* demonstrated its effectiveness.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Mustafa Sevindik: Fundings, Conception, Materials, Data collection and processing, Analysis and Interpretation. **Oguzhan Koçer**: Fundings, Analysis and Interpretation, Supervision, and Writing. **Nuh Korkmaz**: Fundings, Design, Analysis and Interpretation and Literature review. **Mehmet Ali Yüzbaşıoğlu**: Statistics and Design. **Imran Uysal**: Fundings, Analysis and Literature review.

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Research Article

Histopathological evaluation of the effect of microspheres with different natural bioactive components (*Ganoderma lucidum* and *Inula graveolens*) on osteoblastic activity in rats with experimental bone wounds

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Abstract: This study aimed to analyse the effects of microspheres containing *Ganoderma lucidum* and *Inula graveolens* on bone healing in the alveolar socket after tooth extraction in Wistar rats. In this study, chitosan-coated and uncoated hydrogel microspheres were produced using sodium alginate-gelatine by dispersed phase gelling and crosslinking method in microsphere (MS) production. *G. lucidum* (GL) and *I. graveolens* (IG) extracts were entrapped in these microspheres. 126 healthy female rats were randomly divided into 7 different groups. The groups were named according to the microsphere placed in the alveolar socket after extraction. The effects of these microspheres on the healing of the alveolar bone in the groups were evaluated on the 7th day, the 14th day and the 28th day. Immunohistochemical analysis was used to assess bone healing. A statistically significant difference was observed between the negative control group and MS + Chitosan + IG group and between the MS group and MS + Chitosan + IG group in terms of bone formation percentages on the 28th day ($p<0.05$). The results showed that *G. lucidum* and *I. graveolens* in combination with chitosan can enhance the bone healing of the alveolar socket after extraction.

1. INTRODUCTION

Bone tissue, the main structure of the skeletal system, is a specialised type of connective tissue with a calcified extracellular matrix containing characteristic cells (Gartner *et al.*, 2011). This tissue, which consists of structural matrix proteins including type I collagen, bone sialoprotein, osteocalcin, osteonectin, osteopontin, proteoglycans, growth factors and serum proteins, is a dynamic structure with regeneration capacity in which formation and resorption occurs (Lindhe *et al.*, 2009; Gartner *et al.*, 2011; Hollý *et al.*, 2021). There are four types of cells in bone which include osteoblasts, osteocytes, osteoclasts and osteoprogenitor cells (Florencio-Silva *et al.*, 2015). Osteoblasts secrete proteins including type I collagen, osteocalcin, osteonectin and

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signalling proteins including platelet-derived growth factor (PDGF), bone morphogenetic protein (BMP), transforming growth factor-β (TGF-β) which play an important role in bone regeneration (Yoshiko *et al.,* 2007; Kim *et al*., 2020). These proteins play a role in bone regeneration that may occur in the event of alveolar bone injury (Davison *et al*., 2016; Majidinia *et al*., 2018).

In the alveolar socket, which undergoes a series of tissue healing stages after tooth extraction, a gateway is initially formed that facilitates the spread of bacteria. As a result of this threat, a defense line is formed by immune cells. Due to the surgical procedure, it starts to fill with blood containing cells from the severed vessels (Cohen & Cohen-Lévy, 2014). These cells together with platelets form a blood clot covering the socket in the first 24 hours. The inflammatory reaction stimulates the aggregation of cells to form granulation tissue. In the first 72 hours after extraction, the clot starts to disintegrate as granulation tissue starts to leak into the clot from the base and periphery of the extraction socket (Darby *et al*., 2009). An immature and provisional connective tissue matrix is then formed in the socket. After this stage, woven bone and lamellar bone formation occurs (Irinakis, 2006; Shah *et al*., 2019; Min *et al*., 2020; Miranda *et al*., 2020). Bone wound healing process by creating an experimental wound area after tooth extraction has been analysed in previous studies (Okamoto & Russo, 1973; Carvalho *et al*., 1997). This healing process is seen in three histological stages: the exudative stage with clot formation, the proliferative stage with connective tissue formation and the reparative stage including ossification (Rodrigues *et al*., 2016; Hassumi *et al*., 2018). The healing process of the alveolar bone wound area in rats is completed in 28 days (Carvalho *et al*., 1997).

Different materials and treatment methods such as bone grafts (autogenous, allogenic, xenogeneic, and alloplastic bone grafts), demineralised dentin matrix, synthetic materials, platelet-rich plasma application and plant extract therapy are used to increase the regeneration of bone tissue in bone defects and formed wound areas (Troiano *et al*., 2018; Santos *et al*., 2019; Grawish *et al*., 2022; Karayürek *et al*., 2019; Shi *et al*., 2022; Wang *et al*., 2021). *Ganoderma lucidum* Karst (1881) (Ganodermataceae) and *Inula graveolens* (L.) Desf. (Asteraceae) are among the fungi and plants that can be used for regeneration of bone wound site. *G. lucidum* is a fungus species that contains glycoproteins, polysaccharides, triterpenoids, steroids, alkaloids, benzopyran derivatives, benzoic acid derivatives and some minerals such as potassium, calcium, phosphorus, magnesium, selenium, iron, and zinc (Benzie & Wachtel-Galor, 2011; Baby *et al*., 2015; Sohretoglu & Huang, 2018). Due to its anti-inflammatory, antimicrobial, antioxidant and immunostimulant effects, *G. lucidum* has been used in the treatment of diseases such as cancer, immune system disorders, neurodegenerative diseases, hepatitis, hypertension, chronic bronchitis, bronchial asthma (Ma *et al*., 2015; Cör *et al*., 2018; Laçin *et al*., 2019). Studies in rats have reported that *G. lucidum* prevents the loss of bone density and is effective in the treatment of bone defects (Miyamoto *et al.*, 2009; Laçin *et al*., 2019). *I. graveolens* is an erect plant from the Asteraceae family with a sticky texture and a strong odour (Mitic *et al*., 2016). Essential oil and extracts of *I. graveolens* contain polyphenol, tannin, flavonoid, oil, steroidal triterpenoids, sesquiterpene and anthraquinone. These have been reported to give *I. graveolens* antimicrobial, antioxidant, antiproliferative, antipyretic, analgesic, and anti-inflammatory activity (Sevindik & Paksoy, 2017; Al-Snafi, 2018; Koc *et al*., 2021).

Controlled drug release systems are systems that can maintain the active substance regionally or systematically at a predetermined therapeutic rate in the target area for an appropriate period (Tüylek, 2019). Microspheres are now widely used in controlled drug delivery systems (Tüylek, 2017). Microspheres are monolithic microcarriers that carry the active substance in the form of particles at molecular level, have different physicochemical properties and have a diameter size ranging from a few microns to millimetres (Singh *et al*., 2013; Singh *et al.,* 2014). Polysaccharides and natural polymers such as gelatine, chitosan and alginate can be used for production of microspheres. These materials are preferred due to their biocompatibility, biodegradability, and high loading efficiency (Menon *et al*., 2014; Alsmadi et al., 2020). Studies

have shown that mixtures of chitosan-alginate-gelatine polymers give favourable results in controlled release drug systems (Kuo & Wang, 2013; Jia *et al*., 2015; Afzal *et al*., 2018). Chitosan has also been reported to contribute to bone regeneration in drug delivery systems (Kim *et al.*, 2016; Yadav *et al*., 2021).

In this study, chitosan-coated and uncoated polymers were prepared using sodium alginate and gelatine by the dispersed phase gelling and cross-linking method in microsphere (MS) production, and pharmaceutically active extracts of *G. lucidum* and *I. graveolens* were entrapped in these microspheres. The effects of these microspheres on alveolar bone healing after tooth extraction in rats were evaluated on days 7, 14 and 28. Bone wound healing was evaluated by objective criteria using histopathological and immunohistochemical analysis. The study was designed to evaluate the effect of the bioactive components used in regenerating alveolar bone following extraction.

2. MATERIAL and METHODS

2. 1. Preparation of the Bioactive Agent Extract

Dried *G. lucidum* mushrooms were first cut into small pieces and then reduced in size using a laboratory type shredder. The crushed mushrooms were placed in a flask containing ethanol and the first stage extraction was carried out for 24 hours at room temperature in an incubator shaker (24^{o}C, 125 rpm). The mixture was then filtered, and ethanol solution was obtained. The same extraction process was repeated twice more with the solid part (fungus). The ethanol solution containing the extract obtained from the different extraction steps were then combined. The resulting total solution was placed in a rotary evaporator and the ethanol was removed for 72 hours at 40^{o}C. The extract was then transferred to a beaker and subjected to lyophilisation for 48 h. Extract was stored at +4°C until use. The same procedures were followed for *I. graveolens*.

2.2. Preparation of Microspheres

3% solution of sodium alginate (Sigma Aldrich, medium viscosity) and 1% solution of gelatine (Huaxuan, 80-120 bloom) were prepared, mixed and homogenised. For the preparation of loaded spheres, *I. graveolens* and *G. lucidum* extracts were added to the homogenised polymer solution at a rate of 1:20 (w/v). Extracts were homogeneously distributed in the solution by using an ultrasonic sonicator. For the preparation of the unloaded spheres, this step was omitted. After adjusting the pH of the mixture to 7.0, mixture was drawn into the syringe pump injector and dropped into the 0.15 M $CaCl_2$ solution at a speed of 0.1 mL and height of 10 cm under stirring to form microspheres. To produce spheres coated with chitosan, 5 ml of the chitosan solution was added to the $CaCl_2$ solution in this step. This step has been skipped in the case of non-chitosan coated sphere production. Stirring was continued for a further 3 minutes. The spheres were then filtered and the $CaCl_2$ solution removed. They were then washed three times with distilled water, transferred to Falcom tubes and lyophilised at -80°C for 48 hours (Belgin *et al*., 2022)

Following the above protocol, a total of six types of microspheres were prepared: non-chitosan coated microspheres (MS), chitosan coated microspheres (MS + Chitosan), non-chitosan coated microspheres containing *G. lucidum* (MS + GL), chitosan coated microspheres containing *G. lucidum* (MS + Chitosan + GL), non-chitosan coated microspheres containing *I. graveolens* (MS + IG) and chitosan coated microspheres containing *I. graveolens* (MS +Chitosan + IG).

2.3. Experimental Animal Study

2.3.1. *Rat model*

Ethical approval for this study was obtained from Muğla Sıtkı Koçman University Animal Experimentation and Research Centre, Local Ethics Committee for Animal Experimentation (MUDEM-HAYDEK), decision number 19/21. The manuscript was prepared according to ARRIVE guidelines. In the power analysis of the study, at a 95% confidence level (α=0.05),

with 95% power, the minimum sample size was obtained as N=126 for 21 groups and n=6 observations. In this study, 126 female Wistar rats aged 2-3 months were used. The rats were obtained from the Animal Experimentation and Research Centre of Muğla Sıtkı Koçman University. The experiments were performed at this centre. During the study period, all animals were allowed to acclimate to their environment one week before the start of the study. All rats were housed in plastic cages with adequate food and water, a temperature of 19-23°C, humidity of 30-70%, and a 12-hour day/night cycle.

2.3.2. *Designing the experimental model*

3 mg/kg of Xylazine and 90 mg/kg of Ketamine HCl, was used intraperitoneally to anaesthetise the rats. Experimental wounds were created in the alveolar bone of all rats by extraction of the left maxillary incisor. The rats in the study groups received microspheres containing bioactive agents. The microspheres were placed in the alveolar socket in such a way that it was filled and closed. Control and negative control groups were formed. The control groups received chitosan-coated and non-chitosan-coated microspheres without bioactive agents, while the negative control groups received no microspheres (see Table 1).

Table 1. Study groups.

Day 7 Groups	Day 14 Groups	Day 28 Groups
Negative Control (n=6)	Negative Control (n=6)	Negative Control (n=6)
MS+Chitosan (control) (n=6)	MS+Chitosan (control) (n=6)	MS+Chitosan (control) (n=6)
MS (control) (n=6)	MS (control) (n=6)	MS (control) (n=6)
MS+Chitosan+GL (n=6)	MS+Chitosan+GL (n=6)	MS+Chitosan+GL (n=6)
MS+GL (n=6)	MS+GL (n=6)	MS+GL (n=6)
MS+Chitosan+IG (n=6)	MS+Chitosan+IG (n=6)	MS+Chitosan+IG (n=6)
MS+IG (n=6)	MS+IG (n=6)	MS+IG (n=6)

MS: Microsphere, GL: *G. lucidum*, IG: *I. graveolens*

2.3.3. *Collection of samples*

On days 7, 14 and 28 of the study, after anaesthesia with intraperitoneal 3 mg/kg xylazine and 90 mg/kg ketamine HCl, tissue samples were taken from the extracted alveolar bone. Alveolar bone samples were fixed in 10% formaldehyde solution and sent to Muğla Sıtkı Koçman University Faculty of Medicine's Pathology Department for immunohistochemical analysis.

2.4. Immunohistochemical Analysis of Tissues

For histopathological examination, tissues were fixed in 10% neutral buffered formalin, decalcified with 5% EDTA, embedded in paraffin blocks, cut into 4-micron sections, and then stained with haematoxylin and eosin (H&E). After routine microscopic evaluation, 4-micron tissue sections were placed on polylysine coated glass slides (Thermo Scientific) for immunohistochemical staining. Sections were washed in xylene, followed by dehydration in graded ethanol and phosphate buffered saline (PBS). Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide for 20 minutes. Antigen retrieval was performed using 10% citrate buffer and non-specific background staining was blocked by incubation with TBS for 5 minutes. Osteocalcin (Thermofisher PA5-78871) and SPARC osteonectin (GTX133747 IHC-P) antibodies were applied using the Leica Bond-Max fully automated immunohistochemistry device and incubated with secondary antibodies for 20 minutes. Chromogen 3,3'-diaminobenzidine was also incubated for 5 minutes as a substrate for colour development. Each slide was washed in tap water and counterstained with haematoxylin. Osteoblast and osteocyte levels were assessed by light microscopy (BX46 clinical microscope, Olympus, Tokyo, Japan).

2.5. Semi-quantitative Scoring of Histopathologic Parameters

Haematoxylin eosin-stained specimens were supported by immunohistochemical images and bone formation was assessed by the presence of osteoblasts and osteocytes in the

fibroconnective tissue. The percentage of bone tissue formed along the entire alveolar socket surface after tooth extraction was quantified as a percentage.

2.6. Statistical Analysis

Analyses were performed using IBM SPSS 20 statistical analysis software. Data was presented as mean, standard deviation, median, minimum, maximum, percentage, and number. Normal distribution of continuous variables was analysed using Shapiro-Wilk test, Kolmogorov-Smirnov test, Q-Q plot, and Skewness & Kurtosis. The ANOVA test was used when the normal distribution condition was met, and the Kruskal-Wallis test when it was not met, when comparing continuous variables with more than two independent groups. Post-hoc tests after ANOVA were performed using Tukey's test when the variances were homogeneous and Tamhane's T2 test when the variances were nonhomogeneous. For post-hoc tests after Kruskal-Wallis test, Kruskal-Wallis 1-way ANOVA (k samples) test was used. Pearson chi-squared test was used when the expected value was (>5) and Fisher-Freeman-Halton test when the expected value was (<5) for comparisons greater than 2x2 between categorical variables. Statistical significance was $p<0.05$.

3. FINDINGS

3.1. Histopathological Evaluation

Day 7: Increased congested blood vessels, defective bone fragments and foreign body granulation tissue were seen in 2 cases. Increased fibroblastic activity and marked granulation tissue and, to a lesser extent, osteoblastic cell formation was also observed (Figure 1a).

Day 14: A significant increase in osteoblastic activity was observed with a small amount of congested blood vessels and a limited area of inflammation. Osteocyte and osteoblast development in fibroconnective tissue and up to 50% mature bone formation were observed (Figure 1b).

Day 28: Formation of mature bone trabeculae surrounded by up to 85% osteoblastic cell rim within fibroconnective stromal tissue was evident, vasculature was in the form of normal tissue. No inflammation was observed (Figure 1c).

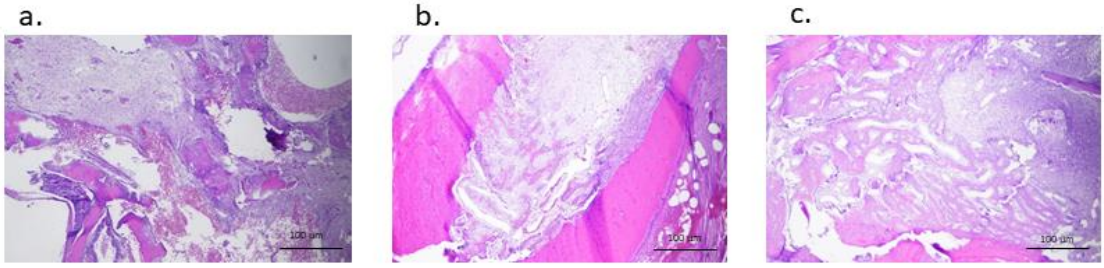

Figure 1. Histopathological evaluation. a. Haematoxylin and eosin staining, original magnification x40 (day 7). Congested blood vessels increased fibroblastic activity and granulation tissue with few osteoblasts. b. Haematoxylin and eosin staining, original magnification x40 (day 14). Reduced congested blood vessels, decreased inflammation, increased osteoblastic activity and initiation of mature bone formation. c. Haematoxylin and eosin staining, original magnification x40 (day 28). Formation of mature bone trabeculae surrounded by a prominent rim of osteoblastic cells.

3.2. Results of Statistical Analysis

According to the results of the study, there was no statistically significant difference in bone formation percentage between both day 7 and 14 groups ($p>0.05$) (Table 2). On day 28, a statistically significant difference was observed between the negative control group and the MS + chitosan + IG group and between the MS group and the MS + chitosan + IG group in terms of bone formation percentage ($p<0.05$) (Table 2). When evaluated in terms of bone formation percentage on different days within the groups, a statistically significant difference was observed in all groups in terms of bone formation percentage on days 7 and 28 ($p<0.05$) (Table 3).

Table 2. Comparing the percentages of bone formation among groups occurring on different days.

		Bone Formation Percentages							
Time	Groups	Mean	Standard Deviation	Median	Minimum	Maximum	Chi-square	*p*	post-hoc
Day 7	Negative Control	2	4	0	0	10	3.165	0.788	
	MS+Chitosan (control)	5	8	0	0	20			
	MS (control)	2	4	0	0	10			
	MS+Chitosan+GL	7	8	5	0	20			
	MS+GL	5	8	0	0	20			
	MS+Chitosan+IG	8	12	5	0	30			
	MS+IG	7	12	0	0	30			
Day 14	Negative Control	15	10	15	0	30	6.793	0.340	
	MS+Chitosan (control)	20	12	20	0	30			
	MS (control)	20	7	20	10	30			
	MS+Chitosan+GL	27	14	30	10	40			
	MS+GL	25	14	25	10	40			
	MS+Chitosan+IG	32	12	30	20	50			
	MS+IG	28	13	20	20	50			
Day 28	Negative Control	44	5	40	40	50	20.607	0.002	(Negative Control-MS+Chitosan+IG), (MS-MS+Chitosan+IG)
	MS+Chitosan (control)	62	15	55	50	80			
	MS (control)	47	8	45	40	60			
	MS+Chitosan+GL	60	11	60	50	80			
	MS+GL	66	9	60	60	80			
	MS+Chitosan+IG	70	9	70	60	80			
	MS+IG	56	9	50	50	70			

(MS: Microsphere, GL: *G. lucidum*, IG: *I. graveolens*)

Table 3. Comparing the percentages of bone formation on different days within the groups.

Groups	Time		Bone Formation Percentages					Chi-square	*p*	Post-Hoc
			Mean	Standard Deviation	Median	Minimum	Maximum			
Negative Control	Day	7	2	4	0	0	10	13.105	0.001	Day 7-28
		14	15	10	15	0	30			
		28	44	5	40	40	50			
MS+Chitosan	Day	7	5	8	0	0	20	12.913	0.002	Day 7-28
		14	20	12	20	0	30			
		28	62	15	55	50	80			
MS	Day	7	2	4	0	0	10	14.558	0.001	Day 7-28
		14	20	7	20	10	30			
		28	47	8	45	40	60			
MS+Chitosan+GL	Day	7	7	8	5	0	20	13.963	0.001	Day 7-28
		14	27	14	30	10	40			
		28	60	11	60	50	80			
MS+GL	Day	7	5	8	0	0	20	13.069	0.001	Day 7-28
		14	25	14	25	10	40			
		28	66	9	60	60	80			
MS+Chitosan+IG	Day	7	8	12	5	0	30	14.190	0.001	Day 7-28
		14	32	12	30	20	50			
		28	70	9	70	60	80			
MS+IG	Day	7	7	12	0	0	30	11.555	0.003	Day 7-28
		14	28	13	20	20	50			
		28	56	9	50	50	70			

(MS: Microsphere, GL: *G. lucidum*, IG: *I. graveolens*)

When comparing bone formation on different days within the groups, a statistically significant difference was found between bone formation on different days within the negative control, MS + chitosan, MS, MS + GL and MS + IG groups (p<0.05) (Table 4). Statistical differences in bone formation within groups were found between day 7-28 in the negative control group, day 7-28 in the MS + Chitosan group, day 7-14 and day 7-28 in the MS group, day 7-14 in the MS + GL group and day 7-14 and day 7-28 in the MS + IG group (p<0.05) (Table 4). There was no statistically significant difference between bone formation in the MS + Chitosan + GL and MS + Chitosan + IG groups at day 7, 14 and 28 (p>0.05) (Table 4).

Table 4. Comparing bone formations on different days within the groups.

Groups	Time		Bone Formation				Fisher Freaman Halton	p	Post-Hoc
			Absent		Present				
			Count	Column N %	Count	Column N %			
Negative Control	Day	7	5	83.3%	1	9.1%	8.598	0.012	7-28(day)
		14	1	16.7%	5	45.5%			
		28	0	0.0%	5	45.5%			
MS+Chitosan	Day	7	4	80.0%	2	16.7%	5.934	0.036	7-28(day)
		14	1	20.0%	4	33.3%			
		28	0	0.0%	6	50.0%			
MS	Day	7	5	100.0%	1	8.3%	10.986	0.002	7-14(day). 7-28(day)
		14	0	0.0%	5	41.7%			
		28	0	0.0%	6	50.0%			
MS+Chitosan+GL	Day	7	3	100.0%	3	20.0%	5.205	0.074	
		14	0	0.0%	6	40.0%			
		28	0	0.0%	6	40.0%			
MS+GL	Day	7	4	100.0%	2	15.4%	7.528	0.015	7-14(day)
		14	0	0.0%	6	46.2%			
		28	0	0.0%	5	38.5%			
MS+Chitosan+IG	Day	7	3	100.0%	3	20.0%	5.205	0.074	
		14	0	0.0%	6	40.0%			
		28	0	0.0%	6	40.0%			
MS+IG	Day	7	4	100.0%	2	16.7%	7.031	0.014	7-14(day), 7-28(day)
		14	0	0.0%	5	41.7%			
		28	0	0.0%	5	41.7%			

MS: Microsphere, GL: *G. lucidum*, IG: I.*graveolens*

When the bone formation of the groups on different days was compared, no statistically significant difference was found between the bone formation of the groups on both day 7 and 14 (p>0.05) (Table 5). On day 28, no statistical comparison could be made as bone formation had occurred in all rats.

Table 5. Comparing bone formations within groups occurring on different days.

		Bone Formation					
		Absent		Present			
Time	Groups	Count	Column N %	Count	Column N %	Fisher Freaman Halton	*p*
Day 7	Negative Control	5	17.9%	1	7.1%	3.176	0.924
	MS+Chitosan	4	14.3%	2	14.3%		
	MS	5	17.9%	1	7.1%		
	MS+Chitosan+GL	3	10.7%	3	21.4%		
	MS+GL	4	14.3%	2	14.3%		
	MS+Chitosan+IG	3	10.7%	3	21.4%		
	MS+IG	4	14.3%	2	14.3%		
Day 14	Negative Control	1	50.0%	5	13.5%	5.193	0.709
	MS+Chitosan	1	50.0%	4	10.8%		
	MS	0	0.0%	5	13.5%		
	MS+Chitosan+GL	0	0.0%	6	16.2%		
	MS+GL	0	0.0%	6	16.2%		
	MS+Chitosan+IG	0	0.0%	6	16.2%		
	MS+IG	0	0.0%	5	13.5%		
Day 28	Negative Control	0	0.0%	5	12.8%	NA	NA
	MS+Chitosan	0	0.0%	6	15.4%		
	MS	0	0.0%	6	15.4%		
	MS+Chitosan+GL	0	0.0%	6	15.4%		
	MS+GL	0	0.0%	5	12.8%		
	MS+Chitosan+IG	0	0.0%	6	15.4%		
	MS+IG	0	0.0%	5	12.8%		

(MS: Microsphere, GL: *G. lucidum*, IG: *I. graveolens*)

4. DISCUSSION and CONCLUSION

Vertical and horizontal resorption of alveolar bone after tooth extraction has become an important issue, especially in combination with the need for dental implants. For this reason, the bone healing process that occurs in the alveolar socket has attracted the attention of researchers in recent years. The alveolar socket healing model after tooth extraction in rats allows the evaluation of different osteopromotive products prior to clinical trials, on the alveolar socket bone regeneration that occurs (Koh *et al*., 2018; Kawecki *et al*., 2022; Sukpaita *et al*., 2024).

In our study, an alveolar socket model was created after extraction of the left central maxillary tooth in rats. Different stages of bone healing in the alveolar socket were observed by histopathological examination on the 7th, 14th and 28th day after tooth extraction. Increased fibroblast activity, dense granulation tissue and osteoblast proliferation were observed on day 7 after extraction. Previous studies have reported that fibroblasts accelerate the closure of the socket after extraction and continue to increase until 7 days after extraction (Vieira *et al*., 2015; Hassumi *et al*., 2018). Granulation tissue is formed by fibroblasts that synthesise collagen and extracellular matrix (Luvizuto *et al*., 2010). This granulation tissue then transforms into a provisional matrix. This contains more matrix and fewer inflammatory cells (Araújo et al., 2015). In our study, fibroblasts and granulation tissue decreased and osteoblastic activity increased on day 14. Hanafiah *et al.* (2021) reported that osteoblastic activity, which began to be observed on day 7 following extraction, peaked on day 14. Osteoblasts then form an osteoid matrix and these osteoids mineralise to form woven bone. Osteoblasts arranged on osteoids and

embedded in the matrix become osteocytes. Olaitan *et al*. (2019) reported that the number of osteocytes in the alveolar socket was higher on day 28 after extraction than on previous days. Hassumi *et al.* (2018) reported that mature bone trabeculae, containing osteocytes that fill most of the alveolar socket, were observed on the 28th day after extraction. In our study, mature trabeculae formation was also seen on day 28 in parallel with these studies.

When analysing the bone formation percentage results of our study, the bone formation percentage in the MS + Chitosan + IG group on day 28 was higher than the negative control and MS groups. As in previous studies, the regenerative contributions of chitosan, which is used as a bioactive polymer in bone tissue engineering applications, to bone wound healing is supported in the results of our study (Kowalczyk *et al*., 2021; Guillén-Carvajal *et al*., 2023). There are however no previous studies on the effect of *I. graveolens* on bone regeneration. Therefore, it is difficult to compare this effect of *I. graveolens* on bone regeneration with the available literature data. Ponticelli et al. have reported antibacterial, cytotoxic, and anti-inflammatory effects of *I. graveolens* (Ponticelli *et al*., 2022). The contribution of *I. graveolens* to bone formation together with chitosan may be associated with these existing effects in the literature. The fact that *G. lucidum* did not make a statistically significant difference in the percentage of bone formation may seem to contradict previous studies (Miyamoto *et al*., 2009; Laçin *et al*., 2019). In a study by Laçin *et al*. (2019) on calvarial defects, used together with bone grafting, *G. lucidum* was administered by gastric lavage. Miyamoto *et al.* (2009) administered *G. lucidum* via a diet containing 0.3% ethanol extracts. In our study, topical application of microspheres to the alveolar sockets after extraction without bone grafting differs from the methods used in previous studies. Therefore, different results may be expected. Another result of our study related to the percentage of bone formation is the statistically significant difference between day 7 and day 28 in all groups. The higher percentage of bone formation on day 28 compared to day 7 is in line with previous studies on socket healing after extraction (Okamoto & Russo, 1973; Carvalho *et al*., 1997; Rodrigues *et al.*, 2016).

In our study, bone formation (present, absent) was compared in addition to the percentage of alveolar socket bone formation after extraction. When the comparison of bone formation on different days within the groups was analysed, no statistically significant difference was found between the bone formation on days 7, 14 and 28 in the MS + Chitosan + GL and MS + Chitosan + IG groups. This shows that *G. lucidum* and *I. graveolens* together with chitosan contribute to bone formation from day 7 onwards. In their study, Yadav *et al.* (2021) showed that chitosan scaffolds induce osteogenic morphogenesis for cell adhesion and proliferation, neovascularisation, mineralisation, and bone regeneration in bone tissue engineering. Kim *et al.* (2016) highlighted the need to develop chitosan hydrogel surfaces that provide a suitable osteogenic microenvironment to facilitate osteogenesis with hydrogels. The synergistic effect of chitosan with *I. graveolens* and *G. lucidum* on alveolar bone formation in our study supports these studies.

Previous studies have shown the antimicrobial and anti-inflammatory effects of *G. lucidum* and *I. graveolens*. However, this study demonstrated the potential of these two bioactive agents for bone regeneration in post-extraction alveolar sockets. Further research is needed as this is the first study to evaluate the effect of these two agents on alveolar bone regeneration.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: Muğla Sıtkı Koçman University, Animal Experimentation and Research Centre, Local Ethics Committee for Animal Experimentation (MUDEM-HAYDEK). 11.01.2021-19/21.

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Ali Batuhan Bayırlı: Conception, Materials, Data collection and processing, Analysis and Interpretation, Writing the Original Draft, and Manuscript Review. **Serhat Sezgin:** Resources, Conception, Analysis and Interpretation, Supervision, and Writing. **Ezgi Eren Belgin:** Materials, Design, Analysis and Interpretation and Writing. **Leyla Tekin:** Analysis and Interpretation and Writing. **Hüseyin Çiçek:** Materials, Analysis and Interpretation, Supervision. **Ayşegül Demirbaş:** Conception, Data collection and processing. **Cankız Gizem Delibalta:** Investigation, and Materials. **Fatma Yılmaz:** Investigation, Supervision, and Validation.

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Research Article

Revealing metabolite diversity in seeds of species belonging to *Orchis* and *Anacamptis* genus

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Abstract: This study aims to compare the chemical compositions of methanol extracts from seeds of 10 different species belonging to the *Anacamptis* and *Orchis* genera, highlighting significant differences among these species. Seeds collected from various locations in Samsun, Muğla, and İzmir during 2022 and 2023 were analyzed using GC-MS. The results revealed various secondary metabolites in seeds of both *Anacamptis* and *Orchis* species. *A. palustris* seeds, hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester was found at a rate of 16.21%, while methyl stearate was found at 11.14%. In contrast, *O. purpurea* seeds contained hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester at 34.94% and methyl stearate at 8.69%. These findings indicate significant variability in the distribution of compounds among species. The rare compound tricyclo [20.8.0.0(7,16)] triacontane, found in *O. provincialis*, contains tricyclic structures with a 1(22),7(16)-diepoxy group, highlighting its potential role in the chemical profile of this species. Additionally, other rare compounds like tricyclo [20.8.0.0(7,16)] triacontane in *O. provincialis* emphasize their potential roles in chemical profiles across different species. This study is considered a significant step towards understanding the similarities and differences in biochemical components of seeds from *Anacamptis* and *Orchis*, thereby contributing to the understanding of their roles in plant physiological adaptations and ecosystem dynamics. The findings provide valuable insights for plant conservation strategies and biological applications.

1. INTRODUCTION

They are distributed globally and can thrive in almost every type of habitat, except deserts, and are found on every continent except Antarctica (Parkins *et al.*,2023). Epiphytic orchids, which make up about a quarter of all described terrestrial orchids, are typically found in tropical and subtropical regions. The abundance, species diversity, and distribution of orchids vary significantly among continents and regions (Baishnab *et al.*, 2024).

Seed morphology offers valuable insights into orchid evolution and adaptations, serving as a key resource for comparative studies due to its genetic conservation. Previous research has employed seed morphology to explore taxonomic, phylogenetic, and phytogeographic relationships among orchid species (Diantina *et al.*, 2020). Monographic studies focus on the

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detailed examination and classification of specific plant groups. Molecular findings by Kretzschmar *et al.* (2007) supported Bateman *et al.* (2003), leading to the identification of the genera Anacamptis, Neotinea, and Orchis (including Aceras) through novel morphological traits such as lip structure, tooth filaments, and stigmatic cavity. Researchers identified 11 species of *Anacamptis* (including 20 subspecies), 21 species of *Orchis* (including 16 subspecies), and 4 species of *Neotinea* (including 2 subspecies). They also noted natural hybrids within the same genus but did not observe hybrids spanning across three genera. Hybridization within *Orchis* was confined to taxa within each subgenus, excluding inter-subgeneric hybrids.

Tyteca *et al.* (2008) proposed a new taxonomic classification of Orchis sensu *lato*, delineating various species into the genera *Herorchis*, *Androrchis*, and *Odontorchis*. They reinstated the previous monotypic status of *Neotinea* and *Anacamptis* genus. While the *Androrchis* genus encompassed all species of the *Orchis* genus, excluding groups with anthropomorphic labels. Subsequently, Tyteca and Klein (2009) embraced the expanded genus of *Anacamptis* and *Neotinea* but reaffirmed the distinct classification of the *Androrchis* genus (Tyteca *et al.*, 2008). Delforge (2009) introduced a new classification of *Orchis* sensu lato. In this proposal, while acknowledging the taxonomic position of the *Orchis* and *Neotinea* genus, they did not support the expanded *Anacamptis* genus, considering it monotypic. Delforge allocated the remaining species to the genera *Herorchis*, *Vermeulenia*, *Anteriorchis*, and the new *Paludorchis*. Researchers have predominantly focused on morphological and molecular classifications. However, distinctions made through biochemical studies could complement phylogenetic research (Delforge, 2006).

Orchid seeds are notably small and lightweight compared to those of other botanical families, with the potential to produce up to a million seeds per capsule (Lee and Yeung, 2023). A distinctive feature of orchid seeds is the presence of a membranous testa with an 'air cavity' surrounding a small spherical embryo, rather than endosperm. The volume of this air cavity varies among specie (Gamarra *et al.*, 2012). Previous investigations have emphasized the diagnostic and phylogenetic significance of specific quantitative and qualitative seed attributes, highlighting a strong correlation between seed micromorphology and molecular phylogeny (Arditti *et al.*, 1979; Clifford & Smith, 1969; Gamarra *et al.*, 2007, 2008).

Preserving orchid seeds is critical for their biochemical processes, which are essential for healthy development and reproductive success. Orchid seeds are distinguished by their unique structures, including air cavities and absence of endosperm, which require specific biochemical processes for proper germination and growth. This includes the presence of suitable microorganisms for seed germination, breakdown of seed coat compounds, and nutrient release for embryo nourishment (Chen *et al.*, 2022; Gao *et al.*, 2022). Additionally, natural antioxidants and protective compounds are crucial for withstanding environmental stresses. Biochemical processes play a significant role in the ecological role of orchid seeds and the sustainability of their populations (Namrata *et al.*, 2022). Therefore, understanding these processes and preserving their natural habitats are essential for the conservation and sustainability of orchid species.

In this context, this study aims to elucidate the similarities and differences in seed chemistry structures of the *Anacamptis* and *Orchis* genus within the orchid family.

2. MATERIAL and METHODS

2.1. Collection of Seed

The seeds of *Orchis* and *Anacamptis* species were harvested upon maturation of the capsules during the years 2022 and 2023. *Anacamptis papilionacea*, *Anacamptis pyramidalis*, *Orchis purpurea*, *Orchis provincialis*, *Orchis mascula*, and *Anacamptis palustris* were collected in 2023 from Samsun in the Central Black Sea region, while *Anacamptis morio* and *Orchis italica* were gathered in 2022 from Muğla, and *Anacamptis sancta* was obtained in 2022 from İzmir. *Orchis punctulate* was procured in 2022 from Antalya. During collection, the seeds were

extracted from the capsules and left to air-dry naturally between cellulose material (or paper) to eliminate moisture. Subsequently, they were preserved in brown bottles at +4°C in the laboratory.

2.2. GC-MS Analysis of Seeds

For gas chromatography-mass spectrometry (GC-MS) analysis, 1 gram of seeds from each *Orchis* and *Anacamptis* species underwent pulverization within a sterile environment. Subsequently, 20 milliliters of 100% methanol were introduced for extraction using the maceration technique at 30°C for 24 hours, following the methodology outlined by Aytar (2024). Following this, the samples underwent centrifugation at 3500 revolutions per minute for 10 minutes, and the resulting supernatant was utilized for GC-MS analysis. The GC-MS analysis was conducted in accordance with the protocol provided by Aytar (2024), utilizing the NIST Standard Reference Database for the analysis.

3. FINDINGS

The GC-MS analyses of the seeds identified various secondary metabolites across different species. The number of bioactive components detected in each species is as follows: 16 in *Anacamptis palustris*, 12 in *Anacamptis morio*, 16 in *Orchis provincialis*, 15 in *Orchis purpurea*, 24 in *Anacamptis papilionacea*, 13 in *Orchis italica*, 16 in *Anacamptis sancta*, 14 in *Orchis punctulata*, 14 in *Anacamptis pyramidalis*, and 12 in *Orchis mascula*.

The GC-MS results for the methanol extract of *Anacamptis palustris* are detailed in Table S1. The major compounds identified in this extract include hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester (16.21%), methyl stearate (11.14%), 2-propenoic acid, 3-(2-hydroxyphenyl)-, (E)- (7.79%), and tetracosamethyl-cyclododecasiloxane (6.79%). The GC-MS results for the methanol extract of *Anacamptis morio* are presented in Table S2. The major compounds identified in this extract include hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester (43.79%), hexadecanoic acid, methyl ester (9.32%), and methyl stearate (7.48%).

The GC-MS results of the methanol extract of *A. papilionacea* are presented in Table S3. According to these results, the major compounds identified were hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester (28.86%), methyl stearate (10.54%), and hexatriacontane (3.98%). The GC-MS results of the methanol extract of *O. provincialis* are presented in Table S4. According to these results, the major compounds identified were hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester (16.21%), methyl stearate (11.14%), 2-propenoic acid, 3-(2-hydroxyphenyl)-, (E)- (7.79%), and tetracosamethyl-cyclododecasiloxane (6.79%).

The GC-MS results of the methanol extract of *A. pyramidalis* are presented in Table S5. According to these results, the major compounds identified were 2,2-dimethoxybutane (27.21%), hydroxyacetic acid, hydrazide (20.56%), 1,3-Dioxolane-4-methanol, 2-ethyl- (9.05%), and Silane, dimethoxymethyl (7.92%). The GC-MS results of the methanol extract of *O. purpurea* are presented in Table S6. According to these results, the major compounds identified were hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester (34.94%), Methyl stearate (8.69%), hexadecanoic acid, methyl ester (7.51%), 2-propenoic acid, 3-(2-hydroxyphenyl)-, (E)- (4.00%), and cyclononasiloxane, octadecamethyl- (3.97%).

The GC-MS results of the methanol extract of *O. italica* are presented in Table S7. According to these results, the major compounds identified were 2,2-dimethoxybutane (33.26%), Di-sec-butyl ether (13.81%), silane, dimethoxymethyl- (10.20%), decane (7.83%), 9,12-octadecadienoic acid (Z,Z)-, methyl ester (5.55%), and hydroxyacetic acid, hydrazide (5.46%).

The GC-MS results of the methanol extract of *O. sancta* are presented in Table S8. According to these results, the major compounds identified were 2,2-dimethoxybutane (29.13%), hydroxyacetic acid, hydrazide (19.88%), 1,3-Dioxolane-4-methanol, 2-ethyl- (8.45%), Propanoic acid, 2-methyl- (6.70%), and 3,5-Dithiahexanol 5,5-dioxide (6.49%). The GC-MS results of the methanol extract of *O. puntlata* are presented in Table S9. According to these

results, the major compounds identified were 2,2-dimethoxybutane (38.62%), 1-pentanol, 5-cyclopropylidene- (13.92%), silane, dimethoxymethyl- (7.00%), and butyl 2-(2-(2-butoxyethoxy) ethoxy) acetate (6.93%). The GC-MS results of the methanol extract of *O. mascula* are presented in Table S10. According to these results, the major compounds identified were 2,2-dimethoxybutane (30.53%), hydrazinecarbothioamide (12.39%), 1,3-dioxolane-4-methanol, 2-ethyl- (11.40%), and propane, 1,1-dimethoxy- (7.15%).

It is observed that both genera have various proportions of major compounds. However, certain species contain specific secondary metabolites exclusively. The distinct secondary metabolites present in species belonging to the *Anacamptis* and *Orchis* genus are illustrated in Figure 1 and Figure 2.

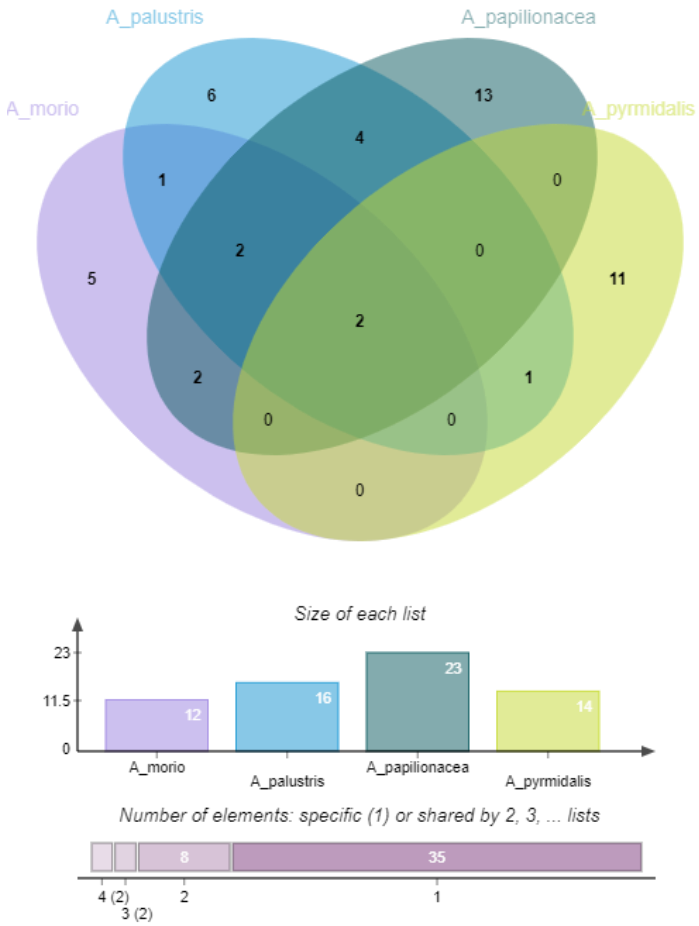

Figure 1. The Venn diagram of species belonging to the *Anacamptis* genus.

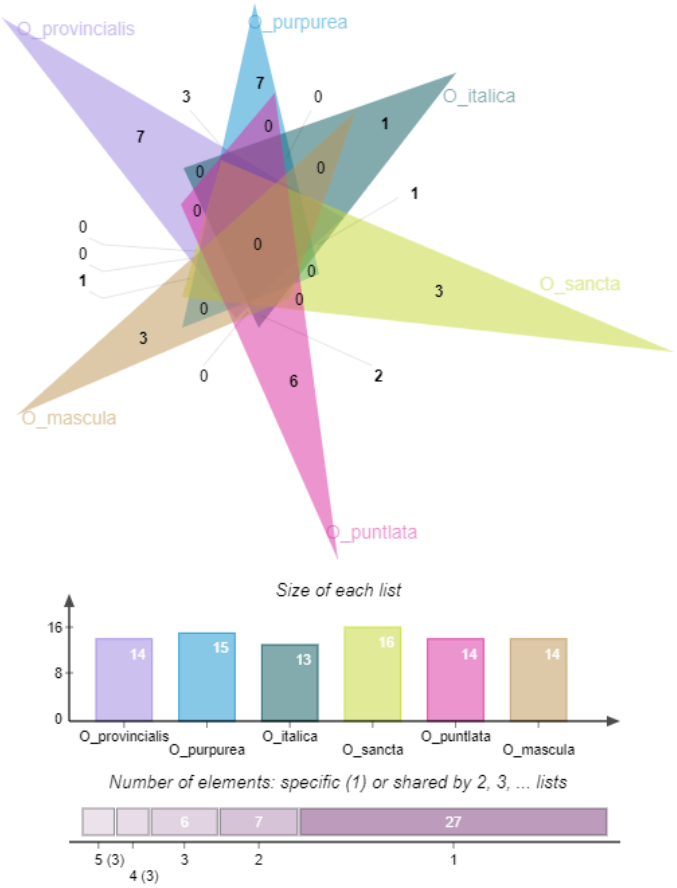

Figure 2. The Venn diagram of species belonging to the *Orchis* genus.

4. DISCUSSION and CONCLUSION

Differences in the percentage composition of major compounds can be attributed to the species and environmental conditions in which plants grow. Variations in chemical composition among plants may be associated with genetic differences, climatic conditions of their habitats, soil properties, and other environmental factors influencing the plants.

2,2-dimethoxybutane, a prominent component in the methanol extracts of plant species such as *O. puntlata*, *O. italica*, *O. sancta*, and *O. mascula*, holds significant percentage proportions. This compound reaches its highest percentage in the methanol extract of *O. puntlata* (38.62%). Similarly, *O. italica*, *O. sancta*, and *O. mascula* exhibit high percentages of 2,2-dimethoxybutane (33.26%, 29.13%, 30.53% respectively). This compound occupies a notable position in the chemical profiles of these plant species, potentially serving as a crucial element in understanding their biological properties and potential pharmacological effects.

Hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester, identified as a significant component in the methanol extracts of plant species such as *A. papilionacea*, *O. purpurea*, *O. provincialis*, and *O. mascula*. This compound attains its highest percentage in the methanol extract of *O. purpurea* (34.94%). Similarly, *A. papilionacea*, *O. provincialis*, and *O. mascula* exhibit high percentages of this compound (28.86%, 16.21%, 11.40% respectively). Methyl stearate, another important component detected in the methanol extracts of plant species such as *O. provincialis*, *A. papilionacea*, *O. purpurea*, and *O. mascula*. This compound reaches its highest percentage in the methanol extract of *O. provincialis* (11.14%). Similarly, *A. papilionacea*, *O. purpurea*, and *O. mascula* show high percentages of methyl stearate (10.54%, 8.69%, 12.39% respectively).

In a study by Aytar *et al.* (2023), hydroxyacetic acid hydrazide was detected at 12.42% in *Anacamptis coriophora* seeds, while it was found to be 5.46% in *O. italica*, 19.88% in *O. sancta*, and 5.59% in *O. mascula* through GC-MS analysis. Additionally, another common

compound, 2,2-dimethoxybutane, was found at a rate of 27.91%, whereas it was 33.26% in *O. italica*, 29.13% in *O. sancta*, 38.62% in *O. puntlata*, and 30.53% in *O. mascula*. In the same study, *A. coriophora* was reported to contain cyclohexasiloxane, dodecamethyl at 1.01%, while it was found to be 1.89% in *O. provincialis* and 1.01% in *O. purpurea*. Furthermore, the compound 9,12-octadecadienoic acid (Z, Z)-, methyl ester was detected in all species except *O. puntlata* and *O. provincialis*.

The rare compound Tricyclo [20.8.0.0(7,16)] triacontane, 1(22),7(16)-diepoxy-found in *O. provincialis* contains tricyclic structures with a diepoxy group, potentially contributing to the chemical profile of the species. Meanwhile, 2-tert-butyl-1,4-dimethoxybenzene present in *O. provincialis* includes a 2-tertiary butyl group and a 1,4-dimethoxybenzene ring, whereas triarachine in *O. purpurea* comprises the special structure of triarachidin. Conversely, 6,6-Diethylhoctadecane, a rare compound in *O. provincialis*, encompasses diethyl groups and hoctadecane structures. Methyl 18-methylnonadecanoate found in *A. papilionacea* includes methyl and methyl nonadecanoate groups, while ethanone, 1-[3-[2-methyl-2-(5-methyl-2-furanyl) propyl]oxiranyl] has a complex structure contributing to understanding the species' metabolic processes and lipid metabolism. Isopropyl palmitate in *A. papilionacea* contains isopropyl and palmitate groups, whereas octadecanal comprises an 18-carbon aliphatic chain. 2,3-Bis[(trimethylsilyl)oxy] propyl icosanoate, a rare silil ester compound found in *A. papilionacea*, includes two trimethylsilyloxypropyl and one icosanoate group. Lastly, Hexatriacontane in *A. papilionacea* constitutes long aliphatic hydrocarbon chains, serving as a significant component of the species' lipid structure. Further exploration of the role of these compounds in determining interspecies differences is warranted. Additionally, studies on the biological activities and effects of these compounds could deepen our understanding of the species' physiology and environmental adaptations.

Previous studies have reported the presence of the tricyclo [20.8.0.0(7,16)] triacontane, 1(22),7(16)-diepoxy compound in various plants. This compound has been observed in white rice at a rate of 0.76% (Kuswaha *et al.* 2021), in *C. corymbosus* root extract at 0.129% (Pauldasan *et al.* 2020), and in *Momordica cymbalaria* at 1.90% (Gopu et al. 2021). 2-tert-Butyl-1,4-dimethoxybenzene is a naturally occurring product found in *Valeriana officinalis*, with available data on its presence in this plant. Additionally, it has been found in *Moringa oleifera* volatile oil at a rate of 0.39%. However, no study has reported the presence of this compound in *Chlorophytum borivilianum*, where it is present at a rate of 4.81% (Chuhang *et al.* 2007). Hexatriacontane, octadecanal, and isopropyl palmitate are natural compounds found in *Camellia sinensis*, *Solanum tuberosum*, and other organisms with available data. In the methanol extract of *Gypsophila pilulifera*, the compound 2,3-Bis[(trimethylsilyl)oxy] propyl icosanoate has been determined to be present in the leaves at a rate of 0.6%. These findings come from a study investigating the chemical composition of endemic and endangered *G. pilulifera* produced by in vitro micropropagation (Ustuner *et al.* 2024).

In conclusion, a comprehensive investigation of these rare chemical compounds could significantly contribute to understanding interspecies differences and physiological adaptations. This study represents an important step toward comprehending the complexity of natural life and developing conservation strategies. Future research into the biological activities and potential applications of these compounds is recommended.

Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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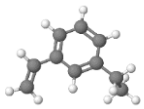
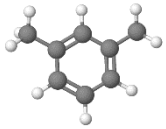
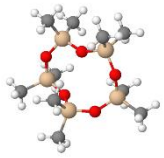
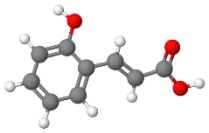
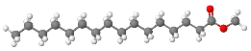
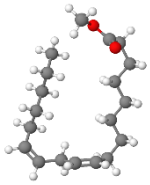
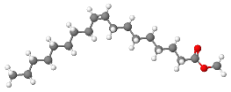
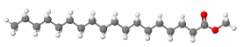
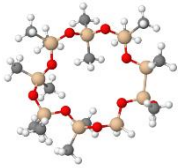
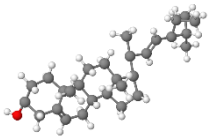
APPENDIX

Supplementary Material

Table S1. The GC-MS analysis results of *A.palustris* seeds.

No	Retention Time (minutes)	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Area (%)	3D Chemical Structure
1	9.569	Benzene, 1-ethenyl-3-ethyl-	$C_{10}H_{12}$	132.20	3.06	
2	11.370	Benzene, 1,3-dimethyl-	C_8H_{10}	106.16	1.59	
3	14.937	Cyclopentasiloxane, decamethyl-	$C_{10}H_{30}O_5Si_5$	370.76	1.20	
4	28.618	2-Propenoic acid, 3-(2-hydroxyphenyl)-, (E)-	$C_9H_8O_3$	164.15	7.79	
5	47.371	Hexadecanoic acid, methyl ester	$C_7H_{14}O_2$	270.45	3.95	
6	49.735	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47	2.34	
7	49.812	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296.48	1.18	
8	50.132	Methyl stearate	$C_{19}H_{38}O_2$	298.50	11.14	
9	50.672	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{14}O_9Si_9$	667.40	5.91	
10	51.881	Stigmasterol	$C_{29}H_{48}O$	412.70	2.74	

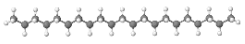
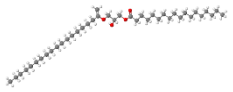
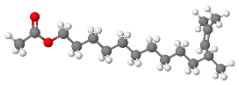
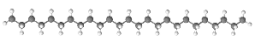
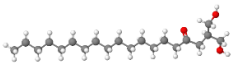
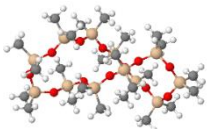
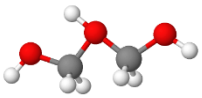
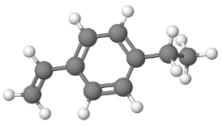
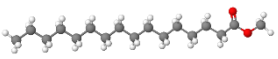
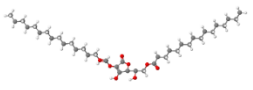
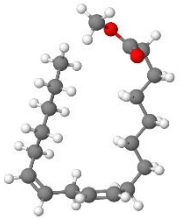
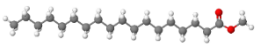
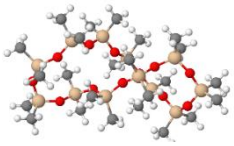
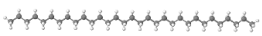
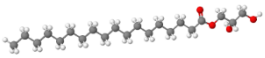
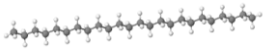
11	51.957	Heneicosane	$C_{21}H_{44}$	296.60	4.22	
12	52.569	1,3-Distearin	$C_{39}H_{76}O_5$	625.02	1.32	
13	53.768	11,13-Dimethyl-12-tetradecen-1-ol acetate	$C_{18}H_{34}O_2$	282.50	1.03	
14	54.392	Pentacosane	$C_{25}H_{52}$	296.48	1.76	
15	54.582	Hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	330.50	16.21	
16	55.226	Tetracosamethyl-cyclododecasiloxane	$C_{24}H_{72}O_{12}Si_{12}$	889.80	6.79	

Table S2. The GC-MS analysis results of *A. morio* seeds.

No	Retention Time (minutes)	Compound Name	Molecular Formula	Molecular Weight (g/mol)	No	Retention Time (minutes)
1	5.087	Glycerin	$C_3H_8O_3$	92.09	1.56	
2	9.515	Benzene, 1-ethenyl-4-ethyl-	$C_{10}H_{12}$	132.20	1.78	
3	47.382	Hexadecanoic acid, methyl ester	$C_7H_{14}O_2$	270.45	9.32	
4	47.988	l-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	652.90	1.00	
5	49.738	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47	2.01	
6	50.134	Methyl stearate	$C_{19}H_{38}O_2$	298.50	7.48	
7	50.634	Tetracosamethyl-cyclododecasiloxane	$C_{24}H_{72}O_{12}Si_{12}$	889.80	3.35	
8	51.956	Heptacosane	$C_{27}H_{56}$	380.70	1.92	
9	52.575	Octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{42}O_4$	358.55	1.15	
10	53.073	Tetracosane	$C_{24}H_{50}$	338.70	1.26	

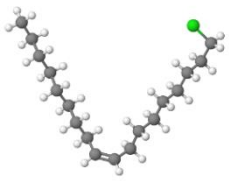
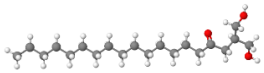
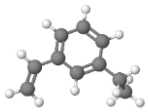
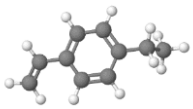
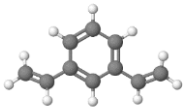
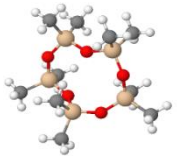
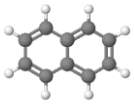
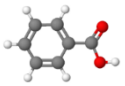
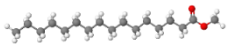
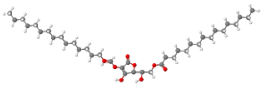
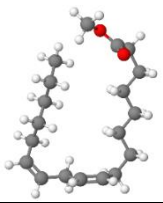
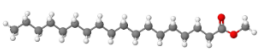
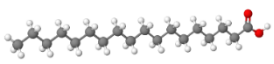
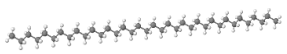
11	53.655	cis-1-Chloro-9-octadecene	$C_{18}H_{35}Cl$	286.92	1.35	
12	54.632	Hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	330.50	43.79	

Table S3. The GC-MS analysis results of *A. papilionacea* seeds.

No	Retention Time (minutes)	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Area (%)	3D Chemical Structure
1	1.383	Benzene, 1-ethenyl-3-ethyl-	$C_{10}H_{12}$	132.20	1.60	
2	10.014	Benzene, 1-ethenyl-4-ethyl-	$C_{10}H_{12}$	132.20	1.27	
3	11.383	Benzene, 1,3-diethenyl-	$C_{10}H_{10}$	130.18	1.59	
4	14.942	Cyclopentasiloxane, decamethyl-	$C_{10}H_{30}O_5Si_5$	370.76	1.08	
5	15.353	Naphthalene	$C_{10}H_8$	128.17	1.28	
6	15.842	Benzoic acid	$C_7H_6O_2$	122.12	1.14	
7	47.378	Hexadecanoic acid, methyl ester	$C_7H_{14}O_2$	270.45	3.64	
8	47.989	l-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	652.90	1.19	
9	49.742	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47	2.83	
10	50.135	Methyl stearate	$C_{19}H_{38}O_2$	298.50	10.54	
11	50.551	Octadecanoic acid	$C_{27}H_{56}$	380.70	1.04	
12	51.520	Hexatriacontane	$C_{36}H_{74}$	507.00	3.98	

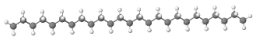
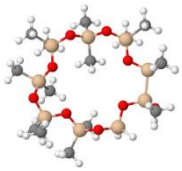
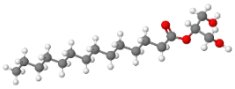
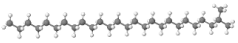
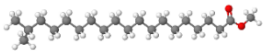
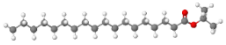
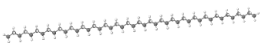
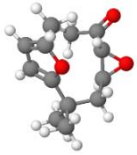
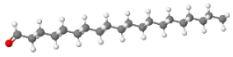
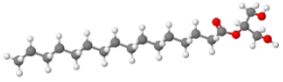
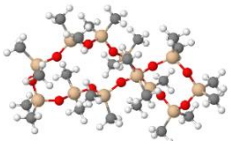
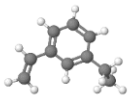
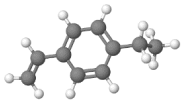
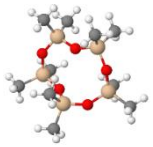
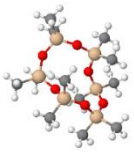
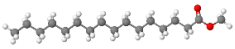
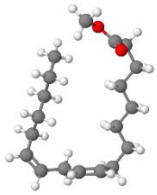
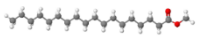
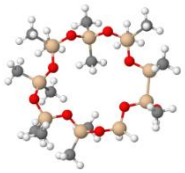
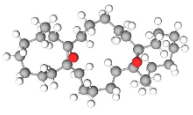
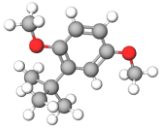
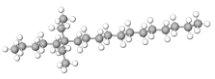
13	54.402	Heneicosane	$C_{21}H_{44}$	296.60	1.23	
14	51.848	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{14}O_9Si_9$	667.40	1.66	
15	51.996	Tetradecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{17}H_{34}O_4$	302.44	1.92	
16	52.155	2-methylhexacosane	$C_{27}H_{56}$	380.70	1.00	
17	52.273	Methyl 18-methylnonadecanoate	$C_{21}H_{42}O_2$	326.60	1.66	
18	52.578	Isopropyl palmitate	$C_{19}H_{38}O_2$	298.50	1.94	
19	52.846	Hexacontane	$C_{60}H_{122}$	843.60	1.07	
20	53.221	Ethanone, 1-[3-[2-methyl-2-(5-methyl-2-furanyl)propyl]oxiranyl]-	$C_{13}H_{18}O_3$	222.28	1.07	
21	53.459	Octadecanal	$C_{18}H_{36}O$	268.50	1.94	
22	54.137	2,3-Bis[(trimethylsilyl)oxy]propyl icosanoate	$C_{29}H_{62}O_4S_2$	531.00	2.11	
23	54.603	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330.50	25.86	
24	55.226	Tetracosamethyl-cyclododecasiloxane	$C_{24}H_{72}O_{12}Si_{12}$	889.80	2.58	

Table S4. The GC-MS analysis results of *O. provincialis* seeds.

No	Retention Time (minutes)	Compound Name	Molecular Formula	Molecular Weight(g/mol)	Area (%)	3D Chemical Structure
1	9.569	Benzene, 1-ethenyl-3-ethyl-	$C_{10}H_{12}$	132.20	1.38	
2	9.969	Benzene, 1-ethenyl-4-ethyl-	$C_{10}H_{12}$	132.20	1.69	
3	14.941	Cyclopentasiloxane, decamethyl-	$C_{10}H_{30}O_5Si_5$	370.76	1.70	
4	24.479	Cyclohexasiloxane, dodecamethyl-	$C_{12}H_{36}O_6Si_6$	444.92	1.89	
5	47.373	Hexadecanoic acid, methyl ester	$C_7H_{14}O_2$	270.45	4.33	
6	49.265	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47	2.09	
8	50.133	Methyl stearate	$C_{19}H_{38}O_2$	298.50	12.37	
9	50.677	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{14}O_9Si_9$	667.40	8.78	
10	51.717	Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy-	$C_{30}H_{52}O_2$	444.70	1.36	
11	52.056	2-tert-Butyl-1,4-dimethoxybenzene	$C_{12}H_{18}O_2$	194.27	2.26	
12	52.569	6,6-Diethylhoctadecane	$C_{22}H_{46}$	310.60	2.06	

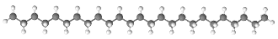
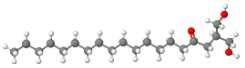
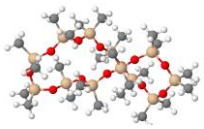
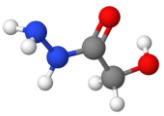
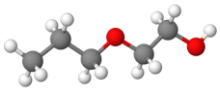
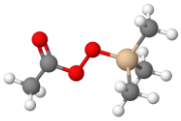
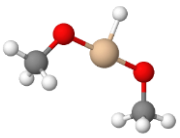
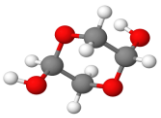
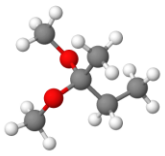
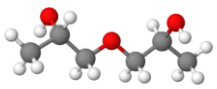
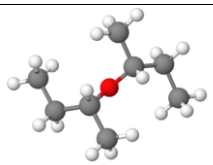
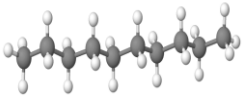
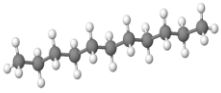
14	54.395	Pentacosane	$C_{25}H_{52}$	296.48	1.41	
15	54.587	Hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	330.50	16.91	
16	55.222	Tetracosamethyl-cyclododecasiloxane	$C_{24}H_{72}O_{12}Si_{12}$	889.80	4.87	

Table S5. The GC-MS analysis results of *A. pyramidalis* seeds

No	Retention Time (minutes)	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Area (%)	3D Chemical Structure
1	3.055	Hydroxyacetic acid, hydrazide	$C_2H_6N_2O_2$	90.08	20.56	
2	3.274	Ethanol, 2-propoxy-	$C_5H_{12}O_2$	104.14	6.65	
3	3.433	Trimethylsilyl ethaneperoxoate	CH_5N_3S	148.23	3.54	
4	3.474	Silane, dimethoxymethyl-	$C_3H_{10}O_2Si$	106.20	7.92	
5	3.695	Glycolaldehyde dimer	$C_4H_8O_4$	120.10	2.49	
6	5.029	2,2-dimethoxybutane	$C_6H_{14}O_2$	118.17	27.21	
7	7.114	2-Propanol, 1,1'-oxybis-	$C_6H_{14}O_3$	134.17	4.82	
8	7.314	1,3-Dioxolane-4-methanol, 2-ethyl-	$C_6H_{12}O_3$	132.16	9.05	
9	10.504	Decane	$C_{19}H_{36}O_2$	296.48	4.33	
10	12.385	Undecane	$C_{11}H_{24}$	156.31	1.64	

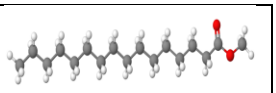
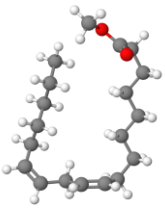
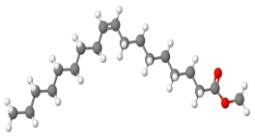
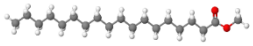
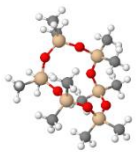
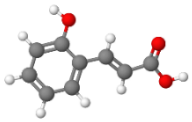
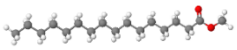
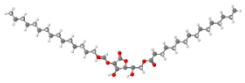
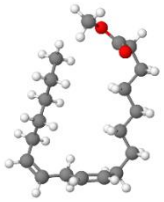
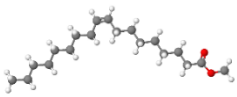
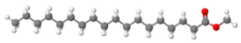
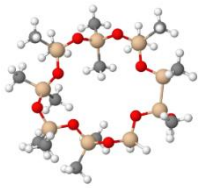
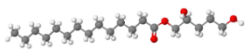
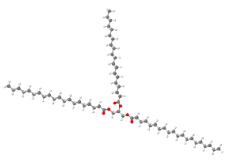
11	34.968	Hexadecanoic acid, methyl ester	$C_7H_{14}O_2$	270.45	1.17	
12	42.423	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47	4.01	
13	42.706	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296.48	1.75	
14	43.880	Methyl stearate	$C_{19}H_{38}O_2$	298.50	1.89	

Table S6. The GC-MS analysis results of *O. purpurea* seeds.

No	Retention Time (minutes)	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Area (%)	3D Chemical Structure
1	24.481	Cyclohexasiloxane, dodecamethyl-	$C_{12}H_{36}O_6Si_6$	444.92	1.01	
2	28.623	2-Propenoic acid, 3-(2-hydroxyphenyl)-, (E)-	$C_9H_8O_3$	164.15	4.00	
3	47.380	Hexadecanoic acid, methyl ester	$C_7H_{14}O_2$	270.45	7.51	
4	47.991	l-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	652.90	1.00	
5	49.738	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47	2.11	
6	49.816	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296.48	1.27	
7	50.134	Methyl stearate	$C_{19}H_{38}O_2$	298.50	8.69	
8	50.684	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{14}O_9Si_9$	667.40	3.97	
9	51.998	Tetradecanoic acid, 2,3-dihydroxypropyl ester	$C_{17}H_{34}O_4$	302.44	1.86	
10	52.573	Triarachine	$C_{63}H_{122}O_6$	975.63	1.08	

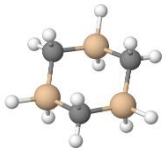
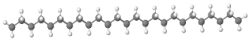
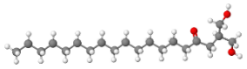
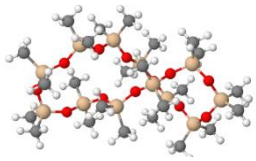
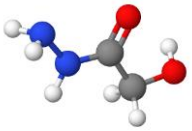
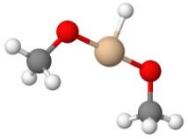
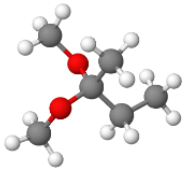
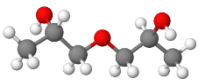
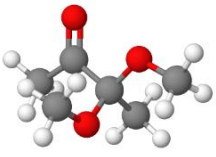
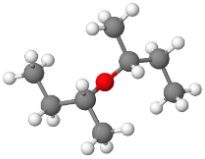
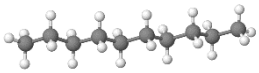
11	53.072	2-methylhexacosane	$C_{27}H_{56}$	380.70	1.67	
12	54.131	1,3,5-Trisilacyclohexane	$C_3H_6Si_3$	126.33	1.01	
13	54.402	Heneicosane	$C_{21}H_{44}$	296.60	1.23	
14	54.632	Hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	330.50	34.94	
15	55.232	Tetracosamethyl-cyclododecasiloxane	$C_{24}H_{72}O_{12}Si_{12}$	889.80	1.80	

Table S7. The GC-MS analysis results of *O. italica* seeds.

No	Retention Time (minutes)	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Area (%)	3D Chemical Structure
1	3.041	Hydroxyacetic acid, hydrazide	$C_2H_6N_2O_2$	90.08	5.46	
2	3.470	Silane, dimethoxymethyl-	$C_3H_{10}O_2Si$	106.20	10.20	
3	4.890	2,2-dimethoxybutane	$C_6H_{14}O_2$	118.17	33.26	
4	7.114	2-Propanol, 1,1'-oxybis-	$C_6H_{14}O_3$	134.17	3.52	
5	7.154	3,3-Dimethoxy-2-butanone	$C_6H_{12}O_3$	132.16	4.88	
6	7.255	Di-sec-Butyl ether	$C_8H_{18}O$	130.22	13.81	
7	10.491	Decane	$C_{19}H_{36}O_2$	296.48	7.83	

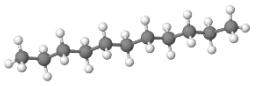
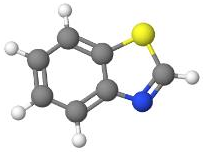
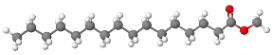
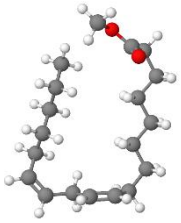
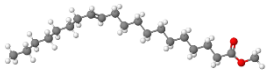
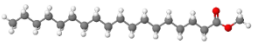
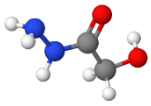
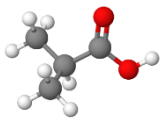
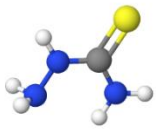
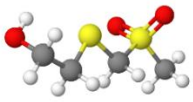
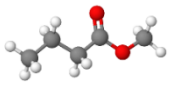
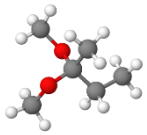
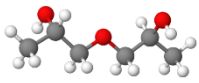
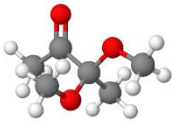
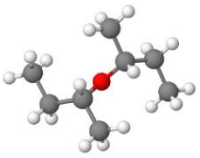
8	12.367	Undecane	$C_{11}H_{24}$	156.31	3.30	
9	14.656	Benzothiazole	C_7H_5NS	135.19	1.99	
10	34.950	Hexadecanoic acid, methyl ester	$C_7H_{14}O_2$	270.45	4.67	
11	42.392	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47	5.55	
12	42.686	10-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.48	1.58	
13	43.870	Methyl stearate	$C_{19}H_{38}O_2$	298.50	2.30	

Table S8. The GC-MS analysis results of *O. sancta* seeds.

No	Retention Time (minutes)	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Area (%)	3D Chemical Structure
1	3.037	Hydroxyacetic acid, hydrazide	$C_2H_6N_2O_2$	90.08	19.88	
2	3.270	Propanoic acid, 2-methyl-	$C_4H_8O_2$	88.10	6.70	
3	3.443	Hydrazinecarbothioamide	CH_5N_3S	91.13	5.71	
4	3.477	3,5-Dithiahexanol 5,5-dioxide	$C_4H_{10}O_3S_2$	170.25	6.49	
5	4.193	Butanoic acid, methyl ester	$C_5H_{10}O_2$	102.13	1.24	
6	4.890	2,2-dimethoxybutane	$C_6H_{14}O_2$	118.17	29.13	
7	7.120	2-Propanol, 1,1'-oxybis-	$C_6H_{14}O_3$	134.17	1.87	
8	7.169	3,3-Dimethoxy-2-butanone	$C_6H_{12}O_3$	132.16	3.72	
9	7.265	1,3-Dioxolane-4-methanol, 2-ethyl-	$C_6H_{12}O_3$	132.16	8.45	

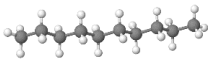
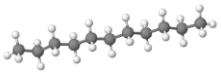
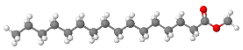
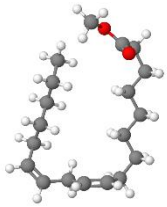
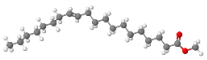
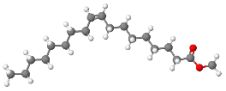
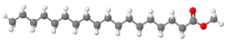
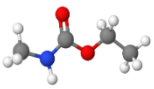
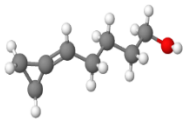
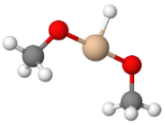
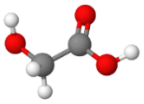
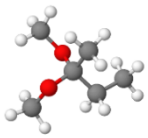
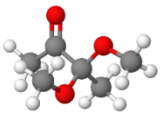
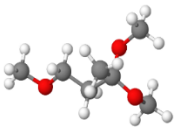
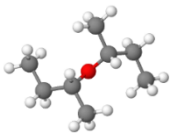
10	10.504	Decane	$C_{19}H_{36}O_2$	296.48	4.39	
11	12.384	Undecane	$C_{11}H_{24}$	156.31	1.82	
12	34.975	Hexadecanoic acid, methyl ester	$C_7H_{14}O_2$	270.45	1.63	
13	42.695	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47	2.99	
14	42.686	10-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.48	1.58	
15	42.695	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296.48	1.26	
16	43.870	Methyl stearate	$C_{19}H_{38}O_2$	298.50	1.91	

Table S9. The GC-MS analysis results of *O. punctulata* seeds

No	Retention Time (minutes)	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Area (%)	3D Chemical Structure
1	3.049	Ethyl N-methylcarbamate	$C_4H_9NO_2$	103.12	5.97	
2	3.125	1-Pentanol, 5-cyclopropylidene-	$C_4H_8O_2$	88.10	13.92	
3	3.453	Silane, dimethoxymethyl-	$C_3H_{10}O_2Si$	106.20	7.00	
4	3.570	Acetic acid, hydroxy-	$C_2H_4O_3$	76.05	1.66	
5	4.890	2,2-dimethoxybutane	$C_6H_{14}O_2$	118.17	38.62	
6	7.120	3,3-Dimethoxy-2-butanone	$C_6H_{12}O_3$	132.16	3.03	
7	7.160	1,3,3-Trimethoxybutane	$C_7H_{16}O_3$	148.20	3.42	
8	7.263	1,3-Dioxolane-4-methanol, 2-ethyl-	$C_6H_{12}O_3$	132.16	4.02	

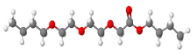
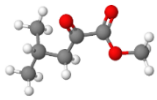
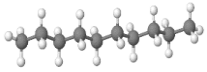
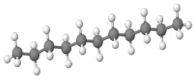
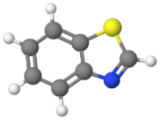
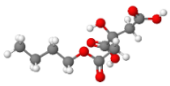
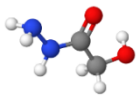
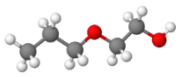
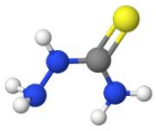
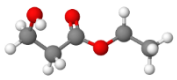
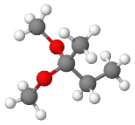
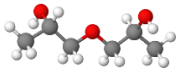
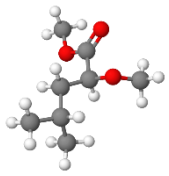
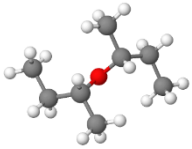
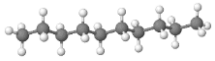
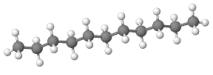
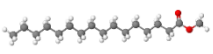
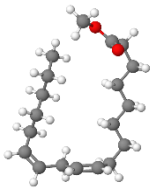
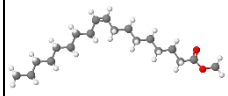
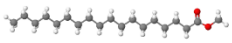
9	7.302	Butyl 2-(2-(2-butoxyethoxy)ethoxy)acetate	$C_{10}H_{20}O_4$	204.26	6.93	
10	9.974	Pentanoic acid, 4-methyl-2-oxo-, methyl ester	$C_6H_{10}O_3$	130.14	1.00	
11	10.500	Decane	$C_{19}H_{36}O_2$	296.48	5.44	
12	12.382	Undecane	$C_{11}H_{24}$	156.31	2.26	
13	14.673	Benzothiazole	C_7H_5NS	135.19	1.30	
14	46.756	Butyl citrate	$C_{18}H_{32}O_7$	360.40	1.57	

Table S10. The GC-MS analysis results of *O. mascula* seeds.

No	Retention Time (minutes)	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Area (%)	3D Chemical Structure
1	3.050	Hydroxyacetic acid, hydrazide	$C_2H_6N_2O_2$	90.08	5.59	
2	3.295	Propane, 1,1-dimethoxy-	$C_5H_{12}O_2$	104.14	7.15	
3	3.463	Hydrazinecarbothioamide	CH_5N_3S	91.13	12.39	
4	3.474	Acetic acid, hydroxy-, ethyl ester	$C_4H_8O_3$	104.10	2.15	
5	5.580	2,2-dimethoxybutane	$C_6H_{14}O_2$	118.17	30.53	
6	7.128	2-Propanol, 1,1'-oxybis-	$C_6H_{14}O_3$	134.17	2.54	
7	7.164	2-Hydroxyisocaproic acid, methyl ether, methyl ester	$C_8H_{16}O_3$	160.21	3.17	
8	7.314	1,3-Dioxolane-4-methanol, 2-ethyl-	$C_6H_{12}O_3$	132.16	11.40	
9	10.503	Decane	$C_{19}H_{36}O_2$	296.48	5.14	
10	12.383	Undecane	$C_{11}H_{24}$	156.31	1.98	
11	34.972	Hexadecanoic acid, methyl ester	$C_7H_{14}O_2$	270.45	5.64	

12	42.418	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47	5.88	
13	42.704	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296.48	1.35	
14	43.876	Methyl stearate	$C_{19}H_{38}O_2$	298.50	2.96	

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Research Article

Biological activities and phenolic content of endemic *Helichrysum artvinense* P.H. Davis et Kupicha (Asteraceae)

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Abstract: Species from the Asteraceae family have been extensively utilized in traditional medicine and as food sources for centuries. They also exhibit important biological activities attributed to their diverse array of phytochemical compounds. This research aimed to determine total phenolic and flavonoid contents of ethanol (EtOH) and methanol (MeOH) extracts of endemic *Helichrysum artvinense* and to reveal its antioxidant, antimicrobial enzyme inhibitory (*α*-glucosidase, *α*-amylase, and tyrosinase) and DNA protective activities. In addition, phenolic compound analyses were conducted using high-performance liquid chromatography (HPLC), establishing a correlation with the aforementioned biological activities. Based on the obtained data, the ethanol (EtOH) extract of the plant demonstrated greater prominence in terms of the screened biological activities. This extract was found to contain significant phenolic components, including epicatechin, chlorogenic acid, and luteolin. Consequently, it appears that the plant has the potential to serve as a natural alternative in both food and pharmacological applications. However, further studies to elucidate the mechanisms underlying the observed biological activities would be beneficial for the product development phase.

1. INTRODUCTION

The rapidly changing living conditions of today have introduced various stressors, leading to an increased incidence of numerous diseases (Pakpour *et al.*, 2021). Various stress-induced reactive oxygen species (ROS) disrupt cellular homeostasis in living organisms (Anwar *et al.*, 2022). The increase of ROS leads to various metabolic diseases such as cancer (with DNA damage), diabetes, aging, inflammation and neurodegenerative diseases (Maritim *et al.*, 2003; Tsao & Deng, 2004; Tepe *et al.*, 2005; Lee *et al.*, 2010; Fu *et al.*, 2011). Therefore, it is crucial to remove these reactive oxygen species (ROS) by natural means. Plants can accomplish this with bioactive compounds (Ebrahimzadeh & Tavassoli, 2015). Plants have already been used throughout history to alleviate or prevent many diseases and have become part of traditional medicine worldwide (Nebrigić *et al.*, 2023). Although the use of synthetic drugs has increased in the modern age, people still prefer natural herbal products to treat diseases due to the negative effects they cause (Gonçalves *et al.*, 2017; Zengin *et al.*, 2018). In fact, studies have shown that

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polyphenols of plants have many biological activities and antioxidant properties (Annadurai *et al.*, 2021; Birsan *et al.*, 2021).

During this period of pronounced global climate change, increased exposure to ultraviolet (UV) rays from the sun can result in advanced pigmentation in humans, leading to serious skin problems (aging, cancer, etc.) (Sheng *et al.*, 2022). UV radiation can stimulate the activity of tyrosinase, leading to an overproduction of melanin in the skin. While it normally helps conserve the skin from UV, excessive melanin synthesis can result in hyperpigmentation, age spots, inflammation, and even skin cancer (Brenner & Hearing, 2008; Pillaiyar *et al.*, 2017). To prevent these problems, it is vital to develop new and effective tyrosinase inhibitors that can help regulate melanin production and prevent its excessive accumulation in the skin. On the other hand, inhibition of enzymes such as alpha-amylase and glucosidase, which are responsible for the digestion of carbohydrates, is also important for solving diabetes, one of the most common health problems of our time (Ogunyemi *et al.*, 2022). Undoubtedly, another of the biggest dangers is the spread of pathogenic microorganisms or their becoming resistant to antibiotics (Gan *et al.*, 2024). Faced with all these problems, it has become essential to identify natural compounds, especially those obtained from plants, and to reveal their potential for use as enzyme inhibitors or antimicrobial agents. Because, considering the possible side effects of the synthetic agents used, the use of plants seems much safer.

Türkiye hosts a remarkable diversity of plant species, with the genus *Helichrysum* from the Asteraceae family being especially prominent (Lahlou *et al.*, 2024). These species have been variously named by the public (e.g. goldenrod, highland flower or immortelle) and are consumed mainly as herbal teas (Acet *et al.*, 2020). *Helichrysum* genus and its members are ethnobotanically valuable and are used as antioxidants, antimicrobials and to alleviate kidney disorders (Sezik *et al.*, 2001; Sala *et al.*, 2003; Albayrak *et al.*, 2010). In recent decades, *Helichrysum* species have been recognized as one of the promising medicinal plants. *Helichrysum* species occupy an important place in the literature with extensive research on their phytochemicals and biological activities. For instance, they have huge properties including cytotoxic, antimicrobial, antioxidant, anti-inflammatory, enzyme inhibitory activities for metabolic and neurodegenerative diseases, and anti-aging (Tepe *et al.*, 2005; Aslan *et al.*, 2007; Gouveia-Figueira *et al.*, 2014; Popoola *et al.*, 2015; Gonçalves *et al.*, 2017; Özcan & Acet, 2018; Acet *et al.*, 2020). Although there are many studies on *Helichrysum* species in the literature, there is limited research on the therapeutic properties of endemic *Helichrysum artvinense* in Artvin region (Eroğlu *et al.*, 2009; Albayrak *et al.*, 2010). Hence, in the present research we proposed to determine the biological activities of EtOH and MeOH extracts of *Helichrysum artvinense* such as *i*- antioxidant activity, *ii*- antimicrobial activity, *iii*- enzyme inhibitory activity, *iv*- DNA protective activity (for the first time), also *v*- phenolic component analysis by HPLC.

2. MATERIAL and METHODS

2.1. Collection of Samples

The plant materials of Helichrysum artvinense P.H. Davis & Kupicha examined in this study were collected from their natural habitat in Artvin Province, Türkiye. Specifically, samples were collected from Ardanuç: Cehennem Deresi Canyon upper sections, along roadsides, and on rocky slopes at an elevation of 660 m (41°08'35.0"N, 42°02'39.0"E), under the collection number Aksu 409, on 3 August 2022. The taxonomic identification and verification of the species were conducted by Dr. Nurşen Aksu Kalmuk, following the taxonomic keys for Helichrysum species as described by Davis (1975) in Flora of Türkiye and the East Aegean Islands. Voucher specimens are preserved at the Medicinal and Aromatic Plants Application and Research Center, Artvin Çoruh University. 5 g of dried and powdered aerial parts of plant were taken and kept in a shaker with 100 mL of EtOH and MeOH for 24 hours, and the solvent was evaporated with an evaporator at 37 °C (Bozkır *et al.*, 2022). For further experiments, the extracts were stored at -20°C.

2.2. Total Phenolic (TPC) and Flavonoid (TFC) Contents

Total phenolic and flavonoid contents in the extracts were determined according to Acet et al. (2020). The results obtained were determined as gallic acid (GAE) and quercetin equivalence (QUE), respectively.

2.3. Determination of Antioxidant Activity

ABTS [(2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] radical scavenging activity of the extracts was assessed by modified method developed by Re *et al*., (1998). Accordingly, 80 µL of the sample was combined with 160 µL of ABTS, allowed to react for 6 minutes, and then measured at 750 nm using a microplate reader. Results were expressed as trolox equivalent (mg TE/g extract). On the other hand, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the extracts was determined by modifying the method developed by Kirby & Schmidt, (1997). 125 µL of DPPH was added to 125 µL of extract solution and after waiting for 45 minutes at room temperature, measurement was made at 490 nm. The results were calculated as trolox equivalent (mg TE/g extract).

The activity of the EtOH and MeOH extracts was determined using the following equation, and then, expressed with IC_{50} value.

$$\%\text{Inhibition} = [(A_{\text{Control}} - A_{\text{Extract}})/A\text{Control}] * 100$$

2.4. Determination of Some Enzyme Inhibitory Activities

Enzyme inhibition activities of plant extracts associated with diabetes were determined using the assay of Acet et al. (2020). The results were stated as acarbose equivalent (mmol g/ extract). Tyrosinase inhibitory activity was determined according to Sarıkürkçü and Zengin (2020). Tyrosinase inhibition activity was given equivalents as kojic acid.

2.5. Determination of Antimicrobial Activity

Disc diffusion and microdilution methods were preferred to detect the antimicrobial activity of the plant extracts (CLSI, 2017). *Salmonella typhimurium* CCM5445, *Escherichia coli* ATCC 29998, *Bacillus cereus* RSKK 709, *Bacillus subtilis* IMG 22, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 13883, methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Enterococcus hirae* ATCC 10541, vancomicine resistance *Enterococcus faecium* DSMZ 13590, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 6538, *Yersinia pseudotuberculosis* ATCC 911, *Streptococcus pyogenes* ATCC 12344, *Candida tropicalis* NRRLL YB-366, *Clavibacter michiganensis* subsp. *michiganensis*, *Xanthomonas* spp. were used.

In both tests, the microorganism concentration was prepared at 0.5 McFarland turbidity. At the end of 48 hours of incubation at 37°C, the zones formed around the discs were measured in the disc diffusion test, while the concentration at which there was no microbial growth in the microdilution test was determined as the MIC value.

2.5. Determination of DNA Protective Activity

DNA protective activity of the extracts was performed by making minor changes to the Çelik Altunoğlu et al. (2022). Firstly, pUC19 plasmid DNA was damaged by using the Fenton solution. Extracts at different doses such as 1, 5, 10, 20 and 40 mg per mL were used. The presence or absence of activity was also observed by electrophoresis of DNA samples in 1% agarose gel.

2.6. Phenolic Compounds

HPLC (Shimadzu, Japan) was used to screen the presence of 20 different phenolic compounds commonly found in this family in the extract. The results of gallic acid, catechin, p-hydroxy benzoic acid, chlorogenic acid, caffeic acid, epicatechin, sirinic acid, vanillin, p-coumaric acid, ferulic acid, benzoic acid, rutin, hesperidin, trans-cinnamic acid, luteolin, kaempferol contents are given as µg/g extract.

2.7. Statistical Analyses

All trials were designed in triplicates. The results were calculated by the SPSS program (One-way ANOVA) (p<0.05 values mean significant).

3. RESULTS and DISCUSSION

TPC and TFC of EtOH and MeOH extracts of *Helichrysum artvinense* were determined by spectrophotometric methods (Table 1). In accordance with the results obtained, EtOH extracts were richer in TPC (62.74 mg GAE/g extract) and TFC (80.75 mg QUE/g extract) than MeOH extracts.

In a study on 15 diverse *Helichrysum* species, including *H. artvinense* species were investigated, revealing that the total phenolic content (TPC) of the methanol (MeOH) extracts was lowest in *H. peshmenianum* (66.75 mg GAE/g extract) and highest in *H. noeanum*(160.63 mg GAE/g extract). Also, the TPC of the MeOH extract obtained from the aerial parts of *H. artvinense* determined as 83.98 mg GAE/g extract (Albayrak *et al.*, 2010). In other study, the TPC and TFC of the ethanol extracts of flower of *H. plicatum* and *H. chionophilum* collected from Gümüşhane were found 535.3 and 424.6 mg GAE/g extract, and 50.9 and 73.6 mg QE/g extract, respectively. In ethanol extracts of the same plants, the TPC was identified as 536.9 and 537.7 mg GAE/g extract and the TFC was 55.4 and 44.6 mg QE/g extract, respectively (Acet *et al.*, 2020). In another study, Gouveia-Figueira (2014) investigated four different *Helichrysum* species and the TPC -TFC were found to be 0.04-121.4 mg GAE/g extract and 0.02-8.2 mg RUE/g extract, respectively. On the other hand, Ebrahimzadeh and Tavassoli (2015) reported the TPC (22.7 mg GAE/g extract) and TFC (9.6 mg QE/g extract) of MeOH extract of *H. pseudoplicatum.* In a study they conducted with *H. pseudoplicatum,* Ebrahimzadeh and Tavassoli (2015) reported that the TPC of the MeOH extract of the plant was 22.7 mg GAE/g extract and the TFC was 9.6 mg QE/g extract. When compared with the literature, it is seen that the TPC and TFC in the present research is lower or over than some studies. However, these differences are thought to be due to the differences in the solvents used. There is limited studies used ethanol as a solvent in the same species. In addition, different secondary metabolite contents in the same plant species may be related to various factors such as climate, collection period, altitude and soil structure.

Table 1. TFC and TPC of plant extracts.

Extracts	TPC (mg GAE/g extract)	TFC (mg QUE/g extract)
EtOH	62.74±0.7[a]	80.75±0.1[a]
MeOH	51.15±0.6[b]	65.03±0.6[b]

Values expressed are the mean ± *SD* of three different measurements. Data shown with different letters in the same column indicate statistically significant differences between the extracts (p<0.05).

Antioxidants are structures that prevent or delay oxidation caused by free radicals (Popoola *et al.*, 2015; Aguilera *et al.*, 2016). ABTS and DPPH methods are frequently used to determine the potential of plants to scavenge free radicals. The results of these experiments are shown in Table 2. As seen in this table, it was detected that the ABTS activity of the ethanol extract (508.036 mgTE/g extract) was higher than that of the methanol extract (450.350 mgTE/g extract). ABTS IC_{50} values also showed parallelism with the equivalence results. While the IC_{50} value of ethanol extract was 0.0284 mg/mL, methanol was determined as 0.0321 mg/mL. On the other hand, in DPPH analysis found that ethanol extract (66.533 mgTE/g extract) was higher than methanol extract (16.024 mgTE/g extract). DPPH IC_{50} values were also in parallel with the equivalence results. While the IC_{50} value of ethanol extract was 0.133 mg/mL, methanol was determined as 0.225 mg/mL. In a study on similar species, ABTS activity in ethanol extracts of flower of *H. plicatum* and *H. chionophilum* collected from Gümüşhane was determined as 89.5 and 66.2 mgTE/g extract, respectively, and IC_{50} values were 0.446 and 0.051 mg/mL. ABTS values in the EtOH extracts of the same plants were found to be 52.6 and 355.3

mgTE/g extract, respectively, and IC_{50} values were 0.574 and 0.348 mg/mL (Acet *et al.*, 2020). Data from the current study indicate that ABTS scavenging activity of extracts is stronger than in the literature. If the DPPH activities in Acet *et al.*, (2020) are compared, it can be seen that the DPPH activity in the EtOH extracts of flower of *H. plicatum* and *H. chionophilum* is 7.9 and 17.7 mgTE/g extract, respectively, and the IC_{50} values are 0.234 and 0.87 mg/mL. DPPH values in the stem ethanol extract of the same plants were found to be 5.8 and 22.0 mgTE/g extract, respectively, and IC_{50} values were 0.381 and 0.679 mg/mL (Acet *et al.*, 2020). The data obtained from the current study show that the DPPH scavenging activity, especially of the EtOH extract, is stronger than the literature. Similarly, the antioxidant capacity of *H. plicatum, H. chionophilum* and *H. arenarium* methanol extracts taken from the Sivas region was reported using the DPPH assay (IC_{50} = 0.0405 and 0.047.6 mg/mL, respectively) (Tepe *et al.*, 2005). In other study, the DPPH IC_{50} value of the MeOH extract obtained using the aerial parts of *H.artvinense* was stated as 0.021 mg/mL (Albayrak *et al.*, 2010). Differences in these values may be due to the location where the plant was taken or the experimental conditions.

Table 2. Antioxidant properties of plant extracts.

Extracts	ABTS (mgTE/g extract)	ABTS IC_{50} (mg/mL)	DPPH (mgTE/g extract)	DPPH IC_{50} (mg/mL)
EtOH	508.036±1.5^{a}	0.0284±0.05^{b}	66.533±0.8^{a}	0.133±0.003^{b}
MeOH	450.350±2.25^{b}	0.0321±0.03^{c}	16.024±0.05^{b}	0.225±0.005^{c}
Trolox	-	0.009539±0.005^{a}	-	0.00528±0.025^{a}

Values expressed are the mean ± *SD* of three different measurements. Data shown with different letters in the same column indicate statistically significant differences between the extracts (p<0.05).

Type-II diabetes, a metabolic disease related to blood sugar levels, is a major health problem (Pari ve Srinivasan, 2010). Around 250 million individuals are projected to be affected by this disease by 2030 (Hwang *et al.*, 2012). From this point of view, the necessity of proper management of this disorder becomes evident. In this regard, control of carbohydrate hydrolyzing enzymes (amylase and glucosidase) is one of the important strategies to cope with the disease (Hu *et al.*, 2013). For this purpose, various synthetic enzyme inhibitors such as acarbose have been produced (Chiasson *et al.*, 2002). However, since these compounds cause significant side effects such as tissue and organ damage in humans, the discovery and use of natural inhibitors have become popular in recent years (Lasano *et al.*, 2019). In addition, tyrosinase inhibitors are used in the treatment of skin disorders such as hyperpigmentation and plants constitute an important source in this regard (Bozkır *et al.*, 2022). In this study, some enzyme inhibition activities of the aerial part of EtOH and MeOH extracts of *Helichrysum artvinense* were investigated. The results are shown in Table 3.

Table 3. Enzyme inhibitory activity of plant extracts.

Extracts	α-amylase inh. (mmolACAE/g extract)	α-glucosidase inh. (mmolACAE/g extract)	Tyrosinase inh. (mgKAE/g extract)
EtOH	533.27±5.5^{a}	24.92±1.5^{ab}	86.05±3.5^{a}
MeOH	489.02±3.5^{b}	25.33±1.25^{a}	22.45±1.05^{b}

Values expressed are the mean ± *SD* of three different measurements. Data shown with different letters in the same column indicate statistically significant differences between the extracts (p<0.05).

There is some research in the literature on the inhibition activities related to diabetes of the Asteraceae family (Spinola ve Castilho, 2017); additionally, studies on the enzyme inhibition activities of *Helichrysum* species were also examined. Accordingly, the α-amylase and α-glucosidase inhibition activity of the ethanol extract of the *Helichrysum stoechas* subsp. *barrelieri*, which was extracted similarly, was found to be 0.59 and 1.63 mmol ACAE/g extract, respectively. Additionally, in this study, tyrosinase enzyme inhibition activity was found to be 183.32 mg KAE/g extract (Zengin *et al.*, 2020). Accordingly, when the data obtained from the current study is compared with the literature, the enzyme inhibition results of the ethanol extract

of the *Helichrysum artvinense*, except tyrosinase, are quite high. In a different study on *H. chionophilum* and *H. plicatum*, the α-amylase enzyme inhibition activity of ethanol extracts of flower was found to be 156.53 and 105.35 mmolACAE/g extract, respectively. On the other hand, the α-glucosidase enzyme inhibition activity of ethanol extracts of stem was determined as 193.36 and 105.12 mmol ACAE/g extract, respectively. Similar evaluations are valid for α-glucosidase inhibition activities. According to the data obtained from the present study, it is seen that ethanol extract is more effective in inhibiting diabetes-related enzymes. As a result, it can be said that the findings are compatible with the literature.

The antimicrobial activity of the extracts was determined by disc diffusion and microdilution tests, and the results are shown in Table 4 (a and b). It was found that the extracts showed antimicrobial activity against all tested microorganisms except *B. cereus*. In addition, it was observed that both plant extracts showed strong antimicrobial activity against *E. faecalis* with MIC value of 16 µg/mL. In a previous study, it was stated that *H. chionophilum* extracts had no activity against *K. pneumoniae*, while *H. plicatum* had an antimicrobial effect against this organism (MIC value 512 µg/mL) (Acet et al., 2020).In the current research, EtOH and MeOH extracts were found to have a similar antimicrobial effect against *K. pneumoniae.* In this context, the results obtained are consistent with the literature. In addition, in a study conducted with *H. artvinense*, generally similar (disk diffusion) activities were observed (Albayrak *et al.*, 2010). MIC values were reported for the first time in this study.

The phenolic components of the extracts were screened using HPLC (Figures 1 and Figure 2) and the findings are given in Table 5. Since the ethanol extract of the plant was more effective than methanol extracts, phenolic content analysis of the ethanol extract was performed. Accordingly, the major phenolic components of the ethanol extract were found to be Epicatechin (2190.5 µg/g extract), Chlorogenic acid (1738.6 µg/g extract) and Luteonin (910.8 µg/g extract). While routine and hesperidin were not found in this extract, other standard phenolics were detected at minor levels. As per the literature, chlorogenic acid and its derivatives are the predominant phenolic acids identified in the plant samples under analysis (Nicolle *et al.*, 2004; Mattila and Hellström, 2007). In the present study, these compounds were also detected in substantial quantities in the tested plant. In this regard, the results taken from the study are compatible with the other scientific studies and it is supposed that the high biological activities exhibited by the plant is due to the phenolic compounds they contain.

The DNA protective activity of the extracts was evaluated by damaging the pUC19 plasmid using Fenton's solution (Figure 3). Different concentrations of extracts were used in the study and no activity was noticed in the MeOH extract. However, DNA protective activity was partially seen at a 20 mg/mL concentration of the EtOH extract. Some studies show that plants have protective activities against DNA damage at various doses (Giri *et al.*, 2017; Bozkır *et al.*, 2022). It has been suggested that phenolic components are related to DNA protective activity (Petersen, 2013). It is possible that some phenolic compounds, also detected in the current study, are responsible for this activity.

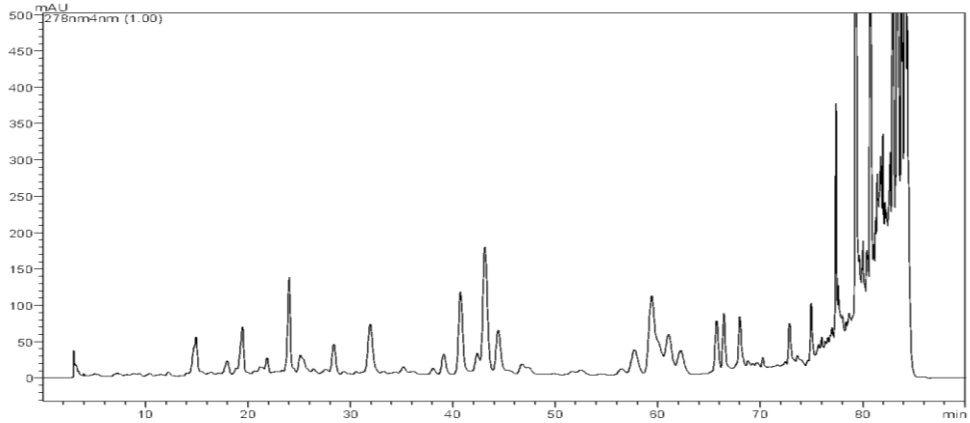

Figure 1. Chromatogram of EtOH extract.

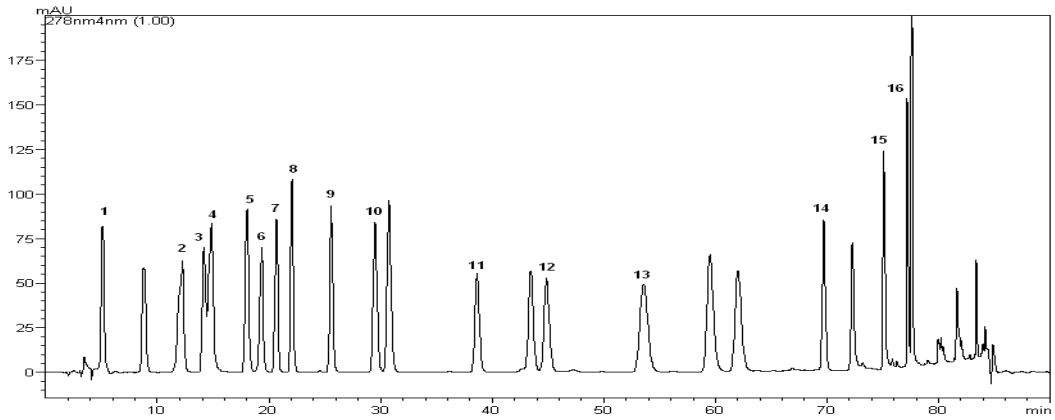

Figure 2. Standard chromatogram**.** Standard chromatogram, 1:gallic acid 2:catechin 3:p-hydroxy benzoic acid 4:chlorogenic acid 5:caffeic acid 6:epicatechin 7:syringic acid 8:vanillin 9:p-coumaric acid 10:ferulic acid 11:benzoic acid 12:rutin 13:hesperidin 14:cinnamic acid 15:luteolin 16:campferol.

Table 4. Antimicrobial activity of *H. artvinense.*
a. Antimicrobial activity (MIC) findings (µg/mL)

	Extracts	
Microorganisms	Ethanol	Methanol
B. cereus	ND	ND
E. coli	64	64
S. thyphi	64	64
K. pneumoniae	512	512
P. aeruginosa	512	512
S. aureus	16	32
MRSA	32	32
C. tropicalis	512	256
B. subtilis	128	128
E. faecium	512	512
E. faecalis	16	16
E. hirae	32	32
Y. pseudotuberculosis	512	128
X. spp.	16	128
C. michiganensis sups. m.	512	256
S. pyogenes	1000	1000

b. Antimicrobial activity (Disc diffusion) findings of the extracts (mm)-800 µg/mL per disc

	Extracts	
Microorganisms	Ethanol	Methanol
B. cereus	13	12
E. coli	-	-
S. thyphi	13	12
K. pneumoniae	13	12
P. aeruginosa	-	-
S. aureus	-	-
MRSA	12	11
C. tropicalis	14	12
B. subtilis	-	-
E. faecium	12	11
E. faecalis	11	12
E. hirae	12	-
Y. pseudotuberculosis	13	12
X. spp.	14	15
C. michiganensis sups. m.	-	-
S. pyogenes	20	17

Table 5. Phenolic compounds of ethanol extract.

No	Phenolic components	Amount (µg/g extract)
		EtOH
1	Gallic acid	34.7±0.001
2	Catechin	265.5±0.5
3	Chlorogenic acid	1738.6±0.4
4	Epicatechin	2190.5±0.05
5	Caffeic acid	224.4±0.02
6	Syringic acid	80.2±0.01
7	*p*-Coumaric acid	84.4±0.25
8	Ferulic acid	71.9±0.1
9	Rutin	*
10	Hesperidin	*
11	*t*-Cinnamic acid	28.0±0.02
12	Luteolin	910.8 ±0.01
13	Kaempferol	385.4±0.006
14	*p*-hydroxy benzoic acid	56.1±0.06
15	Vanilin	150.6 ±0.02
16	Benzoic acid	982.0±0.5

Values expressed are the mean ± SD of three different measurements. *Not detected

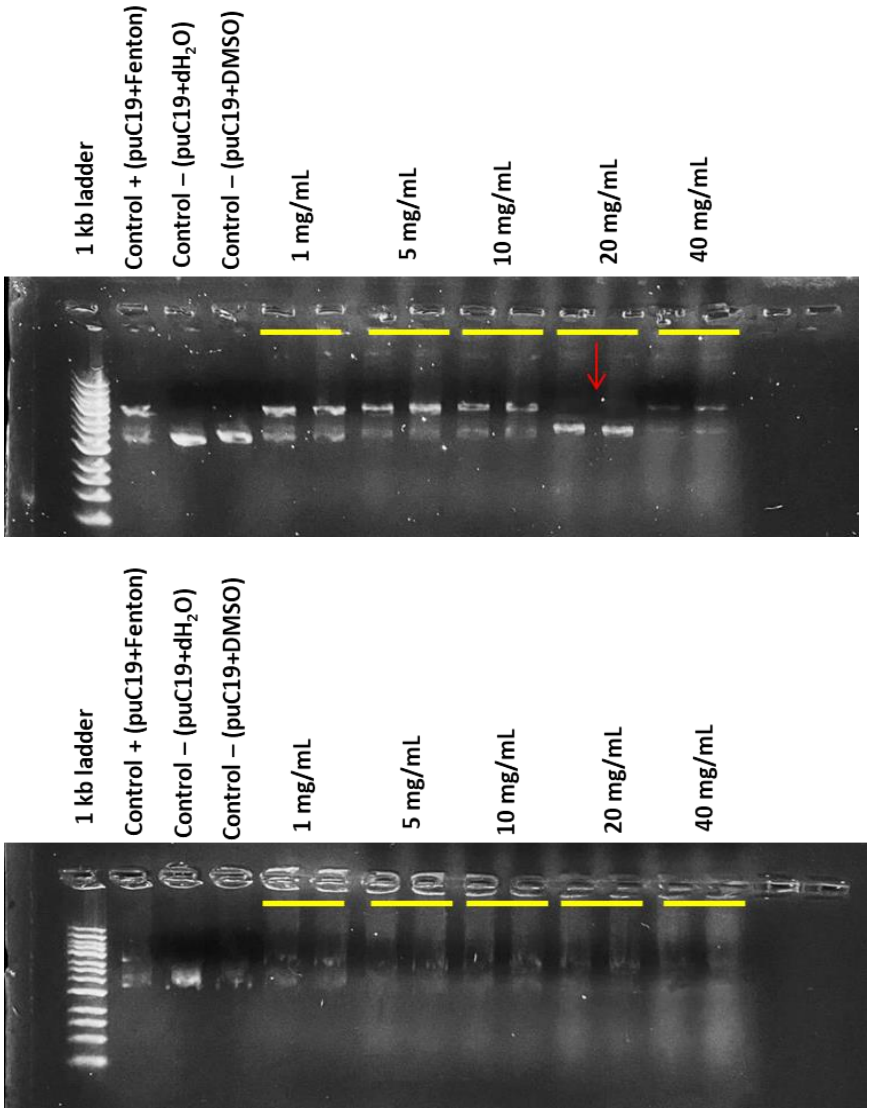

Figure 3. DNA protective activity **a-** ethanol extract, **b-** methanol extract.

4. CONCLUSION

In the study, some biological activities of endemic *Helichrysum artvinense* collected from Artvin province were investigated. With this study, the DNA protective activity of the plant was revealed for the first time. In the study where EtOH and MeOH extracts of the aerial parts of plant were used, it was observed that EtOH extracts were more prominent in terms of the screened properties. Therefore, phenolic components of EtOH extract were revealed by HPLC analysis. Accordingly, epicatechin, chlorogenic acid and luteolin were determined as basic phenolics. At the same time, extracts were demonstrated a high antimicrobial activity against some organisms with a MIC value of 16 µg/mL. It is thought that the high biological activities determined are due to the major and/or minor components of the plant. As a result, the plant in question has the potential to be used in both food and pharmacological fields. However, it seems useful to support the detected biological activity with in vivo studies.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Tuba Acet provided financial support for this study with project and contributed to the experimental studies, data analysis, preparation of manuscript and proofreading; **Kadriye Özcan** participated in antimicrobial experiments and the writing and proofreading; **Nurşen Aksu Kalmuk** provided and determined the plant material used. She also contributed to the critical reading of the article.

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Research Article

Chemical composition and cytotoxicity of *Araucaria heterophylla* (Salisb.) franco essential oils

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Abstract: The Norfolk Island pine, *Araucaria heterophylla* (Salisb.) Franco (Araucariaceae), is a plant that exhibits several pharmacological potentials. Essential oils (EOs) from the plant's fresh stem bark (FRS) and dry stem bark (DRS) were hydrodistilled in an all-glass Clevenger apparatus and further characterized by Gas Chromatography-Mass Spectrometry analysis. Using *Artemia salina* eggs hatched in sea water, the brine shrimp lethality assay was carried out for toxicity. The FRS and DRS yielded 0.33% and 0.29% EOs, respectively, constituting 48 and 42 compounds, representing 94.38% and 84.30% of the total oil fractions. The classes of compounds identified in the FRS and DRS EOs, respectively, include sesquiterpenes (40.8% and 41.36%), oxygenated sesquiterpenes (34.93% and 34.22%), oxygenated monoterpenes (11.58% and 2.84%), diterpenes (3.39% and 2.85%), oxygenated diterpenes (3.68% and 2.29%), and oxygenated triterpenes (0.74%, only in the DRS). The major constituents in the FRS EOs are spathulenol (12.12%), germacrene B (10.63%), dihydroedulan I (10.23%), γ-cadinene (6.90%), (-)-globulol(4.67%), aromadendrene (3.62%) and copaene (3.34%) while spathulenol (16.13%), germacrene B (10.37%), aromadendrene (4.93%), copaene (3.54%), β-panasinsene (3.06%) and guaiol (2.99%) majorly constitute the DRS oil. Constituents common and as well dominant in the two EOs include Spathulenol, Germacrene B, Aromadendrene and Copaene. The result of the cytotoxicity analysis showed that both the fresh and dry essential oils have LC_{50} of 10 ppm. A LC_{50}<100 ppm indicates high toxicity, thus, the EOs possess significant cytotoxicity against *A. salina*.

1. INTRODUCTION

Araucaria heterophylla (Salisb.) Franco (family: Araucariaceae) is a species of conifer. As its common name Norfolk Island pine implies, the tree is endemic to Norfolk Island, one of Australia's external territories, between New Zealand and New Caledonia in the Pacific Ocean. The tree is grown as an outdoor, ornamental plant and the saplings are cultivated as houseplants. The wood of large trees is used in construction, furniture, and ship building. This large evergreen plant has a single upright trunk, tiered branching habit, and a narrow pyramidal or columnar shape. Eventually reaching a height of about 80 feet, the tree purify the air by

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eliminating harmful, volatile organic compounds from the air. The Norfolk Island Pines, *A. heterophylla* brings festive cheer as a live Christmas tree (Stafford, 2016).

Many *Araucaria* species, which are widely used for ornamental and timber purposes worldwide, are evergreen coniferous trees. These plants exhibit several medicinal and pharmacological potentials including antimicrobial (Jaramillo *et al*., 2022; Soliman *et al*., 2023; Verma *et al*., 2014), antioxidant, (Abd-ElGawad, *et al*., 2023; Branco *et al*., 2016), anti-ulcerative, anti-inflammatory, antidepressant, anti-coagulant, antipyretic, neuroprotective effects (Jaramillo *et al*., 2022; Soliman *et al*., 2023). They also possess insecticidal, pesticidal activity and mosquito repellence effect (Baz, *et al*., 2022). Different phytochemicals were documented from these species, including flavonoids, lignans, phenylpropanoids, monoterpenes, sesquiterpenes and diterpenes (Abdel-Sattar *et al*., 2009; Elkady & Ayoub, 2018; Elshamy *et al*., 2020; Michael *et al*., 2010; Soliman *et al*., 2023).

A. heterophylla antitumoral, gastroprotective, anti-inflammatory, antipyretic activities (Abd-ElGawad, *et al*., 2023), as well as uses in respiratory infection, as an emollient, as antiseptic and for rheumatism had been reported. In folk medicine, the plant's aerial parts were used to treat toothache (Aslam *et al*., 2013). The essential oils from the oleoresin is also known for its gastroprotective, anti-inflammatory, antioxidant, and anti-*Helicobacter pylori* potentials (Ali *et al*., 2023). A few isolated compounds identified from the shoot include taxifolin and its 3-O-glucoside derivative, orientin, iso-orientin, vitexin, isovitexin, gallic acid, the labdane diterpenes (labda 8(17),14-diene, 13-epi-cupressic acid and 13-O-acetyl-13-epi-cupressic acid), as well as other diterpenes (Abdel-Sattar *et al*., 2009; Michael *et al*., 2010).

This study however, aimed at comparing the chemical constituents and determining the cytotoxic effect of *A. heterophylla* stem bark essential oil from fresh and dry samples.

2. MATERIAL and METHODS

2.1. Plant Materials and Essential Oils Isolation

A. heterophylla samples were harvested from cultivated plants within the University of Ibadan, Ibadan, Nigeria, in May 2023. Mr. D.P.O. Esimekhuai of the Botany Department, University of Ibadan, identified the plant species (Herbarium Number: UIH-23402), and were deposited in the Department's Herbarium. The air-dried pulverized plant samples were subjected to hydro-distillation for 3 hours in an all glass Clevenger-type apparatus to obtain colourless essential oils. These oils were desiccated over anhydrous sodium sulphate, Na_2SO_4 (Avis chemical), stored in sealed vials under refrigeration prior to analysis.

2.2. Analysis of the Essential Oils

A Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the oils was accomplished with the GCMS-QP2010 Plus (Shimadzu Japan) instrument, with HP-5MS capillary column dimension of 30 m length, 0.25 mm internal diameter and 0.25 µm film thickness. The GC-MS detector was operated in the Electron Ionisation (EI) mode of electron energy = 70 eV, with a scan range of 45-700 amu. The carrier gas was helium at a constant flow rate of 1.61 mL/min. The GC oven program was initial 60 °C, followed by 60-180 °C at a rate of 10°C/min, then held at 180 °C for 2 min, followed by 180-280 °C at a rate of 15 °C/min, then again held at 280 °C for 2 min. The ionisation of sample components was performed in the EI mode (70 eV). The injection port temperature was 250 °C, while the ion source temperature was 200 °C and the interface temperature was 200 °C. 1.0 µl of a diluted sample (1:1 in hexane, v/v) was injected using auto sampler and split mode with a split ratio 25:1.

2.3. Essential Oil Constituents Identification

The essential oils constituents were identified by checking the correlation of their mass spectra with NIST 2017 library data of the GC-MS system and as well by comparison of their retention indices (RI) with the relevant and applicable literature data (Adams, 2007; Joulain & Koenig, 1998). The proportionality of each individual constituent of the essential oil was represented as

the percentage of the peak area relative to the total peak area. The RI-value of each constituent was determined relative to the retention times of a homologous n-alkane series with linear interpolation on the HP-5MS column.

2.4. Cytotoxic Analysis of the Essential Oil Constituents

The brine shrimp lethality test, using *Artemia salina (*brine shrimp) eggs, was employed to determine the level of toxicity in the essential oils (EOs) according to the method by Aboaba *et al*. (2013 & 2014). 200 mL of sea water, collected from the ocean in Lagos State, South-West Nigeria, was poured into a hatching chamber and *A. salina* eggs were added. The hatching chamber is such that two compartments are separated by a perforated partition such that nauplii could swim through from one side, after hatching, to the other side. At room temperature, the eggs were allowed to hatch within 48 hours, the nauplii were attracted to one side of the chamber with a light source and then harvested with a dropping pipette. To test the survival rate of the nauplii, different concentrations of the EOs (1000, 100, 10 µg/mL stock solution each in triplicates) were prepared in sea water. Previously, the non-water soluble EOs were dissolved in 2 mL of dimethylsulfoxide, DMSO (Kermel Chemicals) and 0.5 mL of each of the dose levels introduced into a test-tube to which 4 mL of sea water was added. To prepare the 1000 to 10 µg/mL final concentration of oil extracts, ten (10) nauplii for each concentration were further added to each tube and made up to 5 mL with seawater. A blank solution which serves as reference standard, consisting of DMSO and 10 brine shrimps in sea water, was also prepared. After 24 hours, the total number of deaths was counted and recorded. The death percentage from the data obtained were analysed, using the Prism program for windows, developed by GraphPad Software, Inc., San Diego, CA, USA, version 6.0, to determine the lethal concentration (LC_{50}) expected to kill 50 % of the shrimps, using the formula below.

$$\text{Death percent (\%)} = \frac{\text{Dead nauplii}}{\text{Total naupii}} \times 100$$

3. RESULTS

The fresh stem bark of *A. heterophylla* gave a higher yield of 0.33 % essential oil than the dry stem bark oil with a yield of 0.29 %. As expressed in Table 1, a total of forty-five (45) compounds were obtained in the fresh stem bark oil, representing 94.38 % of the total oil composition. The fresh stem bark essential oil sample is a complex mixture of oxygenated monoterpenes (11.58 %), sesquiterpene hydrocarbons (40.8 %), oxygenated sesquiterpenes (34.93 %), diterpenes (3.39 %), and oxygenated diterpenes (3.68 %). The major compounds identified are Spathulenol (12.12 %), Germacrene B (10.63 %), Dihydroedulan I (10.23 %), γ-Cadinene (6.90 %), (-)-Globulol (4.67 %), Aromadendrene (3.62 %), Copaene (3.34 %), Ledol (3.04 %), Viridiflorol (2.88 %), and δ-Cadinene (2.83 %). Furthermore, in Table 1, a total of forty-three (43) essential oil compounds were obtained in the dry stem bark oil representing 84.30 % of the total oil composition, and were composed of oxygenated monoterpenes (2.84 %), sesquiterpene hydrocarbons (41.36 %), oxygenated sesquiterpenes (34.22 %), diterpenes (2.85 %), oxygenated diterpenes (2.29 %), and oxygenated triterpenes (0.74 %). The main compounds identified are spathulenol (16.13 %), germacrene B (10.37 %), aromadendrene (4.93 %), copaene (3.54 %), β-panasinsene (3.06 %), guaiol (2.99 %), rimuene (2.85 %), δ-cadinene (2.59 %), and β-elemene (2.49 %). Structures of some of these major compounds are represented in Figure 1 below. Common to both fresh and dry essential oil samples, respectively, in high percentage quantity include Spathulenol (12.12 %; 16.13 %, respectively), Germacrene B (10.63 %; 10.37 %, respectively), Aromadendrene (3.62 %; 4.93 %, respectively), Copaene (3.34 %; 3.54 %, respectively) and δ-Cadinene (2.83 %; 2.59 %, respectively).

The Lethal Concentration 50 (LC_{50}) which is the amount of essential oil required to kill 50% of the test organism (*A. salina* nauplii) for *A. heterophylla* fresh and dry stem bark showed similar results with LC_{50} = 10 ppm for both oil extracts.

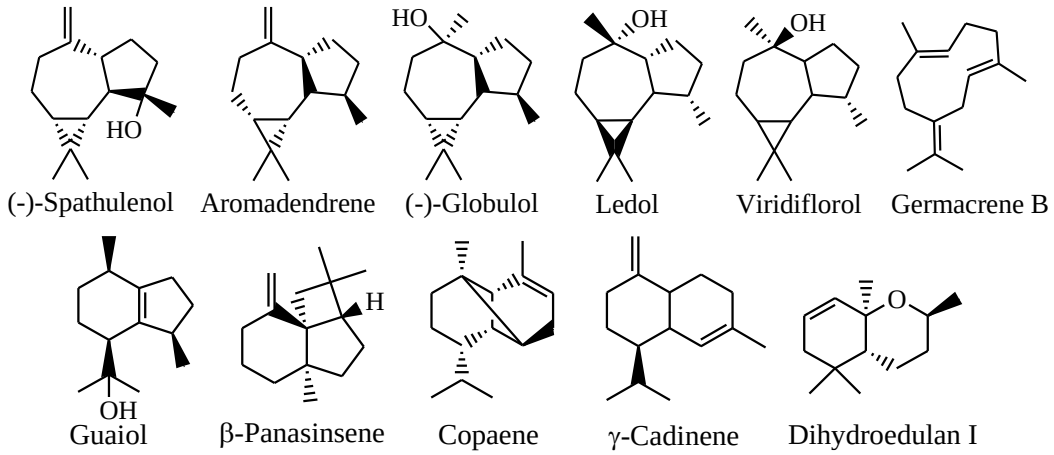

Figure 1. Structures of some major compounds identified in fresh and dry stem bark of *A. heterophylla* essential oils.

Table 1. Chemical composition of *Araucaria heterophylla* essential oils.

S/N	Constituent	Retention Index (LRI)	AHSF % Composition	AHSD % Composition
1	Ylangene	1221	0.71	-
2	Copaene	1221	3.34	3.54
3	Longifolenaldehyde	1293	-	0.29
4	Jasmone	1338	0.21	-
5	β-cubebene	1339	3.01	-
6	Dihydroedulan I	1342	10.23	-
7	α-cubebene	1344	0.86	0.68
8	Isolongifolen-5-one	1354	-	0.36
9	Didehydro-cycloisolongifolene	1385	-	0.38
10	Aromadendrene	1386	3.62	4.93
11	β-Elemene	1398	-	0.77
12	α-pinene	1403	-	0.67
13	β-Gurjunene	1411	1.01	0.69
14	Avermitilol	1411	-	1.37
15	α-Gurjunene	1419	0.31	-
16	Cadala-1(10),3,8-triene	1423	-	1.25
17	5,9-dimethyl-5,8-decadien-2-one	1427	-	0.71
18	γ-Elemene	1431	0.43	0.56
19	1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-cyclohexane	1431	2.51	-
20	(+)-Epi-bicyclosesquiphellandrene	1435	0.44	-
21	γ-muurolene	1435	-	3.7
22	Trans-muurola-4(14)-4-diene	1435	-	2.17
23	α-muurolene	1440	0.17	-
24	β-damascone	1457	-	2.13
25	(-)-β-Elemene	1465	-	2.49
26	β-Eudesmene	1469	0.81	-
27	δ- cadinene	1469	2.83	2.59
28	Valencene	1474	0.72	-
29	β-panasinsene	1474	2.59	3.06
30	1-pentanol,alpha-1-cyclopropene	1479	2.12	0.97
31	β-Caryophyllene	1494	1.07	-
32	Isolongifolene	1494	-	0.63
33	(-)-Globulol	1530	4.67	2.04
34	Viridiflorol	1530	2.88	-
35	Ledol	1530	3.04	-

36	1-[2-(2,2,6-trimethyl- bicyclo)acetic acid	1534	0.91	-
37	Spathulenol	1536	12.12	16.13
38	Ledene alcohol	1541	-	2.47
39	α-calacorene	1547	0.51	0.99
40	γ-cadinene	1565	6.9	1.15
41	α-Caryophyllene	1579	0.38	0.74
42	δ-cadinol	1580	-	1.33
43	Epi-α-muurolol	1580	1.21	-
44	Cubenol	1580	-	1.97
45	α-cadinol	1580	1.74	1.83
46	Selina-6-en-4-ol	1593	-	0.59
47	Guaiol	1598	-	2.99
48	β-selinenol	1598	1.01	-
49	Germacrene B	1603	10.63	10.37
50	Eudesma-4,11-dien-2-ol	1690	0.58	-
51	α-Elemol	1694	-	0.55
52	Rimuene	1726	-	2.85
53	Murolan-3,9(11)-diene-10-peroxy	1729	-	1.81
54	Humulane-1,6-dien-3-ol	1757	0.76	0.31
55	Myristic Acid	1769	0.23	-
56	(+)-Beyerene	1778	2.28	-
57	Kaur-16-ene	1789	0.19	-
58	Sclarene	1891	0.29	-
59	Labda-8(20),14-diene-13,19-diol	1891	2.23	0.92
60	Biformen	1909	0.3	-
61	Aristolene epoxide	1950	0.57	-
62	n-Hexadecanoic acid	1968	0.53	-
63	Elemol	1976	-	0.54
64	Manoyl oxide	1978	-	0.7
65	Sclareol	2016	-	0.33
66	Thunbergene	2072	0.33	-
67	4,5-dimethyl-octahydro-5,7a-isopropenyl	2141	-	0.34
68	Linoleic acid	2183	0.32	-
69	Verticillol	2190	1.03	-
70	Cycloartanol	2338	-	0.74
71	Labda-8(20),12,14-trien-19-oic acid	2900	0.42	-
72	Unknown	-	5.62	15.7
	Class of Terpenoids			
	Oxygenated monoterpenes		11.58 %	2.84 %
	Sesquiterpene hydrocarbons		40.8 %	41.36 %
	Oxygenated sesquiterpenes		34.93 %	34.22 %
	Diterpenes		3.39 %	2.85 %
	Oxygenated diterpenes		3.68 %	2.29 %
	Oxygenated triterpenes			0.74 %
	Unknown derivatives		5.62 %	15.7 %
		Total	100.00	100.00

LRI: Linear retention index on the HP-5MS column
AHSF: *A. heterophylla* fresh sample
AHSD: *A. heterophylla* dry sample

4. DISCUSSION and CONCLUSION

Sample state as well as sample preparation is a crucial process before chemical analysis. It is one of the important processes that determines the physical and chemical properties of samples to be analysed. The drying method, drying time, temperature and even the plant species have effect on the yield of plant volatile contents (Caputo *et al.*, 2022). From various research findings according to Beigi *et al*. (2018), increasing the drying temperature causes decrease in

essential oil yields. Taking into account the storage structures found in different plant organs as well as the volatile constituents embedded, the selection of appropriate drying methods is important. High temperatures, often at ≥ 60^{o}C, can damage cells that store the essential oil in these organs such that the release of volatile compounds is intensified, and result in lower yields after extraction (Nascimento *et al.*, 2021).

Furthermore, constituent denaturing by bond cleavage, rearrangement, or other functional group interconversions (chemical processes) such as oxidation, hydrolysis or dehydration, are another transformations that could take place. The fresh and dry stem bark EOs of *A. heterophylla* showed significant variation in chemical composition, owning to the difference in the physical states of the plant samples involved. It was observed that dehydration of the fresh stem, to obtain the dry sample, decreased the oxygenated monoterpene EO constituents significantly (11.5% composition seen for the fresh and 2.84% for the dry plant EO). Beigi *et al*. (2018) highlighted the effect of drying methods on the yield of essential oils and the main chemical constituents from four chemical groups viz; monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons and oxygenated sesquiterpenes. Hazrati *et al*. (2021) also reported that drying methods and the temperature involved have effect on the transformations essential oil constituents will undergo and be made up of. In addition, the biological properties of the EOs could be influenced (Hazrati *et al*., 2021; Tewari *et al*., 2019).

Many *Araucaria* species have been reported to majorly contain terpenes in their EO composition. The resin essential oil of *A. heterophylla* extracted in Egypt was chemically characterized to mainly contain terpenes (98.23%), and the major constituents were α-pinene (62.57%), β-pinene (6.60%), germacrene D (5.88%), and β-caryophyllene (3.56%) (Abd-ElGawad *et al*., 2023). These major compounds identified in the plant also corroborate what was obtained by Ali *et al*. (2023) as well as what was earlier report by Brophy *et al*. (2000). More so, oils obtained from another *A. heterophylla* foliage from Egypt constitute α-pinene (70.85%), D-limonene (4.26%) and germacrene D (2.99%) as the major constituents (Elkady & Ayoub, 2018). Furthermore, *A. heterophylla* essential oils from different geographic areas were examined to vary in constituents. The Australian *A. heterophylla* leaf essential oil majorly constituted α-pinene and phyllocladene (Brophy *et al*., 2000), while the oil from India was solely dominated by the diterpene hydrocarbons, 13-epidolabradiene and beyerene (Elkady & Ayoub, 2018; Verma *et al*., 2014), as the main constituents. β-Caryophyllene dominated the Hawaiian foliage oil of which β-pinene was obtained in trace quantity (Elkady & Ayoub, 2018). However, comparing these major constituents from previous literatures discussed above with those obtain from this study, some constituents were either found in lesser quantity or not identified from the stem bark oils (even though resins are often secreted from special resin cells in plant stem). There is the place of factors responsible for compound modification and constituents' variation, and thus influence EO composition. Such factors include plant origin or geographical location, nutritional and edaphic influence, genetic makeup, seasonality (temperature, humidity and brightness in weather conditions during maturity), time of harvest (Akande *et al*., 2018; do Nascimento *et al*., 2018; Elkady & Ayoub, 2018) and even method of extraction. An *Araucaria* species, *A. robusta* essential oil, was also reported to contain spathulenol (37 %) as its dominant constituent (Brophy *et al*., 2000).

The lethal concentration (LC) indicates the acute toxicity of a substance. At 50% level of toxicity (LC_{50}), determined by GraphPad Prism 6.0, utilizing the brine shrimp lethality assay as a tool, both *A. heterophylla* fresh and dry stem bark oils showed similar toxicity with LC_{50} of 10 ppm each with upper and lower limits of 10.13 ppm and 9.87 ppm taken as well for the two oils. LC_{50}'s above 1000 ppm, between 500-1000 ppm, and between 100-500 ppm implies a non-toxic, less toxic and high toxic property, respectively (Aboaba *et al*., 2013). For both fresh and dry stem bark oils (LC_{50} = 10 ppm), the lethal concentration is said to be in the high toxic range, indicating the presence of active constituents. Some compounds are commonly present in the two essential oils with relatively similar concentrations to suggest the similarity in

toxicity by a way of synergism. They include spathulenol, germacrene B, aromadendrene, δ-cadinene, copaene (major constituents), γ-elemene, α-cadinol, β-gurjunene and α-cubebene (minor constituents).

The oils from two *Araucaria* plant species (*A. bidwillii* and *A. heterophylla*) exhibited antiproliferative effect in a dose-dependent manner. The oils inhibited proliferation of three different types of human cancer cell lines (Caco-2, Hep-G2, and MCF-7 cells) and the observed cytotoxic effect was comparable with that of the reference drug, Doxorubicin. The essential oil of *A. heterophylla* (the major constituent being α-pinene) had better cytotoxic activity (IC_{50} of 0.7 ppm) of the two species against Hep-G2 cell line. The significant cytotoxic effect on the three cancer cell lines was attributed to their major constituents (Elkady & Ayoub, 2018). However, of further importance are the minor constituents, which could act synergistically to increase the effect of the major compounds. It is worth mentioning that the chloroform extracted resin exudate of *A. heterophylla*, and two diterpene isolates from this resin exudate showed strong and moderate cytotoxic activity, respectively, against breast (MCF7) and colon (HCT116) cancer cell lines. Comparable to that of the reference drug Doxorubicin^R, the strong *in vitro* cytotoxic effect of the resin extract brought the suggestion of possible synergistic effect of the diterpenes present in the resin (Abdel-Sattar *et al*., 2009).

The tricyclic sesquiterpenoid, Spathulenol (5,10-cycloaromadendrane), is an active component of many volatile oils from plants, known to possess various pharmacological activities such as antimicrobial, antioxidant, antiseptic, anti-nociceptive, immunomodulatory and wound healing properties (Manjima *et al*., 2021). Based on a report by do Nascimento *et al*. (2018), *Psidium guineense* essential oil constituents were dominated by spathulenol (one of the dominant compound in this study). Spathulenol was further isolated from the *P. guineense* essential oil. The *P. guineense* oil and isolate, spathulenol, demonstrated moderate to good antiproliferative activity against some human cell lines such as the glioma (U251), breast (MCF-7), ovarian (NCI-ADR/RES), ovarian (OVCAR-3), renal (786-0), prostate (PCO-3), leukaemia (K-562), lung (NCI-H460), colon (HT-29), and keratinocytes (HaCaT) cell lines. It was reported that both *P. guineense* oil and spathulenol were particularly effective against the ovarian (OVCAR-3) cancer cell line and the activity of the essential oil was attributed to synergistic effects associated with other compounds present. According to Ferrer *et al*., (2016), following the chemical characterization of a pooled fraction of extracts from the leaves of *Dasymaschalon dasymaschalum*, strong cytotoxic activities against human lung cancer cell lines (NCI-H187) was reported when (-)-spathulenol was isolated. Furthermore, reports stated that essential oils from many plants enriched in spathulenol exhibited moderate to potent cytotoxic activity against several cancer cell lines such as the human ovarian carcinoma, the human hepatocellular carcinoma and the colorectal cancer (Ferrer *et al*., 2016; Hosseini *et al*., 2021).

The essential oil of *Abrus precatorius* L. Gaertn. was reported to exhibit high cytotoxicity using the brine shrimp lethality test whereby Germacrene B was identified as one of the principal components of the oil. According to Hong *et al*. (2014), Germacrene B exhibit good cytotoxic activity against human ovarian cell line A2780 (Oladimeji *et al*., 2016). Moreover, *Libanotis transcaucasica* Schischk. essential oil, which constitutes germacrene B as the most abundant compound, followed by an isomer of spathulenol (isospathulenol), also exhibited weak to moderate cytotoxic activity in the human cancer cell lines (Shahabipour *et al*., 2013).

The essential oils of Norfolk Island pine obtained from fresh and dry stem bark, were characterized and reported to show potent cytotoxicity on *A. salina*. Because of the difference in the physical state of the plant samples utilized, the fresh and dry stem bark essential oils showed significant variation in their chemical composition. However, similar compounds were identified in both oils as minor and major compounds, which could be said to influence the lethality observed for the oil extracts. Therefore, the remarkable preliminary cytotoxic effect can be considered due to the presence of some plausible cytotoxic compounds. In treating cancer-related diseases, natural essential oils of *A. heterophylla* could hold promise for future

applications. However, further research such as *in vivo* experimental models should be explored to evaluate the effectiveness of the EOs and their constituents.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Precious O. Akinola: Plant sampling and identification, Resources, Experimental studies, Formal analysis, and Writing - original draft. **Akinsola Akande**: Visualisation, Resources, Editing the original draft, Manuscript review. **Sherifat Aboaba**: Conception, Methodology, Resources, Manuscript review, Editing, Supervision and Validation.

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Research Article

Chemical and bioactive potential of the nests of *Polistes nimpha*, *Polistes dominula*, and *Vespa crabro* (Hymenoptera: Vespidae)

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Abstract: This work was accomplished to establish the chemical components and bioactivity potential of the nest materials of *Polistes nimpha* (Christ), *Polistes dominula* (Christ), and *Vespa crabro* (L.). The biological and chemical compounds of materials and their molecular functionalities were detected using FRAP (ferric reducing antioxidant power) method, DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method, and Energy Dispersive X-Ray Analysis. Finally, the bioactivity potentials of nest extracts were assayed and phenolic components were determined. C, N, O, Na, Ca, K, Mg, Al, Si are the elemental components of the nest materials. All nest extracts of three species had high biological activity against nine bacteria and one fungus causing common infections. The maximum antibacterial and antifungal activity was seen when gram-negative *Pseudomonas aeruginosa*, gram-positive *Bacillus cereus*, and gram-positive *Candida albicans* were exposed to ethanol extracts of nest samples in 25 µL. High antioxidant activity can be found in the nest extracts. These extracts might help researchers find novel antifungal and antibacterial substances.

1. INTRODUCTION

Antioxidants are substances that inhibit oxidation, a chemical reaction that produces free radicals which can damage cells. Antioxidants neutralize these free radicals, thereby preventing or reducing cell damage. Antimicrobial activity refers to the ability of a substance to kill or inhibit the growth of microorganisms, such as bacteria, fungi, and viruses. Antimicrobial agents, including antibiotics, antiseptics, and natural compounds like essential oils, play a critical role in preventing and treating infections. This activity is crucial in both medical and food preservation contexts to control harmful microbial growth. All of the eusocial and solitary wasps that are known belong to the Vespidae family. The exquisite nests that Vespidae species use to manage their colonies draw notice. According to research on the nests of eusocial wasps, these nests typically consist of hexagonal cells dangling from a petiole, with the cells typically facing downward (Reeve, 1991; Wenzel, 1998). These cells serve as a breeding environment

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for the wasp as it grows from an egg to an adult. The female wasps construct and enlarge the nests (Jeanne, 1975). The salivary fluids and chewed-up plant and wood fibers make up the paper nest (Evans & West Eberhard, 1970).

In social wasps, the nest is regularly plastered with salivary by the adult wasp to protect the nest from changes in weather conditions (Kudô, 2001). The adult wasp also coats the inside of the brood cell with saliva. The pupating larva secretes and spins the silk. The silk covers the entrance of the brood cell. However, a sterile room is created inside the cell for the pupa to develop, Vespid larvae are exposed to life-threatening pathogens in closed cells (Kirshboim & Ishay, 2020). The possibility of transmission of infections is high due to close contact between wasps living in the colony. Therefore, colonial immunity has developed. Social insects can also produce antimicrobial agents from glands against pathogens (Bot *et al.,* 2002; Turillazzi *et al.,* 2004). Honey, royal jelly, and propolis as bee products have high antimicrobial activity (Anderson *et al.,* 2011). Venom components of some ants, bees, and wasps exhibited antimicrobial activity (Choi & Lee, 2020; El-Seedi *et al.,* 2020; Yacoub *et al.,* 2020; Wen *et al.,* 2021). Nevertheless, there is only a little evidence available about the antibacterial and antioxidant qualities of Vespidae nesting materials.

Materials from the nests of *V. crabro*, *P. dominula*, and *V. crabro germana* were reported to have antibacterial and antioxidant activities (Erturk & Bagdatli, 2019; Ertürk & Şimşek, 2020). Physicochemical properties of the nests of *Vespa orientalis*, *Vespa crabro* (Bagriaçık, 2011); *Polistes gallicus*, *Polistes dominulus*, *Polistes nimpha* (Bagriaçik, 2012, 2013a); *Dolichovespula media*, *Dolichovespula sylvestris* (Bağriaçık, 2013b); *Dolichovespula saxonica* (Ertürk, 2017); *Vespa crabro* (Ertürk & Bağdatlı, 2018) in Türkiye from different regions were previously determined.

In this study, we sought to explain the chemical characteristics and biological activity of the nest components of three species *P. dominula, P. nimpha*, and *V. crabro* found in Türkiye's Black Sea region, as well as to identify the nests' antimicrobial and antioxidant qualities and bioactive potential.

2. MATERIAL and METHODS

2.1. Collecting of Nesting Materials

The nesting materials of P. *nimpha* and P. *dominula* gathered from the stone wall and greenhouse in Terme district of Samsun province (36° 58′ E, 41° 12’ N) and V. *crabro’s* nest from hazelnut tree branches in Trabzon province (39° 43′ E, 41° 00’ N) in July and August 2019. A single nest from each species was researched. Samsun and Trabzon provinces are located in the Black Sea Region of Türkiye with a warm temperate climate, full humidity, and floras (Kottek *et al.,* 2006). Before starting the study, the nests were purified from eggs, pupae, and larvae and stored in the freezer.

2.2. Investigation of Thin Surface Structure

Surface scanning and elemental composition definition analyses of the surface of the nest materials were accomplished with SEM and SEM/EDX techniques with an instrument of SEM-Hitachi, SU1510'. Fixed samples were sputter-coated with a gold layer. Ten to thirty mA electric current was applied during coating for 1 min. little pieces from the hornet and paper wasps nest walls were observed with a stereomicroscope (Leica S8APO).

2.3. Water Vacuum Capacity (%) Determination

The nest examples were cut into small pieces and engrossed in water for one minute. After the submersion process, the samples were reweighed. Percentage capacity of absorption was calculated with the following equation where m1 is the weight of the dried sample before immersion and m^2 is the weight of the sample after immersion: [(m2-m1) / mL] × 100 (Curtis *et al.,* 2005).

2.4. Plant Material and Oral Secretion (%) Determination

The broken pieces of dried nest ingredients were released in 0.5 N KOH solution at 70 °C for two hours. After the process, the samples were filtered with weighed filter papers and kept in a laboratory oven until dry and then the samples were reweighed and the rate of plant material and oral secretion was identified with the following formula where m1 is the weight of the dry sample before the process and m2 is the weight of the sample after the process: Fiber (cellulose) = (m^2 / mL) × 100 (Yamane *et al.*, 1999).

2.5. Oil Content (%) Determination

To calculate the organic compounds like oil and resin in the nests of P. *nimpha*, P. *dominula*, and V. *crabro*, small pieces of paper from the nest were weighed. Each sample was kept engrossed in the petroleum benzine for one hour after which both small pieces of the nest were retrieved. The distinction between the two weights gives us the amount of oily substance. The computation was modified from Yamane *et al.* (1999).

2.6. Bacterial Strains and Growth Conditions

Strains of bacteria and fungi were obtained from ATCC (American Type Culture Collection). The antimicrobial activity of the nest samples was studied using ten bacteria (five gram-positive: *Pseudomonas aeruginosa* ATCC®27853 Gram (-), *Escherichia coli* ATCC®25922 Gram (-), Klebsiella pneumoniae ATCC®13883 Gram (-), Citrobacter freundii ATCC® 43864 (-),*Bacillus subtilis* B209, Gram, Staphylococcus aureus ATCC 6538 Gram (+),*Yersinia enterocolitica* ATCC®27729 Gram (-), *Bacillus cereus* ATCC®10876 Gram (+), *Enterococcus feacalis* ATCC® 29121(+),Mueller Hinton Agar (MHA, Merck) or Mueller Hinton Broth (MHB, Merck) *Candida albicans* ATCC®10231and Sabouraud Dextrose Broth (SDB, Difco) or Sabouraud Dextrose Agar (SDA, Oxoid) were used for growing bacterial and yeast or fungal cells, respectively. For the definition of antibacterial and antifungal efficiency, the diffusion disk plates method was used (Ertürk, 2006). 25µL volume of 46 mg extract 1 mL^{-1} ethanol was used for the analyses.

2.7. Preparation of Extraction of Samples

3 g of a sample placed in an equal volume (30 mL) of 100% methanol was stirred continuously for twenty-four hours at room temperature with a shaker. A syringe filter and filter paper were used to separate the particles (0.45µm). The final concentration of the samples was determined with 100% methanol. The prepared methanolic extract, which was divided into two, was used in antioxidant test and phenolic compound assay.

2.7.1. *Evaluation of total polyphenolic content*

The total polyphenol content (mg GAE/g sample) in the examples was studied using the Folin - Ciocalteu reagent method by Slinkard & Singleton (1977). Following the addition of 680 µL of distilled water to the samples and standards, 400 µL of the 0.2 N Folin-Ciocalteu Reagent, 20 µL of the extract, and the standard solution were added to the mixture. Finally, 400 µL of Na_2CO_3 (10%) was added and vortexed after three minutes had passed. The combination was then allowed to sit at room temperature for 2 hours, after which a UV spectrophotometer was used to measure the mixture's absorbance at 760 nm. The measurement standard utilized was gallic acid. TPC was measured in milligrams of equivalent gallic acid per gram.

2.7.2. *Evaluation of total flavonoid content*

Using the aluminum chloride colorimetry method described by Fukumoto & Mazza (2000), the total flavonoid content (TFC) of extracts was measured. Following that, 2.15 mL of pure methanol was combined with 0.25 mL of both the extracts and the reference solution. 50µL of 10% aluminum chloride and 50 µL of 1M potassium acetate then were added. The mixture's absorbance at 415 nm was measured after 40 min of incubation at room temperature. The

benchmark used was quercetin. The results were presented as milligram quercetin equivalent per gram of material.

2.7.3. *FRAP assays*

The FRAP test was performed using the Benzie & Strain' method (1999). An iron 2, 4, 6-tripyridyl-s-triazine complex (Fe^{3+} -TPTZ) is reduced to its ferrous, colored form (Fe^{2+} -TPTZ) in the presence of antioxidants to create the technique. The method is based on the reduction of to its ferrous, The FRAP reagent contained acetate buffer (300 µM, pH3.6) a solution of 10 µM TPTZ in 40 µM HCl and 20 µM $FeCl_3$. The reagent was prepared daily. 100 µL of samples were mixed with a 3 mL FRAP reagent. The absorbance of the reaction mixture was spectrophotometrically at 593 nm after incubation for 4 min. Standard solutions of $FeSO_4 7H_2O$ (31.25-1000 µM) were used for the calibration curve and the results were expressed FRAP as µmol $FeSO_4.7H_2O$/g.

2.7.4. *DPPH radical scavenging activity*

The method was used for the DPPH assay (Molyneux, 2004). To make the stock DPPH solution, 4 milligram of DPPH was dissolved in 100 mL of methanol. For each sample, 0.75 mL of the extracts were combined with 0.75 mL of the DPPH solution at six different concentrations, and the mixture was vortexed. The mixture's absorbance was measured at 517 nm after the reaction had been allowed to occur at darkroom temperature for 50 min. Trolox served as the reference point for calculations.

2.8. Determination of the Phenolic Composition of Samples by RP-HPLC-UV

20 mL of methanolic extracts that had been prepared for phenolic analysis were received, and they were evaporated using a rotary evaporator at 40° C until dry. 10 mL of acidified distilled water were used to dissolve the residue (pH 2). With consecutive additions of 5 mL of ethyl acetate and 5 mL of diethyl ether, liquid-liquid extraction was performed (Kim *et al.*, 2006). These phases were then dried by rotary evaporation at 40°C (IKA-Werke in Staufen, Germany). The dried sample was redissolved in 2 mL of methanol, passed through RC-membrane syringe filters (0.45 µm), and then injected into the HPLC.

3. RESULTS

3.1. Plant Material (%), Oral Secretion (%), Water Absorption (%), and Oil (%)

The proportion of the plant material to the oral secretion utilized in the building of the nest materials was determined as a percentage. Samples were dowsed in warm KOH solution, the fibers were independent and the oral secretion dissolved. the samples for the *P. dominula* and *V. crabro* nest materials have an analogous proportion of plant material to the oral secretion of % 19.205 and 28.846%, respectively. The amount of oral secretion in nest material of P. *nimpha* was calculated at 30.322%, the water absorption capacity of *P. dominula* and *P. nimpha* is 242.71% and 154.354% while V. *crabro* nest is 283.01%. In addition, the oil percentages of the samples were also determined. The results show that the oil content of the nest of *P. dominula* (15.562 %) is higher than the nest material of *V. crabro (*10.256%). However, the highest oil content was found in *P. nimpha* 18.064 % (Table 1).

Table 1. Water absorption capacity with plant material, oral secretion and oil content of the nests of *V. crabro* (VC) *P. dominula* (PD) and *P. nimpha* (PN).

	(VC)	(PD)	(PN)
Dry weight (mg)	0.312	0.302	0.310
Water absorption capacity (%) determination	1.195 %283.01	1.035 %242.71	0.804 %154.354
Plant material and oral secretion (%) determination	0.222 % 28.846	0.244 % 19.205	0.216 %30.322
Oil content (%) determination	0.280 % 10.256	0.255 % 15.562	0.254 % 18.064

3.2. Surface Properties

3.2.1. Observation of the surface under a light microscope

The basic constructions of the nests' surfaces were monitored with a stereomicroscope. The nest colors of the samples of *P. dominula* and *P. nimpha* were dark brown, black lines, light brown, beige and white highlights whereas the nest colors of the samples of *V. crabro* were brown, light creamcolored tapes with thick stripes, bright brown spots on a dark brown background and rough structure. The honeycomb cover colors of the samples of three wasp species were bright and off-white color. However, the fibers of the *V. crabro* slot had thin, dense, thin spherical structures between the fibers and grooved filaments. The fibers of the nest of *P. nimpha* and *P. dominula* were longer and thicker, there were gaps between the vegetable fibers, the fibers were cross-linked and wavy. Both nest looked very similar to each other. The honeycomb sheaths of the three wasp nests examined were quite different, the fibers were very prominent, and it was found that the structure was persistent on the ground (Figure 1A, 1B, 1C and 1D).

Figure 1A. The outer surface of the section and thickness of the fibers the nestwall of *P. dominula.*

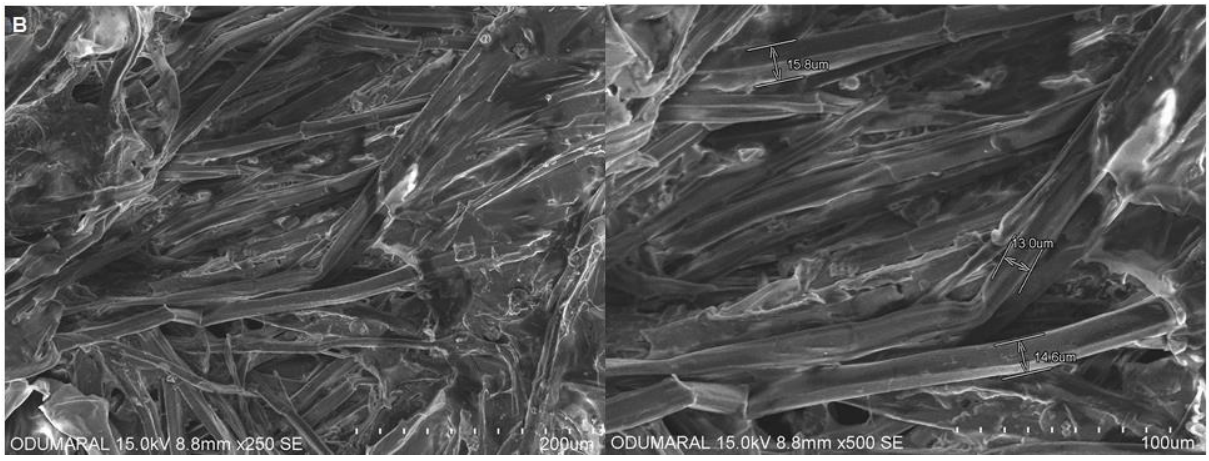

Figure 1B. The inner surface section of the section and thickness of the fibers the nestwall of *P. dominula.*

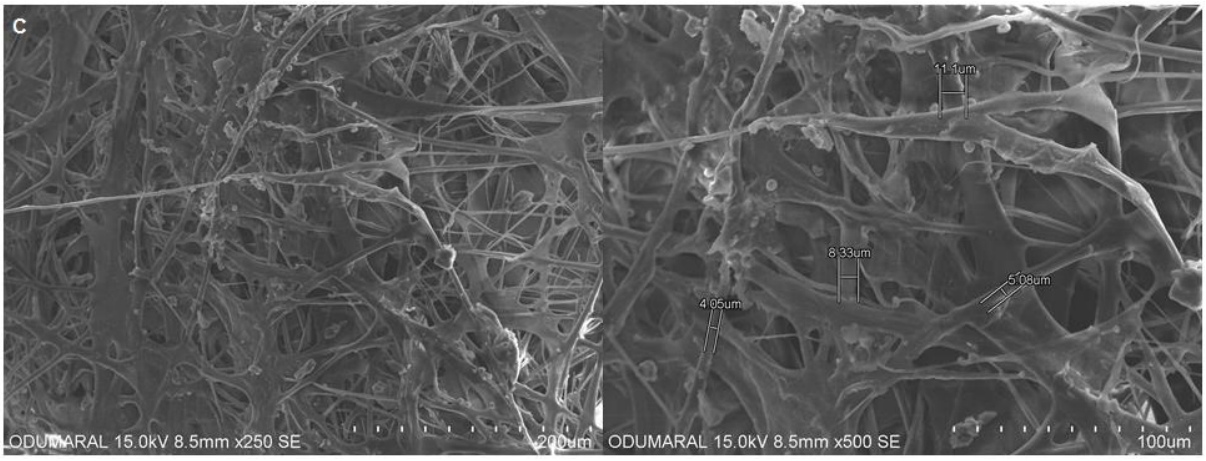

Figure 1C. The thickness of the fibers honeycomb cover of *P. dominula.*

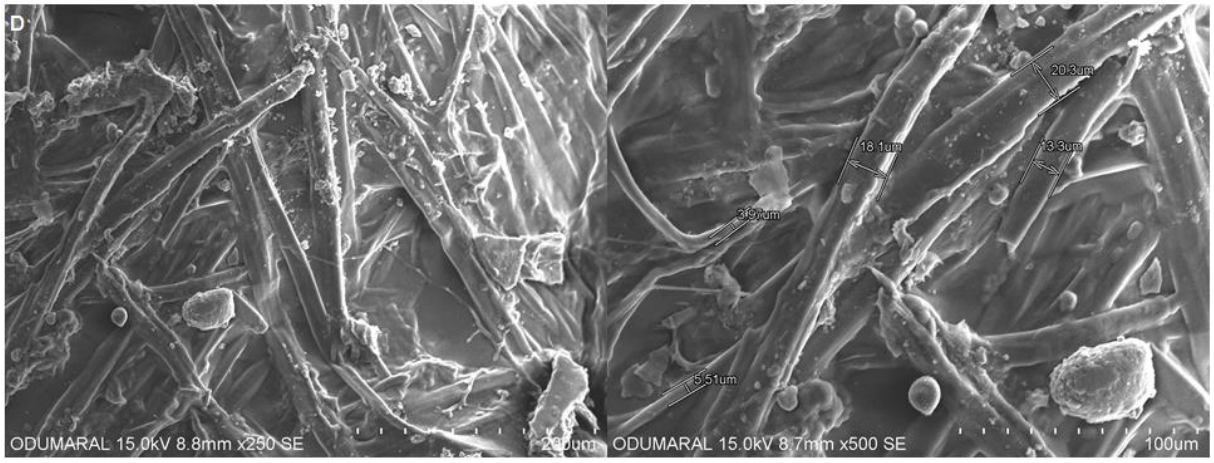

Figure 1D. The outer surface of the section and thickness of the fibers the nestwall of *P. nimpha.*

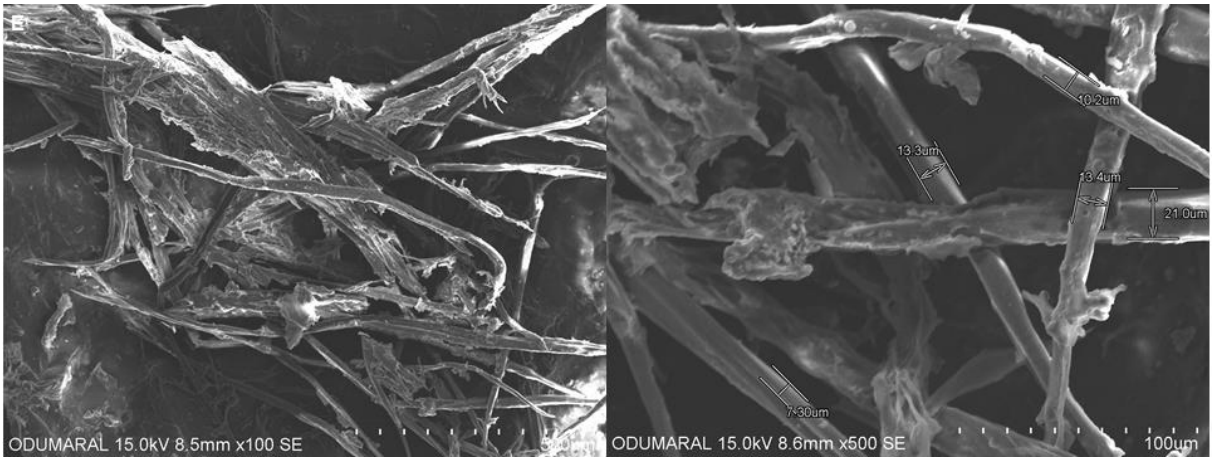

Figure 1E. The inner surface section of the section and thickness of the fibers the nestwall of *P. nimpha.*

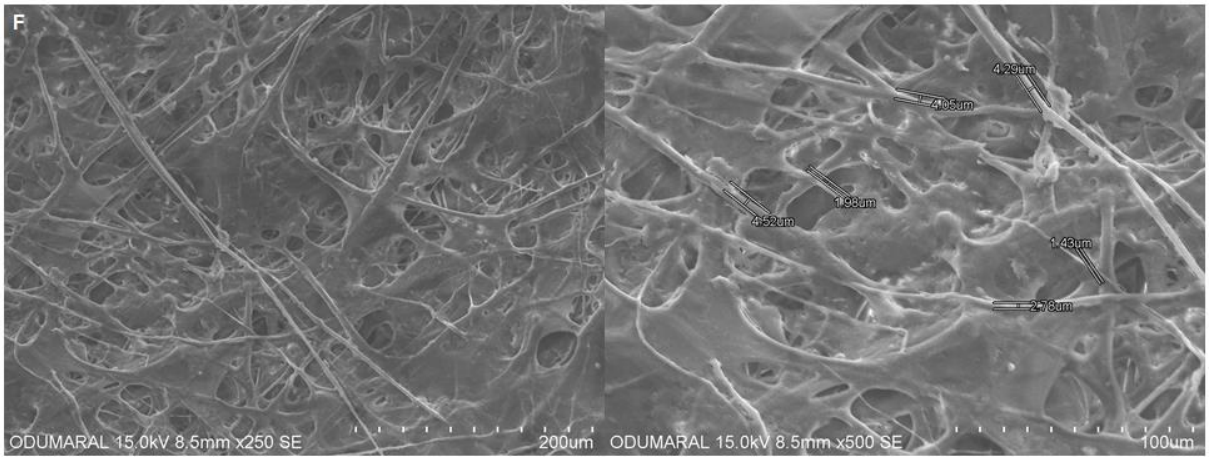

Figure 1F. The thickness of the fibers honeycomb cover of *P. nimpha.*

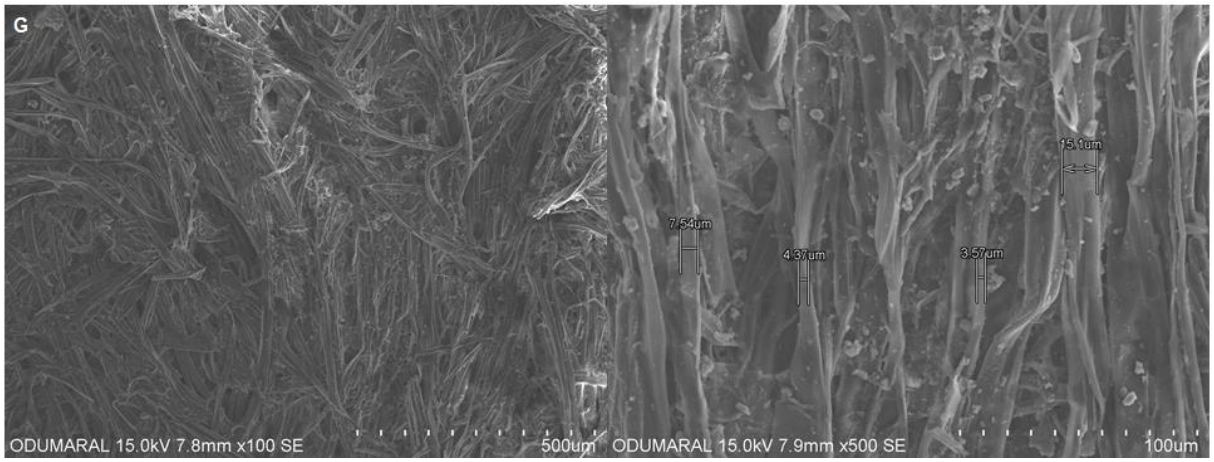

Figure 1G. The outer surface of the section and thickness of the fibers the nestwall of *V. crabro.*

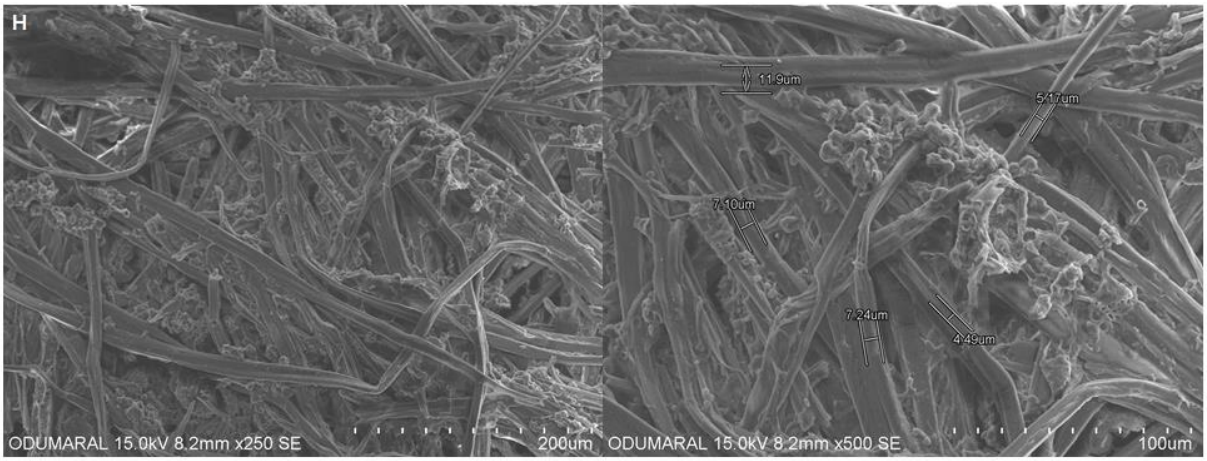

Figure 1H. The inner surface section of the section and thickness of the fibers the nestwall of *V. crabro.*

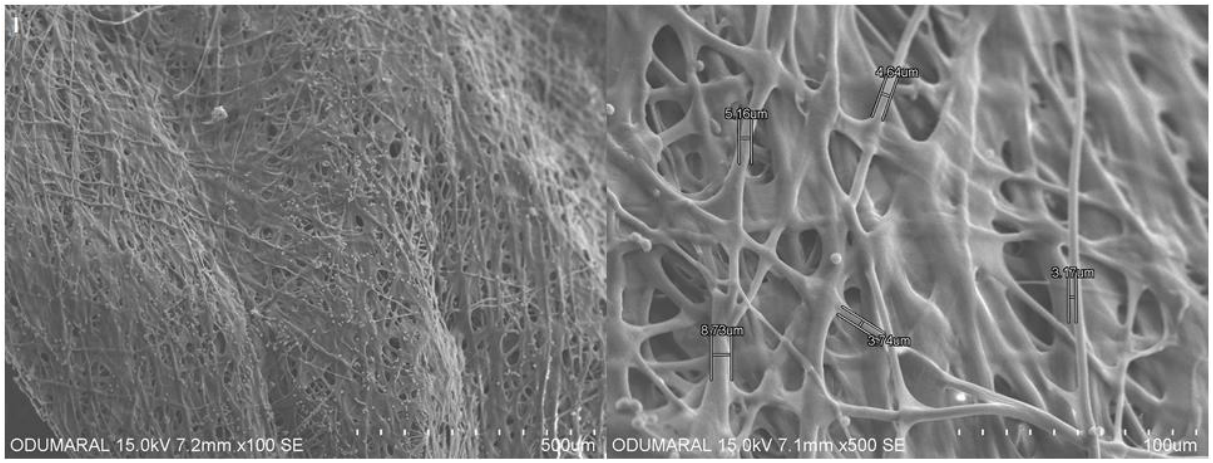

Figure 1I. The thickness of the fibers honeycomb cover of *V. crabro.*

3.2.2. *Observation of surface under SEM*

The oral secretions from *P. dominula*, and *V. crabro* nest were the mixture of salivation and plant fibers was seen as a thin layer. and the saliva including the plant fibers shone as thin varnished beads in the SEM micrographs. Results are as in Table 2. Although *P. nimpha* and *P. dominula* were different species of the same genus, the nest fibers were very different. The oral secretions from *the P. nimpha* nest were seen as a membrane composed of both small and largely organic and inorganic fragments with thick fibers passing through the middle. Many inorganic particles were seen in the SEM micrographs (Figure 1C, 1D) The average fiber thickness of the envelope of *the P. dominula* nest was calculated as 6.03±0.053 µm (min. 5.12±0.543 mm - max. 6.98±0.042 mm) and that of *V. crabro* nest as 7.52±0.052 mm (min. 5.85±0.032 mm - max. 8.15±0.096 mm), (n = 30 for each nest) (Figure 1A, 1B). In contrast, the plant fibers of *P. dominula* were long, thick woody scrapings, that were glued regularly. The plant fibers of *V. crabro* were short, thin woody scrapings. The average fiber thickness of the envelope of *P. nimpha* nest was calculated as 4.10±0.067 µm (min. 3.88±0.345 µm - max. 4.78±8.534 µm) They were intertwined and disordered (Figure 1E-1H). The measurements of the edge length, diameter and depth of the combs' cells of *V. crabro, P. dominula* and *P. nimpha* were as min., max. and average was calculated (Table 3). The surface of the comb of *V. crabro, P. dominula* and *P. nimpha* were medium 254.340 cm^2, 153.860 and 113.040 cm^2 respectively.

Table 2. The thickness of the fibers of the nest (in µm).

Bee species	Big PD			Small PD			TR bee		
Thickness (µm)	outer surface	inner surface	honeycomb cover	outer surface	inner surface	honeycomb cover	outer surface	inner surface	honeycomb cover
Min.	8.3	13.0	4.1	4.0	7.3	1.4	3.6	4.5	3.2
Max.	14.9	15.3	11.1	20.0	21.0	4.6	15.1	11.9	8.7
Avarage	10.9±0.8	14.7±0.7	7.1±0.5	12.8±0.7	13.1±0.1	3.9±0.7	7.7±0.3	5.1±0.6	5.1±0.5

3.3. Antimicrobial Test

The antibacterial and antifungal activities of extracts of the three different nests were assayed in vitro agar disc diffusion method against 9 bacteria and one fungi species. Table 3 summarizes the microbial development inhibition of the ethanol extracts of the screened nests. The three ethanol nest extracts demonstrated antibacterial and antifungal activity. While the ethanol extracts of almost all the nests showed antibacterial and antifungal activity towards one or more bacterium against all the microorganisms used in this study. The alcohol PD-and VC-A extract of the nest demonstrated antibacterial activity (18.59±0.54 and 20.59±0.021 mm 25 μL^{-1} inhibition zone) against S. *aureus*. and B. *ceraus*, respectively, while the alcohol PN-A extract

of the nest demonstrated the highest antibacterial activity (19.13±0,53 -25.66±0.21, mm 25 μL^{-1} inhibition zone) against, S. *aureus* and B. *ceraus*, respectively, and the highest antifungal activity (19.45±0,00 mm 25 μL^{-1} inhibition zone) against C. *albicans*. The ethanol extracts of the investigated nest samples of 25 µL (from 1 mg mL^{-1}) showed maximum antibacterial and antifungal activity against gram-negative *P. aeruginosa*, gram-positive *B. cerausand C. albicans* (see Table 3).

Table 3. Results of antimicrobial screening of hornet and wasp nests' *V. crabro, P. dominula* and *P. nimpha* extracts determined by the agar diffusion method; VC-A, PD-A, PN- A V. *crabro*, P. *dominula* and P. *nimpha* extracts in ethanol respectively

Bacterias	*Vespa crabro*	*Polistes dominula*	*Polistes nimpha*	Ampicillin	Cephazolin	Nystatin
E. coli	14.20±0.54	17.78±0.46	16.80±0.64	19.00±0,00	19.00±0.00	NT
C.freundii	6.00±0.00	6.00±0.00	6.00±0.00	16.53±0.55	16.36±0.06	NT
P. aeruginosa	15.84±0.46	17.66±0.33	16.33±0.56	32.26±0.46	28.33±0.03	NT
K. pneumoniae	14.27±0.78	14.48±0.54	15.70±0.23	15.27±0.10	17.27±0.01	NT
S. aureus	18.59±0.54	18.32±0.72	19.13±0.53	10.76±0.45	6.00±0.03	NT
B. subtilis	16.60±0.73	15.61±0.75	17.81±0.33	32.60±0.32	34.26±0.11	NT
Y.enterocolitica	6.00±0.00	6.00±0.00	6.00±0.00	26.66 ±0.57	34.33 ±0.57	NT
B. ceraus	20.59±0.02	20.50±0.34	25.66±0.21	26.59±0.021	27.50±0.03	NT
C. albicans	19.45±0,00	18.53±0.00	15.50±0.12	NT	NT	17.00±0.00
E. fecalis	14.53±0.55	13.23±0.23	13.32±0.42	32.50±0.06	24.27±0.04	NT

-: no inhibition, NT: Not tested,.*Pseudomonas aeruginosa* ATCC®27853 Gram (-), *Escherichia coli* ATCC®25922 Gram (-), *Klebsiella pneumoniae* ATCC®13883 Gram (-),*Citrobacter freundii* ATCC® 43864 (-),*Bacillus subtilis* B209, Gram, *Staphylococcus aureus* ATCC 6538 Gram (+),*Yersinia enterocolitica* ATCC®27729 Gram (-), *Bacillus cereus* ATCC®10876 Gram (+), *Enterococcus feacalis* ATCC® 29121(+), *Candida albicans* ATCC®10231

3.4. EDX Analysis

The outcomes demonstrate that the main elements of the surfaces are carbon, oxygen and nitrogen followed by potassium and calcium atoms in descending order (see Table 4). Minor amounts of sodium, magnesium, iron, aluminum, chlorine and silicium were also detected. P. *dominula's* inner and outer membrane was not found in nitrogen. In contrast, high levels of nitrogen were found in comb materials of a nest of *P. nimpha* and *V. crabro*. However, nitrogen was found to be highest in the honeycomb cover of all three wasp nests. EDX spectra for the inner surface of *P. dominula* were shown in Figure 2 as a representative example.

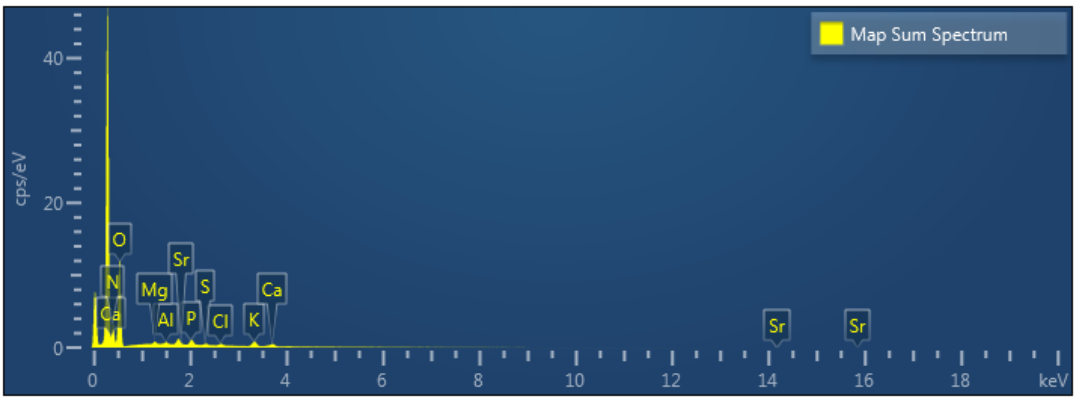

Figure 2. EDX spectrum of the elements embedded in the inner comb surface of *V. crabro (VC) P.nimpha (PN)* and *P. dominula (PD).*

In this essay, elemental analysis was carried out with extracted samples of nest materials. Concerning the outcomes of the elemental analysis (see Table 4), it is shown that the greatest nitrogen content was in VC- PD, honeycomb cover material, while the PN of honeycomb cover material is less than the other nest material. The highest carbon content in the PN-outer surface, the highest oxygen content in PD- inner surface and the highest potassium.

Table 4. EDX analyses of the inner and outer parts of the comb surfaces and honeycomb cover *V. crabro* (VC) *P. nimpha* (PN) and *P. dominula* (PD).

	Weigth %			Weigth %			Weigth %		
Element	*P. dominula* outer surface	*P. dominula* inner surface	*P. dominula honeycomb cover*	*P. nympha* outer surface	*P. nympha* inner surface	*P. nymphaof honeycomb cover*	*V. cobra* outer surface	*V. cobra* inner surface	*V. cobra of honeycomb cover*
C	58.75	54.17	52.03	55.87	52.98	52.71	55.12	52.79	43.10
O	40.16	45.32	27.05	26.85	33.43	29.94	30.89	32.48	30.07
Si	0.23	0.00	0.00	0.32	1.51	0.00	0.09	0.52	0.00
Na	0.11	0.00	0.00	0.00	0.00	0.00	0.03	0.05	0.00
Ca	0.21	0.16	0.00	0.33	0.05	0.42	0.06	0.06	0.08
K	0.22	0.29	0.30	0.54	0.24	0.34	0.04	0.22	0.00
P	0.07	0.06	0.16	0.36	0.10	0.34	0.04	0.14	0.01
S	0.07	0.00	0.18	0.12	0.07	0.09	0.04	0.09	0.06
Mg	0.04	0.00	0.00	0.13	0.00	0.29	0.03	0.07	0.09
Al	0.10	0.00	0.05	0.10	0.00	0.00		0.00	0.00
CI	0.03	0.00	0.16	0.15	0.07	0.41	0.08	0.08	0.38
N	0.00	0.00	20.06	15.11	11.55	15.47	13.47	13.50	26.21
Sr	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00
Total	100	100	100	100	100	100	100	100	100

3.5. Total Phenolic and Total Flavonoid Content

Electron transfer-based methods measure the ability of the antioxidant to reduce oxidants by color change. The antioxidant concentration of the samples and the degree of color change are related. ET-based methods include total Folin-Ciocalteu reagent and total phenolic method, iron ion reducing antioxidant power (FRAP), DPPH (2,2-diphenyl 1-picrylhydrazyl). In this study, nest materials belonging to *P. nimpha and P. dominula* species collected from Samsun *and V. crabro* species collected from Trabzon were used.

To study, nest materials belonging to *P. nimpha* and *P. dominula* species collected from Samsun *V. crabro* specie collected from Trabzon. The phenolic contents of 3.121 *P. nimpha*, 1.881 *P. dominula* and 0.635 mgGAE / g *V. crabro* were determined. For Total flavonoid content, *P. nimpha* had the highest amount of TFC (0.853), followed by *P. dominula* 0.425 mgQE/g while *V. crabro* could not be found (see Table 5).

Table 5. Total Polyphenol, Flavonoid Content, FRAP and DPPH tests results of samples.

Samples	TP (mgGAE/g sample)	TF (mgQE/g sample)	FRAP (µmol$FeSO_4 7H_2O$/g	DPPH SC_{50} (mg/mL)
P. *nympha*	3.121±0.017	0.853±0.002	24.645±0.104	3.185±0.061
P. *dominula*	1.881±0.054	0.425±0.006	21.639±1.356	4.325±0.027
V. *crapro*	0.635±0.067	-	11.978±0.075	6.338±0.029
Trolox				0.0004±0.000

3.6. FRAP and DPPH Assays

The FRAP test is a measure of the sample's Fe^{+3} Fe^{+2} reduction capacity. The samples from the *P. nimpha* and *P. dominula* showed a high ferric ion reducing capacity, 24.645 and 21.639 µmol$FeSO_4 7H_2O$/g whereas *V. crabro* the low 11.978 µmol$FeSO_4 7H_2O$/g (see Table 5).

The radical scavenging ability of samples was determined by DPPH decoloration assays in comparison with Trolox. The DPPH radical scavenging test measures the free radical scavenging activity of samples in solution, by granting one electron (Huang *et al.*, 2005). The radical scavenging activity of the samples was found to be 3.185 *P. nimpha*, 4.325 *P. dominula*, and 6.338 *V. crabro* mg / mL (see Table 5).

3.7. Phenolic Compounds

High-performance liquid chromatography (HPLC) is the most widely used for the characterization of polyphenolic compounds. In this study, the phenolic composition of the samples was determined by RP-HPLC-UV. Epicatechin, resveratrol, and rutin could not be detected in all three samples, while other components were detected in samples at different rates (see Table 6). As major components were protocatechuic acid, chrysin, myricetin, *P. nimpha* catechin, protocatechuic acid, chrysin, caffeic acid, *P. dominula* chrysin, myricetin was detected in *V. crabro*. In all three samples, chrysin was detected as the major component.

Table 6. Phenolic compounds of samples.

Standards	*P. nympha* (µg phenolic/g sample)	*P. dominula* (µg phenolic/g sample)	*V. crapro* (µg phenolic/g sample)
Gallic Acid	2.043	1.302	n.d.
Protocatechuic Acid	35.304	35.256	2.651
p-OH Benzoic Acid	13.208	13.284	2.461
Catechin	28.644	20.418	6.413
Caffeic Acid	10.144	42.022	7.045
Syringic Acid	15.382	21.931	4.778
Epicatechin	n.d.	n.d.	n.d.
p-Coumaric Acid	28.002	15.106	5.123

Table 6. Continues.

Ferulic Acid	11.606	8.284	16.849
Rutin	n.d.	n.d.	n.d.
Myricetin	30.627	15.456	26.400
Resveratrol	n.d.	n.d.	n.d.
Daidzein	1.821	n.d.	2.391
Luteolin	17.282	10.573	9.476
t-Cinnamic Acid	n.d.	0.423	0.751
Hesperetin	1.791	2.001	3.026
Chrysin	30.068	22.326	29.652
Pinocembrin	18.054	4.617	3.541
CAPE	20.615	1.685	4.301

*n.d.:not detected.

4. DISCUSSION and CONCLUSION

The topic materials of the work are *P. nimpha*, *P. dominula* and *V. crabro* nests. This work primarily consists of several parts. In the first step, the physicochemical feature of the nest materials was investigated in terms of water absorption capacities, oral secretion, plant lift material ratios and oil substance percentage. Then, SEM and stereomicroscope were used to establish the surface of nest properties, and the surface chemical content was established by EDX technique and elemental decomposition techniques were used. At the last stage, antioxidant and antimicrobial activity works were accomplished with the extracts of the materials in distinct polarities. Bagriacik (2011), determined the water absorption capacity of the nest materials of Turkish origin as 91% and 100% for *V. orientalis* and *V. crabro*, respectively. Ertürk & Bağdatlı (2019), the water absorption capacities of the nests were 100% and 53.19% for *P. dominula* and *V. crabro* nest materials, respectively. In this work, the water absorption capacities of the nests were 283.01%, 242.71% and 154.354% for *V. crabro*, *P. dominula* and *P. nimpha* nest materials, respectively. The material from which bee nests are built generally depends on the ecological and vegetative vegetation it depends on. The results we found in our study may be different from other studies and the use of different plant species in the nest. The water absorption capacity of nest material of *V. crabro* and *P. dominula* is very similar to each other. According to the oil content, plant material, oral secretion, and oil content (%) determination, plant material-oral secretion ratios of the samples nest material of *V. crabro* and *P. nimpha* were similar, *V. crabro* 28.846%, %30.322 % whereas *P. dominula* sample of nest material was different 19.205. However, the oil content was similar between the two paper wasps, but the oil rate of *V. crabro* nest material was very different detected. These results indicated that the plant material, oral secretion and oil content of nest materials of *P. nympha*, *P. dominula* and *V. crabro* are not related to the species, but the fact that the nest depends on the environment and depends on the plant. It is expected that these two species have different nest characteristics. The fact that species belong to the same genus does not mean that the nest structures will have the same structure. This situation could be emphasized if changes in nest characteristics could be observed in different populations of the same species.

Paper wasps structure umbrella-figurated nests hanging underneath fringe and protrusion. Bald-faced hornets construct large, football-shaped nests. Construction begins with finding suitable support for the nest – a window shutter, a tree branch, or a root in the case of subterranean nests. regardless of where a wasp constructed its nest or what figured the nest is, the process wasps handling to establish their nests is usually similar.

Paper wasps and hornets all build paper nests, even though the dimensions, figures, and positions of their nests differ. Most social wasps construct combs from herbal plant fiber, mud and oral secretions (Wenzel, 1991). The comb consists of a particular unit, the cell, which is generally hexagonal and contains only one offspring. This modular planning and organized

structure are influential amongst wild social wasps and some bees. Despite these modular structures, the combs of wasp image large variety, both inter-and intra-particularly. At present, the problem of microbial strength is rising. Therefore, some precautions should be taken, such as controlling antibiotic use, conducting modern work to better understand the genetic mechanisms of resistance, and continuing research for new synthetic or inherent drugs. The aim is to propose antimicrobial drugs that are suitable and influential for the patient. In this research, all three nest extracts have potential against microorganisms as antimicrobial compounds. So, they can be used in the cure of contagious diseases reasoned by resistant microorganisms. Therefore, our outcomes once again highlight the significance of effective native extracts against antibiotic-resistant bacteria that pose a threat to human health. To the best of our knowledge, this is the first comprehensive study on the nesting materials of all three species originating from the Black Sea region: *Vespa crabro*, *Polistes dominula,* and *Polistes nimpha*.*V. crabro, P. dominula* and *P. nimpha*. The nest materials of the three species with the same origin have distinct specifications like physical appearance, water absorption capacity, the proportion of plant material to oral secretion, and their bioactive and spectral features. Found some choice of foundresses in correlation to these three parameters. Nests are most frequently started on *Hypericum* spp., *Tanacetum* spp., *Daucus spp.,* and *Achillea* spp., plants. These plants have antimicrobial properties. The highest antimicrobial activity of the essential oil of *Tanacetum walteri* was observed against *Staphylococcus aureus, Enterococcus faecalis* and *Klebsiella pneumoniae* with MIC value of 0.63 mg/mL. In some studies, bees start building nests with more Hypericum spp., Tanacetum spp., Daucus spp., and Achillea spp., plants. Therefore, these plants are found in bee nests. These herbs have antimicrobial properties. The highest antimicrobial activity of *Tanacetum walteri* essential oil was observed against *Staphylococcus aureus, Enterococcus faecalis* and *Klebsiella pneumoniae* with a MIC value of 0.63 mg / mL (Ghaderi & Sonboli, 2019). Finally, aqueous solutions of *Hypericum perforatum* teas were found to be antimicrobial against gram-positive bacteria with specific activity against methicillin-resistant strains of Staphylococcus aureus (Reichling *et al.,* 2001). (Ghaderi & Sonboli, 2019). Latterly, hydrous solutions of *Hypericum perforatum* teas were found to be antimicrobial effective against gram-positive bacteria with special activity towards methicillin-resistant strains of *Staphylococcus aureus* (Reichling *et al.,* 2001). In conclusion, the three nest ethanol extracts have an extensive spectrum of efficiency against a panel of bacteria accountable for the most common bacterial diseases. These promising extracts open the possibility of evidence of new clinically influential antibacterial and antifungal compounds.

Determination of the activities of products with antioxidant properties is important in the use of many areas. In this study, especially the previously unstudied *P. nimpha, P. dominula* and *V. crabro* were determined of biological activities.

The total phenolic content of samples as gallic acid equivalent, *P. nimpha* was found 3.121 mg/g, *P. dominula* for the sample to 1.881 mg/g and *V. crabro* for the sample 0.635 mg/g. In another study total phenolic content was found of *V. crabro* and *P. dominula* nests collected from the Black Sea region ranged from 6.41 to 11.93 mg GAE/g (Ertürk & Bagdatli, 2019). The total phenolic content of the samples in our present study was found to be lower in the literature. Total flavonoid content was determined in the study was 0.853, 0.425 mgQE/g *P. nimpha* and *P. dominula* respectively, while the amount of flavonoid substance in *V. crabro* was not detected.

One of the antioxidant methods is the most widely used FRAP method. This study measured Fe^{+3} Fe^{+2} reduction capacity in the samples. FRAP results were determined linearly proportional to total phenolic and flavonoid content. Again, the highest activity was determined 24.645 µmol$FeSO_4 7H_2O$/g in P. *nympha* bee nests.

The DPPH has been widely used to determine the free radical scavenging ability of the samples. The free radical scavenging activity of the samples depends on their hydrogen-donating ability (Silva *et al.,* 2006). The highest effective sample extract was *P. nimpha*, *P. dominula* and the

lowest was *V. crabro* with SC_{50} 3.185, 4.325 and 5.87 mg/mL, respectively. In the study conducted by Ertürk & Bagdatli (2019), DPPH results of *Vespa crabro* and *Polistes dominula* nests were found to be an experiment and IC_{50} values for BHA and ascorbic acid are 175.87 ± 1.34 and 156.87 ± 1.62 µg ml^{-1}, respectively.

In this study, phenolic contents were determined using an RP-HPLC-UV device. HPLC analysis of phenolic compounds in wasp nests from the Black Sea region in Türkiye showed that many phenolic compounds were present in all wasp nests analyzed.

The concentrations of individual phenolic compounds in wasp nests are presented in Table 6. As major components were found chrysin, myricetin in V. *crabro*. Chrysin was determined as a major component in three samples. Chrysin and Myricetin were found as major components in *V. crabro*. On the other hand, Chrysin was found to be the main component in all three samples. It has been found in studies conducted with chrysin flavonoids that it has strong anti-inflammatory and antioxidant properties and promotes cell death by inhibiting cell cycle progression (Samarghandian *et al.*, 2011). Protocatechuic acid was found 35.304 and 35.256 µg phenolic/g sample in a nest of *P. nimpha* and *P. dominula* nest, respectively. Chrysin was found in 29.652 µg phenolic/g sample in *V. crapro* bee nest. Chrysin is found in excess amounts in plants which were reported to have many biological activities including antibacterial, antioxidant, antiinflammatory, antiallergic, anticancer, antiestrogenic activities (Babu *et al.*, 2006). Chrysin has an inhibitory effect on the tyrosinase enzyme (Kubo *et al.*, 2000). Accordingly, it can be thought that wasp nests in the study may be a potential tyrosinase inhibitor.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Zehra Can: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. Supervision, and Validation. **Ömer Erturk**: Methodology, Formal Analysis, and Writing -original draft. **Mustafa Yaman**: Methodology, Formal Analysis, and Writing -original draft.

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Research Article

Chemical compositions of essential oils, antimicrobial effect and antioxidant activity studies of *Hyoscyamus niger* L. from Türkiye

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Abstract: This study investigated the essential oil components of *Hyoscyamus niger* L. and their antimicrobial and antioxidant properties. Essential oils were extracted separately from the aerial parts and seeds of the plant using the hydrodistillation method. Antimicrobial activity was evaluated using the disk diffusion method, while antioxidant activity was assessed by measuring the total antioxidant status (TAS), total oxidant status (TOS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity. The primary essential oil components of the aerial parts were identified as phytol (52.09%) and hexahydrofarnesyl acetone (19.66%). Hydrodistillation of the seeds yielded 0.7% (v/w) yellow oils, comprising 41 components that accounted for 99.0% of the oil. The major components in the seed oils were hexahydrofarnesyl acetone (46.36%) and hexanal (9.05%). Methanol extracts of the aerial parts demonstrated inhibitory effects on pathogenic microorganisms, with inhibition zones ranging from 13±0.46 to 32±0.11 mm. The TAS and TOS values of the methanol extracts were calculated as 3.77±0.0 mmol and 6.94±0.0 µmol, respectively. The DPPH radical scavenging activity increased with rising extract concentrations. These findings highlight the potential antimicrobial and antioxidant applications of *H. niger* essential oils and methanol extracts.

1. INTRODUCTION

Today, medicinal plants continue to play a vital role in modern medicine and pharmacy, serving as valuable natural sources of pharmaceutical raw materials due to their essential oils and secondary metabolites (Akbaş *et al.*, 2020). The Solanaceae family, which includes many important medicinal plants, is a diverse plant family consisting of 98 genera and approximately 2,700 species. Plants from the Solanaceae family have been utilized for centuries by humans as sources of food, ornamental plants, and medicinal remedies (Guha & Maheshwari, 1966). This family is particularly notable for its richness in alkaloids with potent pharmacological effects, such as scopolamine, atropine, and hyoscyamine, making it highly significant both medically and economically (Maiti *et al.*, 2002).

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Hyoscyamus niger L. (black henbane) is an annual or biennial herbaceous plant belonging to the Solanaceae family. It is commonly found in rocky areas, uncultivated lands, and especially along roadsides (Orbak *et al.*, 1998; Li *et al.*, 2011; Yücel & Yılmaz, 2014). This plant is a valuable resource for the pharmaceutical industry due to its alkaloid content, which includes some of the oldest drugs used in medicine (Orbak *et al.*, 1998; Pudersell *et al.*, 2012; Dehghan *et al.*, 2012). Traditionally, *H. niger* has been used to treat various ailments, including stomach pains, ulcers, and kidney and liver disorders, and is known for its analgesic and antispasmodic effects (Tanker *et al.*, 1998; John *et al.*, 2010). However, the consumption of its roots and leaves can lead to poisoning, as some of its secondary metabolites paralyze the nerve endings of the parasympathetic system in humans (Orbak *et al.*, 1998).

In addition to its medicinal applications, *H. niger* has a history of being used as a hallucinogen. This usage dates back to ancient times, as evidenced by ancient Greek writings, and the plant's hallucinogenic properties were introduced to Europe in the Middle Ages. The seeds and leaves of *H. niger* contain hyoscyamine, scopolamine, and other tropane alkaloids, which are responsible for its hallucinogenic effects as well as symptoms like ataxia and hypertension (Ugur, 2013). Due to its toxicity, *H. niger* is considered extremely poisonous and undesirable for consumption, and it has been historically used as a poison (Haas, 1995).

Despite its toxic nature, recent studies have highlighted potential therapeutic applications of *H. niger*. For instance, Kosari *et al*. (2021) conducted a clinical study in Iran in which both a placebo and a methanolic extract of *H. niger* were administered to 50 participants. The extract was given three times a day for six days, and the results showed beneficial effects in improving COVID-19 symptoms. Symptoms such as dry cough, shortness of breath, sore throat, chest pain, fever, dizziness, headache, abdominal pain, and diarrhea were reported to have improved more significantly in the group receiving *H. niger* extract combined with propolis than in the placebo group.

This study aims to determine the biological activity characteristics of *H. niger*, a medicinal plant. With the growing need for natural therapeutic resources against diseases, identifying the biological properties of plants that grow naturally in specific regions can support their use as valuable pharmaceutical raw materials. In this study, the essential oil properties, antimicrobial activity, and antioxidant properties of *H. niger* are evaluated together for the first time.

2. MATERIAL and METHODS

2.1. Collection of the Plant Material

The flowering period of *H. niger* occurs from May to June. During this period, plant materials were collected from the Baskil district of Elazığ province and preserved through shade drying. Following the collection, the plant materials were identified at the Fırat University Herbarium (FUH) using the Flora of Turkey as a reference. The identification was carried out by Şemsettin Civelek. The general appearance of the plant is presented in Figure 1.

2.2. Isolation and Analysis of Essential Oils

Essential oils were extracted from shade-dried *H. niger* plants using the hydrodistillation method. Approximately 250 g of dried plant material was boiled in a Clevenger apparatus for 4 hours, and the essential oils were collected through distillation. The extracted essential oil was stored at +4°C until further analysis (Demirpolat, 2023).

The essential oils were analyzed using gas chromatography-flame ionization detection/mass spectrometry (GC-FID/MS) with an HP 7890A GC system, 5975C MS, and a flame ionization detector (FID) at the Bingöl University Research Laboratory. A series of n-alkanes were employed as reference points to calculate retention indices (RI). The mass spectrometer was operated at 70 eV, scanning a mass range of 35–425 m/z. Compound identification was carried out by comparing the retention indices and mass spectra of the samples with those in the Wiley-NIST W9N11 libraries.

Figure 1. General view of *H. niger*.

2.3. Determination of the Antimicrobial and Antioxidant Effect

2.3.1. *Antimicrobial analysis*

The antimicrobial effect of the methanol extract of the plant sample was evaluated using the disk diffusion method (Collins & Lyne, 2004). Various bacteria, yeast, and dermatophyte fungi were used as test microorganisms in this study. All microbial cultures were obtained from the Microbiology Laboratory culture collection of the Department of Biology, Faculty of Science, Firat University. The microorganisms used in the study, along with their strain numbers, are listed in Table 2.

Bacterial strains were inoculated into Nutrient Broth and incubated at 35±1°C for 24 hours, yeast strains were inoculated into Malt Extract Broth (MEB) and incubated at 25±1°C for 48 hours, while dermatophyte fungi were inoculated into Glucose Sabouraud Broth (GSB) and incubated under the same conditions. The prepared bacterial, yeast, and fungal cultures (10^6 bacteria/mL, 10^4 yeast/mL, 10^4 fungi/mL) were inoculated into Mueller Hinton Agar (MHA), Sabouraud Dextrose Agar (SDA), and Potato Dextrose Agar (PDA), respectively, at a concentration of 1%. After thorough mixing, the inoculated media were poured into sterile petri dishes.

Disks impregnated with 100 µl of the plant extract were gently placed onto the solidified media in the petri dishes. The plates were kept at 4°C for 1–2 hours to allow the diffusion of the extract and subsequently incubated. Plates containing bacteria were incubated at 37±0.1°C for 24 hours, while those containing yeasts were incubated at 25±0.1°C for 72 hours. Different standard disks were used as controls: Streptomycin sulphate (10 µg/disc) for bacteria and Nystatin (30 µg/disc) for yeasts. The resulting inhibition zones were measured in millimeters (mm).

2.3.2. *Antioxidant assays*

In this phase of the study, the total oxidant status (TOS) and total antioxidant status (TAS) of the plant samples were measured using Rel Assay kits. Trolox was used as the calibrator for TAS, while hydrogen peroxide served as the calibrator for TOS. The TAS value was expressed as mmol Trolox equivalent/L, and the TOS value was expressed as µmol H_2O_2 equivalent/L (Erel, 2004; 2005).

The antioxidant activity of the plant's methanol extract was also assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity method (Cuendet *et al.*, 1997; Kirby & Schmidt, 1997). A stock solution of the plant extract was prepared in methanol at a

concentration of 1 mg/mL, and this solution was serially diluted to generate the DPPH calibration curve. From the prepared solutions, 40 µL was mixed with 160 µL of DPPH solution. The mixture was kept in the dark for 30 minutes to prevent photodegradation, and this process was repeated for all concentrations. Methanol was used as the control.

At the end of the incubation period, absorbance values were measured at a wavelength of 570 nm using an ELISA reader. The percentage of inhibition was calculated using the following formula:

$$I\ (\%) = (A_{control} - A_{sample} / A_{control}) \times 100$$

This methodology enabled a quantitative evaluation of the antioxidant activity of the plant extract.

2.4. Statistical Analysis

The results obtained from antimicrobial and antioxidant analyses were statistically evaluated using the SPSS 22.0 package program and $p<0.05$ was considered significant.

3. FINDINGS

3.1. Essential Oil Studies

The composition of the essential oils extracted from the aerial parts and seeds of *H. niger* is presented in Table 1, which details the percentage composition and retention indices of the identified components. The seeds of *H. niger* were hydrodistilled separately, yielding 0.5% (v/w) of yellow oils. The analysis of the seed essential oils identified 41 components, accounting for 99.0% of the total oil composition. The major components were hexahydrofarnesyl acetone (46.36%) and hexanal (9.05%). Similarly, the aerial parts of the plant were hydrodistilled, yielding 0.4% (v/w) of light yellowish oils. The analysis identified 22 components in the essential oils from the aerial parts, representing 97.51% of the total oil composition. The predominant component was phytol (52.09%), followed by hexahydrofarnesyl acetone (19.66%). Further analysis of the essential oils revealed the presence of sesquiterpenes in both the seed and aerial part oils, highlighting the diverse chemical profile of *H. niger*.

Table 1. Constituents of the essential oils from *H. Niger*.

No	R.Time	Area	Name	*H. niger* seeds	*H. niger* aerial parts
1.	5.159	48718	1-Pentanol	0.82	-
2.	5.618	14757	3-Hexanone	0.56	-
3.	5.761	58489	2-Hexanone	0.99	-
4.	5.945	25972	3-Hexanol	0.44	
5.	**6.033**	**534856**	**Hexanal**	**9.05**	**0.11**
6.	8.647	112848	1-Hexanol	1.91	-
7.	9.497	55310	2-Heptanone	0.94	-
8.	9.942	73828	Heptanal	1.25	-
9.	12.240	45719	Hydroperoxide, 1-ethylbutyl	0.77	0.45
10.	12.459	96863	Undecanone	1.64	-
11.	12.605	29708	Benzaldehyde	-	0.22
12.	13.261	44576	1-Heptanol	0.75	-
13.	13.693	43030	Amyl vinyl carbinol	0.73	-
14.	13.904	30506	2,3-Octanedione	0.52	-
15.	14.224	175344	Furan, 2-pentyl-	2.97	-
16.	14.786	105944	Octanal	1.79	-
17.	15.129	30388	Cloroben	0.51	-
18.	16.610	50606	3-Octen-2-one	0.86	-
19.	17.541	90244	Octenal	1.53	-
20.	18.310	55427	1-Octanol	0.94	-
21.	19.918	144251	Nonanal	2.44	-

22.	22.712	50773	2-Nonenal, (E)-	0.86	-
23.	25.031	32352	Decanal	0.55	-
24.	27.750	73534	2-Decenal, (E)-	1.24	-
25.	28.249	75682	Silane	1.28	-
26.	30.345	186050	2,4-Decadienal, (E,E)-	3.15	-
27.	32.529	72146	2-Undecenal	1.22	-
28.	33.020	116446	2-Octenal, 2-butyl-	1.97	-
29.	37.239	28290	6-Undecanol	0.48	-
30.	38.006	55612	trans-β-Ionone	0.42	-
31.	43.130	43977	Tetradecanal	0.74	-
32.	45.410	27984	Hexadecamethylcyclo octasiloxane	0.47	-
33.	45.546	52687	1-4-γ-H-Pregna	0.89	-
34.	45.971	55390	2,2,6,7-Tetramethyl-10-oxatricyclo[4.3.0.1(1,7)]decan-5-one	0.94	-
35.	46.496	35940	2-Pentadecanone	0.61	-
36.	46.509	50401	Nonadecane	-	0.38
37.	47.079	87288	Tetradecanal	1.48	0.31
38.	50.552	78277	2-Ethylhexyl salicylate	-	0.59
39.	50.827	63244	Tetradecanal	1.07	
40.	51.040	43682	1-Dodecanol, 2-octyl-	0.74	
41.	51.633	70940	Neophytadiene	-	0.53
42.	**51.860**	**2683617**	**Hexahydrofarnesyl acetone**	**46.36**	**19.19**
43.	52.295	74391	Versalide	-	0.56
44.	52.528	63003	2-Pentadecanone, 6,10,14-trimethyl-	-	0.47
45.	52.720	35371	Phthalic acid	-	0.27
46.	**53.747**	**95393**	**Heneicosane**	**-**	**7.02**
47.	54.443	228392	Farnesyl acetone	-	1.71
48.	54.653	91116	Hexadecanoic acid, methyl ester	-	0.68
49.	55.413	204847	Isophytol	-	1.54
50.	57.106	34439	2-methyloctacosane	-	0.26
51.	57.352	116782	Spathulenol	-	0.88
52.	60.193	88314	8,11-Octadecadienoic acid, methyl ester	1.49	0.60
53.	60.391	74347	Oleic acid	1.26	-
54.	60.509	48743	γ-Stearolactone	0.82	-
55.	**60.884**	**7121450**	**Phytol**	**0.96**	**52.09**
56.	61.683	109259	α-ıonone	-	0.82
57.	62.019	46342	Neophytadiene	-	0.35
58.	63.413	526003	Pentacosane	-	3.90
59.	63.621	392579	Adenine	-	2.90
60.	64.032	110852	Phytol, acetate	-	0.83
61.	64.277	191580	Sulfurous acid	-	1.44
			Total	99.99	98.52

3.2. Antimicrobial Effect

The antimicrobial activity of *H. niger* methanol extract against the tested microorganisms is summarized in Table 2. The extract produced inhibition zones of 32±0.1 mm against *E. coli*, 21±0.2 mm against *S. aureus*, 20±0.5 mm against *K. pneumoniae*, 19±0.2 mm against *B. megaterium*, 23±0.1 mm against *C. albicans*, 20±0.4 mm against *C. glabrata*, 13±0.4 mm against *Trichophyton* sp., and 16±0.2 mm against *Epidermophyton* sp. Among these, the methanol extract showed the strongest antimicrobial activity against *E. coli* (see Table 2).

Statistical analysis revealed that the antimicrobial effects of the methanol extract against *E. coli*, *S. aureus*, *K. pneumoniae*, *B. megaterium*, *C. albicans*, and *C. glabrata* were similar (Table 2, $p > 0.05$). However, the results for *Trichophyton* sp. and *Epidermophyton* sp. were significantly different (Table 2, $p < 0.05$). When compared with the control group, the differences in antimicrobial activity across all tested microorganisms were statistically significant (Table 2, $p < 0.05$).

Table 2. Antimicrobial effect of *H. niger*.

Microorganism	*H. niger*	Standart Control
Escherichia coli (ATCC25322)	32±0.1[a]	30±0.2[b]
Staphylococcus aureus (ATCC25923)	21±0.2[a]	20±0.1[a]
Klebsiella pneumoniae (ATCC700603)	20±0.5[a]	19±0.0[a]
Bacillus megaterium (DSM32)	19±0.2[a]	25±0.0[b]
Candida albicans (FMC17)	23±0.1[a]	25±0.2[b]
Candida glabrata (ATCC66032)	20±0.4[a]	20±0.1[a]
Trichophyton sp.	13±0.4[a]	22±0.0[b]
Epidermophyton sp.	16±0.2[a]	25±0.4[b]

[a, b] Values shown with the same letters in each line are not different from each other, Values are means of three replicates ±SD

3.3. Antioxidant Activity

The TAS value of the *H. niger* methanol extract at a concentration of 1 mg/mL was measured as 3.77±0.0 mmol, while the TOS value at the same concentration was recorded as 6.94±0.0 µmol. These results indicate that the TAS value of the plant was notably high, whereas the TOS value was within the normal range (see Table 3).

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the methanol extract at 1000 µg/mL was determined to be 73.07±0.02%. In contrast, the antioxidant effect at 125 µg/mL was very low (see Table 4). The results demonstrate that the DPPH radical scavenging effect of *H. niger* methanol extracts increases proportionally with higher extract concentrations.

Table 3. TAS and TOS values of *H. niger*.

	TAS (mmol Trolox equiv./L)	TOS (µmol H2O2 equiv./L)
H. niger-MetOH	3.77±0.0[a]	6.94±0.0[b]

[a, b] Values shown with different letters in each line are different from each other ($p<0.05$), Values are means of three replicates ±SD

Table 4. Percent inhibition of the DPPH radical of root and leaf parts of *H. niger*.

	H. niger -MetOH
1000 µg/mL	73.07 ±0.02[d]
500 µg/mL	56.73 ±0.07[c]
250 µg/mL	27.88 ±0.44[b]
125 µg/mL	16.82 ±0.29[a]

[a, b, c, d] Means in the same column with the different superscripts are significantly different ($p<0.05$), Values are means of three replicates ±SD

4. DISCUSSION

In this study, the essential oil components of the aerial parts and seeds of *Hyoscyamus niger* were analyzed using GC-MS. The essential oils were found to be complex mixtures of sesquiterpenes and hydrocarbons. Compared to previous studies, such as Ting-guo (2013), who identified 29 components in *H. niger* leaves through HPLC, including palmitic acid (28.30%), linoleic acid (26.85%), oleic acid (14.39%), hexanal (10.24%), and stearic acid (1.81%) as major compounds, the current study showed similar results with hexanal (9.05%) and oleic acid (1.26%) identified. Differences in other components like palmitic and linoleic acids could be attributed to geographical and climatic variations, as well as the methods and equipment used.

Additionally, Ayari-Guentri *et al.* (2017) analyzed the aerial parts of *H. muticus L.* subsp. *falezlez* and found borneol (76.47%) as the main compound, followed by bornyl acetate (4.6%) and 1,3-bis(2-cyclopropyl-2-methylcyclopropyl) but-2-en-1-one (4.19%), with an oil yield of 0.50% (v/w). These compounds were absent in this study, likely due to variations in

geographical regions and ecological conditions, which significantly influence the composition of essential oils.

In a separate study, the methanolic extracts of *H. niger* and *H. reticulatus* were analyzed using HPLC, revealing and quantifying three major phenolic compounds: chlorogenic acid, rutin, and quercetin-3-O-glucoside-rhamnoside-rhamnoside-rhamnoside (Jassbi *et al.*, 2014). Additionally, the oil and methanol extract of *H. niger* demonstrated antinematode activity (Kareem, 2020). The ethanol extract of *H. niger* seeds exhibited significant insecticidal activity against *Aphis laburni* and *Lucilia sericata* (Küpeli *et al.*, 2020; Wang *et al.*, 2006). The findings of this study align with existing literature. Secondary metabolites produced in plants during infections are critical defense mechanisms against pathogens and display substantial biological activity. The quality, quantity, and diversity of these secondary metabolites can vary based on habitat, soil, climatic conditions, and the specific plant organ where they are synthesized (Moradi *et al.*, 2020; Balogun *et al.*, 2017).

Hexahydrofarnesyl acetone was identified as the major constituent of the *H. niger* seed essential oil in this study. This compound is known for its antibacterial, anti-nociceptive, and anti-inflammatory properties (Wei, 2016). The methanol extract of *H. niger* demonstrated the highest antimicrobial effect, which can be attributed to the presence of hexahydrofarnesyl acetone. As a key compound in all the oils extracted in this study, hexahydrofarnesyl acetone exhibited strong antimicrobial activity against both gram-positive and gram-negative bacteria (Filipowicz *et al.*, 2003). Additionally, essential oil from *Equisetum arvense* rich in hexahydrofarnesyl acetone has been shown to inhibit a broad spectrum of bacterial and fungal species (Radulovic *et al.*, 2006).

Phytol, a diterpene alcohol derived from chlorophyll, was identified as a major component in the essential oil of the aerial parts of *H. niger* in this study. Phytol, widely used as a food additive and in medical applications, has promising antischistosomal properties. It also exhibits antinociceptive, antioxidant, anti-inflammatory, and antiallergic activities. Furthermore, phytol has demonstrated antimicrobial effects against *Mycobacterium tuberculosis* and *Staphylococcus aureus* (de Moras *et al.*, 2014). These findings underscore the potential pharmacological applications of the major constituents of *H. niger* essential oil. As a result of the current study, phytol, which constitutes 52.09% of the essential oil of *H. niger*, was found in sufficient quantities to serve as a significant resource for future research.

Previous studies have demonstrated the antimicrobial properties of *H. niger* seeds against various microorganisms, with inhibition zones ranging from 9.6 mm to 26.2 mm. Notably, the strongest antimicrobial effect was observed against *Escherichia coli* (Dulger and Dulger, 2015). Additional research has reported antifungal activity for *H. niger* (Dulger *et al.*, 2010a). Methanol extracts obtained from the seeds of this species exhibited strong antimicrobial activity (25.0 mm) against *Staphylococcus aureus* (Dulger *et al.*, 2010b). Similarly, alkaloid extracts derived from the flower stems and roots of *H. niger* showed potent antimicrobial effects (Chalabian *et al.*, 2002). Crude protein extracts of the same species were tested against *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*, yielding inhibition zone diameters of 14 mm, 15 mm, 14 mm, and 20 mm, respectively (Mateen *et al.*, 2015).

Moreover, *H. reticulatus* seed extract has demonstrated antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Staphylococcus aureus*, and *Yersinia enterocolitica* (Akbaş *et al.*, 2020). It has also been established that the leaf parts of *H. niger* exhibit significant antimicrobial effects (Hossain *et al.*, 2021).

When comparing the antimicrobial results of *H. niger* with findings from the literature, some variations are evident. These differences may be attributed to factors such as the specific plant species studied, the extraction methods and concentrations used, and the microorganisms tested. Alkaloids, glycosides, flavonoids, and their derivatives—commonly found in plant extracts—are primarily responsible for the antimicrobial effects observed (Akbaş *et al.*, 2020; Ayari-

Guentri *et al.*, 2017; Benhouda *et al.*, 2014; Dulger *et al.*, 2010a-b; Dulger and Dulger, 2015; Elsharkawy *et al.*, 2018). These factors, along with the ecological and environmental conditions of the plants, influence the observed antimicrobial activity.

Hajipoor *et al*. (2015) investigated the antioxidant activity of the aerial parts of *H. niger* extracts using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging and iron-reducing antioxidant power (FRAP) assays. They reported that the DPPH radical scavenging effect of the plant's methanol extract was 2% at a concentration of 10 mg/mL, with this value gradually increasing at higher concentrations. Similarly, Koçpınar (2020) examined the heavy metal reduction properties (FRAP and CUPRAC) and DPPH radical scavenging activity of *H. niger*, confirming that its antioxidant activity was stronger than its radical scavenging and heavy metal reduction effects.

Jassbi *et al*. (2014) studied the free radical scavenging activity of seven fractions of *H. niger* alkaloidal extracts using the DPPH assay. Among these, only one fraction demonstrated moderate free radical scavenging activity compared to positive and negative controls. In another study, Ayari-Guentri *et al*. (2017) evaluated the DPPH radical scavenging activity of methanolic extracts from different parts of *H. muticus L. subsp. falezlez*. The highest IC50 value was observed in the stem (0.54 ± 0.19 mg/mL), followed by the leaf (0.65 ± 0.29 mg/mL) and the flower (0.92 ± 0.39 mg/mL).

When these results are compared with the literature, it is evident that antioxidant effects vary depending on the plant species, the specific plant parts used, the habitat where the plants were collected, and the presence of bioactive components in the plant material. These factors significantly influence the observed antioxidant activity.

5. CONCLUSIONS

The highest antimicrobial effect of *H. niger* was observed against *E. coli*. When the results of this study are compared with previous studies, variations are evident, which can be attributed to differences in the plant parts used, the microorganisms tested, the solvents applied, and the concentrations studied. The antioxidant effect of the species was found to be significant at a concentration of 1 mg/mL. However, comparisons with the literature reveal some discrepancies, likely due to differences in the plant materials, solvent types, and concentrations used.

Hyoscyamus species are recognized for their significant pharmacological effects, largely due to their phytochemical content. A thorough investigation of these plants, particularly their phytochemical composition, is essential to provide valuable contributions to the scientific literature. *H. niger* is a medicinal plant deserving of further study for this purpose. The bioactive compounds in *H. niger*, especially hexahydrofarnesyl acetone, and phytol, identified in the essential oils from its seeds and aerial parts, may offer promising therapeutic potential.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Şule Inci: Antimicrobial and Antioxidant studies, Writing-original draft. **Pelin Yılmaz Sancar:** Investigation, Resources, Visualization, Formal Analysis and Writing-original draft. **Azize Demirpolat:** Essential oil studies, Writing-original draft. **Sevda Kırbağ:** Antimicrobial-Antioxidant studies, Statistical analysis, Supervision, Validation and Writing-original draft. **Şemsettin Civelek:** Supervision, Validation and Writing-original draft.

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Research Article

Phytochemical profiling, molecular docking and ADMET prediction of essential oil of *Ocimum basilicum*

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Abstract: Essential oils are widely used in pharmacology, cosmetics, and food industries, and they also have biological activities such as antioxidant, anti-inflammatory, anti-rheumatic, and antimicrobial. *Ocimum basilicum* (basil) plant has a rich content of essential oils. Hence, the stem, leaf, and flower parts of the *O. basilicum* were analyzed freshly on the RSH/GC-MS device to determine the essential oil content. As a result of the analysis, α-elemene, linalool, and eucalyptol were detected as the main components. It was observed that the highest linalool content was in the flower part at 47.85%, and the eucalyptol content was in the leaf part at 44.00%. Additionally, it was determined that the α-elemene content was highest in the flower part with 12.49%. According to the analysis results, high amounts of linalool, eucalyptol, and α-elemene were detected. The inhibitory properties of these compounds against the DNA gyrase enzyme were investigated by molecular docking. MolDock score (-78.72, -47.50, -88.86) and binding energy (2.9, 4.6, 4.0 kcal/mol) of linalool, eucalyptol, and α-elemene compounds were determined respectively. According to the ADME/T properties of the molecules examined; The α-elemene did not show any toxic effects. As a result, the eucalyptol compound may be used as an inhibitor against the DNA gyrase enzyme. In addition, it can contribute to the economy by obtaining essential oils from the non-consumable flowers and stem parts of the basil plant and increasing its usability in industries such as cleaning, cosmetics, etc.

1. INTRODUCTION

Plants have been exploited for medicinal purposes and food since ancient times. Moreover, they reveal significant biological activity due to their possessing of secondary metabolites (Erenler, *et al.*, 2023; Hadjra *et al.*, 2023; Khodja *et al.*, 2023). Furthermore, plants play an important role in regulating ecosystems and are thus known to influence biological processes. From the past to the present, aromatic and medicinal plants have been widely used to sweeten meals and make them more delicious (Yaglioglu *et al.*, 2022; Zerrouki *et al.*, 2022). Today, developing technology has made it possible for aromatic and fragrant plants to be used not only in the

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kitchen, but also in cosmetics, the pharmaceutical industry, food additives, and many other areas (Topçu *et al.*, 1999). *Ocimum* includes more than 30 species based on their morphology, color of flowers, growth habits, chemical composition, and characteristics of their leaves and stems (Nusret *et al.*, 2020). It grows widely in Asia, America, and Africa. *Ocimum basilicum* (OB) in traditional medicine; It has been reported that it is used as a flavoring agent, deodorizer in oral and dental health, against bacterial skin infections in wounds, cough, headache, dewormer, diarrhea, and cancer treatment (Ahmed *et al.*, 2019).

Essential oils are important secondary metabolites used in many fields including pharmaceuticals, cosmetics, and spices (Boulechfar *et al.*, 2022; Erenler, *et al.*, 2023). Essential oils have been reported to display a large variety of biological activities (Karan *et al.*, 2018a; Karan *et al.*, 2018b). Volatile components are molecules that generally contain terpenes, aldehyde, and alcohol groups. The presence of these chemical groups in different proportions in different parts of the plants affects the odors, tastes, and therapeutic properties of the plants (Bayir *et al.*, 2014; Kaya *et al.*, 2014; Türkmen *et al.*, 2014). The main essential oil components of the basil plant have been reported to contain α-pinene, β-pinene, methyl cavicol, 1,8 cineole, L-linalool, and o-cymene (Purushothaman *et al.*, 2018). GC, GC-MS, and Headspace GC-MS are effectively used to determine the compounds in plants. The OB, which grows widely in Türkiye, is consumed as a spice due to its intense pleasant smell and flavoring properties (Telci *et al.*, 2006).

At harvest, the flower and stem parts are usually thrown away. By comparing the non-edible parts of OB with the edible parts, it is hoped to increase their use in pharmacology, perfumery, cosmetics, aromatherapy, and the food industry and to provide new economic benefits to those working in agriculture. For this purpose, the stem, leaf, and flower parts of OB were analyzed separately by RSH/GC-MS in our previous study (Gök & Başar, 2023). In this study, the interactions of the main components detected in the RSH/GC-MS analysis with the antibacterial enzyme (DNA gyrase) were determined by molecular docking. In addition, the pharmacokinetic properties (ADME/T) of these main components were investigated. Therefore, they are expected to provide information about the inhibitory properties and pharmacokinetic properties of these molecules.

2. MATERIAL and METHODS

2.1. Plant and Sample Preparation

OB was freshly collected in Siirt (Siirt University Kezer Campus) at coordinates of 37°57'56"N 41°51'01"E. The plant was divided into stem, leaf, and flower parts. 0.5 g samples of the stem, leaf, and flower were added to a 25 mL headspace bottle without drying and then placed in the chamber of the RSH/GC-MS device for analysis.

2.2. RSH/GC-MS Analysis

The sample vial was heated at 130 °C for 30 min in the triplus RSH oven. It was delivered to GC/MS with an injection volume of 2.5 mL from the heated vial. The analysis was carried out by ISQ mass spectroscopy (Thermo Fisher Scientific, Austin, TX) and trace 1310 gas chromatography. The process was held at an initial temperature of 80 °C for 2 minutes, then heated to 240 °C by increasing 4 °C/min and held at 240 °C for 25 minutes. The ion source and detector temperature were set at 250 °C and the sample injection volume was set at 1.5 mL. Helium (1.2 mL/min) was used as carrier gas. Thermo TG-WAXMS with GC column (60 m × 0.25 mm ID × 0.25 µm) was used for sensitive separation. The mass spectral scan range was set to 55–300 (Amu) (Gök & Başar, 2023). Components were identified by scanning the NIST demo, Wiley7, Wiley9, redlip, mainlip, and WinRI libraries (Benguedouar *et al.*, 2022).

2.3. Molecular Docking Application

3D structures and minimum energy of the linalool, eucalyptol, and α-elemene were carried out in the ChemDraw software. The 3D protein structure of DNA gyrase (PDB ID: 1KZN) was

selected from the protein data bank (RCSB PDB: Homepage). The search area of the enzyme was determined as coordinates X: 19.07, Y: 29.61, Z: 34.87, and the rasidus was determined as 29.00 Å, and the molecules interacted in this area. Linalool, eucalyptol, and α-elemene with enzyme interactions were determined using the Molegro Virtual Docker (MVD) program (Başar *et al.*, 2023). The 2D and 3D images of the interactions were taken with the BIOVIA Discovery Studio Visualizer program. Also, The AutoDock Vina program was used to calculate the binding affinities (Yenigün *et al.*, 2024; Başar *et al*., 2024a).

2.4. ADME/T Application

In the RSH/GC-MS analysis, ADME/T calculations were utilized for prediction in pharmacokinetics to investigate the absorption, distribution, role in metabolism, excretion from the body, and whether there are toxic effects of the most common components in the body. These parameters are SwissADME (https://www.swissadme.ch/), Molinsiparation (https://www.molinspiration.com), Molsoft (https://molsoft.com/mprop/), Peo (https://www.organic-chemistry.org/prog/peo/) and pKCSM (https://biosig.lab.uq.edu.au/pkcsm/prediction) programs. ADME/T utilizes *in silico* techniques to better understand and predict how drugs will affect the body. It can optimize clinical use, reduce unwanted side effects, focus research on development, and improve alternative treatments (Pires *et al.*, 2015; Başar *et al*., 2024b; Ipek *et al*., 2024).

3. RESULTS

In our study, the essential oil content of the OB plant, which we had previously presented as a report, was determined by RSH-GC/MS (Gök & Başar, 2023). As a result of the analysis, the interactions of the molecules determined as the main constituent with the enzyme DNA gyrase, which is known as an antibacterial enzyme, were determined by molecular docking application, and the binding energies were calculated using Autodock vina. In addition, the parkinetic properties of the molecules were calculated using the online application ADME/T.

The volatile components of the body, leaf, and flower of the OB were presented. The stem part included linalool (32.68%), eucalyptol (21.44%), and α-elemene (3.17%), and the leaf part contained the eucalyptol (44.00%), linalool (40.34%) and α-elemene (2.48%). The flower consisted of linalool (47.85%), eucalyptol (24.16%), and α-elemene (12.49%) (Figure 1 and Table 1) (Gök & Başar, 2023).

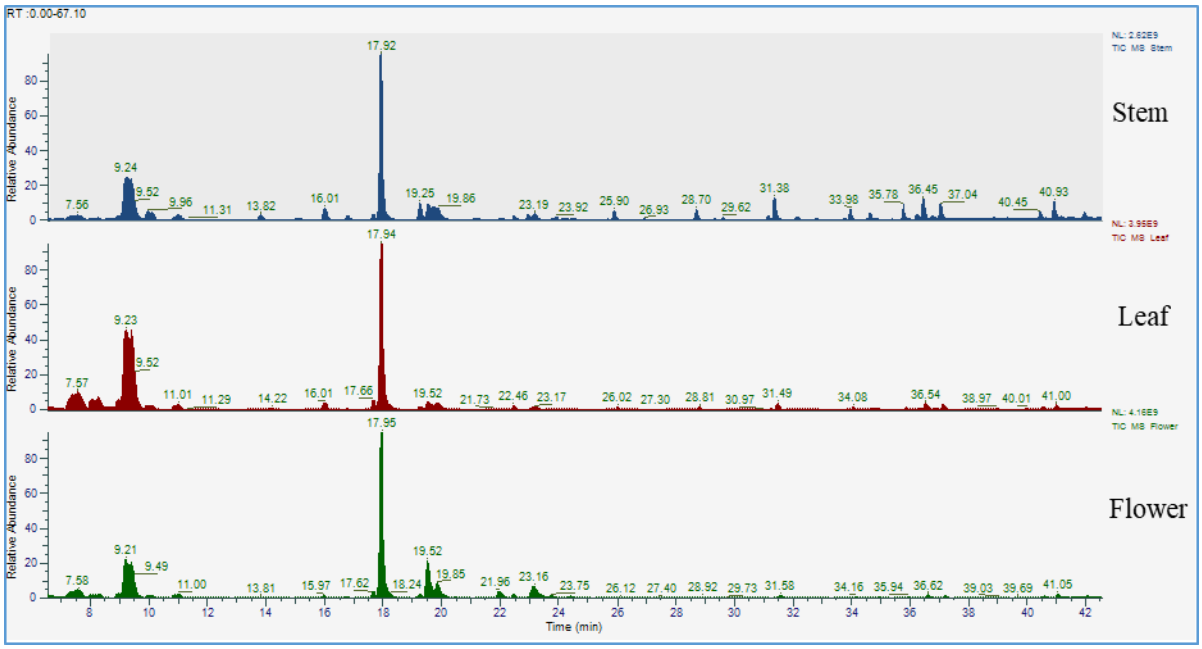


Figure 1. RSH/GC-MS chromatograms of the body, leaf and flower of OB (Gök & Başar, 2023).

Table 1. Results of volatile components of OB (Gök & Başar, 2023).

No	Compound	RT	RI[a]	RI[b]	Body%	Leaf %	Flower %
1	Eucalyptol	9.24	1211	1237	21.44	44.00	24.16
2	*β*-Ocimene	9.96	1254	1268	2.16	-	-
3	1,3,6-Octatriene,3,7-dimethyl-(E)-	10.10	1258	1273	1.46	-	-
4	*α* -Terpinolene	10.87	1294	1304	-	0.83	-
5	1,5,5-Trimethyl-6-methylene-cyclohexene	11.01	1338	1309	2.15	1.49	1.18
6	Nonanal	13.81	1408	1411	1.24	-	-
7	Fenchyl acetate	16.01	1482	1482	3.05	2.00	-
8	Decanal	16.79	1505	1508	1.13	-	-
9	Camphor	17.67	1531	1539		1.88	1.62
10	Linalool	17.92	1547	1547	32.68	40.34	47.85
11	Bornyl acetate	19.26	1591	1591	3.03	-	-
12	*α*-Elemene	19.55	1605	1601	3.17	2.48	12.49
13	Calarene	19.71	1610	1607	2.69	-	-
14	Caryophyllene	19.85	1614	1612	2.22	2.09	4.37
15	*α*-Humulene	21.95	1681	1685	-	-	2.58
16	L-*α*-Terpineol	22.46	1690	1702	-	0.96	0.92
17	Dodecanal	22.96	1718	1721	1.13	-	-
18	Valencene	23.19	1728	1729	1.54	-	4.84
19	Tridecanal	25.90	1822	1827	1.97	-	-
20	Tetradecanal	28.70	1933	1933	1.99	-	-
21	Pentadecanal	31.38	2041	2041	4.33	1.47	-
22	Hexadecanal	33.98	2137	2147	1.69	-	-
23	cis-11-Hexadecenal	34.64	2159	2175	1.07	-	-
24	2-Heptadecanone	36.23	2243	2245	1.18	-	-
25	Heptadecanal	36.45	2247	2254	3.86	1.42	-

RT: Retention time**, RI[a]:** Covarx index literature (Cadwallader & Xu, 1994; Adams, 2007), **RI[b]:** Covarx index experimental results

3.1. Molecular Docking

DNA gyrase is a bacterial enzyme that functions to reduce the molecular tension created by winding during DNA replication. It belongs to the topoisomerases class of enzymes that control the topological transitions of DNA (Reece & Maxwell, 1991). OB essential oils are known to show high antibacterial activity (Al Abbasy *et al.*, 2015). The linalool, eucalyptol, and *α*-elemene were determined to be high concentrations in OB essential oil. Therefore, the interactions of corresponding compounds with the DNA gyrase enzyme were investigated (Figure 2).

Linalool consisted of one conventional hydrogen bond with residues VAL43 and seven alkyl interactions with amino acids such as VAL43, VAL71, VAL167, VAL120, ILE78 within DNA gyrase (Figure 2 and Table 2). The interactions of the linalool molecule with DNA gyrase were calculated as -78.72 (MolDock score), and binding energy was detected as -2.90 kcal/mol. The eucalyptol molecule contained one carbon-hydrogen bond with residues THR165 and ten alkyl interactions with amino acids, VAL43, ALA47, ILE78, VAL71, VAL167, VAL120 in DNA gyrase (Figure 2 and Table 2). The MolDock score of the interaction of the eucalyptol with DNA gyrase was calculated as -47.50, and the binding energies were calculated as -4.60

kcal/mol.

The thirteen alkyl interactions of α-elemene with amino acids such as VAL43, ALA47, ILE78, VAL71, VAL167, VAL120, within DNA gyrase were observed (Figure 2 and Table 2). The MolDock score of the interactions of the α-element molecule with DNA gyrase was calculated as -88.86, and the binding energies were calculated as -4.00 kcal/mol. According to a molecular docking study; eucalyptol may be used as an inhibitor against the DNA gyrase enzyme. The accuracy of these studies can be checked in an *in vitro* environment.

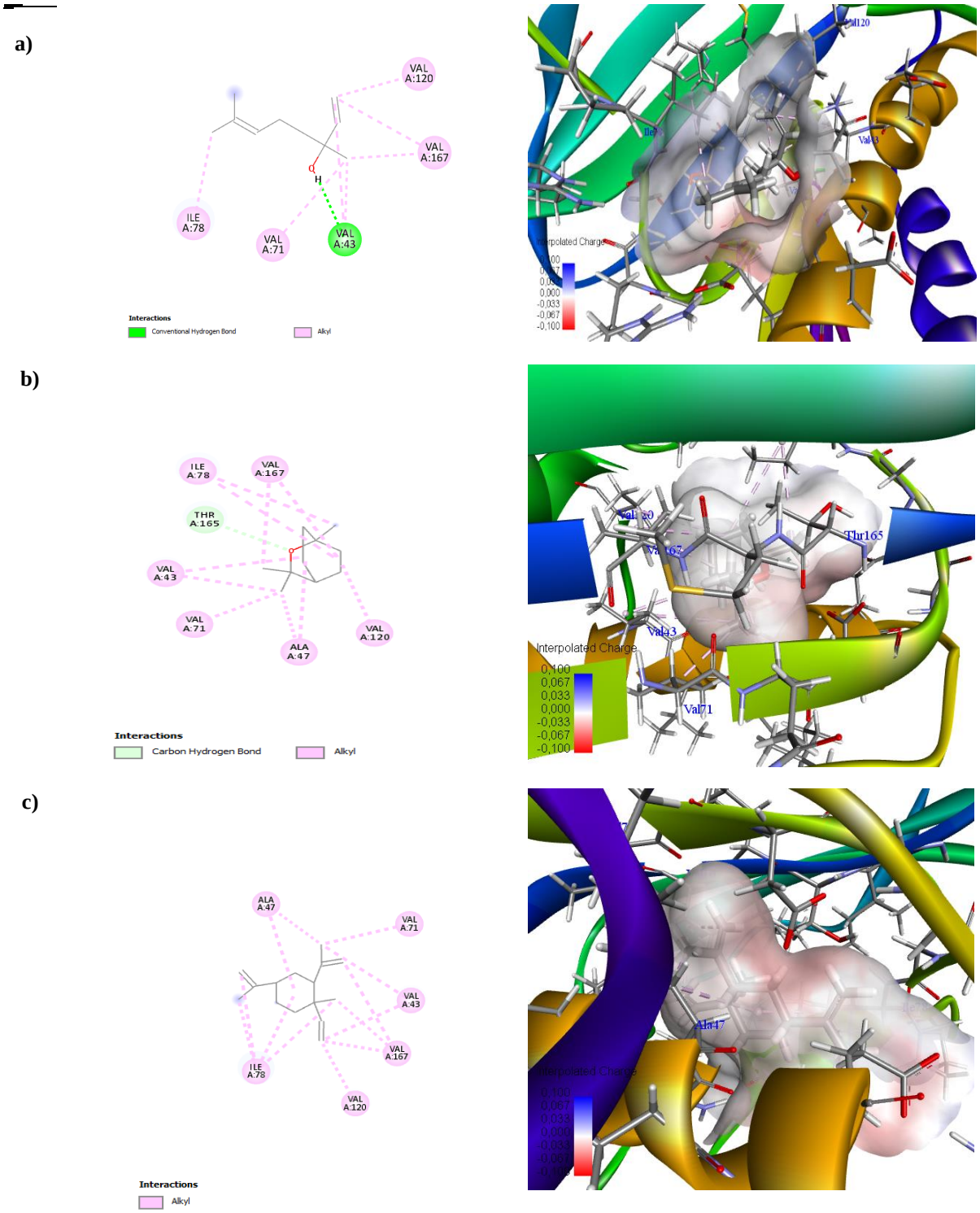


Figure 2. 2D images and 3D interpolated load view of a) linalool b) eucalyptol c) α-elemene with DNA gyrase interaction.

Table 2. DNA gyrase-compounds interaction categories, species and molecular docking distance.

Conpound Name	Aminoacid Names	Distance	Bond Types
Linalool	VAL43	1.183206	Hydrogen Bond (Conventional)
	VAL43	4.54811	Hydophobic (Alkyl)
	VAL71	4.29064	Hydophobic (Alkyl)
	VAL167	3.91122	Hydophobic (Alkyl)
	VAL43	4.91913	Hydophobic (Alkyl)
	VAL120	3.95533	Hydophobic (Alkyl)
	VAL167	4.00937	Hydophobic (Alkyl)
	ILE78	4.73652	Hydophobic (Alkyl)
Eucalyptol	THR165	2.3919	Hydrogen Bond (Carbon)
	VAL43	5.34605	Hydophobic (Alkyl)
	ALA47	4.44641	Hydophobic (Alkyl)
	ALA47	3.4129	Hydophobic (Alkyl)
	ILE78	5.16506	Hydophobic (Alkyl)
	VAL120	5.10748	Hydophobic (Alkyl)
	VAL167	4.71775	Hydophobic (Alkyl)
	ILE78	4.91334	Hydophobic (Alkyl)
	VAL167	4.0741	Hydophobic (Alkyl)
	VAL43	5.34768	Hydophobic (Alkyl)
	VAL71	3.94457	Hydophobic (Alkyl)
α-Elemene	ALA47	5.4813	Hydophobic (Alkyl)
	ALA47	2.91057	Hydophobic (Alkyl)
	ILE78	5.0842	Hydophobic (Alkyl)
	ILE78	4.79639	Hydophobic (Alkyl)
	VAL167	4.45664	Hydophobic (Alkyl)
	VAL43	3.48572	Hydophobic (Alkyl)
	VAL120	3.33498	Hydophobic (Alkyl)
	VAL167	3.87033	Hydophobic (Alkyl)
	ILE78	5.16772	Hydophobic (Alkyl)
	ILE78	4.81986	Hydophobic (Alkyl)
	VAL43	5.21836	Hydophobic (Alkyl)
	VAL71	4.07398	Hydophobic (Alkyl)
	VAL167	4.66851	Hydophobic (Alkyl)

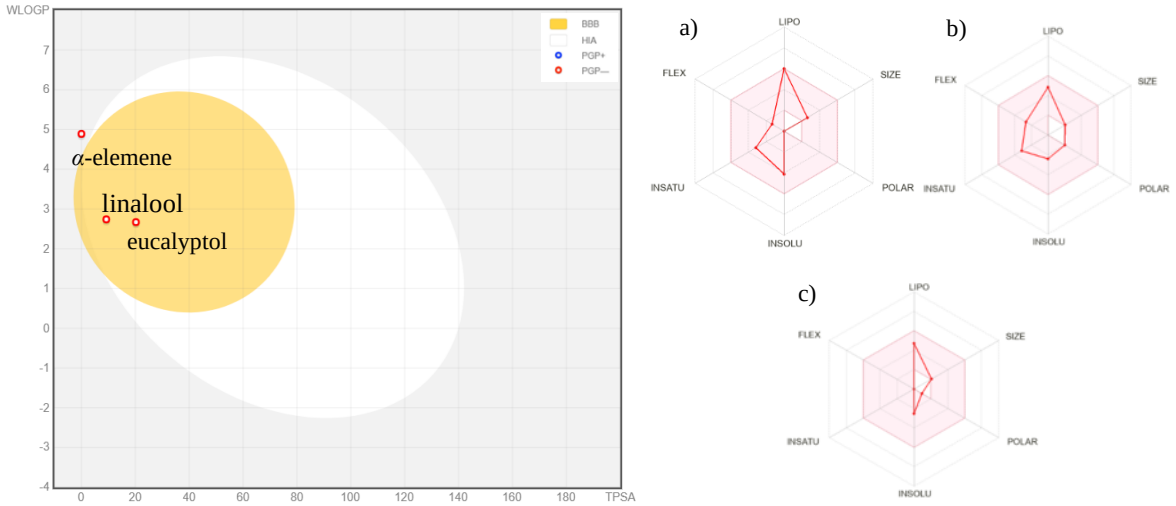
3.1. ADME/T Results

The evaluation of ADME/T properties (absorption, distribution, metabolism, excretion, and toxicity) is considered an important step in drug development. Thus, a substance, which may be a drug, is absorbed and distributed in the body within a certain period to ensure effective metabolism (Bruna *et al.*, 2022). ADME/T evaluation can be completed after estimating the physicochemical properties and following Lipinski's “rule of five”(Ye *et al.*, 2018). Lipinski's "rule of five" states that the compound has a molecular weight ≤ 500, logP ≤ 5, number of hydrogen bond donors ≤ 5, and number of hydrogen bond acceptors ≤ 10. In biological systems, the LogP value is taken into account in the distribution of molecules between phases (oil, water) (Znati *et al.*, 2019).

Analysis of the boiled egg plot (Figure 3) shows that eucalyptol and linalool have high gastrointestinal absorption (GIa) and blood-brain barrier permeability (BBBp). In contrast, the *α*-elemene compound has low GIa and BBBp. P-glycoprotein (P-gp) is a membrane protein that removes compounds from cells. Drug-like compounds should not be P-gp substrates. According to the predictions in this regard, the investigated compounds fulfilled this condition. According

to the bioavailability radar table of the ingredients, they exhibited better scores than the standard references used (Figure 3). The fact that the molecules are in the pink region indicates high bioavailability and similarity to the drug (Znati *et al.*, 2019). In addition, cytochrome P450 (CYP), derived from pharmacokinetically related proteins, can be excreted via the kidneys following the polarization of oxidized molecules and may play a role in the oxidative metabolism of compounds.That is, the studied components did not appear to interact with CYP isozymes (Table 3) (Bruna *et al.*, 2022). In addition, bioavailability values of 0.55 indicate that they have more drug-like properties and high usability as drugs (Table 3).

The bioactivity values of the compounds that were detected in large quantities to identify biological targets are shown in Table 3: Ligand of a G protein-coupled receptor (GPCR), ligand of a nuclear receptor, kinase, protease enzyme inhibitor and modulator of an ion channel. In addition, the bioactivity values of these molecules are grouped as active, moderately active, or inactive.



The pink zone is the suitable physicochemical space for oral bioavailability. LIPO (lipophilicity), POLAR (polarity), INSOLU (insolubility), INSATU (insaturation), FLEX (flexibility). The gastrointestinal tract is illustrated by the white of a boiled egg, the blood-brain barrier by the yolk, and chemicals expected to be P-glycoprotein substrates are represented by the blue dot.

Figure 3. Boiled egg graph and bioavailability radar graph of the main components of RSH/GC-MS analysis of OB, *α*-elemene (a), eucalyptol (b), linalool (c).

If the bioactivity score value is greater than 0.00, the molecule is assumed to be active; if the score value is between -0.50 and 0.00, it is assumed to be moderately active; if the score value is less than -0.50, the molecule is assumed to be inactive (Znati *et al.*, 2019).The compounds eucalyptol and linalool were found to be significantly bioactive as modulators of ion channels, and only the elemental compound was predicted to have a moderate effect. On the other hand, eucalyptol was postulated to be an excellent nuclear receptor ligand and a moderate general enzyme inhibitor. In protease inhibition, only the *α*-elemene compound was found to be moderately inhibitory, while the compounds eucalyptol and linalool were found to be strongly inhibitory (Table 3).

Determining the toxicity of chemical compounds is of utmost medical importance (Srivastava, 2021). It is also an in-silico approach to predict the risks of particular toxicity such as mutagenicity, tumor formation, irritation, and reproductive efficacy. No risk of tumorigenicity, reproductive toxicity, irritation, and mutagenicity was predicted for the elemental compound (Table 3).

Table 3. Pharmacokinetic properties of the main components of OB plant RSH/GC-MS analysis.

Name	MW (g/mol)	logP	GIa	BBB_p	nHA	nHD	nRB	P-gp	IA (Human) %	CL_{tot} (Log mL/min/kg)	VD_{ss} (Human) Log L/kg
α-Elemene	204.36	4.89	Low	0.77	0.00	0.00	2.00	No	96.83	1.39	0.58
Eucalyptol	154.25	2.74	High	0.37	1.00	0.00	0.00	No	96.50	1.01	0.50
Linalool	154.25	2.67	High	0.61	1.00	1.00	4.00	No	93.65	0.45	0.11

Name	GPCR ligand	Kinase inhibitor	Ion channel modulator	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor	Oral Acute Toxicity (LD_{50})	CYP substrate
α-Elemene	-0.55	-0.86	-0.14	0.49	-0.64	0.26	1.60	-
Eucalyptol	-0.93	0.01	-1.60	-1.07	-0.90	-0.15	2.01	-
Linalol	-0.73	0.07	-1.26	-0.06	-0.94	0.07	1.80	-

Name	Mutagenicity	Irritation	Tumorigenicity	Reproductive	Bioavailability Score
α-Elemene	LR	LR	LR	LR	0.55
Eucalyptol	HR	LR	LR	HR	0.55
Linalol	HR	LR	HR	LR	0.55

MW: Molecular weight, **LogP:** logarithmic ratio of partition coefficient, **CYP:** human cytochrome P450, **VDss:** volume of distribution, **CLtot:** total clearance (hepatic and renal clearance), Number of hydrogen bond acceptors, Number of hydrogen bond donors, Number of rotatable bonds, GIa: Gastrointestinal absorption, **BBBp:** Blood-brain barrier permeant, **P-gp:** P-glycoprotein substrate, **IA:** Intestinal absorption, **LR:** Low risk, **MR:** Medium risk, **HR:** Higher risk.

4. DISCUSSION and CONCLUSION

Linalool was determined as the first main component in the stem (32.68%) and flower (47.85%) and the second main component in the leaf (40.34%). The second main component, eucalyptol molecule, was found in the leaf (44.00%), stem (21.44%), and flower (24.16%). According to the results, it was observed that 44% eucalyptol was found in the leaf parts, which are mostly consumed as spices, and linalool and α-elemene were high in the flower and stem parts. The main component of OB essential oils was reported as linalool (31.6-69.87%) (Hussain *et al.*, 2008). GC-MS analysis of OB revealed linalool (44.18%), 1,8-cineole (13.65%), eugenol (8.59%), methyl cinnamate (4.26%), iso-caryophyllene (3.10%) and α-cubebene (4.97%) as the main constituents of the essential oil (Ismail, 2006). The main constituents of OB were reported as linalool (48.4%), 1,8-cineole (12.2%), eugenol (6.6%), methyl cinnamate (6.2%), α-cubebene (5.7%), caryophyllene (2.5%), β-ocimene (2.1%) and α-farnesene (2.0%) (El-Soud *et al.*, 2015). GC/MS analysis of OB revealed that the main components of the essential oil are geranial (35.5%) and cis-citral (26.2%) (Barua *et al.*, 2023). Linalool has been reported as the major constituent of the essential oil of OB originating (Telci *et al.*, 2006). Linalool is not only used in perfumes, cosmetics, food, and detergents but also has anti-inflammatory, analgesic (pain-relieving), antispasmodic (muscle relaxant), DNA-protective and antimicrobial properties (Mitić-Ćulafić *et al.*, 2009). In addition to its use in the pharmaceutical industry and cosmetics, the eucalyptol compound has been reported to have anti-inflammatory, analgesic (pain-relieving), antispasmodic (muscle relaxant), antioxidant, and antimicrobial activities against chronic upper respiratory tract infections.

According to the MolDock results of linalool, eucalyptol, and α-elemene compounds, moldock scores were detected as -78.72, -47.50, -88.86 respectively, and binding energies were calculated as 2.9 kcal/mol, 4.6 kcal/mol, 4.0 kcal/mol respectively. The eucalyptol compound may be used as an inhibitor against the DNA gyrase enzyme. According to the ADME/T results of these components, it was observed that eucalyptol and linalool components passed the blood-brain barrier, while the other components did not pass through both the blood-brain barrier and the gastrointestinal system. While it was determined that the elemental compound had no toxic effects, it was noted that other components had toxic effects. It was also determined that these components did not interact with cytochrome P450 enzymes. In summary, these components can be used as medicine. But further studies should be carried out. The essential oil content extracted from flowers and stems is therefore widely used in perfumery, cosmetics, aromatherapy, and the food industry. The extraction of essential oils from the OB and their processing into products with high added value can make an economic contribution.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Yunus Başar: Investigation, Software, Visualization, Formal Analysis, Design, and Writing-original draft. **Mesut Gök:** Investigation, Resources, Formal Analysis, and Literature review. **Ramazan Erenler:** Methodology and Supervision. **İbrahim Demirtaş:** Methodology, Supervision, and Validation.

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Research Article

Bioactivity-guided isolation and quantification of Chlorogenic acid from *Calystegia silvatica* (Kit.) Griseb. (Convolvulaceae)

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Cytotoxic activity.

Abstract: This study aimed to investigate the bioactivity-guided isolation and quantification of chlorogenic acid from *Calystegia silvatica*, a plant traditionally utilized for its medicinal properties. The most cytotoxic aqueous sub-extract was subjected to open column chromatography to assess its cytotoxic activity against MCF-7 breast cancer cells using a resazurin reduction assay. The structure of the most active pure compound was determined to be chlorogenic acid (IC_{50}= 36.44 ± 2.18 µg/mL) using 1D and 2D NMR spectroscopy. A validated high-performance liquid chromatography-diode array detector (HPLC-DAD) method was employed for the quantification of chlorogenic acid, which resulted in a content of 10.05 mg/g crude extract. The results indicate the potential of *Calystegia silvatica* as a source of chlorogenic acid with cytotoxic activity.

1. INTRODUCTION

The use of medicinal plants in drug discovery has gained significant attention because of their rich bioactive and phytochemical compounds that have the potential to be used in the development of drugs for various diseases. These natural products have been recognized as important sources for the development of new pharmaceuticals and have traditionally been used in indigenous medicine. In this context, the Convolvulaceae family is an important group in drug research. *Calystegia silvatica*, also known as "large bindweed," is a medicinal plant belonging to the Convolvulaceae family that is traditionally used in Turkish folk medicine to alleviate knee pain (Sener *et al*., 2023). Although there has been limited phytochemical analysis of *C. silvatica*, various compounds such as fatty acids, phenols, sterols, ketones, hydrocarbons, and other organic compounds and volatile compounds in its essential oil were identified by gas chromatography–mass spectrometry (GC-MS) studies (Derbak *et al.*, 2023; Youssef *et al.*, 2023). Its sub-extracts exhibit cytotoxic effects against various cancer cell lines, with the stem

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extract showing moderate antitumor properties against cervical, prostate, and breast cancer cell lines, whereas the leaf extract exhibits weak antitumor properties (Youssef *et al*., 2023).

Cytotoxicity research is crucial for drug discovery, particularly because of the limitations of conventional chemotherapeutic agents in terms of their efficacy, side effects, and resistance. Preliminary investigations are essential to evaluate the potential of plant-derived compounds as alternative therapeutic options. In the context of breast cancer, which is the most common cancer in women, assessing the cytotoxic activity of plant extracts on various types of breast cancer cells could provide insights (Chen *et al*., 2018). In this study, we targeted the MCF-7 breast cancer cell line and conducted a bioactivity-guided isolation study to identify the cytotoxic effects. Chlorogenic acid, a significant secondary metabolite, was isolated and quantified by HPLC-DAD. We used a resazurin reduction assay to assess cytotoxic activity in the MCF-7 mammalian breast cancer cell line. Chlorogenic acid showed potential as an effective cytotoxic agent against MCF-7 cells.

2. MATERIAL and METHODS

2.1. Bioactivity-Guided Isolation

2.1.1. *Plant material*

The aerial parts of *Calystegia silvatica* were collected from Bostancı village in Trabzon (at an altitude of 85 meters) on June 23, 2016. The voucher specimen was deposited at the Herbarium of Ankara University Faculty of Pharmacy in Ankara, Türkiye, and assigned the identification number "AEF 26802." The species was identified by Prof. Dr. Ufuk Özgen, a professor of Pharmacognosy.

2.1.2. *Preparation of extract and subfractions*

The aerial parts of *Calystegia silvatica* (CS) were air-dried and powdered (433 g), and then extracted three times with methanol (2 liters for 8 hours each time). The resulting combined extracts were evaporated using a rotary evaporator at 40°C to obtain a crude residue. The dry methanolic extract (37 g) was then suspended in a mixture of water and methanol (9:1), and partitioned with chloroform (300 mL × 2) and ethyl acetate (300 mL × 2) to obtain the remaining aqueous phase. The resulting fractions were then lyophilized to obtain dry extracts, yielding chloroform (9.5 g), ethyl acetate (1.42 g), and the remaining aqueous phase (26 g), which was tested for its antiproliferative activity on MCF-7 mammalian breast cancer cell line (ER+ and PR+).

2.1.3. *Bioactivity-guided isolation studies*

Bioactivity-guided isolation led to the lyophilized aqueous phase, which exhibited the most cytotoxic activity among all sub-extracts. Consequently, the residual aqueous phase was dissolved in water and processed through reverse-phase silica gel column chromatography. Gradient elution was carried out using a solvent system consisting of H_2O: CH_3OH solvent system (99:1 to 0:100) for purification. A total of 53 fractions were obtained. Thereafter, the fractions were subjected to TLC using a solvent system of EtOAc:CH_3OH:H_2O (7:2:1), and similar fractions were combined into 4 main fractions. The results of the antiproliferative activity on MCF-7 cells enabled the identification of combination fractions 36-39 for further purification. The fraction was dissolved in methanol and separated on a Sephadex column through isocratic elution with 100% methanol. 19 fractions were obtained, and 3 subfractions were combined using thin-layer chromatography under the same conditions. The most effective fraction for cytotoxic activity was chlorogenic acid (CHA), which was purified from the combined fractions 11-15 (Figure 1). The structure of chlorogenic acid was determined using Nuclear Magnetic Resonance (NMR) spectroscopy. The compounds were dissolved in deuterated methanol (CD_3OH), and NMR spectra were obtained using a Bruker Ascend 400 MHz NMR spectrometer.

2.2. HPLC Analysis

2.2.1. *HPLC conditions*

Quantitative analysis was conducted utilizing a newly validated High-Performance Liquid Chromatography (HPLC) method that employed a C18 column (4.6 × 150 mm, 5 µm) in conjunction with a gradient program consisting of a two-solvent system: A (100% methanol) and B (2.5% v/v acetic acid in deionized water, adjusted to pH 2.65). The solvents were delivered at a constant flow rate of 1.2 mL/min, and the analysis was monitored using a diode array detector that scanned wavelengths between 240 and 320 nm. The gradient program was as follows: 0.01 min 15% A, 85% B; 4 min 30% A, 70% B; 7 min 40% A, 60% B; 12 min 50% A, 50% B; 0% B; 20 min 0% A, 100% B.

2.2.2. *Method validation*

The method was validated for linearity, recovery, precision, and selectivity in accordance with ICH guidelines (Singh, 1996) Five calibration curves were used to evaluate linearity, which included five different concentrations of CHA (6.25-100 g/mL) with five repetitive data points. To obtain a linear regression equation and determine the correlation coefficients (Table 1), the peak areas were plotted against different CHA concentration ranges. To measure the recovery, the percentage concentration of CHA was analyzed in triplicate at three different concentrations. Recovery was calculated by dividing the percentage of known quantities by the mean and standard deviation. Precision was evaluated by measuring intra- and inter-day precision for three different concentrations of CHA, and the relative standard deviation (% RSD) of retention times and % peak areas were determined for two separate days. The selectivity of the method was assessed by comparing the chromatograms.

2.2.3. *Quantitative analysis*

A calibration curve was used to determine the CHA content of the CS main methanol extract. The new HPLC method was used to run the extract in triplicate to perform quantitative analysis. The results were expressed as µg/mL.

2.3. Cytotoxic Activity Test

The cytotoxic effect of all sub-extracts and fractions was evaluated by bioactivity-directed resazurin induction assay on MCF-7 breast cancer.

Resazurin Reduction Assay

This assay is based on the conversion of resazurin to resorufin within viable cells. Non-viable cells cannot show blue staining because they lack metabolic capacity, which prevents the reduction of resazurin. To conduct this assay, 0.5 x 10^4 adherent cells were allowed to attach overnight, and 1 x 10^4 suspension cells per well were seeded in 96-well plates with varying concentrations of the test substance to obtain a total volume of 200 µL/well. After a 72-hour incubation period and the addition of resazurin (Sigma-Aldrich) for 4 hours, staining was measured using a plate reader (Infinite 200 M Plex-Tecan, Türkiye) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each assay was performed independently three times (Kuete *et al*., 2016). Doxorubicin HCl (Sigma-Aldrich) was used as a positive control.

3. FINDINGS

3.1. Bioactivity-Guided Isolation Studies

NMR data indicated that the isolated compound is chlorogenic acid (Figure 1).1H-NMR (CD_3OD, 400 MHz): δ 7.59 (d, *J* = 16.2 Hz, H-7'), 7.07 (bs, H-2'), 6.96 (d, *J* = 7.3 Hz, H-6'), 6.79 (d, *J* = 6.3 Hz, H-5'), 6.31 (d, *J* = 15.9 Hz, H-8'), 5.39 (t, *J* = 10.1 Hz, H-5), 4.13 (bs, H-3), 3.69 (d, *J* = 9.6 Hz, H-4), 2.27-2.07 (m, H-6), 1.94-2.04 (m, H-2) (Figure 2). ^{13}C-APT NMR (CD_3OD, 100 MHz): δ 179.57 (C-7), 167.81 (C-9'), 148.12 (C-4'), 145.46 (C-7'), 145, 40 (C-

3'), 126.42 (C-1'), 121.54 (C-6'), 115.10 (C-5'), 114.15 (C-2'), 113.70 (C-8'), 76.32 (C-1), 73.75 (C-4), 71.70 (C-5), 71.15 (C-3), 39.23 (C-6), 37.72 (C-2) (Figure 3). NMR data were confirmed in previous studies (Yang *et al*., 2015).

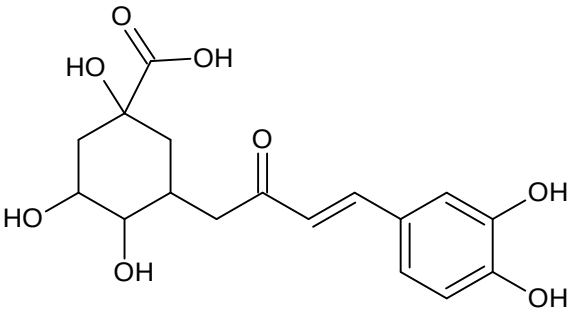

Figure 1. Molecular structure of chlorogenic acid.

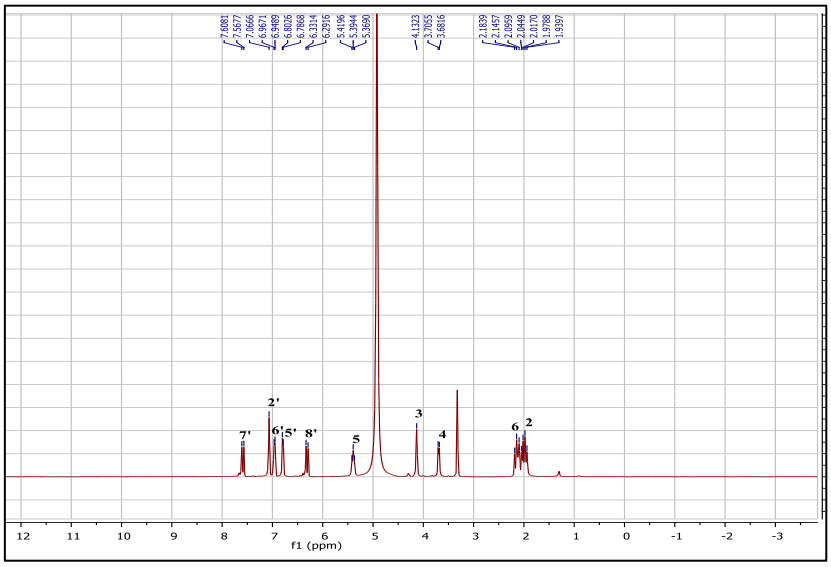

Figure 2. ^{1}H-NMR Spectrum of chlorogenic acid (CD_3OD, 400 MHz).

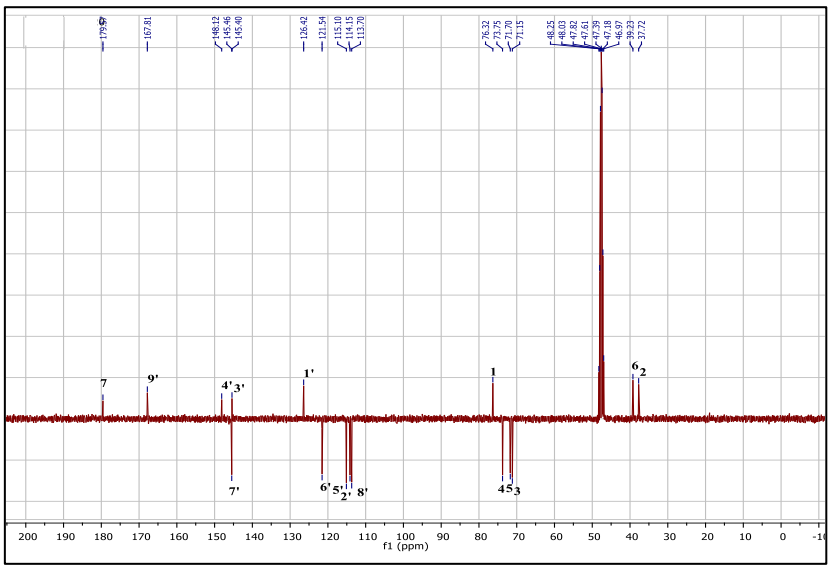

Figure 3. ^{13}C-APT NMR Spectrum of chlorogenic acid (CD_3OD, 100 MHz).

3.2. Results of HPLC Analysis

The different parameters of the new HPLC method for validation are shown in Table 1. The method showed good linearity (R^2>0.99) over the assayed concentration range (6.25-100 µg/mL) (Table 1, Figure 4). The relative standard deviation (RSD) values for intra- and inter-day precision were 0.20% and 0.83% for retention time and 0.33% and 0.35% for peak area, respectively. The accuracy for quality control varied from 98.77% to 101.78% (SD<0.81%) (see Table 1).

Table 1. Validation parameters of new HPLC method for detection of CHA in *Calystegia silvatica* methanol extract.

Retention time (min.)		Regression coefficient (R^2)	
3.23		0.9911	
Retention time (% RSD)		Peak area (% RSD)	
Intra-day	Inter-day	Intra-day	Inter-day
0.33	0.35	0.20	0.83
% Recovery (Mean ± SD)			
12.5 µg/mL	50 µg/mL	100 µg/mL	
101.78 ± 0.3010	98.77 ± 0.8177	100.83 ± 0.2013	

The quantitative analysis of CHA in the *C. silvatica* extract was performed based on the peak areas of the chromatograms of the sample using the calibration curve (Figure 3). Quantitative analysis by HPLC showed that the CHA content of the extract was expressed as mg/g crude extract (Figure 4). CHA content was detected as 10.05 mg/g crude extract.

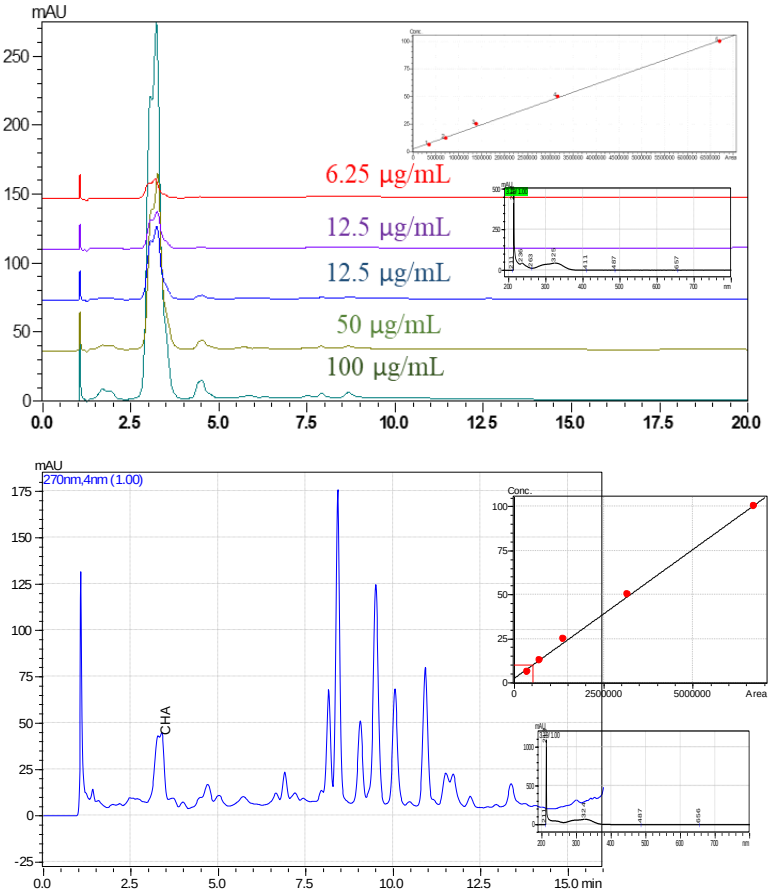

Figure 4. HPLC chromatograms and calibration curves for CHA, methanol extract of *Calystegia silvatica*.

3.3. Results of Cytotoxic Activity Test

The cytotoxic activity of the main methanol and sub-extracts of *Calystegia silvatica* was assessed based on IC_{50} values obtained through the resazurin method by measuring fluorescence. The main methanol extract displayed the lowest IC_{50} value of 124.70 ± 3.25 µg/mL, followed by the aqueous extract at 136.76 ± 5.36 µg/mL, while the ethyl acetate and chloroform extracts demonstrated higher IC_{50} values of 249.41 ± 2.58 µg/mL and 256.09 ± 3.98 µg/mL, respectively (Table 2). The aqueous extract was identified as the most active and suitable sub-extract for isolation studies. Subsequent fractionation of the active fractions obtained from column chromatography led to the isolation of chlorogenic acid compound with an IC_{50} value of 36.44 ± 2.18 µg/mL (Figure 5). The positive control doxorubicin demonstrated an IC_{50} value of 0.0522 ± 0.001 µg/mL.

Table 2. IC_{50} value of main methanol and sub-extracts of *Calystegia silvatica.*

	MeOH	*Aqueous*	*EtOAc*	*Chloroform*
IC_{50} (µg/mL)	124.70 ± 3.25	136.76 ±5.36	249.41 ± 2. 58	256.09 ± 3.98

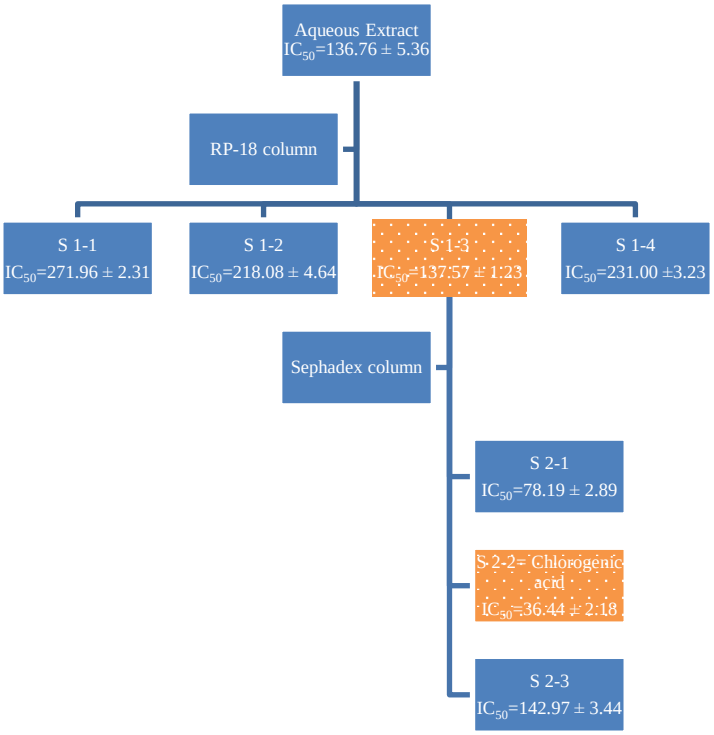

Figure 5. Bioactivity guided isolation scheme by using the cytotoxicity results on MCF-7 breast cancer cell line (IC_{50}: The concentration of a substance required to inhibit cell viability by 50%, µg/mL)

4. DISCUSSION and CONCLUSION

The study on the bioactivity-guided isolation and quantification of chlorogenic acid from *Calystegia silvatica* represents the first phytochemical isolation study on this plant, highlighting the presence of this important secondary metabolite. By focusing on the cytotoxic activity of the *C. silvatica* fractions against MCF-7 breast cancer cells and utilizing a bioactivity-guided isolation approach, the research sheds light on the bioactive potential of natural compounds for therapeutic applications. The quantification of chlorogenic acid using a validated HPLC-DAD method further strengthens the study's methodology and credibility.

MCF-7 breast cancer cells, known for their estrogen and progesterone receptor positivity (ER+ and PR+), are crucial in studying hormone-responsive breast cancer treatments. Previous studies have shown that the stem extract of *C. silvatica* exhibited an IC_{50} of 172 ± 15 µg/mL in

the same cancer cell line using the MTT method (Youssef *et al*., 2023). We evaluated the entire aerial part of the plant using the resazurin reduction assay, which has been demonstrated to be relatively inexpensive, sensitive, and non-invasive compared to tetrazolium assays (Petiti *et al.*, 2024). Our findings indicated that the main methanol extract of *C. silvatica* exhibited the lowest IC_{50} value of 124.70 ± 3.25 µg/mL, suggesting a potent inhibitory effect. Further investigation into the sub-fractions revealed that the aqueous extract was more active compared to the ethyl acetate and chloroform extracts, with IC_{50} values of 136.76 ± 5.36 µg/mL, 249.41 ± 2.58 µg/mL, and 256.09 ± 3.98 µg/mL, respectively. Consequently, our research focused on the aqueous extract for further isolation studies.

Subsequent fractionation of the active aqueous extract from column chromatography led to the isolation of chlorogenic acid, which showed a significantly lower IC_{50} value of 36.44 ± 2.18 µg/mL (102.9 ± 6.1 µM) according to the resazurin reduction assay. This high cytotoxic activity on MCF-7 breast cancer aligns with previous studies indicating its effectiveness in inducing apoptosis, DNA damage, reducing MMP levels, and activating the PKC signaling (Deka *et al.*, 2017; Suberu *et al.*, 2014). Chlorogenic acid has also been extensively studied for its various biological activities, including antioxidant properties (Sato *et al*., 2011), reducing cardiovascular disease and diabetes risk, and exhibiting antibacterial and anti-inflammatory effects (Farah & Lima, 2019). Considering its significant biological effects, quantification of this biologically active compound in the main methanol extract of *C. silvatica* was conducted using a newly developed and validated HPLC method. Notably, while green coffee is traditionally recognized as a primary source of chlorogenic acid (50-120 mg/g extract), our study demonstrates that *C. silvatica* also contains substantial levels of this compound (10.05 mg/g extract) (Ayelign & Sabally, 2013; Dado *et al.*, 2019; Farah *et al.*, 2008).

In conclusion, the research on chlorogenic acid from *C. silvatica* presents promising findings regarding its cytotoxic potential against breast cancer cells. Future studies could focus on the isolation and characterization of additional bioactive compounds from *C. silvatica*, as well as investigating the molecular mechanisms underlying its cytotoxic activity.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Merve Yüzbaşıoğlu Baran: Investigation, Methodology, Resources, Writing-Editing. **Sıla Özlem Şener:** Investigation, Resources, Methodology, Validation, Formal Analysis, Writing-original draft. **Şeyda Kanbolat:** Investigation, Methodology, Formal Analysis. **Merve Badem:** Investigation, Methodology, Formal Analysis. **Ufuk Özgen:** Resources, Methodology, Supervision, Writing-original draft.

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Research Article

Molecular identification and phytochemical profiling of selected medicinal plants in Bongabon, Nueva Ecija, Philippines

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Abstract: The study focuses on the five medicinal plants used by the local people residing in Calaanan, Bongabon Nueva Ecija Philippines. The study aimed to investigate using DNA-based identification, phytochemical screening, and antioxidant analysis of the plant's ethanolic extract. The selected five plants were initially identified by a taxonomist and molecularly identified using the *rbc*L gene marker. These plants were identified as *Scoparia dulcis, Vachellia fernasiana, Centella asiatica, Sapindus saponaria,* and *Ocimum tenuiflorum*. The extracts of the plants underwent Fourier Transform Infrared spectroscopy (FTIR) analysis to determine the functional group present in each plant and further analysis led to Thin Layer Chromatography (TLC) to unveil the presence and absence of the plant's secondary metabolites. The phytochemical profiles revealed the presence of essential oils, phenols, fatty acids, anthraquinones, anthrones, coumarins, flavonoids, and tannins. The results from the phytochemical analysis demonstrated the chemical diversity of the plant, prompting further investigations into its various bioactive properties. Further, the plants were subjected to 2,2'diphenyl-1-1picrylhydrazyl (DPPH) radical scavenging assay using a 1000ppm concentration of crude extracts, results revealed a range from 22.71% to 79.01% radical scavenging activity compared to the control which is at 83.56%. Collectively, this study reveals the accurate identity, phytochemical profile, and antioxidant activity of the medicinal plants.

1. INTRODUCTION

Plants were long recognized as vital sources of novel pharmacologically active compounds, which played a crucial role in drug development within the pharmaceutical industry. Their historical significance in treating various diseases underscored their importance in medical science (Boy *et al*., 2018; Veeresham *et al*., 2012) Plant-based traditional medicines remained

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widespread due to their affordability, efficacy, and minimal side effects in treating common ailments. In addition, plant extracts emerged as a valuable resource for the discovery of new compounds and the development of new pharmaceutical products.

In the Philippines, medicines and health services from physicians and other healthcare professionals are difficult to obtain in a geographically distant plunge location where people are physically or economically constrained (Lazarte, 2020). More than 12,000 different plant species may be found in the Philippines, of which, 1500 are used in traditional medicine by the indigenous peoples (Dela Cruz & Ramos 2006; Tantengco *et al*., 2018). Indigenous communities in the Philippines have used plants to cure a variety of illnesses, from less serious to potentially fatal ones (Balangcod & Balangcod, 2015).

The utilization of plants for medicinal purposes has been an important part of the cultural heritage of Philippine provinces for centuries. Through generations, indigenous and local communities have passed down invaluable knowledge about the practical use of local flora, offering effective remedies for common ailments. Depending on the location, the inaccessibility of the people communities adopted a deep dependence on natural resources, highlighting the importance of being familiar with the plants and their deep understanding of the efficacy and safety of the plants. The study identified five medicinal plants, *namely Scoparia dulcis, Vachellia fernasiana, Centella asiatica, Sapindus saponaria,* and *Ocimum tenuiflorum* confirmed by its DNA with matched sequences on the GenBank database. Before the conduct of this study, an initial survey for the knowledge and practices of the locals and collection of medicinal plants was provided by the research under the project of the Department of Science and Technology - Regional Office III of the Philippines.

The Barangay Calaanan in Bongabon, Nueva Ecija Philippines was surrounded by agricultural lands, rivers, and mountains. The residents live close to a mountainous region abundant with a diverse range of plants suitable for medicinal purposes. The distant location of the local residents from the community prompted them to make use of medicinal plants. However, the utilization of medicinal plants carries potential risks, as excessive dosages or the presence of harmful effects can lead to serious health issues shortly after consumption. The danger arises from the challenge some locals face in accurately discerning the safe quantity of medicinal plants to use, thus running the risk of encountering toxic or even life-threatening effects later on. In the interest of providing proper information for the locals, the goal of this research was to identify plants using molecular techniques, particularly those unknown to them. Despite their existing knowledge about the medicinal applications of the plants, understanding their bioactive constituents was similarly important. To validate their medicinal uses, the study also determined the antioxidant potential of the collected medicinal plants.

2. MATERIAL and METHODS

2.1. Molecular Identification

2.1.1. *DNA extraction*

The plant leaves were obtained in February 2022, from Barangay Calaanan in Bongabon, Philippines. Samples and a photograph voucher were initially identified by a taxonomist at Central Luzon State University. The extraction of DNA was carried out using Cetyltrimethylammonium bromide (CTAB) method followed by the published paper of Abdel-Latif and Osman (2017).

2.1.2. *PCR amplification*

Polymerase Chain Reaction (PCR) amplification of the nuclear ribosomal DNA was carried out using a primer pair of ribulose 1,5-biphosphate carboxylase *rbc*L forward (5′-ATGTCACCACAAACAGAGACTAAAGC-3′) and *rbc*L reverse (5′-GTAAAATCAAGTCC ACCRCG-3′) (CBOL Plant Working Group *et al*., 2009). The gene *rbc*L had the strongest characterization among plastid genes. It was now simple to retrieve across land plants owing to advances in primer design, and it was ideal for recovering high-quality bidirectional sequences

(Fazekas *et al*., 2008; CBOL Plant Working Group *et al*., 2009). The PCR reaction was composed of a 25µL mixture (manufacturer's protocol) containing the following: 1µL of genomic DNA, 1µL of each rbcL primer, 12.5µL of 1X GoTaq® Green Master Mix (Promega Corporation, USA), and 9.5µL of nuclease-free water. Samples were run into Veriflex™ 96-Well Thermal Cycler (Applied Biosystems, California, USA), and the conditions were programmed as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 51.2°C for 30 sec, extension at 72°C for 60 sec, and a final cycle of extension at 72°C for 5 min. The quality of PCR amplicons was checked following the same method described in the preceding section. The PCR product was quantified using Qubit 4 Fluorometer (Invitrogen Thermo Fisher Scientific, USA) and PCR purified before sequencing.

2.1.3. *DNA sequencing and sequence alignment*

Unpurified PCR products were sent to Apical Scientific in Selangor Malaysia for PCR Product Cleaning and DNA sequencing. The validated sequence data generated using the forward primer and reverse primer were edited and aligned using a pairwise alignment tool, and consensus sequences were conducted using the BioEdit 7.2 (Hall, 1999) application. The nucleotide sequence comparisons were performed using the standard nucleotide Basic Local Alignment Search Tool (BLASTN) against the National Center for Biotechnology Information (NCBI) GenBank database.

2.2. Phytochemical Analysis

2.2.1. *Preparation of extracts*

The air-dried samples were ground and sieved into a powder form using an automated grinder. Ten grams (10 g) of powdered sample were soaked in 90% ethanol for three days with frequent agitation. After three days, the soaked samples were filtered using a No. 2 Whatman filter. The filtrates were concentrated to dryness using a digital rotary evaporator (DLAB™, Flinn Scientific, Canada). The resulting extracts were used for various analyses and assays.

2.3. Phytochemical Analysis using FTIR Spectroscopy

To confirm the presence of secondary metabolites determined in phytochemical analysis using the TLC method, the ethanolic extract of selected medicinal plants was analyzed using Fourier Transform Infrared Spectroscopy (FTIR) at the Central Instrumentation Facility, De La Salle University, Laguna Campus.

2.4. Phytochemical Analysis using Thin Layer Chromatography

The phytochemical screening of the ethanolic extract of collected medicinal plants was determined at the Chemistry Laboratory of the Center for Natural Sciences at St. Mary's University, Bayombong, Nueva Vizcaya. Phytochemical screening was performed to detect the secondary metabolites of the extract. The extract was spotted on a 7 x 4 cm marked and labeled TLC (thin layer chromatography) plate. This was done in the developing chamber using an acetate-methanol (7:3) mixture. To test the separation of the different substances, the spots for specific metabolites were observed using TLC plates that were subjected to UV light and a hot plate. Vanillin-sulfuric acid reagents were used to detect the presence of phenols, steroids, triterpenes, and essential oils. A methanolic potassium hydroxide was used to detect secondary metabolites such as anthraquinones, coumarins, and anthrones. The phenolic compounds and tannins were determined using the potassium ferricyanide-ferric chloride reagent. Lastly, Dragendorff's reagent was used to identify the presence of flavonoids (Guevara, 2005).

2.5. Screening of the Antioxidant Property

The 2,2'diphenyl-1-1picrylhydrazyl (DPPH) radical scavenging activity and the total phenolic content of the collected medicinal plant ethanolic extracts were also performed at the Chemistry Laboratory of the Center for Natural Sciences at St. Mary's University, Bayombong, Nueva Vizcaya.

The radical scavenging activity of bioactive chemicals extracted from medicinal plants was determined using the DPPH assay adapted from the methods of Kolak *et al*., 2006. Concentrated extract was used as a stock solution, and aliquots were taken to create 1000 ppm dilutions and 1000 ppm Catechin (1mg/mL) as controls. In a separate plastic cuvette, 4mL of 0.1 mM DPPH solution was combined with 1mL of prepared stock solution. The reactions were carried out in triplicate. The prepared mixtures were incubated in the dark for 30 minutes at 37°C. The absorbance measurements were checked at 517 nm using a UV-VIS spectrophotometer. Lower absorbance of the reaction mixture determined higher free radical scavenging activity. The radical scavenging activities were compared to Catechin activity, and the following equation was used to calculate the ability to scavenge the DPPH radical:

$$\%\ Radical\ Scavenging\ Effect = [\ (A_{control} - A_{sample}) / A_{control}\ ]\ x\ 100$$

Where $A_{control}$ is the absorbance of the control which is the DPPH without the sample. A_{sample} is the absorbance of the test sample containing the DPPH sample. Catechin was used as the positive control.

3. FINDINGS

3.1 Molecular Identification and Plant Assessment

In the present study, the five plants (Figure 1) declared by the local people of Bongabon Nueva Ecija, Philippines as medicinal plants were molecularly identified. The BLAST (NCBI-Genbank) tool was employed to determine the homology of the plant samples (see Table 1). According to the Consortium for the Barcode of Life (2009), an optimal DNA barcode marker should be easily amplifiable, exhibit sufficient variability for species identification, and offer maximum discrimination among species. Hence, PCR amplification of *rbc*L forward and reverse primer pairs was used to establish and produce a single band of approximately 570 base pairs.

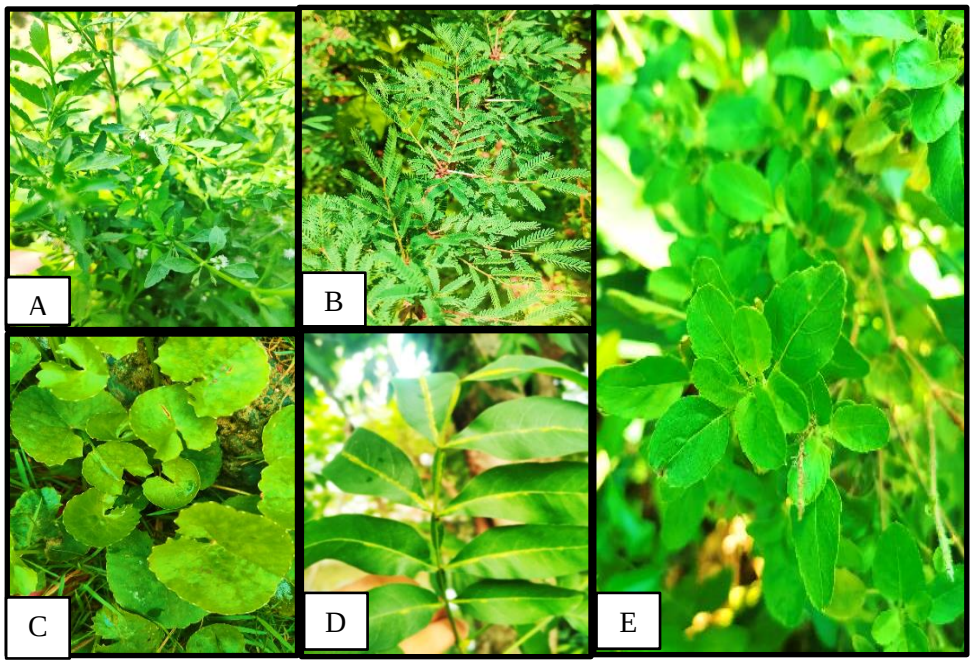

Figure 1. Photograph image of the five collected plants (A. *Scoparia dulcis* B. *Vachellia farnesiana* C. *Centella asiatica* D. *Sapindus saponaria* E. *Ocimum tenuiflorum*)

Table 1. BLAST analysis results or ribosomal DNA sequences of the five collected plants.

Sample Code	BLASTn Identity	% Identity	Query Cover	Accession Code
CB2	*Scoparia dulcis*	99.82	100	MZ958832.1
CB3	*Vachellia farnesiana*	99.82	100	MN592492.1
CB4	*Centella asiatica*	99.82	100	MN854377.
CB9	*Sapindus saponaria*	99.64	99	AY724366.1
CB10	*Ocimum tenuiflorum*	99.82	100	NC.043873.1

Based on the gathered data from the interview conducted by the Central Luzon Health and Research Department (CLHRDC) team, it was reported that the listed plants had been declared by locals to have been used as remedies for various diseases. The collected plants comprised primarily shrubs and trees; however, according to available literature, not all parts of these plants were utilized for medicinal purposes. To support, the consolidation of data from books and online sources was also cited to support these findings (see Table 2).

The *S. dulcis* is a compact shrub, with its roots and leaves historically utilized for a diverse range of remedies, including addressing dysentery and fever. On the other hand, the bark, roots, and leaves of *V. farnesiana* shrub were employed for treating sore throats and treats skin diseases. Similarly, *C. asiatica*, another shrub in the collection, was consumed for dysentery and colds, focusing specifically on the use of its leaves. *Ocimum tenuiflorum,* another shrub, had its roots and leaves applied for the treatment of conditions such as coughs, bronchitis, fungal infections, and malaria. In contrast, the lone tree in the assortment, *S. saponaria*, boasts a comprehensive use of its bark, fruit, roots, and leaves as remedies for external wounds, lesions caused by fungi, and ulcers.

The assortment of these five plants showcased a diverse selection of applications in addressing various diseases. Remarkably, many of these plants were commonly found in close proximity to households, highlighting the richness of natural resources in the study area and the accessibility of those plants for the local population to use as cost-effective remedies. This underscored the importance of traditional knowledge and the potential of local flora in providing healthcare solutions.

Table 2. List of the Medicinal Plants Species Collected in Calaanan, Bongabon Nueva Ecija.

Species	**Medicinal Value**	**Parts Used**	**Medicinal References**
Local Name: Culantro Family: Plantaginaceae Scientific name: *Scoparia dulcis* Leaf shape: Lanceolate Leaf margin: Serrate Leaf venation: Pinnate Habit: Shrub	gastralgia, diarrhea, and dysentery, fever, cough	root, leaves	1
Local Name: Aroma Family: Acacieae Scientific name: *Vachellia farnesiana* Leaf shape: Compound Leaf margin: Entire Leaf venation: Pinnate Habit: Shrub	astringent, sore throats, antispasmodic, skin diseases	bark, leaves, roots	1, 2

Local Name: Takip kuhol Family: Scientific name: *Centella asiatica* Leaf shape: Reniform Leaf margin: Serrate Leaf venation: Palmate Habit: Shrub	skin diseases, sclerotic wounds, fever, colds, dysentery	leaves	1, 2
Local Name: *Unknown* Family: Sapindaceae Scientific name: *Sapindus saponaria* Leaf shape: Compound Leaf margin: Entire Leaf venation: Pinnate Habit: Tree	ulcers, external wounds, skin lesions caused by fungi	bark, fruit, roots, leaves	1, 2
Local Name: Biday Family: Sapindaceae Scientific name: *Ocimum tenuiflorum* Leaf shape: Elliptical Leaf margin: Serrate Leaf venation: Pinnate Habit: Shrub	cough, bronchitis, asthma, malaria, dysentery, stress situations, worm infestations, superficial fungal infections, and as diuretic	bark, fruit, roots, leaves	1

Note: Plant families, followed by simple description, habit (T = tree, S = shrub, H = herb, V = vine, E = epiphyte) and medicinal references (1 = www.stuartxchange.org/CompleteList.html, 2 = www.tkdlph.com, 3 = other sources)

3.2. Phytochemical Analysis (Fourier Transform Infrared Spectroscopy)

Infrared (IR) spectroscopy serves as a valuable spectroscopic technique when combined with High Performance Thin Layer Chromatography (HP-TLC). Its notable advantages include the minimal or absence of sample preparation, making it an environmentally friendly and reagent-free tool that delivers results within a few minutes (Dytkiewitz & Morlock, 2008; Agatonovic-Kustrin, & Morton, 2020). The chemical bonds and functional groups present in the crude extracts of the leaves were predicted using the FTIR. Figures 1-5 represent the FTIR spectrum and Table 2 is the interpretation of the chemical bonds in the 5 collected plant extracts. Each functional group possesses a unique assigned wave number (cm^{-1}), which the FTIR spectrometer reads. The presence of a peak at specific wavelengths signifies the presence of the corresponding functional group. The FTIR spectrum confirmed the presence of the 8 functional groups (see Table 3). These were the alcohols or phenols group, alkanes or methyl group, carbonyl group, aromatic compounds, methyl or methylene group, alkanes group, ethers or esters group and amines or amides group.

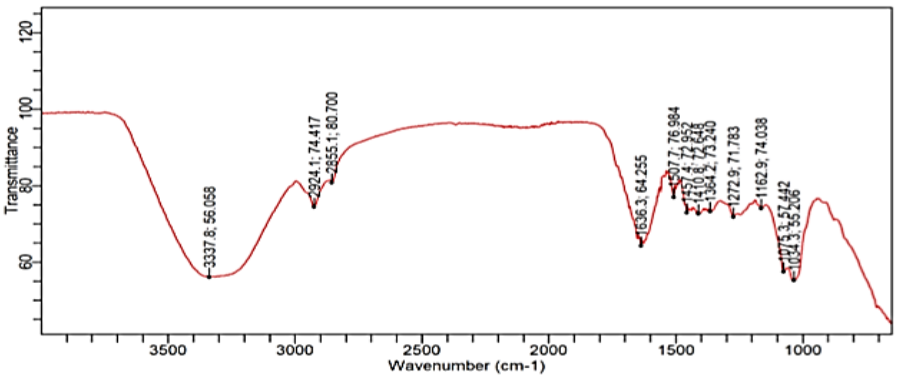

Figure 2. FTIR Spectrum of *Scoparia dulcis* leaves ethanolic crude extract.

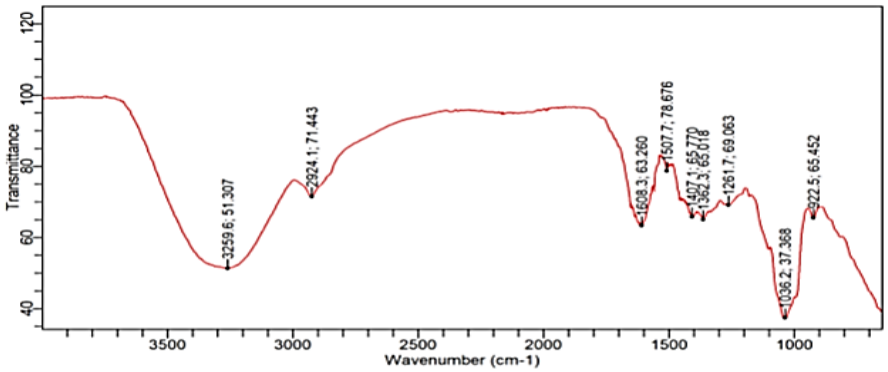

Figure 3. FTIR Spectrum of *Vachellia fernasiana* leaves ethanolic crude extract.

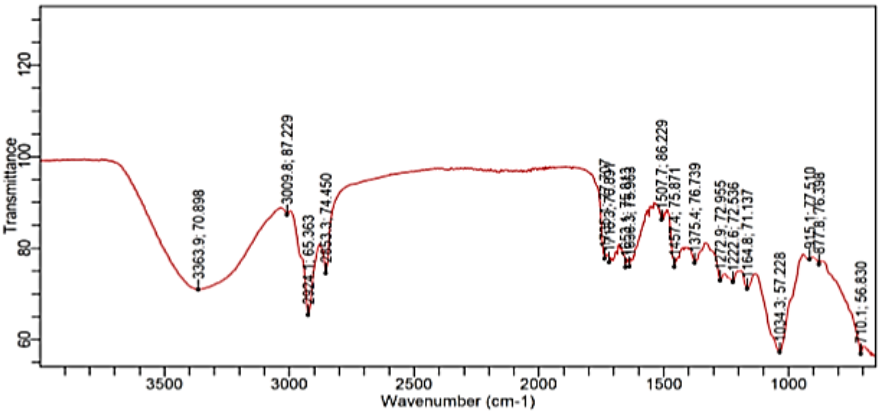

Figure 4. FTIR Spectrum of *Centella asiatica* leaves ethanolic crude extract.

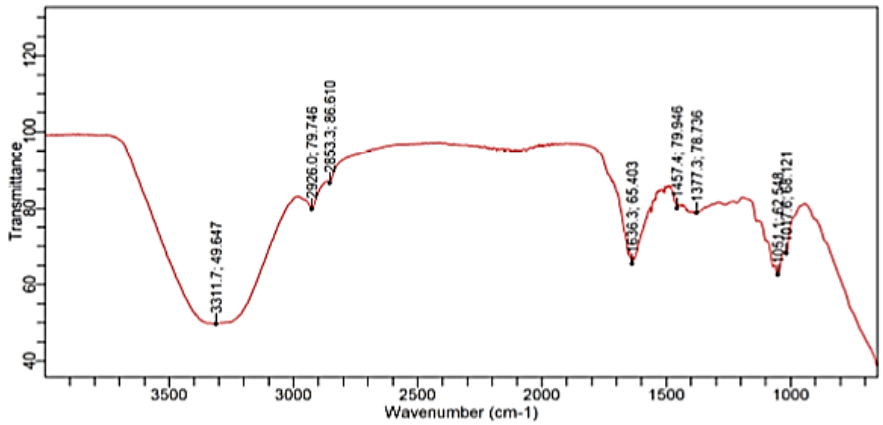

Figure 5. FTIR Spectrum of *Sapindus saponaria* leaves ethanolic crude extract

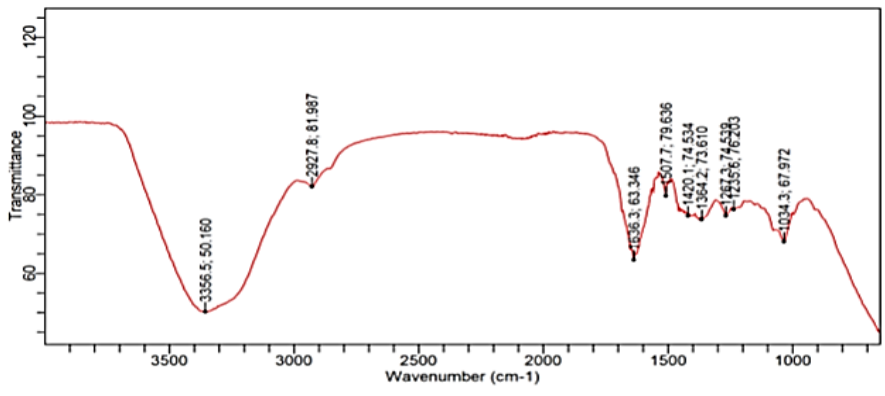

Figure 6. FTIR Spectrum of *Ocimum tenuiflorum* leaves ethanolic crude extract

Table 3. FTIR spectral peak values, functional groups and the possible presence of secondary metabolites of the five plants' ethanolic leaf extract.

Plant Samples	Peaks (cm-1)	Molecular Vibration	Functional Group	Possible Presence	References
Scoparia dulcis	3337.8	O-H stretching	Alcohol or Phenols group	Phenols, Tannins, Fatty Acids	1, 5
	2924.7	C-H stretching	Alkanes or Methyl group	Alkaloids	2
	1636.4	C=O stretching	Carbonyl group	Anthraquinones, Tannins, Flavonoids	2, 5
	1507.7	C=C stretching	Aromatic compounds	Flavonoids, Alkaloids, Saponins	2
	1457.4	C-H bending	Methyl or Methylene group	Flavonoids, Tannins	3
	1162.9	C-O stretching	Ethers or Esters group	Flavonoids, Coumarins	2
	1075.3	C-N stretching	Amines or Amides group	Alkaloids	4
	1034.3	C-H bending	Aromatic compounds	Essential oils	5
Vachellia fernasiana	3259.6	O-H stretching	Alcohol or Phenols group	Phenols, Tannins, Fatty Acids	1, 5
	2924.1	C-H stretching	Alkanes or Methyl group	Alkaloids	2
	1608.3	C=C stretching	Aromatic compounds	Flavonoids, Alkaloids, Saponins	2
	1362.3	C-H bending	Methyl or Methylene group	Flavonoids, Tannins	3
	1036.2	C-H bending	Aromatic compounds	Essential oils	5
Centella Asiatica	3363.9	O-H stretching	Alcohol or Phenols group	Phenols, Tannins, Fatty Acids	1,5
	3009.8	C-H stretching	Alkanes or Methyl group	Alkaloids	2
	1718.3	C=O stretching	Carbonyl group	Anthraquinones, Tannins, Flavonoids	2, 5
	1507.7	C=C stretching	Aromatic compounds	Flavonoids, Alkaloids, Saponins	2
	1457.4	C-H bending	Methyl or Methylene group	Flavonoids, Tannins	3
	1162.9	C-O stretching	Ethers or Esters	Coumarins, Flavonoids	2
	1222.6	C-N stretching	Amines or Amides group	Alkaloids	4
	1034.3	C-H bending	Aromatic compounds	Essential oils	5
	710.1	C-H out-of-plane bending	Aromatic compounds	Essential oils	6
Sapindus saponaria	3311.7	O-H stretching	Alcohol or Phenols group	Phenols, Tannins, Fatty Acids	1, 5
	2926.0	C-H stretching	Alkanes or Methyl group	Alkaloids	2
	1636.3	C=O stretching	Carbonyl group	Anthraquinones, Tannins, Flavonoids	2, 5
	1457.4	C-H bending	Methyl or Methylene group	Flavonoids, Tannins	3
	1272.9	C-H bending	Alkanes	Alkaloids	4
	1051.1	C-O stretching	Ethers or Esters	Coumarins, Flavonoids	2
	1017.6	C-N stretching	Amines or Amides group	Alkaloids	4
Ocimum tenuiflorum	3356.5	O-H stretching	Alcohol or Phenols group	Phenols, Tannins, Fatty Acids	1, 5
	2927.8	C-H stretching	Alkanes or Methyl group	Alkaloids	2
	1636.3	C=O stretching	Carbonyl group	Anthraquinones, Tannins, Flavonoids	2, 5
	1507.7	C=C stretching	Aromatic compounds	Flavonoids, Alkaloids	2
	1420.1	C-H bending	Methyl or Methylene group	Flavonoids, Tannins	3
	1267.3	C-N stretching	Amines or Amides group	Alkaloids	4
	1034.3	C-H bending	Aromatic compounds	Essential oils	5

**References used:* 1. Shahidi, F., & Ambigaipalan, P. (2015); 2. Megawati *et al*., (2023); 3. Sahayaraj *et al*., (2015) 4. Mohanapriya *et al*., (2019); 5. Mwangi *et al*., (2024) 6. Topala L. & Ducu T. (2017)

3.3 Phytochemical Analysis (Thin Layer Chromatography)

In the study, samples were subjected to thin-layer chromatography, revealing the presence and absence of the 14 plant constituents tested in each plant leaf crude ethanolic extract (see Table 4). The preliminary FTIR analysis conforms with the qualitative phytochemical screening using TLC. The detected phytochemical compounds were recognized for their valuable significance in both industrial and medicinal fields. The results from the phytochemical analysis demonstrated the chemical diversity of the plant, prompting further investigations into its various bioactive properties. Based on the analysis, each plants have 7-9 phytochemicals present. Tons of records showed the benefits of these compounds detected from the five plants.

Table 4. Secondary metabolites present in the five medicinal plants.

Plant Constituents	*S. dulcis*	*V. farnesiana*	*C. asiatica*	*S. saponaria*	*O. tenuiflorum*
Essential Oils	+	+	+	+	+
Triterpenes	-	-	-	-	-
Sterols	-	-	-	-	-
Phenols	+	-	+	+	+
Fatty Acids	+	+	-	-	+
Sugars	-	-	-	+	-
Anthraquinones	+	+	+	+	+
Coumarins	-	-	-	+	+
Anthrones	-	+	+	+	-
Tannins	+	+	+	+	+
Flavonoids	+	+	-	+	-
Steroids	+	+	+	+	+
Alkaloids	+	+	-	-	-
Amino acids	-	+	+	-	+

**(+) Present (-) Absent*

3.4 Antioxidant Activity

The plants were subjected to a DPPH radical scavenging assay using a 1000ppm concentration of crude extracts. From the 5 samples tested (see Table 5), results showed that *O. tenuiflorum* had the highest percentage of radical activity at 79.01% that were comparable to the positive control Catechin which was at 83.56%. These samples were followed by *V. farnesiana* at 76.55%, *C. asiatica* at 73.38%, *S. dulcis* at 42.33%, and lastly *S. saponaria* at 22.71%.

Table 5. Percentage radical activity of the plant samples in comparison with the control.

Plant Samples	% Radical Scavenging Activity
Scoparia dulcis	42.33
Vachellia farnesiana	76.55
Centella asiatica	73.38
Sapindus saponaria	22.71
Ocimum tenuiflorum	79.01
Catechin (Control)	83.56

4. DISCUSSION and CONCLUSION

Traditional approaches for identifying plants involve using our senses (taste, sight, smell, touch), as well as examining their physical characteristics (shape, color, texture) through macroscopic and microscopic methods. Moreover, chemical profiling techniques such as TLC, HPLC-UV, and HPLC-MS are employed. The accuracy of chemical profiles can be influenced by physiological and storage conditions. Thus, authentication at the DNA level offers enhanced

reliability. Subsequently, the development of DNA-based markers plays a significant role in verifying the authenticity of medicinal plants (Techen *et al*., 2014). Due to inadequate morphological identification, unfamiliarity, confusion surrounding species identity due to similar local names, and the absence of local name confirmation, molecular identification of the plants is conducted.

For the purpose of mainly identification, the present study only used the rbcL primer pair since the CBOL Plant Working Group chose *rbc*L as the core barcode because of its significant advantage in terms of PCR success scores (China Plant BO; Li *et al*., 2011; Olivar *et al*., 2014). Results showed that the plant has 99% above identity and 99% - 100% query cover using the primer pair which indicates the sequence divergence between the collected plants and related species in the Genbank repository of NCBI. According to the study of Pearson (2013), identifying homologous sequences through sequence similarity searching stands as a pivotal and highly informative initial step in analyzing newly acquired sequences. The vast comprehensiveness of modern protein sequence databases ensures that over 80% of metagenomic sequence samples commonly exhibit substantial similarity with proteins cataloged within these databases.

Following proper identification, the plants underwent phytochemical analyses. Plants serve as the most potent sources of drugs, with many pharmaceutical products originating from traditional ethnomedicine practices. The indigenous wisdom concerning medicinal plants held by local communities represents a vital source of information that consistently contributes to contemporary herbal remedies (Balangcod *et al*., 2012). Plants can produce a large number of diverse bioactive compounds. Plants containing beneficial phytochemicals may supplement the needs of the human body by acting as natural antioxidants (Clemens-Pascual *et al*., 2022). With the progress of phytochemical research, more and more plant constituents have been isolated and identified (De Leon *et al*., 2018).

Based on the peaks of the five plants, the FTIR spectrum confirmed the presence of the 8 functional groups. The molecular vibration of O-H stretching at a range of 3259.6-3363.9 cm^{-1} and CH stretching at the range of 2853.3-3009.3 cm^{-1} (Oliveira *et al*., 2016) was found to be present uniformly in the five plant samples. The O-H and C-H were common and noticeable in diverse chemical environments within secondary metabolites, encompassing alcohols, phenols, aldehydes, ketones, carboxylic acids, and ethers. This broad spectrum of functional groups allows plants to produce secondary metabolites containing a diverse array of chemical structures and biological activities. These activities cover antioxidant, antimicrobial, antiviral, anticancer, and anti-inflammatory properties (de Sousa *et al*., 2023).

In addition, the carbonyl group of C=O stretching (George *et al*., 2022) was also found in the plants with the exception of *V. fernasiana*. With the possible presence of fatty acids, anthraquinones, tannins, flavonoids, and steroids, according to the study of Mamari and Hamad (2021) compounds containing carbonyl groups frequently exhibit pharmacological properties beneficial for medicinal use. Specifically, these groups can contribute to antioxidant, antimicrobial, anti-inflammatory, and anticancer activities. Another important functional group is the aromatic compound of C=C stretching at the peak range of 1507.7-1608.3 cm^{-1} (Teclegeorgish *et al*., 2021) was also present in the plants except for *S. saponaria;* the presence of phenols, unsaturated fatty acids, anthraquinones, flavonoids, alkaloids in this group may exhibit antioxidant, anti-inflammatory, antimicrobial, antiviral, anticancer, analgesic, and neuroprotective activities, making them valuable candidates for drug discovery and development (Gu *et al*., 2014).

The methylene functional group of C-H stretching with a peak range of 1362.3-1457.4 cm^{-1} (Md Salim *et al*., 2021) was again found to be present in all the plants that have the possible presence of tannins and flavonoids. A review from the study of Atanasov *et al*., (2021) mentioned that methyl groups are prevalent in natural products obtained from plants, and play a significant role in the extensive array of bioactive compounds utilized for medicinal purposes.

Traditionally employed in alternative medicine, these compounds persist as crucial reservoirs of potential drug leads and pharmaceutical agents. Furthermore, the alkanes group of C-H bending with a peak of 1272.9 was only detected in *S. dulcis* and *C. asiatica*. Within this group, it is expected to have the possible presence of terpenoids, alkaloids, and steroids. According to Hussein *et al*., (2021), many traditional medicinal plants contain alkane-rich secondary metabolites that have been used for centuries in herbal remedies and traditional medicine systems worldwide.

Moreover, ether groups of C-O stretching at the peak range of 1051.1-1164.8 cm^{-1} (Devi *et al*., 2019) have been detected in *S. dulcis, C. asiatica*, and *S. saponaria*. It is expected to have a presence of coumarins and essential oils in this functional group. Many plant-derived ethers exhibit valuable biological activities relevant to medicinal applications. These activities may include antioxidant, antimicrobial, anti-inflammatory, analgesic, and sedative properties, among others. Ethers can also serve as bioactive components in traditional herbal remedies (Oluwapelumi *et al*., 2023). Another important functional groups were the amines or amides with C-N stretching at the peak ranges of 1017.6-1235.6 cm^{-1} (Devi *et al*., 2019) a presence of alkaloids was expected in this group and it was observed in *S. dulcis* and *C. asiatica.* These plants were used by a huge number of population in the Philippines and were very well known to be consumed for different medicinal applications supporting that the plants had various of study conducted. Amines are organic compounds characterized by the presence of a nitrogen atom bonded to one or more alkyl or aryl groups (Ertl *et al*., 2020). Many drugs contain amine functional groups, which can participate in hydrogen bonding, receptor interactions, and enzymatic reactions (Ishtiaq *et al*., 2020).

Lastly, the lowest peak recorded at 710.1 cm^{-1} represents the functional group of the aromatic compound with a vibration of C-H out of plane bending (Nortije *et al*., 2024). The possible presence of essential oils is expected to be found in the plant containing this peak. The presence of these functional groups provides valuable insight into the chemical properties and potential biological activities of the plant samples.

Moreover, plant samples were also subjected to thin-layer chromatography, revealing the presence and absence of the 14 plant constituents tested in each plant leaf crude ethanolic extract. Based on the analysis, each plants have 7-9 phytochemicals present. The phytochemicals showed diverse compounds like essential oils, phenols, flavonoids, anthraquinones, coumarins, tannins and alkaloids. Tons of records showed the benefits of these compounds detected from the five plants.

The results from the phytochemical analysis demonstrated the chemical diversity of the plant, prompting further investigations into its various bioactive properties. Hence, the conduct of antioxidant activity was also initiated and confirmed high levels of radical scavenging activity in all five plants. Plants contain various natural compounds like polyphenols, flavonoids, glutathione, vitamin E (α-tocopherol), vitamin C, and other metabolites. These molecules have the ability to scavenge free radicals, neutralize singlet and triplet oxygen, decompose peroxides, inhibit enzymes, and work together to prevent and treat diseases (Latayada and Uy, 2016). The build-up of harmful free radicals in the body can lead to various health conditions such as ischemia, asthma, arthritis, inflammation, neurodegeneration, Parkinson's disease, Down syndrome, the aging process, and potentially dementia (Mundhe *et al*., 2011; Kaur & Mondal 2014).

To further understand the specific chemical components of plants, researchers are carrying out assays such as antioxidant screenings. These tests help determine the true efficacy of traditional remedies passed down through folklore (Uy & Villazorda, 2015). Traditionally, antioxidant activity was linked to the presence of phenolic compounds in plants, which were considered secondary metabolites along with flavonoids. While phenolic compounds were directly associated with antioxidant activity, flavonoids also played a role in this effect. Their mechanism involved hydrogen donation and scavenging of free radicals. This scavenging

ability enabled them to interact with reactive oxygen species (ROS), which could cause oxidative stress and tissue damage. By reacting with ROS, phenolic compounds and flavonoids prevented oxidative stress, thereby promoting wound healing. Additionally, other plant metabolites besides phenols and flavonoids could contribute to antioxidant activity and wound healing (da Silva *et al*., 2006; Sharma *et al*., 2012; Oliveira *et al*., 2016). The results found in the present work could be related to phenols, flavonoids and other metabolites. Thus, this study provides necessary information for the medicinal plants usually consumed and used by the locals for their safety and awareness.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: Central Luzon State University Ethical Clearance Code: 2023-5192.

Authorship Contribution Statement

Dana Theresa De Leon: Investigation, resources, visualization, software, formal analysis, and writing - original draft. **Arwil Nathaniel Alfonso**: Investigation, resources, visualization. **Angeles De Leon**: Supervision, and validation. **Jerwin Undan**: Supervision, and validation.

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Research Article

Evaluation of the *in-vitro* anti-inflammatory activity of *Malva sylvestris* leaves extract

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Abstract: The primary aim of this research endeavor was to thoroughly evaluate and explore the potential anti-inflammatory properties exhibited by leaf extracts obtained from the *Malva sylvestris* plant species. In order to investigate the anti-inflammatory capabilities, a comprehensive set of *in vitro* experimental procedures was carried out to meticulously examine and gauge the extract's efficacy to stabilize human erythrocyte cell membranes under various stressful conditions, including exposure to varying concentrations of sodium chloride (NaCl), elevated temperatures, and oxidation induced by hypochlorous acid (HOCl). Additionally, the extract's potential to inhibit the denaturation of albumin, a process linked to inflammation, was evaluated. The findings revealed that the aqueous *Malva sylvestris* leaf extract exhibited notable anti-inflammatory properties by protecting red blood cell membranes from disruption caused by hypotonic NaCl solutions, heat stress, and oxidative damage from HOCl. These results suggest that the extract possesses significant anti-inflammatory potential and could be utilized as a natural remedy to mitigate inflammatory processes within the body. In summary, the aqueous extract derived from *Malva sylvestris* leaves demonstrated remarkable anti-inflammatory activity in vitro, making it a promising candidate for further exploration and potential therapeutic applications in the management of inflammatory conditions.

1. INTRODUCTION

The exploration of medicinal flora for their potential bioactive constituents has a rich legacy in traditional medicine practices, providing a fertile ground for modern medical advancements (Hill, 2022). Natural compounds have garnered increasing attention due to their emerging role as alternative antioxidant sources to synthetic counterparts. Radicals generated from external factors and metabolic processes pose a threat to biological molecules, ranging from minor tissue damage to cellular demise. The body employs antioxidant molecules, notably polyphenols, to combat this oxidative stress (Akbari *et al*., 2022).

Polyphenolic compounds, with their unique chemical architectures, act as effective electron donors, neutralizing free radicals and reactive oxygen species, thereby exhibiting antioxidant and anti-inflammatory properties (Mucha *et al*., 2021). In this context, *Malva sylvestris*,

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commonly referred to as mallow, is a perennial plant belonging to the Malvaceae family. Despite its historical use in traditional medicine (Panchenko, 2022), the anti-inflammatory capacities of *Malva sylvestris* leaves remain relatively underexplored. This study aims to investigate the anti-inflammatory potential of the aqueous extract of *Malva sylvestris* leaves.

2. MATERIAL and METHODS

The leaves used for extraction were collected from *Malva sylvestris* plants in the Tizi-Ouzou region of Algeria during the month of December 2022. The plant specimens were properly identified and authenticated by Professor SAADOUN-SMAIL Noria, a Botanist with the Department of Plant Biology at Mouloud Mammeri University of Tizi-Ouzou. A voucher sample of the identified plant material was deposited and preserved at the university's facilities (Voucher specimen: FSBSA/MK/2122).

The harvested leaves were air-dried and subsequently ground into a fine powder. This powdered material was stored away from light exposure at room temperature until the extraction process began. To prepare the extract, 20 grams of the powder was soaked in 200 milliliters of distilled water and allowed to macerate for a 24-hours at room temperature. Following this maceration step, the liquid portion was isolated by filtration and then lyophilized to obtain the dried extract.

2.1. Erythrocyte Suspension

Blood samples were obtained from healthy volunteers (ethical clearance certificate: CHU-UMMTO-N°0104/2024) and centrifuged for 10 minutes at 2,000 rpm and 4°C. The resulting red blood cells (RBCs) were washed three times with a saline buffer (PBS, 0.9% NaCl).

2.2. *In-vitro* Anti-inflammatory Studies

2.2.1. *Hypotonic induced hemolysis*

To assess the extract's ability to stabilize erythrocyte membranes, an experimental methodology involving hypotonic solution-induced hemolysis was employed. This approach followed the procedural guidelines previously described by de Freitas *et al*. (2008). Fourty microliter (40 µl) of the previously washed erythrocyte solution was introduced into different tubes containing a buffered hypotonic solution (PBS, pH 7.4) with varying NaCl concentrations (0.1%, 0.3%, 0.5%, 0.7% and 0.9%) and extract concentrations (18,75, 37,5,75, 150, 300, 600 and 1200 µg/mL). After 30 minutes of slow homogenization at 37°C, the tubes were centrifuged for 10 minutes at 2,000 rpm, and the absorbance was determined at 540 nm (UV-visible spectrophotometer MEDLINE MD2000).

2.2.2. *Heat-induced hemolysis*

Following the method described by Sakat *et al*. (2010), a 2% erythrocyte solution was added to a buffered phosphate saline solution at a pH of 7.4, 1 milliliter of extract (18,75, 37,5,75, 150, 300, 600 and 1200 µg/mL) and were incubated for 30 minutes at 56°C. After cooling, the tubes were subjected to centrifugation for 10 minutes at 2000 rpm. Following this step, the supernatant from each tube was carefully collected, and its absorbance was measured spectrophotometrically at a wavelength of 560 nanometers to quantify the extent of hemolysis that had occurred.

2.2.3. *Oxidant-induced hemolysis*

Following the technique detailed by Suwalsky *et al*. (2007) and Chandler *et al*. (2013). Various quantities of the extract (200, 400, 600, 800 and 1000 µg/mL) were added to 1 mL of a 5% erythrocyte solution (PBS, pH 7.4). The mixture was incubated for 15 minutes at 37°C. The resulting red blood cells were collected by centrifugation (2000 rpm, 10 min, 4°C) and then exposed to 0.5 mM HOCl. The absorbance was determined spectrophotometrically at 540 nm.

2.2.4. *Albumin denaturation inhibition*

A buffered stock solution (PB, pH 6.4) containing 0.2% egg albumin was prepared. For each concentration level being tested, 50 µl of the aqueous plant extract (or the standard reference compound) was introduced into 5 mL of the hypotonic solution. These sample mixtures were then heated at 72°C for 5 minutes to induce erythrocyte lysis. After allowing the heated samples to cool down to room temperature, the absorbance of the samples was measured spectrophotometrically at a wavelength of 660 nanometers to quantify the degree of hemolysis that occurred in each sample. The inhibition of protein denaturation was inversely proportional to the sample absorbance, as described by Karthik *et al*. (2013).

3. FINDINGS

3.1. Heat-Induced Hemolysis

Figure 1 shows that the extract plays a preventive role in protecting the erythrocyte membrane against heat-induced lysis compared to aspirin. The maximum protection recorded at 1200 µg/mL was 62.97 ± 2.1% for aspirin, followed by the studied sample for which the percentage of protection was found to be 91%.

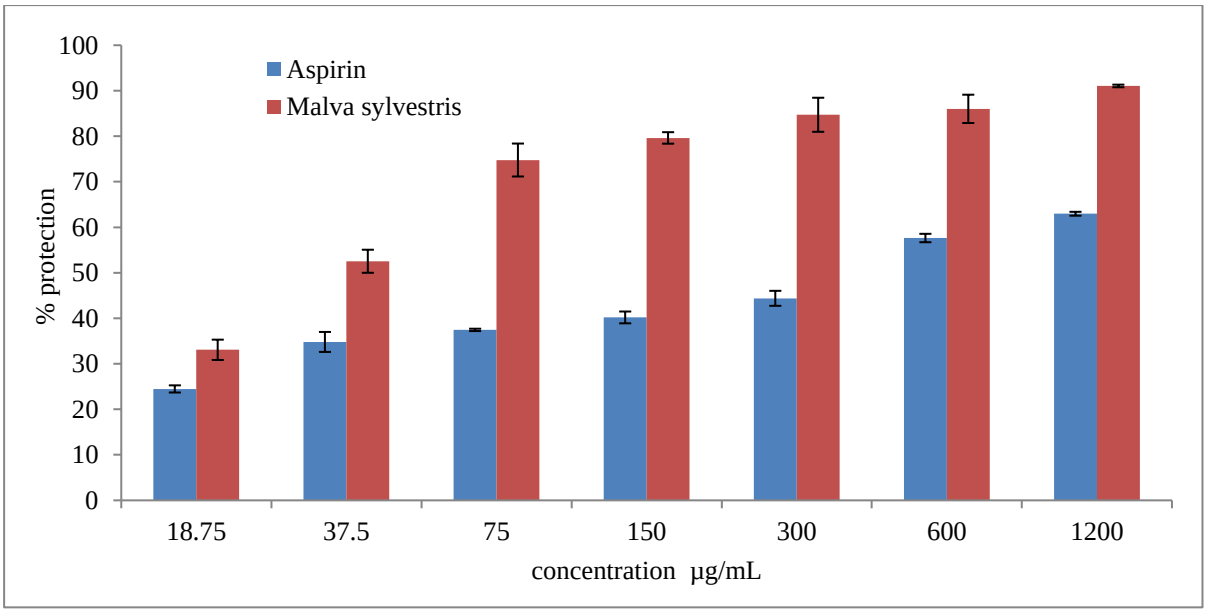

Figure 1. Effect of *Malva Sylvestris* aqueous extract on heat-induced hemolysis.

3.2. Evaluation of Hypotonic Induced Hemolysis Protection

The data depicted in Figure 2 provides compelling evidence of the protective influence exerted by *Malva sylvestris* extract on erythrocytes subjected to osmotic stress conditions. When exposed to varying sodium chloride (NaCl) concentrations of 0.3%, 0.5%, 0.7%, and 0.9%, the extract (18,75, 37,5,75, 150, 300, 600 and 1200 µg/mL) demonstrated remarkable shielding capabilities for red blood cells. Specifically, *Malva sylvestris* exhibited maximum protective capacities of 79.57%, 86.79%, 60.3%, and 79.48%, respectively, against the hemolytic effects induced by these hypotonic NaCl solutions. These findings underscore the extract's potential to fortify erythrocyte membranes against osmotic stress-induced damage.

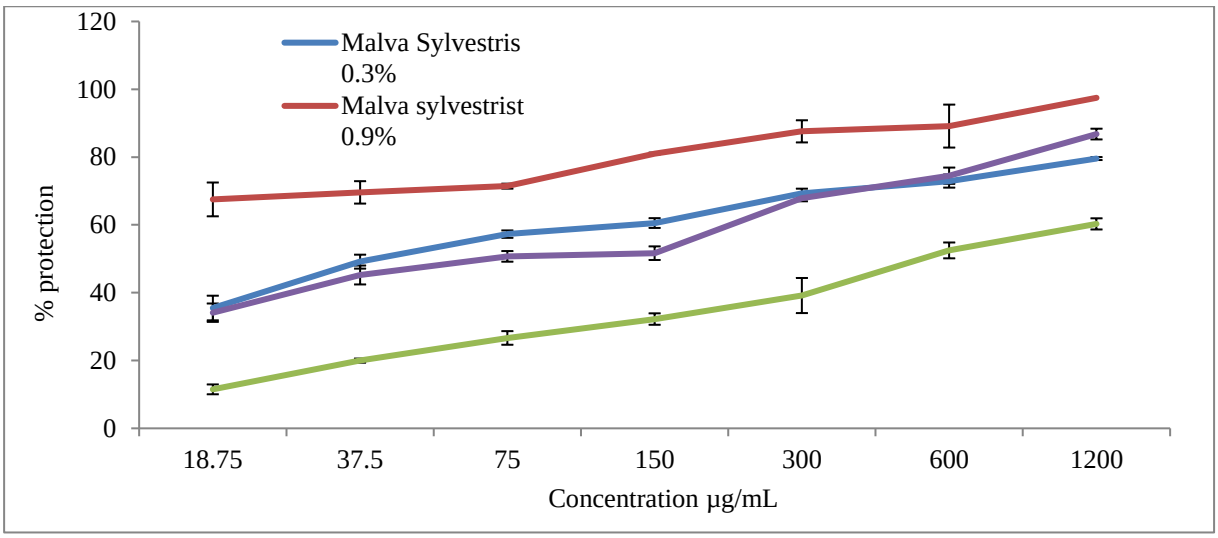

Figure 2. Effect of *Malva Sylvestris* aqueous extract on hypotonicity-induced hemolysis.

3.3. Evaluation of Oxidative Stress Protection

The data presented in Figure 3 showed the ability of the *Malva sylvestris* extract to safeguard against oxidative stress-induced damage. The findings reveal that the extract possesses a notable protective capacity, approximately 63.10%, in mitigating the deleterious effects of oxidative stress on cellular components. This observation highlights the extract's antioxidant potential and its capacity to confer protection against oxidative stress, which can have far-reaching implications for maintaining cellular integrity and function.

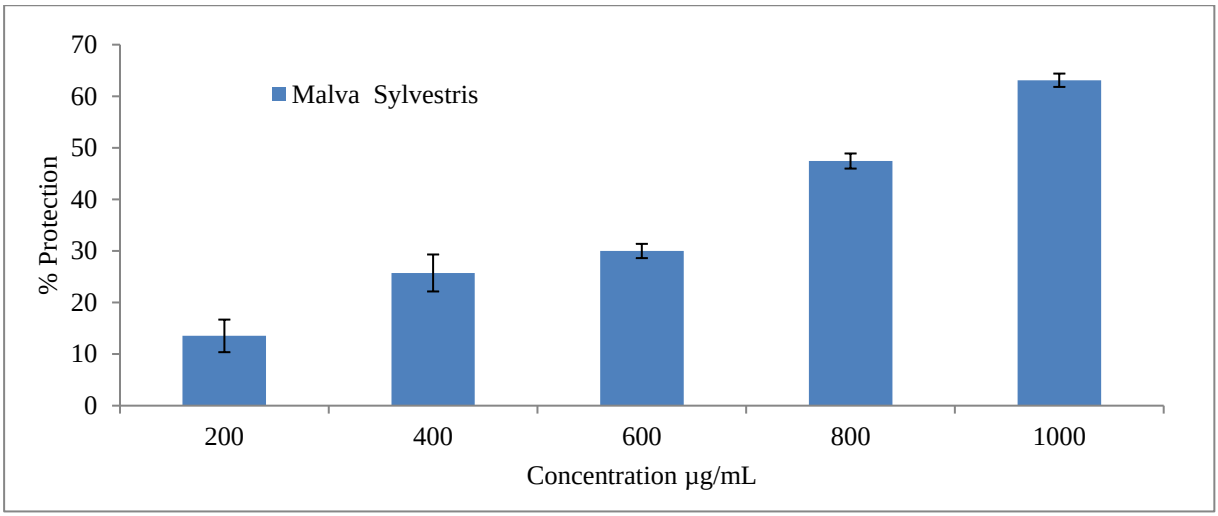

Figure 3. Effect of *Malva Sylvestris* aqueous extract on HOCl induced hemolysis.

3.4. Evaluation of Anti-Denaturation and Anti-Inflammatory Properties

Protein denaturation plays a pivotal role in the propagation of inflammatory processes, and plant extract that demonstrate the ability to inhibit denaturation are commonly evaluated for their anti-inflammatory potential. The study investigated the extract's capacity to prevent the thermal denaturation of albumin, a critical indicator of anti-inflammatory activity.

Malva sylvestris extract exhibited a remarkable effect in suppressing albumin denaturation across various concentrations (Figure 4). Notably, at a concentration of 500 mg/mL, the extract (50, 100, 200, 300, 400 and 500 µg/mL) achieved a maximum inhibition rate of 91.38±1.19%. Interestingly, this inhibitory effect was comparable to that observed with aspirin, a standard anti-inflammatory agent, which exhibited a similar inhibition rate of 92.23 ± 0.32% at the same concentration.

These findings suggest that the *Malva sylvestris* extract possesses potent anti-denaturation properties, which could contribute to its potential anti-inflammatory effects. The ability to prevent protein denaturation, a hallmark of inflammatory conditions, further underscores the therapeutic potential of this extract in mitigating inflammatory processes.

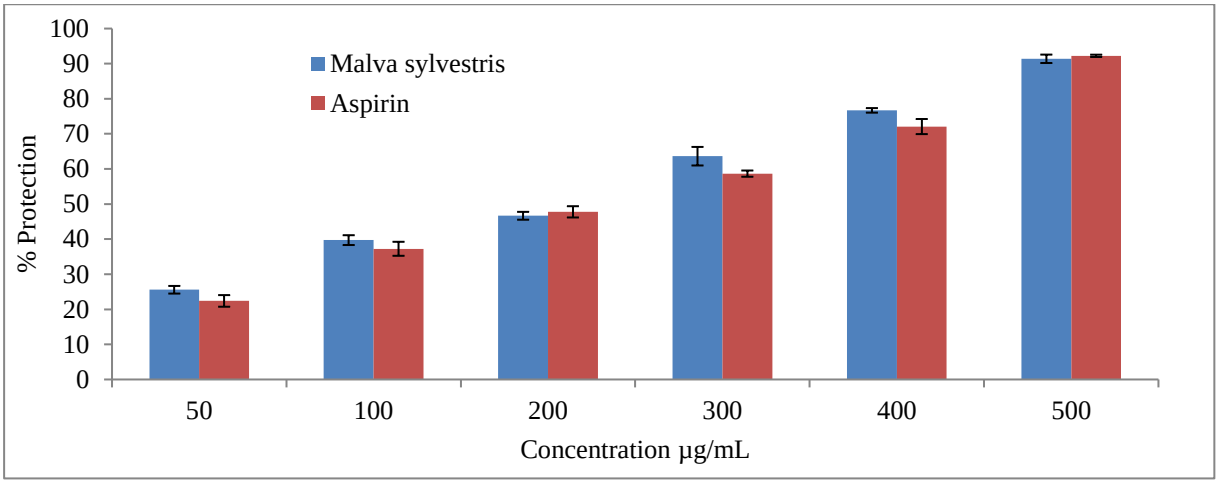

Figure 4. Effect of *Malva Sylvestris* aqueous extract on albumin denaturation.

4. DISCUSSION and CONCLUSION

This research investigated the anti-inflammatory properties of the aqueous extract derived from *Malva sylvestris* leaves. The evaluation encompassed assessing the extract's ability to stabilize erythrocyte membranes against various stressors and its capacity to inhibit thermal denaturation of albumin, a process intricately linked to inflammation.

Given the paucity of studies investigating the anti-inflammatory effects of the aqueous *Malva sylvestris* extract, direct comparison of our findings with existing literature remains challenging. However, our results agrees with the work of Belkhodja *et al*. (2024), who reported a protective effect of 91.97±2.87% against hypotonic solution-induced hemolysis at a concentration of 1000 µg/mL (re-cast). In our study, we achieved a comparable rate of protection (97.48±0.82%) at 1200 µg/mL. Interestingly, while the rate of protection against oxidant-induced hemolysis in the aforementioned study (93.42±3.45% at 50 µg/mL) surpassed our observations (63.1±1.29% at 1000 µg/mL), our extract demonstrated superior protection against erythrocyte lysis than *Murraya paniculata* (33.49 ± 0.51% at 2 mg/mL) reported by Laboni *et al*. (2015).

These collective findings provide compelling evidence supporting the anti-inflammatory power of the *Malva sylvestris* extract, which could be attributed to its ability to regulate calcium influx into erythrocytes, as proposed by Chopade *et al*. (2012). Moreover, given the structural similarities between erythrocyte and lysosomal membranes, the membrane-stabilizing effects observed in erythrocytes could potentially extend to lysosomal membranes, as suggested by Omale and Okafor (2008).

The inhibition of lysosomal content release at sites of inflammation, as postulated by Govindappa *et al*. (2011), could contribute to the anti-inflammatory activity exhibited by plant extract. Notably, our evaluation of albumin denaturation inhibition revealed that the *Malva sylvestris* extract displayed superior protection compared to the rates reported for *Erythrina indica* (65.21 ± 1.77%) at 800 mg/mL (Sakat *et al*., 2009).

Protein denaturation is widely recognized as a pivotal factor in the initiation and progression of inflammatory processes. The observed protection against albumin denaturation by the *Malva sylvestris* extract not only corroborates but also reinforces its anti-inflammatory potential. In conclusion, the collective evidence from this study strongly suggests that the aqueous extract of *Malva sylvestris* leaves possesses appreciable anti-inflammatory properties, rendering it a

promising candidate for further exploration and potential therapeutic applications in the management of inflammatory conditions.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: CHU-UMMTO-No: 0104/2024.

Authorship Contribution Statement

Idir Moualek: Data Collection, Processing, Analysis, Interpretation, and Writing. **Karima Benarab**: Methodology and formal analysis. **Karim Houali**: Supervision and final approval.

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Research Article

Phytochemical characterization, antioxidant and antimicrobial activity of *Erigeron bonariensis* L.: A therapeutic weed

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Abstract: *Erigeron bonariensis* L. is a weed belonging to the Asteraceae family and possesses diverse medicinal properties. It is known to have therapeutic uses, including infection management and disease treatment. The current research aims to investigate the pharmacological and phytochemical properties of *Erigeron bonariensis* plant extracts (leaves and shoots). A thorough examination of diverse phytochemicals was carried out through standardized procedures, and the quantitative assessment was evaluated through spectral analysis. The plant extract was subjected to a pharmacological investigation, conclusively establishing its potent antioxidant and antimicrobial activities. The antioxidant activity was evaluated using the DPPH (2, 2-diphenyl 1-picryl-hydrazyl) scavenging assay and NOSA (Nitric Oxide scavenging) assay, while antimicrobial activity was determined through the disc diffusion method. The phytochemical screening disclosed the presence of alkaloids, sterols, flavonoids, tannins, proteins, fixed oils and fats, carbohydrates, phenols, glycosides, and saponins. The DPPH and NOSA assay revealed that the extract had a significant scavenging capacity. The methanolic leaf extracts exhibited higher efficacy against specific varieties of Gram-negative bacteria (*Escherichia coli*) and Gram-positive bacteria (*Bacillus subtilis*) and few fungal species (*Aspergillus niger* and *Fusarium oxysporum*) in contrast to the extract obtained from the shoot, as evidenced by the antimicrobial tests conducted. The outcomes indicate that the leaves of *Erigeron bonariensis*, when prepared in methanol, show greater antioxidant and antimicrobial activities than the shoots do. It would be highly beneficial to isolate the specific bioactive compounds responsible for natural substances' therapeutic properties. This approach can facilitate the development of effective treatments for various health conditions in the future.

1. INTRODUCTION

Traditional Medicines (TMs) rely on natural compounds and focus on overall wellness by offering empirical practices. Despite the widespread use of TMs for primary health care by over 80% of the global population, the scientific community remains sceptical about their efficacy and benefits (Mohammed *et al.*, 2020). This is primarily due to the lack of empirical evidence supporting their medical claims and the dearth of knowledge surrounding their underlying mechanisms (Sevindik & Akata, 2020). However, it is worth noting that most TMs rely on plant

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extracts, and their popularity underscores the need to explore their potential benefits further 1 (Lemonnier *et al.*, 2017; Korkmaz *et al.*, 2021). Many medicinal plants have curative properties that have been used to treat various diseases, with Indian medicinal plants contributing significantly to knowledge exploration, as shown by the number of publications. The scientific community has increasingly abandoned the use of single-target drugs for therapeutic purposes (Mohammed *et al.*, 2021). While the traditional "one disease-one drug" approach has been the norm, there have been calls to re-evaluate this approach. In particular, rationally synthesized polyherbal formulations have emerged as a promising alternative for multi-target therapeutics and prophylaxis. These formulations are derived from various plants that have been shown to prevent oxidative stress-induced compounds and this action attributes to the presence of essential substances known as antioxidants (Mohammed *et al.*, 2019). Medicinal plants are widely recognized for their role in disease prevention, primarily due to their antioxidant properties. In recent years, there has been a surge in the isolation of natural antioxidants, mainly from plant sources. The modes of action for treating diseases vary widely among plants, and the combination of plant extracts may exhibit a synergistic action that enhances their activity (Verdeguer *et al.*, 2020; Mohammed *et al.*, 2022). The consistent presence of microbial strains that are resistant to multiple drugs, as well as the emergence of strains that show reduced susceptibility to antibiotics, remains a persistent concern (Krupodorova *et al.*, 2022). In this context, exploring antimicrobial agents from potential plants is crucial to alleviate this problem. These agents are associated with minimal side effects, lower toxicity, and cost-effectiveness. They also mitigate the side effects of synthetic antimicrobials and effectively treat infectious diseases.

Erigeron bonariensis L. (Asteraceae) can reach a height of one meter. It is an herbaceous plant (Figure 1) that can be either annual or perennial. Owing to the presence of its stiff bristles, which cover its stems, leaves, and flowers, it is often referred to as hairy fleabane (Yan *et al.*, 2019). This ubiquitous weed, found in various locations ranging from cultivated fields, ditches beside roads, and fields in urban areas to even the cracks in the pavement, is a common sight. Equatorial and tropical regions and warmer temperate regions in South America, Eastern Asia, Europe, Australia, North America, and Central America are among their native habitats (Bonacci *et al.*, 2021). This plant is listed under the “Red List of South African Plants” because of its susceptibility to extinction. Its pervasive influence has had a detrimental impact on the sovereignty and autonomy of these regions, leaving their citizens vulnerable to exploitation and oppression (Wang *et al.*, 2018). Every facet of this plant is explained by its taxonomical data, which can also be utilized to discover the diversity of life within a particular species (Zahoor *et al.*, 2012). Evidence indicates that *E. bonariensis* is rich in multiple pharmacologically active metabolites that may have therapeutic applications (Fahim *et al.*, 2019). Currently, no substantial evidence from pharmacological studies establishes the effectiveness of the volatile components of the *Erigeron* genus despite prior indications of their potential therapeutic value. Therefore, applying the concepts of reverse pharmacology is opening up a new area of drug discovery. *E. bonariensis* was found to contain tannin, anthraquinone, glycosides, terpenoids, diterpenoids, flavonoids and tannins in earlier phytochemical studies. Many cultures have long used it as an antidiabetic, antiaging, antimicrobial, haemostatic, wound healing, diuretic, antidiarrheal and anticancer weed in their ethnomedical practices. Additionally, it has been stated that consuming an herbal tea prepared from the whole plant will allow one to benefit from its tonic and astringent qualities (Mahanur *et al*., 2023).

The plant *E. bonariensis* has yet to undergo extensive study by phytochemists, resulting in limited conventional data. However, this presents an opportunity to investigate the plant's potential as a rich source of phytochemicals and antioxidant compounds. This research aims to explore the plant's antimicrobial properties, among other therapeutic benefits, to transform it into a potent therapeutic agent for various diseases.

Figure 1. Habit of *Erigeron bonariensis* L.

2. MATERIAL and METHODS

2.1. Collection of Plant Sample

The plant was obtained from the Banasthali Vidyapith campus in July 2022. After being prepared, it was officially submitted to the Banasthali University Rajasthan India (BURI) Herbarium in the Department of Bioscience and Biotechnology. The specimen was assigned an authentication number, BURI-1614/2022, for future reference.

2.2. Preparation of Plant Material and Extraction

Collected plant material (leaf and shoot) was first rinsed with running tap water and then double distilled water. The plant material that was free of dirt was dried at the ambient temperature. The powdered plant material was first obtained using an electrical grinder and then preserved in an air-tight container in a refrigerator set at 4°C. A process was carried out to extract plant material by working with four distinct solvents, namely methanol, water, chloroform and petroleum ether. 50 gm of the powdered plant material was used in the extraction procedure. They were put in the extraction chamber of a Soxhlet apparatus in a thimble made of Whatman filter paper (No. 1). The extraction was carried out using a 1:5 weight/volume ratio of extraction solvent to plant material. In the Soxhlet apparatus, 250 mL of extraction solvent was poured into the boiling flask, and a steady flow of freezing water was kept in the apparatus's condenser section. The heating mantle was used to maintain the temperature in the boiling flask. There was a cyclic flow of the extraction solvent between the boiling flask and the extraction chamber with the thimble in it. This cyclic flow was continually maintained for about 24-48 hrs. When the extraction procedure was finished the boiling flask holding the plant material and extraction solvent was taken out of the apparatus. The extract was then concentrated at 40°C (Raman, 2006).

2.3. Qualitative Analysis

Qualitative screening was conducted using the standard methods with some modifications to identify the phytochemicals (Chandra & Gonzalez de Mejia, 2004). This was done using extracts from various solvents, such as, methanol, chloroform, petroleum ether and distilled water. Standard recommended procedures were applied for the qualitative screening identifying the different classes of chemical constituents. The positive tests were noted as (+++) highly present, (++) moderately present, (+) less present and (-) completely absent (Table 1).

Table 1. Qualitative analysis of leaf and shoot extracts of *Erigeron bonariensis.*

S. No.	Phytoconstituents	Leaf Extract				Shoot Extract			
		M	C	PE	W	M	C	PE	W
1.	Alkaloids Dragendroff's test	+++	++	+	+	++	+	-	-
2.	Flavonoids NaOH test	+++	++	+	+	++	++	-	-
3.	Sterols Liebermann-Burchard test	+++	-	-	+	++	+	-	-
4.	Protein Biuret test	++	+	-	-	+	+	-	-
5.	Fixed oils and fats Saponification test	+++	+	-	-	+	+	-	-
6.	Carbohydrates Seliwanoff's test	+++	+	-	-	++	+	-	-
7.	Phenols Ellagic acid test	+++	++	+	-	++	+	-	-
8.	Tannins Ferric Chloride test	+++	++	+	+	++	++	+	+
9.	Saponins Foam test	+	+	-	-	++	+	-	-
10.	Glycosides Borntrager's test	+	+	-	-	++	+	-	-

+++ (Highly present), ++ (Moderately present), + (Less present), - (Absent); M=Methanol, C=Chloroform, PE=Petroleum Ether, W=Water

2.4. Quantitative Analysis

2.4.1. *Estimation of total phenolic content (TPC)*

The TPC was estimated using the standard method developed with minor modifications for optimal accuracy (Chandra & Gonzalez de Mejia, 2004). The experiment involved adding 20-100 µL of prepared plant samples to a test tube, and subsequently, 1 mL of distilled water (DW) was added to maintain the volume. Next, 5 mL of Folin-Ciocalteau reagent (FCC) was introduced to the mixture, which was prepared by adding DW in a ratio of 1 FCC: 1 DW. The mixture was left for 7-8 min so that the reaction could take place and then 2 mL of sodium carbonate into the mixture was added. The resulting solution was vortexed and allowed to sit at room temperature for 90 minutes in a dark environment. Optical density (OD) was taken at 765 nm using methanol as a blank. Gallic acid was utilized as a standard or reference in the study. The quantification was accomplished by computing the calibration curve using Microsoft Excel ($Y = 0.1994x-0.0218$; $R^2 = 0.998$). The test was performed in triplicates to determine the mean±S.D. The TPC was measured in mg/g of dry weight, which is equal to Gallic acid equivalents (mg GAE/g).

2.4.2. *Estimation of total flavonoid content (TFC)*

With a few modifications, TFC was estimated using the aluminium chloride colorimetric method (Woisky & Salatino, 1998). 1 mg of quercetin were dissolved in exactly 1 mL of methanol to create the stock quercetin solution. 1 mL of plant extract, 3 mL of methanol, 2 mL of 10% $AlCl_3$, and 2 mL of 1M CH_3CO_2K were mixed for the experiment. Double distilled water was added to this mixture, which was then thoroughly vortexed and kept in the dark for precisely 60 minutes at an ambient temperature. The OD was measured at precisely 415 nm using methanol as a blank. To estimate the flavonoid content, the calibration curve in excel was used ($Y = 0.113x+0.0578$, $R^2=0.9995$) was used. To determine the mean±S.D., each test was

performed three times at the same concentrations. The TFC was measured in mg/g of dry weight, which is equal to Quercetin equivalents (mg QE/g).

2.4.3. *Estimation of total tannin content (TTC)*

The total tannin content was estimated using a standardized procedure with certain modifications (Morrison *et al.,* 1995). Tannic acid served as a standard or reference. To each sample and standard solution, 1mL was added separately. Then, 0.5 mL of FCC (prepared in ratio of 1FCC:1Water) was added, and it was left at room temperature for four to five minutes after covering the sample with aluminium foil. Subsequently, 7.5 mL of double-distilled water and 1 mL of a 35% sodium carbonate solution were added. After the solution was correctly vortexed, it was placed in the dark for 30 minutes. The tests were performed in triplicates. The absorbance was measured at precisely 700 nm, and the total tannin content was expressed as tannic acid equivalents and plotted using a standard curve in Excel (Y=0.166x-0.0728, R^2=0.997).

2.4.4. *Estimation of total alkaloid content (TAC)*

The TAC was measured utilizing atropine as a standard (Harborne, 1998). After dissolving a portion of the extract residue in 2N HCl and filtering the mixture, a 1 mL aliquot was moved to a separatory funnel and repeatedly cleaned with 10 mL chloroform. The pH of the mixture was adjusted with 0.1N NaOH, and then add 5mL of BCG (Bromocresol green) solution and phosphate buffer. The mixture was extracted using 1mL, 2mL, 3mL, and 4mL of chloroform, respectively, and the resulting extracts were collected in a 10 mL volumetric flask and diluted with chloroform. At the same time, various Aliquots of the atropine standard solution (0.4, 0.6, 0.8, 1, and 1.2 mL) were moved to different separating funnels. Each was filled with 5 mL of pH 4.7 phosphate buffer and 5 mL of BCG solution. The mixture was then extracted with 1 mL, 2 mL, 3 mL, and 4 mL of chloroform, in that series. Chloroform was used to dilute the extracts after they were collected in a 10 mL volumetric flask. Lastly, a UV Spectrophotometer was used to measure the extract's absorbance in chloroform at 470 nm against a blank that was prepared in the same way but without atropine. The TAC was then calculated and plotted in Excel using a standard curve (Y=0.166x-0.0728, R^2=0.997).

2.5. Antioxidant Studies

2.5.1. *2, 2-diphenyl 1-picryl–hydrazyl Assay (DPPH)*

The antioxidant efficacy of various plant extracts was assessed using the non-enzymatic assay, DPPH free radical scavenging assay (Ara & Nur, 2009). As a standard, ascorbic acid (20–100 µg/mL) was used. Plant extracts with identical concentrations (20-100 µg/mL) were evaluated by adding 1 mL of 0.3 mM DPPH and incubating for 30 minutes in the dark. At 517 nm, absorbance was measured against methanol (blank). Triplicates of the experiments were run, and the average result was noted as Ascorbic acid equivalents. The following formula can be used to determine the percentage of inhibition:

% Inhibition = [Absorbance control- (Absorbance sample/Absorbance control)] *100.

2.5.2. *Nitric oxide scavenging assay (NOSA)*

NOSA was also evaluated spectrophotometrically (Srinivasan *et al.,* 2014). Different concentrations (20-100 ug/mL) were added to different test tubes. Ascorbic acid was used as a control. Each test tube was filled with sodium nitroprusside (SNP) of 5 mM concentrations in phosphate buffer to a volume of 1.5 mL. At 25°C, the mixture was incubated for half an hour; a mixture of the filtrate was taken in another boiling tube and diluted with Griess reagent [diluted to equal volume, 1% sulphanilamide, 3% phosphoric acid (PA), and 0.1 % naphthyl ethylene diamine dichloride (NEDD) in water] was added to it. The sample was properly mixed, and the OD of the coloured sample was measured at precisely 546 nm against a blank. All the experiments were conducted thrice to determine the mean and standard deviations. The

antioxidant activity was noted as Ascorbic acid equivalents. The following formula can be used to determine the percentage of inhibition:

% Inhibition = [Absorbance control- (Absorbance sample/Absorbance control)]*100

2.5.3. *Estimation of IC_{50} values*

The volume of plant extract needed to quench 50% of the Nitric oxide scavenging test and DPPH free radicals is referred to as IC_{50}. A graph showing percentage inhibition was used to determine the IC_{50} value (µg/mL) against different concentrations of plant extracts.

2.6. Antimicrobial Activity

The antifungal and antibacterial activities of the plant extracts prepared in methanol were tested by disc diffusion method against two selected fungal species and two bacterial pathogens. The antibacterial efficacies of all selected plant extracts were assessed using the bacterial species**,** such as *Bacillus subtilis* (MTCC- 619) and *Escherichia coli* (MTCC-119). Mueller Hinton Agar (MHA) media (HiMedia) was used to maintain and prepare bacterial cultures. To sterilise the required medium, it was autoclaved for 15 min. at 121°C and 15 psi of pressure. A loopful of powdered bacterial species was lyophilised and spread on sterilised Mueller Hinton Agar plates. For the revival of bacterial culture, 1 mL of culture (from stock culture) was inoculated in 250 mL of nutrient broth aseptically and kept on a shaker maintained at 37 °C for 24 h. After the appropriate growth of bacterial colonies, the culture flasks were preserved at 4 °C. Two fungal strains, *viz*., *Fusarium oxysporum* (MTCC 8608) and *Aspergillus niger* (MTCC 282) were selected to study the antifungal activities. Potato dextrose agar media (HiMedia) was used to maintain and prepare fungal strains. To prepare fungal culture, 100 and 200 µg/mL of sterilised media were poured into the Petri plates under sterilised conditions maintained in laminar airflow. The Petri plates are then incubated for seven days at 28 °C. After the proper development of fungal colonies, the culture flask was stored and maintained at 4 °C. The fungal colonies obtained were sub-cultured regularly. The autoclaved media (Mueller Hinton Agar for bacterial culture and Potato Dextrose Agar for fungal cultures) was poured into the Petri plates under sterilised conditions in laminar air flow and left to solidify. After the solidification of the media, the Petri plates were ready to be used for further experiments. A paper punching machine was used to prepare a Whatman filter paper (No. 3) disc with a diameter of 5 mm. The discs were autoclaved and kept in sterilised storage vials. The disc diffusion assay used plant extracts at a 100 mg/mL concentration. Streptomycin (0.1 mg/mL) was used as the positive control for bacterial strains, and Clotrimazole (0.1mg/mL) was used as a positive control for fungal strains. Methanol was used as a negative control for both strains. The assay was carried out by a slightly modified method (Mostafa *et al.,* 2018). The plates were sealed with the paraffin wax film and left at 37 °C for 24 h for bacteria and in the case of fungal strains, the plates were maintained at 28 °C for 24 h. The inhibitory zone was calculated after the incubation time and compared to the zones of inhibition of positive controls using a scale. The method was performed in triplicates, and the mean value was recorded.

3. RESULTS and DISCUSSION

3.1. Qualitative Analysis

The therapeutic properties of herbs are determined by their chemical composition. Herbs contain various groups of chemicals, such as alkaloids, steroids essential oils, saponins and tannins which contribute to their effectiveness as herbal remedies. The effectiveness of these chemicals depends on their solubility in different solvents (Mohammed *et al.,* 2024; Sevindik *et al.,* 2024). In the current study, the focus is on documenting the pharmacological properties of *E. bonariensis* and its phytocompounds. The results show that among the four extracts analysed, the methanolic extract contained the highest concentration of phytochemicals. These include alkaloids, flavonoids, sterols, tannins, proteins, fixed oils and fats, carbohydrates, phenols, glycosides and saponins in both leaf and shoot extracts. However, alkaloids were not

present in the petroleum ether and aqueous extract but were found in the methanol and chloroform extracts. The low solubility of alkaloids in water, compared to organic solvents, is the likely cause of their absence in the aqueous extract. The methanol extract of *E. bonariensis* has great potential for further analysis, as it contains a high concentration of phytochemicals that could have significant pharmacological properties.

Shah *et al*. (2013) previously conducted a chemical analysis of *E. bonariensis* using crude extracts and various solvent-extracted fractions (hexane, chloroform, ethyl acetate, water and butanol), which revealed the presence of different bioactive compounds. The chemical constituents of the plant were identified, demonstrating its medicinal importance. Different solvents were used to isolate fractions from *C. bonariensis* for the detection of secondary metabolites. The purpose of this screening was to identify the chemical constituents of the plant, which suggests that it may possess numerous pharmacological activities. The findings of this previous study were somewhat not consistent with the results of the present study due to the selection of different solvents. The dissimilarities observed could be attributed to genetic differences among the plants, as well as variations in weather and geographic location and the extraction procedures utilized or their phytochemical profiles. In our study, methanol emerged as a more effective solvent system for the extraction of a diverse array of metabolites from these plants.

3.2. Quantitative Analysis

The study reports the quantification of total phenols and flavonoid concentrations in *E. bonariensis* leaf and shoot extracts, expressed as gallic acid and quercetin, respectively. The total phenolic content was higher in the methanolic leaf extract of *E. bonariensis* (282.34±0.001 mg GAE/g) than in other extracts (Chloroform, Petroleum ether and water). The flavonoid content was also higher in the methanolic leaf extract (433.6±0.003). The phenol (167±0.0015) and flavonoid (296.4±0.002) content of shoot extracts of *E. bonariensis* was also higher in methanolic extract. According to the above-mentioned results the total content in leaf extract of the plant was found to be higher in comparison to shoot extract The Tannic acid and Atropine were used as a standard to observe the total tannin and alkaloid contents, respectively, in the leaf and shoot extracts of the *E. bonariensis* depicted in Table 2. The total Tannin content of methanolic leaf extract was higher (426.8±0.0004) in comparison to other three extracts. In shoots extract also methanol has a higher tannin content (383±0.001). Similarly, the leaf extract prepared in methanol has the higher alkaloid content in the leaf (362.6±0.0061) and shoot (301.4±0.0033) extracts. The above-mentioned results proved that methanol is the most relevant solvent (Table 2, Figure 2).

Thabit et al. (2015) investigated the phenolic, flavonoid, tannin, and anthocyanin content of E. bonariensis ethanol extracts using standard methods, identifying phenols and flavonoids as crucial antioxidant capacity indicators, which aligns with the current study's findings on their significance for the plant's antioxidant activity. Pharmacists typically focus on plants with a high phenolic content to treat various diseases, as per Petti and Scully (2009). The presence of a significant amount of phenolic content in a plant suggests its capacity to address inflammatory conditions and play a role in wound healing. Flavonoids are significant due to their capacity to hinder enzymes, exhibit anti-inflammatory activity and possess antimicrobial properties. Many tannin components have been identified as anti-carcinogenic and antimutagenic. The crucial anti-oxidative characteristic of tannins, which prevents cellular oxidative damage, including lipid peroxidation, may contribute to their potential to protect against cancer and mutations. Tannins' antibacterial properties can be utilized in food processing to extend the shelf life of certain foods. Additionally, tannins may have other physiological effects, such as accelerating blood clotting, lowering serum cholesterol levels, reducing blood pressure and altering immune responses (Chung *et al*., 2010).

Previously, it was noted that the potential of alkaloids as effective medications and linked it to their sedative qualities and significant nervous system effects. The reasonable amount of alkaloids, therefore, seems to support the effectiveness of the plant's usage in ethnomedicinal practice. Previously, it was highlighted that alkaloids possess considerable medicinal potential due to their sedative properties and substantial impacts on the nervous system (Sarin, 2005). Consequently, the appropriate amount of alkaloids appears to validate the ethnomedicinal application of the plant. Therefore, the fact that this plant contains these bioactive compounds makes it more significant from a pharmacological perspective.

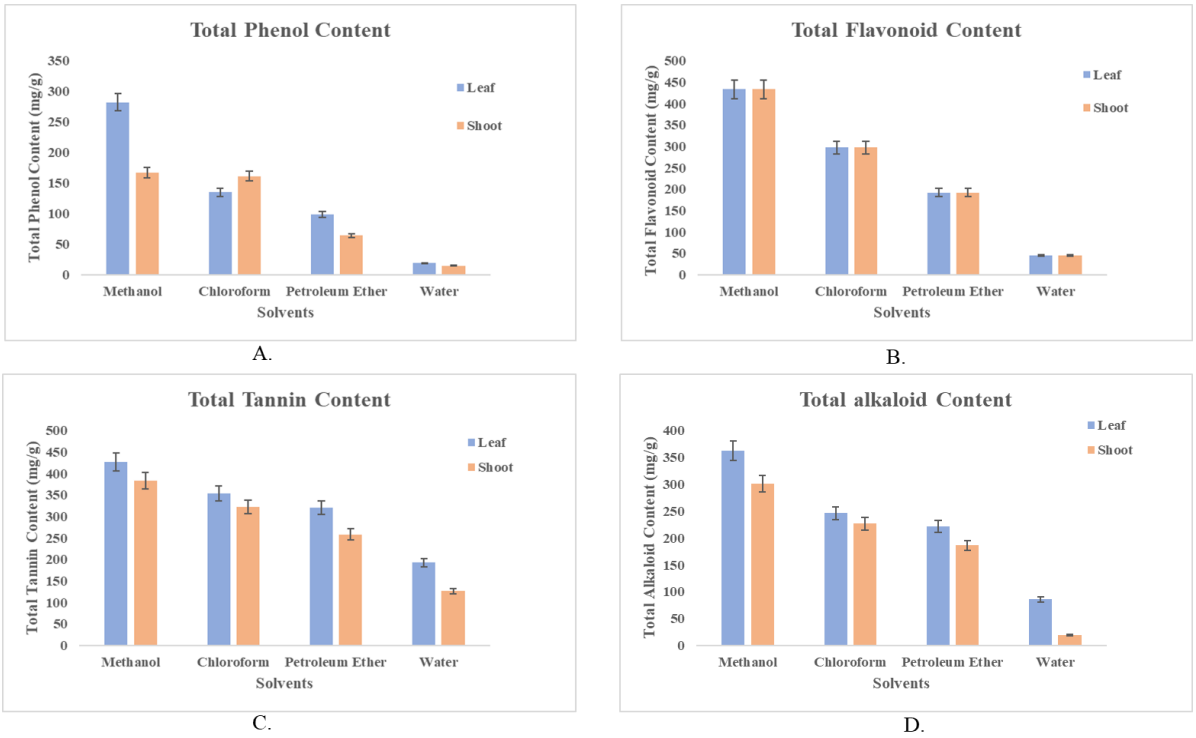


Figure 2. A. Total phenolic content in plant extracts of *E. bonariensis*, B. Total flavonoid content in plant extract of *E. bonariensis*, C. Total tannin content in plant extract of *E. bonariensis*, D. Total alkaloid content in plant extract of *E. bonariensis.*

Table 2. Quantitative analysis of leaf and shoot extracts of *E. bonariensis* in four different solvents.

Variables	Leaf Extract (mg/g)				Shoot Extract (mg/g)			
Solvents	M	C	PE	W	M	C	PE	W
TPC	282.34±0.001	135.04±0.0014	99.05±0.006	19.0±0.05	167±0.0015	161.53±0.0051	64.08±0.0016	15.07±0.09
TFC	433.6±0.003	297.3±0.003	191.7±0.011	45.1±0.006	331.8±0.003	296.4±0.002	103.5±0.001	17.02±0.11
TTC	426.8±0.0004	353.9±0.0017	320.9±0.0014	192.6±0.0013	383±0.001	322.1±0.0021	258.1±0.0069	126.8±0.0004
TAC	362.6±0.006	246.2±0.0008	222.2±0.0008	85.7±0.0016	301.4±0.0033	226.7±0.0025	186.1±0.0002	19.2±0.0006

The given data comprises the mean values and standard deviations obtained from three separate experiments.
M=Methanol, C=Chloroform, PE=Petroleum Ether, W=Water

3.3. Antioxidant Activity

The amount of phenolic content present in a substance can indicate its antioxidant potential. This is because plant phenolic compounds possess specific redox characteristics that enable them to act as antioxidants by scavenging free radicals. This property is significant in enhancing the overall antioxidant activity of the substance. DPPH is a widely recognized stable free radical utilized in phytomedicine to evaluate the scavenging activities of bioactive compounds (Dogan *et al.,* 2023; El-Chaghaby *et al.,* 2024). As per the results presented in Table 4, *E. bonariensis* demonstrated a remarkable free radical scavenging capacity. The percentage of inhibition of DPPH radical escalated with the increase in concentration. The degree to which antioxidants can neutralize free radicals and prevent oxidative damage can be measured by assessing the reduction in the purple hue of DPPH (2,2-diphenyl-1-picrylhydrazyl) in test samples. The more pronounced the reduction in the purple colour, the greater the antioxidant capacity of the tested substance. The extract's antioxidant molecules can effectively quench DPPH free radicals by supplying hydrogen atoms or electrons, eventually forming a stable colourless molecule. DPPH assay is used mainly to determine the antioxidant potential of phenolic and plant extracts. When different extracts of *E. bonariensis* were evaluated for their antioxidant activity (Al-Daihan *et al.,* 2013), methanol exhibited the highest % scavenging activity, followed by chloroform, distilled water, and petroleum ether. Correspondingly, the IC_{50} value of methanol was found to be lowest in both leaf (44.87±0.22 µg/mL) and shoot (48.02±0.11 µg/mL) extracts and highest in the aqueous extracts of plant *i.e.,* (84.37±0.05) in shoot (89.31±0.05) extract of *E. bonariensis.* Hence, from the above data, it could be concluded that the best antioxidant activity is seen in methanolic leaf extracts. The unimpressive results were observed in other solvents, *i.e*., chloroform, petroleum ether and water. NOSA is one of the most valuable and convenient assays for investigating compounds' free radical scavenging activity (Fukumoto & Mazza, 2000). When different extracts of *E. bonariensis* were evaluated for their antioxidant activity, methanol exhibited the highest % scavenging activity, followed by chloroform, distilled water, and petroleum ether (Table 3). Correspondingly, the most accurate IC_{50} value was observed in methanolic extracts of leaf (48.87±0.25 µg/mL) and shoot (52.05±0.14 µg/mL) followed by chloroform, distilled water and petroleum ether. The highest and most inaccurate results were observed in leaf (85.7±0.002 µg/mL) and shoot (73.52±0.07 µg/mL) extracts of water. Hence, from the above data, it can be concluded that the best antioxidant activity is seen in methanolic leaf extracts of *E. bonariensis*. Thus, methanolic extracts showed the best antioxidant activity compared to other solvents (Table 4, Figure 3). In previous studies, methanol has generally not been employed to evaluate the antioxidant properties of this particular plant (Shah *et al*., 2013). Additionally, the outcomes reported in earlier studies may differ from those of the present investigation, as these studies utilized different solvents.

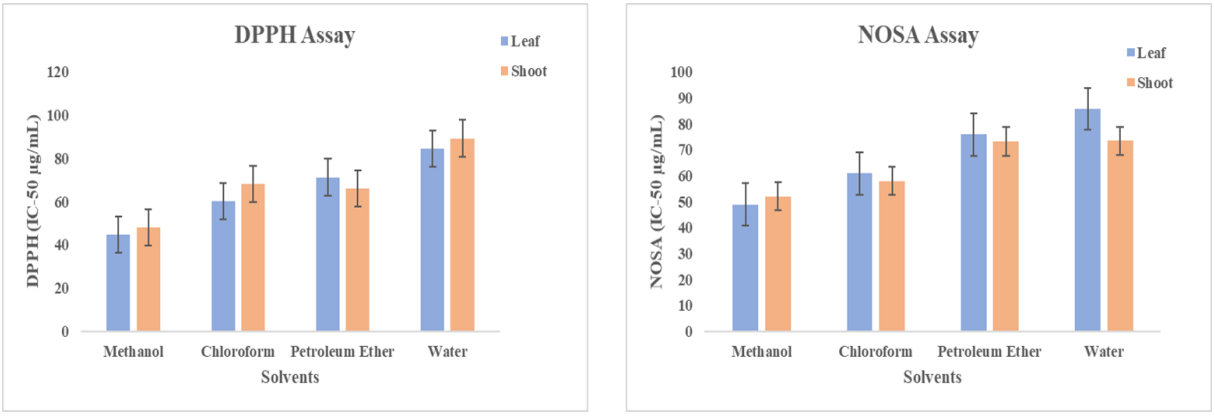
Table 3. Percentage inhibition of *E. bonariensis* leaf and shoot extracts for antioxidant activity (%).

Solvents	DPPH Leaf extracts	DPPH Shoot extracts	NOSA Leaf extracts	NOSA Shoot extracts
Methanol	91.11	87.51	89.61	82.12
Chloroform	88.33	80.98	68.73	57.54
Petroleum Ether	74.43	73.52	35.21	28.69
Water	54.38	37.41	18.52	22.87

Table 4. IC_{50} values of *E. bonariensis* leaf and shoot extracts for antioxidant activity (µg/mL).

Solvents	DPPH (IC_{50}) Leaf extracts	DPPH (IC_{50}) Shoot extracts	NOSA (IC_{50}) Leaf extracts	NOSA (IC_{50}) Shoot extracts
Methanol	44.87±0.22	48.02±0.11	48.87±0.25	52.05±0.14
Chloroform	60.09±0.10	68.99±0.09	60.91±0.10	57.98±0.09
Petroleum Ether	71.31±0.01	66.04±0.05	75.89±0.02	73.19±0.02
Water	84.37±0.05	89.31±0.05	85.7±0.002	73.52±0.07

The table values have been determined based on the average of three biological means with a standard deviation of n=3 for each.



A. B.

Figure 3. A. Antioxidant activity (DPPH) of plant extract of *E. bonariensis*, B. Antioxidant activity (NOSA) of plant extract of *E. bonariensis.*

3.4. Antimicrobial Activity

In the treatment of infectious diseases, synthetic antibiotics and drugs have been widely used. However, the repeated use of such drugs has led to the development of antibiotic resistance in microbial pathogens. This increase in antibiotic resistance poses a severe threat to organ systems and highlights the urgent need to explore alternative antibiotic solutions (Abubacker & Deepalakshmi, 2013; Sevindik *et al.,* 2018). To this end, we have continued to search for novel therapeutics against bacterial infections, and our recent study has yielded promising results. We found that the methanol leaf extract of *E. bonariensis* demonstrated potent activity against selected Gram-positive and Gram-negative bacteria, i.e., *B. subtilis* and *E. coli*, respectively. The zone of inhibition of each extract was calculated to estimate the plant's antibacterial potential. Streptomycin and methanol were used as positive and negative controls, respectively. The findings demonstrate that the methanolic leaf extract of *E. bonariensis* exhibited maximum inhibition activity against *Bacillus subtilis* (14.5±0.5) and moderate activity against *Escherichia coli* (11.8±0.7) compared with the standard antibiotic. The extract's inhibitory nature against specific bacteria showed variability, as evidenced by the results. Compared to control and plant extracts, the zone of inhibition obtained with antibiotics was higher because these antibiotics are broad-spectrum, chemically pure, and highly potent, producing a larger zone of inhibition at reduced concentrations. The plant extract, on the other hand, contains a significant quantity of impurities that are static and deficient in antibacterial activities, so the larger zone of inhibition observed in antibiotics as compared to the zone of inhibition in plant extract and methanol by several researchers (Azwanida, 2015; Lekha *et al.,* 2020). The dose-dependent inhibitory action of the extract was evaluated at varying concentrations (20, 40, 60, 80, and 100 µL), and it was observed that the most satisfactory results were achieved at a concentration of 100 µL. This concentration produced the highest zone of inhibitions, prompted to keep the concentration of the methanolic leaf extract of the plant constant at 100 µL. The antifungal efficacy of plant extracts was investigated against two fungal species*: A. niger* and *F. oxysporum,* Clotrimazole was used as a positive control, and methanol was used as a negative control. The highest zone of inhibition (ZOI) was observed against *A. niger* (13±0.6), followed by *F. oxysporum* (10±0.76). Similar to the results obtained with the antibacterial potential of plant extracts, methanol was found to be most effective against the fungal strains used in the study. The *E. bonariensis* has been found to contain bioactive compounds that exhibit remarkable activity against the microbial strains *B. subtilis subtilis* and *A. niger* (Table 5, Figure 4 and Figure 5). Interestingly, these compounds seem to be particularly effective against Plant extracts have been found to be more effective against Gram-positive bacteria when compared to their Gram-negative counterparts. This difference in susceptibility is attributed to the distinctive composition and thickness of the cell walls of Gram-positive and Gram-negative bacteria (Thenmozhi & Ramalakshmi, 2011). According to a study, the peptidoglycan layer

found in Gram-positive bacteria makes them more susceptible to the effects of plant extracts. This is because the cell walls of these bacteria are easily penetrated by phytochemicals such as flavonoids, alkaloids, tannins, and phenols. These compounds are thought to interfere with the bacterial cell wall and bind with extracellular proteins, thereby inhibiting their growth. Specifically, tannins have been found to possess the ability to precipitate microbial proteins, which leads to insufficient proteins available for bacterial growth. Similarly, fungus development may have been repressed due to the presence of phenols in the extract, which caused hyphae to swell, leak plasma, become distorted, show abnormal branching or fusion, and wrinkle (Huang & Chung, 2003). A recent study on ethyl acetate crude extracts of *C. bonariensis* sourced from Yemen and Tanzania has shown greater antimicrobial activity than the results obtained from the current study. This difference may be attributed to the varying geographical locations where *C. bonariensis* grows. Additionally, it is important to consider the potential impact of genetic variations among the tested microorganisms (Nsindagi *et al*., 2023). In the present study, methanol proved to be the best solvent for studying the antimicrobial activity. The methanolic leaf extract showed more prominent results in the present study than in previous studies.

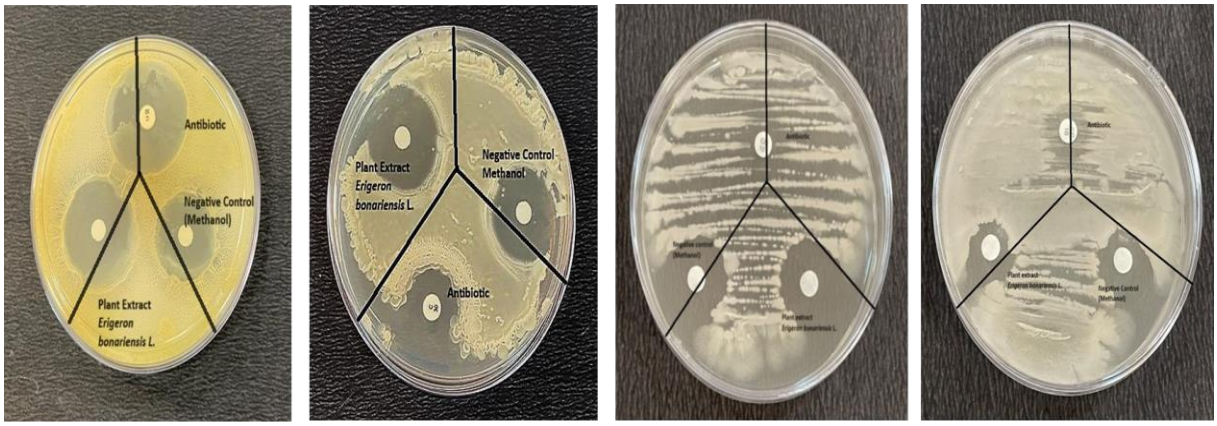


Figure 4. Antimicrobial activity of methanolic leaf extract of *E. bonariensis* against bacterial and fungal strains (A. *Escherichia coli*, B. *Bacillus subtilis*, C. *Aspergillus niger*, D. *Fusarium oxysporum).*

Table 5. Antimicrobial activity of methanolic leaf extract of the *E. bonariensis* using disc diffusion methods.

S. No.	Bacterial and Fungal strains	Zone of inhibition (mm)		
		Plant Extract	Antibiotic	Control
1.	*Escherichia coli*	11.8±0.7	22.6±2.0	8±1.0
2.	*Bacillus subtilis*	14.5±0.5	25±1.0	12±1.0
3.	*Aspergillus niger*	13±0.76	16±0.1.52	16±1.15
4.	*Fusarium oxysporum*	10±0.6	8.3±0.57	11±1.52

The table values have been determined based on the average of three biological means with a standard deviation of n=3 for each.

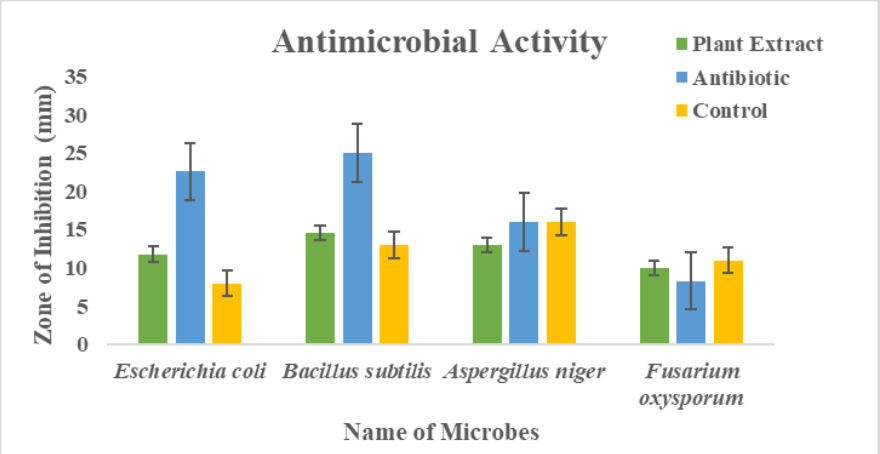


Figure 5. Antimicrobial activity of methanolic leaf extract of *E. bonariensis* against different bacterial and fungal strains.

4. CONCLUSION

E. bonariensis is a plant that presents a global challenge in its eradication due to its persistent nature. Despite its prevalence, there has been limited research on the phytochemical, antioxidant, and antimicrobial properties of plants. However, the present study has identified several phytochemical components in the plant extract that contribute to its antioxidant and antimicrobial properties, lending scientific credibility to its use in traditional medicine. The outcomes of this study indicate that the methanolic plant extract exhibited more phytochemicals and antioxidant activity in comparison to other extracts thus suggesting that it serves as the optimal source for the isolation of bioactive compounds for incorporation into standard medical practices and pharmaceutical production. The methanolic leaf extract displayed more pronounced antimicrobial activity, indicating that this plant is suitable for the manufacturing of antimicrobial drugs. Therefore, this resilient weed has demonstrated remarkable therapeutic properties that make it valuable for the pharmaceutical industry.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Supriya Kumari Sharma: Conceptualization, methodology, plant species collection, extraction and writing, original draft preparation. **Afroz Alam**: plant species identification, supervision, critical review of draft and editing.

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Research Article

Effect of ZnO nano priming on germination and root length of soybean seeds (*Glycine max* L.)

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Abstract: Nano-priming is a pioneering method of treating seeds that improves seed germination, growth, and yield by imparting resilience to several plant stressors. Zinc oxide (ZnO) is a nanomaterial with a specific surface area, high pore volume, low toxicity, and an extended lifetime, and used in nano-priming. This study aimed to determine the effect of ZnO nanoparticles (NPs) on seed germination and root length in determining the optimum concentration of ZnO-NPs for soya plants. The transmission of electron microscopy (TEM) and zeta potential measurements were used to characterize ZnO-NPs. Soya seeds were treated with different concentrations of ZnO-NPs (0, 250, 500, 1000 and 2000 mgL^{-1}) for 24 h. to determine the optimum concentration of ZnO-NPs for selected variants. After priming, the germination percentage and root length of each treatment were measured. The effect of ZnO nanoparticles (in soya plants was investigated by comparing them with seeds germinated in a control (hydro-priming) medium. The investigation demonstrated that the high concentration of ZnO NPs had an adverse impact on both seed germination and root length. Based on this, it was suggested that studies should be conducted including different concentrations of ZnO nanoparticles, which are thought to have a complex structure, to understand the mechanism of action, to find the appropriate concentration for soybean plants, and to increase seed germination.

1. INTRODUCTION

Seed priming, which often occurs during the first stage of germination, triggers metabolic processes that result in a higher percentage of germination and a faster emergence rate (Nile *et al*., 2022). At the same time, this process increases the ability of seeds to survive abiotic challenges and provides for their survival (Khan *et al*., 2023). Seed priming techniques often used include hydropriming (using water), Osmo priming (using polyethylene glycol and inorganic salts), hormonal priming, and nutritional priming. (Paparella *et al.*, 2015; Sytar *et al*., 2019). Classic hydro priming involves soaking the seeds in water before drying them. So, seeds absorb water rapidly, increasing germination rates, and seedlings emerge uniformly.

Over the last decade, the use of nanoparticles for nutrient priming has significantly increased, surpassing other seed preparation methods (Zain *et al*., 2023). The process of nano priming forms nanopores in the embryo, aiding in water absorption (Gupta *et al*., 2024). In addition, nanoparticle (NP) stimulates amylase, which in turn promotes seed germination, causing starch

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degradation. Nano-priming mechanisms in seeds may speed up the growth of roots by changing the transcription of genes that control metabolic processes, such as making phytohormones (Imtiaz *et al*., 2023). Nanoparticles' most important roles in seed preparation are their ability to create electron exchange and their higher surface interaction capacity with different parts of plant cells and tissues. Different metal-based nanoparticles, along with biogenic and polymeric nanoparticles, have been used as seed pretreatment agents in nutrient priming. These include Ag, Au, Cu, Fe, FeS2, TiO2, Zn, and ZnO NPs. Among them, researchers are interested in Zn NPs because they are different from other metal oxide nanoparticles in that they can photocatalyze and photo-oxidize chemical and biological species (Chikkanna *et al*., 2019). Zinc doesn't dissolve well in soil, so plants don't have any of it. Zn NPs applications can solve this problem by providing plants with a form of zinc that dissolves better and is easier to use because it is very reactive. Rameshraddy *et al*. (2017) have demonstrated that priming seeds with Zn NPs enhances their Zn content, thereby promoting improved seedling growth and yield.

Nanoparticles' size, zeta potential, type, and concentration (Santás-Miguel *et al*., 2023) can either help seeds germinate or stop them from doing so (Alobaiddy & Zorer Çelebi, 2022; Das *et al.,* 2020; Hassanisaadi *et al.,* 2022; Li *et al*., 2021). In addition to NP characteristics, the effect of ZnO nanoparticles on plants also varies with plant age and species (Burman *et al*., 2013). ZnO nanoparticles have been shown to aid in the growth of plants such as mung bean (*Vigna radiata*), chickpea *(Cicer arientinum*), cucumber (*Cucumis sativus*), alfalfa *(Medicago sativa* L.), and tomato (*Solanum lycopersicum*) (Mahajan *et al*., 2011; de la Rosa *et al.*, 2013). Research on the exposure of ZnO NPs should include many plant species and variants since the impacts of ZnO NPs are unique to plants (Wang *et al.,* 2023). Further investigation is required to fully comprehend the consequences of NPs and reveal their concentration and variant dependence. The possibility that the ZnO NPs may have beneficial effects on the germination process makes the studies valuable in this area.

Given their economic significance, practical convenience, and various variants, soybeans are ideal model plants to test the impacts of these nanoparticles. Before the widespread application of ZnO NPs in agricultural environments, it is necessary to develop empirical models based on controlled experiments and field research. There is a limited amount of research that particularly examines the biological impacts of ZnO NPs on the process of seed germination and the development of various plant varieties (Faizan *et al.,* 2020; Thounaojam *et al*., 2021). The studies indicate that high concentrations such as 500 mg/kg (Yoon *et al*., 2014), 500 mgL (Lopez-Moreno *et al.*, 2010) and 400 mg/kg (Yusefi-Tanha *et al*., 2020) of ZnO NP negatively affects the growth and development of soybeans. On the other hand, low concentrations 50 mgL (Gaafar *et al.,* 2020), <160 mg/kg (Yusefi-Tanha *et al*., 2020) effect positively root development and seed production, as well as 200 mg/kg the optimum root development in soybeans (Yusefi – Tanha *et al*., 2022). There are still unresolved scientific uncertainties surrounding the influence of nanoparticles on plants, namely different soybean variants with higher concentrations. Therefore, this research used a highly economic variant of *Glycine max* L. as a model to describe the effects of ZnO NPs on seed germination and root length.

The study's goal was to determine whether we could use different amounts of ZnO NPs as nano priming agents instead of hydropriming to aid in soya seed germination and improve their metabolic activity. The research further aimed to evaluate the influence of ZnO NPs on soybean plants and ascertain the optimal dosage for maximum effectiveness. We can use ZnO NPs to increase the Zn concentration in soybean seeds, which enhances their nutritional quality and speeds up germination. This approach may serve as an alternative to traditional hydropriming methods.

2. MATERIAL and METHODS

2.1. Plant Material

In our research, we used ANP 2018 seeds from a widely cultivated and easily grown soybean variety. The variety's thousand-grain weight is 121–131 g, and the growing period is near the middle (I. product: 135–140 days, II. product: 108–112 days) (ANP 2018, 2023). We obtained the variety of seeds from the Eastern Mediterranean Agricultural Research Institute Directorate.

2.2. Characterization of ZnO Nanoparticles

The ZnO NP used in the research was purchased from Nanography Turkey (https://shop.nanografi.com.tr). The nanoparticles that are provided have a purity level of at least 99 percent and an average size of less than 100 nanometers. The characterization research of ZnO nanoparticles included assessing the dimensions and distribution of the particles. The study was performed at Canakkale Onsekiz Mart University Science and Technology Application and Research Centre (COBİLTUM) utilizing a JEOL JEM-1400 PLUS Transmission Electron Microscope (TEM) model. The Zetasizer apparatus at the Turkish Energy, Nuclear, and Mining Research Institute (TENMAK) was used to estimate the zeta potential, which measures the level of repulsion or attraction between particles.

2.3. Seed Priming Method

This study used two distinct methodologies to investigate the effects of water and nanoparticles on seed preparation: Hydropriming, which simply employed water, and nano-priming, which included the dispersion of ZnO nanoparticles in water. The seeds were mixed in a magnetic stirrer in a 0.1% sodium hypochlorite solution for surface sterilization for ten minutes. Following sterilization, the seeds were thoroughly rinsed three times with deionized water to eliminate any traces of chlorine. Subsequently, the seeds were left to dry naturally (Mohamed *et al*., 2019). To manufacture the seeds, ZnO nanosuspensions with varying concentrations (0, 250, 500, 1000, and 2000 $mgL^{-1)}$ were produced by dispersing the particles in deionized water using ultrasonic vibration (200 W, 37 kHz) for 30 minutes (Hòe *et al.*, 2018). The seeds that had been prepared were thereafter rinsed 3–4 times (with each rinse lasting 3 minutes) using distilled water and then dried until they regained their initial moisture content. Subsequently, the seeds were packed in polythene bags and kept at ambient temperature until their next use.

2.4. Seed Germination Parameters

The seeds were placed inside a petri dish with a diameter of 10 cm, resting on two filter paper discs. The germination experiment was carried out in three replicates, and each treatment group consisted of a total of 100 seedlings. The seeds were incubated in an oven at a temperature of 24 ± 2 °C to initiate germination.

2.4.1. *Screening of different concentrations of ZnO NPs and priming time for seed priming*

To determine the ideal concentrations of nanoparticles for experimental purposes, four solutions were created with ZnO NPs at concentrations of 250 mgL^{-1}, 500 mgL^{-1}, 1000 mgL^{-1}, and 2000 mgL^{-1}. Soybean seeds were soaked in ZnO NP solutions at varying concentrations for 24 h at room temperature with continuous aeration and shaking. Hydroprimed seeds were soaked in deionized water for the same period (Mohamed *et al*., 2019). Each petri dish was filled with ten seeds and coated with filter paper. The dishes were then sealed with parafilm tape. Ultimately, all petri dishes were incubated in an oven at a temperature of 24 ± 2 °C. The germinating seeds were quantified using a binocular stereo microscope based on the appearance of a 2 mm root. The germination percentages and root lengths in mm were measured at time intervals of 15, 21, 24, 48, 72, and 96 hours (Hòe *et al*., 2018).

2.5. Statistical Analysis

We presented the data as the mean and standard deviation of three replicates for each treatment. They were then put through a two-way analysis of variance (ANOVA) using the R and R Studio (R Studio Team, 2020; R Core Team, 2021). We used a two-way ANOVA test to evaluate the

effect of two grouping variables (treatment and duration) on a response variable, germination percentage or root length. Four ZnO NP solutions and the untreated control group comprised the five levels of the first factor, which related to the concentrations the seeds encountered (treatment). The second factor is related to temporal measurement, specifically the six-time intervals during which we conducted the measurements. The ANOVA assumptions were checked using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variance. Next, we performed a square root transformation on the root length data to meet the required assumptions. We used Duncan's multiple-range test to validate the statistical significance of the average difference between certain pairs of groups. (Açıkgöz *et al*., 2004).

3. FINDINGS

3.1. Characterisation of ZnO Nanoparticles

Transmission electron microscopy (TEM) analysis of ZnO NPs at various concentrations revealed that the ZnO NPs had an average size of 30-50 nm and exhibited a spherical morphology. Furthermore, the process of combining or gathering together in the cluster of sponge-like particles was documented and shown in Figure 1. The analysis revealed that a solution containing 250 mgL^{-1} of ZnO NPs had an average zeta potential of 11 ± 3.82 mV (Figure 2a). The solutions containing 500 mg/L^{-1}, 1000 mg/L^{-1}, and 2000 mg/L^{-1} of ZnO nanoparticles exhibited average zeta potentials of 11.1 ± 3.36 mV (Figure 2b), 9.25 ± 3.23 mV (Figure 2c), and 7.79 ± 3.10 mV (Figure 2d), respectively

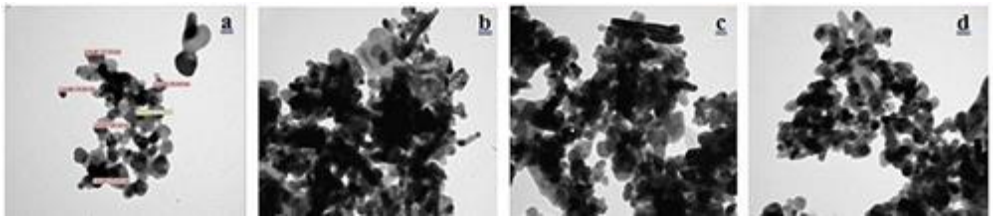

Figure 1. TEM micrograph of dispersion and dispersion of ZnO suspension at four different concentrations (250, 500, 1000, 2000 mgL^{-1}). (x 300000) **(a)** 250 mgL^{-1}, **(b)** 500 mgL^{-1} particle aggregation, **(c)** 1000 mgL^{-1} particle aggregation, **(d)** 2000 mgL^{-1} particle aggregation.

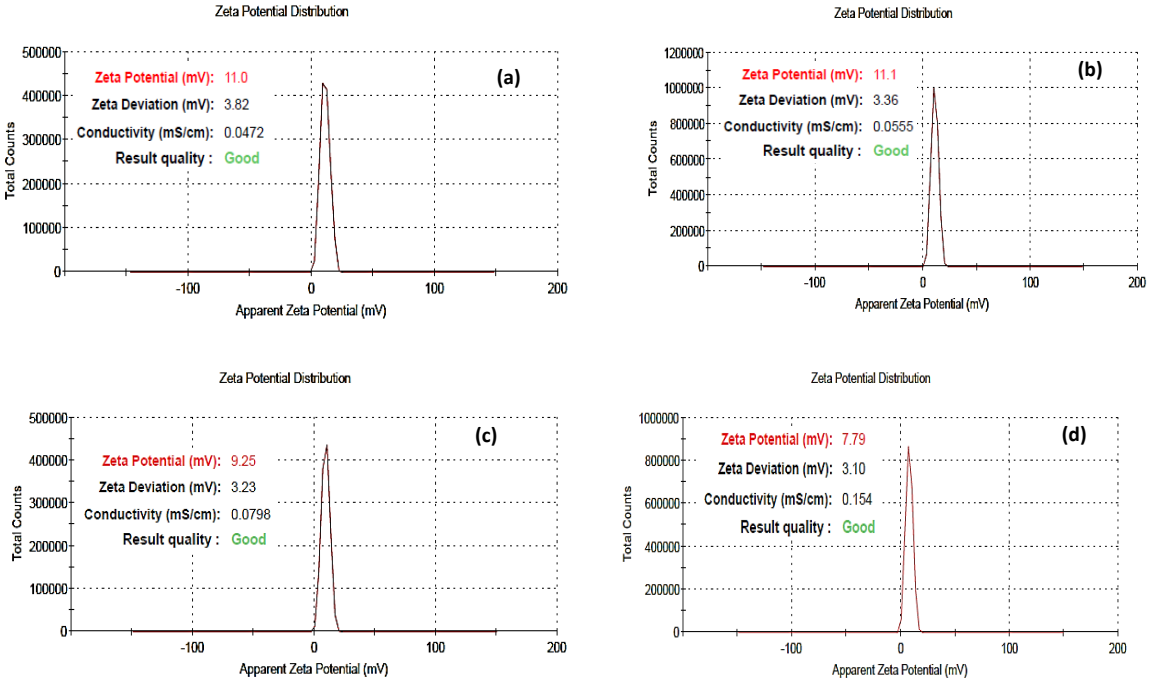

Figure 2. Zeta potential values of ZnO suspension at four different concentrations (250, 500, 1000, 2000 mgL^{-1}). (a) 250 mgL^{-1}, (b) 500 mgL^{-1}, (c) 1000 mgL^{-1}, (d) 2000 mgL^{-1}.

3.2. Accumulation of ZnO Nanoparticles in Seed Coat

The images of soya seeds treated with different concentrations of ZnO nanoparticles at the end of 24 hours were examined by Scanning Electron Microscopy (SEM). It was observed that as the number and concentration of nanoparticles penetrating the seed increased, the accumulation in the seed coat increased significantly in parallel (see Figure 3).

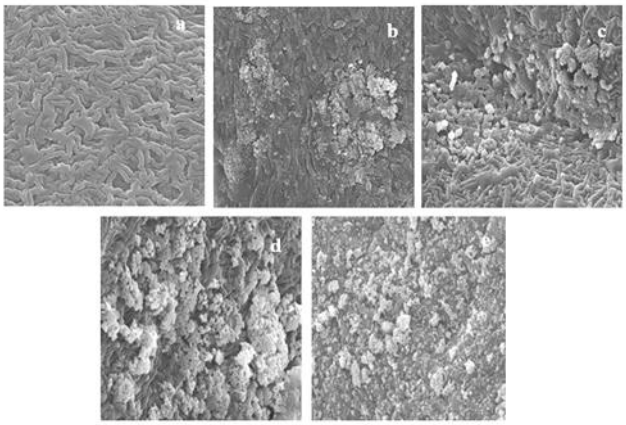

Figure 3. SEM micrograph of nanoparticle accumulation in seed coat. (x 5000) (a) Untreated, (b) 250 mgL^{-1}, (c) 500 mgL^{-1}, (d) 1000 mgL^{-1}, (e) 2000 mgL^{-1}.

3.3. Effect of ZnO Nanoparticle on Germination of Soybean

The germination percentage progressively increased with time in both the untreated and treatment groups. However, the maximum value was seen at the 72nd and 96th hours (Table 1 and Figure 4). The lowest germination percentage was seen at the 15th hour in both the untreated and treated groups with varying doses of ZnO NPs. Overall, the germination percentages quickly rose after 48 hours in both the untreated and treatment groups. The germination percentage indicated variability during 96 hours of the experiment (F=58.16, df=6, p<0.001).

Table 1. Effect of ZnO nanoparticle on germination of soya bean seeds.

Variety	ZnO NP Concentration (mgL^{-1})	Germination Percentage (%)						
		15th hour	18th hour	21st hour	24th hour	48th hour	72th hour	96th hour
ANP2018	0	60^{a}	72^{a}	74^{a}	79^{a}	83^{a}	90^{a}	91^{a}
	250	19^{b}	37^{b}	37^{b}	44^{b}	57^{b}	61^{b}	69^{b}
	500	20^{b}	35^{b}	39^{b}	44^{b}	67^{b}	67^{b}	78^{ab}
	1000	18^{b}	26^{b}	33^{b}	40^{b}	55^{b}	63^{b}	79^{ab}
	2000	20^{b}	32^{b}	38^{b}	48^{b}	57^{b}	67^{b}	82^{ab}
MSE (Mean Square Error)		90.2	97.13	110.4	99.3	119.6	42.3	50.4

*There is no difference between the values shown with the same letters.

Similarly, the germination percentage varied within treatments in all temporal measurement groups, and this variation was statistically significant (F=51.25, df = 4, p<0.001). The untreated group significantly contributed to this variation. The germination percentage of the untreated group was higher than that of the treatment groups, and the differences between the germination percentage of the untreated group and that of the treatment groups were statistically significant for all temporal measurement groups (p<0.001). Among treatment groups, the germination percentage of seeds exposed to high concentrations of ZnO NP was high compared to the seeds exposed to low concentrations at the 96th hour of the experiment. Among treatment groups, the germination percentage of seeds exposed to high concentrations of was high compared to the seeds exposed to low concentrations at the 96th hour of the experiment (see Table 1).

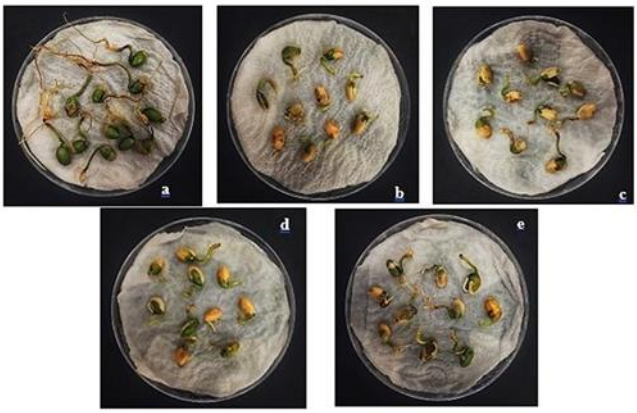

Figure 4. Effect of ZnO nanoparticle on germination percentage of soya bean seeds at the endof 96th hour **a:** control, **b:** 250 mgL^{-1}, **c:** 500 mgL^{-1}, **d:** 1000 mgL^{-1}, **e:** 2000 mgL^{-1}.

3.4. Effect of ZnO Nanoparticle on Root Length of Soybean

Root lengths obtained in the study are presented in Table 2. The root length got longer in the untreated group until they reached their longest point at 96 hours (Figure 5). There was no significant variation in root lengths between the 15th and 24th hours ($p > 0.05$, Table 2). The findings were consistent across all different ZnO NP treatments, indicating that there were no significant changes seen between the 15th and 48th hours ($p > 0.05$). In all treatments, the length of the roots exhibited growth at 72 hours, with the exception of the untreated group. The growth accelerated at the 48th hour in the untreated group (see Table 2). At the 96th hour, all treatment groups, including the untreated group, achieved the highest value. There was a significant difference among the root lengths of all experimental groups ($F = 112.7$, $df=4$, $p<0.001$). Root length was highest at the untreated group ($p<0.001$). The mean root length in the untreated group was longer than that in the all-treatment groups at the all-temporal measurement groups ($p<0.001$).

Table 2. Effect of ZnO nanoparticle on root length of soya bean seeds.

		Average Root Length (cm)						
Variety	ZnO NP Conct. (mgL^{-1})	15^{th} hour	18^{th} hour	21^{th} hour	24^{th} hour	48^{th} hour	72^{th} hour	96^{th} hour
ANP 2018	0	0.56 ± 0.13^{a}	0.73 ± 0.22^{a}	0.81 ± 0.22^{a}	0.81 ± 0.21^{a}	1.91 ± 0.98^{a}	2.90 ± 1.61^{a}	7.22 ± 5.30^{a}
	250	0.24 ± 0.13^{b}	0.32 ± 0.16^{b}	0.32 ± 0.16^{b}	0.33 ± 0.15^{b}	0.71 ± 0.3^{b}	0.96 ± 0.58^{b}	1.44 ± 1.12^{b}
	500	0.28 ± 0.14^{b}	0.36 ± 0.21^{bc}	0.36 ± 0.21^{bc}	0.37 ± 0.20^{bc}	0.6 ± 0.31^{b}	1.03 ± 0.47^{b}	1.68 ± 1.42^{b}
	1000	0.24 ± 0.11^{b}	0.35 ± 0.19^{bc}	0.43 ± 0.20^{bc}	0.44 ± 0.19^{bc}	0.81 ± 0.4^{b}	0.93 ± 0.53^{b}	1.51 ± 1.29^{b}
	2000	0.29 ± 0.09^{b}	0.43 ± 0.18^{c}	0.45 ± 0.21^{c}	0.45 ± 0.20^{c}	0.78 ± 0.3^{b}	0.95 ± 0.55^{b}	1.52 ± 1.47^{b}
MSE (Mean Square Error)		0.002	0.003	0.003	0.004	0.009	0.02	0.33

*There is no difference between the values shown with the same letters.

Figure 5. Effect of ZnO nanoparticle on root length of soya bean seeds at the end of 96^{th} hour.

4. DISCUSSION and CONCLUSION

Zeta potential analysis measured the stability of ZnO NPs at different concentrations in this study. The ZnO NPs we studied were moderately stable and dispersed. The ZnO NP parts in the suspension can be used as a seed priming agent (Ateş, 2018). In our study, we measured the zeta potential lower than what Sharma *et al*. (2022) recorded. Furthermore, our study measured the zeta potential positively. There were functional groups on the surface of NP that gave it a positive charge. These groups consisted of hydroxyl (OH) or amine (NH_2) groups. Therefore, the higher pH values (alkaline) of the ZnO NP environment also explain the positive zeta potential (Hunter *et al.*, 1981). This phenomenon contributed to their stability, dispersion, and interactions with biological systems. On the other hand, low stability increases the possibility of particles adhering to one another (Hidayat Chai *et al*., 2018). Our results indicate that ZnO NPs exhibited lower stability at a concentration of 2000 $mgL^{-1,}$ leading to increased particle agglomeration. The SEM visualization supports this conclusion.

Seed germination and early seedling production are crucial phases in plant growth. The positive and detrimental effects of ZnO-NP use on plant development depend on the concentration used (Faizan *et al*., 2020; Rajput *et al*., 2021). In a study with maize and wheat, it was confirmed that germination percentage increased at lower ZnO NP concentrations (100 mgL^{-1}), but decreased variably at higher levels (150–200 - 200 mgL^{-1}) (Srivastav *et al*., 2021). In our study, seeds showed germination in all treatments, suggesting that ZnO NPs had a positive effect. ZnO NPs significantly increase seed water uptake compared to conventional hydropriming (El-Saadony *et al*., 2021; Rai-Kalal &Jajoo, 2021). The studies demonstrate that rapid absorption of water molecules and ZnO nanoparticles during nano-priming allows them to enter the seed through the seed coat's cell wall, leading to the production of ROS, a signal for fast seed germination (Mahakham *et al.,* 2017; Sharma *et al.,* 2021). As a result of ZnO nanocoating applications, increased seed water uptake promotes metabolic activity. Taking in water accelerates the process of breaking down starch by activating germination enzymes such as α-amylase. This makes seed germination much better in the early stages. Nano priming controls the aquaporin and α-amylase genes, making them better at quickly taking in water from the seed, breaking down starch, making more soluble sugar, starting GA signaling pathways, and getting rid of ROS to help seeds germinate faster and healthier (Khan *et al.,* 2023).

Researchers found that priming with ZnO NP increased the germination percentage in soybean (Montanha *et al.,* 2020) and wheat (Munir *et al*., 2018). However, Rosa *et al.* (2013) found that ZnO NPs at a concentration of 1600 ppm slowed the germination of Cucumis sativus (cucumber) by 10% and sped up the germination of *Solanum lycopersicum* (tomato) and *Medicago sativa* (black clover) by 20% and 40%, respectively. In a different study, it was found that high levels of ZnO NPs reduce the growth and germination of pepper seeds and seedlings, showing that ZnO NPs are toxic (García-López *et al.,* 2018). The results of our study are consistent with the literature indicating that high concentrations of ZnO NP reduce the percentage of germination in soybeans. Seeds treated with high concentrations of ZnO nanoparticles may have nanoparticle deposits on the outer surfaces. This situation may naturally block the pores of the seed coat, disrupting the water uptake necessary for germination. As a result, the seed is unable to absorb water sufficiently (Johns & Cahill, 2018). This event disrupts metabolic processes such as enzyme activation, protein synthesis, RNA synthesis, and cell division, among others. Accordingly, cellular oxidative stress begins to increase, and the antioxidant defence system weakens. This may delay the germination of seeds.

As in the germination percentage, previous studies reported that the root length of plants exposed to low ZnO NP concentrations (for example, 10 mgL^{-1}) increased (Itroutwar *et al*. 2020, Nemček *et al*. 2020, Youssef & Elamawi 2020). It is known that zinc is needed to make certain hormones, like auxins and gibberellins, which explains why the length of the radicle and plumule grows when ZnO NPs are present. Consequently, the radicle and plumule length of NP-exposed seeds increase. Zinc is essential for the biosynthesis of endogenous hormones,

including auxins and gibberellins, which explains the increase in radicle and plumule length in the presence of ZnO NPs. Consequently, the radicle and plumule length of NP-exposed seeds increase (Cakmak, 2008; Prasad *et al*., 2012). On the other hand, the toxic effect of the high concentration of ZnO NP (1,000 $mgL^{-1)}$ reported as reducing the root length of corn and cucumber (Zhang *et al*., 2015). Also, research has linked high levels of ZnO NP (100–200 mgL^{-1}) to phytotoxicity by creating chromosomal errors, micronuclei, and vacuolated nuclei (Youssef & Elamawi 2020). Our study's result is consistent with this literature: high concentrations of ZnO NP exposure negatively affect root length in soybeans.

We used the nano-priming method to test ZnO nanoparticles on soy bean seeds at 15, 21, 24, 48, 72, and 96 hours to see how they changed the number of seeds that germinated and the length of the roots. The concentrations we tested were 0, 250 mgL^{-1}, 500 mgL^{-1}, 1000 mgL^{-1}, and 2000 $mgL^{-1.}$ ZnO had near-spherical structures with an average particle size of 30–50 nm. In solution, the zeta potential values of four different suspensions of ZnO NPs showed moderate stability and dispersion. At high concentrations, these characterized ZnO NPs had a negative effect on germination percentages and root lengths compared to the control groups. This statement is compatible with the results of previous studies on various plant species and varieties. These findings can help us understand the mechanism of action of high-concentration ZnO nanoparticles. However, given the intricate nature of the impact mechanisms, further research is necessary prior to agricultural application to boost plant growth and mitigate adverse effects.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: Canakkale Onsekiz Mart University/ Postgraduate Education Institute Ethics Committee Scientific Research Ethics Committee, E-84026528-050.01.04-2200303364.

Authorship Contribution Statement

Burcu Akbay: Investigation, resources, visualization, software, formal analysis, and writing - original draft. **F. Sevil Yalçın**: Methodology, supervision, validation, review and editing.

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Research Article

Patulin and phenolic content in commercial fruity baby foods on the Turkish market

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Abstract: This study aimed to determine baby foods' safety, antioxidant content, and color. In this study, the mycotoxin patulin, which is toxic to humans, and phenolics (chlorogenic acid, p-coumaric acid, epicatechin), which are beneficial to human health and color, were analyzed in baby foods containing fruit puree. The patulin content in 12 baby foods purchased from the market in Türkiye was below 8.45 µg/kg. Patulin found in baby foods comes from spoiled fruit. By performing this study, the quality of fruits used in baby foods was evaluated. Fruits also have chlorogenic acid, p-coumaric acid, and epicatechin phenolics. These phenolics in baby foods were 9.316-598.428 µg/kg, 0.953-14.166 µg/kg, and 0.471-20.35 µg/kg, respectively. L*, a*, and b* color values of 12 baby foods were found between 12.77-23.00, 1.44-11.19, and 10.50-11.01, respectively.

1. INTRODUCTION

Patulin (see Figure 1) is a mycotoxin produced by *Penicillium* and *Aspergillus* molds, often found in moldy fruits and their products. The highest allowable amount of patulin in baby food is 10 µg/kg, according to the Turkish Food Codex (2011). In an experiment on mice, patulin's lethal dose (LD50) value was 5 mg/kg. The level of toxic effects was determined as 43 µg/kg according to the Provisional Maximum Tolerable Daily Intake Value and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The highest level of patulin that can be taken daily is 0.4 mg/(kg body weight x day). Determining patulin levels in foods is very important for consumers' health. The Codex Alimentarius and the Food and Drug Administration (FDA) have set the highest permissible level of 50 µg/kg for apple juices and other apple-containing food products. Patulin content in baby foods and apple puree is subject to regulations set by the authorities. In the European Union (EU, 2006), this upper limit was determined to be about 10 µg/kg for baby foods and apple puree. Research conducted in European Union countries, such as Cuba, South Africa, Iran, and Türkiye, found that the amount of patulin in apple juices differed. In most studies, patulin levels were below the Codex Alimentarius limits in Iran and Türkiye. In Australia, patulin levels exceeded the European Union limit (Li *et al.*, 2007).

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Patulin is known as polyketide lactone (4-hydroxy-4H-furo (3,2-c) pyran-2 (6H)-1) (Baert *et al*., 2007). It is colorless and crystalline, and its melting point is 110ºC. It is stable with a temperature of 105-125ºC and pH 3.5-5.5. The degree of disintegration increases with increasing levels of pH. At pH six and 100ºC, only 50% of patulin is broken. Therefore, it is not enough to complete the pasteurization for the inactivation of patulin (Gonzales-Osnaya *et al*., 2007). In one study, patulin in 120 homogenized baby foods was examined. Baby foods were purchased from the supermarket in Italy between 2008 and 2009. Sixty of these samples were apple flavored (40 from traditional agriculture, 20 from organic agriculture), and 60 were selected as mixed fruit flavors (40 from traditional agriculture and 20 from organic agriculture). Patulin levels in all of the samples were below the limit. In 22 samples, the amount of patulin was close to the limit (9µg/kg) (Bonerba *et al*., 2010). Funes & Resnik (2009) determined that there was patulin contamination in apples and products purchased from supermarkets in Argentina. In this study, 21.6% of 51 products were found contaminated (17-221 µg/kg, average 61.7 µg/kg), and the highest patulin level was determined as 123 µg/kg in 50% contaminated apple puree. The level of patulin in apple puree should be reduced. Zaied *et al*. (2013) examined the patulin content of 85 apple products sold in supermarkets in Tunisia in 2013. This study compared the patulin contamination in apple products and determined the factors accelerating mycotoxin formation. The contamination level was defined as an average of 20 µg/L in the range of 0-167µg/L in all samples. 28% of baby foods exceeded the allowable limit for patulin. Barreira *et al*. (2010) examined the level of patulin in 144 apple foods (76 of them homogenized apple puree) sold in markets in Portugal. Patulin was detected in 33 samples (23%) in the 1.2-42µg/kg range. Patulin was determined in five homogenized apple purees (7%). Janotova *et al*. (2011) reported that all stages of apple puree production led to patulin reduction, particularly pulping, which reduced patulin levels by 29-80% of its original content. Marín *et al*. (2011) reported that patulin content in fruit products purchased from a market in Spain was below EC limits.

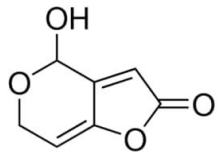

Figure 1. Formula of patulin.

Phenolic substances contain one or more hydroxyl groups in their aromatic ring (Shadidi & Naczk, 1995). In this respect, it is known that the simple phenolic substance is benzene, which contains one hydroxyl group and other phenolic substances derived from it (Cemeroğlu & Acar, 1986; Saygı, 2017; Saygi, 2021a; Saygi, 2021b). Phenolic substances are divided into two groups: simple phenolic substances and polyphenols. Phenolic substances commonly found in fruits and vegetables are examined in hydroxybenzoic acids, hydroxy-cinnamic acids, and flavonoids. Flavonoids are divided into five subgroups. These are catechins, anthocyanidins, flavanols, flavonoids, and proanthocyanins (Cemeroğlu & Acar, 1986). Phenolic acids vary according to the position and number of the hydroxyl group in the ring chain. These compounds have a strong antioxidant effect against free radicals that cause cancer and cardiovascular diseases (Yu *et al*., 2002). Phenolics effectively protect against oxidative stress in the human body (Papas, 1998). It is known that fruits are richer than vegetables in terms of phenolic substances. In addition, phenolic substances are present in almost every fruit and vegetable (Shadidi & Naczk, 1995). The main phenolic compounds in apple juice are procyanidins, epicatechin, chlorogenic acid, p-coumaric acid, phloretin 2-xylo glucoside, and phlorizin (Suárez *et al*., 1998).

Santos *et al*. (2014) examined the change in the phenolic profiles of baby food prepared from green vegetables during storage. Phenolic content was determined at the beginning and end of storage for ten days. It was found that storage affects the phenolic content of baby foods at

different rates. Reyes *et al*. (2007) found a prolonged change in the total phenolic content of fresh fruits during storage. As a result of this change, the amount of ascorbic acid and phenolic compound decreased.

This study aimed to ensure an overview of patulin contamination, determine the antioxidant value, and provide data on the color values of fruity baby foods in Türkiye (produced in Türkiye, Hungary, and Poland). The assessment of antioxidant potential is of crucial importance in foods. The study about patulin content, phenolics, and color value in baby foods containing fruit puree purchased from a local market in Türkiye has not been performed so far. No study has examined patulin and phenolics in baby foods produced in Türkiye, Hungary, and Poland. Additionally, no publications perform all three analyses on baby foods.

2. MATERIAL and METHODS

2.1. Materials

Twelve baby fruit puree from four companies were purchased from a market in Türkiye. These fruit purees are given in Table 1.

Table 1. Fruity baby food samples.

Company	Samples
A	Carrot Apple Puree, Apple Peach Puree, and Apple Pear Puree
B	Mixed Fruit Oats, Organic Mixed Fruit, and Organic Apple Peach
C	Organic Peach Puree, Organic Apple Puree, and Organic Apple and Banana Puree
D	Organic Peach Apple Puree, Organic Cereal Mixed Fruit Puree, and Organic Fruit Cocktail

2.2. Patulin Analysis

The sample was homogenized. It was filtered through a coarse filter pan, and 10 mL of the filtrate was taken and placed in the separating funnel. Then, 20 mL of ethyl acetate was added and shaken for 1 minute. After performing phase separation, the ethyl acetate phase (clear phase on top) was transferred to another container. The yellowish phase at the bottom was treated twice with ethyl acetate, and then, the eluates were combined. The remaining aqueous phase was discarded. Agitation was carried out three times with 20 mL of ethyl acetate. 4 mL of 1.5% sodium carbonate was added over the combined ethyl acetate phases and shaken within 1 minute, and the underlying phase was taken in a beaker. 15 g of sodium sulfate was weighed in the upper phase and collected in an evaporator flask. The phase into the beaker was brought into the separatory funnel and shaken by adding 10 mL of ethyl acetate. The ethyl acetate phase was filtered through 15 g of sodium sulfate-weighed filter paper. This process was done once again. All ethyl acetate phases were collected in an evaporator flask. Ethyl acetate, the evaporator extraction solution, was blown off. 2 mL of mobile phase was added to the residue in the evaporator flask, mixed well, and put into the vial. Samples were analyzed by HPLC (Shimadzu-SIL-20AHT, Japan) at 276 nm with a flow rate of 0.7 mL/min according to the method of AOAC 2000.02 with slight modifications. The separation column was octadecylsilane (ODS), (3.5µ, 250x4.6mm). Analysis was done in duplicate.

2.3. Phenolic Analysis

The homogenized sample (1g) was extracted in 1000 mL of 80% methanol for 20 min in an ultrasonic bath and filtered. The residue was washed with 50 mL of 100% methanol. Extraction and washing processes were repeated. The solution mixture was evaporated at 40ºC. 1 mL of methanol was added and injected into HPLC (Shimadzu Prominence Brand, Japan). Phenolic compounds of samples were analyzed according to the method reported by Caponio *et al*. (1999) with some modifications (A: 3% Formic acid B: Methanol). Analysis was done in duplicate.

2.4. Color Analysis

The samples ' color values (L*, a*, b*) were determined using an X-rite instrument (USA). Analysis was done in duplicate.

2.5. Statistical Analysis

Data related to patulin, phenolic acids, and color values was statistically analyzed according to the Duncan test. The difference between groups was significant at $p < 0.05$. All data were analyzed using IBM Statistics SPSS 24.

3. FINDINGS

3.1. Patulin

Patulin level in all samples was found below 8.45µg/kg, below the upper limit of patulin in baby foods (10µg/kg). Ritieni (2003) reported that two samples of ten apple-based baby foods purchased from a supermarket in Italy were contaminated with 17.7 and 13.1 µg/L patulin.

3.2. Phenolic Compounds

The quantity of phenolic compounds in 12 baby foods is given in Table 2. LOD, wavelength, and retention times of phenolic standards are shown in Table 3. A chromatogram of baby foods is given in Figure 2. The highest chlorogenic acid was 598.428 µg/kg belonging to the organic apple puree of Company C, while the lowest chlorogenic acid was 9.316 µg/kg belonging to mixed fruit and oat of Company B. Chlorogenic acid in carrot and apple puree of Company A was statistically similar to that of apple and peach puree of Company C, apple and pear puree of Company A and organic cereal mixed fruit puree of Company D. The highest *p*-coumaric acid was 14.166 µg/kg belonging to organic peach and apple puree of Company D, while the lowest *p*-coumaric acid was 0.953 µg/kg belonging to apple and pear puree of Company A. *p*-coumaric acid in the organic peach puree of Company C was statistically similar to that of the organic apple puree of Company C. *p*-coumaric acid in apple and peach puree of Company A was statistically similar to that of mixed fruit oat of Company B, organic apple peach puree of Company B and organic cereal mixed fruit puree of Company D. *p*-coumaric acid in organic apple and banana puree of Company C was statistically similar to that of organic fruit cocktail of Company D. *p*-coumaric acid in carrot and apple puree of Company A was statistically similar to that of apple and pear puree of Company A. The highest epicatechin was 20.35 µg/kg belonging to the organic apple and banana puree of Company C, while the lowest epicatechin was 0.471 µg/kg belonging to mixed fruit oat of Company B. Epicatechin of the organic apple and peach of Company B was statistically similar to that of organic apple puree of Company C, organic peach apple puree of Company D and organic fruit cocktail of Company D. Epicatechin in an organic peach puree of Company C was statistically similar to that of organic cereal mixed fruit puree of Company D. Epicatechin in apple and peach puree of Company A was statistically similar to that of apple and pear puree of Company A, mixed fruit oat of Company B and organic mixed fruit puree of Company B. Similar results were reported by Casado *et al*. (2019). Casado *et al*. (2019) investigated two different extraction techniques for four baby foods purchased from a local pharmacy in Portugal. Casado *et al*. (2019) reported that chlorogenic acid in four baby foods containing bananas, apples, multi fruits with cereals, or chicken, beef, and vegetables was not detected, 815 µg/kg, 569 µg/kg, and 194 µg/kg, respectively. Epicatechin of those baby foods was 153µg/kg, 469µg/kg, 293µg/kg, and not detected, respectively. *P*-coumaric acid of those baby foods was 67µg/kg, 32.6µg/kg, 64µg/kg, and 65µg/kg, respectively. Osminaski *et al*. (2008) reported that the chlorogenic acid of apple purees was 63-200µg/kg, while epicatechin was 28.8-143µg/kg. Talcott & Howard (1999) reported that strained carrots had chlorogenic acid between 2.04 and 55.25µg/kg.

3.3. Color Values

L*, a*, and b* color values of baby foods are given in Table 4. L* color values of 12 baby foods ranged between 12.77 and 23.00. a* color values of 12 baby foods ranged between 1.44 and 11.19. b* color values of 12 baby foods ranged between 10.50 and 18.01. The highest L* color value was 23.00 belonging to the organic apple and banana puree of Company C, while the lowest L* color value was 12.77 belonging to the carrot and apple puree of Company A. The highest a* color value was 11.19 belonging to carrot and apple puree of Company A, while the lowest a* color value was 1.44 belonging to organic apple puree of Company C. The highest b* color value was 18.01 belonging to the carrot and apple puree of Company A, while the lowest b* color value was 10.50 belonging to the organic apple puree of Company C. Similar results were reported by Oszmiański *et al*. (2008). Oszmiański *et al*. (2008) reported that apple purees' L*, a*, and b* color values ranged between 47.99-60.98, 2.74-10.33, and 11.17-20.74, respectively.

Table 2. Phenolic content of fruity baby foods (µg/kg).

Fruity baby foods	Chlorogenic acid (µg/kg)	*p*-coumaric acid (µg/kg)	Epicatechin (µg/kg)
Company A Carrot and Apple Puree	66.316^{f}	1.282^{e}	3.219^{d}
Company A Apple and Peach Puree	71.041^{f}	6.662^{c}	1.349^{e}
Company A Apple and Pear Puree	79.867^{f}	0.953^{e}	0.638^{e}
Company B Mixed Fruit Oats	9.316^{h}	4.911^{c}	0.471^{e}
Company B Organic Mixed Fruit	40.388^{g}	7.027^{c}	1.304^{e}
Company B Organic Apple and Peach	449.842^{b}	6.115^{c}	9.301^{b}
Company C Organic Peach Puree	96.337^{e}	11.984^{b}	5.395^{c}
Company C Organic Apple Puree	598.428^{a}	10.459^{b}	9.230^{b}
Company C Organic Apple and Banana Puree	119.331^{d}	2.955^{d}	20.354^{a}
Company D Organic Peach and Apple Puree	286.252^{c}	14.166^{a}	9.054b
Company D Organic Cereal Mixed Fruit Puree	76.208^{f}	6.736^{c}	6.279^{c}
Company D Organic Fruit Cocktail	87.362^{e}	2.083^{d}	9.437^{b}

Values within columns with similar letters are not significantly different (Duncan's test).

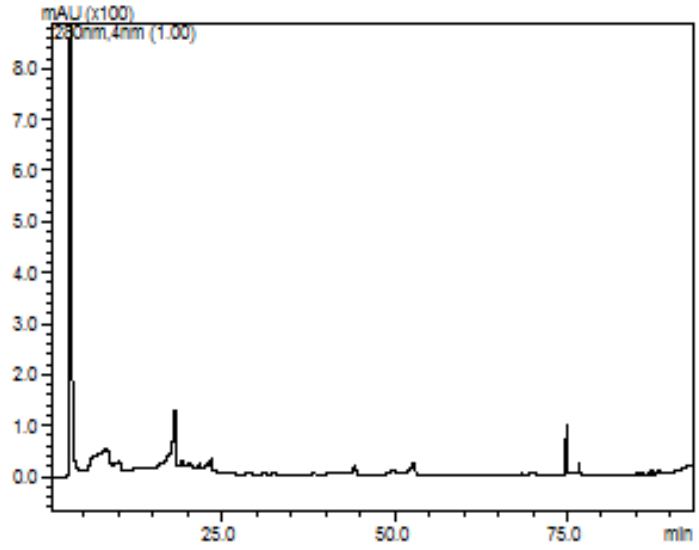

Figure 2. Chromatogram of phenolics in fruity baby food (Company A apple pear puree).

Table 3. LOD, wavelength, and retention times of phenolic compounds.

Standards	LOD (µg/g)	Wavelength (nm)	Retention times (min)
Chlorogenic acid	0.01	320	18.2
p-coumaric acid	0.01	320	26.1
Epicatechin	0.43	260	21.3

LOD: Limit of detection

Table 4. Color values (L*, a*, b*) of fruity baby foods.

Fruity baby foods	L*	a*	b*
Company A Carrot and Apple Puree	12.77^{e}	11.19^{a}	18.01^{a}
Company A Apple and Peach Puree	15.67^{d}	5.50^{c}	14.31^{b}
Company A Apple and Pear Puree	14.91^{d}	7.16^{b}	17.46^{a}
Company B Mixed Fruit Oats	17.35^{c}	2.14^{e}	11.13^{d}
Company B Organic Mixed Fruit	20.13^{b}	3.28^{d}	16.97^{a}
Company B Organic Apple and Peach	20.89^{b}	3.84^{d}	10.87^{d}
Company C Organic Peach Puree	16.42^{c}	3.38^{d}	12.24^{c}
Company C Organic Apple Puree	14.60^{d}	1.44^{f}	10.50^{d}
Company C Organic Apple and Banana Puree	23.00^{a}	2.87^{e}	12.72^{c}
Company D Organic Peach and Apple Puree	16.07^{d}	5.00^{c}	14.56^{b}
Company D Organic Cereal Mixed Fruit Puree	16.54^{d}	3.67^{d}	14.06^{b}
Company D Organic Fruit Cocktail	15.21^{d}	4.16^{d}	13.78^{b}

Values within columns with similar letters are not significantly different (Duncan's test).

4. DISCUSSION and CONCLUSION

The presence of patulin in apple juice indicates the microbiological quality of the raw material (Acar *et al*.,1998). Patulin in baby foods comes from fruit, which is spoilage. The daily intake of patulin is essential for babies and children who are sensitive to its toxic effects. According to the Turkish Food Codex, the maximum limit for patulin in baby foods is 10 µg/kg. It was concluded that the raw material of baby foods produced in Türkiye, Hungary, and Poland was of high quality. The patulin content did not exceed 8.45 µg/kg. Similar results were reported by Bonerba *et al.* (2010). Bonerba *et al*. (2010) reported that the patulin content in 120 baby foods purchased at a market in Italy was less than 10 µg/kg. The low level of patulin in Italian apple products is a parameter used to evaluate fruit quality, and the process is of a high standard (Ritieni, 2003). Patulin, an undesirable natural contaminant of fruits, can indicate fruit quality and processes (Burda, 1992; Gökmen & Acar, 1998).

The main phenolic compounds in apples were chlorogenic acid, *p*-coumaric acid, phloretin-2'-O-glucoside, phloretin-2'-O-xyloglucoside, (+)-catechin, (-)-epicatechin, and procyanidins (Wojdyło et al., 2007). Therefore, epicatechin, chlorogenic acid, and *p*-coumaric acid were found to have high ratios in organic apple-based fruit puree analyzed in this study. Apple contains considerable amounts of polyphenols, contributing to total antioxidant activity (Khanizadeh *et al*., 2007), and may reduce the risk of cardiovascular disease, coronary, and mortality risk (Knekt *et al*., 2000).

Determining the level of patulin in baby foods gives information about the quality of fruits used in baby food production. Children are exposed to patulin toxicity more than adults. Therefore, the amount of patulin in baby foods was investigated. The level of patulin was found below the upper limit (10 µg/kg) in all baby foods belonging to four companies. Phenolic contents were observed at a high rate in baby foods belonging to four companies. The highest amount of chlorogenic acid, p-coumaric acid, and epicatechin was determined in the organic apple puree of Company C, the organic peach and apple puree of Company D, and the organic apple and banana puree of Company C, respectively. The brightness values of the baby foods belonging

to the four companies were close. There is no study about the patulin content, phenolic compounds, and color of baby foods purchased in Türkiye. The results obtained from this study will make a significant contribution to the creation of a detailed database.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Seda Yalçın: Design Analysis, Interpretation, and Writing. **Sevgül Coşkun:** Conception, Materials, and Literature Review

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Research Article

Anticancer effects of sodium selenate in human neuroblastoma, breast cancer, and melanoma cells

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Abstract: Sodium selenate (Na_2SeO_4) is one of the oxidized inorganic forms of selenium. Effects on cytotoxicity, total antioxidant level, total oxidant level, oxidative stress, and genotoxicity status and its anticancer effect on SH-SY5Y human neuroblastoma, MCF-7 human breast cancer, and 451Lu human melanoma cells were investigated in this study. Sodium selenate exhibited a highly cytotoxic effect at all concentrations (0.078125 - 10 mg/mL) against SH-SY5Y, MCF-7, and 451Lu cancer cell lines. In addition, sodium selenate reduced the total antioxidant levels, increased the total oxidant levels (except for SH-SY5Y), and induced oxidative stress significantly in SH-SY5Y, MCF-7, and 451Lu cells. However, in agarose gel electrophoresis images, it was observed that sodium selenate did not have any genotoxic effect on SH-SY5Y, MCF-7, and 451Lu cancer cells. Sodium selenate can be used in cancer treatment because of its antioxidant, as well as pro-oxidant and anticancer properties, which depend on the concentrations used.

1. INTRODUCTION

Sodium selenate (Na_2SeO_4) is a microelement among the oxidized inorganic forms of selenium. The high bioavailability of sodium selenate not only allows it to play important roles in biological processes but also provides various health benefits. Sodium selenate serves as a selenium source in the human body and participates in the structure of enzymes such as glutathione peroxidases (GPx), thioredoxin reductases (TrxR), selenoprotein P and iodothyronine deiodinases (DIO), which play a critical role in the antioxidant defense system and provide protection against oxidative damage (Arnér, 2009; Brigelius-Flohé & Maiorino, 2013; Burk & Hill, 2015; Köhrle, 2000; Rayman, 2012).

Oxidative stress occurs as a result of the imbalance between free radicals and reactive oxygen species (ROS) that occur as a result of various biochemical events in the human body and cellular antioxidant defense systems. It is known that oxidative stress plays an important role in the pathogenesis of many diseases, including cancer (Birben *et al.*, 2012). However, recent studies have shown that oxidative stress may have anticancer effects under certain conditions (Gorrini *et al.*, 2013).

In addition to its antioxidant properties, sodium selenate increases oxidative stress depending on the dose and application method and can also show anticancer effects against various types of cancer through increased oxidative stress. It is stated in the literature that sodium selenate

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triggers apoptosis in cancer cells and stops the cell cycle and tumor growth (Cao *et al.*, 2019). In addition, it is stated in the literature that sodium selenate has antiproliferative effects against cancer cells at low concentrations, while it has toxic effects on cancer cells at high doses (Foster & Sumar, 1997). Sodium selenate has become a molecule that has attracted attention in recent years due to its properties (Rayman, 2012).

Cancer is the second most common cause of death worldwide. In 2022, there were almost 20 million new cases of cancer and 9.7 million deaths from cancer. Estimates suggest that approximately one in five men or women will develop cancer in their lifetime, while about one in nine men and one in 12 women will die from it. Brain, and central nervous system cancer was the 19th most frequently diagnosed cancer in 2022, responsible for almost 321476 new cases, with 1.6% of all cancers globally. Brain, and central nervous system cancer was also one of the leading causes of cancer death, with an estimated 248305 deaths (2.6%). Female breast cancer was the second most frequently diagnosed cancer in 2022, responsible for almost 2.3 million new cases, or one in eight cancers world-wide (11.6% of all cancers globally). Female breast cancer was also one of the leading causes of cancer death, with an estimated 665684 deaths (6.9%). Melanoma of skin cancer was the 17th most frequently diagnosed cancer in 2022, responsible for almost 331647 million new cases, with 1.7% of all cancers globally. Melanoma of skin cancer was also one of the leading causes of cancer death, with an estimated 58645 deaths (0.6%) (Bray *et al.*, 2024). The anticancer activity of sodium selenate has been studied in some cancer cells (Corcoran *et al.*, 2010; da Costa *et al.*, 2023; Tsukamoto *et al.*, 2013).

In this study, the effects of sodium selenate on the total antioxidant, oxidant, and oxidative stress status of SH-SY5Y human neuroblastoma, MCF-7 human breast cancer, and 451Lu human melanoma cells and its cytotoxic effect on these indicated cancer cell lines were investigated. No studies have yet been conducted on the anticancer effects of sodium selenate on the SH-SY5Y human neuroblastoma, MCF-7 human breast cancer, and 451Lu human melanoma cells. Our study aims to give an idea to the cancer research to be made with sodium selenate.

2. MATERIAL and METHODS

2.1. Chemicals and Reagents

RPMI 1640 medium, Cell Viability Detection Kit-8 (CVDK-8), Phosphate Buffered Saline (PBS) (1x), pH:7.4, Genomic DNA Isolation Kit, ClearBand 6x DNA Loading Dye, ClearBand SAFE DNA Gel Stain Solution, 20,000x, TBE 10x, and 100bp DNA ladder were procured from EcoTech Biotechnology, Erzurum, Türkiye. Total antioxidant status (TAS) and total oxidant status (TOS) assay kits were purchased from Rel Assay Diagnostics, Mega Tıp, Gaziantep, Türkiye. Fetal bovine serum (FBS) and penicillin-streptomycin solution were procured from Gibco Life Technologies, Paisley, UK. Sodium selenate and all other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Cell Culture

Human neuroblastoma SH-SY5Y (ATCC: CRL-2266), human breast cancer MCF-7 (ATCC: HTB-22), and human melanoma 451Lu (RRID: CVCL_6357) cell lines were used in this study. The related cells were cultured in T25 flasks using RPMI 1640 medium supplemented with 10% fetal bovine serum and 0.5% penicillin-streptomycin antibiotic solution. The cells were grown in an incubator at 37 °C with 5% CO_2 and subcultured every two or three days.

2.3. Sodium Selenate Treatment

Experiments were started when the cells reached sufficient density (70-80%). The SH-SY5Y, MCF-7, and 451Lu cells were trypsinized, harvested, and then counted using a Thoma hemocytometer. 1×10^4 cells per well in a 100 µL medium were seeded in a 96-well plate for WST-8 cell viability, total antioxidant status (TAS), and total oxidant status (TOS) assays. For

DNA fragmentation assay, 1×10^6 cells per well in a 2 mL medium were seeded in a 6-well plate. After 24 hours, the media of SH-SY5Y, MCF-7, and 451Lu cells were removed, and for WST-8 cell viability assay; a new RPMI 1640 medium including 10% fetal bovine serum, 0.5% penicillin-streptomycin antibiotic solution, and varying concentrations of sodium selenate (from 0 to 10 mg/mL), for other assays; a new RPMI 1640 medium including 10% fetal bovine serum, 0.5% penicillin-streptomycin antibiotic solution, and 10 mg/mL sodium selenate added to cells. The cells were then subjected to an additional 24 hours of incubation in an incubator at 37 °C with 5% CO_2.

2.4. WST-8 Cell Viability Assay

Cell Viability Detection Kit-8 (WST-8 / CVDK-8) was used to determine the cytotoxic effect of sodium selenate on the SH-SY5Y, MCF-7, and 451Lu tumor cell lines. For this, at the end of the 24-hour incubation period, 5 µl of water-soluble WST-8 tetrazolium salt was added to each well of the cells in 96-well plates and incubated for 3 h. The absorbance was measured at 450 nm using a microplate reader (Rel Assay Diagnostics, BK-EL10C, Mega Tıp, Gaziantep, Türkiye). The cell death was determined using the following formula:

$$\text{Cell death (\%control)} = [1- ((OD_{sample}- OD_{blank}) / (OD_{control}- OD_{blank}))] \times 100$$

A nonlinear regression graph was plotted between % cell death and Log10 concentration and the calculation of IC_{50} value was determined using GraphPad Prism software 5.01 (GraphPad, San Diego, CA). Each assay was repeated in triplicate and the results were given as mean ± SD of independent experiments.

2.5. Total Antioxidant Status (TAS) Assay

The effect of sodium selenate on the total antioxidant levels of SH-SY5Y, MCF-7, and 451Lu tumor cells was assessed using the Total Antioxidant Status (TAS) Assay Kit, following the manufacturer's instructions with some modifications. Briefly, the cell culture media belonging to control and experimental groups of SH-SY5Y, MCF-7, and 451Lu cell lines in each well in the 96 well plates were collected in Eppendorf tubes according to their respective groups at the end of the 24-hour incubation period. 12 µL of each of the samples from Eppendorf tubes, standards, and dH_2O were added to the respective well of 96 well plates. 200 µL reagent 1 was then added, mixed well, incubated at room temperature for 30 seconds in the dark, and measured spectrophotometrically at 660 nm absorbance. The initial obtained values of absorption were recorded as A1. Subsequently, 30 µL of reagent 2 was added, thoroughly mixed, and then left to incubate at room temperature for 10 minutes in the dark. The final step involved measuring the spectrophotometric readings at 660 nm. The second obtained absorbance values were recorded as A2. The results were calculated according to the following formula, and expressed as µmol Trolox Equiv./L.

TAS (µmol Trolox Equiv./L) = ([ΔAbs H_2O- ΔAbs Sample] / [ΔAbs H_2O- ΔAbs Standard]) x1000

ΔAbs of standard or sample or H_2O = A2– A1

2.6. Total Oxidant Status (TOS) Assay

The effect of sodium selenate on the total oxidant levels of SH-SY5Y, MCF-7, and 451Lu tumor cells was assessed using the Total Oxidant Status (TOS) Assay Kit, following the manufacturer's instructions with some modifications. Briefly, the cell culture media belonging to control and experimental groups of SH-SY5Y, MCF-7, and 451Lu cell lines in each well in the 96 well plates were collected in Eppendorf tubes according to their respective groups at the end of the 24-hour incubation period. 30 µL of each of the samples from Eppendorf tubes, and standards were added to the respective well of 96 well plates. 200 µL reagent 1 was then added, mixed well, incubated at room temperature for 30 seconds in the dark, and measured spectrophotometrically at 530 nm absorbance. The first obtained absorbance values were recorded as A1. 10 µL reagent 2 was then added, mixed well, incubated at room temperature

for 10 minutes in the dark, and measured spectrophotometrically at 530 nm absorbance. The second obtained absorbance values were recorded as A2. The results were calculated according to the following formula, and expressed as μmol H_2O_2 Equiv./L.

$$\text{TOS (μmol } H_2O_2 \text{ Equiv./L)} = (\Delta\text{Abs Sample} / \Delta\text{Abs Standard}) \times 10$$

ΔAbs of standard or sample= A2– A1

2.7. Oxidative Stress Index (OSI)

The effect of sodium selenate on the oxidative stress levels of SH-SY5Y, MCF-7, and 451Lu tumor cells was determined using the following formula;

$$\text{OSI (arbitrary unit)} = \text{TOS (μmol } H_2O_2 \text{ Equiv./L)/TAS (μmol Trolox Equiv./L)} \times 100$$

2.8. DNA Isolation

The SH-SY5Y, MCF-7, and 451Lu cells treated with or without sodium selenate were trypsinized, harvested, and washed with PBS. Genomic DNAs of SH-SY5Y, MCF-7, and 451Lu cells were obtained using the Genomic DNA Isolation Kit, following the manufacturer's instructions.

2.9. Analysis of DNA Fragmentation Using Agarose Gel Electrophoresis

25 μL DNA was loaded with 5 μL ClearBand 6x DNA Loading Dye on a 1.5% agarose gel (containing 5μl of ClearBand SAFE DNA Gel Stain Solution, 20,000x) and run in TBE buffer (90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA, pH 8.0) for 50 minutes at 80 V. After agarose gel electrophoresis, the gel was photographed under UV illumination. 100bp DNA ladder was used as marker.

2.10. Statistical Analysis

All measurements were repeated three times, and GraphPad Prism 5.01 software was applied for statistical analysis. Comparable datasets were assessed, and the analyses were conducted by using two-tailed Student's *t*-test. The data are represented by the mean ± S.D. from 3 independent experiments and are statistically significant at $p < 0.05$.

3. RESULTS

Sodium selenate increases cell death in SH-SY5Y human neuroblastoma, MCF-7 human breast cancer, and 451Lu human melanoma cells. The cytotoxic effects of sodium selenate on SH-SY5Y, MCF-7, and 451Lu cells were examined with the WST-8 assay. SH-SY5Y, MCF-7, and 451Lu cells were treated with sodium selenate at concentrations ranging between 0 (untreated, control) and 10 mg/mL for 24 h. The results showed that sodium selenate significantly inhibited the viability of SH-SY5Y, MCF-7, and 451Lu cells at all concentrations ($^{****}p < 0.0001$ vs 0 mg/mL), and the cytotoxic effect increased as the concentration increased (Figure 1). The cell death rates caused by sodium selenate at concentrations ranging from 0 to 10 mg/mL applied to SH-SY5Y cells were determined to be approximately 0, 66.39, 85.86, 91.03, 92.16, 92.76, 93.54, 94.53, and 95.37%, respectively (Figure 1A). The cell death rates resulting from the application of sodium selenate at concentrations ranging from 0 to 10 mg/mL to MCF-7 cells were approximately 0, 26.31, 26.69, 40.52, 57.26, 87.88, 97.53, 96.86, and 98.04%, respectively (Figure 1B). The cell death rates resulting from the application of sodium selenate at concentrations ranging from 0 to 10 mg/mL to 451Lu cells were as follows: 0, 81.95, 82.01, 87.52, 93.15, 96.30, 97.37, 98.08, and 99.21, respectively (Figure 1C).

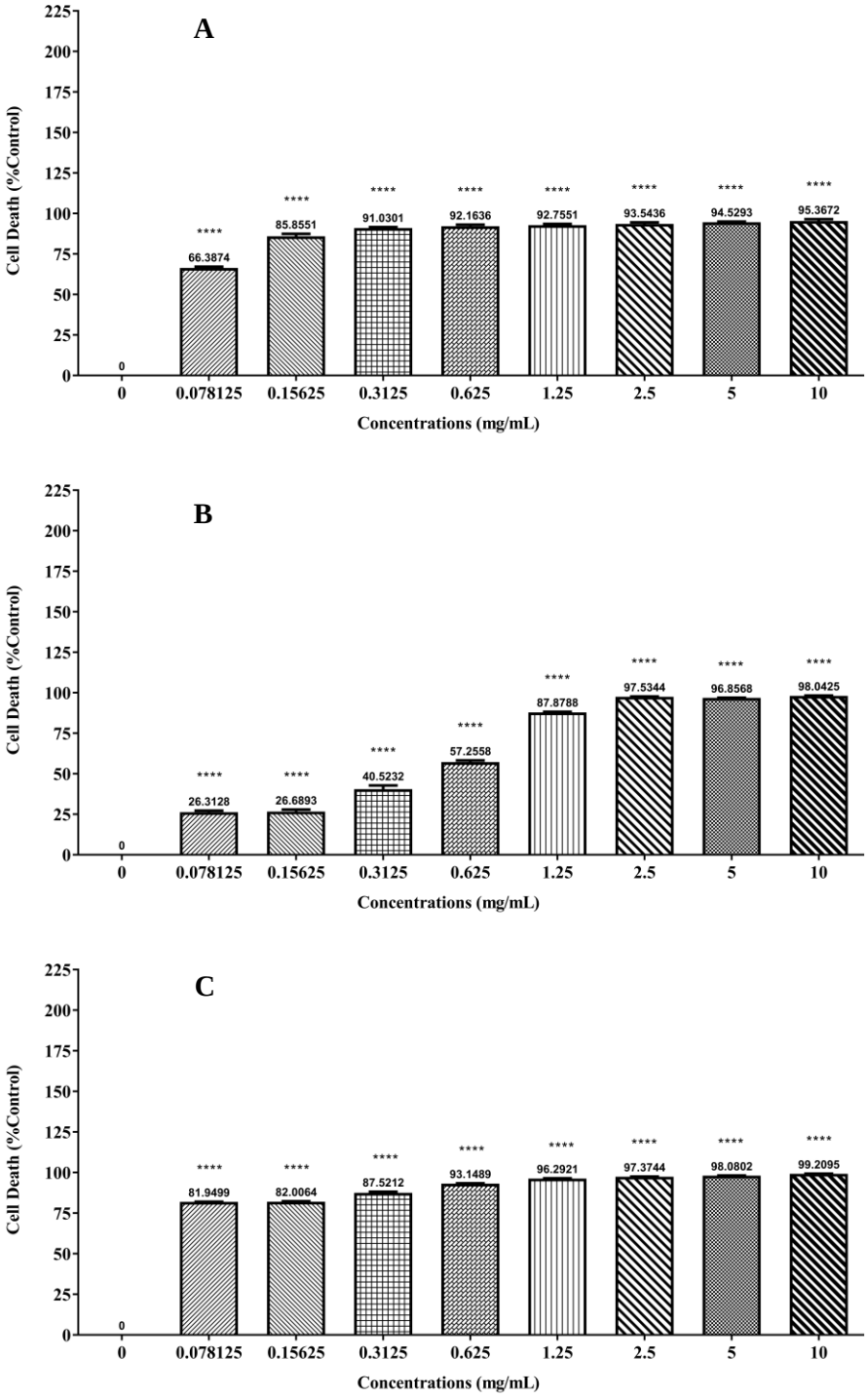

Figure 1. Cell death of SH-SY5Y human neuroblastoma **(A)**, MCF-7 human breast cancer **(B)**, and 451Lu human melanoma cells **(C)** (% of the control) after incubation with sodium selenate.

The IC50 values of sodium selenate in the SH-SY5Y, MCF-7, and 451Lu cells were found to be 0.0507 ± 0.004, 0.4554 ± 0.0152, and 0.0036 ± 0.0002 mg/mL, respectively. It was observed that sodium selenate significantly inhibited cell viability at all applied concentrations (0.078125-10 mg/mL), and also had a maximum cytotoxic effect at a concentration of 10 mg/mL on SH-SY5Y, MCF-7, and 451Lu cells. For this reason, the concentration value of 10 mg/mL, where sodium selenate showed maximum cytotoxic effect, was used in all remaining assays of the study.

Sodium selenate reduces total antioxidant levels in SH-SY5Y, MCF-7, and 451Lu cells. The effects of sodium selenate on total antioxidant levels of SH-SY5Y, MCF-7, and 451Lu cells were examined. The cells were treated with sodium selenate at concentrations of 0 mg/mL

(untreated, control) and 10 mg/mL for 24 hours. The total antioxidant levels in sodium selenate untreated (0 mg/mL) and treated (10 mg/mL) SH-SY5Y, MCF-7, and 451Lu cells were approximately 511.74 and 452.27 µmol Trolox Equiv./L, respectively, for SH-SY5Y; approximately 432.71 and 392.02 µmol Trolox Equiv./L, respectively, for MCF-7; approximately 437.40 and 351.33 µmol Trolox Equiv./L, respectively, for 451Lu (Figure 2). The results indicated that 10 mg/mL of sodium selenate, when compared with 0 mg/mL, significantly decreased the total antioxidant levels in SH-SY5Y, MCF-7, and 451Lu cells (*** p <0.001, $^{\#\#}$ p <0.01, and $^{++}$ p <0.01 vs 0 mg/mL) at the rates of 11.62, 9.40, and 19.67%, respectively.

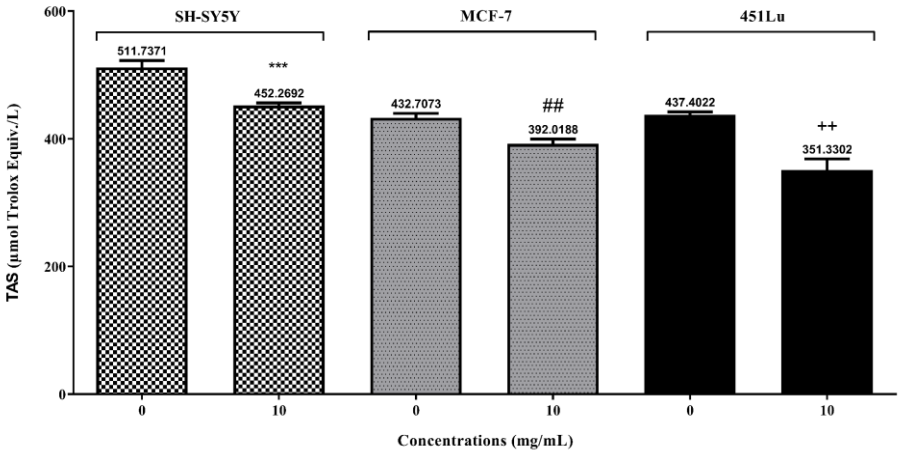

Figure 2. Total antioxidant status of SH-SY5Y human neuroblastoma, MCF-7 human breast cancer, and 451Lu human melanoma cells (% of the control) after incubation with sodium selenate.

Sodium selenate induces total oxidant levels in MCF-7, and 451Lu cells. The impact of sodium selenate on the total oxidant levels of SH-SY5Y, MCF-7, and 451Lu cells was investigated. The cells were treated with sodium selenate at concentrations of 0 mg/mL (untreated, control) and 10 mg/mL for 24 hours. The total oxidant levels in sodium selenate untreated (0 mg/mL) and treated (10 mg/mL) SH-SY5Y, MCF-7, and 451Lu cells were approximately 3.89 and 3.65 µmol H_2O_2 Equiv./L, respectively, for SH-SY5Y; approximately 2.54 and 3.63 µmol H_2O_2 Equiv./L, respectively, for MCF-7; approximately 2.54 and 4.00 µmol H_2O_2 Equiv./L, respectively, for 451Lu (Figure 3). The results indicated that 10 mg/mL of sodium selenate compared with 0 mg/mL statistically did not significantly change the total oxidant levels in the SH-SY5Y cell line, while increasing the total oxidant levels in MCF-7, and 451Lu cells (^{ns} p> 0.05, $^{\#\#\#}$ p <0,001, and $^{+++}$ p <0.001 vs 0 mg/mL) at the rates of 42.67, and 57.32%, respectively.

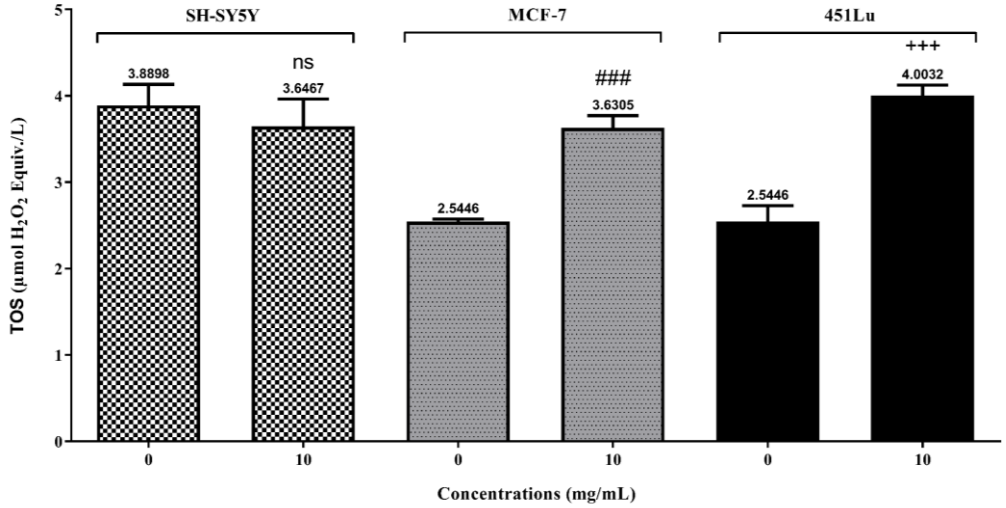

Figure 3. Total oxidant status of SH-SY5Y human neuroblastoma, MCF-7 human breast cancer, and 451Lu human melanoma cells (% of the control) after incubation with sodium selenate.

Sodium selenate induces oxidative stress in SH-SY5Y, MCF-7, and 451Lu cells. The effects of sodium selenate on oxidative stress of SH-SY5Y, MCF-7, and 451Lu cells were determined by the relative total oxidant levels to the total antioxidant levels of these cells, and the results were expressed as arbitrary units. Oxidative stress levels in the untreated control (0 mg/mL) groups of SH-SY5Y, MCF-7, and 451Lu cells were approximately 0.76, 0.59, and 0.58, respectively, while in the 10 mg/mL of sodium selenate-treated groups of these cells were in order of, approximately 0.81, 0.93, and 1.14 (Figure 4). The results indicated that 10 mg/mL of sodium selenate compared with 0 mg/mL statistically significantly increased the oxidative stress levels in the SH-SY5Y, MCF-7, and 451Lu cells (^{ns} p> 0.05, $^{###}$ p <0.001, and $^{+++}$ p <0.001 vs 0 mg/mL) at the rates of 6.65, 57.00, and 96.08 %, respectively.

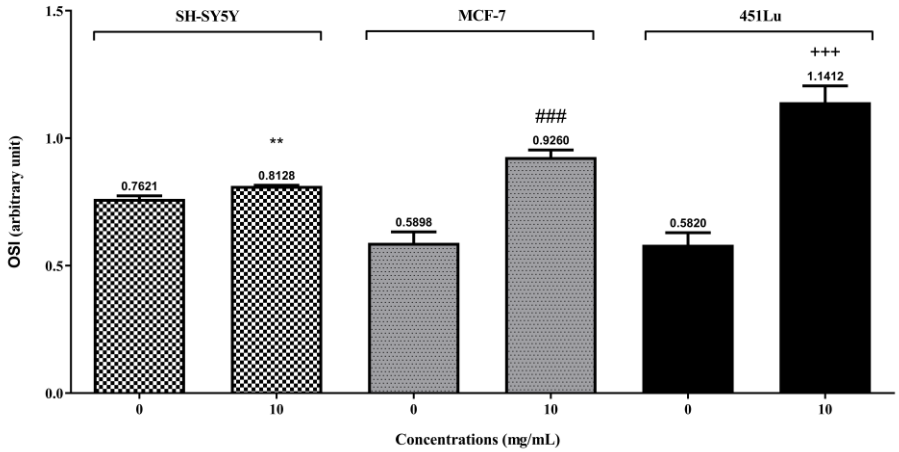

Figure 4. Oxidative stress index of SH-SY5Y human neuroblastoma, MCF-7 human breast cancer, and 451Lu human melanoma cells (% of the control) after incubation with sodium selenate.

Sodium selenate did not have a genotoxic effect on genomic DNAs obtained from SH-SY5Y, MCF-7, and 451Lu cells. The genotoxic effect of sodium selenate on the SH-SY5Y, MCF-7, and 451Lu cells was evaluated by subjecting genomic DNAs to agarose gel electrophoresis (Figure 5).

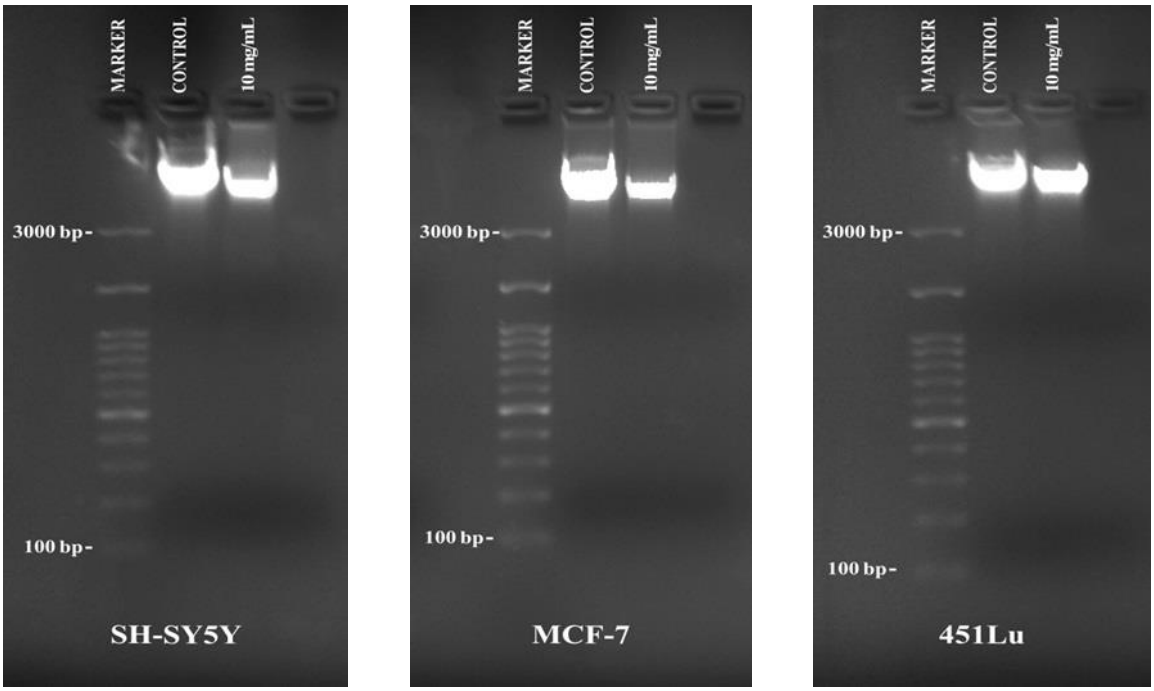

Figure 5. Agarose gel electrophoresis of genomic DNA from untreated (control) and 10 mg/mL sodium selenate treated SH-SY5Y human neuroblastoma, MCF-7 human breast cancer, and 451Lu human melanoma cells.

No degradation, such as DNA laddering or smearing, was observed in the integrity of DNA extracted from SH-SY5Y, MCF-7, and 451Lu cells treated with 10 mg/mL sodium selenate. In the control and sodium selenate treated samples, no migration of genomic DNAs was observed. After being subjected to electrophoresis, the DNA was almost still found to be localized at the starting point. This indicated well-preserved genomic integrity of the DNA isolated from SH-SY5Y, MCF-7, and 451Lu cells that were not treated with sodium selenate.

4. DISCUSSION and CONCLUSION

The effects of selenium on human neuroblastoma, breast cancer, and melanoma cells have not been clearly elucidated. Given the anti-tumor effects of selenium, the study focused on the effects of sodium selenate, an inorganic selenium compound, on SH-SY5Y human neuroblastoma, MCF-7 human breast cancer, and 451Lu human melanoma cells. Recent studies have reported that selenium compounds exhibit dose-dependent cytotoxic (da Costa *et al.*, 2023; Yang *et al.*, 2018), antioxidant, or pro-oxidant effects (Misra *et al.*, 2015) in many cancer cells, triggering intracellular ROS and oxidative stress (Misra *et al.*, 2015; Zeng *et al.*, 2012), and demonstrating strong anticancer effects through both apoptotic (Rikiishi, 2007; Yang *et al.*, 2018) and non-apoptotic mechanisms (Sanmartín *et al.*, 2012). It was hypothesized that exposure to sodium selenate would lead to a cytotoxic effect, induce oxidative stress, and apoptosis or non-apoptotic events in SH-SY5Y, MCF-7, and 451Lu cells. Therefore, the study analyzed the effects of sodium selenate exposure on cell viability. The results showed that sodium selenate effectively reduced the viability of SH-SY5Y, MCF-7, and 451Lu cells. In the study carried out by da Costa *et al.* (2023), the authors, consistent with our findings, observed that sodium selenate reduced cell viability in breast cancer BT-549 and MDA-MB-231 cell lines. In addition, Tsukamoto *et al.* (2013) reported in their paper that sodium selenate induced cytotoxicity in the human colon adenocarcinoma DLD-1 cell line, and it showed an anti-cancer effect. Oxidative stress can be defined as a condition that occurs as a result of the disruption of the balance between intracellular oxidant and antioxidant molecules (Preiser, 2012). Selenium and its inorganic (e.g., sodium selenate) and organic compounds can act as a “double-edged sword”; depending on the dosage, they may exhibit antioxidant or pro-oxidant properties (Radomska *et al.*, 2021). According to the results of our study, sodium selenate was observed to have pro-oxidant properties on SH-SY5Y, MCF-7 and 451Lu cell lines when applied at a concentration of 10 mg/mL depending on the dose. This concentration significantly reduced intracellular total antioxidant activity in all cells, while it increased intracellular total oxidant levels in MCF-7 and 451Lu cells except for the SH-SY5Y cell line, and significantly increased oxidative stress in these cells. Selenium compounds might induce cell death through pathways other than apoptosis, involving both internal and external mechanisms (Radomska *et al.*, 2021). Non-apoptotic events, such as cell cycle arrest (Chen *et al.*, 2019; Sanmartín *et al.*, 2012; Sinha & El-Bayoumy, 2004), necrosis (Sinha & El-Bayoumy, 2004), autophagy (Sanmartín *et al.*, 2012), ferroptosis (Subburayan *et al.*, 2020), necroptosis (Misra *et al.*, 2015), entosis (Khalkar *et al.*, 2018), anoikis (Gandin *et al.*, 2018; Jiang *et al.*, 2001), NETosis (Zhang *et al.*, 2020), or mitotic catastrophe (Sanmartín *et al.*, 2012), may also occur. Among the types of cell death caused by selenium compounds, ferroptosis appears to be a particularly intriguing process (Mou *et al.*, 2019). In ferroptosis, oxidative stress increases significantly due to the increase in intracellular oxidant molecules and the decrease in antioxidant defenses. This process leads to the accumulation of lipid peroxides in the cell membrane. As a result, a non-apoptotic cell death occurs, and DNA fragmentation is not typically observed in this cell death. This cell death mechanism, unlike the classical apoptotic pathways, is associated with a particularly iron-dependent process and is a type of programmed cell death in which oxidative damage is intensified (Mou *et al.*, 2019; Ursini & Maiorino, 2020). Choi *et al.* (2015) showed that sodium selenate (Na2SeO4) induced cell growth inhibition via G2/M phase cell cycle arrest in MDR oral squamous carcinoma cells KBV20C. In our agarose gel electrophoresis results, none of the sodium selenate-treated SH-SY5Y, MCF-7, and 451Lu cells was not showed DNA band-type

phenotypes of apoptotic (DNA ladder) or necrotic (DNA smear). To sum up, used concentrations (10 mg/mL) of sodium selenate in this study caused a decrease in antioxidant activity, an increase in oxidative stress, high cytotoxic activity, and non-apoptotic and non-necrotic DNA band phenotypes on SH-SY5Y, MCF-7, and 451Lu cells. Therefore, all these findings suggest that sodium selenate exhibits anticancer activity in these cells through non-apoptotic ferroptosis cell death, depending on the dose. In conclusion, further molecular studies are needed to better elucidate the way in which sodium selenate exhibits anticancer activity on SH-SY5Y, MCF-7, and 451Lu cancer cells.

Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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