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Phytochemical analyses of *Ebenus haussknechtii* flowers: Quantification of phenolics, antioxidants effect, and molecular docking studies

Ramazan Erenler [1,2]*, İlyas Yıldız [1], Esma Nur Geçer [2], Aslı Yıldırım Kocaman [1], Mehmet Hakkı Alma [1], İbrahim Demirtas [1], Yunus Başar [1], İbrahim Hosaflıoğlu [1], Lütfi Behçet [3]

[1]Research Laboratory Practice and Research Center, Igdir University, 7600, Igdir, Türkiye

[2]Dept. of Chemistry, Faculty of Arts and Sciences, Tokat Gaziosmanpaşa University, 60240 Tokat, Türkiye

[3]Dept. of Molecular Biology and Genetics, Faculty of Arts and Sciences, Bingol University, Bingol, Türkiye

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ABSTRACT

Plants have been benefited as medicine and food since ancient times. After the discovery of spectroscopy, bioactive compounds in plants have been elucidated and have been utilized in drug development. *Ebenus haussknechtii* has been utilized for traditional medicine. In this study, *Ebenus haussknechtii* flowers were extracted in methanol and quantification of phenolics of this extract was conducted by LC-MS/MS. Antioxidant effect of *E. haussknechtii* flowers was carried out using DPPH free radical scavenging assay, ABTS radical cation scavenging assay, and hydroxyl radical scavenging assay. Quantitative analysis revealed that shikimic acid (0.77 mg/g extract), protocatechuic acid (0.61), catechin (0.34), hydroxybenzaldeyde (0.32) were determined as major products. Hence, the interaction of shikimic acid and DNA gyrase enzyme was calculated theoretically. Moreover, MolDock score, and binding affinity were determined as -73.64 and -5.5 kcal/mol respectively. *Ebenus haussknechtii* flowers displayed good antioxidant activity. In DPPH assay, the extract displayed good activity with the value of 7.27 ± 0.173 (IC_{50}, µg/mL). Moreover, the flower extract exhibited the outstanding ABTS activity with a value of 6.62 ± 0.23 (IC_{50}, µg/mL) in comparison to the extract BHA (7.58 ± 0.15, IC_{50}, µg/mL).

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Ebenus haussknechtii çiçeklerinin fitokimyasal analizleri: Fenoliklerin miktarının belirlenmesi, antioksidan etkisi ve moleküler yerleştirme çalışmaları

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Antioksidan aktivite

ÖZET

Bitkilerden eski çağlardan beri ilaç ve gıda olarak yararlanılmaktadır. Spektroskopinin keşfinden sonra bitkilerde bulunan biyoaktif bileşikler aydınlatılmış ve ilaç geliştirmede kullanılmaya başlanmıştır. *Ebenus haussknechtii* geleneksel tıpta kullanılmaktadır. Bu çalışmada, *Ebenus haussknechtii* çiçekleri metanol içerisinde ekstrakte edilmiş ve bu ekstraktın fenoliklerinin miktar tayini LC-MS/MS ile yapılmıştır. *E. haussknechtii* çiçeklerinin antioksidan etkisi, DPPH serbest radikal giderme testi, ABTS radikal katyon giderme testi ve hidroksil radikal giderme testi kullanılarak gerçekleştirildi. Kantitatif analizde şikimik asit (0,77 mg/g ekstrakt), protokatekuik asit (0,61), kateşin (0,34), hidroksibenzaldeidin (0,32) ana ürünler olduğu belirlendi. Böylece şikimik asit ile DNA giraz enziminin etkileşimi teorik olarak hesaplandı. Ayrıca MolDock skoru ve bağlanma afinitesi sırasıyla -73,64 ve -5,5 kcal/mol olarak belirlendi. *E. haussknechtii* çiçekleri iyi antioksidan aktivite sergiledi. DPPH testinde ekstrakt 7.27 ± 0.173 (IC_{50}, µg/mL) değeriyle iyi aktivite sergiledi. Ayrıca çiçek ekstraktı, BHA (7.58 ± 0.15, IC_{50}, µg/mL) ile karşılaştırıldığında 6.62 ± 0.23 (IC_{50}, µg/mL) değeriyle olağanüstü ABTS aktivitesi sergiledi.

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*Corresponding author; e-mail: rerenler@gmail.com

1. INTRODUCTION

The use of plants for medicine and food purposes goes back as far as human history, but with the improvement of spectroscopy in the 19th century, plants have become a subject of science (Cragg et al., 1997; Sahin Yaglioglu et al., 2013; Topçu et al., 1999). After this development, many bioactive compounds have been isolated from plants and elucidated, and their biological activities have been investigated (Aksit et al., 2014; Aydin et al., 2016; Elmastas et al., 2016). This situation attracted the attention of synthetic chemists, and they succeeded in synthesizing many natural compounds and modified natural compounds (Cakmak et al., 2006; Erenler et al., 2005, 2007; Erenler et al., 2004). They also increased the effectiveness of natural compounds by functionalizing them (Lu et al., 2014; Ökten et al., 2013). Thus, the rapidly developing pharmaceutical industry had the opportunity to renew itself and develop new drugs to combat various diseases.

The plants include primary and secondary metabolites (Erenler, Atalar, et al., 2023; Y. B. Karan et al., 2024; Cennet Yaman et al., 2024). Primary metabolites are essential compounds produced by plants for basic life functions. These metabolites are involved in processes such as growth, development, and energy making. Secondary metabolites, on the other hand, are not directly involved in basic metabolic processes but often play crucial roles in ecological interactions, defense mechanisms, and signaling. They often have pharmaceutical, agricultural, or industrial significance (Guemidi et al., 2024; Khodja et al., 2023).

Free radicals are highly reactive molecules (T. Karan et al., 2024). This electron configuration makes them unstable and highly reactive, as they seek to gain stability by donating or accepting electrons from other molecules, causing a chain reaction of oxidative damage (Gecer et al., 2023). Free radicals can be generated within the body as part of normal metabolic processes, such as during cellular respiration or immune response (Erenler, Karan, & Bozer, 2023). They can also be produced in response to external factors such as exposure to ultraviolet radiation, pollution, cigarette smoke, and certain chemicals (Erenler & Hosaflioglu, 2023). Antioxidants, which can neutralize free radicals by donating electrons without becoming reactive themselves, play a crucial role in mitigating oxidative stress and preventing cellular damage (Erenler, Chaoui, et al., 2023; Erenler, Gecer, et al., 2023). A diet rich in antioxidant-containing foods, such as fruits, vegetables, nuts, and seeds, can help combat the harmful effects of free radicals and promote overall health and well-being (Atalar et al., 2023; Dag et al., 2023). Antioxidants are molecules that neutralize harmful free radicals (Karan et al., 2022; Sahin Yaglioglu et al., 2022; C Yaman et al., 2022).

Ebenus haussknechtii has been utilized for traditional medicine. *Ebenus* species were reported to display significant biological activities including antifungal, antibacterial, antioxidant, and anticonvulsant. In Turkey, *Ebenus* species were distributed around the Mediterranean and Anatolia regions (Hayta et al., 2014).

Herein, methanol extract of *Ebenus haussknechtii* flowers was prepared, and a quantitative analysis of phenolic compounds was carried out. Moreover, the antioxidant activity of this extract was conducted. Shikimic acid was determined as a major product, so a molecular docking study was executed on this compound.

2. MATERIAL and METHODS

Plant material

Ebenus haussknechtii was collected from Bingol in July 2022 and was identified by Dr. Lutfi Behcet, Bingol University, a voucher specimen was deposited in the herbarium at the same university (No: 20798).

Extraction

Ebenus haussknechtii leaves (5.0 g) were macerated with methanol (120 mL) for 24 hours at room temperature. After filtration, the solvent was evaporated by reduced pressure to yield the crude extract (0.5 g) (Houari et al., 2022).

LC-ESI-MS/MS analysis

The leaves of Ebenus haussknechtii were subjected to LC-MS/MS analysis to quantify bioactive compounds (Agilent Technologies 1260 Infinity II). *Ebenus haussknechtii* leaf extract (50 mg) in Eppendorf was mixed with methanol (1.0 mL). The hexane was added and centrifuged at 10000 rpm for 15 minutes. The methanol phase (100 µL) was diluted by adding water (450 µL) and methanol (450 µL). After filtration (0.22 µm filter), the solution was injected into the device. Formic acid (0.1%) and ammonium formate (5.0 mM) in water A, formic acid (0.1%) and ammonium formate (5.0 mM) in methanol B were used as the mobile phase. The gradient program was set as 20% for 1-5 min, 55% for 6-15 min, 85% for 16-25 min and 5% for 25-30 min for mobile phase B. The injection volume was 5.12 µL and the flow rate was 0.40 mL/min. The capillary voltage was 4000 V and the column temperature was 40°C. 39 standard compounds were used for the analysis (Figure 1) (Erenler, Karan, & Hosaflioglu, 2023).

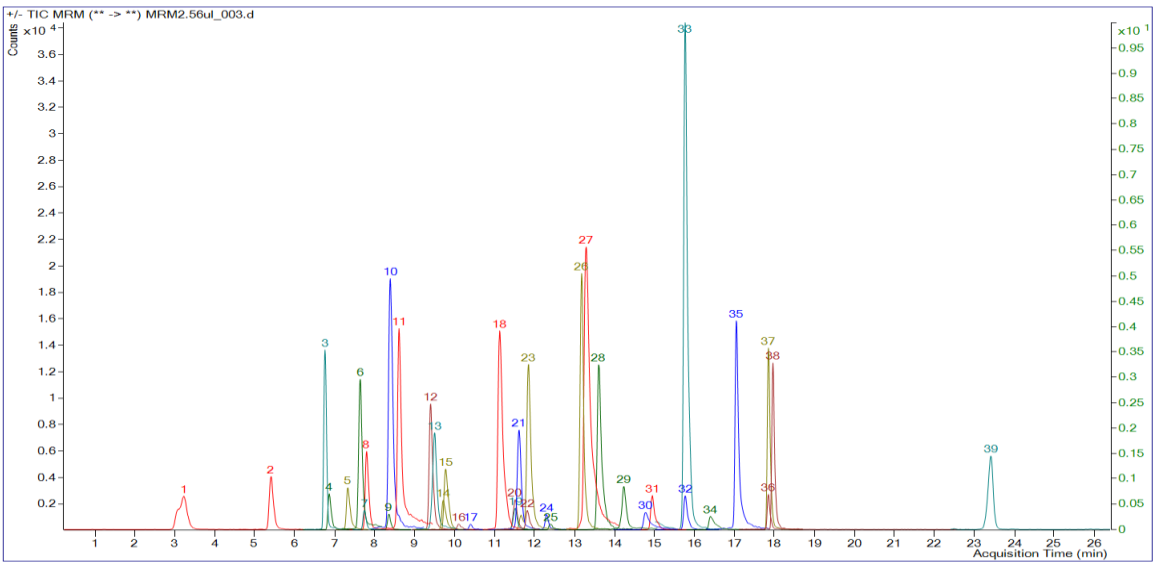

Figure 1. The MRM chromatogram of 39 standard compounds by using LC-MS/MS (1-Gallic acid, 2-Protocatechuic acid, 3-Epigallocatechin, 4-Catechin, 5-Chlorogenic acid, 6-*4*-Hydroxybenzaldehyde, 7-Vanillic acid, 8-Caffeic acid, 9-Syringic acid, 10-Caffeine, 11-Vanillin, 12-*p*-Coumaric acid, 13-Salicylic acid, 14-Taxifolin, 15-Resveratrol, 16-*trans*-ferulic acid, 17-Sinapic acid, 18-Scutellarin, 19-*o*-Coumaric acid, 20-Coumarin, 21- Protocatechuic ethyl ester, 22- Rutin, 23- Isoquercitrin, 24- Hesperidin, 25- Quercetin-*3*-*D*-xyloside, 26- Kaempferol-*3*-glucoside, 27- Fisetin, 28- Baicalin, 29- *trans*-Cinnamic acid, 30- Quercetin, 31- Naringenin, 32- Hesperetin 33- Morin, 34- Kaempferol, 35- Baicalein, 36- Luteolin, 37- Biochanin A, 38- Chrysin, 39-Diosgenin).

DPPH free radical scavenging assay

The samples at different concentrations (4-60 µg/mL) from stock solution were treated with DPPH$^{\bullet}$ solution in ethanol (1.0 mL, 0.26 mM) and then vortexed. The mixture was incubated at rt for 30 min. An absorbance measurement was performed with a spectrophotometer (517 nm). The results were calculated as IC_{50} (Gecer & Erenler, 2022).

$ABTS^{\bullet+}$ radical cation scavenging assay

The stock solution of samples (0.25 mg/mL) were prepared, and phosphate buffer was made then the reaction was carried out in this buffer solution. The different concentration of samples (4-60 µg/mL) was reacted with $ABTS^{\bullet+}$ solution. The measurement was executed by a spectrophotometer (734 nm). The results were calculated as IC_{50} (Gecer, Erenler, et al., 2022).

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was carried out using the *E. haussknechtii* flowers. The hydrogen peroxide (1.0 mL, 40 mM) was mixed with the phosphate buffer (40 mM, 2.4 mL, pH 7.4), and then the sample solution (100 µL) was added to the buffer solution and incubated for 10 minutes. Absorbance measurement was carried out (230 nm) (Erenler, Yaman, et al., 2023).

Molecular docking application

The drawing, 3D structures, and minimum energy of the shikimic acid were calculated in the ChemDraw software. The enzymes chosen for this docking study were DNA gyrase [PDB ID: 1KZN] in the Protein Data Bank. Molecule-enzyme interactions were observed with the Molegro Virtual Docker (MVD) program. Images of interactions (2D, 3D) were captured with BIOVIA Discovery Studio Visualizer. Additionally, binding affinity was calculated with the AutoDock Vina program (Başar et al., 2024).

Statistical analysis

The statistical analysis was conducted by GraphPad Prism (version 8.00). One-way ANOVA followed by Tukay multiple comparison test was carried out. The results were expressed as mean values ± standard deviation ($P < 0.05$).

3. RESULTS and DISCUSSIONS

Ebenus haussknechtii flowers were extracted in methanol, after the removal of methanol the crude extract was yielded. The quantitative analysis of bioactive compounds in methanol extract was carried out by LC-MS/MS. Shikimic acid (0.77 mg/g extract), Protocatechuic acid (0.61 mg/g extract), catechin (0.34 mg/g extract), Hydroxybenzaldeyde (0.32 mg/ g extract) were determined as major products (Table 1).

Table 1. Quantitative analysis of natural compounds in *Ebenus haussknechtii* flowers by LC-MS/MS (mg/g extract)

No	Compound	RT	Amount
1	Shikimic acid	1.2654	0.773
2	Gallic acid	3.4401	0.232
3	Protocatechuic acid	5.4826	0.612
4	Catechin	6.9819	0.343
5	Chlorogenic acid	7.3728	0.143
6	Hydroxybenzaldeyde	7.7280	0.321
7	Vanillic acid	7.8442	0.227
8	Syringic acid	8.2417	0.112
9	Vanillin	8.4197	0.32
10	o-coumaric acid	9.2680	0.45
11	Hesperidin	11.8041	0.332
12	Isoquercitrin	11.9065	0.213
13	Fisetin	13.2761	0.003
14	Luteolin	17.9513	0.144

Abbreviation: nd: not detected, RT: retention time.

Ebenus haussknechtii includes naturally occurring compounds that are significant for pharmaceuticals and food. Shikimic acid is existed commonly in plants due to the intermediate of the shikimic acid pathway. Shikimic acid serves as a crucial starting material in the synthesis of oseltamivir, which is a widely used antiviral medication for treating and preventing influenza infections. Shikimic acid is obtained by certain plants, chemical synthesis, and microbial fermentation. (Ghosh et al., 2012). Protocatechuic acid is a kind of phenolic acid. The presence of protocatechuic acid in pigmented onion scales helps to inhibit the growth of *Colletotrichum circinans*, thereby protecting the onion plants from the detrimental effects of the fungal disease. This natural defense mechanism highlights the importance of phytochemicals in plant health and disease resistance, and it underscores the potential applications of such compounds in agriculture for disease management and crop protection (Kakkar et al., 2014).

Ebenus haussknechtii flowers were found to have a considerable antioxidant effect. In DPPH free radical scavenging activity, this extract displayed the same activity (7.27 ± 0.173, IC_{50}, µg/mL) with the standard BHA (7.10 ± 0.55, IC50, µg/mL). In ABTS cation radical scavenging effect, the same trend was observed. The flower extract revealed excellent activity with a value of 6.62 ± 0.23 (IC50, µg/mL) in comparison to the extract BHA (7.58 ± 0.15, IC_{50}, µg/mL). The flower extract displayed a lower hydroxyl radical scavenging activity (11.53 ± 0.35, IC_{50}, µg/mL) than that of the standard BHA (8.58 ± 0.15, IC_{50}, µg/mL) (Figure 2).

There is an accord between the present study and the reported work. The antioxidant activity of *Althaea officinalis* flowers was investigated and these flowers displayed a good antioxidant effect. (Elmastas et al., 2004). The flavonoids were isolated from *Allium vineale* and they revealed great antioxidant activity (Demirtas et al., 2013). Another study was conducted on *Echinops orientalis* and the compounds isolated from this plant demonstrated a high antioxidant effect (Erenler et al., 2014). The mint genotypes were reported to show considerable antioxidant effects. (Elmastaş et al., 2015). The bioactive compounds were isolated and identified from *Echinacea purpurea* and *Echinacea pallida* and these compounds displayed excellent antioxidant activity (Erenler et al., 2015).

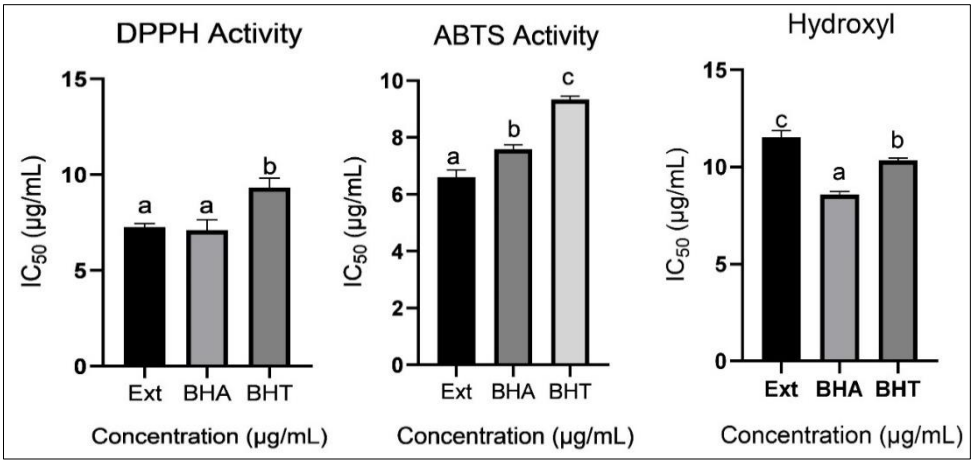

Figure 2. Antioxidant activity of *Ebenus haussknechtii* flowers. Means (three replicates) followed by different letters (a, b, and c) express a statistical difference ($P < 0.05$).

Due to the major compound of shikimic acid of *Ebenus haussknechtii* flowers, the interaction of shikimic acid and DNA gyrase enzyme was calculated theoretically.

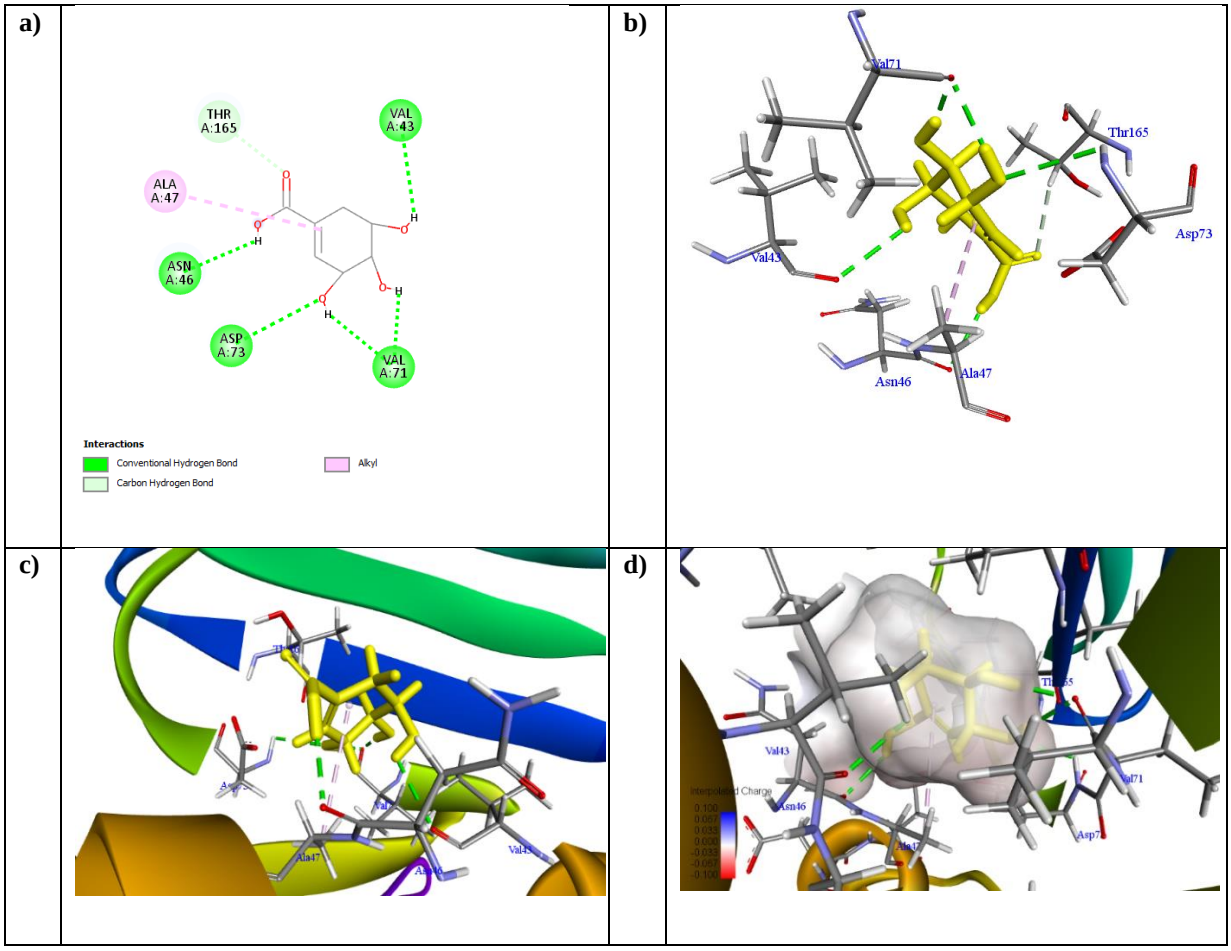

Figure 3. Shikimic acid interaction with DNA gyrase, a) 2D images b) general view c) 3D images d) interpolated load view

Shikimic acid interacted with DNA gyrase by five conventional hydrogen bonds with amino acids ASP73, ASN46, VAL71, and VAL43, one carbon-hydrogen bond with amino acid THR165, and one alkyl with amino acid ALA147 (Figure 3, Table 2). Shikimic acid with DNA gyrase was calculated as a MolDock score of -73.64, with binding energies of -5.5 kcal/mol.

Table 2. Interaction categories, types, and distances of molecular insertion of the shikimic acid molecule with DNA gyrase

No	Name	Distance	Category	Type	Transmitter	From Chemistry	Receiver	To Chemistry
1	A:ASP73:HN - :[001:O4	3.0128	Hydrogen Bond	Conventional Hydrogen Bond	A: ASP73:HN	H-Donor	:[001:O4	H-Acceptor
2	:[001:H7 - A:ASN46:O	2.28384	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H7	H-Donor	A: ASN46:O	H-Acceptor
3	:[001:H8 - A:VAL71:O	1.77862	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H8	H-Donor	A: VAL71:O	H-Acceptor
4	:[001:H9 - A:VAL71:O	2.14128	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H9	H-Donor	A: VAL71:O	H-Acceptor
5	:[001:H10 - A:VAL43:O	2.66703	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H10	H-Donor	A: VAL43:O	H-Acceptor
6	A:THR165:HB - :[001:O2	2.92677	Hydrogen Bond	Carbon Hydrogen Bond	A: THR165:HB	H-Donor	:[001:O2	H-Acceptor
7	A:ALA47 - :[001	4.69407	Hydrophobic	Alkyl	A:ALA47	Alkyl	:[001	Alkyl

4. CONCLUSION

Quantitative analysis of phenolic compounds of *Ebenus haussknechtii* flowers was conducted and shikimic acid was found as a major product. *Ebenus haussknechtii* displayed outstanding antioxidant activity, so, this plant has the potential to be used in food and pharmaceuticals. The theoretical study presented that shikimic acid which is the major product has an inhibition effect on DNA gyrase.

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Phytochemical Content and *In Silico* Molecular Docking Studies of *Achillea biebersteinii* and *A. millefolium* Plants

Fatih Gül [1]*, Yunus Başar [1], İbrahim Demirtas [1,2]

[1] Research Laboratories Application and Research Center (ALUM) Iğdır University, 76000, Iğdır, Türkiye
[2] Department of Pharmaceutical Chemistry, Faculty of Pharmacy Ondokuz Mayıs University, 55139, Samsun, Türkiye

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ABSTRACT

The genus *Achillea* L. belongs to *Asteraceae*, the largest family of vascular plants. *Achillea* species is a medicinal plant widely used in traditional medicine due to the bioactive compounds it contains that are widely distributed worldwide. In this study, we aimed to examine the biological activity potential of the plants by analyzing the phytochemical content of two *Achillea* species growing on the land of Iğdır University campus. In this study, the volatile and phenolic contents of flower, and stem-leaf parts of *A. biebersteinii* and *A. millefolium* harvested in different periods were determined by chromatographic methods (GC-MS/MS and LC-MS/MS). As a result of the GC-MS analysis, it was observed that the presence of high levels of eucalyptol (43.22%) in the *A. millefolium* plant and that the harvest time dramatically changed the rates of volatile components in the *A. biebersteinii* plants. The LC-MS/MS analysis showed that the main constituent in all plant materials was chlorogenic acid. In addition, the main component, chlorogenic acid interactions with the xanthine oxidase enzyme were determined by *in silico* molecular docking. Chlorogenic acid interaction with xanthine oxidase was calculated of binding energies (-8.0 kcal/mol) and MolDock score (-130.96).

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Achillea biebersteinii ve *A. millefolium* Bitkilerinin Fitokimyasal İçeriği ve *In Silico* Moleküler Doking Çalışmaları

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Ksantin oksidaz

ÖZET

Achillea L. cinsi, damarlı bitkilerin en büyük familyası olan *Asteraceae* familyasına aittir. *Achillea* türleri, dünya çapında yaygın olarak bulunan, içerdiği biyoaktif bileşikler nedeniyle geleneksel tıpta yaygın olarak kullanılan tıbbi bir bitkidir. Bu çalışmada Iğdır Üniversitesi kampüsü arazisinde yetişen iki *Achillea* türünün fitokimyasal içeriğini analiz ederek bitkilerin biyolojik aktivite potansiyelini incelemeyi amaçladık. Bu çalışmada, farklı dönemlerde hasat edilen *A. biebersteinii* ve *A. millefolium*'un çiçek, gövde-yaprak kısımlarının uçucu ve fenolik içerikleri kromatografik yöntemlerle (GC-MS/MS ve LC-MS/MS) belirlendi. GC-MS analizi sonucunda *A. millefolium* bitkisinde yüksek düzeyde eucalyptol (%43.22) varlığının olduğu ve hasat zamanının *A. biebersteinii* bitkisinde uçucu bileşenlerin oranlarını önemli ölçüde değiştiği görüldü. LC-MS/MS analizinde, tüm bitki materyallerindeki ana bileşenin klorojenik asit olduğunu gösterdi. Ayrıca ana bileşen, klorojenik asit ile ksantin oksidaz enzimi etkileşimleri, *in silico* moleküler yerleştirme yöntemi ile belirlendi. Ksantin oksidaz ile klorojenik asit etkileşiminin, bağlanma enerjisi (-8,0 kcal/mol) ve MolDock skoru (-130,96) hesaplandı.

2024

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*Corresponding author; e-mail: fatihgul3018@gmail.com

1. INTRODUCTION

Plants have developed an effective defense system against their enemies and pests. This defense is based on various phytochemical compounds (Wallander & Albert, 2001). Plants have been used for centuries as medicine in the treatment of human diseases, and the compounds extracted from these plants are known as secondary metabolites (Demirci et al., 2018).

The *Asteraceae* family is found almost everywhere in the world. It is widespread in America and Mexico, in the Brazilian Andes, in the Mediterranean region, on the Asian continent, and in South Africa and Australia (Bremer, 1993). The *Asteraceae* family consists of 136 genera and 1195 species in the flora of Türkiye and there are a total of 446 endemic species (Arabaci, 2012). The genus *Achillea L.* belongs to the Anthemideae of the *Asteracea*e family. Although the genus *Achillea* occurs in almost all regions of our country, it is particularly widespread in the region of northern and eastern Anatolia. The endemic species are widespread in the Eastern Anatolia region (Güneş & Ozhatay, 2000). *Achillea* is commonly known to the public as "yarrow". In Turkey, the genus *Achillea* consists of a total of 46 species belonging to 6 sections. (Arabaci, 2012). *Achillea* species growing in Turkey; wound healing, anti-inflammatory, antipyretic, antidepressant, antihypertensive, astringent, antiseptic, antimicrobial, diuretic, in the treatment of respiratory diseases (in pregnant women), antispermatogenic and antidiabetic, antitumor and hepatoprotective, as well as analgesic, muscle relaxant, digestive, kidney-strengthening and It is reported to be used in the treatment of liver diseases, as an appetite stimulant, carminative, menstrual regulator, for the treatment of hemorrhoids and protection against pests (Candan et al., 2003; Karamenderes et al., 2002; Popovic et al., 2002; Saeidnia et al., 2011).

Among the *Achillea* species, *A. biebersteinii* and *A. millefolium* species grow in the Ağrı Mountain flora of Iğdır province in Turkey (Zeynalov, Y., & Türkoğlu, M., 2016). The main volatile components of *A. biebersteine* volatiles have been reported to be 1,8-cineole, camphor, piperitone, p-cymene, β-edesmol, and ascaridol (Bariş et al., 2006). Moreover in *A. millefolium*, it has been reported that the main volatile components are eucalyptol, camphor, α-terpineol, β-pinene, and borneol (Candan et al., 2003).

Xanthine oxidase is a key enzyme in purine catabolism, in which xanthine and hypoxanthine are converted to uric acid (Page et al., 1998). It is known to reduce oxygen, forming reactive oxygen sources such as hydrogen peroxide and superoxide. It also plays a role in the formation of peroxynitrite, which has a strong antimicrobial effect by reducing nitrite to nitrite oxide (Martin et al., 2004). Xanthine oxidase can catalyze the reduction of molecular oxygen to superoxide anion and hydrogen peroxide. This reagent (superoxide anion, hydrogen peroxide) is present in various oxygen sources. It has been reported to cause ischemia-reperfusion injury in tissues (Granger et al., 1986). It has also been reported that hyperactivity of this enzyme and deficiency of detoxification mechanisms are associated with diseases such as oxidative stress, inflammation, diabetes, and cardiovascular diseases (Abd-ElGawad et al., 2024).

In this study, the phenolic content (LC-MS/MS) and the volatile oil content (GC-MS/MS) of the flower stem/leaf parts of the species *A. biebersteinii* and *A. millefolium* were determined. In addition, the interactions of the molecule, which was identified as the main component in the LC-MS/MS analysis of these species and which is said to have an anti-inflammatory effect, with xanthine oxidase were theoretically calculated by molecular

docking. It is anticipated that investigating the phytochemical content and inhibitory properties of *A. biebersteinii* and *A. millefolium* will provide insight into areas such as pharmaceuticals and cosmetics.

2. MATERIAL and METHODS

Plants

A. biebersteinii (15 may, 15 June 2023) and *A. millefolium* (15 June 2023) harvested at the Iğdır University Şehit Bülent Yurtseven campus (Figure 1). Species identification was made by Prof. Dr. Ahmet Zafer Tel at the Faculty of Agriculture, Department of Agricultural Biotechnology. The collected plants were dried in a cool place without sunlight.

a) **b)**

Figure 1. Natural appearance of *Achillea*, a) *A. millefolium*, b) *A. biebersteinii* (Dr. Yunus BAŞAR)

Extraction

Dried 3 different samples of *A. biebersteinii* May (AB1), *A. biebersteinii* June (AB2) and *A. millefolium* flowers and stem leaves were ground to powder. 100 grams of each samples were placed in separate 1 liter bottles and ethyl acetate was added. The extraction process was continued for 1 week. Then the solvent-extract mixture was filtered and the solvent was removed using a rotary evaporator. The crude extract was then obtained.

GC-MS/MS analysis

The volatile content of *A. biebersteinii* and *A. millefolium* was determined using GC-MS analysis using Agilent (7890 A GC System, 5975 C by Triple-Axis Detector MS) device. The instrument had an Agilent J&W HP-5 ms UltraInert 5%-phenyl)-methylpolysiloxane (30 m × 0.25 mm × 0.25 µm) GC column (Başar et al., 2024). For headspace analysis, samples were placed in the oven of the instrument (Agilent 7000 A GC/MS Triple Quad with 7890 GC, 7693 Autosampler, and 7697A Headspace Sampler). Headspace conditions: The oven temperature was set to 130 °C, the loop temperature was set to 140 °C, the transfer line temperature was set to 145 °C, and the

vial equilibration was set to 30 minutes. GC conditions: The initial column oven temperature was set to 60 °C and then increased to 220 °C at a rate of increase of 4 °C/min and held for 10 minutes. The Wiley software, the NIST library, and NIST MS Search 2.2 were used for the qualitative determination of the components (Yenigün et al., 2024a).

LC-MS/MS analysis

An LC-MS/MS instrument with mass spectrometry (MS) in combination with liquid chromatography (LC) under the brand name Agilent 6460 Triple Quad was used to determine the phenolic content of *A. biebersteinii* and *A. millefolium* of flowers and steam-leafs of ethyl acetate extracts. A flow rate of 0.400 mL/min was set for eluents A (pure water + 5 mM NH_4HCO_2 + 0.1% CH_2O_2) and B (MeOH + 5 mM NH_4HCO_2 + 0.1% CH_2O_2). 50 mg of each sample was weighed. It was then dissolved in 1 mL methanol and 1 mL hexane was added (to remove non-polar solvents) and waited for phase formation. 100 µL of the methanol phase was removed and filtered into the vial, and then 900 µL of methanol was added the monitoring process was performed and the samples were placed in the apparatus (Başar et al., 2023).

Molecular Docking Studies

The interaction of chlorogenic acid with xanthine oxidase (PBD ID: 3NRZ) was investigated theoretically. The structure of chlorogenic acid was drawn with Chem-Draw, refined to the lowest energy, and saved in Mol2 format. Molecule-enzyme interactions were observed using the program Molegro Virtual Docker 6.0.1 (MVD). 2D and 3D representations of the interactions were obtained using the BIOVIA Discovery Studio Visualizer (İpek et al., 2024; Yenigun et al., 2024b).

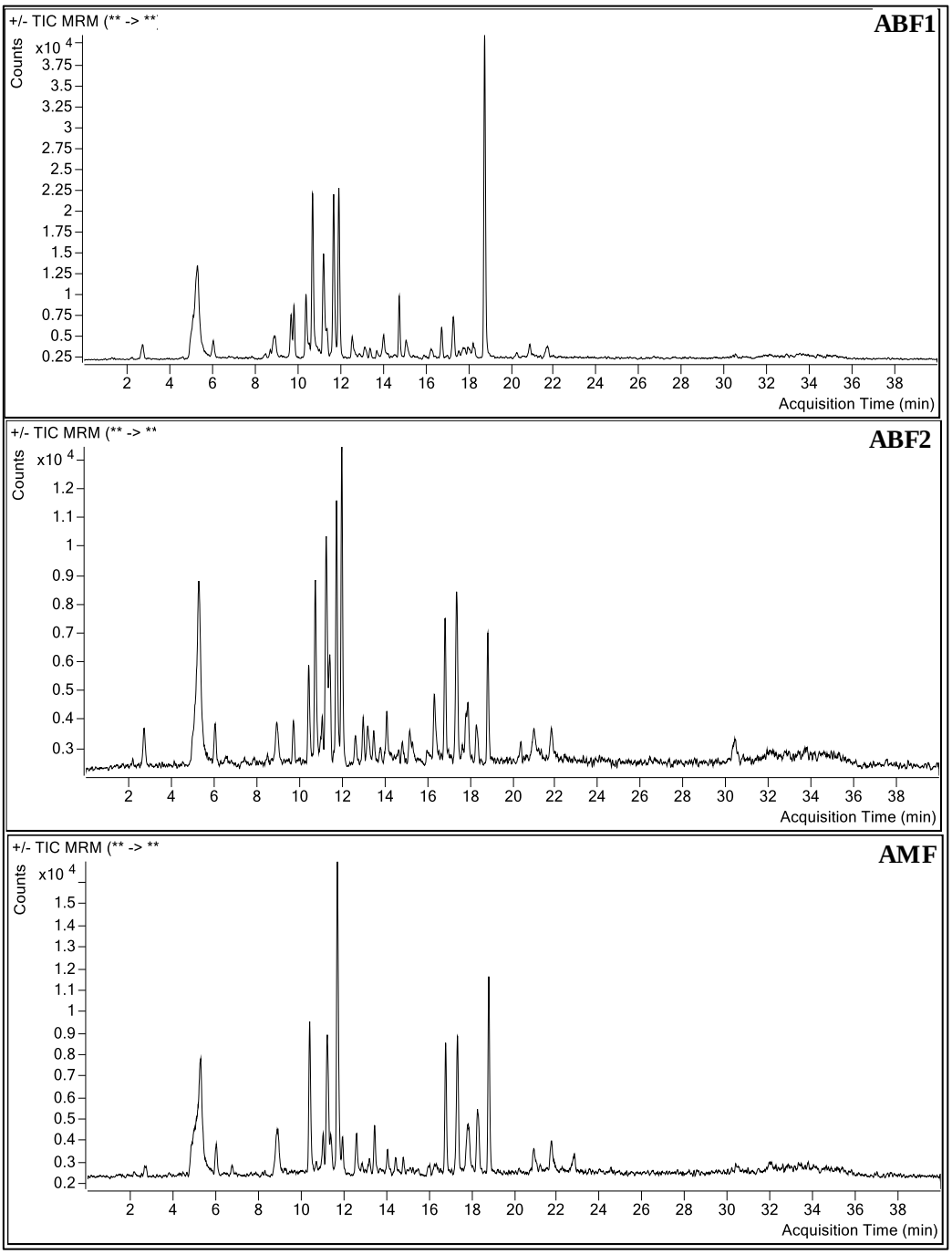
3. RESULTS and DISCUSSIONS

Ethyl acetate extracts were obtained from flower, stem, and leaf parts of the species *A. biebersteinii* and *A. millefolium* collected at different times. The phytochemical content of the extracts was analyzed by LC-MS/MS. In addition, the volatile oil content of the flower parts was determined using the headspace method on the GC-MS/MS instrument. The results of the interactions of chlorogenic acid, which was determined as the main component in the analysis of phenolic content, with BSA were theoretically determined by molecular docking.

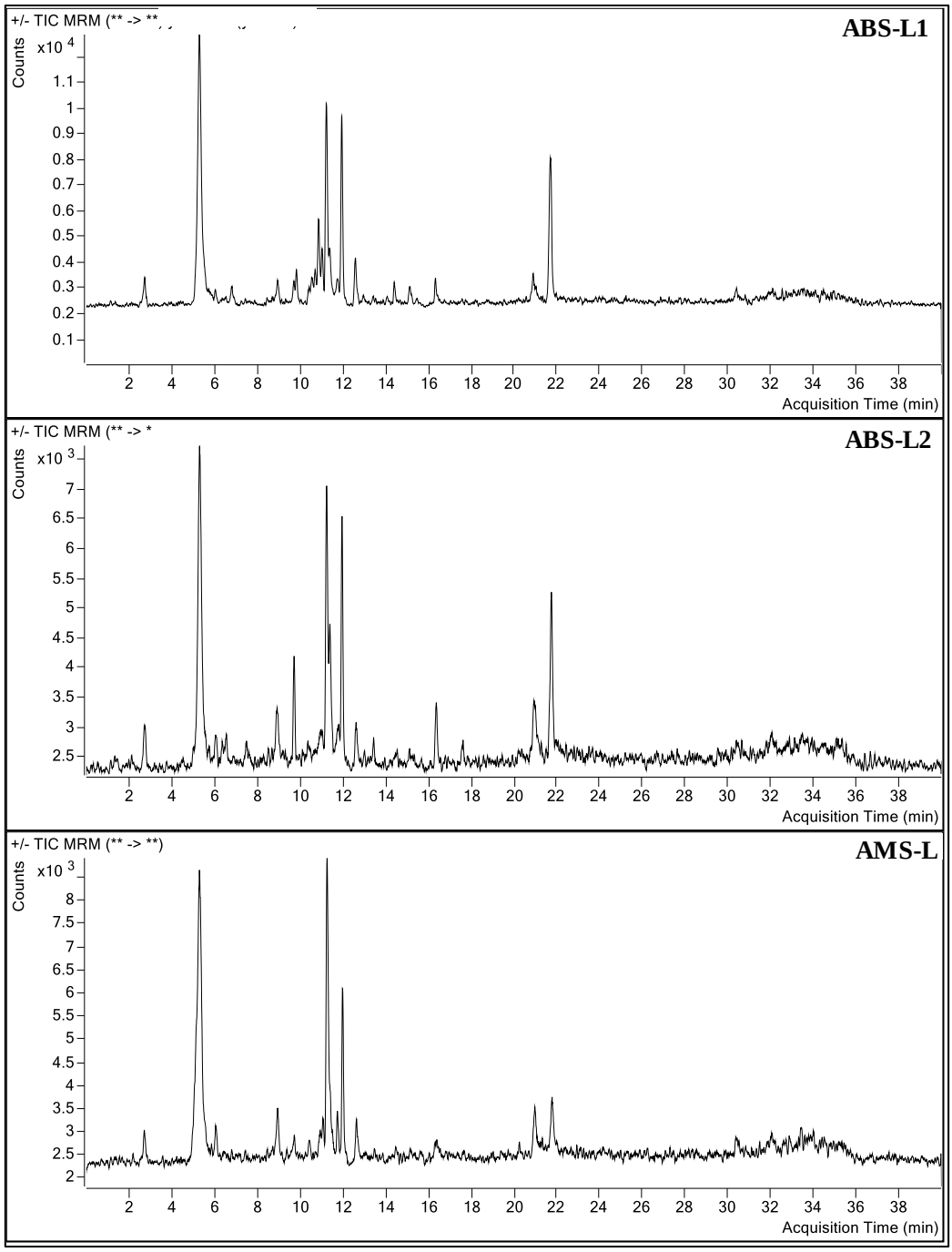
LC-MS/MS results

The phenolic content of different parts of *A. biebersteinii* and *A. millefolium* plants was determined using LC-MS/MS (Figure 2-Figure 3). According to the results, in ABF1; chlorogenic acid (8535.71 µg/g extract), quercimetrin (597.71 µg/g extract), shikimic acid (73.08 µg/g extract), in ABS-L1; chlorogenic acid (2872.09 µg/g extract), cynarin (100.17 µg/g extract), shikimic acid (53.04 µg/g extract) were detected in the highest amount (Table 1). In ABF2; chlorogenic acid (4035.84 µg/g extract), cynarin (208.11 µg/g extract), quercimetrin (192.52 µg/g extract), in ABS-L2; chlorogenic acid (6174.62 µg/g extract), cynarin (67.20 µg/g extract), shikimic acid (53.80 µg/g extract) were determined in the highest amount (Table 1). In AMF; chlorogenic acid (2848.87 µg/g

extract), cynarin (70.22 µg/g extract), shikimic acid (53.04 µg/g extract), in AMS-L; chlorogenic acid (4502.28 µg/g extract), cynarin (245.60 µg/g extract), shikimic acid (53.24 µg/g extract) were detected in the highest amount (Table 1). Similar studies have confirmed that the main component in the different parts of the two species is chlorogenic acid. (Kostevski et al., 2016). In addition to its antioxidant, antibacterial, antitumor, and anti-inflammatory effects, chlorogenic acid is also reported to have liver-protecting, kidney-protecting, nerve-protecting, and vascular-protecting effects (Wang et al., 2022).



ABF1: *A. biebersteinii* flower may, **ABF2:** *A. biebersteinii* flower june, **AMF:** *A. millefolium* flower
Figure 2. LC-MS/MS chromatogram of *A. biebersteinii* and *A. millefolium* of flowers



ABS-L1: *A. biebersteinii* stem-leaf may, **ABS-L2:** *A. biebersteinii* stem-leaf june,
AMS-L: *A. millefolium* stem-leaf

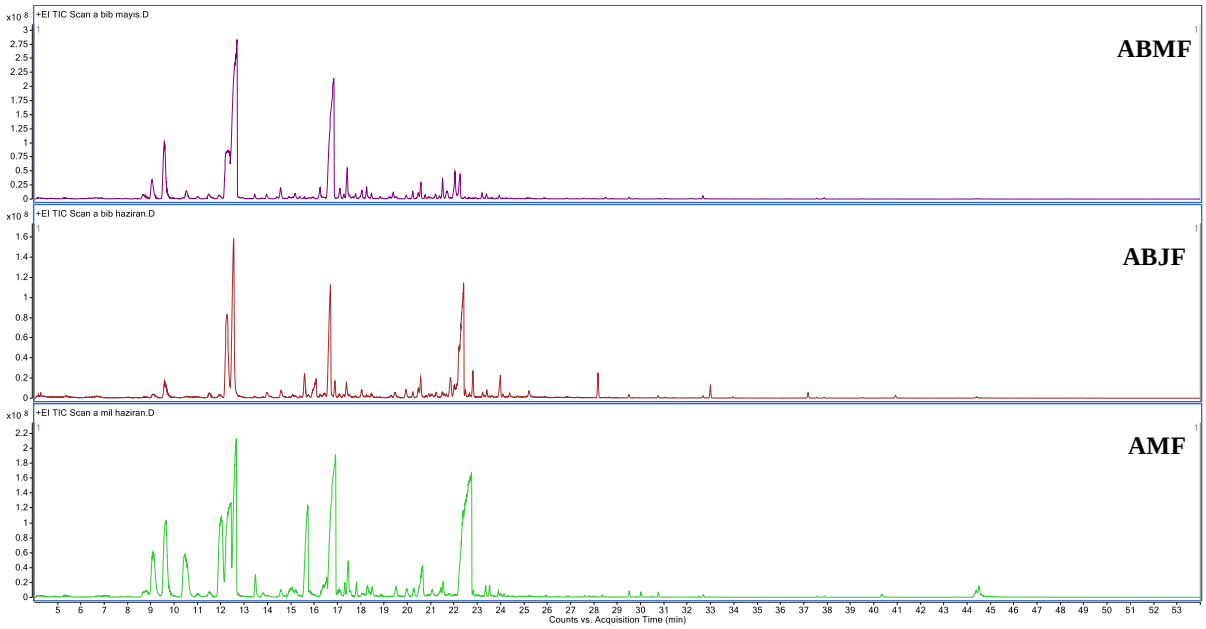
Figure 3. LC-MS/MS chromatogram of *A. biebersteinii* and *A. millefolium* of stem-leaf

Table 1. Phenolic content of *A. biebersteinii* and *A. millefolium* flower and leaf-stem (µg/g extract)

No	*Compound*	*RT (min.)*	*ABF1*	*ABF2*	*AMF*	*ABS-L1*	*ABS-L2*	*AMS-L*
1	*Ascorbic Acid*	*1.29*	*16.64*	*17.36*	*21.74*	*20.78*	*22.10*	*16.62*
2	*Shikimic acid*	*1.18*	*73.08*	*60.94*	*53.16*	*53.04*	*53.80*	*53.24*
3	*Protocatechuic acid*	*2.75*	*17.45*	*22.11*	*2.40*	*6.97*	*1.40*	*4.61*
4	*Gentisic acid*	*3.28*	*8.06*	*7.01*	*7.17*	*7.49*	*7.26*	*6.85*
5	*Catechin*	*4.14*	*9.87*	*10.99*	*11.97*	*10.33*	*10.29*	*10.65*
6	*Chlorogenic acid*	*5.29*	*8535.71*	*4035.84*	*2848.87*	*2872.09*	*6174.62*	*4502.28*
7	*4-Hydroxybenzaldehyde*	*5.76*	*1.07*	*1.58*	*1.45*	*1.03*	*0.83*	*0.69*
8	*Vanillic acid*	*5.98*	*-*	*3.54*	*-*	*-*	*-*	*-*
9	*Caffeic Acid*	*6.07*	*25.55*	*14.20*	*16.30*	*2.40*	*0.77*	*3.72*
10	*Epicatechin*	*6.83*	*5.60*	*5.84*	*5.89*	*6.30*	*5.65*	*6.07*
11	*Syringic acid*	*6.98*	*29.41*	*29.92*	*28.74*	*32.20*	*28.14*	*28.00*
12	*Salicylic Acid*	*8.95*	*39.97*	*13.12*	*29.73*	*-*	*-*	*0.45*
13	*trans-Ferulic acid*	*9.57*	*4.66*	*5.34*	*3.54*	*3.77*	*3.29*	*3.35*
14	*Quercimetrin*	*10.75*	*597.71*	*192.52*	*12.29*	*1.44*	*29.25*	*1.30*
15	*Coumarin*	*10.62*	*8.64*	*3.12*	*3.33*	*3.23*	*3.51*	*4.33*
16	*Scutellarin*	*11.20*	*-*	*-*	*15.60*	*-*	*-*	*1.98*
17	*Cynarin*	*11.43*	*62.37*	*208.11*	*70.22*	*100.17*	*67.20*	*245.60*
18	*Hyperocide*	*11.52*	*-*	*-*	*2.71*	*1.60*	*-*	*3.14*
19	*Quercetin-3-glucoside*	*11.99*	*55.95*	*30.29*	*0.01*	*-*	*20.02*	*1.11*
20	*Isoquercitrin*	*11.99*	*56.11*	*30.24*	*3.68*	*11.64*	*20.24*	*8.12*
21	*Quercetin*	*15.19*	*52.90*	*23.88*	*-*	*2.41*	*16.07*	*1.88*
22	*Naringenin*	*15.30*	*8.55*	*12.89*	*3.35*	*-*	*0.78*	*1.13*
23	*Tamarixetin*	*17.65*	*16.32*	*9.26*	*5.70*	*7.99*	*4.29*	*1.72*
24	*Chrysin*	*21.00*	*19.83*	*23.57*	*19.22*	*21.49*	*17.85*	*15.86*
25	*Flavon*	*21.81*	*2.72*	*2.04*	*2.74*	*4.91*	*10.95*	*2.22*

RT: Retention time, **ABF1:** *A. biebersteinii* flower may, **ABF2:** *A. biebersteinii* flower june, **AMF:** *A. millefolium flower*, **ABS-L1:** *A. biebersteinii* leaf-stem may, **ABS-L1:** *A. biebersteinii* leaf-stem june, **AMS-L:** *A. millefolium* leaf-stem

GC-MS/MS Results



***ABMF**: A. biebersteinii May flower, **ABJF**: A. biebersteinii June flower, **AMF:** A. millefolium flowers.*

Figure 4. GC-MS/MS chromatogram of *A. biebersteinii* and *A. millefolium* of flowers

The volatile oil content of flower parts of *A. biebersteinii* and *A. millefolium* species was determined by the headspace method in GC-MS/MS (Figure 4 and Table 2). According to the results in AB1; isoascaridol (30.15%), camphor (17.39%), eucalyptol (12.89%), m-cymene (8.82%), in AB2; isoascaridol (22.91%), camphor (15.54%), o-cymene+ p-cymene (10.73%), eucalyptol (10.60%), 4-carene (7.53%), camphene (6.84%), sabinene (4.72%), α-pinene (4.14%), in A. millefolium; eucalyptol (43.22%), cis-chrysanthenyl acetate (7.93%), camphor (7.87%), sabinene (3.39%), m-cymene (3.26%), α-pinene-isomer (3.03%) were determined in the highest amount. The main components of the two species were consistent with similar studies (Kostevski et al., 2016).

Table 2. Volatile oil of A. biebersteinii and *A. millefolium* flower (%)

No	***RT***	***Compound Name***	***ABF1***	***ABF2***	***AMF***
1	*5.33*	*Hexanal*	*-*	*0.16*	*-*
2	*8.67*	*Tricyclene*	*0.18*	*0.75*	*0.73*
3	*9.05*	*α-Pinene-isomer*	*0.79*	*-*	*3.03*
4	*9.56*	*α-Pinene*	*2.06*	*4.14*	*-*
5	*9.63*	*Camphene*	*-*	*6.84*	*-*
6	*10.53*	*Sabinene*	*0.12*	*4.72*	*3.39*
7	*11.00*	*3,3-Dimethyl-6-methylenecyclohexene*	*0.18*	*-*	*-*
8	*11.46*	*α-Phellandrene*	*0.32*	*0.35*	*-*
9	*11.91*	*α-Terpinene*	*0.39*	*-*	*-*
10	*12.03*	*4-Carene*	*-*	*7.53*	*-*
11	*12.21*	*m-Cymene*	*8.82*		*3.26*
12	*12.43*	*o-Cymene + p-Cymene*	*-*	*10.73*	*-*
13	*12.47*	*Eucalyptol*	*12.89*	*10.60*	*43.22*
14	*13.44*	*γ-Terpinene*	*0.09*	*0.78*	*0.67*
15	*13.70*	*Sabinene hydrate, cis*	*-*	*0.19*	*1.00*
16	*13.94*	*Linalool oxide*	*0.41*	*-*	*-*
17	*14.55*	*p-Cymenene*	*0.67*	*0.36*	*0.29*
18	*14.91*	*Linalool*	*0.26*	*-*	*0.70*

No	*RT*	*Compound Name*	*ABF1*	*ABF2*	*AMF*
19	*15.08*	*Nonanal*	*-*	*-*	*0.45*
20	*15.17*	*Thujone*	*1.34*	*-*	*-*
21	*15.38*	*1,5,8-p-Menthatriene*	*0.15*	*-*	*-*
22	*15.56*	*Chrysanthone*	*0.15*	*-*	*0.54*
23	*15.73*	*α-Thujone*	*-*	*5.84*	*-*
24	*15.76*	*cis-2-Menthenol*	*0.29*	*-*	*0.69*
25	*15.96*	*2-Propylcyclohexanone*	*2.78*	*-*	*-*
26	*16.26*	*1,2,3,4,5,8-Hexahydronaphthalene*	*0.24*	*-*	*-*
27	*16.39*	*trans-Pinocarveol*	*-*	*-*	*1.19*
28	*16.40*	*Cyclooctanone*	*-*	*0.62*	*-*
29	*16.68*	*Camphor*	*17.39*	*15.54*	*7.87*
30	*17.09*	*Sabinaketone*	*-*	*-*	*0.74*
31	*17.10*	*trans-Chrysanthemol*	*1.34*	*-*	*-*
32	*17.26*	*Pinocarvone*	*0.18*	*0.30*	*1.97*
33	*17.38*	*endo-Borneol*	*2.42*	*1.10*	*1.86*
34	*17.51*	*2-Caren-4-ol*	*0.46*	*-*	*-*
35	*17.76*	*4-Terpinenol*	*0.16*	*0.25*	*0.67*
36	*18.03*	*p-Cymen-8-ol*	*1.38*	*-*	*1.07*
37	*18.23*	*α-Terpineol*	*0.48*	*0.29*	*2.17*
38	*18.45*	*Myrtenal*	*0.20*	*-*	*0.76*
39	*18.50*	*α-Thujenal*	*-*	*0.18*	*-*
40	*18.82*	*cis-Piperitol*	*0.15*	*-*	*-*
41	*18.92*	*Verbenone*	*0.12*	*-*	*0.39*
42	*19.37*	*2-Hydroxycineole*	*0.26*	*-*	*0.51*

No	*RT*	*Compound Name*	*ABF1*	*ABF2*	*AMF*
43	*19.45*	*Ascaridole*	*0.74*	*-*	*-*
44	*19.50*	*Ascaridole epoxide*	*-*	*0.31*	*-*
45	*19.93*	*Cuminal*	*0.53*	*-*	*0.51*
46	*19.97*	*Dihydro carveol neo*	*-*	*0.27*	*-*
47	*20.23*	*Ascaridole-isomer*	*0.25*	*-*	*-*
48	*20.27*	*Ascaridole*	*-*	*0.24*	*-*
49	*20.46*	*Piperitone oxide*	*0.56*	*-*	*-*
50	*20.50*	*Piperitone oxide isomer + 7-Oxabicyclo[4.1.0]heptan-2-one, 3-methyl-6-(1-methylethyl)-*	*-*	*1.83*	*-*
51	*20.55*	*6-Isopropyl-3-methyl-7-oxabicyclo[4.1.0]heptan-2-one*	*0.89*	*0.22*	*-*
52	*20.66*	*cis-Chrysanthenyl acetate*	*-*	*-*	*7.93*
53	*20.75*	*2,5-Bornanedione*	*0.31*	*-*	*-*
54	*21.03*	*trans-Ascaridol glycol*	*1.01*	*0.31*	*0.31*
55	*21.11*	*4-Thujen-2-α-yl acetate*	*-*	*-*	*0.93*
56	*21.20*	*1,7-Octadiene-3,6-diol, 2,6-dimethyl-*	*0.53*	*-*	*-*
57	*21.39*	*Cyclopropanemethanol, 2,2-dimethyl-3-(2-methyl propenyl)-, acetate, trans-*	*1.18*	*-*	*-*
58	*21.51*	*Bornyl acetate*	*1.12*	*0.45*	*1.42*
59	*21.64*	*Thymol*	*0.16*	*-*	*0.77*
60	*21.81*	*Bicyclo[3.1.1]hept-2-en-4-ol, 2,6,6-trimethyl-, acetate*	*2.71*	*-*	*0.49*
61	*21.85*	*Carvacrol*	*0.49*	*-*	*-*
62	*22.39*	*Isoascaridol*	*30.15*	*22.91*	*0.96*
63	*22.65*	*2-Methyl-5-(propan-2-ylidene)cyclohexane-1,4-diol*	*0.41*	*-*	*-*
64	*22.81*	*Cyclopentaneacetaldehyde, 2-formyl-3-methyl-α-methylene-*	*-*	*-*	*0.49*
65	*23.36*	*γ-Terpineol acetate*		*0.25*	

No	*RT*	*Compound Name*	*ABF1*	*ABF2*	*AMF*
66	*23.85*	*Eugenol*	*0.09*	*0.14*	*-*
67	*25.90*	*Caryophyllene*	*-*	*-*	*0.95*
68	*26.96*	*Humulene*	*-*	*-*	*0.10*
69	*27.77*	*Germacrene D*	*0.06*	*-*	*0.55*
70	*28.52*	*β-Bisabolene*	*-*	*-*	*0.22*
71	*29.51*	*Terpinyl propionate*	*0.25*	*-*	*-*
72	*29.80*	*Nerolidol*	*-*	*-*	*0.51*
73	*29.84*	*Terpinyl propionate-isomer*	*0.07*	*0.16*	*-*
74	*29.93*	*Caryophyllene oxide*	*-*	*-*	*0.45*
75	*30.02*	*Benzyl angelate*	*-*	*0.12*	*-*
76	*30.08*	*Viridiflorol*	*-*	*-*	*1.39*
77	*30.64*	*Spathulenol*	*0.06*	*-*	*0.32*
78	*30.75*	*Piperityl angelate, cis*	*0.19*	*-*	*-*
79	*30.81*	*Patchoulane*	*-*	*-*	*1.09*
80	*32.20*	*α-acorenol*	*0.19*	*-*	*0.71*
81	*32.30*	*Caryophylladienol II*	*-*	*-*	*-*
82	*32.50*	*Methyl jasmonate*	*0.10*	*-*	*-*
83	*32.68*	*13-Tetradecanolide*	*0.20*	*-*	*0.62*
84	*32.75*	*α-Eudesmol*	*-*	*-*	*1.02*
85	*33.22*	*α-Santalal*	*-*	*-*	*0.18*
86	*33.64*	*ent-Germacra-4(15),5,10(14)-trien-1β-ol*	*-*	*-*	*0.67*
87	*37.56*	*Hexahydrofarnesyl acetone*	*0.06*	*-*	*0.24*
88	*37.88*	*Palmitoleic acid*	*0.12*	*-*	*-*
89	*40.34*	*Gerany-p-cymene*	*0.06*	*-*	*-*

Molecular Docking Results

Chlorogenic acid has been reported to have antioxidant, antibacterial, antitumor, and anti-inflammatory effects as well as hepatoprotective, renoprotective, neuroprotective, and vasoprotective effects (Wang et al., 2022). Chlorogenic acid is known to have an antioxidant effect; its interaction with xanthine oxidase was determined by molecular docking. The inhibitory properties were investigated by calculating mold score and binding energies. Its inhibitory properties were investigated by calculating the MolDock score and binding energies.

Table 3. Interaction categories, types, and distances of molecular insertion of the chlorogenic acid with xanthine oxidase

No	Name	Distance	Category	Type	From Chemistry	To Chemistry
1	L:LYS754:HZ1 - :[001:O5	2.0507	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
2	:[001:H8 - :[001:O2	1.90921	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
3	:[001:H16 - C:VAL764:O	2.06686	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
4	:[001:H17 - C:ARG793:O	1.73615	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
5	:[001:H18 - L:LYS754:O	1.92604	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
6	C:ILE596:HA - :[001:O8	2.24509	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
7	L:LYS754:HA - :[001:O1	2.66327	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
8	:[001:H3 - L:TYR599:OH	2.38793	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
9	C:LYS792:HN - :[001	2.72295	Hydrogen Bond	Pi-Donor Hydrogen Bond	H-Donor	Pi-Orbitals
10	:[001 - C:CYS593	4.74597	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
11	:[001 - C:LYS792	4.52128	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl

Chlorogenic acid interacted with xanthine oxidase by five conventional hydrogen bonds with amino acid LYS754, VAL764, ARG793, three carbon-hydrogen bonds with amino acid ILE596, LYS754, TYR599, one pi-donor hydrogen bond with amino acid LYS792, and two alkyls with amino acid CYS593, LYS792 (Figure 5 – Table 3). Chlorogenic acid interaction with xanthine oxidase was calculated as a MolDock score of -130.96, with binding energies of -8.0 kcal/mol.

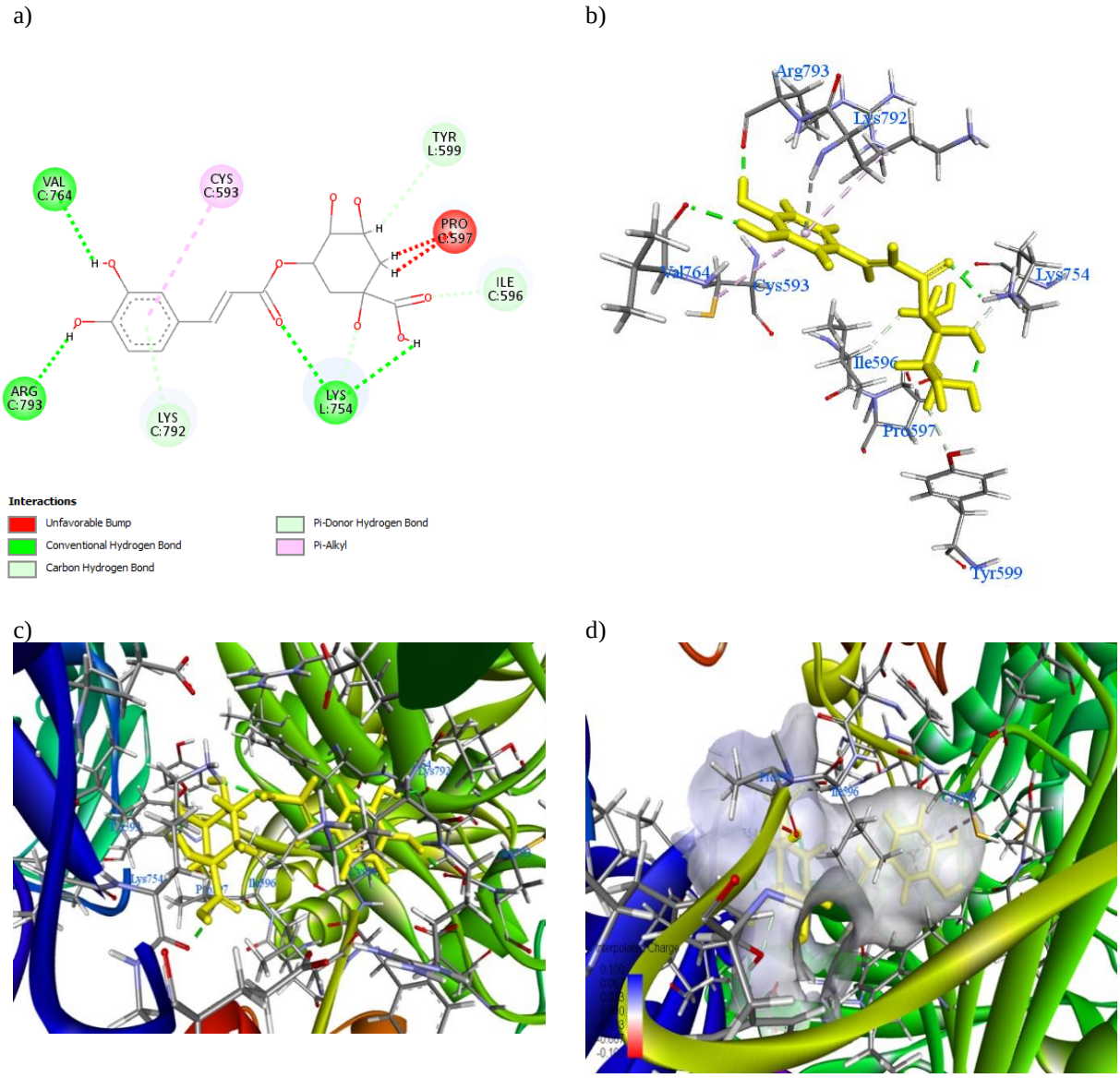


Figure 5. Chlorogenic acid interaction with xanthine oxidase, a) 2D images, b) interaction images c) 3D images, d) interpolated load view

4. CONCLUSION

Achillea is commonly known to the public as "yarrow". It has a high content of volatile oils and phenols. In our study, the volatile and phenolic components of the leaves-stems, and flowers of two different Achillea species were determined by chromatographic methods. According to the results of LC-MS/MS analysis of A. biebersteinii and A. millefolium, the main component in the leaves, stems and flowers was found to be chlorogenic acid. In addition, GC-MS analysis revealed that the presence of high content of eucalyptol (43.22%) in the A. millefolium plant and the time of harvest (May and June) drastically altered the levels of volatiles (especially m-cymene, α-thujone, 4-carene) in the A. biebersteinii plants. The ability of chlorogenic acid to be an inhibitor of the enzyme xanthine oxidase was investigated by an in silico molecular docking application. According to the results of molecular docking, chlorogenic acid could be an inhibitor of xanthine oxidase, but it is expected that these results will be supported by *in vitro* and *in vivo* studies in future studies.

Author Contributions

All the authors equally contributed to this work. They all read and approved the final version of the paper.

Conflict of Interest

All the authors declare no conflict of interest.

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