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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.

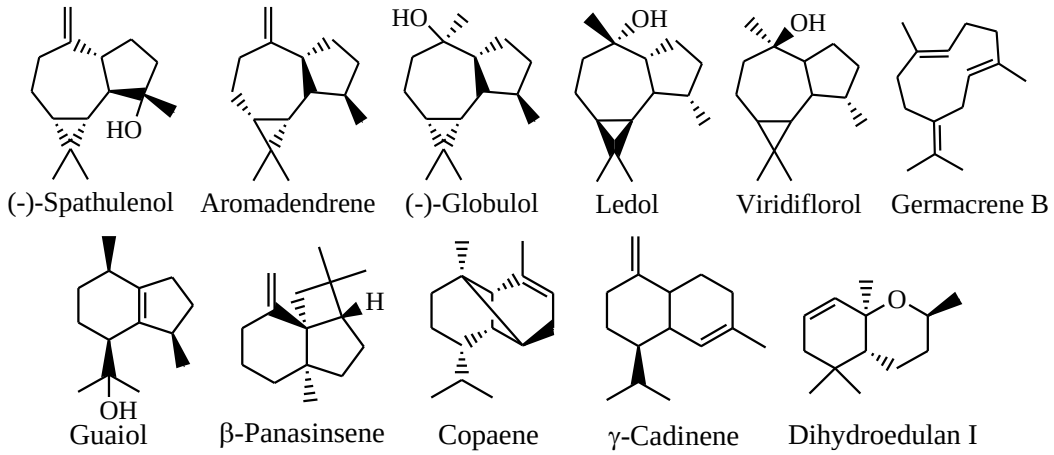

Figure 1. Structures of some major compounds identified in fresh and dry stem bark of *A. heterophylla* essential oils.

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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