Journal of Taibah University Medical Sciences (2021) 16(2), 231–239

Taibah University

Journal of Taibah University Medical Sciences

www.sciencedirect.com

Original Article

Composition and pharmacological activity of essential oils from two imported *Amomum subulatum* fruit samples

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Received 17 August 2020; revised 11 October 2020; accepted 19 October 2020; Available online 11 November 2020

الملخص

أهداف البحث: في هذا العمل حاولنا فصل وتحديد تركيبة الزيت العطري والخصائص الدوائية لنوعين من ثمار حب الهيل الأسود المستوردة التي تم شراؤها من المتاجر المحلية الهندية والسعودية للتأكد من علاقة التركيب الكيميائي بالتأثيرات العلاجية.

طرق البحث: لقد تم استخلاص الزيت العطري من عينات ثمار حب الهيل الأسود الهندية والسعودية باستخدام طريقة التقطير المائي، ثم تم تحديد تركيب الزيت العطري بواسطة جهاز مطياف الكتلة المرتبط بالكروماتوغرافيا الغازية. بعدها تم تقييم فاعلية تلك الزيوت كمضاد جرثومي ضد البكتيريا سالبة الجرام (الزائفة الزنجارية والإشريكية القولونية وأسينوباكتر بومائي) باستخدام طريقتي تحديد قيمة الحد الأدنى من التركيز المثبط وتحديد التركيز الأدنى القاتل للجراثيم. هذا بالإضافة إلى أنه قد تم تحديد الفاعلية المضادة للأكسدة باستخدام طريقة مكافحة الجذور الحرة التي يسببها مركب (٢,٢-ثنائي الفينيل ١- بيكريل الهيدرازيل) مع الفاعلية المضادة للالتهابات بواسطة فحص مثبطات الألبومين البقري وذلك لتقييم التأثيرات الدوائية لتلك الزيوت.

النتائج: لقد أظهرت نتائج تحليل الزيوت العطرية المستخلصة بواسطة جهاز مطياف الكتلة المرتبط بالكروماتوغرافيا الغازية لكلتا العينتين وجود ٥٦ مكونا ذا فاعلية حيوية بنسب مختلفة حيث كانت المركبات الرئيسة في عينات الزيوت المستخلصة الهندية منها والسعودية هي: ١.٨- سينول (بتركيز ٤٤.٢٤٪ و٤٦.٢٢٪ على التوالي) ومركب الفا-تيربينول (بتركيز ٧.٤٧٪ و٧.٠٤٪ على التوالي) ومركب تيربينين-٤-ول (بتركيز ٥.٠١٪ و٤.٨٣٪ على التوالي) ومركب جرانيول د (بتركيز ٤.٠٥٪ و٣.٥٤٪ على التوالي) وبيتا-بينين (بتركيز ٣.٣٨٪ و٣.٩٨٪ على التوالي). كما لوحظ نشاط فائق مضاد للجراثيم ضد السلالات المختارة في كلتا العينتين بحد أدنى من التركيز المثبط الذي تراوح بين ٠.٥-١٪. كما كان التأثير المضاد للأكسدة ذو نشاط معتدل في كلتا العيّنتين. علاوةً على ذلك لقد أظهرت عينات السوق الهندي والسعودي قيمتي التأثير المضاد للالتهابات المعرفة بتركيز المادة الموافقة للتثبيط النصفي بنسبة ٥٣.١٢٪ و٥٥.٢٦ ميكروغرام/مل على التوالي في اختبار تثبيط تمسخ الألبومين. مما يشير إلى إمكانات مضادة للالتهابات مماثلة أو شبيهة لتأثير دواء الإيبوبروفين.

الاستنتاجات: أن مكونات تركيبة الزيتين من كلتا العيّنتين متشابهتين نوعيا ولكن مع وجود بعض الاختلافات الكمية لكل مركب تم التعرف عليه. كما أنه لم يلحظ أي اختلافات كبيرة في الخصائص الدوائية لهما مما يتطلب إجراء المزيد من الدراسات لمزيد من التأكيد.

الكلمات المفتاحية: حب الهيل الأسود؛ العينات المسوّقة؛ الزيوت الأساسية؛ الكروماتوغرافيا الغازية؛ النشاط الدوائي

Abstract

Objective: This work attempted to isolate, identify, and correlate the composition of essential oils (EOs) and pharmacological properties of two imported *Amomum subulatum* fruit samples. These samples were collected from Indian and KSA local supermarkets to ensure consistency in their therapeutic effects.

Methods: EOs were extracted from Indian and KSA *A. subulatum* fruit samples using a hydro-distillation method and identified by gas chromatography-mass spectrometry (GC–MS). Antimicrobial activity against gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, and *Acinetobacter baumannii*) was determined using minimum inhibitory (MIC) and minimum bactericidal concentration methods. Antioxidant and anti-inflammatory activities were determined using a 2,2-diphenyl-1-picrylhydrazyl-induced free radical assay, and a bovine albumin inhibitory assay, respectively. These analyses were performed to evaluate the pharmacological activities of the substances.

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Peer review under responsibility of Taibah University.

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 https://doi.org/10.1016/j.jtumed.2020.10.007

Results: GC–MS retention times of both samples demonstrated 56 bioactive ingredients with different percentages. The principal bioactive compounds in the Indian and Saudi Arabian EO samples were 1,8-cineole (44.24% and 46.22%, respectively), α-terpineol (7.47% and 7.04%, respectively), terpinen-4-ol (5.01% and 4.83%, respectively), geraniol D (4.05% and 3.54%, respectively), and β-pinene (3.38% and 3.98%, respectively). Superior antimicrobial activity against the selected strains was observed for both samples, with an MIC range of 0.5%–1%. Antioxidant assays demonstrated moderate activity in both samples. Moreover, the Indian and Saudi Arabian samples exhibited IC_{50} values of 53.12% and 55.26 μg/mL, respectively, in albumin denaturation inhibition assays. This indicated an outstanding anti-inflammatory potential comparable to ibuprofen.

Conclusions: The composition of EOs from both samples exhibited similar qualitative but different quantitative variability. No major variations in the pharmacological properties of EOs were observed. More studies are essential for further validation of our study findings.

Keywords: *Amomum subulatum*; Essential oils; Gas-chromatography; Marketed samples; Pharmacological activity

Introduction

Greater cardamom (*Amomum subulatum*, F: Zingiberaceae) is native to India, Nepal, and Bhutan. The fruit of this plant is mainly used as a flavoring agent in the cuisines of those countries and is considered as an aphrodisiac in the Middle East. The seeds of this plant are used as diuretics, astringents, and appetisers.[1] *A. subulatum* is mainly grown as a cash crop in Eastern Nepal, India (Sikkim, West Bengal, Uttarakhand, Assam, Nagaland, Himachal Pradesh, and Arunachal Pradesh), and Southern Bhutan. Fruit or seeds of this plant are generally used for the treatment of cough, nausea, vomiting, congestive jaundice, gonorrhoea, headache, ischemic heart disease, pulmonary tuberculosis, and skin cancer, as well as in producing antioxidant and anti-inflammatory compounds.[2]

The fruit of *A. subulatum* contains 2%–3% essential oils (EOs). The main component of the EOs is the oxygenated monoterpene 'eucalyptol' or 1,8-ceniole (65%–80%), the concentration of which varies across cultivars and geographical conditions of cultivation.[1] Variable chemical composition has been reported by different investigators. Kaskoos et al. reported 1,8-cineole, β-myrcene, α-terpineol and terpinen-4-ol as the major constituents of EO in the Indian (Sikkim) varieties,[3] whereas Satyal et al. reported 1,8-cineole, alpha- and beta-pinene, and alpha-terpineol as the major consituents in the Nepalese varieties.[4] In another study, Joshi et al. reported 1,8-cineole, α-terpineol, limonene, nerolidol,4-terpineol, δ-terpineol, δ-3-carene, β-myrcene and germacrene in the EO of Himachal Pradesh (Indian) cultivars.[5] Shrestha reported a completely different composition of EOs of Nepalese cultivars, consisting of mainly α-terpineol, terpine-4-ol, pinocarvone, nerolidol, and pinocarveol.[6] Hence, the composition of EOs in the known cultivars has been reported to be variable. Since then, *A. subulatum* has become one of the most widely investigated plants. Active compounds, including both simple and oxygenated monoterpenes and sesquiterpenes, have been reported in the EO of *A. subulatum* fruits.[7,8]

The EOs of this plant exhibit antifungal and antibacterial activities. Variable potency of antimicrobial activity has been reported for similar microbial strains.[9,10] Additionally, anticancerous, nematocidal, and insecticidal activities have been reported by several researchers.[3,4,8] In addition to the EOs, different solvent-soluble extracts of the fruit have been investigated for food preservative, anti-scabies, anticancer, immune suppression, and various other properties.[11,12]

Thus, the composition and pharmacological properties of the EOs of *A. subulatum* are still the subjects of extensive research interests. In the current study, we investigated and compared the EO composition of *A. subulatum* fruit available in local Indian and Saudi Arabian markets to assess the uniformity in the composition and pharmacological properties of the EOs by assaying *in vitro* antimicrobial activity against unique gram-negative microbial strains, by assessing antioxidant, and anti-inflammatory activities that have not been reported to date.

Materials and Methods

Sample collection and authentication

Fruit of *A. subulatum* was purchased from India (New Friend's Colony, East Delhi) and KSA (Al Kharj, Riyadh Region). Fruit samples were deposited and authenticated in an herbarium (2020/3/SOP/AS/027), dated 02-03-2020 at the School of Pharmacy, Sharda University, Greater Noida (UP). Fruits were pulverized using a grinder. Approximately 150 g of each ground sample was stored in a wide-mouth airtight amber coloured glass container for further study.

Hydrodistillation and percentage yield

A Clevenger-type apparatus (10 mL volume capacity, lighter than water, and fitted with a condenser), chiller (Buchi B-741, Switzerland), 2000 mL round bottom flask (RBF), and heating mantle (2000 mL) were used for the extraction of EOs. The powder (50 g) was transferred into the RBF, 1 L distilled water was added, the apparatus was fixed, the chiller was switched on, and the temperature was adjusted to 70 °C. The process was continued for 3 h, the volume of extracted EO was recorded, and the percentage yield was calculated. The experiment was repeated 3 times, and the percentage ± standard deviation was determined. The extracted EOs of each sample were then dried using

anhydrous Na_2CO_3 and stored in amber coloured borosilicate glass vials at 4 °C for further analysis of sample composition and antimicrobial activity.

Gas chromatography–mass spectrometry analysis

The identification of metabolites in the EOs of the Indian and Saudi Arabian samples was performed using gas chromatography (GC) (HP 5890, Hewlett–Packard, Agilent Technologies, Palo Alto, CA, USA) coupled to a mass spectrometer (MS) (HP 5972A) equipped with a flame ionisation detector. An HP-5 MS (30 m × 250 μm × 0.25 μm film thickness) capillary column was used. The temperatures of the injector and detector were maintained at 270 °C and 300 °C, respectively. The oven temperature was initially set at 40 °C for 1 min, and then increased at a rate of 10 °C/min to 110 °C, maintained for 1 min, again increased at the rate of 10 °C/min to 300 °C, and then held for 5 min. Three microliters (3 μL) of the diluted sample (10% in acetone) was injected at a split ratio of 1:100. The flow rate for the carrier gas (helium) was adjusted to 3.0 mL per min. The process was repeated 3 times for each EO. The scan mass ranged from 50 to 550 m/z. Normal scanning was used for EO spectra.

Linear retention indices (RIs) of metabolites were calculated using a homologous series of n-alkanes (C8–C30) under similar conditions of temperature-programmed GC. Metabolites were identified by comparing linear RIs with those reported in the literature[13] and mass spectra with those of NIST 05 and Wiley 275 inherent mass spectral library.

Analysis of different classes of terpenes

From the GC–MS spectra for each sample, the structure of each composition was identified.[5,14] The composition of each sample was divided into different groups of terpenes such as monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, diterpene hydrocarbons, and non-terpenes.

Estimation of antibacterial activities of EOs

Micro-organisms

The *in vitro* antimicrobial activity of EOs was assessed in the 3 selected gram-negative bacterial strains, namely *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 35218), and *Acinetobacter baumannii* (ATCC BAA747). The strains were sub-cultured on Mueller–Hinton medium (MHA) at 37 °C prior to antibacterial assays.

Disc diffusion method

The antimicrobial activity of *A. subulatum* EOs was compared using a disc diffusion method.[15] MHA plates were inoculated with 0.1 mL of appropriately diluted (2.5×10^{-6} CFU/mL) freshly grown cultures. Sterile discs (6-mm in diameter) impregnated with 10 μL of EOs were mounted. Solvent and MH broth were used as controls. Plates were incubated for 18–24 h at 37 °C to assess growth inhibition around the disc. All assays were performed in triplicate, and inhibition zone diameters were measured in mm following the CLSI guidelines.[16]

Minimum inhibitory/bactericidal concentrations

Serial EO sample dilutions of 0.125%, 0.25%, 0.5%, 1%, 2%, 4%, and 8% in analytical grade ethanol were used to determine MIC values by the broth macrodilution method.[17] The lowest EO concentration at which there was no visible growth following incubation was considered to be the MIC. The minimum bactericidal concentration (MBC) was determined by subculturing 100 μL from each negative test tube on agar following a previous method.[15] The experiment was repeated 3 times and the lowest concentration with no visible growth after 24 h incubation at 35 °C was considered to be the MBC. Means ± standard deviation values were calculated using Excel 2013 (Microsoft).

Free radical scavenging (2,2-diphenyl-1-picrylhydrazyl) assay

The *in vitro* antioxidant properties of EOs were analyzed by determining the free radical scavenging (FRS) ability induced by 2,2-diphenyl-1-picrylhydrazyl (DPPH) using a previous method with certain modifications.[8] The DPPH-cation (10 mL; 0.1 mM) was prepared in methanol. Different dilutions of EOs and ascorbic acid (62.5.1000 μg/mL) were prepared in the same solvent separately. Approximately 2 mL each of different dilution of the mixture and DPPH solutions were vortexed and kept for 30 min at 37°C. After incubation, the optical absorbance (Ab) was recorded against a blank (a mixture of 2 mL DPPH and 2 mL methanol) using a UV-VIS spectrophotometer at 517 nm. The test was performed in triplicate. The percentage FRS ability of EOs was compared using the following equation:

$$\% \text{ Inhibition of DPPH-cation} = [(1 - Ab_{sample}/Ab_{control}) \times 100]$$

Inhibition of albumin (BSA) denaturation assay

The *in vitro* anti-inflammatory activity of EOs was assessed using a bovine soluble albumin (BSA) denaturation method, with certain modifications in the process followed by Gunathilake et al..[18] To perform the assay, seven dilutions (ranging from 6.25 to 100 μg/mL) of EOs and standard (Ibuprofen) were prepared in phosphate-buffered saline (PBS; pH = 6.8). Aliquots of 100 μL sample or standard, 1000 μL of 1% BSA, and 1400 μL of PBS were mixed thoroughly, and the reaction mixture was incubated at 37 °C for 15 min, heated at 72 °C for 5 min, and then cooled. The optical absorbance was measured at 660 nm against a blank containing a mixture of 1000 μL and 1500 μL of BSA (1%) and PBS, respectively, using a UV-VIS spectrophotometer. The test was performed in triplicate, and the percentage of protein denaturation (inhibition) by EOs was compared using the following equation:

$$\% \text{ inhibition BSA} = [(1 - Ab_{sample}/Ab_{control}) \times 100]$$

Statistical analysis

All experiments were repeated three times. Regression-analysis was used to estimate the IC_{50} values for

antioxidant and anti-inflammatory activities. All analyses were performed using Microsoft (MS 2010) Excel software.

Results

Percentage yield of EO

Hydrodistillation using Clevenger apparatus of fruit samples of *A. subulatum* from local Saudi Arabian and Indian markets yielded 1.9. ± 0.24% and 1.7. ± 0.11% of EO, respectively, and the colour of both samples was light yellow.

Composition of EOs

Table 1 presents the composition of EOs obtained from Saudi Arabian and Indian samples of *A. subulatum* fruit. The oils of *A. subulatum* samples were characterized by a high percentage of volatile components (97.01%−98.68%). The percentage of eucalyptol (1,8-Cineole), was identified as the main compound in the EOs of both Saudi Arabian (46.22%) and Indian (44.24%) samples. The EOs of Saudi Arabian and Indian samples also contained α-terpineol (7.04% and 7.47%, respectively), terpinen-4-ol (4.83% and 5.01%, respectively), β-pinen (3.98% and 3.38%, respectively), trans-p-mentha-1(7),8-dien-2-ol (3.54% and 2.34%, respectively), α-selinene (2.91% and 3.14%, respectively), β-myrcene (2.53% and 2.26%, respectively), and linalool (2.08% and 2.14%, respectively) in considerable amounts. Other compounds constituting less than 2%, namely α-pinene, γ-terpinene, α-terpinolene, cis-carveol, limonene, and β-selinene, were also identified in both samples. Compounds such as perillaldehyde, trans-geranic acid methyl-ester, methyl cinnamate, isoledene, elemene, and tetrasiloxane decamethyl were identified only in Saudi Arabian samples, whereas bicyclo[4.4.0]decane, α-springene, and arsenous acid were identified only in Indian samples.

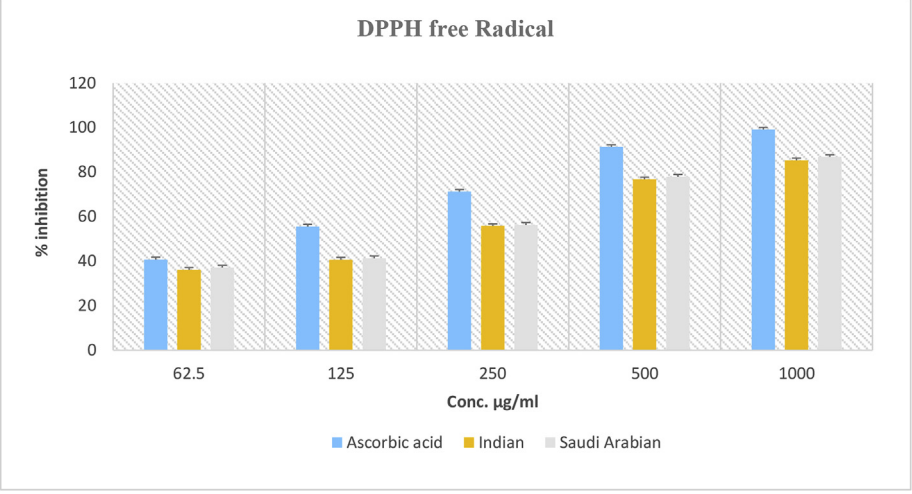

Figure 1: Antioxidant activity of essential oils from *A. subulatum* (Indian and Saudi Arabian samples), and ascorbic acid using DPPH FRS assay.

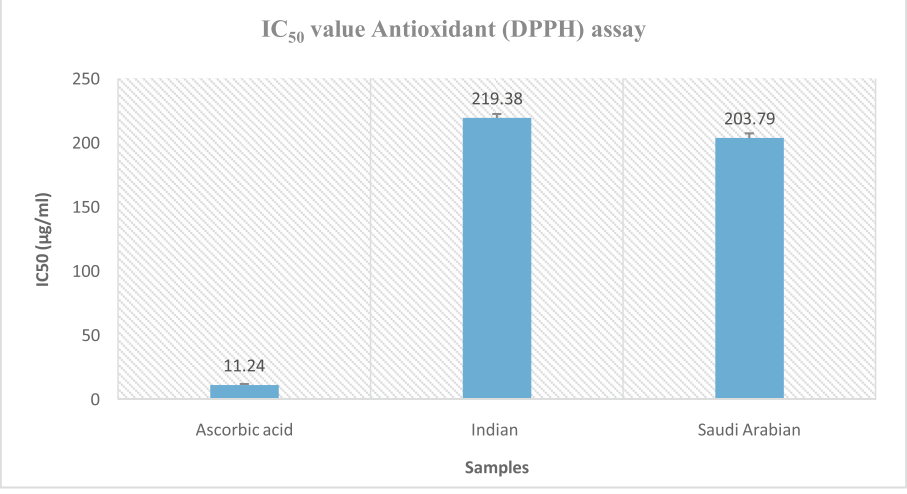

Figure 2: IC50 value of DPPH FRS assays of essential oils from *A. subulatum* (Indian and Saudi Arabian samples), and ascorbic acid.

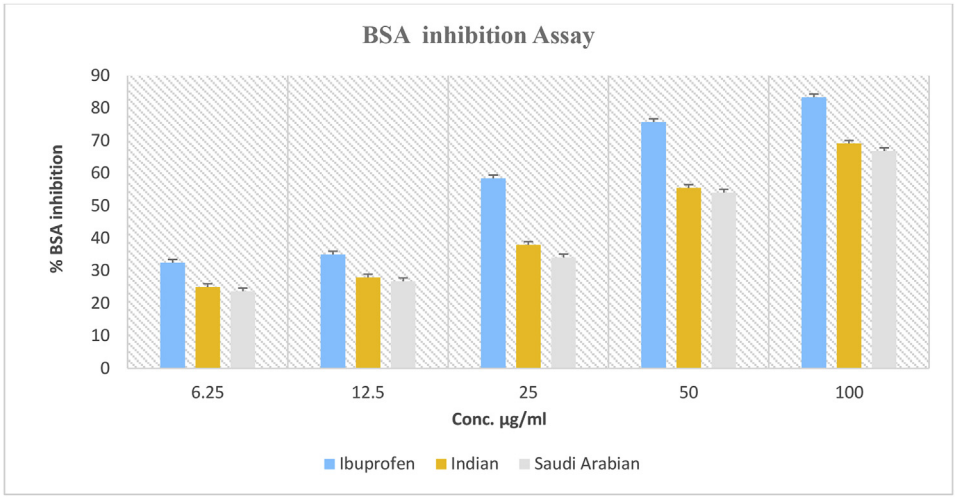

Figure 3: BSA inhibitory assays for essential oils from *A. subulatum* (Indian and Saudi Arabian samples), and Ibuprofen.

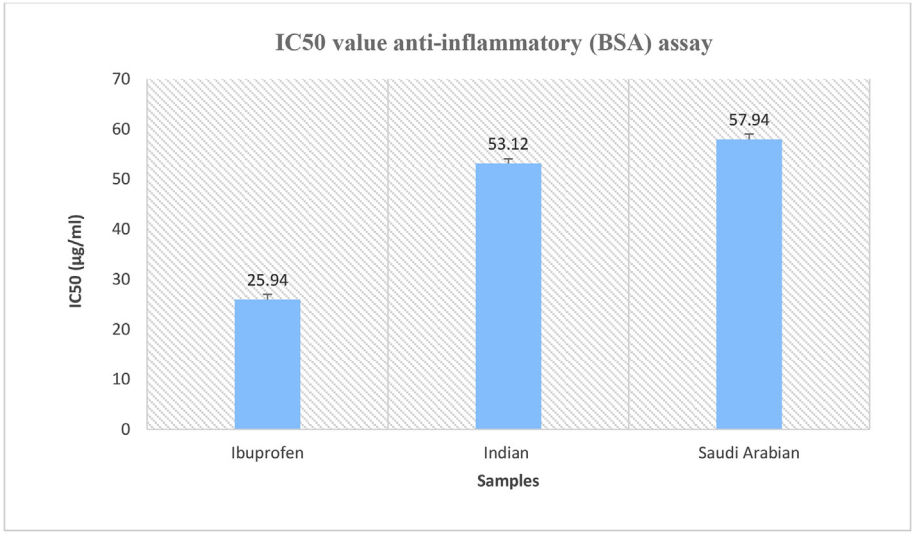

Figure 4: IC50 value of BSA inhibitory assays for essential oils from A. subulatum (Indian and Saudi Arabian samples), and Ibuprofen.

Table 1: Composition of volatile oil hydro-distilled from the Indian and Saudi Arabian *A. subulatum*.

Composition (A)		Percentage Area (B)		RI Lit. (C)	RI Exp. (D)
Monoterpene hydrocarbons		Indian	Saudi Arab		
1.	β-Thujene	0.28	0.3	925	928
2.	α-Pinene	1.41	1.87	932	938
3.	β-Pinene	3.38	3.98	974	976
4.	β-Myrcene	2.53	2.26	988	992
5.	3-Carene	0.26	0.26	1005	1011
6.	D-Limonene	1.13	1.29	1024	1028
7.	beta.-Ocimene	0.17	0.18	1048	1055
8.	γ-Terpinene	1.64	1.79	1054	1058
9.	α- Terpinolen	1.62	1.71	1086	1088
10.	p-Mentha-1,3,8-triene	0.34	0.30	1118	1121
% Monoterpene hydrocarbons		12.76%	13.94%		
Oxygenated monoterpene					
11.	1,8 Ceniole	44.24	46.22	1026	1032
12.	cis-Sabinene hydrate	0.34	0.33	1065	1070

(*continued on next page*)

Table 1 (*continued*)

Composition (A)		Percentage Area (B)		RI Lit. (C)	RI Exp. (D)
Monoterpene hydrocarbons		Indian	Saudi Arab		
13.	Linalool	2.14	2.08	1095	1093
14.	trans-Sabinene hydrate	0.59	0.55	1098	1099
15.	cis-p-Menth-2-en-1-ol	0.31	0.29	1124	1118
16.	p-Mentha-1(7),3-diene	0.46	0.41	1160	1161
17.	p-Mentha-1,5-dien-8-ol	1.16	1.06	1170	1166
18.	Terpinen-4-ol	5.01	4.83	1180	1077
19.	p-Menth-1-en-9-al	0.23	0.23	1191	1188
20.	α-Terpineol	7.47	7.04	1194	1193
21.	cis-Carveol	0.86	0.70	1206	1202
22.	2-exo-Hydroxy-8-cineole	0.31	0.23	1222	1228
23.	trans-Carveol	0.18	0.16	1226	1231
24.	cis Citral	0.49	0.43	1227	1235
25.	D-Carvone	0.81	0.29	1241	1245
26.	Cis-Geraniol	4.05	3.54	1256	1254
27.	trans Citral	0.63	–	1260	1256
28.	L-Perillaldehyde	–	0.11	1271	1273
29.	trans-Bornyl acetate	0.33	0.29	1290	1285
30.	trans-Pinocarveyl acetate	t	0.12	1309	1302
31.	trans-Geranic acid methyl ester	–	1.26	1322	1323
32.	trans-p-mentha-1(7),8-diene-2-ol	2.46	2.13	1332	1333
33.	α-Terpinyl propionate	0.63	0.60	1428	1420
% Oxygenated monoterpene		72.79%	72.9%		
Sesquiterpenes hydrocarbons					
34.	β-Elemene	0.45	0.40	1389	1392
35.	10s,11s-Himachala-3(12),4-diene	0.15	0.11	1399	1402
36.	Aromadendrene	0.77	0.38	4140	1437
37.	Isoledene	–	0.24	1457	1460
38.	α-Elemene	–	0.11	1477	1475
39.	Germacrene D	0.28	0.24	1476	1481
40.	γ-Muurolene	0.96	0.57	1488	1485
41.	α-selinene	3.14	2.91	1489	1486
42.	β-selinene	1.15	1.00	1496	1495
43.	Bisabolene	0.18	t	1499	1505
44.	(−)-.α-Panasinsen	0.19	0.16	1527	1530
% Sesquiterpenes hydrocarbons		7.27%	6.18%		
Oxygenated sesquiterpenes					
45.	Nerolidol	2.35	2.40	1560	1565
46.	Farnesol	t	0.11	1740	1727
% Oxygenated sesquiterpenes		2.43%	2.51%		
Non-terpenoid					
47.	Tetrasiloxane, decamethyl-	–	1.17	1033	1035
48.	Octanal	0.27	0.27	1068	1073
49.	1,3,7-Nonatriene, 4,8-dimethyl-	0.39	0.39	1105	1113
50.	Cyclododecene, (Z)-	0.11	t	1193	1190
51.	Bicyclo[4.4.0]decane	0.12	–	1241	1244
52.	Methyl cinnamate	–	0.74	1375	1381
53.	4,8,12-trimethyltrideca-1,3,7,11-tetraene	0.24	0.16	1581	1592
54.	Estrone methyl ether	0.13	0.11	2045	2060
55.	9-Octadecenoic acid (Z)-, methyl ester	0.31	0.26	2096	2172
56.	Arsenous acid, tris(tert-butyldimethylsilyl) ester	0.19	–	–	2249
% Non-terpenoid		1.76%	3.15%		

T = trace (less than 0.09%), (−) = not detected, (A): Composition of total essential oils (computed from the total GC peak area), (B): Percentage area, (C): RI exp. − KI Kovats' retention index relative to C8−C3 and 2n-alkanes. (D): RI Lit-KI Kovats' retention index.[13]

Antibacterial activity

In the present study, the assessed EOs exhibited superior antimicrobial potency against all selected microbes; however, the level of microbial growth inhibition was found to be dependent on the concentration of EOs and the microbial strain involved (Table 2).

Antioxidant activities

The FRS activity against DPPH-induced free radicals in Indian and Saudi Arabian samples at 1000 μg/mL was approximately 85.27% and 86.86%, respectively (Figure 1). The EOs of both samples exhibited similar antioxidant contents. The IC_{50} values of EOs for the Indian samples

Table 2: Antimicrobial activity of essential oils extracted from Indian and Saudi Arabian *A. subulatum*.

Gram-negative bacteria	Al-Mehran (KSA)			AS (Delhi)		
	ZI(MM)	MIC (% V/V)	MBC (% V/V)	ZI(MM)	MIC (% V/V)	MBC (% V/V)
P. aeruginosa	15.00 ± 0.00	0.5	1	15.00 ± 0.81	0.5	1
E. coli	16.00 ± 0.00	0.5	1	14.66 ± 0.94	0.5	2
A. baumanii	12.33 ± 0.94	1	4	12.66 ± 0.47	1	4

Zone of inhibition (ZI, mean ± SD of triplicates).

(219.38 μg/mL) were marginally higher than the Saudi Arabian samples (203.79 μg/mL) (Figure 2).

Anti-inflammatory activity

The percent inhibition of protein denaturation by Ibuprofen was approximately 83.33% at 100 μg/mL. The EOs of Indian and Saudi Arabian *A. subulatum* samples exhibited inhibition of 69.09% and 66.81%, respectively (Figure 3). The IC_{50} value for Ibuprofen was 25.94%, whereas the EOs of Indian and Saudi Arabian *A. subulatum* samples exhibited IC_{50} values of 53.12 μg/mL and 57.94 μg/mL, respectively (Figure 4).

Discussion

The EO yields of both *A. subulatum* samples were similar to those reported in Sikkim and Himachal Pradesh (India) cultivars and in local varieties,[5] but lower than that of Nepalese varieties.[2,4]

The results of the current study demonstrated a high proportion of oxygenated monoterpenes, such as 1,8-cineole, α-terpineol, terpinen-4-ol, trans-p-mentha-1(7),8-dien-2-ol, and linalool in the extracted EOs. The obtained compositions are dissimilar to those reported in the literature. Kaskoos et al. reported high amounts of monoterpenes, including eucalyptol (77.4%) and β-myrcene (5.0%), and low amounts of α-terpineol (4.9%), terpinen-4-ol (2.3%), and caryophyllene (2.3%).[3] Bhandari et al. also reported a completely different composition of EOs in a sample obtained from Uttarakhand (India) and demonstrated high amounts of eucalyptol (73.27%), along with α-terpineol, limonene, α-terpinyl acetate, α-pinene, and other compounds.[19] Noumi et al. analysed EOs of *A. subulatum* fruits obtained from Jeddah, KSA, and demonstrated the presence of 1,8-cineole (41.7 ± 1.6%), similar to our results; however, other reported components exhibited only qualitative similarity with our results while exhibiting quantitative variations.[10] Different types of terpenes, such as monoterpenes, sesquiterpenes, and diterpenes, resulting from the combination of 2, 3, and 4 units of isoprene (C5 units), respectively, exhibit a wide range of pharmacological activities and represent a large class of natural products.[14] Several studies have reported a similar range of different classes of terpenoids.[5,10,20] In several studies, quantitative variation in the composition of *A. subulatum* EO has been attributed to changes in the environmental, geographical, and genetic variability of the cultivated area.

EOs from Indian and Saudi Arabian samples exhibited higher antibacterial activity against gram-negative bacteria, with inhibition zones ranging from 15 to 12.33 mm. The present data demonstrated that *A. subulatum* EOs exhibit antimicrobial inhibitory activity against all selected gram-negative bacteria in the range of 0.5%v/v−1%v/v for MIC and 1%−4% for MBC. No major differences were observed between the samples. Among bacteria, *P. aeruginosa* and *E. coli* exhibited equal susceptibility to EOs obtained from both samples, whereas *A. baumannii* exhibited less susceptibility. Bachir and Benali demonstrated slightly higher sensitivity of gram-negative bacteria to EOs compared with that of gram-positive bacteria.[21]

Generally, gram-positive bacteria are more sensitive to antibiotics and EOs than gram-negative bacteria. The lower sensitivity of gram-negative bacteria could be attributed to their additional cell walls, which act as barriers to restricting the entry of hydrophobic compounds. Contrary to the general myth, Mith et al., after studying the effects of 15 commercial EOs against 8 bacterial strains, reported that a majority of EOs exhibit activity against gram-positive bacteria; however, *Origanum majorana* EO was more active against gram-negative bacteria than gram-positive bacteria.[15] The antibacterial activity of *A. subulatum* is due to the presence of active antibacterial components in the EO.

Antimicrobial components identified in the oil of both samples included 1,8-cineole, α-pinene, β-pinen, geranic acid methyl-ester, β-myrcene, nerolidol, γ-terpinene, and α-terpinolen. These compounds may contribute to the activity against gram-negative bacteria. Eucalyptol (1,8-cineole) is the major compound identified in *A. subulatum*. Hendry et al. studied the antimicrobial activity of 1,8-cineole against gram-negative bacteria, including *E. coli* and *P. aeruginosa*, and reported higher activity of 1,8-cineole against gram-negative bacteria. Overall, their results indicated that EOs containing 1,8-cineole are more active than 1,8-cineole alone.[22] Li et al. conducted a study regarding EOs of *C. longepaniculatum* leaf containing 1,8-cineole (48.55%) and reported excellent activity against gram-negative bacteria, which may be due to the hydrophobicity of 1,8-cineole.[23]

Other constituents, such as α-terpineol, terpinen-4-ol, linalool, limonene, p-menthane chemotype, α-pinene, α-selinene and β-selinene, nerolidol, terpinene, and terpinolene also contribute to antibacterial activity against gram-negative bacteria.[24–28] Several other studies have also demonstrated the antimicrobial activity of *A. subulatum* EO against gram-negative bacteria.[4,6,9,10,29] Dose-dependent increases in antioxidant effects of both samples with increasing concentration was also observed ($p < 0.001$). Ascorbic acid (standard) exhibited greater antioxidant activity than EOs obtained from both samples. The antioxidant activity of *A. subulatum* is mainly due to the presence of a high content of 1,8-cineole,[30] α-terpineol,[31] terpinen-4-ol,[32]

β-pinene and α-pinene,[33] linalool,[34] and β-myrcene.[30] Furthermore, our results are aligned with reports by several other investigators. Such studies also directly support the antioxidant potential of *A. subulatum* EO.[8,35] Protein denaturation % inhibition is normally the degree of protein stabilization measured against the control. The anti-inflammatory drug Ibuprofen and EOs showed a reduction in protein denaturation, and confirmed the anti-inflammatory activity of *A. subulatum* fruit Eos which may be helpful in the management of inflammatory conditions.[36] A previous study demonstrated concentration-based bovine albumin denaturation inhibition by EOs, which is consistent with our study.[37] The higher anti-inflammatory activity of Indian *A. subulatum* samples compared with KSA samples may be due to the presence of higher percentages of 1,8-cineole, α-terpineol, β-pinene, α-pinene, linalool, among several other components.[32–38]

Conclusions

EOs from the fruit of *A. subulatum* obtained from KSA and India exhibited qualitatively similar, but quantitatively different, compositions. No significant differences in pharmacological properties were observed while correlating the activities of both samples. Overall, higher antibacterial activity against selected gram-negative bacteria, moderate antioxidant activity comparable to that of standard ascorbic acid, and excellent anti-inflammatory activity similar to Ibuprofen were observed in both samples. Thus, EO of *A. subulatum* may be a suitable candidate as a novel alternative antibacterial and anti-inflammatory agent. Further studies involving marketed samples are required to confirm the useful pharmacological properties.

Recommendations

Further pharmacological evaluations should be carried out to determine the extent of other medicinal properties of *A. subulatum* EOs from different geographical origins.

Source of funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

This study does not contain any experimental research with humans or animals performed by any of the authors. Ethical approval was exempted.

Authors' contributions

AA collected samples, conducted the research, interpreted the data, and wrote the entire manuscript. VS conceived and designed the study, provided logistic support, and revised a draft of the article, as well as interpreted the data of the article. Both authors have critically reviewed and approved the final draft, and are responsible for the content and similarity index of the manuscript.

Acknowledgments

The authors are grateful to Dr. Raees, College of Pharmacy, King Saud University, Riyadh, for advice regarding antioxidant and antimicrobial activity, and Dr. Afroz Bakht, College of Science, Prince Sattam Bin Abdulaziz University, Al-Kharj, for advice regarding GC–MS analysis.

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How to cite this article: Alam A, Singh V. Composition and pharmacological activity of essential oils from two imported *Amomum subulatum* fruit samples. J Taibah Univ Med Sc 2021;16(2):231–239.