Original Article

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

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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.

Un挠ياً على ذلك فقد أظهرت عينات السوق الهندي والعسدي فييمي التأثير المضاد للالتهابات المعززة بتركيز المادة المذابة التفاعل الشمسي بنسبة 82.12% و55.62% ميكرورايميل على التوالي في اختبار تنبؤ النمو البكتيري. كما أن الزراعة في المناطق المعتدلة أو شديدة القذار دواء الأعورونين.

الاستنتاجات: أن مكونات تركيبية القنبتين من كلتا العينتين متشابهين نوعاً و لكن مع وجود بعض الاختلافات الكمية للمركب تم الدراسة عليه. كما أنه لم يلاحظ أي اختلافات كبيرة في الخصائص البدنية لهما مما يتطلب إجراء المزيد من الدراسات لمزيد من التأكد.

الكلمات المفتاحية: حب الحيل الهندي; العينات المضادة للالتهابات; الزروب الأساسي; الكرموتوغرافيا الغازية; النشاط الدوائي

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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 IC₅₀ 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

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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, gonorrhea, 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-cineole (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 alphaterpineol as the major constituents 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, pino-carvone, nerolidol, and pino-carvone.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 monoterpene and sesquiterpene, 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$_2$CO$_3$ and stored in amber coloured boro-silicate 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 1500 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 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. The composition of each sample was divided into different groups of terpenes such as monoterpen hydrocarbons, oxygenated monoterpenes, sesquiterpen 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. 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.

**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. 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. 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. 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:

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

**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. 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:

\[
\% \text{ inhibition BSA} = \left(1 - \frac{Ab_{sample}}{Ab_{control}}\right) \times 100
\]

**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-terpinol (7.04% and 7.47%, respectively), terpinen-4-ol (4.83% and 5.01%, respectively), β-pinene (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, isoleadene, 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](image1.png)

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

![Figure 2](image2.png)

**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*.

| Monoterpene hydrocarbons | Composition (A) | Percentage Area (B) | RI Lit. (C) | RI Exp. (D) |
|--------------------------|-----------------|---------------------|-------------|-------------|
|                          |                 | Indian   | Saudi Arab |             |             |
| 1. b-Thujene             |                 | 0.28     | 0.3        | 925         | 928         |
| 2. a-Pinene              |                 | 1.41     | 1.87       | 932         | 938         |
| 3. b-Pinene              |                 | 3.38     | 3.98       | 974         | 976         |
| 4. b-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. y-Terpine             |                 | 1.64     | 1.79       | 1054        | 1058        |
| 9. a-Terpinolene         |                 | 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 Cineole          |                 | 44.24    | 46.22      | 1026        | 1032        |
| 12. cis-Sabinene hydrate |                 | 0.34     | 0.33       | 1065        | 1070        |

(continued on next page)
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 \( \mu g/mL \) 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...
koos et al. reported high amounts of monoterpenes, 
which are dissimilar to those reported in the literature. Kas-
and linalool in the extracted EOs. The obtained composi-
tions are a high proportion of oxygenated monoterpene,
including eucalyptol (73.27%), along with 1,8-cineole,
limonene, terpinen-4-ol, trans-p-mentha-1(7),8-dien-2-ol,
and linalool in the extracted EOs. The obtained composi-
tions are dissimilar to those reported in the literature. Kas-
koos et al. reported high amounts of monoterpene,
including eucalyptol (77.4%) and β-myrcene (5.0%), and low
amounts of 1,8-cineole (4.9%), terpinen-4-ol (2.3%),
and caryophyllene (2.3%).3 Bhandari et al. also reported a
different composition of EOs in a sample obtained from
Uttarakhand (India) and demonstrated high amounts of eucalyptol (73.27%), along with 1,8-cineole,
limonene, terpinen-4-ol, and other compounds.18 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 re-
results; however, other reported components exhibited only
gative EOs in a sample obtained from Uttarakhad (India) and demonstrated high amounts of eucalyptol (73.27%), along with 1,8-cineole,
limonene, terpinen-4-ol, and other compounds.18

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%–1% v/v for MIC
and 1%–4% for MBC. No major differences were observed
between the samples. Among bacteria, P. aeruginosa and E.
colii exhibited equal susceptibility to EOs obtained from
both samples, whereas A. baumanii exhibited less suscepti-
bility. 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 gen-
ereal myth, Mith et al., after studying the effects of 15 com-
mercial EOs against 8 bacterial strains, reported that a
majority of EOs exhibit activity against gram-positive bac-
teria; however, Origanum majorana EO was more active
against gram-negative bacteria than gram-positive bacte-
ria.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-pinene, geranic acid
methyl-ester, β-myrcene, nerolidol, γ-terpinene, and α-
terpinol. 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 bacte-
ria, which may be due to the hydrophobicity of 1,8-cineole.23

Other constituents, such as α-terpinene, 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.29–31 Dose-
dependent increases in antioxidant effects of both samples with increasing concentration was also observed (p < 0.001).
Ascorbic acid (standard) exhibited greater antioxidant ac-
tivity 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-terpinen,31 terpinen-4-ol.32

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).
β-pinene and α-pinene, linalool, and β-myrcene. Furthermore, our results are aligned with reports by several other investigators. Such studies also directly support the antioxidant potential of *A. subulatum* EO. 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.

**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.

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**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.

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