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Original Article

Chemical composition and *in-vitro* antioxidant and antimicrobial activity of the essential oil of *Citrus aurantifolia* L. leaves grown in Eastern Oman

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الملخص

اهداف البحث: يدرس هذه البحث مضادات الأكسدة، ونشاط مضادات الميكروبات والتركيب الكيميائي للزيت الأساسي الذي تم عزله من الأجزاء الهوائية لحمض أورانتيفوليا.

طرق البحث: تم جمع الأوراق الطازجة لحمض أورانتيفوليا من مزارع محلية في مدينة صور (المنطقة الشرقية) لسلطنة عمان خلال الفترة من يونيو- يوليو، ٢٠١٥. استخدمت طريقة التقطير المائي لعزل الزيت الأساسي. واستخدم الغاز اللوني- قياس الطيف الكتلي للتعرف ولتحديد كمية المكونات الكيمائية في الزيت. استخدمت طريقة الجرف الحرة للجذور في – المختبر لتحديد نشاط مضادات الأكسدة للزيت الذي تم عزله من أوراق الليمون، بينما تم تقييم النشاط المضاد للجراثيم ضد البكتيريا إيجابية الغرام والبكتيريا سلبية الغرام بطريقة انتشار القرص.

النتائج: تم تحديد ما مجموعة ثلاثة وثلاثين مركبا كيميائيا وتم العثور على د- ليمونين (٦٣.٣٥٪) ليكون المكون الأساسي. تم تحديد مكونات رئيسة أخرى بنسب مختلفة. كما أظهر الزيت الأساسي لحمض أورانتيفوليا نشاطا ممتازا مضادا للجراثيم ضد المكورات العنقودية الذهبية ونشاط متوسطا ضد السلالات الإشريكية القولونية. وأظهر الزيت كذلك نشاطا واعدا لمضادات الأكسدة في المختبر ولكنه أظهر نشاطا مضادا للجراثيم متوسطا.

الاستنتاجات: يتميز الزيت الأساسي لأوراق الليمون العماني بمحتوى عالي من د- ليمونين الذي يجعله مفيدا في الغذاء، والصناعات الدوائية والعطور.

الكلمات المفتاحية: النشاط المضاد للجراثيم؛ حمض أورانتيفوليا؛ الزيت الأساسي؛ د- ليمونين

Abstract

Objectives: This study investigated the chemical composition and the antioxidant and antimicrobial activities of the essential oil isolated from the aerial parts of *Citrus aurantifolia* L.

Methods: Fresh *Citrus aurantifolia* L. leaves were collected from farms in Sur city, located in the Al-Sharqia (Eastern) region of the Sultanate of Oman, during June–July of 2015. The essential oil was isolated using hydrodistillation. Gas chromatography–mass spectrometry (GC–MS) was used to identify and quantify the chemical constituents of the oil. An *in-vitro* 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method was used to determine the antioxidant activity of the isolated oil from the lime leaves while a disc diffusion method was used to evaluate the antibacterial activity against Gram-negative and Gram-positive bacteria.

Results: Thirty-three chemical compounds were identified, with D-limonene (63.35%) forming the major constituent. Other prominent constituents include 3,7-dimethyl-2,6-octadien-1-ol (7.07%), geraniol (6.23%), *E*-citral (4.35%), *Z*-citral (3.29%), and β-ocimene (2.25%). The essential oil of *Citrus aurantifolia* L. leaves showed excellent antibacterial activity against *Staphylococcus aureus* and moderate activity against pathogenic *Escherichia coli* strains. The oil exhibited promising *in-vitro* antioxidant activity (IC_{50} value = 21.57 μg/mL) but showed moderate antibacterial activities.

Conclusions: The essential oil from Omani lime leaves is characterized by a high D-limonene content, making

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it useful for food, pharmaceutical, and perfumery industries.

Keywords: Antibacterial activity; *Citrus aurantifolia*; D-limonene; Essential oil; GC–MS

Introduction

Citrus aurantifolia (L.) Swingle or Omani lime (Family: Rutaceae), is a major citrus crop in the Sultanate of Oman in terms of cultivation, production, and consumption.[1] This edible and medicinal plant is native to Southeast Asia and was widely cultivated in the tropics and subtropics before its introduction to the African and European continents through Oman. It is a perennial, flowering, evergreen tree, which is 3–5 m in height. Its stem is unusually slender and branched, with sharp thorns and spines. Leaves are alternately arranged and elliptical to oval in shape with rounded teeth on their perimeters; they usually measure 4–6 cm in length by 2.5–4.5 cm in width. The tree's flowers are white in color and have a strong aroma. The lime fruits are round in shape (3–5 cm in diameter), and green to yellow in color with a thin skin; they are juicy, aromatic, and acidic in nature.[2] The fruit is known as lime in English and lomi or limah in Arabic. The Omani lime variety resembles the Indian, Mexican, or Floridian key lime.[3]

Citrus aurantifolia L. is a very popular and valued citrus species in the Gulf region due to its nutritional qualities, distinct flavor, and health benefits. Various parts of the plant are used in traditional medicine to treat cataract, cold, sore throat, fever, chest pain, earache, headache, stomach ailments, and edema, and it is considered an antiseptic, anthelmintic, mosquito repellent, anti-scurvy, astringent, digestive, and appetite stimulant, among others.[4] Lime juice and its essential oil are also commonly used in the food, drug, and cosmetic industries because of their medicinal properties and fragrance. The traditional and pharmacological uses of *Citrus aurantifolia* L. plants are attributed to the presence of secondary plant metabolites including flavonoids, coumarins, and terpenoids.[5–7]

Biologists have recently become increasingly interested in the useful biological activities of essential oils, especially their broad antimicrobial abilities against a wide range of pathogenic microbes.[8] This antimicrobial activity is primarily due to their complex chemical composition, including substances belonging to a broad range of chemical classes including terpenes, aldehydes, alcohols, esters, phenols, ethers, and ketones.[9,10] Thus, understanding the chemical constitution of volatile natural essential oils could prove a viable approach to identify and develop novel antimicrobial agents to overcome the problem of antimicrobial drug resistance.[11]

An extensive literature review revealed that very little is known about the chemical composition and antimicrobial activity of the essential oil of *C. aurantifolia* L. leaves grown in Eastern Oman. Hence, the goals of this study were to (1) analyze the composition of the essential oil of *C. aurantifolia* leaves by GC–MS and (2) investigate the antioxidant and antibacterial activity of the isolated oil.

Materials and Methods

Chemicals and test microorganisms

Chemicals and reagents were obtained from a local supplier. A medium-size glass Clevenger apparatus made by Borosil®, India, was used to isolate the essential oil. To evaluate the antibacterial activity, two pathogenic bacterial strains (*Escherichia coli*-ATCC 8739, Gram-negative and *Staphylococcus aureus*-ATCC 29213, Gram-positive) were obtained from the Department of Natural Sciences, Oman Medical College, Sultanate of Oman.

Collection of lime leaves

Fresh *C. aurantifolia* L. leaves grown in Sur city, which is located in the Al-Sharqia region of the Sultanate of Oman, were collected from farms in June–July of 2015. The leaves were identified by a subject expert from Oman Medical College, and a voucher specimen (PHAR425/2015/4) was deposited in the Department of Pharmacy, Oman Medical College, Oman.

Isolation of essential oil by hydro-distillation method

C. aurantifolia leaves (150 g) were washed under running water to remove dust and insects and then cut into small pieces to increase their surface area. The material was transferred to a 1-L round bottom flask and covered with a sufficient quantity of water (approximately 700 mL). Hydro-distillation in a Clevenger apparatus for 6 h yielded a strongly aromatic light green volatile oil.[3] The oil was separated from the aqueous layer, collected in plastic sample tubes, dried over anhydrous sodium sulfate, and stored in the dark at 4 °C until further use. The yield of the isolated essential oil was calculated based on the weight of the fresh leaves.

Gas chromatography–mass spectrometry analysis

A small portion of the essential oil was diluted in diethyl ether to determine the chemical composition using a gas chromatography–mass spectrometry (GC–MS) instrument equipped with an auto sampler, including a Perkin Elmer Clarus 600 GC system fitted with a Rtx-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; maximum temperature = 350 °C) which was coupled to a Perkin Elmer Clarus 600C MS at SQU. Ultra-high purity helium (99.9999%) was used as a carrier gas at a constant flow of 1.0 mL/min. The injection, transfer line, and ion source temperatures were 280, 260, and 260 °C, respectively. The ionizing energy was 70 eV. The electron multiplier (EM) voltage was obtained using auto-tune. All data were obtained by collecting a full-scan mass spectrum within the range of 40–550 amu. The injected sample volume was 1 μL with a split ratio of 100:1. The oven temperature was programmed to begin at 60 °C and heat at a ramp rate of 3 °C/min to a final temperature of 280 °C, which was held for

2 min. Unknown compounds were identified by comparing the experimental spectra with those in mass spectrum libraries (NIST 2011 v.2.2 and Wiley, 9th edition).

Identification of volatile constituents of the essential oil

Volatile constituents were identified based on their retention time relative to *n*-alkanes (C_6–C_{24}), with corresponding literature data in conjunction with that available in mass spectrometry libraries (NIST 2011 v.2.3 and Wiley, 9th edition).

In vitro anti-oxidant activity

The *in vitro* free radical scavenging activity of the essential oil of lime leaves was determined with DPPH using a slightly modified adaptation of a previously reported method.[12] Briefly, 50 µL solutions of the essential oil in ethyl acetate at various concentrations (5–50 µg/mL) were measured using a micropipette and added to 2.95 mL of a DPPH/ethyl acetate solution (0.01 mM) in a test tube. After 30 min in the dark at room temperature 23–28°C, the absorbance (A_t) of the reaction mixture was measured at 517 nm on a UV–Vis spectrophotometer (UV Analyst-CT 8200). Ethyl acetate was used as a blank while DPPH solution was used as the control (A_b). The % inhibition of DPPH radical was calculated using the formula $[(A_b - A_t/A_b) \times 100]$. The IC_{50} value was also calculated using the plot of percentage inhibition versus sample concentration.

Evaluation of antibacterial activity

The antibacterial activity of the lime leaves essential oil was evaluated against Gram-positive and negative pathogenic bacteria, *S. aureus* and *E. coli*, respectively. The antibacterial activity was determined using the disc diffusion method with standard Mueller Hinton agar (MHA) media.[13] Sterile filter paper discs (6 mm in diameter) were impregnated with 5 and 10 µL of pure extracted essential oil and then placed on inoculated petri plates. The plates were then incubated at 37 °C for 24 h before measuring the diameter of the zone of inhibition (clear zone) around the disc. The antimicrobial activity of the test samples was compared with that of the positive control, Ampicillin (25 µg/disc). All experiments were performed in triplicate.

Results and discussion

Yield and chemical composition of Omani lime leaf essential oil

The slightly green essential oil was obtained in a 0.53% v/w (0.8 mL) yield (calculated from the fresh weight of the leaves). The results of the GC–MS analysis, including the content and composition of the lime essential oil, are presented in Table 1. A total of 30 chemical substances out of 33 were identified (90.9%). D-limonene (Figure 1) formed the major component (63.35%), followed by 3,7-dimethyl-2,6-octadien-1-ol (7.07%), geraniol (6.23%), *E*-citral (4.35%), *Z*-citral (3.29%) and β-ocimene (2.25%). These results confirm that the Omani lime leaves belong to the limonene chemotype. In a similar study of various lime species, Lota et al., detected a total of 59 chemical constituents with D-limonene, pinene, and sabinene as the major components, followed by citronellal, geranial, linalool, and neral.[14] Lawal et al., analyzed the essential oil of *C. aurantifolia* grown in the Lagos state of Nigeria and determined that D-limonene (45%) and geranial (38%) form the chief constituents in the oil.[15]

Antioxidant activity

A number of studies have recommended the use of essential oils in the food and drug industries as natural antioxidants because of the combination of their promising antioxidant activities and relatively safe toxicological profiles.[16] In 2000, Choi et al., tested the antioxidant activity of 31 essential oils from citrus fruits and found them to be similar or better antioxidants than Trolox.[17] Prompted by these and other studies, a simple and reliable *in vitro* assay method using DPPH free radicals was used to investigate the antioxidant potential of the essential oil isolated from lime leaves from the Al-Sharqia region. The results of the antioxidant assay, which are presented in Table 2, indicate that in concentrations

Table 1: Chemical composition of the essential oil isolated from Al-Sharqia lime leaves.

S. No.	Compound name	RT (min)	KI	% composition
1.	α-Pinene	5.152	924.24	1.7485
2.	Sabinene	6.063	974.36	0.2596
3.	β-Pinene	6.16	911.26	0.3195
4.	D-Limonene	7.558	1026.03	63.3539
5.	Trans-β-Ocimene	7.774	1039.02	0.4397
6.	β-Ocimene	8.078	1028.74	2.2450
7.	β-Thujene	8.403	1060.86	0.0625
8.	Isoterpinolene	9.302	1092.08	0.0503
9.	Linalool	9.692	1090.2	1.6491
10.	UI	10.386	1127.01	0.0476
11.	Limonene oxide	10.7	1136.90	0.0563
12.	Trans-Limonene oxide	10.841	1141.35	0.0749
13.	Citronellal	11.307	105.06	0.8985
14.	Terpinen-4-ol	12.174	1183.4	0.0562
15.	Cis-Verbenol	12.271	1186.46	0.0885
16.	L-α-Terpineol	12.64	1222.44	0.2821
17.	3,7-dimethyl-2, 6-Octadien-1-ol	13.821	1234.81	7.07054
18.	Z-Citral	14.157	1236.01	3.2880
19.	Geraniol	14.666	1261.05	6.2331
20.	E-Citral	15.11	1267.35	4.3481
21.	UI	17.83	1361.21	0.0439
22.	Neryl acetate	18.046	1368.14	0.6930
23.	Geranyl acetate	18.653	1387.59	1.8138
24.	β-Elemene	18.935	1396.63	0.1800
25.	Trans-Caryophyllene	19.78	1407.75	1.6035
26.	Humulene	20.82	1458.94	0.2702
27.	(−)-Germacrene D	21.644	1486.22	0.0965
28.	UI	22.424	1512.72	0.1776
29.	α-Springene	23.41	1547.20	0.0958
30.	γ-Elemene	23.865	1563.11	0.2437
31.	Caryophyllene oxide	24.613	1565.53	0.4910
32.	Spathulenol	26.01	1639.92	0.2303
33.	α-Bisabolol	27.506	1694.72	0.0777

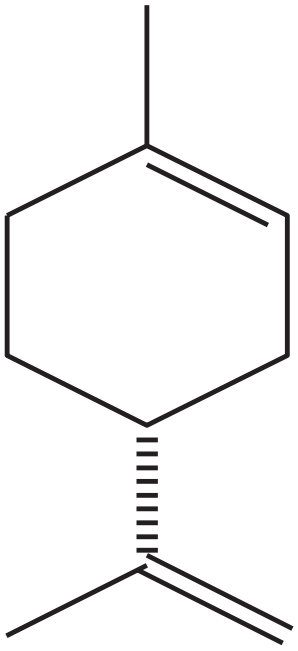

Figure 1: Chemical structure of D-limonene.

Table 2: *In- vitro* anti-oxidant activities of lime essential oil and ascorbic acid by the DPPH method.

Concentration (μg/mL)	% inhibition of DPPH	
	Ascorbic acid	Lime oil
5	42.18 ± 3.46	27.9 ± 1.20
10	70.06 ± 2.09	63.23 ± 0.27
25	92.89 ± 0.81	85.93 ± 1.99
50	93.83 ± 0.19	87.54 ± 0.36
IC_{50} value	13.68	21.87

Values are mean ± SD, n = 3.

of 5−50 μg/mL, the oil has a comparable antioxidant activity (27.9−87.54%) to that of a reference compound, ascorbic acid (42.18−93.83%). The free radical scavenging activities of the test and reference compounds increased as concentrations increased; however, ascorbic acid (IC_{50} = 13.68) was found to be approximately 1.5 times as potent as the essential oil based on the IC_{50} value.

Antibacterial activity

Essential oils have been well known to exert antimicrobial activity and are potential candidates for the development of antimicrobial agents from alternative sources.[18,19] Because of their lipophilic nature, essential oils can interact with and alter the permeability of the cell membrane in microorganisms, eventually leading to the microorganism's death.[20] The antimicrobial spectrum of a volatile oil invariably depends upon its chemical composition. In the present study, D-limonene was found to be the major chemical constituent of the citrus oil, contributing to its sharp aroma and antibacterial actions.[21] The results of the antibacterial activity analysis (reported as the diameter of the zone of inhibition, Table 3) indicate that the essential oil exerts dose-dependent activity with more pronounced effects against *S. aureus* (5.8−7.9 mm) than in *E. coli* (1.7−3.1 mm). However, its antibacterial activity is much weaker than that of the positive control, ampicillin.

Table 3: Antibacterial activity of the essential oil isolated from lime leaves.

Essential oil/Standard	Conc/disc	Inhibition zone (mm)[a] against microbes	
		S. aureus	*E. coli*
Al- Sharqia region	5 μL	5.8 ± 1.5	1.7 ± 0.2
	10 μL	7.9 ± 1.2	3.1 ± 0.6
Ampicillin	25 μg	19.3 ± 1.2	19.0 ± 0.0

[a] Values are mean ± SD; n = 3.

Citrus oils are generally recognized as safe (GRAS) and therefore, lime essential oil can be used as a natural flavoring agent or food additive for its aroma as well as its antioxidant and antibacterial activities. D-limonene, the major constituent of lime oil, is an effective gastroprotective agent[22]; therefore, lime oil may be used in combination with anti-inflammatory agents to overcome their gastrotoxicity. Lime essential oil can also be explored as an alternative to conventional therapy for some common ailments.

Conclusions

A GC−MS analysis of lime leaf essential oil detected 33 volatile chemical compounds, three of which remain unidentified (9.1%). D-limonene was found to be the major constituent, confirming the limonene chemotype of the Al-Sharqia lime variety. The lime leaves oil demonstrated concentration-dependent inhibition of DPPH radicals with an IC_{50} value of 21.87 μg/mL. Its *in-vitro* free radical scavenging activity was nearly comparable to that of ascorbic acid at a concentration of 50 μg/mL. On the other hand, the oil had moderate anti-bacterial activities. Further, more detailed studies are recommended to explore the potential of *C. aurantifolia* L. leaves essential oil as a food preservative and a source of natural antioxidants.

Conflicts of interest statement

The authors have no conflict of interest to declare.

Authors' contribution

SAK designed the experiment, analyzed the results and edited the manuscript; TA performed the antioxidant activity and wrote the manuscript; MSA, NMA, and SSA performed the experiment, collected the data, and analyzed the results (all three authors contributed equally). All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jtumed.2017.12.002.

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