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CHEMICAL COMPOSITION, ANTIFUNGAL AND ANTIBACTERIAL ACTIVITIES OF ESSENTIAL OIL FROM *LALLEMANTIA ROYLEANA* (BENTH. IN WALL.) BENTH.

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ABSTRACT

In this study, the volatile constituents from the aerial parts of *Lallemantia royleana* Benth. have been measured by gas chromatography and gas chromatography–mass spectrometry. The antimicrobial activity was determined by both the disk diffusion method and the microbroth dilution method. Thirty-seven compounds were identified and the main constituents were *trans*-pinocarvyl acetate (26.0%), pinocarvone (20.0%), β-pinene (1.5%), (*E*)-β-ocimene (4.1%), terpinolene (1.1%), linalool (3.4%), *trans*- pinocarveol (1.6%), 3-thujen-2-one (5.1%), myrtenal (1.5%), verbenone (7.1%), *trans*-carveol (5.3%), *cis*-carveol (3.5%), pulegone (4.4%), carvacrol (1.6%), dihydrocarvyl acetate (2.5%) and β-cubebene (2.1%). Antifungal screening of the essential oil of *L. royleana* showed that the oil significantly inhibited the growth of *Candida albicans* and *Aspergillus niger* (minimum inhibitory concentration [MIC] = 3.1 and 2.5 μg/mL, respectively). Antibacterial screening of *L. royleana* essential oil showed that all assayed concentrations significantly inhibited the growth of *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* at $P < 0.05$. MIC for *S. aureus*, *B. subtilis* and *K. pneumoniae* were 5.6, 4.8 and 3.5 μg/mL, respectively; *L. royleana* oil was inactive against *P. aeruginosa* in this study. Therefore, *L. royleana* essential oil has antifungal and antibacterial activities, corroborating the traditional therapeutic uses of this plant, and can be used in the therapy of infectious diseases as well as an antimicrobial additive in foods.

PRACTICAL APPLICATIONS

Today, many classes of synthetic antifungal and antibacterial drugs are used in medicine to treat infections. However, these antifungal and antibacterial therapies are showing diminishing effectiveness because of the emergence of drug-resistant strains. Hence, using effective natural antifungal and antibacterial agents with fewer side effects is a promising approach to combat fungal and bacterial infection and diseases in medicine, agriculture and related fields. In this study, we have demonstrated the essential oil from *Lallemantia royleana* (Benth. in Wall.) Benth. to be a novel natural material to inhibit the fungal (*Candida albicans* and *Aspergillus niger*) and bacterial (*Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) growth, and we have evaluated the chemical composition of *L. royleana* essential oil as an antifungal and antibacterial agent.

INTRODUCTION

In the past, healing herbs were the primary medicinal agents used by people. Knowledge of the properties and beneficial effects of traditional herbal medicines coupled with basic human nature has led to their increasing use. Even in recent years, medicinal plant therapy is greatly used for treatment of many human and animal diseases. Herbs continue to be a very important resource due to their complex molecular compositions. The herbs produce ingredients through secondary metabolism and many of these derivatives (e.g., phenolic compounds, coumarins, alkaloids, tannins, terpenoids, glycosides, flavonoids and isoflavonoids) have antimicrobial properties (Sharifi Rad *et al.* 2013). The natural products derived from tradi tional herbs or essential oils have been used for their antioxidant, antibacterial, antiparasitic, antifungal, antiviral, anti-dermatophyte, anti-*Fusarium* cytotoxic activities, and for food safety (Sharifi Rad *et al.* 2014; Akter *et al.* 2014; Bhagwata and Datara 2014; El-Sawia *et al.* 2014; Horn and Duraisingh 2014; Hoseini Alfatemi *et al.* 2014; Marčetić *et al.* 2014; Tangarife-Castaño *et al.* 2014; Znati *et al.* 2014).

Lallemantia royleana (Benth.) Benth. of the Lamiaceae is an important medicinal plant that is originally native to tropical Asia, throughout Iran, India, Pakistan and Afghanistan. *L. royleana* seeds are ethnobotanically well founded throughout the world for the therapy of inflammation, respiratory problems and abscesses (Abdulrasool *et al.* 2011; Abbas *et al.* 2012). Traditionally, it is a specific ordinary exercise that local people use native plants to treat infectious diseases. These plant products or native plants are designated as ethnomedicine. However, there have been few studies on the mechanisms of action and photochemistry of these important medicinal plants. However, traditional knowledge does report that these plants possess ability to ameliorate infectious diseases. Today, these ethnomedicines have been receiving important consideration by researchers and pharmaceutical industries to complement or replace current chemotherapies.

This study has been performed to investigate the chemical composition and antifungal and antibacterial activities of essential oil from *L. royleana* aerial parts.

MATERIALS AND METHODS

Plant Materials and Essential Oil Preparation

The aerial parts of *L. royleana* were obtained in July 2013 from wild plants in the mountains of Meymand (coordinates: 28°52′04″N and 52°45′12″E), Firuzabad County, Fars Province, Iran. The plant was taxonomically identified by a botanist at the herbarium of Pharmacognosy Department of the Faculty of Pharmacy affiliated to Shahid Beheshti University of Medical Sciences of Iran. The aerial parts of plant materials were dried in the shade for 3 days. For the essential oil, the dried aerial parts (leaves, stems and flowers) (100 g) were hydrodistilled for 4 h utilizing an all-glass Clevenger-type apparatus in accordance with the method outlined by the British Pharmacopeia (British Pharmacopeia 1998). The essential oil obtained was dried over anhydrous sodium sulfate (Sigma-Aldrich St. Louis, MO, USA) and stored at 4C before gas chromatography–mass spectrometry (GC–MS) analysis and further assays.

The Oil Components Separation and Identification

In this study, gas chromatographic (GC) analysis was carried out using a Perkin-Elmer gas chromatograph, Model 8500, equipped with a FID detector and a BP-1 capillary column (30 m × 0.25 mm, film thickness 0.25 μm). Oven temperature was performed as follows: 50–250C at 3°/min; 210–240C at 20°/min and maintained for 9 min, injector temperature 250C; detector temperature, 265C; carrier gas, N_2 (1 mL/min); split ratio of 1:50. The GC–MS analysis was carried out using Hewlett-Packard 6890 mass selective detector coupled with a Hewlett-Packard 6890 gas chromatograph operating at 70 eV ionization energy, equipped with a cross-linked 5% PH ME siloxane HP-5MS capillary column (30 m × 0.25 mm i.d. × 25 μm film thickness) with He as the carrier gas and split ratio 1:50. Retention indices (RIs) were determined using retention times of *n*-alkanes that were injected after the essential oil under the same chromatographic conditions. Compounds were identified by comparison of mass spectral fragmentation patterns and RIs (HP-5) with Wiley 7n.L Mass Spectral Library (Wiley, New York, NY), Adams Library, and Mass Finder 2.1 Library data published mass spectra data (Joulain *et al.* 2001; Adams 2007; Mc Lafferty 2009). The relative percentages of the components of the essential oil were obtained according to the peak area in the chromatogram (Shibamoto 1987).

Antifungal and Antibacterial Assays Using Disk Diffusion Method and Minimum Inhibitory Concentration Determination

In this study, the essential oils were assayed against two fungi (*Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 9142) and two gram-positive bacteria (*Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633) and two gram-negative bacteria (*Klebsiella pneumoniae* ATCC 10031 and *Pseudomonas aeruginosa* ATCC 9027). The fungi and bacteria were cultured at 37C for 14–24 h and the densities were adjusted to 0.5 McFarland standards at A_{530} nm (10^8 cfu/mL). The antimicrobial assays were performed by the disk diffusion method (Bauer *et al.* 1996). About 100 μL

of the microbial suspensions (10^8 cfu/mL) were spread on nutrient agar (Merck, Darmstadt, Germany) plates (100 × 15 mm). Disks (6 mm diameter) were impregnated with 10 μL of different concentrations of essential oil (10, 20, 40, 60, 80 and 100 μg/mL) and placed on the inoculated agar. All the inoculated plates were incubated at 37C for 24 h. In this study, we used positive control disks comprised of ketoconazole, ampicillin and gentamicin (10 mg/disk) for fungi, and gram-positive and gram-negative bacteria, respectively. In addition, we used dimethyl sulfoxide as the negative control. Antimicrobial activity was assessed by measuring the zone of inhibition.

Minimum inhibitory concentration (MIC) was determined using serial dilutions of the essential oils (0–100 μg/mL) using microdilution test confirmed by Clinical and Laboratory Standards Institute (Wayne 2006). The bacteria and fungi strains were suspended in Luria–Bertani media and the densities were regulated to 0.5 McFarland standards at 530 nm (10^8 cfu/mL). The bacteria and fungi suspensions (100 μL) and the essential oil (100 μL) were added to microtiter plates and incubated at 37C for 24 h. In this study, sterility control was medium without bacteria and fungi. Growth control was medium with bacteria and fungi but without essential oil. The growth in each well was compared with that of the growth in the control well. The MICs were visually detected in comparison with the growth in the control well and delineated as the lowest concentration of the ingredients with >95% growth inhibition.

Statistical Analysis

The essential oil was prepared in triplicate for chemical characterization, and antifungal and antibacterial tests. Data were subjected to analysis of variance following a completely random design to determine the least significant difference at $P < 0.05$ using SPSS v. 11.5 (IBM SPSS, New York, USA).

RESULTS

The chemical composition of *L. royleana* is shown in Table 1. Analysis of the essential oil showed the main ingredients were *trans*-pinocarvyl acetate (26.0%), pinocarvone (20.0%), β-pinene (1.5%), (*E*)-β-ocimene (4.1%), terpinolene (1.1%), linalool (3.4%), *trans*-pinocarveol (1.6%), 3-thujene-2-one (5.1%), myrtenal (1.5%), verbenone (7.1%), *trans*-carveol (5.3%), *cis*-carveol (3.5%), pulegone (4.4%), carvacrol (1.6%), dihydrocarvyl acetate (2.5%) and β-cubebene (2.1%).

The antifungal and antibacterial results of disk diffusion test are summarized in Tables 2 and 3, respectively. The essential oil showed a significant inhibition at all assayed concentrations of the growth of *C. albicans* and *A. niger* at $P < 0.05$. MICs for *C. albicans* and *A. niger* were 3.1 and 2.5 μg/mL of essential oil, respectively. In addition, *L. royleana* oil significantly inhibited the growth of gram-positive bacteria, *S. aureus* and *B. subtilis*, as well as the gram-negative bacterium, *K. pneumoniae*. MICs for *S. aureus*, *B. subtilis* and *K. pneumoniae* were 5.6 ± 0.7, 4.8 ± 0.5 and 3.5 ± 0.0, respectively. *P. aeruginosa*, however, was only marginally inhibited in the disk diffusion assay and showed no activity in the broth dilution assay (MIC > 100 μg/mL).

TABLE 1. COMPOSITION OF THE ESSENTIAL OIL OF *LALLEMANTIA ROYLEANA*

No.	Name of compound	RI	Relative % in essential oil
1	Sabinene	979	0.6
2	β-Pinene	981	1.5
3	3-Octanone	983	0.4
4	Myrcene	992	0.2
5	*p*-Cymene	1022	0.1
6	Limonene	1028	0.8
7	1,8-Cineole	1034	0.3
8	(*Z*)-β-Ocimene	1039	0.5
9	(*E*)-β-Ocimene	1050	4.1
10	γ-Terpinene	1059	0.1
11	Isobutanol	1063	0.7
12	Terpinolene	1084	1.1
13	Linalool	1099	3.4
14	Butanol	1102	0.1
15	Dehydro-sabina ketone	1116	0.4
16	*iso*-3-Thujanol	1129	0.8
17	*trans*-Pinocarveol	1138	1.6
18	Sabina ketone	1153	0.5
19	Pinocarvone	1164	20.0
20	3-Thujen-2-one	1169	5.1
21	Myrtenal	1190	1.5
22	Myrtenol	1194	0.1
23	Verbenone	1205	7.1
24	*trans*-Carveol	1220	5.3
25	*cis*-Sabinene-hydrate acetate	1222	0.3
26	*cis*-Carveol	1230	3.5
27	Pulegone	1245	4.4
28	*trans*-Sabinene-hydrate acetate	1255	0.4
29	Bornyl acetate	1289	0.3
30	*trans*-Sabinyl acetate	1295	0.4
31	Carvacrol	1303	1.6
32	*trans*-Pinocarvyl acetate	1304	26.0
33	Dihydrocarvyl acetate	1333	2.5
34	α-Longipinene	1352	0.2
35	β-Cubebene	1396	2.1
36	α-*trans*-Bergamotene	1440	0.1
37	Spathulenol	1587	0.2

RI, retention index.

DISCUSSION

Sefidkon *et al.* (2006) analyzed the essential oils of aerial parts of the *Lallemantia peltata* (L.) Fisch. et Mey. from Iran. They

TABLE 2. ANTIFUNGAL ACTIVITY OF ESSENTIAL OIL OF *LALLEMANTIA ROYLEANA* AGAINST TWO FUNGI STRAINS

Essential oil (μg/mL)	*Candida albicans*	*Aspergillus niger*
10	6.2 ± 0.0g	7.7 ± 0.2g
20	12.8 ± 0.4e	13.1 ± 0.0e
40	19.9 ± 0.5d	20.5 ± 0.0d
60	31.4 ± 0.1c	36.2 ± 0.0c
80	44.9 ± 0.0b	49.5 ± 0.3b
100	59.5 ± 0.3a	64.9 ± 0.4a
DMSO (negative control)	4.5 ± 0.2h	4.5 ± 0.4h
Ketoconazole (μg/mL)	9.7 ± 0.1 f	9.7 ± 0.2 f
MIC	3.1 ± 0.0	2.5 ± 0.1

Note: Data are expressed as means ± SD of inhibition zone diameter (mm) for different concentration of essential oil, controls and minimum inhibitory concentration (MIC) (μg/mL). The values with different letters within a column are significantly different ($P < 0.05$; LSD).
DMSO, dimethyl sulfoxide; MIC, minimum inhibitory concentration.

collected plants at full flowering stage from two provinces (Hamedan and West Azerbaijan) of Iran. Thirty-six components were identified that approximately constituted more than 91.1% of the oil from Hamedan and 90.4% of the oil from West Azerbaijan. The main compounds of the essential oils were germacrene D (42.5 and 49.9%), β-caryophyllene (20.6 and 26.0%), germacrene B (5.6 and 1.6%), β-elemene (0.2 and 5.1%) and α-farnesene (4.2 and 0.3%).

Morteza-Semnani (2006) investigated the essential oil of flowering aerial parts of *Lallemantia iberica* Fisch. et C.A. Mey., collected from the suburb of Larijan, north of Iran. The major constituents identified in this oil were *p*-cymene (22.1%), isophytol (19.8%), α-cadinol (11.1%), 3-octanol (8.1%), caryophyllene oxide (7.4%) and terpinen-4-ol (5.7%).

Baser *et al.* (2000) analyzed the oil of *L. peltata* (L.) Fisch. et Mey. from Turkey. Thirteen compounds were recognized representing all of the constituents detected. The major constituents of the oil were germacrene-D (27.4%), (*E*)-β-ocimene (20.1%) and geijerene (12.0%).

Amanzadeh *et al.* (2011) studied the essential oils from the aerial parts of *L. iberica*, collected in two stages (flowering and post-flowering) from plants in Hashtgerd of Iran. Thirty-six chemical components of the essential oils were characterized in the flowering stage with β-cubebene (19.55%), linalool (18.71%), spathulenol (18.04%), β-caryophyllene (11.11%), geraniol (3.50%) and bicyclogermacrene (3.46%) as the major compounds. About 39 components of the post-flowering essential oil were identified with the major constituents caryophyllene oxide (38.77%), linalool (15.15%), germacrene-D (7.03%), β-caryophyllene (5.61%), β-bourbonene (4.96%) and geraniol (4.34%).

The composition of *L. royleana* essential oil in this study is in marked contrast to that reported previously by Ghannadi and Zolfaghari (2003). In the previous report, a sample collected from Kolahghazi (Isfahan Province, Iran), the essential oil was dominated by verbenone (16.4%) and *trans*-carveol (9.8%). These two components were identified in the present work (7.1 and 5.3%, respectively). However, the large concentrations of pinocarvone and pinocarvyl acetate in this sample from Meymand (Fars Province, Iran) were not detected in the Kolahghazi sample. Thus, these two samples likely represent distinct chemotypes of this medicinal plant.

Mahmood *et al.* (2013) have investigated the antibacterial activity of *L. royleana* (Benth.) indigenous to Pakistan. That study showed that *L. royleana* seeds possess significant antibacterial potential against *S. aureus*, *Escherichia coli* and *Enterobacter cloacae*, and complement the results of this present study.

TABLE 3. ANTIBACTERIAL ACTIVITY OF ESSENTIAL OIL OF *LALLEMANTIA ROYLEANA* AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA STRAINS

Essential oil (μg/mL)	*Staphylococcus aureus*	*Bacillus subtilis*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
10	29.3 ± 1.1 f	32.2 ± 0.1 f	24.31 ± 0.2 f	2.9 ± 0.2g
20	38.4 ± 0.1e	41.16 ± 0.1e	27.8 ± 0.9e	3.61 ± 0.0 f
40	41.1 ± 1 d	46.11 ± 0.0d	31.9 ± 0.4d	4.22 ± 0.4e
60	46.3 ± 0.2c	51.31 ± 1.0c	38.1 ± 0.0c	5.81 ± 0.1d
80	49.81 ± 0.0b	57.66 ± 0.8b	42.5 ± 0.1b	6.21 ± 0.0c
100	54.12 ± 0.9a	61.3 ± 0.9a	44.3 ± 1.1a	7.71 ± 0.0b
DMSO (negative control)	3.12 ± 0.0h	2.21 ± 0.0h	2.15 ± 0.0h	1.00 ± 0.0h
Ampicillin	13.9 ± 0.5g	12.8 ± 0.3g	–	–
Gentamicin	–	–	11.8 ± 0.2g	12.7 ± 0.0a
MIC	5.6 ± 0.7	4.8 ± 0.5	3.5 ± 0.0	>100

Note: Data are expressed as means ± SD of inhibition zone diameter (mm) for different concentration of essential oil, controls and minimum inhibitory concentration (MIC) (μg/mL). The values with different letters within a column are significantly different ($P < 0.05$; LSD).
DMSO, dimethyl sulfoxide; MIC, minimum inhibitory concentration.

It is not clear what components in the essential oil of *L. royleana* are responsible for the antimicrobial activity. To our knowledge, neither of the two major components, pinocarvone or *trans*-pinocarvyl acetate, have been individually screened for antimicrobial activity. Nevertheless, the essential oil of *Myrothamnus flabellifolius*, which was rich in pinocarvone and *trans*-pinocarveol, was shown to be both antifungal and antibacterial (Viljoen *et al.*, 2002). *L. royleana* oil is a complex mixture, so synergistic effects of the various components may also be important.

Over the years, humans have used various species of fungi for the production of foods such as blue cheese, soy sauce, and tempeh (Leistner 1990), and for the production of antibiotics (Kück *et al.* 2014). However, several fungi produce toxic metabolites, mycotoxins that can cause acute, subacute or chronic toxicity in animals and humans. The most notorious of the mycotoxins are the aflatoxins, which are produced by several species of *Aspergillus*. Aflatoxins have been shown to be carcinogenic and teratogenic, and can be a major foodborne problem in developing countries where proper food storage is not available (Williams *et al.* 2004).

C. albicans is the most common cause of opportunistic fungal infections and diseases that are prevalent in all parts of the world. Different clinical forms of *C. albicans* infections often occur in people with underlying disorders. This yeast, as part of the normal flora in humans and many other animals, is found capable of causing candidiasis when transferred from one host to another. Therefore, intervention and control measures for combating candidiasis are important.

Bacteria, viruses, fungi, parasites, heavy metals and chemicals can cause food poisoning. The most common bacteria associated with food poisoning include *S. aureus*, *Salmonella*, *Clostridium perfringens*, *Campylobacter*, *Listeria monocytogenes*, *Bacillus cereus* and *E. coli*. These bacteria responsible for more than 90% of food poisoning cases are commonly found in raw foods. Control of these pathogens can be achieved by cooking food thoroughly and avoiding recontamination after cooking and can prevent the occurrence of infections and food poisoning. Staphylococcal food poisoning is an intoxication that is caused by the consumption of foods including adequate amounts of one or more preformed enterotoxins (Le Loir *et al.* 2003). The foods that have been often implicated in staphylococcal intoxication include milk and dairy products, poultry and egg products, salads, bakery products, sandwich fillings, and meat and meat products (Tamarapu *et al.* 2001).

B. subtilis is an omnipresent bacterium usually found in water, air, soil and decomposing plant residue. The bacterium produces an endospore that enables it to endure excessive conditions of heat and desiccation in the environment. *B. subtilis* produces a multiplicity of proteases and other enzymes that allow it to degrade a variety of natural substrates and help to nutrient cycling (Hemila *et al.* 1989).

Salad vegetables have been discovered to be contaminated with some foodborne pathogens such as *Enterobacter cloacae* and *Klebsiella* (Odumeru *et al.* 1997; Van Gerwen *et al.* 1997). *K. pneumoniae* was also identified as a significant foodborne pathogen in fresh produce (Hamilton *et al.* 2006). Because of the high contamination rate of salad vegetables with food pathogens, it is essential to control the hygienic level associated with these products to decrease or control risk of foodborne disease (Francis *et al.* 1999).

P. aeruginosa is a widespread environmental bacterium. It can be found frequently in high numbers, in ordinary food, particularly vegetables. Additionally, it can be recovered in low numbers in drinking water. *P. aeruginosa* can cause a wide range of infections, and is a leading cause of disease in immunocompromised individuals. In particular, it can be a serious pathogen in hospitals (Hardalo and Edberg 1997).

In conclusion, the essential oil of *L. royleana* has a guarantee to be a beneficial medication for fungal, gastrointestinal and cutaneous diseases due to human pathogenic microorganisms. In addition, screening for bioactive phytochemicals from extracts of *L. royleana* should be performed in search of novel chemotherapeutic agents.

CONFLICT OF INTEREST

The authors declare no financial or other conflicts of interest.

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