Agr. Nat. Resour. 53 (2019) 148–153

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Antifungal activity of *Pelargonium graveolens* essential oils from different geographical locations against *Candida albicans*

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Article Info

Article history:
Received 29 Nov 2017
Revised 1 July 2018
Accepted 29 July 2018
Available online 30 April 2019

Keywords:
Candida albicans,
Citronellol,
Geraniol,
Geographical location,
Pelargonium graveolens

Abstract

Vulvovaginal candidiasis, an acute inflammatory disease, is caused by *Candida* sp. especially *C. albicans*. The antifungal activity was studied using micro-broth dilution assay of 16 samples of *P. graveolens* essential oils from different geographical locations (Germany, $n = 5$; India, $n = 3$; Iran, $n = 8$) and different combinations of geraniol and citronellol (10:90; 20:80; 30:70; 40:60; 5:50; 60:40; 7:30; 80:20; 90:10; 95:5, respectively) against 72 clinical isolates of *C. albicans* from vaginal discharges. All nine combinations of geraniol and citronellol tested had the same anti-candidal effects against *C. albicans*. *P. graveolens* essential oil E20 (geraniol, 95%), and E9 (citronellol, 48%; geraniol, 5.1%; citronellol fromate, 16.2%) had minimal inhibitory concentration values (means ± Standard Deviation) of 0.497 ± 0.085 and 1.149 ± 0.083 µL/mL, respectively. Although geraniol and citronellol played an important role in the anti-candidal activity of *P. graveolens* essential oils, other minor components may also have roles in its anti-candidal activity.

Introduction

Vulvovaginal candidiasis, an acute inflammatory disease, is associated with intense pruritus, vaginal discharge, an erythematous vulva and dyspareunia and up to 75% of women of reproductive age experience vulvovaginal candidiasis (Sobel, 2002). *Candida albicans*, which is isolated in 85% of cases, is the largely prevalent etiological agent of vulvovaginal candidiasis (Cassone, 2015). Short-term local or single dose oral therapies are prescribed for 90% of infections (Dovnik et al., 2015). The appearance of azole-resistant isolates of *C. albicans* (Whaley et al., 2016) and adverse effects of antifungal agents (Bodey, 1992) have encouraged researchers to find new antifungal agents among the essential oils.

Pelargonium graveolens essential oil has been known as a potent antifungal agent (Leite et al., 2015; Mahboubi et al., 2018; Pereira Fde et al., 2015; Rosato et al., 2008, 2009) and its antifungal activity has been confirmed against *C. albicans* (Rosato et al., 2008, 2009; Mahboubi et al., 2018) and dermatophytes (Pereira Fde et al., 2015). Although, studies have exhibited the relation between the geraniol and citronellol contents of *P. graveolens* essential oils and antifungal activity (Leite et al., 2015; Pereira Fde et al., 2015), to date, there has been no study comparing the chemical composition of *P. graveolens* essential oils from different geographical origins and their relation with anti-candidal activities. Consequently, the current study evaluated the anti-candidal activity of 16 samples of *P. graveolens* essential oils from Germany ($n = 5$), India ($n = 3$) and Iran ($n = 8$) and the main components of their chemical profiles.

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https://doi.org/10.34044/j.anres.2019.53.2.08

Materials and Methods

Plant materials, essential oils and chemical analysis

The essential oils from Germany (n = 5) and India (n = 3) were purchased from markets, but the essential oil samples from Iran were prepared by gathering the aerial parts of *P. graveolens* at full flowering stage from different geographical regions of Iran (Fig. 1). The plants aerial parts were dried and cut into small pieces and then were prepared using a hydro-distillation method with a Clevenger-type apparatus for 3 hr. The essential oil for each sample was gathered and dried. The chemical compositions of essential oils were determined using gas chromatography in the Agilent technology (HP) 6890 system, with a capillary column (HP-5MS; 60 m × 0.25 mm) and a film thickness 0.25 µm. The oven temperature program was initiated at 40°C, held for 1 min, then raised up to 230°C at a rate of 3°C/min and held for 10 min. Helium was used as the carrier gas at a flow rate 1.0 mL/min. The detector and injector temperatures were 250°C and 230°C, respectively.

Chemical compounds

Amphotericin B (10 µg), ketoconazole (15 µg), clotrimazole (10 µg) discs (Rosco Diagnostica A/S) and amphotericin B powder (Sigma-Aldrich), (-)-β-citronellol (27483, Sigma-Aldrich), geraniol (48798, Sigma-Aldrich) were used in this study.

Microbial strain

The study was conducted on 72 clinical isolates of *C. albicans* obtained from vaginal discharges. The strains were cultured on Sabauraud dextrose agar medium, chloramphenicol and gentamycin, and were identified as *C. albicans* on the basis of some biochemical tests (color of colony, positive germ tube, chlamydospore formation, absorption of carbohydrate and fermentation tests according to Mahboubi and Kazempour (2016). Polymerase chain reaction (PCR) was performed using primers for ITS1 (5′-TCCGTAGGTGAACCTGCGG-3’) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). Each PCR reaction contained 2.5 µL of 10x PCR buffer, 0.4 µM primers, 1.5 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 1.25 U of Taq polymerase, template DNA and dH_2O up to 25 µL. The initial DNA denaturation was performed at 95°C for 5 min. The cycle consisted of a denaturation step at 95°C for 30 s, an annealing step at 55°C for 30 s and an extension step at 72°C for 1 min, with a final extension of 72°C for 5 min. Then 10 µL of each PCR product was directly digested with 5 U (1 µL) of the *Msp*I, 1.5 µL of the digestion buffer and 2.5 µL of dH_2O and then was incubated at 37°C for 180 min. The digested fragments were electrophoresed through 2% agarose gel and then visualized using DNA green safe (Roshan et al., 2014). The antibiogram profiles of clinical isolates were determined using the Kirby-Bauer method for amphotericin B, ketoconazole and clotrimazole. *C. albicans* ATCC 10231 was used as the control strain.

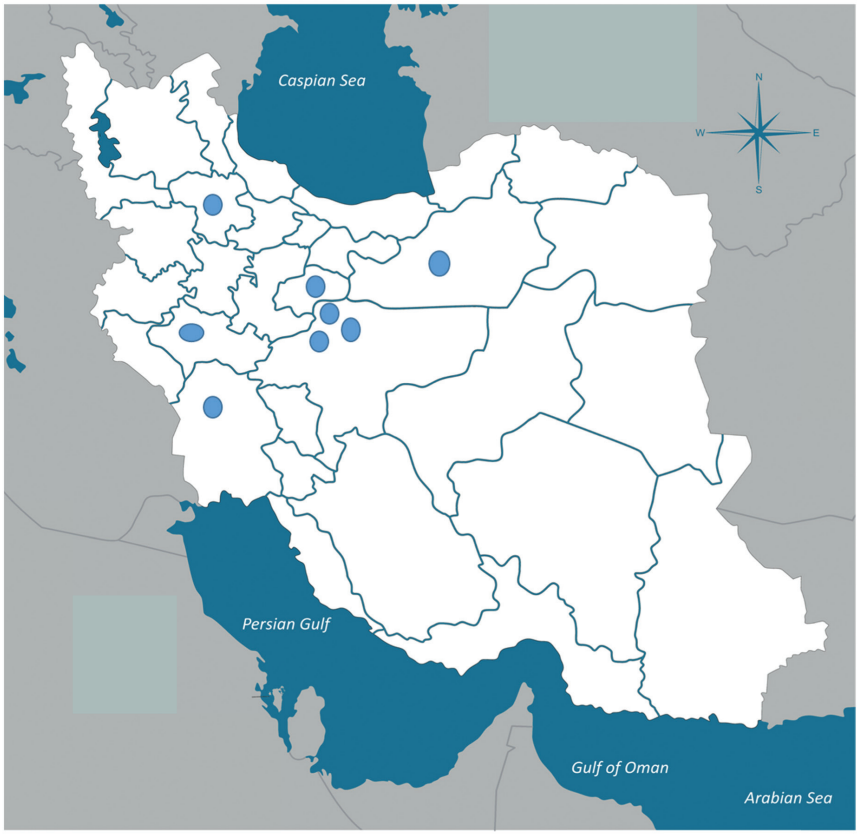

Fig. 1 Sampling locations in Iran of *Pelargonium graveolens* aerial parts

Anti-candidal activity of essential oils using micro-broth dilution assay

The anti-candidal activities of *P. graveolens* essential oils were evaluated using micro-broth dilution assay. At first, the anti-candidal activity of all the samples of *P. graveolens* essential oils (n = 16) was evaluated against *C. albicans* ATCC 10231. Due to the different profiles of the chemical composition of the essential oils and their antimicrobial activities against a standard strain of *C. albicans*, the anti-candidal activity of *P. graveolens* essential oils (E19, E20, E9, E10, E22) were compared with combinations of geraniol: citronellol (10:90; 20:80; 30:70; 40:60; 60:40, 7:30; 80:20; 90:10; 95:5, respectively) against clinical isolates of *C. albicans* (n = 72). Amphotericin B and *C. albicans* ATCC 10231 were used as the control. For the micro-broth dilution method, the candidal suspensions were prepared by suspending the colonies in normal saline and adjusting the turbidity to 0.5 McFarland (1×10^6 colony forming units (CFU)/mL). The candidal suspension was diluted in RPMI 1640 (1×10^4 CFU/mL). The essential oils or geraniol:citronellol combinations were dissolved in dimethyl sulfoxide and diluted serially in RPMI 1640 (32–0.125 µL/mL). 100 µL of diluted essential oils were poured in each well of 96-well micro titer plates. Then, 100 µL of diluted candidal suspension was added to each well. The plates were incubated at 22.5 ± 2.5°C for 48 h. The first well with no visual turbidity was reported as the minimal inhibitory concentration (MIC µL/mL) and the lowest concentration with no growth on Sabauraud dextrose agar was reported as the minimal fungicidal concentration (MFC) (Mahboubi and Kazempour, 2016). All experiments were performed in triplicate.

Statistical analysis

The data of the MIC and MFC values were analyzed using one-way analysis of variance facilitated by the SPSS software package (version 17; Chicago, IL, USA). Differences between means were tested using Duncan multiple range test at a significance test level of 0.05.

Results and Discussion

Antibiogram profile of clinical isolates of C. albicans

All 72 clinical isolates were sensitive to amphotericin B with mean ± SD inhibition zones of 24.5 ± 1.25 mm, while 94.3% and 94.2% of isolates were sensitive to ketoconazole and clotrimazole, respectively, 1.4%, and 5.7% had intermediate sensitivity to ketoconazole and clotrimazole, respectively, and 4.3% of isolates were resistant to ketoconazole.

Chemical compositions of P. graveolens essential oils

The main components of *P. graveolens* essential oils from Germany (Table 1) were identified as: limonene (0–7.8%), linalool (0–5.9%), borneol (0–1.8%), α-terpineol (0.21–3.3%), citronellol (29.5–42.5%), geraniol (10.6–20%), citronellyl formate (1.4–7.9%) and geraniol formate (0.63–3.7%). Therefore, citronellol, geraniol, limonene and citronellyl formate were the main components of the German *P. graveolens* essential oils.

The Indian *P. graveolens* essential oils (Table 1) contained: α-pinene (0–1.9%), limonene (0–1.0%), linalool (0.7–11.0%), borneol (0–1.3%), α-terpineol (0–0.6%), citronellol (0.2–42.6%), geraniol (7.9–95.6%), citronellyl formate (0.3–5.5%) and geraniol formate (1–3.4%). Citronellol was the primary component of *P. graveolens* from India. The other main components varied depending on the source of the samples.

Linalool (0.8–2.2%), citronellol (39.9–53%), geraniol (3.4–8.4%), citronellyl formate (10.9–16.2%) and geraniol formate (1.2–3.0%) were the dominant components of the Iranian samples (Table 2). Thus, citronellol and citronellyl formate were the main components of *P. graveolens* essential oils from Iran, followed by geraniol.

Table 1 Chemical composition of *P. graveolens* essential oil from Germany (n = 5) and India (n = 3)

P. graveolens	RI	Percentage of components in each essential oil							
		E1	E2	E3	E15	E16	E19	E20	E4
α-Pinene	935	-	0.10	4.70	0.30	0.30	0.10	-	1.90
Limonene	1033	7.80	0.12	5.00	0.30	0.20	0.20	-	1.00
Linalool	1102	5.30	1.90	13.40	5.80	5.90	2.30	0.70	11.00
Borneol	1164	0.62	0.12	-	1.80	1.70	1.30	-	-
α-Terpineol	1200	2.10	0.21	3.30	0.70	0.70	-	-	0.60
Citronellol	1230	34.70	42.50	29.50	36.80	35.90	42.60	0.20	30.20
Geraniol	1256	17.90	9.90	10.60	20.00	19.50	7.90	95.60	21.90
Citronellyl formate	1276	1.40	5.80	7.90	7.30	7.30	5.05	0.30	5.00
Geraniol formate	1304	0.63	1.40	2.60	3.70	3.70	1.00	1.60	3.40
Citronellol/geraniol	-	1.90	4.30	2.70	1.84	1.84	5.30	0.00	1.30
Citronellol+geraniol	-	52.60	52.40	40.10	56.80	55.40	50.50	95.80	52.10
Geographical region	-	Germany (E1–E19)						India (E20, E4)	

RI = retention index; E = essential oil.

Table 2 Chemical composition of *P. graveolens* essential oils from different locations in Iran

P. graveolens	Percentage of components in each essential oil							
	E21	E6	E7	E9	E8	E10	E11	E22
α-Pinene	0.80	0.23	0.2	0.30	0.20	0.10	0.40	0.30
Limonene	0.20	0.13	0.07	-	0.15	0.10	0.20	0.10
Linalool	2.20	1.00	1.45	1.60	1.00	1.30	0.80	1.30
Borneol	0.90	0.20	0.15	0.10	0.20	0.20	0.20	0.14
α-Terpineol	0.20	0.14	0.15	0.13	0.16	0.20	0.13	0.20
Citronellol	48.30	41.00	53.00	48.00	46.30	45.60	45.60	39.90
Geraniol	8.40	8.00	7.10	5.10	3.90	5.30	3.40	4.20
Citronellyl formate	10.90	12.70	12.60	16.20	12.30	11.80	14.10	13.40
Geraniol formate	1.70	2.10	1.60	3.00	1.20	2.40	1.60	2.80
Citronellol/geraniol	5.75	5.13	7.40	9.40	11.80	8.60	13.40	9.50
Citronellol+geraniol	56.70	49.00	60.10	53.10	50.20	50.90	49.00	44.10
Yield	0.12	0.08	0.09	0.11	0.09	0.11	0.11	0.11
Region	NoorAbad	Zanjan	Mashhad-e-ardehal	Delijan	Barzook	Lathoor	Shahrood	Dezfool

E = essential oil.

It has been reported that the transplanting time has significant effects on the essential oil composition from *P. graveolens,* with Indian *P. graveolens* essential oils, which were gathered in different months, containing citronellol (21.3–28.7%), geraniol (23.1–38.4%), citronellyl formate (6.3–8.3%) and geranyl formate (3.3–4.3%) as their main components (Verma et al., 2010). The cultivar and method of distillation (Kaul et al., 1995), the distilled organ (Mallavarapu et al., 1997), the age of leaves (Rajeswara Rao et al., 1993), geographical regions (Rajeswara Rao et al., 1990), seasonal changes (Rajeswara Rao et al., 1996), oil storage (Kaul et al., 1997) and the type of distillation unit (Kahol et al., 2001) could change the chemical composition of *P. graveolens* essential oils. The ratio of citronellol to geraniol determines the quality of *P. graveolens* essential oil and high quality *P. graveolens* essential oil has this ratio equivalent to one (Southwell et al., 1995). Therefore, the *P. graveolens* essential oils from the current study could not be considered as high quality.

Anti-candidal activity of P. graveolens essential oils against C. albicans

Screening the anti-candidal activity of *P. graveolens* essential oils against *C. albicans* ATCC 10231 showed that *P. graveolens* essential oils (n = 16) with regard to their anti-candidal effects belonged to four subsets. The highest anti-candidal activity was observed for *P. graveolens* essential oils E10, E20, E9 and E22, while *P. graveolens* essential oils E16 and E3 had less activity against *C. albicans* ATCC 10231 (Table 3). The *P. graveolens* essential oils with high anti-candidal effects were evaluated against clinical isolates of *C. albicans* (n = 72) and combinations of geraniol and citronellol at different ratios were screened against these clinical strains. The results showed that different ratios of geraniol and citronellol (20:80; 30:70; 40:60; 50:50; 60:40; 70:30; 80:20; 90:10, respectively) had the same anti-candidal effects against clinical isolates of *C. albicans* (Table 4). Furthermore, these anti-candidal activities were comparable with *P. graveolens* essential oil E20 with 95% geraniol (MIC and MFC values were 0.497 ± 0.085 µL/mL and 0.774 ± 0.105 µL/mL). Geraniol/citronellol at ratio 10:90 had weaker anti-candidal activity (MIC = 1.054 ± 0.079 µL/mL; 1.391 ± 0.098 µL/ml) than that of other ratios of geraniol/citronellol. *P. graveolens* essential oil samples E20 and E9 had high sensitivity against clinical isolates of *C. albicans*, followed by E22, and E10. Citronellol and geraniol as the main components of *P. graveolens* essential oil play important roles in the anti-candidal activities (Leite et al., 2015). The MIC_{90} for geraniol against clinical isolates of *C. albicans* was reported as 16 µg/mL and the anti-candidal activity of geraniol was not related to cell wall or ergosterol binding (Leite et al., 2015), while the MIC values of geraniol and citronellol against *Trichophyton rubrum* were reported being between 16–256 µg/mL and 8–1024 µg/mL, respectively (Pereira Fde et al., 2015). Citronellol and geraniol inhibited ergosterol biosynthesis and caused leakage of intracellular materials in *T. rubrum* (Pereira Fde et al., 2015). Therefore, the high content *P. graveolens* E20 was responsible for the high anti-candidal effects. Based on the total concentrations of geraniol and citronellol, it was expected that E15, E21 and E7 would have high anti-candidal effects, but the results showed the opposite. Indeed, geraniol, citronellol and other compounds like linalool, citronellol formate or different ratios of these compounds may play critical roles in the anti-candidal effects. *P. graveolens* E9 with citronellol (48%), geraniol (5.1%), citronellyl formate (16.2%) and geraniol formate (3%) had high anti-candidal effects against clinical isolates of *C. albicans*. Some of the *P. graveolens* essential oils tested had higher contents of citronellol than *P. graveolens* essential oil E9, but their anti-candidal effects were lower. Investigation of the chemical composition of *P. graveolens* E9 by further analysis (gas chromatography–mass spectrometry) showed the presence of carvacrol (1.3%) and β-caryophyllene (3.0%)

as minor components of its essential oil. These minor components have prominent antimicrobial effects (Chami et al., 2005; Dahham et al., 2015); therefore, these minor components play important roles in anti-candidal activity. In another study with commercial six *P. graveolens* essential oils against 32 clinical isolates, no differences were observed among the essential oils. In addition, the citronellol/geraniol ratio for *P. graveolens* essential oils were in the range 0.15–1.8 and the number of clinical strains was smaller than in the current study (Mahboubi et al., 2018). The current study, for the first time, demonstrated that different combinations of citronellol/geraniol except for GC10:90 had the same anti-candidal activity against clinical isolates of *C. albicans*.

Although geraniol and citronellol as the main components of *P. graveolens* essential oils play an important role in their anti-candidal activities against clinical isolates, different combinations of geraniol and citronellol did not result in significant differences in their anti-candidal activities. Furthermore, the minor components of *P. graveolens* essential oils such as carvacrol or β-caryophyllene had an effect on their activities.

Table 3 Effects of *P. graveolens* essential oils and main components against *C. albicans* ATCC 10231 using micro-broth dilution assay

	P. graveolens	MIC (µg/mL)	MFC (µg/mL)
Subset 1	E10	0.625±0.0	1.330±0.0
	E20	0.375±0.0	1.160±0.0
	E9	0.458±0.0	0.916±0.0
	E22	0.500±0.0	1.500±0.0
Subset 2	E1	0.830±0.0	2.000±0.0
	E2	0.916±0.0	1.830±0.0
	E6	0.830±0.0	1.500±0.0
	E7	0.830±0.0	1.500±0.0
	E8	0.916±0.0	1.580±0.0
	E21	0.916±0.0	1.660±0.0
	E19	0.750±0.0	1.250±0.0
Subset 3	E4	1.250±0.0	1.330±0.0
	E11	1.000±0.0	2.000±0.0
	E15	1.160±0.0	2.160±0.0
Subset 4	E16	1.500±0.0	3.330±0.0
	E3	1.580±0.0	2.660±0.0

MIC = minimal inhibitory concentration; MFC = minimal fungicidal concentration.
Values are presented as mean ± Standard Deviation.
Means in each subsets are significant different at $p < 0.05$.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgement

The authors thank Mrs. Laleh Hejazi for financial and spiritual support during the project. This study was supported by grant number 9510 in medicinal plants at the Research Center of Barij, Kashan, Iran.

Table 4 Anti-candidal effects of *P. graveolens* essential oils and main components against clinical isolates *C. albicans* (n = 72) using micro-broth dilution assay

P. graveolens	MIC (µg/mL)	MFC (µg/mL)
E20	0.497±0.085^{a}	0.774±0.105^{a}
GC20:80	0.603±0.080^{a}	0.827±0.100^{a}
GC30:70	0.737±0.080^{a}	0.946±0.100^{a}
GC40:60	0.641±0.080^{a}	0.856±0.100^{a}
GC50:50	0.484±0.080^{a}	0.696±0.100^{a}
GC60:40	0.728±0.080^{a}	0.926±0.100^{a}
GC70:30	0.651±0.080^{a}	0.878±0.100^{a}
GC80:20	0.465±0.080^{a}	0.686±0.100^{a}
GC90:10	0.545±0.080^{a}	0.814±0.100^{a}
GC95:5	0.753±0.080^{a}	0.910±0.100^{a}
GC10:90	1.054±0.079^{b}	1.391±0.098^{b}
E9	1.149±0.083^{b,c}	1.656±0.103^{c}
E22	1.362±0.080^{c,d}	1.647±0.100^{c}
E10	1.490±0.080^{d}	1.846±0.100^{c}

GC = geraniol-citronellol; E = essential oil; MIC = minimal inhibitory concentration; MFC = minimal fungicidal concentration.
Values are presented as mean±SD.
Means in the same column superscripted with different letters are significant different at $p < 0.05$.

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