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Phytochemical Investigation and Antifungal Activity of *Daucus littoralis* Smith sub sp. *hyrcanicus* Rech.f

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ABSTRACT

Daucus littoralis Smith subsp. *hyrcanicus* Rech.f. (Caspian carrot) is an endemic species in the north of Iran. The aim of this study was to report the separation and structural elucidation of constituents from the ethyl acetate extract of fruits, which have not been reported previously. A phenylpropanoid (myristicin), a sesquiterpenoid (acorenone B) and two fatty acids (palmitic and oleic acid) were identified by GC and GC/MS. Also acorenone B and triolein were isolated by different chromatographic methods and identified by ^{1}H, ^{13}C-NMR and MS spectra. Furthermore, the antifungal activity of the Fruits Ethyl Acetate (FEE) and Fruits Methanol Extracts (FME) were investigated against vulvovaginal isolates of *Candida albicans*. The results showed that both FEE and FME had antifungal activity for standard *Candida* (MIC 6.25 and 25 mg mL^{-1}, respectively) as well as for the isolates with MIC ranged 25-100 mg mL^{-1}.

Key words: *Daucus littoralis* Smith subsp. *hyrcanicus* Rech.f., acorenone B, triolein, antifungal

INTRODUCTION

Daucus littoralis Smith subsp. *hyrcanicus* Rech.f. (Apiaceae), well known as Caspian carrot, is an annual or perennial plant growing exclusively in the north of Iran (Mazandaran and Guilan provinces) (Yousefbeyk *et al*., 2014b). The genus *Daucus* is distributed in Europe, Africa, West Asia, North America and Australia. Among the plants of this genus, the essential oil of *D. carota* has been used in traditional medicine as antibacterial and antifungal agents (Tavares *et al*., 2008). So far the antimicrobial activity of the essential oil from aerial parts of *D. carota* was investigated against *Bacillus subtilis*, *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Penicillium expansum* (Maxia *et al*., 2009). Furthermore, the antifungal activity of *D. carota* L. subsp. *carota* and *D. carota* L. subsp. *halophilus* against yeasts, dermatophytes and *Aspergillus* strains were investigated (Maxia *et al*., 2009; Tavares *et al*., 2008). In addition, *D. carota* extract

has a long history of usage in treating hepatic and renal insufficiency as well as skin disorders like burns and furunculous (Maxia *et al*., 2009). In other studies, anthelmintic, hepatoprotective, cytotoxic, antioxidant and iron-chelative activities have been reported for *D. carota* (Maxia *et al*., 2009).

Recently, composition of essential oils from leaves, fruits, flowers and roots of *D. littoralis* subsp. *hyrcanicus* has been investigated. Sesquiterpene hydrocarbons like germaceren D (22- 36%) and acorenone B (19.7-57.5%) were reported as major components. Also high amount of myristicin was identified in leaves/stems, roots and fruits essential oils (8.6, 9.5 and 15.2%, respectively) (Yousefbeyk *et al*., 2014b). Phytochemical investigation of fruits eventuated in identification of β-sitosterol, stigmasterol, caryophyllene oxide, β-amyrin, quercetin 3-O-β-glucoside, quercetin 3-O-β-galactoside and luteolin in this plant (Yousefbeyk *et al*., 2014a).

The aim of this study was to report the separation and structural elucidation of constituents from the fruits extract of *D. littoralis* subsp. *hyrcanicus*, which have not been reported previously. Furthermore, the antifungal activity of the fruits was investigated against vulvovaginal isolates of *Candida albicans*.

MATERIALS AND METHODS

General procedures: ^{1}H and ^{13}C-NMR spectra were acquired using a Bruker Avance TM500 DRX (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer with tetramethylsilane as an internal standard and chemical shifts are given in δ (ppm). Column chromatography was accomplished using silica gel (70-230 and 230-400 mesh) (Merck, Germany). Silica gel 60 F254 precoated plates (Merck, Germany) were used for TLC. The spots were detected by spraying anisaldehyde-H_2SO_4 (Sigma-Aldrich, Germany) reagent followed by heating. The HPLC separations were carried out on a Knauer system (Smart line system, Germany) connected to a photodiode array detector. The MPLC separations were carried out on a Buchi system. All the solvents, standards and reagents were obtained from Merck (Germany).

Plant material: The fruits were collected from Bandar-e-Anzaly sea coast, province of Guilan, north of Iran, June 2013. A voucher specimen of plant (6734-TEH) was deposited in Herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

Extraction and isolation: The fruits (1 kg) were powdered and extracted successively with ethyl acetate and methanol by percolation method. The fruit ethyl acetate extract (FEE) (88 g) was subjected to column chromatography over a silica gel column (70-230 mesh, 10×3 cm), using a mixture of $CHCl_3$: AcOEt (9:1) as mobile phase and afforded 10 fractions (A-J). Among these fractions, the fraction C was pale yellowish oil with characteristic odor. The fatty acids composition was determined as methyl esters following the procedures described by Metcalfe and Schmitz (1961). Briefly, 12 mL of methanol solution of boron trifluoride (BF_3) 10% was added to sample under reflux condition and maintained for 2 min. The solution was extracted three times with 50 mL of hexane at room temperature. The hexane layer was separated and dried over anhydrous Na_2SO_4 (Metcalfe and Schmitz, 1961). The sample was analyzed by GC and GC/MS methods.

Furthermore, the fraction C subjected to MPLC (Buchi). The eluted ratio was adjusted with 50: 50 (hexane: CH_3Cl) and delivered to the silica gel column (5 cm) for 60 min (flow-rate: 6 mL min^{-1}, UV detector (λ: 254 nm) to give fractions C_a-C_i. The fraction C_c was selected for more

purification by HPLC. The HPLC system consisted of a Knauer instrument (Smart line system, Germany) with a diode array detector. The column was a normal phase semi-preparative (250×18 mm i.d.) with a particle size of 5 nm (Eurospher). The eluents were hexane and CH_3Cl with a flow rate of 3 mL min^{-1}. The gradient condition was: 0-5 min, hexane 80%, CH_3Cl 20%; 5-35 min, hexane 75%, CH_3Cl 25%. The UV-vis spectra were recorded between 210 and 500 nm. The compounds 1 and 2 were purified with this method.

GC and GC/MS analysis of oily fraction of FEE extract: Oil sample analysis was performed on an Agilent 6890 gas chromatography equipped with Flame Ionization Detector (FID). A capillary column HP-5ms (30×0.25×0.25 μm film thickness) was used. The column temperature program was as follows: 5 min isothermal at 50°C, increased to 240°C at a rate of 3°C min^{-1}, increased to 300°C at a rate of 15°C min^{-1} and finally held at this temperature for 3 min. Injection temperature was 290°C. Injection volume was 1.0 μL. Helium was the carrier gas and the split ratio was 25:1.

GC/MS analysis was performed with an Agilent 6890 gas chromatograph with a capillary column HP-5 ms (30×0.25×0.25 μm). Temperature program was as follows: 5 min at 50°C, increased to 240°C at a rate of 3°C min^{-1}, then, increased to 300°C at a rate of 15°C min^{-1} and finally held at that temperature for 3 min. Injection temperature was 290°C. Injection volume was 1.0 μL. Helium was the carrier gas and the split ratio was 25:1. The MS was operated at 70 eV ionization energy and the interface temperature was 220°C. Mass range was 50-500 Da. Retention indices were calculated using standard hydrocarbons (C8-C30 n-alkanes, injected in the same conditions of the samples). Identification of each component was performed by comparison of their mass spectra with Wiley library or authentic compounds in our laboratory and those published in the references and confirmed by comparison of their calculated retention indices, with those of authentic compounds or data published in the literature (Adams, 2004). Quantitative data were obtained from the electronic integration of the FID peak areas.

Preparation of inoculum

Isolates: Seven clinical vulvovaginal isolates of *C. albicans* were investigated in this study. The isolates were kindly provided by Drug and Food Control Laboratory of Faculty of Pharmacy, Tehran University of Medical Sciences. All strains were stored at -80°C in the appropriate medium. Identification of the isolates was based on gross colony characteristics and microscopic morphology of their micro and macroconidia and accessory structures. *Candida albicans* ATCC 1023 was used as the control. Before performing antifungal susceptibility testing, each isolate was inoculated onto Sabouraud Dextrose Agar (SDA) for 24 h at 25°C to ensure optimal growth characteristics and purity.

Antifungal susceptibility test: Antifungal susceptibility tests for clotrimazole, ketoconazole and nystatin were used to determine the susceptibility of isolates and standard *Candida albicans* according to CLSI 2012 (Pakshir *et al*., 2011). The antifungal agents were obtained as standard powders and stock solutions were prepared by dissolving the powders in their specific solvents (DMSO, water and ethanol). After that, they were loaded into blank paper at the following concentrations: Ketoconazole 15 μg $disc^{-1}$, clotrimazole 10 μg $disc^{-1}$ and nystatin 100 μg $disc^{-1}$. The inoculum was adjusted to 10^6 CFU mL^{-1} and spread on agar plate by a sterile swab. Four discs

Table 1: Criteria of susceptibility and resistance of antifungal discs

	Zone diameter (mm)		
Parameters (μg $disc^{-1}$)	Sensitive	Dose dependent	Resistance
Nystatin (100)	≥25	17-24	≤16
Clotrimazole (10)	≥20	12-19	≤11
Ketoconazole (15)	≥30	23-29	≤22

Table 2: GC and GC/MS analysis of oily fraction obtained from fruit ethyl acetate extract of *D. littoralis* subsp. *hyrcanicus*

RT[a]	Component	%	RI_s[b]	RI_l[c]	Fragmentation peaks (m/z)
25	Myristicin	9.9	1519	1523	192, 177, 165, 147, 131, 119, 103, 91, 77, 65, 53, 41
29.4	Acorenone B	44.5	1698	1704	220, 205, 191, 177, 164, 150, 135, 121, 109, 93, 82, 69, 55, 41
33.6	Palmitic acid methyl ester (Hexadecanoic, C16:1)	3.8	1895	1911	270, 255, 239, 227, 213, 199, 185, 171, 157, 143, 129, 115, 101, 87, 74, 55, 43
37.4	Oleic acid methyl ester (9-ctadecanoic acid (Z), C18: 1)	41.7	2106	2117	294, 278, 264, 247, 235, 222, 207, 194, 180, 166, 151, 137, 123, 110, 96, 81, 67, 55, 41

[a]Retention time, [b]Retention indices calculated for sample, [c]Retention indices in literature. Values (%) given are the mean of three replicated values

were placed onto each plate and incubated in 25°C. The inhibition zones were recorded after 48 h of incubation. Criteria of susceptibility and resistance of antifungal disks were measured according to following Table 1.

MIC and MFC determination: Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of the extracts were evaluated by broth microdilution method with visible growth observed by using 96 U-shaped wells plates (Yousefbeyk *et al*., 2014b). A stock concentration of 200 mg mL^{-1} from each extract was prepared in DMSO. Then, two-fold serial dilution of the stock solution of each extract (100 mL) was prepared by using Sabouraud Dextrose Broth (SDB) (100 mL, each) in ten wells. The stock microbial suspension with two-fold test inoculum was prepared in SDB from 24 h old culture. Then, aliquot of 100 mL of twofold test strain inoculum was added to each well to reach the final inoculum size of 5×10^5 CFU mL^{-1}. The MFC was determined by quantitative subculture of 100 mL from each clear well onto SDA agar plates. Plates were incubated at 25°C for 48 h. The MFC is defined as the lowest of extracts concentration that results in more than 99.9% killing of the bacteria being tested (Golfakhrabadi *et al*., 2014).

RESULTS AND DISCUSSION

In this study, the FEE of *D. littoralis* subsp. *hyrcanicus* was investigated for major components. The oily fraction obtained from FEE was analyzed by GC and GC/MS methods after preparing methyl ester derivations. The results demonstrated the presence of a phenylpropanoid, myristicin (1), a sesquiterpenoid, acorenone B (2) and two fatty acids, palmitic and oleic acid methyl esters (3-4) (Table 2).

Moreover, the FEE was used for isolation and purification of main components by different chromatography methods. The isolated compounds (Fig. 1) were identified as acorenone B (2) and triolein (5). Compounds 2 and 5 were identified by comparison of their spectroscopic data (^{1}H-NMR, ^{13}C-NMR and MS) with those in the literatures and authentic compounds from our laboratory (Vlahov *et al*., 2008; Wolf *et al*., 1976).

Acorenone B (2): ^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm): 0.78 (3H, d, J = 6.7 Hz, isopropyl methyl), 0.87 (3H, d, J = 6.7 Hz, isopropyl methyl), 0.97 (3H, d, J = 6.7 Hz, 4-CH_3), 1.76 (3H, m, 8-CH_3), 2.09 (1H, m, H-10), 2.23 (1H, d, J = 16.5 Hz, H-6), 2.29 (1H, m, H-10), 2.7 (1H, d, J = 16.5 Hz, H-6), 6.67 (1H, m, H-9).

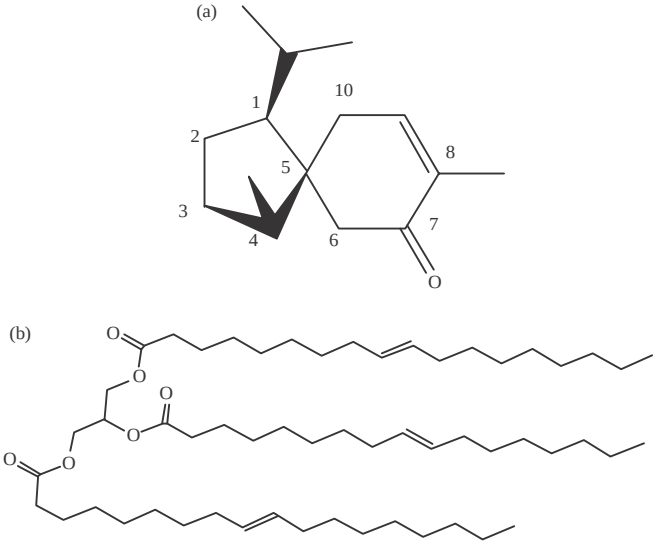

Fig. 1(a-b): Structures of compounds 2 and 5 isolated from *D. littoralis* sub sp. *hyrcanicus* including, (a) Acorenone B (2) and (b) Triolein (5)

Triolein (5): ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm): 0.88 (9H, t, H-18), 1.64 (6H, m, H-3), 2.02 (12H, m, H-8, H-12), 2.33 (6H, m, H-3), 4.1 (2H, dd, J = 10 Hz, CH_2O), 4.3 (2H, dd, J = 10 Hz, CH_2O), 5.27 (1H, m, CHO), 5.36 (6H, m, H-9). ^{13}C-NMR (125 MHz, CDCl3), δ (ppm): 14.1 (C-18), 22.17 (C-12 to C-17), 24.86 (C-3), 27.25 (C-8, C-11), 29.09 (C-4 to C-7), 29.75 (C-4 to C-7), 33.99 (C-2), 34.17 (C-2), 62.1 (CH_2O), 68.88 (CHO), 128.91 (C-9), 130.54 (C-10), 173.13 (C-1).

Acorenone B is a sesquiterpenoid which is reported in the essential oil of plants like *Bothriochloa intermedia* (Zalkow *et al*., 1980), *Angelica lucida* (Lawrence and Morton, 1974), *Euphorbia macrorrhiza* (Lin *et al*., 2012*)*, *Levisticum persicum* (Shafaghat, 2011*)* and *Acorus calamus* (Balakumbahan *et al*., 2010). This compound has been identified in the essential oil of *D. littoralis* subsp. *hyrcanicus* as a major compound (Yousefbeyk *et al*., 2014b). In a study, the antimicrobial, anti-inflammatory, antioxidant, antidiarrheal, antiulcer, antispasmodic, immunosuppressant and mitogen inhibitor activities of *Acorus calamus* are referred to acorenone and other constituents in the essential oil (Divya *et al*., 2011).

Triolein is a triglyceride derived from a glycerol and three unites of mono-unsaturated fatty acid oleic acid (18:1n-9). This compound represents 4-30% of olive oil (Thomas, 2002; Shubert and Leyba, 2013). Oleic acid has been showed several beneficial effects, such as prevention of ischemic cardiovascular disease, acting as a potent inhibitor of Platelet Aggregating Factor (PAF) (Massimo *et al*., 2009), endothelial vasomodulatory and antioxidant activities (Visioli *et al*., 2005). Also, studies showed that oleic acid had antibacterial activity against *Staphylococcus aureus*, *Micrococcus kristinae*, *Bacillus megaterium* and *Pseudomonas phaseolicola* (Dilika *et al*., 2000) as well as antifungal activity against *Crinipellis pernicosa* and *Pythium ultimum* (Pohl *et al*., 2011). In a study, antifungal activities of four essential oils of *D. carota* L. subsp. *carota* in different vegetative phases of the plants collected in Portugal (sample 1 and 2) and in Italy (sample 3 and 4) were assessed against yeasts, dermatophyte and *Aspergillus* strains. The results showed that the essential oils had more sensibility to *Cryptococcus neoformans* and dermatophyte strains (MIC 0.16-0.64 μL mL^{-1}) compared with *Candida* and *Aspergillus* strains.

Table 3: Antifungal susceptibility test of standard and vaginal isolates of *Candida albicans* to antifungal drugs including clotrimazole, ketoconazole and nystatin

	Zone diameter[a] (mm)		
	Nystatin (100 μg disc^{-1})	Clotrimazole (11 μg disc^{-1})	Ketoconazole (15 μg disc^{-1})
C. albicans ATCC 10231	21.5 DD[b]	21 S	27.5 DD
C. albicans 1	23 DD	0 R	0 R
C. albicans 2	24 DD	20.5 S	24.5 DD
C. albicans 3	23 DD	18 DD	24.5 DD
C. albicans 4	24 DD	11 R	0 R
C. albicans 5	25 S	0 R	0 R
C. albicans 6	25.5 S	4.5 R	0 R
C. albicans 7	22.5 DD	19 DD	24.5 DD

[a]Mean of three assays, [b]Susceptibility: S, Sensitive, DD: Dose dependent, R: Resistant

Table 4: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of ethyl acetate and methanol extracts of *D. littoralis* subsp. *hyrcanicus* against standard and vaginal isolates of *Candida albicans*

	FEE		FME	
	MFC	MIC	MFC	MIC
C. albicans ATCC10231	6.25[a]	12.5	12.5	12.5
C. albicans 1	>100	>100	>100	>100
C. albicans 2	25	100	25	50
C. albicans 3	100	<100	50	100
C. albicans 4	100	>100	100	>100
C. albicans 5	>100	>100	>100	>100
C. albicans 6	100	100	100	>100
C. albicans 7	>100	>100	>100	>100

MIC and MBC were determined by broth micro dilution method and expressed in mg mL^{-1} (W/V), Key to extracts employed: FEE: Fruits ethyl acetate extract, FME: Fruits methanol extract, MFC: Minimum fungicidal concentration, MIC: Minimum inhibitory concentration

In this study, antifungal activity of FEE and FME of *D. littoralis* subsp. *hyrcanicus* was investigated against some vulvovaginal isolates of *C. albicans*. Criteria for susceptibility to used antifungal drugs have been summarized in Table 1. Antifungal susceptibility tests of isolates and standard *C. albicans* for clotrimazole, ketoconazole and nystatin showed in Table 3. The results of antifungal test demonstrated that both FEE and FME had antifungal activity against standard *Candida* (MIC 6.25 and 25 mg mL^{-1}, respectively) as well as the isolates with MIC ranged 25-100 mg mL^{-1}. Both methanol and ethyl acetate extracts showed better antifungal activity against standard *C. albicans* than isolated strains. In addition, isolates C_2 was more sensitive to FEE and FME (MIC 25 mg mL^{-1}) than other isolates (Table 4).

Our previous study revealed the presence of β-Amyrin in the FEE (Yousefbeyk *et al*., 2014b). It has reported that β-Amyrin had moderate anti-microbial activity against *S. aureus* and *C. albicans* (MIC: 0.5 and 1.02 mg mL^{-1}, respectively) (Rivero-Cruz *et al*., 2009). The antifungal activity of the FEE can be due to the presence of this compound. Also high amount of phenolic compounds and flavonoids like quercetin 3-O-β-glucoside, quercetin 3-O-β-galactoside and luteolin has been reported in FME. The presence of these compounds explains the antifungal activity of FME.

CONCLUSION

The phytochemical investigation of *D. littoralis* subsp. *hyrcanicus* fruits ethyl acetate extract resulted in isolation of two compounds including a sesquiterpenoid, acorenone B and a triglyceride, triolein. Also myristicin, palmitic and oleic acid were isolated and identified by GC/MS. The results

of antifungal assays showed that both FEE and FME had antifungal activity against standard *Candida* (MIC 6.25 and 25 mg mL^{-1}, respectively) as well as the isolates with MIC ranged 25-100 mg mL^{-1}.

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