30 درصد تخفیف نوروزی ویژه کارگاه‌ها و فیلم‌های آموزشی

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آموزش مهارت های کاربردی در ندوین و پذیرش مقاله

پش
Isolation and characterization of steroids, phthalide and essential oil of the fruits of *Kelussia odoratissima* Mozaff., an endemic mountain celery

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**Abstract**

*Kelussia odoratissima* Mozaff. is an Iranian endangered endemic edible plant with enormous use in the middle region of Iran as food and spice especially yogurt seasoning, and as medicinal herb for anti-inflammatory and cardiovascular purposes. Although antioxidant, anti-inflammatory and antilipidemic effects of this plant have so far been studied, but chemical analyses of the non volatile constituents of the plant have not yet been reported. Therefore, identification of chemical constituents of different parts of plant was investigated in the present study. In this direction, two steroids including stigmasterol and β-sitosterol and one phthalide namely 3-butyliden-4,5-dihydrophthalide were isolated from the plant fruits. In addition, the essential oil composition of the fruits was studied. Thirty eight compounds were identified, of which the major components were found to be Z-ligustilide (29.2%), germacrene-B (15.9%) and germacrene-D (15.5%). According to the previous bioassays of the isolated compounds such as anti-inflammatory and antilipidemic effects of phthalides and steroids, a chemical-biological relation can be postulated.

**Keywords:** Apiaceae; Stigmasterol; β-Sitosterol; Z-Ligustilide; 3-Butyliden-4,5-dihydrophthalide; Germacrene

**INTRODUCTION**

Apiaceae have aroused interest in food science because of its common used plants such as celery, carrot, fennel, caraway and dill. Coumarins, polyacetylenes, flavonoids, sesquiterpenes and phthalides are among the important chemical constituents of this family along with biologically active essential oil (1-3).

*Kelussia* is a monotypic endemic genus to Apiaceae, and *K. odoratissima* is wild growing in high altitude of western Zagros mountains, Iran (4). In some studies, its previous name *Amirkabiria odoratissima* has been used. As an endangered plant, it has recently drawn more attention for its regular cultivation. Being called “karafs koohi” (mountain celery) or “keloss” in the region and bearing a unique flavorful smell, *K. odoratissima* is vastly used as spice especially in yogurt. Pickles of small leaves and shoots are common in Chaharmahal Bakhtiari province in south west of Iran. It is believed in folk medicine to be effective as anti-inflammatory and sedative, and to treat hypertension (interview with local people).

The antioxidant activity of the methanolic extract of the plant has been evaluated using β-carotene bleaching assay, reducing power, thiocyanate, accelerated oxidation of sunflower oil, and DPPH radical-scavenging and it was effective in some assays (5,6). In spite of its *in vitro* and *in vivo* efficiencies in lipid profile (7) the results could not be confirmed in the clinical trial, except for an increase in HDL (8). Furthermore, providing rabbits’ food with aerial parts of the plant, suggested bearing beneficial effects to prevent development of fatty streak (9). Besides, gastric acid secretion has been reduced meaningfully in rats fed with *K. odoratissima*, but pepsin secretion was unaffected (10).
Polyphenolic extract and essential oil were tested for fibrinolytic effects, and the latter was effective on thrombolytic agent (11). Essential oil showed a pronounced antibacterial effect as well (12). In folkloric medicine the use of this plant is proposed in inflammatory conditions which were confirmed in vitro by carrageenan test (13).

In spite of consumption of keloss as food additive and flavor and several pharmacological effects reported earlier, chemical identification of non volatile constituents of the plant except for clarification of fatty acid pattern and total phenolic content (6) have not yet been reported.

MATERIALS AND METHODS

General instrument

Medium pressure liquid chromatography (MPLC) was performed on a Büchi apparatus equipped with two pump modules 601/605, a pump manager (C-615) and a fraction collector C-60 using silica gel (70-230 mesh) columns. IR spectrum was recorded on a Rayleigh WQF-510 FTIR instrument. 1H NMR was recorded on a Brucker (500 MHz) instrument, using CDCl3 as the solvent and TMS as the internal standard. EI-MS spectrum was recorded on Hewlett Packard 5972A mass spectrometer. Compounds on the TLC (Silicagel 60 GF254 precoated plates, Merck) were detected at 254 and 365 nm followed by KOH/ethanol, vaniline- sulfuric acid or cerium sulfate molibdate as spraying reagents (2). All reagents and solvents were purchased from Merck (Germany). GC/MS analysis was carried out on a Hewlett-Packard 6890 gas chromatograph fitted with a fused silica HP-5MS capillary column (30 m × 0.25 mm; film thickness 0.25 μm). The oven temperature was programmed from 60 to 280°C at 4°C/min increment. Helium was used as the carrier gas at a flow rate of 2 ml/min with inlet pressure set at 17.7 psi and injection volume of 0.1 μl. The gas chromatograph was coupled to a Hewlett-Packard 5972A mass selective detector. The EI-MS operating parameters were as follows: ionization voltage, 70 eV; ion source temperature, 200°C. Identification of components of the volatile oil was based on retention indices relative to n-alkanes and computer matching with the Wiley 275.L library, as well as comparison of the fragmentation patterns of the mass spectra with those reported in the literature (14,15).

Plant material

The fruits of K. odoratissima were collected in July 2008 from Zardkooh mountain which is located in Charmahal Bakhtiari province (south western of Iran), at an altitude of ca. 2400 m above sea level. The plant identity was confirmed by the Botany Department of Isfahan University and a voucher specimen of the plant (No. 2022) was deposited in the herbarium of the School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

Extraction and isolation of non-volatile compound

Ground fruits (300 g) were extracted with 600 ml of n-hexane for 6 h using Soxhlet apparatus which left 74 g crude extract (24.6%). The extract was then solubilized in methanol, kept in -20°C overnight and filtered which resulted in 30 g filtrate. The filtrate was purified using medium pressure liquid chromatography (MPLC) on a normal silica 60 A (15-40 μ) column eluted with heptane-ethylacetate (10:0 to 0:10) to get 14 fractions as A1-N1. The fractions were pooled according to the TLC profile using chromogenic reagents including vaniline-sulfuric acid and cerium sulfate molibdate as spraying reagents (2). All reagents and solvents were purchased from Merck (Germany). GC/MS analysis was carried out on a Hewlett-Packard 6890 gas chromatograph fitted with a fused silica HP-5MS capillary column (30 m × 0.25 mm; film thickness 0.25 μm). The oven temperature was programmed from 60 to 280°C at 4°C/min increment. Helium was used as the carrier gas at a flow rate of 2 ml/min with inlet pressure set at 17.7 psi and injection volume of 0.1 μl. The gas chromatograph was coupled to a Hewlett-Packard 5972A mass selective detector. The EI-MS operating parameters were as follows: ionization voltage, 70 eV; ion source temperature, 200°C. Identification of components of the volatile oil was based on retention indices relative to n-alkanes and computer matching with the Wiley 275.L library, as well as comparison of the fragmentation patterns of the mass spectra with those reported in the literature (14,15).
Isolation of essential oil

Plant material was hydrodistilled in a Clevenger-type apparatus for 3 h according to the method recommended in the British Pharmacopoeia (16). The volatile oil was dried over anhydrous sodium sulfate and stored in sealed vial and stored at 4°C until analysis. The yield of oil was calculated based on the dried weight of the plant material.

Compound I

Stigmasterol; colorless needle like crystals, \(^1\)HNMR (CDCl\(_3\), 500 MHz, \(J\) in Hz): Table 1; \(^{13}\)C NMR (CDCl\(_3\), 125 MHz) Table 1; (+) EI-MS \(m/z\) 412 [M]\(^+\), 397 [M-CH\(_3\)]\(^+\), 394 [M-H\(_2\)O]\(^+\). FT-IR (KBr): \(\nu_{\text{max}}\) = 3320, 2946, 2854, 1648, 1600, 1450, 1220, 890.

Compound II

\(\beta\)-sitosterol; colorless needle like crystals; m.p. 139°C; HNMR (CDCl\(_3\), 500 MHz, \(J\) in Hz): Table 1; (+) EI-MS \(m/z\) 414 [M]\(^+\), 396, 381. FT-IR (KBr): \(\nu_{\text{max}}\) = 3319, 2946, 2854, 1640, 1600, 870.

RESULTS

Using a combination of winterization, medium pressure chromatography and recrystallization, three compounds were obtained in pure state from hexane extract of \(K.\) odoratissima.

According to the \(^1\)HNMR spectrum of compound I, the multiplet signal at \(\delta\) 3.71 was assigned to the resonance of methine proton at C-3 which is a common figure in 3-OH bearing triterpenoids and steroids. Additional data such as methyls (\(\delta_H \) 0.9-1.2, \(\delta_C \) 19-20, \(m/z\) 397 [M-CH\(_3\)]\(^+\)), OH strong stretch bond at 3471 cm\(^{-1}\) in IR spectrum and [M-H\(_2\)O]\(^+\) ion fragment at \(m/z\) 394 confirmed the initial

Table 1. Selected \(^1\)HNMR and \(^{13}\)CNMR data of isolated compounds.

| Compound I | Compound II | Compound III |
|------------|-------------|--------------|
| \(C/H\)   | \(\delta_C\) | \(\delta_H\) | \(C/H\) | \(\delta_H\) | \(H\)  | \(\delta_H\) |
| 3         | 72.2        | 3.71         | 3         | 3.71         | 4     | 2.60, 2H, \(J=13.5\) |
| 5         | 141.1       | ----         | 6         | ----         | 5     | 2.45, 2H, m         |
| 6         | 122.1       | 5.30         | 6         | 5.36         | 6     | 5.92, 1H, m         |
| 22        | 138.7       | 4.91         | 8         | 5.20, 1H, \(J=7.9\) |
| 23        | 129.7       | 5.12         | 9         | 2.38, 2H, \(J=7.9\) |
| 10        | 1.20, 2H, m |              | 11        | 0.92, 3H, \(J=7.5\) |

Fig. 1. Structures of isolated pure compounds. I. stigmasterol; II. \(\beta\)-sitosterol; III. ligustilide (3-butyliden-4,5-dihydropthalide)

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proposal which is in accordance with stigmasterol. There are two unsaturation bonds represented by one broad singlet (δ_H 5.30, δ_C 122.1) and two doubled doublets (δ_H 5.12, δ_C 138.7 and δ_H 4.91, δ_C 129.7), well in consistent with H-6, H-22 and H-23 of stigmasterol (Fig. 1), respectively (17).

The characteristic feature of its mass fragmentation, except the molecular ion at 412 is the presence of a fragment ion peak at m/z 271 due to the loss of the side chain of stigmasterol (Fig. 1) followed by the loss of two hydrogen atoms (Table 1).

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Compound II, was assigned as β-sitosterol (Fig. 1) according to Rayleigh IR library comparing the finger print region with confirmation from mass data, with molecular ion at m/z 414 and m/z 396 for [M-H2O]+. Further IR characteristics were stretch absorbing features for OH (3471 cm⁻¹), C=C (1671 cm⁻¹) and unsaturated CH (after 3000 cm⁻¹).

EI-mass analysis defined the molecular ion peak of compound III at m/z 190. It showed to contain an α-β unsaturated lactone because of stretching absorption at 1750 cm⁻¹ in IR spectrum, and two further unsaturated bonds as below: H-6 and H-7 were attached to unsaturated carbons with peaks at δ 5.98 (dt, J = 4, 9.5 Hz) and 6.20 (dt, J = 2, 9.5 Hz), respectively. H-8 was coupled with two protons of C-9 and appeared as a triplet at δ_H 5.2 ppm (Table 1).

The absorption sharp band at 1059 cm⁻¹, typical of C-O bond and multiple weak sharp peaks at 1300-1600 cm⁻¹, characteristics of stretching absorption of C-C(=O)-O of α-β unsaturated esters (15) supported the elucidation.

The remaining unsaturation degree goes well with an extra cycle which is a cyclohexane bearing H-4 and H-5 as multiplets at δ 2.4-2.6, to represent the molecule as a phthalide called ligustilide (3-butyliden-4,5-dihydrophthalide) (Fig. 1) (18). The ion fragment at m/z 161, due to the loss of an ethyl group and a characteristic feature of fragmentation with the presence of a fragment ion peak at m/z 134 due to the loss of the side chain of the compound, supported the identification.

**DISCUSSION**

To the best of our knowledge, the only previous investigation on the non-volatile constituents of *K. odoratissima* fruits concerned identification of fatty acid pattern and total phenolic contents (6). Here, we reported the isolation and identification of two sterols and a phthalide from fruits of *K. odoratissima*.

In order to explain the health promoting effects of various fruits and vegetables, focus has primarily been on vitamins, minerals, fibers and antioxidants, but still remains to be clear which components are responsible for these beneficial effects of food plants. A possible explanation could be that plants contain other bioactive compounds, i.e. compounds having a direct or indirect effect on living tissue *in vitro* and/or *in vivo*, which provide benefits to the health, even though they are not essential nutrients (1). Although application of “keloss” as a yogurt and pickle...
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Table 2. Percentage composition of the oil of *Kelussia odoratissima* fruits analyzed by GC/MS.

| No. | Compound                | %  | RI  |
|-----|-------------------------|----|-----|
| 1   | α-pinene                | 0.6| 935 |
| 2   | camphene                | t  | 949 |
| 3   | sabinene                | t  | 972 |
| 4   | β-pinene                | t  | 977 |
| 5   | myrcene                 | 0.1| 987 |
| 6   | α-phellandrene          | 0.3| 1001|
| 7   | δ-3-carene              | 0.1| 1007|
| 8   | β-phellandrene          | 9.2| 1028|
| 9   | cis-β-ocimene           | 3.8| 1038|
| 10  | trans-β-ocimene         | t  | 1047|
| 11  | γ-terpinene             | t  | 1059|
| 12  | α-terpinolene           | t  | 1085|
| 13  | allo-ocimene            | 0.1| 1126|
| 14  | Unknown                 | 1.0| 1155|
| 15  | citronellol             | 0.1| 1225|
| 16  | bornyl acetate          | t  | 1283|
| 17  | lavandulyl acetate      | 0.1| 1286|
| 18  | citronellyl acetate     | 0.2| 1351|
| 19  | α-copaene               | 1.1| 1373|
| 20  | β-elemene               | 2.3| 1388|
| 21  | β-caryophyllene         | 0.4| 1415|
| 22  | linalyl butyrate        | 0.4| 1420|
| 23  | γ-elemene               | 3.4| 1430|
| 24  | α-guaiene               | 0.5| 1436|
| 25  | α-humulene              | 0.3| 1450|
| 26  | germacrene-D            | 15.5| 1477|
| 27  | β-selinene              | 0.7| 1483|
| 28  | δ-selinene              | 1.3| 1492|
| 29  | α-murolene              | 0.7| 1496|
| 30  | trans-β-guaiene         | 1.2| 1497|
| 31  | germacrene-A            | 1.5| 1500|
| 32  | δ-cadinene              | 5.8| 1521|
| 33  | Unknown                 | 1.1| 1525|
| 34  | cadina1,4-diene         | 0.9| 1529|
| 35  | α-cadinene              | 0.9| 1536|
| 36  | germacrene-B            | 15.9| 1554|
| 37  | germacrene-D-4-ol       | t  | 1574|
| 38  | cis-3-butyldiene phthalide| 1.3| 1667|
| 39  | 3-butyldiene-4,5-dihydrophthalide| 29.2| 1734|
| 40  | 3N-butyl phthalide      | t  | 1815|

T = trace (<0.05%)
RI : Retention indices determined on HP-5MS capillary column
RT : Retention time (min)
% : Calculated from TIC data

seasoning is mainly because of its pleasant smell, but the regional beliefs about anti-flatulence and stomach tonic effect of the plant which is now attributed to its high amount of phthalides in addition to evidences of being effective on *Helicobacter pylori* (19) and inflammation play an important role (20). Besides, effects of phytosterols on blood
cholesterol (21) and inflammation (22), and decreasing effect of phthalides on platelet aggregation would propose a clue to the folk belief regarding its use in cardiovascular diseases. Z-ligustilide, the pure isolated phthalide has shown antifungal effects (23).

The percent of essential oil was found to be 0.8%. The chemical composition of the K. odoratissima volatile oil is presented in Table 2. The compositions are listed in order of their elution on the DB-5 column. Thirty eight compounds were identified, of which the major components were found to be Z-ligustilide (29.2%), germacrene-B (15.9%) and germacrene-D (15.5%). Omidbaigi and coworkers (24) reported higher amount of ligustilide in essentioal oil of the aerial parts of K. odoratissima once compared to our findings. While considerable amount of ligustilide and piperitone epoxide were reported as other constituents in the aerial parts of K. odoratissima, here we found germacrene B and D in about 15% of the total essential oil. The plant sample used in our study and the one used by Omidbaigi (24) were both collected from Zagros mountain chain in Iran, but from different places and heights. The essential oil of K. odoratissima has shown fibrinolytic (11) and antibacterial effects (12). Overall, essential oil of Apiaceous plants has shown various biological and nutritional efficiencies (25,26).

Since phytosterols are effective on blood cholesterol (21) and inflammatory processes (22) in human, and phthalides could decrease platelet aggregation (27-28) and were effective on Helicobacter pylori (19) and inflammation (20), a rational relation between the major plant constituents and its proposed pharmacological effects may be postulated. Since it is an edible plant, further biological analyses on these and other isolated compounds are proposed.

The major components of the essential oil have been previously reported from Apiaceous plants. Ligustilide have been identified in edible and medicinal plants like celery (Apium graveolens), Angelica sinensis and lovage (Levisticum officinale) (29). Germacrene B has been found in Ferula spp (30) and Germacrene D in Prangos asperula (31). The essential oil of the fruits of K. odoratissima consisted of thirteen monoterpen hydrocarbons (15.2%), five oxygenated monoterpenes (0.8%), sixteen sesquiterpene hydrocarbons (53.5%) and one oxygenated sesquiterpene (trace amount). Three phthalide compounds were also consisted 30.5% of the oil. As it could be concluded, the oil of K. odoratissima fruit was characterized by a high content of sesquiterpene hydrocarbons and phthalides, respectively.

CONCLUSION

Kelussia odoratissima is a good source of pharmacologically active phthalides and steroids. Previous cardioprotective and antihyperlipidemic effects of the plant could be attributed to these constituents.

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