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Research Article

Antioxidant Activity and Isolation of Luteoline from Centaurea behen L. Grown in Iran

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Flavonoids are secondary metabolites providing Ultraviolet-visible (UV) spectroscopy protection and color in almost all terrestrial plants and fruits. They have a fused ring system consisting of an aromatic ring and a benzopyran ring with a phenyl substituent. As their biological activities have an impact on human health, they serve as target molecules in the development of new drugs. The objective of this research was to study the antioxidant activity and chemical analysis of the luteoline from *Centaurea behen* L. (Compositae family). The aerial parts of powdered and dried *C. behen* were extracted with methanol (MeOH) in a Soxhlet apparatus over a period of 2 days. The concentrated total extract was extracted with petroleum ether, diethylether, and methanol. From the methanol extract of the aerial parts of *C. behen*, the flavonoid derivative (luteoline) was identified. The aerial parts' extract demonstrated effective antioxidant activity measured in terms of half-maximal inhibitory concentration (IC_{50}). The product extract has been isolated by UV, column chromatography (CC), and preparative high-performance liquid chromatography (HPLC). The structures involved were elucidated by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and heteronuclear multiple-bond correlation (HMBC) spectra. The compound identified had not been reported in previous studies of *C. behen* L.

1. Introduction

Throughout time, many plants have been used for the treatment of mental problems. A good number of these have been alkaloid-containing plants, as alkaloids are known to have a strong interaction with receptors in the central nervous system (CNS). In recent years, however, it has become clear that flavonoids may also play a role in the action of enzymes on the receptor systems of the brain, exerting various effects on the CNS, including prevention of the neurodegeneration associated with Alzheimer's and Parkinson's diseases [1].

Flavonoids possess a variety of biological activities in addition to their effects on the CNS. They have attracted attention as free radical scavengers with antioxidant activity. They are yellow, blue, or red and function to afford UV protection for plants and as pollination aids by providing specific colors or patterns to flowers. More than 6000 flavonoids have been identified.

Plants in which flavonoids are thought to or have been proven to be active constituents include species with a long history of use in traditional folk medicines in Europe. For centuries chamomile flowers (*Matricaria recutita* L., Asteraceae family) have been used for their calming effect, which is due to their apigenin content [2]. We studied the mechanism of nanocapsules of *Matricaria recutita* by the emulsion-diffusion process [3, 4]. This study showed that nanocapsules of plant extract can be effective in medicinal drugs. Other monoterpenes produced an oxidation reaction, releasing energy to fuel biological cycles [4]. There are eight species of the genus *Carduus* (Asteraceae family) growing wild in Iran [5, 6]. Previous chemical investigations on *Carduus* species have shown the presence of flavonoids, steroids, and triterpenoid constituents [7]. The biological transformation of the natural plant product mycene by *Pseudomonas aeruginosa* and of citral by *Aspergillus niger* demonstrated hydroxylation [3, 8]. A report on the methanol extract of *Tanacetum parthenium* (Asteraceae family) from Northwest Iran identified the flavonoids flavonol, kaempferol, fisetin, and naringenin [9]. In addition, identification of the flavonoids from *Zosimia*

Table 1: HMBC connectivities for the assignment luteoline.

Step	Connectivity
(1)	OH5-C4
(2)	OH3′-C4′
(3)	OH3′-C2′
(4)	C2-H6′
(5)	H2′-C4′
(6)	H2′-C2
(7)	C10-H3
(8)	H8-C10
(9)	C8-H6
(10)	H8-C6

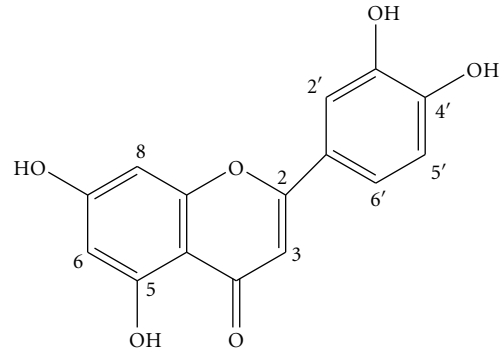

Figure 1: Structures of 5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4H-chromen-4-one.

absinthifolia (Umbelliferae family) and *Galium verum* (Rubiaceae family) is found in the literature [10, 11]. Our study of *Salvia glutinosa* (Lamiaceae family) found high flavonoid content [12].

Previous chemical investigations on *Centaurea* species have shown the presence of flavonoids [13]; sesquiterpene lactones, especially guaianolides [14–16]; germacranolide-type sesquiterpene lactones [13]. Sesquiterpene lactones have been reported to have multiple beneficial biological effects, including cytotoxic, antibacterial, anti-inflammatory, and hypotensive. A study of flowering and aerial parts of *Centaurea africana* Lamk var. *africana* (Bonnet) M., a species endemic to Algeria and Tunisia, isolated a new acylated flavonoid glucoside [17]. Other studies of *Centaurea africana* have reported on compounds extracted from the flowering and aerial parts [18, 19]. To the best of our knowledge, this is the first report on the flavonoids extracted from the aerial parts of *C. behen* L. from Iran and their antioxidant activities.

2. Methods

2.1. General Experimental. The IR spectra were determined on a Bruker Tensor 27 spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AM 300 spectrometer. Column chromatography was performed over silica gel (70–230 mesh, Merck) using petroleum ether, AcOEt, and methanol gradients as eluents. UV spectra were recorded on a Perkin Elmer Lambda 12 spectrophotometer, and mass spectra were recorded on an AEI MS-50 spectrometer.

2.2. Plant Materials. The aerial parts of *Centaurea behen* L. were collected in June 2009 from Givi, Khalkhal Road (Ardabil province) in the northwest of Iran, at an altitude of 1400 m. A voucher specimen (No. 1563) has been deposited at the Herbarium of the Agriculture Research Centre, Ardabil, Iran.

2.3. Extraction and Isolation. Dried and finely powdered *Centaurea behen* L. aerial parts (600 g) were treated with methanol using a Soxhlet extractor over a period of 2 days. The concentrated total extract (72 g) was extracted with petroleum ether, diethylether (Et_2O), and methanol. A part of the Et_2O portion (3 g) was subjected to silica gel column chromatography (70–230 mesh, Merck), eluted with an equivalent petroleum ether, diethylether, and methanol stepwise gradient to obtain 32 fractions (15 mL each). After the evaporation of the solvent, fractions 7–13 were chromatographed over silica gel with the Et_2O : MeOH mixture to provide 16 subfractions. Subfraction 7 (145 mg) was rechromatographed on silica gel into 12 fractions (20 × 15 mL) using as eluents an 8.5 : 1.5 Et_2O : MeOH mixture. The combined fractions 6 to 10 (24 mg) were further purified on a preparative TLC to give compound **1** (14 mg) [20].

2.4. Antioxidant Activity Tests

2.4.1. DPPH Assay. The DPPH assay was carried out using a modified form of the method used by Cheung et al. [21]. In brief, 0.5 mL of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in ethanol (0.1 mM) was added to 1 mL of extracts in different concentrations (50–800 μg mL^{-1}) and left in the dark for 10 min. The absorbance of the resulting solution was recorded on a spectrometer at 520 nm against a blank of alcohol. Vitamin C was used as the reference antioxidant. DPPH scavenging activity was expressed as IC_{50} values (μg extract mL^{-1}) for comparison. The IC_{50} value of each sample was defined as the concentration of sample required for a 50% decrease in absorbance of the blank [22].

2.4.2. Determination of Total Phenolic Compounds. Total phenolics of the aerial parts of *C. behen* were determined by methods described in the literature, using the Folin-Ciocalteau reagent with gallic acid as the calibration standard (both obtained from Sigma-Aldrich). An aliquot (0.1 mL) of extract solution containing 1 mg of extract was transferred to a volumetric flask; 46 mL of distilled water and 1 mL of Folin-Ciocalteau reagent were added, and the flask was thoroughly shaken. After 3 min, 3 mL of a solution of 7% Na_2CO_3 was added, and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 765 nm.

3. Results

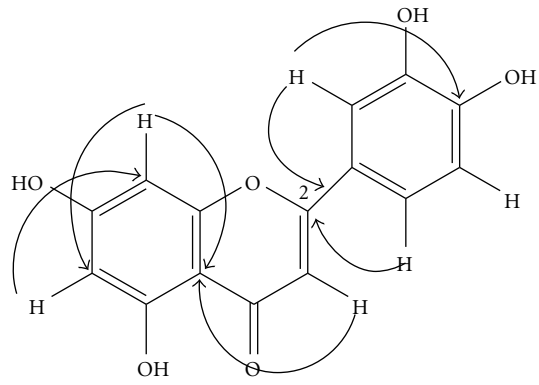

Figure 2: The selected HMBC correlation of of 5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4H-chromen-4-one.

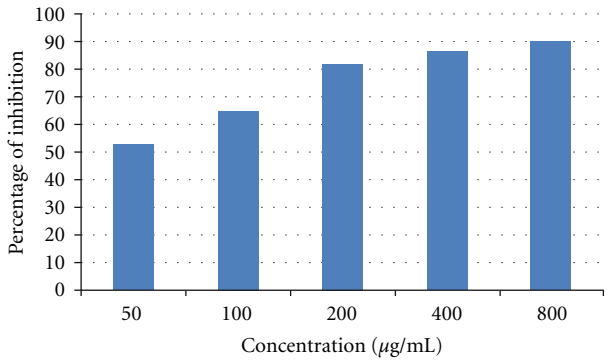

Figure 3: Reducing power of aerial parts extract of 5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4H-chromen-4-one.

3.1. Chemical Component Identified. Luteoline (**1**) was isolated from a $CHCl_3 : CH_3OH$ (3 : 1) extract of *C. behen* (Figure 1). Metabolite (**1**) had the same molecular formula; $C_{15}H_{10}O_6$ (MW = 286.047738) was obtained on the basis of ^{13}C NMR and mass spectra analysis. ^1H and ^{13}C-NMR spectra for (**1**) were shown at 6.20 (H-1 and H-2), 6.43 (H-3), 6.44 (H-4), 7.40 (H-5), 8.90 (H-6), 6.98 (H-5′), and 10.09 (OH-7 to OH-4′) (Figure 1). Four hydroxyl groups were observed on the spectrum, which were assigned to 10.09 (H-7 to H-4′) in ^1H NMR. Also some signals ^{13}C NMR were δ133 (C-1′), 124 (C-2′), 155 (C-3′), 163 (C-4′), 122 (C-5′), 133 (C-6′), 155 (C-2), 113 (C-3), 193 (C-4), 162 (C-5), 100 (C-6), 170 (C-7), 101 (C-8), 179 (C-9), 111 (C-10)146.5, 163.9, 166.4, and 147.2.

The combination HMBC correlation spectral data of Figure 2 indicated the presence of groups at 6.98 (H-5′) and 10.09 (H-7 to H-4′) (Table 1). Four hydroxyl groups were observed on the spectrum, which were assigned to 10.09 (H-7 to H-4′) in ^1H NMR and 170, 163, 170, and 163 in ^{13}C NMR.

3.2. Amount of DPPH. The antioxidant activity of the compound was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH. The *C. behen* extract was able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine with an IC_{50} value of 200 ± 7.0 $\mu g\,mL^{-1}$. The concentration of the positive control Vitamin C required to scavenge 50% of the free radical was 260 ± 8.0 $\mu g\,mL^{-1}$. In this research, we used Vitamin C for measurement DPPH and compare together (Figure 3).

3.3. Total Phenol Content. Total phenol compounds, as determined by folin Ciocalteau method, are reported as gallic acid equivalents by reference to standard curve. The plant had good total phenol contents and they may cause the antioxidative activities of the *C. behen.* Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities. In our investigation the highest total phenolic content was found in the *C. behen*. We experiment all data and replicated three times.

4. Discussion

4.1. Chemical Component Identified. Various species of the genus *Centaurea* have been the object of phytochemical investigations, which have showed their wealth of bioactive secondary metabolites, in particular flavonoids [23, 24] and sesquiterpene lactones [25–28].

For this study, the aerial parts of *Centaurea behen* were collected from Givi, Khalkhal Road (Ardabil province) in the northwest of Iran. This plant contained flavonoid compounds and showed antioxidant activity, indicating potentially wide applications in formulation of medicinal drugs. Extracts of the aerial parts of *C. behen* obtained using a Soxhlet extractor were chromatographed on silica gel columns. The compound obtained was identified as compound (**1**). The structure of the compound was established by chemical and spectral analysis, including UV and ^1H and ^{13}C NMR as well as by comparing their spectroscopic data with those reported in the literature. The mass spectrum presented at m/z 286 according to the molecular formula $C_{15}H_{10}O_6$. The ^1H NMR spectrum ($CDCl_3$) showed groups at 6.98 (H-5′) and 10.09 (H-6′ to H-4′). The spectrum also showed a one-proton singlet signal at 10.09 (H-4′) in ^1H NMR, which may be the flavonoid skeleton. Other signals of the ^{13}C NMR spectrum were shown at δ124 (C=), 163 (O–C=), and a doublet at dH 5.13 (J = 7.6 Hz). Significant HMBC correlations were observed between H-2′ and C-4′, and between H-3 and C-5, confirming the location of the OH groups. The UV spectrum exhibited absorption maxima at 283 nm and 369 nm, which are characteristic absorption bands of a flavonoid skeleton as reported in the literature concerning 5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4H-chromen-4-one [29]. The isolated flavonoid has a weight of 14 mg. That showed good yield for luteoline. In other research, we identified a sesquiterpene with low yield [20]. So in next fraction, we found glycoside luteline but we cannot separate by HPLC because that was not suitable amount. So in this plant were other flavonoids with trace small.

4.2. Antioxidant Activity. Antioxidant activity of the compound **(1)** was determined by two different test systems, DPPH and total flavonoid. In the DPPH method, the antioxidants react with the stable free radical, DPPH (deep violet color), and convert it to DPPH with discoloration. The degree of discoloration indicates the free radical scavenging potentials of the sample/antioxidant. It has been found that known antioxidants such as cysteine, glutathione, ascorbic acid, and polyhydroxy aromatic compounds (hydroquinone, pyrogallol, etc.) reduce and decolorize DPPH due to their hydrogen-donating ability. Synergistic effects of phenolic acids, for example, rosmarinic acid and polyphenols as well as other chemicals such as flavonoids, could also be taken into account for the radical scavenging activity observed in the methanol extracts.

The total phenolic content of *C. behen* extract was measured by the Folin-Ciocalteau method. Total phenol compound, as determined by the Folin-Ciocalteau method, are reported as gallic acid equivalents by reference to the standard curve. The highest total phenolic content was found in the *C. behen* extract [2, 12, 30]. Figure 3 shown to stable free radical DPPH method is an easy, rapid, and sensitive way to survey the antioxidant activity of a specific compound or plant extracts. The capacity of plant extract to scavenge DPPH was measured and the results are shown in Figure 3. The antioxidants react with DPPH, a purple-colored stable free radical, and convert it into a colorless α-α-diphenyl-β-picryl hydrazine. The amount of reduced DPPH could be quantified by measuring the decrease in absorbance at 517 nm. CS extract reduced DPPH radicals in a dose-dependent manner. IC_{50} of the standard compound, Vitamin C was $260 \pm 8.0\,\mu g\,mL^{-1}$. As can be seen in Figure 3, the extract at $72\,mg\,mL^{-1}$ scavenged about >80% of DPPH radicals and had an IC_{50} value of $59\,mg\,mL^{-1}$. So the extract showed potency than the controls in this study. The DPPH scavenging ability of the extract may be attributed to its hydrogen donating ability.

5. Conclusions

In this work we performed a chemical analysis of luteoline extracted from the aerial parts of *C. behen* and studied its antioxidant activity. We identified the flavonoid derivative compound **(1),** which was isolated by UV, CC, and HPLC. The structure was elucidated by ^{1}H and ^{13}C NMR and HMBC spectra. The compound identified had not been reported in our previous studies. Radical scavenging activity exhibited was determined to be $IC_{50} = 200 \pm 7.0\,\mu g\,mL^{-1}$.

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