Original Article

Isolation and Identification of Two Phenolic Compounds from a Moderately Cytotoxic Fraction of *Cousinia verbascifolia* Bunge

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Abstract

Background: Little information is available about chemical components of the *Cousinia* genus. A primary cytotoxicity screening on *Cousinia verbascifolia* showed moderate cytotoxic activity against OVCAR-3 ovarian and HT-29 colon cancer cells. Therefore, the aim of this study is a phytochemical investigation to identify the compounds responsible for this bioactivity. **Materials and Methods:** Extraction was done through percolation and fractionations by reverse phase column chromatography and normal column chromatography. Using standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay Fr.b8 with moderate cytotoxicity was selected for identification of major components. Fr.b8 was subjected to polyamide column chromatography. More purification was done using a new modified recycle high-performance liquid chromatography (HPLC) with flow splitter. **Results:** Two known compounds: Apigenin (flavone) and caffeic acid (phenolic acid) were obtained from phenolic bioactive fraction for the first time from this plant. **Conclusions:** Apigenin and caffeic acid with known antitumor and matrix metalloproteinase inhibitory effects seem to be the bioactive components responsible for moderate cytotoxicity of phenolic fraction. Recycle HPLC following with flow splitting is a new method useful for isolation of closely eluted compounds in HPLC chromatogram.

Keywords: *Apigenin, Asteraceae, caffeic acid, colon cancer, Cousinia verbascifolia, ovary cancer, recycle high-performance liquid chromatography*

Introduction

Plant kingdom provides a rich and wonderful source of biologically active compounds. There are about 250,000–500,000 plant species on earth, out of which only about 20% have been submitted to biological and phytochemical screening.[1-3] Hence, a huge number of plant species still remain to be studied and described in terms of biological and phytochemical profiles.

Cousinia, one of the largest genera of *Asteraceae* with 600–700 species, is one of the most diverse genera in central and South-West Asia. About 250 species of the genus are distributed throughout Iran, comprising about 200 endemic species.[4-7] Based on phytochemical studies on a few *Cousinia* species chemical constituents including acetylenes, triterpenes, steroids, sesquiterpene lactones, and flavonoids were reported.[8-17] However, little information is available about the phytochemical and biological properties of the species grow in Iran. In a phytochemical study on the aerial parts of three Iranian species of the genus *Cousinia* (*Cousinia picheriana*, *Cousinia piptocephala*, and *Cousinia canescens*), all three species yielded guianolide class sesquiterpens including 10 (14), 11 (13)-guiadien, 12, 6-olide, 4 (15), 10 (14)-guiadien, 12, 6-olide, and 4 (15), 10 (14), 11 (13)-guiatrien, 12, 6-olide derivatives mainly differed in functions on C-3, C-4, C-15, and ester located on C-8. In addition, *C. canescens* contained oxygenated bisabolene derivatives.[9] In another study on cytotoxicity of total ethanol extract of seven Iranian *Cousinia* species against fibrosarcoma WEHI 164 cancer cells, they showed medium cytotoxicity with inhibitory concentration ranged between 18.4 ± 0.59 and 87.9 ± 0.58 μg/mL. In the same study on fibrosarcoma WEHI 164 cancer cells, most of the *Cousinia* species had considerable inhibitory effect on matrix metalloproteinase (MMP) activity.[18]

Cousinia verbascifolia Bunge is endemic to Kopetdagh (NE Iran and S Turkmenistan), and could be seen

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in open areas, scrublands, and stony slopes.[6] It is a monocarpic biennial plant, up to 45 cm high, stems simple or branched from the base, leaves lyrate with spiny-dentate margin, flowers 50–150; corolla pink, rose or purple, 20–35 mm long. The flowering period of the plant is May to July.[6] In a primary study by authors, different fractions of *C. verbascifolia* showed medium cytotoxicity against OVCAR-3 and HT-29 cells.[20] In this present study, due to the observed inhibitory properties of semi-polar fractions of *C. verbascifolia*'s extract against cancer cells, phytochemical investigation on these semi-polar fractions were carried out using a new modified recycle high-performance liquid chromatography (HPLC) technique which was used in the purification process.

Materials and Methods

General

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance AV 400, using pyridine-d6 as solvent. Electrospray ionization mass spectrometry (ESI-MS) spectra were measured in negative mode on Shimadzu 2010EV LC-MS system (Shimadzu, Japan).

Chromatographic conditions

Column chromatography runs were performed using silica gel, 63–200 μm (Merck), polyamide DC6 (MACHEREY-NAGEL, Duren, Germany), and reverse phase chromatography was done by LiChroprep RP-18, 40–60 μm (Merck). High performance thin layer chromatography was performed on silica gel GF-254 plates (Merck KGaA, Darmstadt, Germany). Plates were developed by natural product reagent (1% methanolic diphenyl-boric acid-ethanolamine) and visualized by ultraviolet (UV) fluorescent colors at 254/366 nm UV lamps. Recycle-high pressure liquid chromatography was done on a Waters HPLC apparatus (Waters Assoc., Milford, MA, USA), at 250 nm using silica gel column (YMC-Pack SIL, 250 mm × 20 mm, YMC Co., Kyoto, Japan).

Plant material

Flowering aerial parts of *C. verbascifolia* (*Asteraceae*) were collected from Salehabad, Torbat-e Jam in Razavi Khorasan Province, Iran, in May 2013. It was identified by Dr. Iraj Mehregan. A voucher specimen (No. 2838) was deposited at the Herbarium of the Pharmacognosy Department, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, I. R. Iran.

Extraction, isolation, and purification

Air dried aerial parts of *C. verbascifolia* (3.5 kg) were extracted via percolation method using methanol as solvent. Filtration and in vacuum concentration of methanol extract resulted in a green gum which was subjected to reverse phase chromatography for removing fats and chlorophylls using methanol:water (80:20) as solvent. Defatted fraction (41.3 g) was then subjected to a silica gel column chromatography using hexane/acetone as mobile phase with increasing polarity (5→50%) that afforded 11 fractions (Fr.b1-8). These fractions were studied for cytotoxic activity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide standard method against ovarian and colon cancer cells.[19,20] Detailed description of this study is published by authors elsewhere.[20] The cytotoxicity screening revealed that Fr.b2, Fr.b4, and Fr.b8 were the most cytotoxic fractions.[20] Fr.b2 contained mainly fatty acids and common steroids which were discarded. Inspired from ^{1}H-NMR spectra, Fr.b4 contained sesquiterpene lactones but with inadequate amounts for further analysis that were excluded from the study. Finally, bioactive fraction, Fr.b8, was selected for identification of major components responsible for the observed cytotoxic effects. Fr.b8 was then subjected to polyamide DC6 column (40 cm × 2 cm) using chloroform:methanol (2→20%) with increasing polarity to yield fractions Fr.b8a-d. After thin layer chromatography visualization, Fr.b8d eluted by chloroform:methanol (88:12) with positive reaction to natural product reagent was selected and injected into the recycle HPLC system using YMC-SIL column (25 cm × 2.5 cm), particle size: 10 μm, mobile phase: chloroform:methanol (85:15), injection volume: 2 ml, flow rate: 3 ml/min, chart rate: 0.1 cm/min, and detector wavelength: 250 nm.

In water, 501 HPLC pump a recycle valve and a flow splitter were incorporated in HPLC system between detector and HPLC pump, so that the chromatographic profile could reenter the column after passing through the detector. This process resembles using a longer column and thus provides more theoretical plates leading to increasing the column efficiency and resolution. In the case of observing tailing in the peaks, flow is splitted before reentering the column. Finally, during any cycle in which the desired resolution is achieved, samples are collected. In this process, after three recycles following the flow splitting (80% to pump and 20% to waste), the peaks were sufficiently resolved to compounds A, B, and C [Figure 1]. Compound B was excluded from the study because of insufficient purity, and compounds A and C were subjected to mass and NMR spectroscopy which led to the identification of compounds 1 (30 mg) and 2 (10 mg) [Figure 2].

Results

Two known compounds were obtained from phenolic bioactive fraction for the first time from this plant. Their physical properties are given bellow:

Compound 1

Pale yellow powder, ^{1}H-NMR in pyridine-d6 (400 MHz) ppm: 7.76 (d, 2H, J = 8.9 Hz), 6.84 (d, 2H, J = 8.8 Hz), 6.50 (s, ^{1}H), 6.37 (d, 1H, J = 2.1 Hz), 6.11 (d, 1H, J = 2.1 Hz); ^{13}C-NMR (100 MHz, pyridine-d6) δ_C: 163.8 (C2), 104.4 (C3), 183.2 (C4), 106.5 (C4a),

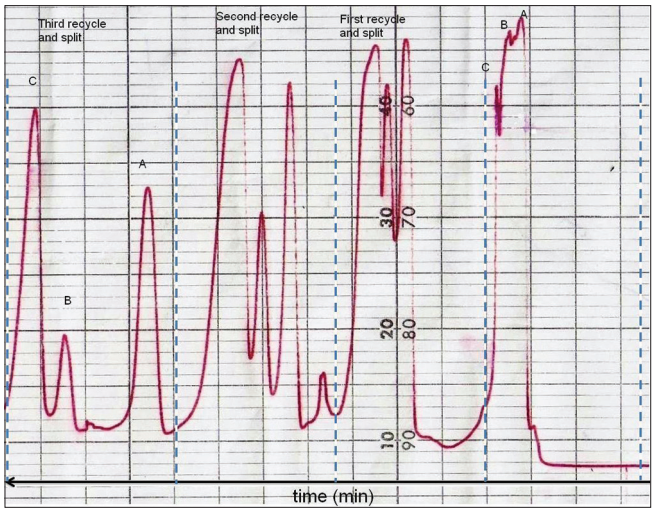

Figure 1: Isolation of apigenin (A) and caffeic acid (C) from *Cousinia verbascifolia* by implementing recycle high-performance liquid chromatography technique in the purification process. Fraction Fr.b8d was injected into the recycle high-performance liquid chromatography system using YMC-SIL column (10 µm, 25 cm × 2.5 cm) using chloroform:methanol (85:15) at 250 nm. After three recycles, the peaks were sufficiently resolved to compounds A, B, and C

163.2 (C5), 100.5 (C6), 166.4 (C7), 95.3 (C8), 159.0 (C8a), 122.8 (C1'), 129.4 (C2'), 117.3 (C3'),163.3 (C4'), 117.3 (C5'), 122.8 (C6'). ESI negative mass m/z 269 (M–1).

Compound 2

White powder, ^{1}H-NMR in pyridine-d6 (400 MHz) ppm: 9.63 (d, 1H, J = 15.9 Hz, H-7), 9.16 (d, 1H, J = 1.4 Hz, H-2), 8.73 (d, 1H, J = 8.1 Hz, H-5), 8.71 (dd, 1H, J = 1.6 Hz, J = 8.2 Hz, H-6), 8.33 (d, ^{1}H, J = 15.8 Hz, H-8); ^{13}C-NMR (100 MHz, pyridine-d6) δ_C: 171.7 (C1), 116.7 (C2), 148.7 (C3), 147.9 (C4), 117.8 (C5), 122.7 (C6), 146.2 (C-7), 117.8 (C-8), 170.6 (C-9). ESI negative mass m/z 179 (M–1).

Discussion

Compound 1 was isolated as a pale yellowish solid with positive reaction to $FeCl_3$ and natural product reagents. Molecular formula was determined by NMR data and negative ESI-MS m/z 269 (M–1) as $C_{15}H_{10}O_5$. Eleven degrees of unsaturation and NMR data suggested the presence of one carbonyl carbon, seven olefin bands, and therefore, three rings in the skeleton. The UV spectrum showed absorption maxima at 265 and 340 nm that are characteristic of flavones.[8] The ^{1}H-NMR spectrum displayed meta-coupled doublets at δ 6.11 (1H, d, J = 2 Hz) and 6.37 (1H, d, J = 2 Hz) described to H-6 and H-8. In addition, ortho-coupled proton signals at δ 6.84 (^{2}H, d, J = 8. 5 Hz) and 7.76 (2H, d, J = 8.5 Hz), corresponding to H-5',3' and H-6',2' as well as δ 6.50 (^{1}H, s) corresponding to H-3 proton, indicated that compound 1 is a flavone derivative. Comparison of the spectral data with those published before allowed us to establish the structure of compound 1 as 4',5,7-trihydroxyflavon or apigenin.[21]

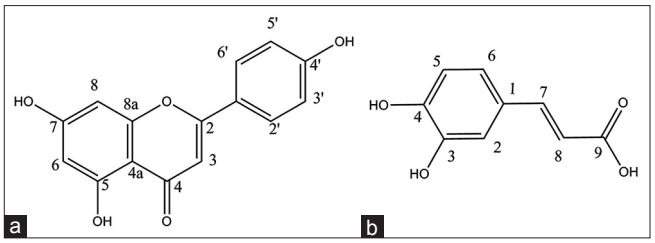

Figure 2: Apigenin (a) and caffeic acid (b) isolated from *Cousinia verbascifolia* Bunge

Compound 2 was yielded as a white solid with positive reaction to $FeCl_3$. The six degrees of unsaturation derived from positive HR-ESI-MS m/z 181.0495 (calcd. for $C_9H_8O_4$ + H^+, 181.0495) and NMR data suggested the presence of one carbonyl carbon, four olefin bands, and therefore, one ring in the skeleton. The ^{1}H-NMR spectra displayed meta-coupled doublets at δ 9.16 (^{1}H, d, J = 1.4 Hz) as well as ortho-coupled proton signals at δ_H 8.73 (1H, d, J = 8.1 Hz) and 8.71 (1H, d, J = 1.6, 8.2 Hz), described to H-2, H-6 and H-8. The above ABX spin coupling system resembled 3,4-disubstituted benzyl derivatives.[22] In addition, two trans-olefin protons at δ_H 9.63 (1H, d, J = 15.9 Hz) and 9.63 (1H, d, J = 15.9 Hz) with relatively large coupling constant J = 15.8 Hz and acidic proton at down field aria at δ_H 10.1 ppm were observed. Comparison of the spectral data with those published before allowed us to establish the structure of compound 2 as 3-(3,4-dihydroxyphenyl)-2-propenoic acid or caffeic acid.[23,24]

Numerous studies have reported anticancer properties of both apigenin and caffeic acid through various mechanisms and pathways.[25,26] In a report by Wang *et al*., they investigated the cell growth inhibition and cell-cycle arrest caused by apigenin against HT-29 colon cancer cell line. The results showed that apigenin induces a cell cycle arrest at G2/M phase, but this inhibitory effect on cancer cells appeared to be cytostatic, not cytotoxic.[27] Antitumor effects of caffeic acid against colon and hepatocarcinoma cell lines are also reported through MMP-9 inhibitory effects.[28-30] There are many evidence supporting those selective MMP inhibitors are effective in prevention and treatment of cancer.[28-32] Caffeic acid and its derivatives selectively inhibited MMP-2 and -9.[29] In a similar study, hepatocarcinoma cells with phorbol ester (PMA)-induced MMP-9 expression were also suppressed followed by caffeic acid derivatives treatment. Their oral administrations significantly reduced the hepatic metastasis.[28,29] Apigenin has also been reported to inhibit MMP-9 secretion.[33] The IC_{50} of apigenin and caffeic acid against HT-29 colon cancer cell line was reported as 50 and 60 µM, respectively.[27,34] In a previous study, Shahverdi *et al*. demonstrated cytotoxicity and MMP inhibitory effects of *C. verbascifolia*.[18] These results together with aforementioned properties of apigenin and caffeic acid may explain the moderate cytotoxicity of *C. verbascifolia* phenolic fraction.

Conclusion

Two phenolic compounds: Apigenin (flavone) and caffeic acid (phenolic acid) were isolated for the first time from *C. verbascifolia.* Both of these compounds possess antitumor and MMP inhibitory effects. Thus, the bioactivities observed from *C. verbascifolia* in the previous studies might be due to the presence of these two phenolic compounds. Moreover, recycle HPLC following with flow splitting is a new method useful for isolation of closely eluted compounds in HPLC chromatogram.

Limitation of the study

The limitation of this study was inadequate amounts of sesquiterpene lactones in fraction Fr.b4 for further purification and elucidation. One another limitation of this study was the sample size due to the low yield of the extract. Although apigenin and caffeic acid seems to be responsible for observed cytotoxic effects but more phytochemical investigation with larger amounts of the plant material is suggested for identification of sesquiterpene lactones as another possible compounds responsible for cytotoxic activities in this plant.

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Conflicts of interest

There are no conflicts of interest.

References

1. Dutt R, Garg V, Madan AK. Can plants growing in diverse hostile environments provide a vital source of anticancer drugs. J Cancer Ther 2014;10:13-37.
2. Niño J, Narváez DM, Mosquera OM, Correa YM. Antibacterial, antifungal and cytotoxic activities of eight *Asteraceae* and two *Rubiaceae* plants from Colombian biodiversity. Braz J Microbiol 2006;37:566-70.
3. Pan L, Chai HB, Kinghorn AD. Discovery of new anticancer agents from higher plants. Front Biosci (Schol Ed) 2012;4:142-56.
4. Attar F, Djavadi SB. A taxonomic revision of *Cousinia*, sect. *Cynaroides* (*Asteraceae*, *Cardueae*) in the flora of Iran. Iran J Bot 2012;16:130-84.
5. Mehregan I. Systematics, Phylogeny and Biogeography of *Cousinia* (*Asteraceae*). Ph.D, Thesis, Fachbreich Biologie Der Johannes Gutenberg-Universitat Mainz; 2008.
6. Mehregan I, Kadereit JW. Taxonomic revision of *Cousinia* sect. Cynaroideae (*Asteraceae*, *Cardueae*). Willdenowia 2008;38:293-362.
7. Zare M, Khosravi AR, Joharchi MR. Distribution patterns of the genus *Cousinia* (*Asteraceae*) in Iran. Iran J Bot 2013;19:127-41.
8. Bohlmann F, Burkhardt T, Zdero C. Naturally Occurring Acetylenes. London: Academic Press; 1973.
9. Alberto Marco J, Juan FS, Albiach R, Rustayian A, Habibi Z. Bisabolene derivatives and sesquiterpene lactones from *Cousinia* species. Phytochemistry 1993;32:395-400.
10. Rustaiyan A, Niknejad A, Sigari H, Ahmadi A. Guaianolides from *Cousinia onopordioides*. Fitoterapia 1981;52:31-2.
11. Rustaiyan A, Sharif Z, Sadjadi A. Two farnesol derivatives from *Cousinia adenostica*. Phytochemistry 1987;26:2635-6.
12. Turdumambetov K, Rakhimov DA, Malikova MK. Oligo-and polysaccharides from *Cousinia umbrosa*. Molbank 2007;43:308-9.
13. Turdumambetov K, Plekhanova NV, Rakhimov DA, Yagudaev MR. Glucofructans of *Cousinia polycephala*. Molbank 1989;25:371-2.
14. Plekhanova NV, Turdumambetov K, Sudnitsyna IG. Carbohydrates of *Cousinia*. Molbank 1983;19:603-4.
15. Ulubelen A, Tuzlaci E, Mericli A. Triterpenic and steroidal compounds from *Cousinia canescens*. Fitoterapia 1986;57:269-70.
16. lubelen A, Tuzlaci E. Flavonoids and terpenoids from *Cousinia eriocephala*. Fitoterapia 1988;59:350.
17. Azimova S, Glushenkova A. Lipids, Lipophilic Components and Essential Oils from Plant Sources. London: Springer; 2012. p. 110-3.
18. Shahverdi AR, Khorramizadeh MR, Attar F, Saadat F, Vahid S, Ghahraman A. Concomitant chemopreventive and antibacterial effects of some Iranian plants from the genus *Cousinia* (*Asteraceae*). Braz J Pharmacogn 2007;17:325-30.
19. Zarei SM, Ayatollahi AM, Ghanadian M, Kobarfard F, Aghaei M, Choudhary MI, *et al*. Unusual ingenoids from *Euphorbi*a *erythradeni*a Bioss. With pro-apoptotic effects. Fitoterapia 2013;91:87-94.
20. Sajjadi SE, Ghanadian M, Haghighi M, Mouhebat L. Cytotoxic effect of *Cousinia verbascifolia* Bunge against OVCAR-3 and HT-29 cancer cells. J Herb Med Pharmacol 2015;4:15-9.
21. Markham KR. Techniques of Flavonoid Identification. Vol. 31. London: Academic Press; 1982.
22. Ghanadian M, Sadraei H, Yousuf S, Asghari G, Choudhary MI, Jahed M. New diterpene polyester and phenolic compounds from *Pycnocycla spinosa* Decne. Ex Boiss with relaxant effects on KCl-induced contraction in rat ileum. Phytochem Lett 2014;7:57-61.
23. Kelley CJ, Harruff RC, Carmack M. Polyphenolic acids of *Lithospermum ruderale*. II. Carbon-13 nuclear magnetic resonance of lithospermic and rosmarinic acids. J Org Chem 1976;41:449-55.
24. Andary C, Wylde R, Laffite C, Privat G, Winternitz F. Structures of verbascoside and orobanchoside, caffeic acid sugar esters from *Orobanche rapum-genistae*. Phytochemistry 1982;21:1123-7.
25. Lefort ÉC, Blay J. Apigenin and its impact on gastrointestinal cancers. Mol Nutr Food Res 2013;57:126-44.
26. Rocha LD, Monteiro MC, Teodoro AJ. Anticancer properties of hydroxycinnamic acids. – A review. Cancer Clin Oncol 2012;1:109-21.
27. Wang W, Heideman L, Chung CS, Pelling JC, Koehler KJ, Birt DF. Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines. Mol Carcinog 2000;28:102-10.
28. Rao CV, Desai D, Kaul B, Amin S, Reddy BS. Effect of caffeic acid esters on carcinogen-induced mutagenicity and human colon adenocarcinoma cell growth. Chem Biol Interact 1992;84:277-90.
29. Chung TW, Moon SK, Chang YC, Ko JH, Lee YC, Cho G, *et al*. Novel and therapeutic effect of caffeic acid and caffeic acid

phenyl ester on hepatocarcinoma cells: Complete regression of hepatoma growth and metastasis by dual mechanism. FASEB J 2004;18:1670-81.

30. Chiang EP, Tsai SY, Kuo YH, Pai MH, Chiu HL, Rodriguez RL, *et al.* Caffeic acid derivatives inhibit the growth of colon cancer: Involvement of the PI3-K/Akt and AMPK signaling pathways. PLoS One 2014;9:e99631.
31. Li NG, Shi ZH, Tang YP, Duan JA. Selective matrix metalloproteinase inhibitors for cancer. Curr Med Chem Anticancer Agents 2009;16:3805-27.
32. Nemunaitis J, Poole C, Primrose J, Rosemurgy A, Malfetano J, Brown P, *et al.* Combined analysis of studies of the effects of the matrix metalloproteinase inhibitor marimastat on serum tumor markers in advanced cancer: Selection of a biologically active and tolerable dose for longer-term studies. Clin Cancer Res 1998;4:1101-9.
33. Lindenmeyer F, Li H, Menashi S, Soria C, Lu H. Apigenin acts on the tumor cell invasion process and regulates protease production. Nutr Cancer 2001;39:139-47.
34. Ng SH. Characterization of Colon Cancer Cell Culture Based Screening Assay to Study Effects of Phenolic Acids. Thesis for Ph.D, University of Saskatchewan, Saskatoon, USA; 2011.