Three phenolic glycosides and immunological properties of *Achillea millefolium* from Iran, population of Golestan

*[1]Yassa N., [1]Saeidnia S., [2]Pirouzi R., [1]Akbaripour M., [3]Shafiee A.

[1]Department of Pharmacognosy, Faculty of Pharmacy and Medicinal Plant Research Center, Tehran University of Medical Sciences. [2] Faculty of Pharmacy, Islamic Azad University, Tehran. [3]Department of Medicinal Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran.

Received 28 May 2006; Revised 2 Aug 2006; Accepted 5 Aug 2006

ABSTRACT

From MeOH extract of aerial parts of *Achillea millefolium* L. collected from Golestan province of Iran, three glycosylated phenolic compounds, luteolin 7-O-glucoside, apigenin 7-O-glucoside and caffeic acid glucoside were isolated and identified by spectroscopic analyses. Immunological properties of different fractions of plant extract were studied on humoral immune system of BALB/c mice by Microhaemagglutination test. Among these fractions only two fractions at 125 mg kg^{-1} and 61.5 mg kg^{-1} showed a significant decrease in the anti- SRBC titer of mice (P<0.05). The immunological properties of the latter fractions may be due to glycosylated derivatives of caffeic acid.

Keywords: *Achillea millefolium,* Asteraceae, Flavonoids, Humoral Immune System

INTRODUCTION

The genus *Achillea* (Asteraceae) comprises 115 species, which are mainly distributed in Europe, Asia, and North America but it is an introduced genus in the New World (1). This genus is well known for medicinal properties such as anthelmintic, anti- inflammatory and anti-microbial effects (2, 3). These plants have been traditionally used for treatment of fever, asthma, bronchitis, cough, skin inflammation, jaundice and other liver disease in Iran (4). *Achillea millefolium* L. is one of nineteen herbaceous species growing in north of Iran (5).

Previous phytochemical studies showed three methoxylated flavones from *A. millefolium* (USA origin) which were identified as casticin, artemetin and 5-hydroxy-3, 6, 7, 4′-tetramethoxy-flavone (6). Also *A. millefolium* has been found to be rich in 7-O and 7-malonyl glycosides of two flavones, apigenin and luteolin (7, 8). The results of effects of *A. millefolium* and *A. talagonica* essential oils on humoral immune responses in experimental animals indicated that essential oil of *A. millefolium* had immunosuppressive activity on primary humeral responses (9). This result was of interest because the oil of *A. millefolium* has been used to treat neuralgia and rheumatic pains in traditional medicine (10).

Literature survey showed that there was no report about the flavonoid constituents of the Iranian species and in the present study the isolation and identification of phenolic contents and immunological properties of *A. millefolium* methanolic extract is reported.

MATERIALS AND METHODS

Plant material

A. millefolium subsp. *millefolium* was collected in August 2003 during the flowering stage, from Golestan province near to TV and Radio Tower of Derazno in north part of Iran. A voucher specimen (No. 13607) has been deposited at the private herbarium of Dr. H. Akhani, housed in the Department of Biology, Faculty of Sciences, Tehran University, Tehran, Iran.

Experimental animals

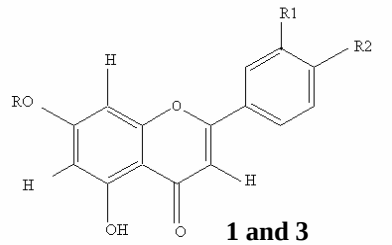
BALB/c albino female mice (17-22g) purchased from Pastor Institute of Iran. The mice received a standard pellet diet and water ad libitum, and were maintained under standard environmental conditions (20 ºC ±3, 12h light/dark cycle).

Instruments and Materials

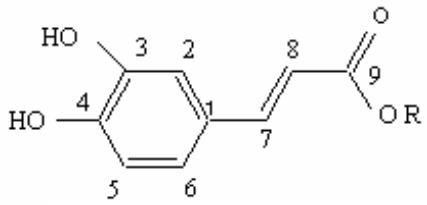
^{1}H and ^{13}C-NMR spectra were measured on a Brucker (300 MHz) spectrometer with TMS as an internal standard, and chemical shifts are given in δ (ppm). Mass Spectra (EIMS) were determined on a Finnigan TSQ-MAT 70 at 70 eV. Ultra violet spectra were recorded on a Shimadzu UV-160A instrument.

Chromatographic papers were purchased from Whatman Company. All compounds for UV

Correspondence: yasa@tums.ac.ir



1 and 3



2

Compound	R	R1	R2
1	Glu	OH	OH
3	Glu	H	OH

Figure 1. Structure of compounds: **1** luteolin 7-O-glucoside**; 2** caffeic acid glucoside and **3** apigenin 7-O-glucoside

spectral shift reagents, natural product reagent and sugar indicator, solvents, glacial acetic acid, authentic samples of sugars and concentrated NH_3 were purchased from Merck Company.

Extraction

Aerial parts (leaves, stems and capitols) of *A. millefolium* were dried in shadow and cut into small pieces. Powdered crude material (150g) was extracted by percolation method with methanol at room temperature. The liquid extract was evaporated and dried under vacuum to give a gummy extract (10.8g %, fraction A). The crud material was extracted with petroleum ether (discarded), chloroform (1.95g %, fraction B) and ethyl acetate (0.93g %, fraction C). The residue (7.82g %) was fraction D. Aqueous extract of powdered plant (50 g) was prepared by maceration followed by evaporation of extract at low temperature and pressure (13.18g %, fraction E).

Microhaemagglutination test

Several groups, each consisting of 6 mice, were used. The treated groups were injected intra peritoneally with different doses(31.2, 62.5, 125, 250 and 500 mg kg^{-1}/day in 0.5 ml of solvent) of each fraction A, B, C, D and E. Control group received i.p. injection of saline (0.5 ml). The test and control groups injected for 6 consecutive days. The native group received nothing. On the 8th day, all except the natives mice were injected (i.p.) 2×10^8 sheep red blood cells (SRBC). After 6 days, bloods were collected from the retro-orbital plexus of animals. Serums were separated and Hemagglutinating Antibody titer (HA) was determined by the microtiter plate method (4).

Isolation procedure

Because of the immunosuppressive activity of the residue (fraction D), a part of this extract (1g) was submitted to paper chromatography (PC). The chromatograms were developed descendingly in the long direction of Whatman 3 MM chromatographic papers in the chromatocab using BAW (n-butanol: acetic acid: water, 4:1:5) as eluent for 12 hours (12) to obtain flavonoid lines as follow: line1 (R_f = 0.39, purple before and after using NH_3 at 366nm), line 2 (R_f = 0.73, blue color before and after using NH_3 at 366nm), line 3 (R_f = 0.68, purple before and after using NH_3 at 366nm).

For further purification, compounds were subjected to PC with 15% acetic acid as eluent (12) to separate compounds 1 (23 mg, R_f = 0.15, purple before and after using NH_3 at 366nm), 2 (250 mg, R_f = 0.38, blue before and after using NH_3 under 366nm) and 3 (32 mg, R_f = 0.22, purple before and after using NH_3 at 366nm). Detection of flavonoids was carried out under ultraviolet lamp (366 nm) and then treatment with Natural Product reagent (13).

Compound **1**: *Luteolin-7-O-glycoside*, ^{1}H-NMR (DMSO d_6): δ 7.42 (1H, *bs*, $H_{2'}$), 7.39(1H, *bd*, *J* = 8.4 Hz, $H_{6'}$), 6.92(1H, *d*, *J* = 8.4 Hz, $H_{5'}$), 6.81(1H, *d*, *J* = 2 Hz, H_8), 6.73(1H, *s*, H_3), 6.44(1H, *d*, *J* = 2 Hz, H_6), 5.05(1H, *d*, *J* = 6.6 Hz, Glu $H_{1'}$), 3.5-5.05(6H, m, sugar protons); ^{13}C NMR (DMSO d_6): δ 182.1(C-4), 163.8 (C-2), 162.8 (C-7), 161.2 (C-5), 157.0 (C-9), 149.8 (C-4′), 145.8 (C-3′), 121.4 (C-1′), 120.2 (C-6′), 116.0 (C-5′), 113.5 (C-2′), 105.1 (C-10), 103.2 (C-3), 99.6 (C-6) 95.0 (C-8) and sugar: 98.0 (C-1"), 78.4 (C-2"), 77.1 (C-5"), 75.8 (C-3"), 69.6 (C-4"), 60.6 (C-6"); EIMS: m/z 286 [aglycon fragment]$^+$,152 [A1]$^+$, 137 [B_2]$^+$, 109 [B2- CO]$^+$; UV λ_{max} nm in MeOH: 346, 258; +NaOMe 407, 267; +$AlCl_3$ 422, 320sh, 265; +$AlCl_3$/HCl 390, 375, 273; +NaOAc 400, 362, 258; +NaOAc/BO_3H_3 370, 258.

Compound **2:** *Caffeic acid gluoside*, ^{1}H-NMR (DMSOd6): *δ* 7.49 (1H, *d*, *J*= 15.4 Hz, H_7), 7.06 (1H, *d*, *J* = 1.5 Hz, H_2), 6.91(1H, *dd*, *J* = 8.4, 1.5 Hz, H_6), 6.78 (1H, *d*, *J* = 8.4 Hz, H_5), 6.21 (1H, *d*, *J* = 15.4 Hz, H_8), 5.27(1H, *d*, *J* = 6.6 Hz, Glu $H_{1'}$), 3.42-5.27 (6H, m, sugar protons); ^{13}C-NMR

Table1. The results of different fractions of *A. millefolium* extract on antibody titer against control.

No. of groups	groups and doses (mg/kg)	No. of alive mice	Mean ± SD
1	Native [1]	6	93 ± 13*
2	Control (NS/DMSO) [2]	6	1108 0 ± 3082
3	Control (NS) [3]	6	8533 ± 2778
4	Fraction **A** 31.5	5	7480 ± 1378
5	Fraction **A** ≥ 62.5	0	-
6	Fraction **B** 31.5	5	12140 ± 2280
7	Fraction **B** ≥ 62.5	0	-
8	Fraction **C** 31.5	4	11080 ± 3082
9	Fraction **C** ≥ 62.5	0	-
10	Fraction **D** 62.5	5	7680 ± 1478
11	Fraction **D** 125	5	1520 ± 353*
12	Fraction **D** ≥ 250	0	-
13	Fraction **E** 31.5	5	9240 ± 4206
14	Fraction **E** 62.5	5	1408 ± 313*
15	Fraction **E** ≥ 125	0	-

* Results with P <0.05 (against control), [1] Negative control (mice received nothing)
[2, 3] Positive control (mice received NS or NS/DMSO)
NS = normal saline, NS/DMSO = 20% DMSO in normal saline

(DMSOd6+ $CDCl_3$): δ 170.1 (C-9), 150.2 (C-4), 149.62 (C-3), 146.3 (C-7), 128.3 (C-1), 125.1 (C-6), 118.4 (C-8), 117.5 (C-5), 115.1(C-2) and sugar: 60.7 (C-6'), 69.1 (C-4'), 79.1 (C-3'), 74.6 (C-2', 5'), and 102.4 (C-1'); EIMS: m/z 179 [aglycon fragment -H]$^+$, 163 [179 -O]$^+$, 136 [163 +H -CO_2]$^+$; UV λ_{max} nm in MeOH: 330, 299.

Compound **3:** *Apigenin-7-O-glycoside*, ^{1}H-NMR (DMSO d6): δ 7.94 (2H, *d*, *J* = 8.4Hz, H-$_{2',6'}$), 6.94(2H, *d*, *J* = 8.4Hz, H $_{3',5'}$), 6.87 (1H, *s*, H $_3$), 6.83 (1H, *bs*, H $_8$), 6.43 (1H, *bs*, H $_6$), 5.08(1H, *d*, *J* = 6.8 Hz, Glu $H_{1'}$), 3.25-5.08(6H, *m*, sugar protons); ^{13}C-NMR (DMSO d6): δ 181.7(C-4), 164.0 (C-2), 162.3 (C-7), 161.3 (C-4′), 160.9 (C-5), 156.8 (C-9), 128.4 (C- 2',6′), 120.7 (C-1′), 115.9 (C-3′,5'), 105.3 (C-10), 103.0 (C-3), 99.2 (C-6), 94.4 (C-8) and sugar: 97.7 (C-1"), 77.1 (C-5"), 76.9 (C-3"), 76.2 (C-2"), 69.6 (C-4"), 60.4 (C-6"); EIMS: m/z 270 [aglycon fragment]$^+$, 256 [270 -CH_2]$^+$, 153 [A1+H]$^+$, 121 [B2]$^+$, 94 [B2 +H -CO]$^+$; UV λ_{Max} nm in MeOH: 335, 270, +NaOMe 393, 302sh, 273, 259sh; +$AlCl_3$ 388, 348, 302, 278; +$AlCl_3$/HCl 386, 347, 300, 278; +NaOAc 385, 360, 270 ; +NaOAc/BO_3H_3 335, 268.

Identification of sugars

About 10mg of each compound was dissolved in 2N hydrochloric acid (10ml) using a minimum amount of MeOH to affect complete solution. The solution was heated on a steam bath for 45 min and then cooled and extracted thoroughly by shaking with diethyl ether. Evaporation of the aqueous layer yielded a pale yellow powder, which was paper chromatographed in a solvent system of ethyl acetate: pyridine: water (12:5:4), alongside with some of the more common sugars such as glucose, rhamnose, xylose, arabinose and galactose. The chromatogram was dried and sprayed uniformly with a solution of p-anisidine hydrochloride and sodium hydrosulfite in methanol and n-butanol according to the literature (13). The sprayed chromatogram was then heated for 10 min, during which time the sugar spots turned brown.

RESULTS AND DISCUSSION

Dried aerial parts of *Achillea millefolium,* collected from north of Iran, were extracted and fractionated with different solvents to give fractions A-E. Separated fractions (A, B, C, D and E) of *A. millefolium* were evaluated in BALB/c mice, immunized by sheep red blood cells, in order to find the effects of active principles on the hemagglutinating antibody titer. Among them only fraction D (at 125 mg kg^{-1}) and E (at 61.5 mg kg^{-1}) showed a significant decrease in the anti-SRBC titer of BALB/c mice (Table 1). Based on the statistical analysis of Kruskal-Wallis, there is a significant difference between various doses of fraction D (P< 0.03) and E (P< 0.02) in the diminution of antibody titer.

Because of the immunosuppressive activity of the residue (fraction D), this fraction was fractioned

by paper chromatography to obtain three components. Isolated compounds were identified as luteolin-7-O-glucoside (**1**), caffeic acid gluoside (**2**) and apigenin-7-O-glucoside (**3**) (Figure 1). All these components were glycosides and the results obtained from acidic hydrolysis showed the presence of glucose (at the same R_f in comparison to authentic samples of glucose) as a sugar part of each compound (12). Their spectral data showed good agreement with those of literature data (12, 14-16) and confirmed the structure of compound **1** as: 5, 7, 3′, 4′- tetrahydroxy flavone (glycosylated at position 7) and the structure of compound **3** as: 5, 7, 4′- trihydroxy flavone (glycosylated at position 7) and the structure of compound **2** as: 3′, 4′- dihydroxy phenolic acid (glycosylated at position 9).

7-O-Glucosylated flavones (derived from apigenin and luteolin) are reported previously from *A. millefolium* of other origin (17) but caffeic acid glycoside is reported for the first time from this Iranian species. Glycosylated derivatives of caffeic acid are responsible for different biological activities such as suppression of humoral and cellular immunity (11). The residue of methanol extract of *A. millefolium* was rich of this component therefore it could be inhibitor of the immune system.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Research Center of Medical Sciences. The authors wish to thank Dr. H. Akhani for collection and identification of plant materials.

REFERENCES

1. Bremer K. Asteraceae: Cladistics and Classification. Oregon: Timber Press; 1994
2. Zargari A. Medicinal Plants. 5th ed. Tehran: Tehran University Publication; 1992; p. 528
3. Rustaiyan A, Masoudi S, Yari M. The essential oils of *Achillea aucheri* Boiss. and *A. Kellalensis* Boiss. et Hausskn. from Iran. *J. Essent. Oil Res*. 1999; 11: 19-20
4. Rezaeipoor R, Saeidnia S, Kamalinejad M. Immunosuppressive activity of *Achillea talagonica* on humoral immune responses in experimental animals. J. Ethnopharm. 1999; 65: 273-276
5. Podlech D, Huber-Morath A, IranshahrM, Rechinger KH. *Compositae* VI- *Anthemideae*. In: Flora Iranica (Rechinger KH, ed.) No. 158, Ackademiche Druck- u.Verlagsanstalt, Graz; 1986; pp. 62-64
6. Falk AJ, Smolanski AJ, Bouer L, Bell CL. Isolation and identification of three new flavones from *Achillea millefolium* L. J. Pharmaceutical Sci. 1975; 64(11): 1838-1842
7. Glasl S, Mucaji P, Warner I, Presser A, Jurenitsch J. Sesquiterpense and flavonoid aglycones from a hungarian taxon of the *Achillea millefolium* group. Z. Naturforsch [C]. 2002; 57(11-12): 976-82
8. Gherase F, Miron A, Zagnat M, Hancianu M, Stanescu U, Grigorescu E. Phytochemical investigations regarding the flavonoidic compounds of some species belonging to the *Achillea millefolium* L. group. Rev. Med. Chir. Soc. Med. Nat. Iasi. 2004; 108(1):177-180
9. Saeidnia S, Yassa N, Rezaeipoor R. Comparative investigation of the essential oils of *Achillea talagonica* Boiss. and *A. millefolium* L. chemical composition and immunological studies. J.Essent.Oil Res. 2004; 16: 262-265
10. Zargari A. Medicinal Plants. 5th Ed. Tehran University Publication, Tehran, Iran, 1992; 3: pp 106-112
11. Sasaki H, Nishimura H, Morota T, Chin M, Mitsuhashi H, Komatso Y, Maruyama H, Tu GR, Wei H, Xiong YL. Immunosuppressive principle of Rehmannia glutinosa var.hueichingensis. Planta Med. 1989; 55: 458- 462
12. Mabry TJ, Markham KR, Thomas MB. The Systematic Identification of Flavonoids. New York: Springer- Verlage.1970
13. Wagner H and Bladt S. Plant Drug Analysis. 2nd ed., Berlin: Springer; 1996
14. Kasaj D, Krenn L, Prinz S, Hufner A., Haslinger E., Yu SS, Kopp. Flavone and Flavonol glycosides from *Achillea pannonica* Scheele. Z. Naturfurschung (C), 2001; 56 (7): 521-525
15. Okamura N, Haraguchi H, Hashimoto K, Yagi A. Flavonoids in *Rosmarinus officinalis* leaves**.** Phytochemistry, 1994; 37(5):1463- 1466
16. Lim EK, Higgins GS, Li Y and Bowles DJ. Regioselectivity of glucosylation of caffeic acid by a UDP-glucose: glucosyltransferase is maintained in planta. Biochem. J. 2003; 373: 987–992
17. Guedon D, Abbe P, Lamaison JL. Leaf and flower head flavonoids of *Achillea millefolium* L. subspecies. Biochemical Systematics and Ecology, 1993; 21(5): 607- 611