Immunosuppressive principles from *Achillea talagonica,* an endemic species of Iran

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ABSTRACT

Background and the purpose of study: *Achillea talagonica* Boiss. (Asteraceae) grows in the western and central parts of Iran. This plant has long been used in traditional medicine as an anti-inflammatory agent for treatment of rheumatic pain. Previously, the immunosuppressive activity of the aqueous extract of this endemic plant in experimental animals was reported. In this research, isolation of the main immunologically active components of *A. talagonica,* which were effective on humoral immune responses in BALB/c mice is elucidated.

Methods: In order to find the main immunosuppressive components of *A. talagonica,* methanol and methanol-water (80% and 50% v:v) extracts were injected to BALB/c mice and the hemagglutinating antibody titer was assayed after immunization with SRBC (sheep red blood cells). Guided by this assay, active principles were separated by chromatographic methods.

Results: Isolated compounds were identified as caffeic acid 9-O-glucoside (**1**), quercetin (**2**), luteolin (**3**), 3'-methoxy luteolin (**4**), proline (**5**) and choline (**6**) by comparison of their spectral data with those of reported in literatures. Immunosuppressive property of choline (5 $mgkg^{-1}$) was comparable to those of prednisolone (10 $mgkg^{-1}$); although, quercetin (20 $mgkg^{-1}$) and caffeoyl glucoside (20 $mgkg^{-1}$) decreased anti-SRBC titer in comparison with control groups.

Major conclusion: Immunosuppressive effects of *A. talagonica* are due to some components belonging to betaine, flavonol and phenoilc esters.

Keywords: *Achillea talagonica*, Asteraceae, immunosuppressive, choline, caffeoyl glycoside, quercetin

INTRODUCTION

Immunomodulatores, which are also known as biological response modifiers, are substances that modify the immune responses in the body and include corticosteroids, which have immunosuppressive activity (1). Some plants of traditional medicine have been shown to possess immunosuppressive activity (2) and it has been reported that: *Sideritis foetens* Clem which has a labdane diterpene (andalusol) inhibited the hemolytic activity of the classical complement pathway and lymphocyte proliferation (3), flavonol glycosides from *Epimedium hunanense* (Hand.-Mazz.). enhanced lymphocyte proliferation (4) and phenethyl alcohols isolated from *Rehmannia glutinosa* L. reduced the hemolytic plaque- forming cells (5).

Achillea genus (Asteraceae) comprises 115 species, which are mainly distributed in Europe, Asia, and North Africa (6) and *A-talagonica* Boiss is one of the endemic Iranian species, which grows in the western and central parts of Iran (7) and has long been used in traditional medicine as an anti-inflammatory agent for treatment of rheumatic pains (8). Previously the immunomodulation activities of several Iranian medicinal plants in experimental animals were reported (9-11) and it was shown that although aqueous extract of *A. talagonica* has immunosuppressive activity, its volatile oil was not effective. Also, the

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essential oil of *A. millefolium* has been found to be an inhibitor of antibody production (12). In the present study, immunologically guided separation and identification of the main active components of *A. talagonica* on humoral immune responses in BALB/c mice is described.

MATERIALS AND METHODS

Plant material

Aerial parts of *Achillea talagonica* Boiss. (Asteraceae) was collected from Talegan area in Tehran Province during May and was identified by M. Kamalinejad. A voucher specimen (No: A-424-2) has been deposited at the Herbarium of the Faculty of Pharmacy, Mazandaran University of Medical Sciences.

Instruments and chemicals

^{1}H and ^{13}C-NMR spectra were measured on a Varian 400 unity plus 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. Ultraviolet spectra were recorded on a Shimadzu UV-160A instrument.

Chromatographic paper was purchased from Whatman Company, and all reagents, solvents and authentic samples used in this study were purchased from Merck Company. Prednisolone was obtained from Abu-Reihan Pharmaceutical Company, Tehran, Iran.

Extraction and fractionation

Dried and powdered aerial parts (650 g) of the flowered plant were percolated with methanol at room temperature and the extract was concentrated under low pressure and temperature (30 g, fraction A). The marc was extracted again with 80% and then 50% (v/v) methanol-water and fractions were evaporated under vacuum to give 6g (fraction B) and 12 g (fraction C) residues respectively. Fraction A was extracted again with petroleum ether (discarded) then with chloroform and ethyl acetate, solvents evaporated in vacuum to give 3.7 g (fraction A1), 5.3 g (fraction A2), respectively and 21 g of residue (fraction A3).

Purification and Identification of compounds

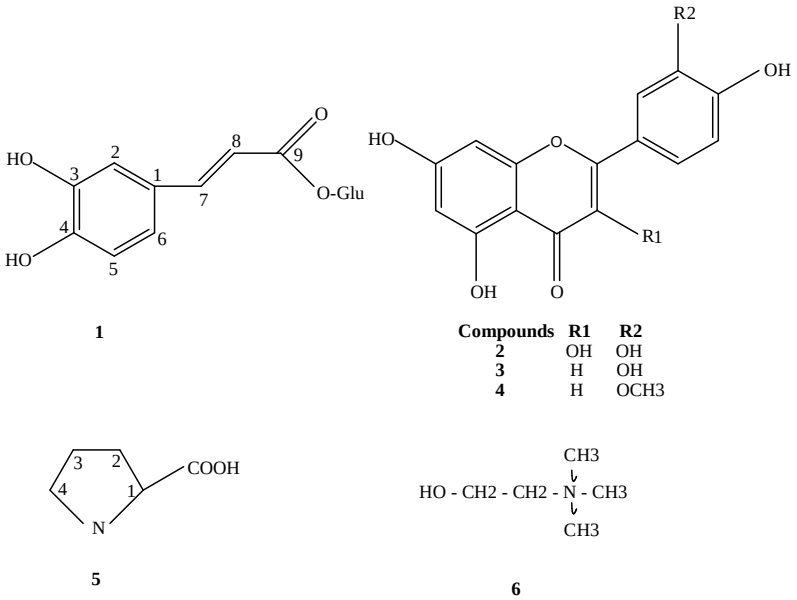
A part of fraction A3 (750 mg) was subjected to paper chromatography (PC). The chromatogram was developed descending in the long direction of Whatman (3 mm) chromatographic paper in the chromatocab using 2% acetic acid. The separated lines on the chromatograms were detected under Ultraviolet Lamp (366nm) before and after treatment with NH3 vapor and Natural Product reagent, and the resulting fractions were assigned as A3-1 (82 mg) and A3-2 (334 mg). A3-1 was chromatographed with BAW (n-butanol: acetic acid: water, 4: 1: 5) as eluent to give compound **1** (60 mg). Residue of the fraction of A3-2 was subjected to further fractionation by PC, with TBA (t-butanol: acetic acid: water, 3: 1: 1) as a solvent system to separate compounds **2** (23 mg), **3** (250 mg) and **4** (32 mg). For further purification, later compounds were chromatographed on Whatman chromatographic paper using 15% acetic acid as a mobile phase.

Fractions B (1 g) and C (2 g) were subjected to PC (BAW as solvent) to obtain two fractions (B1 and C1) which were detected by Dragendorff reagent as yellow and purple spots, respectively. In order to separate B1and C1, the samples were chromatographed on sephadex LH_{20} (MeOH-water 50:50 as eluent) to obtain impure compounds **5** and **6**, respectively. The isolated compounds after concentration, were subjected to column chromatography on silica gel 60 (Merck Co. mesh 30-70) using CH_2Cl_2-MeOH (5: 3) acidified to pH 2-3 as eluent to obtain the pure compounds, **5** (15 mg) and **6** (120 mg) .

***Compound* 1:** *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 (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 %: 180(52), 179(62), 163(100), 136(80), 121(20); UV λ_{max} nm in MeOH: 330, 299.

***Compound* 2:** *Quercetin*, ^{1}H-NMR (DMSO d6): δ 8.02 (1H, *bs*, $H\text{-}_{2'}$), δ 7.59 (1H, *d*, *J* = 8.4Hz, $H\text{-}_{6'}$), 6.88(1H, *d*, *J* = 8.4Hz, $H_{5'}$), 6.40 (1H, *br*, H_{8}), 6.19 (1H, *br*, H_{6}); ^{13}C-NMR (DMSO d6): δ 176.2(C-4), 163.7 (C-7), 160.9 (C-5), 160.4 (C-4′), 156.8 (C-9), 156.7(C-2'), 149.6 (C-2), 136.2 (C-3), 131.7 (C-6′), 109.2(C-1′), 106.8(C-5'), 103.6 (C-3′), 102.9 (C-10), 98.0 (C-6), 93.4 (C-8); EIMS (40 eV): m/z %: 302(100), 273(10), 153(10), 137(18); UV λ_{Max} nm in MeOH: 370, 299sh, 256; +NaOMe 425, 303(sh), 261 (dec); +$AlCl_3$ 448, 292(sh), 271; +$AlCl_3$/HCl 425, 360, 293(sh), 270; +NaOAc 360, 292(sh), 270; +NaOAc/BO_3H_3 381, 299(sh), 261.

***Compound* 3:** *Luteolin*, ^{1}H-NMR (DMSO d_6): δ 7.40(1H, *bd*, *J* = 8.4 Hz, $H_{6'}$), 7.38 (1H, *bs*, $H_{2'}$), 6.88(1H, *d*, *J* = 8.4 Hz, $H_{5'}$), 6.60(1H, *s*, H_3),6.43(1H, *d*, *J* = 2 Hz, H_{8}), 6.18(1H, *d*, *J* = 2



1 **5** **6**

Figure 1. Structure of compounds: **1** caffoyl glucoside; **2** quercetin; **3** luteolin; **4** chrysoeriol; **5** proline; **6** choline.

Hz, H $_{6}$); ^{13}C- NMR (DMSO d_6): δ 182.1(C-4), 164.5 (C-7), 164.4(C-2), 161.9 (C-5), 157.7 (C-9), 150.1 (C-4′), 146.2 (C-3′), 122.0 (C-1′), 119.4 (C-6′), 116.5 (C-5′), 113.8 (C-2′), 104.2 (C-10), 103.3 (C-3), 99.3 (C-6) 94.3 (C-8); EIMS (40 eV.): m/z % 286 (100), 152 (10) 137 (15); 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, 268; +NaOAc/BO_3H_3 370, 258.

***Compound* 4:** *Chrysoeriol (3'-methoxy luteoline)*, ^{1}H-NMR (DMSO d_6): δ 7.30 (1H, *bs*, $H_{2'}$), 7.27(1H, *d*, *J* = 8.4 Hz, H $_{6'}$), 6.78(1H, *d*, *J* = 8.4 Hz, H $_{5'}$), 6.48(1H, *s*, H $_{3}$), 6.36(1H, *bs*, H $_{8}$), 6.07(1H, *bs*, H $_{6}$), 3.83(3H, *s*, OCH_3); ^{13}C NMR (DMSO d_6): δ 182.1(C-4), 167.3 (C-7), 164.7 (C-2), 162.2 (C-5), 158.3 (C-9), 148.3 (C-3′), 147.2 (C-4′), 121.0 (C-1′), 119.5 (C-6′), 116.9 (C-5′), 113.7 (C-2′), 103.7 (C-10), 102.8 (C-3), 100.2 (C-6) 94.9 (C-8), 56.5(OCH_3); EIMS: m/z %: 300(17), 286(70), 151(18), 153(30), 148(10), 134(20); UV λ_{max} nm in MeOH: 356, 268; +NaOMe 407, 267; +$AlCl_3$ 401, 360(sh), 293(sh), 271, 261(sh); +$AlCl_3$/HCl 401, 357, 293(sh), 277, 257(sh); +NaOAc 365, 322(sh), 269; +NaOAc/BO_3H_3 425(sh), 370, 293(sh), 260.

***Compound* 5:** *Proline*, ^{1}H-NMR (Pyridine d_5): δ 3.71 (1H, *dd*, *J* = 6.3, *J* = 8.7 Hz, H_1), 2.08(1H, *m*, H $_{2}$), 1.87(1H, *m*, H $_{2'}$), 1.76(2H, *m*, $H_{3, 3'}$), 3.19(1H, *m*, H $_{4}$), 3.03(1H, *m*, H $_{4'}$); EIMS: m/z%: 115(8), 87(8), 71(5), 70(100), 68(17).

***Compound* 6:** *Choline*, ^{1}H-NMR (DMSO d_6 + D_2O): δ 3.82 (2H, *m*, , H_1), 3.37(2H, *m*, H $_{2}$), 3.10(9H, *s*, CH_3); ^{13}C-NMR (DMSO d_6 + D_2O): δ 55.08 (C-1), 67.01 (C-2), 53.14 (3×CH_3); EIMS: m/z%: 104(100), 88(18), 59(5), 45(5) (see Fig. 1).

Experimental animals

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

Microhaemagglutination test

Groups of animals, each of 6 mice, were treated by intraperitoneall injection (i.p.) of different doses (31.5, 62.5, 125 and 250 mg kg^{-1}) of each fraction of B, C, A1, A2 and A3 and isolated compounds at different doses (1, 5, 10, 20, 30 mg kg^{-1}). Control groups received i.p. injection of 0.5 ml of normal saline (NS) or NS+DMSO (8:2 v/v). The test and control groups were treated for 6 consecutive days and the native group received nothing. On the 8th day, all mice except the natives were injected (i.p.) 2 × 10^8 sheep red blood cells (SRBC). After 6 days, bloods were collected from the retro-orbital plexus, serums were separated and Hemagglu-tinating Antibody titer (HA) was determined by the microtiter plate method (9, 12). Prednisolone was used as a positive control.

Statistical analyses

Comparison between groups was made by two sided Mann-Whitney U Statistic Test and then, one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Differences with $P< 0.05$ between experimental groups were considered statistically significant.

Table 1. Effects of some fractions of *Achillea talagonica* on the hemagglutinating antibody titer in BALB/c mice immunized with SRBC.

Groups	Doses mg/kg	Alive mice	[b]Antibody titer, Mean ± SD	[a]P.value
Native	-	6	93.0 ± 13.3	0.003
[c]Control (NS)	-	6	8960.0 ± 2623.2	<0.003
[c]Control (NS-DMSO)	-	6	11080.0 ± 3082.6	<0.003
Fraction A1 ($CHCl_3$)	31.5	5	12032.0 ± 7393.0*	0.64
Fraction A1	≥ 62.5	0	-	-
Fraction A2 (EtOAc)	31.5	5	8760.0 ± 1610.6*	0.43
Fraction A2	≥ 62.5	0	-	-
Fraction A3 (Residue)	31.5	5	6144.0 ± 1736.2*	0.519
Fraction A3	62.5	5	1280.0 ± 350.5	0.0001
Fraction A3	125	3	80.0 ± 0.0	0.002
Fraction A3	250	0	-	-
Fraction B (MeOH 80+water 20)	31.5	6	3200.0 ± 640.0	0.006
Fraction B	62.5	6	453.3 ± 86.8	0.0001
Fraction B	125	6	320.0 ± 71.5	0.0001
Fraction B	250	4	200.0 ± 40.0	0.0001
Fraction C (MeOH 50+water 50)	31.5	6	6144.0 ± 736.3*	0.519
Fraction C	62.5	6	266.6 ± 33.7	0.0001
Fraction C	125	5	176.0 ± 39.1	0.0001
Fraction C	250	3	120.0 ± 40.0	0.002
Quercetin	20	6	1813.3 ± 347.1	0.004
Caffeoyl glycoside	20	6	1173.3 ± 305.4	0.004
Choline	5	5	360.1 ± 25.2	0.003
Prednisolone	5	5	2120±222.3	0.006
Prednisolone	10	5	825.1 ± 125.2	0.003

[a]Analysis of data was based on Mann-Whitney U test ($p < 0.05$ is significant) and multiple comparison Dunet Test ($\alpha = 0.001$).
[b]All data showed significant decrease in the anti-SRBC titer compared with control group except those of specified by star.
*Not significant data *vs* control.
[c]Control groups (mice received normal saline (NS) and NS+DMSO instead of extract) showed significant increase ($p < 0.003$) in antibody titer compared to native (mice received nothing), representing a successful immunization of mice with SRBC antigen.

RESULTS AND DISCUSSION

Different fractions of *Achillea talagonica* (fractions B, C, A1, A2, and A3) were evaluated in BALB/c mice immunized by SRBC, in order to find the effective principles on the hemagglutinating antibody titer. Fraction B and A3 showed a significant decrease in the anti-SRBC titer of BALB/c mice at 125 and 250 mg kg^{-1} (Table 1). The immunological guided fractionation led to isolation of compounds **1-4** from fraction A3 by paper chromatography. These isolated compounds were identified as caffeic acid 9-O-glucoside (**1**), quercetin (**2**), luteolin (**3**) and 3'-methoxy luteolin (**4**) by a comparison of their spectral data (H-NMR, ^{13}C-NMR, UV, IR and Mass) with those reported in literatures (13-17). Fractions B and C at all doses inhibited production of hemagglutinating antibody (Table 1). Further purification of these two fractions led in isolation of compounds **5** and **6**. The compounds on treatment with Dragendorff reagent gave yellow and purple spots, respectively which are specific reactions of quaternary amino acid derivatives of betaine type. The ^{1}H and ^{13}C-NMR spectral data of these compounds in DMSO+D_2O and pyridine-d_5 showed good agreement with those reported in references as proline (**5**) and choline (**6**) (18-19).

Immunosuppressive activity of isolated components was tested again in BALB/c mice using above described method. Among components, quercetin (20 mg kg^{-1}), caffeic acid 9-O-glucoside (20 mg kg^{-1}) and choline (5 mg kg^{-1}) showed a significant decrease in the anti- SRBC titer of mice compared to control groups. Luteolin, 3'-methoxy luteolin and proline showed no significant activity at any doses (Table 1).

In this study only the polar fractions of *A. talagonica* which has shown immunosuppressant activity was investigated (9). Choline, the main quaternary amine principle of this plant, which was determined in aqueous methanol (80% and 50%) showed a good inhibitory on antibody production (19). It seems that choline (5 mg kg^{-1}) which had activity similar to prednisolone as a standard immusuppressive drug (10Mg/kg $^{-}$1) is responsible for the immunosuppressive effect of fractions B and C. There is no report on the activity of choline on primary humoral responses. Caffeic acid-9-O-glucoside is one of the phenolic glycosides, which are classified into phenethyl alcohol or phenyl propanoid glycosides. Jionosides A_1 and B_1 isolated from *Rehmannia glutinosa* are two samples of this class which have shown *in vivo* immunosuppressive activity attributed to the phenethyl alcohol moiety of the molecule (5). Caffeic acid-9-O-glucoside isolated from A3 fraction showed inhibitory activity comparable to Prednisolone.

Naturally occurring plant flavonoid, quercetin, was less active than other effective components. A review of literature revealed that quercetin prevented the UV-induced suppression of the contact hypersensitivity and reduced percentage of CD8+ cells in spleen and lymph nodes (20). Also it has been shown that quercetin inhibits both *in vitro* generation and effecter function of alloantigen specific cytotoxic T lymphocytes (21). In such a flavonol aglycone, presence of a double bond at position C-2-3, a keto group at C-4, B ring hydroxylation and /or a free hydroxyl group at C-3 may be responsible for the activity (21- 22).

In conclusion, this study confirms the presence of flavonols aglycones, phenethyl alcohol glycosides and quaternary N containing compounds in the polar extracts of *A. talagonica* which have immunosuppressive activity comparable to Prednisolone and suggests further phytochemical investigation of the plant to find other minor immunosuppressive components.

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