Cytotoxic Flavonoid from *Achillea talagonica* Bioss.

Saeidnia S (Ph.D.)[1]*, Moradi-Afrapoli F (Pharm.D.)[2], Gohari AR (Ph.D.)[1], Malmir M (M.Sc.)[1]

1- Medicinal plants Research center, Medicinal Sciences, University of Tehran, Tehran, Iran
2- Department of Pharmacognosy, Faculty of Pharmacy, Medicinal Sciences, University of Tehran, Tehran, Iran
*Correspondence author: Medicinal Plants Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran, P.O.Box: 14155 – 6451
Tel: +98-21-66959090, Fax: +98-21-66461178
E-mail: soodabehsaeidnia@hotmail.com

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Abstract

Background: ***Achillea talagonica*** **(Asteraceae) is an endemic herbaceous plant in Iran with small yellow flowers and several times pinnately divided leaves in worm shape. The plant was found to be cytotoxic in our previous studies.**

Materials and Methods: ***A. talagonica*** **was collected in May 2005 from Talegan in north of Iran during the full flowering stage. Dried aerial parts of the plant were reduced in to small pieces and percolated with ethyl acetate for 72 hours. The extract obtained was filtered and then concentrated under reduced pressure and completely dried by a freeze dryer. Column chromatography of the extract on silica gel and sephadex LH-20 yielded in isolation of three main components. Cytotoxic activity was evaluated against the *Artemia salina* larvae by the Brine Shrimp Cytotoxicity Bioassay.**

Results: **Isolated compounds were identified as β-sitosterol (1), salvigenin (5- hydroxy 4', 6, 7- three methoxy flavone) (2) and santoflavon (5- hydroxy 4', 3', 6, 7- tetra methoxy flavone) (3). NMR data of all the isolated compounds showed good agreement with literature data.**

Conclusions: **In our previous studies ethyl acetate extract of *A. talagonica* showed cytotoxic activity in brine shrimp lethality assay. The results of BST assay indicated that only santoflavone (3) showed a good cytotoxic activity (LC_{50} = 15 µg/ml) against *A. salina* larvae so this compound seems to be responsible for the extract toxicity of *A. talagonica* against *Artemia* nauplii.**

Keywords: **Asteraceae, *Achillea talagonica*, Santoflavone, Salvigenin, Cytotoxic**

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Introduction

The genus *Achillea* (Asteraceae) comprises more than 100 species which are mainly distributed in northern hemisphere [1]. Since the Trojan War (1200 BC) different species of *Achillea* have been used extensively by many cultures as the herbal remedy [2]. Several traditional usages for *Achillea* were reported in Iran such as treatment of fever, asthma, skin inflammation, jaundice and other liver ailments. This genus is chemically characterized by the frequent accumulation of sesquiterpene lactones [3], alkamids [4] and 3 or 7-glycosilated flavonoids [5]. It was also shown to contain free flavonoid aglycons accumulated externally on leaves and stems together with other lipophilic materials [6].

Achillea talagonica is an endemic herbaceous plant in Iran with small yellow flowers and several times pinnately divided leaves in worm shape [7]. The plant was found to be cytotoxic in our previous studies. Among different extracts of *Achillea talagonica* which were tested under the Brine Shrimp Cytotoxicity (BST) method, ethyl acetate extract was considered to be the most active fraction [8]. Methanol and aqueous-methanol extracts of *A. talagonica* have shown immune suppressive activity on primary humeral responses [9]. 1, 8 cineol (9.7 %) and camphor (21.9 %) have been recognized to be the main constituents of the essential oil of *A. talagonica* in analysis by GC and GC/MS [10]. Phytochemical constituents of the plant have not been mentioned in literature. Here, we report the isolation and identification of the main flavonoid aglycones which show cytotoxic activity in BST assay.

Materials and methods

Plant material and extraction

A. talagonica was collected in May 2005 from Talegan in north of Iran during the full flowering stage. It was identified by M. Kamalinejad (Department of Pharmacognosy, Faculty of Pharmacy, Shaheed Beheshti University of Medical sciences) and a voucher specimen has been deposited at the herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences. Shade dried aerial parts of the plant (700 g) were reduced in to small pieces and percolated with ethyl acetate for 72 hours. The extract obtained (9 g) was filtered and then concentrated under reduced pressure. It was completely dried by a freeze dryer.

Experimental

^{1}H- and ^{13}C-NMR spectra were measured on a Brucker Avance 500 DRX (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer with tetramethylsilane as an internal standard and chemical shifts are given in δ (ppm). MS data were recorded on Agilent Technology (HP) instrument with 5973 Network Mass Selective Detector (MS model). Silica gel $60F_{254}$ pre-coated plates (Merck) were used for TLC. The spots were detected by spraying anisaldehyde-H_2SO_4 reagent followed by heating.

Separation process

A part of ethyl acetate extract (5 g) was submitted to silica gel column chromatography (normal phase) with Hexane: $CHCl_3$ (7:3, 1:1), $CHCl_3$, $CHCl_3$: EtOAc (1:1, 1:2) and EtOAc successively, to give 6 fractions (F_1-F_6). F_2 (1.1 g) was chromatographed on silica gel with Hexane: $CHCl_3$ (7:3, 3:7) and 8 sub fractions were obtained (F_{2a}-F_{2h}). Purification of compound **1** (17 mg) took place by submitting F_{2h} to sephadex LH-20 CC with MeOH: $CHCl_3$ (8:2). Fractionation of F_3 (351 mg) on silica gel with Hexane: $CHCl_3$ (7:3, 1:1), EtOAc and MeOH gave three sub fractions (F_{3a}-F_{3c}). Chromatography of F_{3a} on sephadex LH-20 with MeOH: EtOAc (6:4) yielded in isolation of compound **2**. Submitting F_4 on sephadex LH-20 with MeOH : EtOAc (4:6)

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resulted in purification of compound **3** (10mg). The isolated compounds were finally identified using spectroscopic methods.

β-sitosterol (**1**). ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 0.68 (3H, *s*, H-18), 0.81 (3H, *br s*, H-26), 0.82 (3H, *br s*, H-27), 0.84 (3H, *br s*, H-24b), 0.92 (3H, *d*, *J*= 6.7 Hz, H-21), 1.01 (3H, *s*, H-19), 3.52 (1H, *m*, H-3), 5.35 (1H, *m*, H-6). ^{13}C-NMR (125 MHz, $CDCl_3$): δ_C (from C-1 to C-27) 37.3, 31.7, 71.8, 42.3, 140.8, 121.7, 31.9, 31.9, 50.2, 36.5, 21.1, 39.8, 42.3, 56.8, 24.3, 28.3, 56.1, 11.9, 19.8, 36.2, 18.8, 34.0, 26.1, 45.8, 29.2, 19.0, 19.4, 23.1 (C-24a), 12.0 (C-24b).

Salvigenin (2): MS m/z (%): 312.8 (100), 328 (98), 180 (31), 134 (20.5), 133 (45.5); H-nmr (DMSO-d_6, 500 MHz) *δ*: 6.95 (1H, *s*, H-3), 6.96 (1H, *s*, H-8), 7.12 (2H, *d*, J = 8.8 Hz, H-3', 5'), 8.07 (2H, *d*, J = 8.8 Hz, H-2',6'), 3.74, 3.87, 3.93 (each 3H, *s*, OMe); ^{13}C-nmr (DMSO-d_6) *δ*: 163.6 (C-2), 103.3 (C-3), 182.3 (C-4), 152.6 (C-5), 131.9 (C-6), 158.6 (C-7), 91.6 (C-8), 152.7 (C-9), 105.1 (C-10), 122.7 (C-1'), 128.4 (C-2'), 114.6 (C-3'), 162.4 (C-4'), 114.5 (C-5'), 128.4 (C-6'), 60.1 (6-OMe), 56.5 (7- OMe), 55.7 (4'-OMe).

Santoflavon (3): H-nmr ($CDCl_3$, 500 MHz) *δ*: 6.65 (1H, s, H-3), 6.60 (1H, s, H-7), 7.38 (*d*, J = 2Hz, H-2'), 7.02 (*brd*, J = 8.5 Hz, H-5'), 7.57 (*dd*, J = 8.5, 2 Hz, H-6'), 3.98, 4.02, 4.03, 4.04 (each 3H, *s*, OMe); ^{13}C-nmr ($CDCl_3$) *δ*: 164.4 (C-2), 104.9 (C-3), 183.0 (C-4), 153.5 (C-5), 132.5 (C-6), 159.2 (C-7), 91 (C-8), 153.6 (C-9), 106.6 (C-10), 124.3 (C-1'), 109.4 (C-2'), 149.8 (C-3'), 152.2 (C-4'), 111.7 (C-5'), 120.5 (C-6'), 61.2, 56.7, 56.6, 56.5 (4 OMe).

Brine Shrimp Lethality Assay (BSA)

Brine shrimp (*Artemia salina*) eggs were purchased from the Shilat Center (Tehran). The eggs were hatched in a flask containing 300 ml artificial seawater made by dissolving distilled water. The flask was well aerated with the aid of an air pump, and kept in a water bath at 29-30 ˚C. A bright light was left on. The nauplii hatched within 48h. The extracts and pure compounds were dissolved in normal saline. Different concentrations were obtained by serial dilution. Solution of each concentration (500 µl) was transferred into clean 24 wells plates via a pipette, and aerated seawater having 10-20 nauplii (500 µl) was added. A check count was performed, and the number alive noted after 24h. The mortality end point of the bioassay was determined as the absence of controlled forward motion during 30 seconds of observation. The controls used were seawater and berberine hydrochloride (LC_{50} = 26µg/ml). Lethality percentage was determined and LC_{50} calculated based on Probit Analysis with 95% of confidence interval [11].

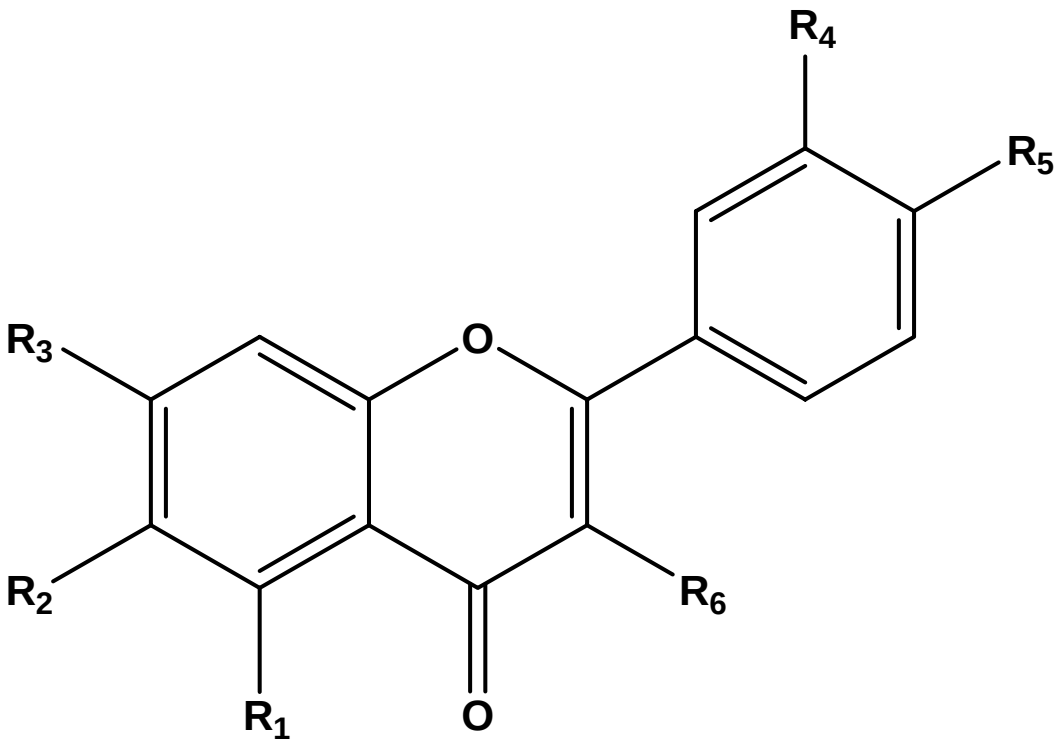
Results and discussion

Full flowering aerial parts of *Achillea talagonica* were extracted by ethyl acetate. Column chromatography of the extract on silica gel and sephadex LH-20 yielded in isolation of three main components which identified as β-sitosterol (**1**), salvigenin (5-hydroxy 4', 6, 7- three methoxy flavone) (**2**) and santoflavon (5- hydroxy 4', 3', 6, 7- tetra methoxy flavone) (**3**) (Fig.1). NMR data of all the isolated compounds showed good agreement with literature data [12, 13, 14].

External accumulation of poly methyl ether flavonoids has been frequently reported for Asteraceae family [6]. Salvigenin and santoflavon were identified in some other *Achillea* species such as *A. depressa*, *A. ochroleuca, A. santolina* and *A. wilhelmsii* [15, 16] but their existence in none of the Iranian *Ahillea* species was reported up to now. In many cases a correlation between preferred

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Salvigenin: R_1= OH, R_2= OMe , R_3= OMe, R_4= H, R_5= OMe, R_6= H

Santoflavone: R_1= H, R_2= OMe , R_3= OH, R_4= OMe, R_5= OMe, R_6= OMe

Fig. 1- Structures of the isolated flavonoids from *A. talagonica*

habitat and production of excreted flavonoids has been noted, indicating that these compounds may be of ecological significance [6].

In our previous studies ethyl acetate extract of *A. talagonica* showed cytotoxic activity in brine shrimp lethality assay [8]. Here, the results of BST assay indicate that only santoflavone (**3**) showed a good cytotoxic activity (LC_{50} = 15 µg/ml) against *A. salina* Larvae. Anti-cancer activity of santoflavon against KB and P-388 cells was previously reported, so this compound seems to be responsible for extract toxicity against *Artemia* nauplii. It has also displayed bacterial inhibitory effects against gram negative and gram positive organisms [17]. β- sitosterol has been identified in several *Achillea* species. It has been effective in reducing symptoms of benign prostate hyperplasia [18], also appears to modulate the immune function, inflammation and the pain levels by controlling the production of inflammatory cytokines. B - sitosterol has been reported neither genotoxic nor cytotoxic on the sister chromatid exchanges (SCE) and cellular proliferation kinetics (CPK) models [19].

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