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Isolation of chemical constituents from *Filago vulgaris* and antiproliferative activity of the plant extract and its flavonoid against human tumor cell lines

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Abstract: Phytochemical investigation of the whole plant of *Filago vulgaris* Lam. (Asteraceae) resulted in the isolation and characterization of seven compounds, including a rare methoxylated flavonol (araneol), tetrahydrofurofuranolignans (pinoresinol and syringaresinol), *p*-hydroxybenzaldehyde, vanillin, vanillic acid and scopoletin. The structures of the compounds were determined by NMR and mass spectroscopy. All compounds were first obtained from this species and reported for the genus *Filago*. Our results demonstrate that highly methoxylated flavonols lacking substituents on ring B and lignans can be regarded as taxonomic markers for the tribe Inuleae. The lipophilic extract of *F. vulgaris* was found to have antiproliferative activity against HeLa cells (62.1±0.9% inhibition at 30 µg/ml), and araneol was highly effective against this tumour cell line (IC_{50} 8.36 µM).

Keywords: *Filago vulgaris,* Asteraceae, araneol, lignans, antiproliferative activity.

INTRODUCTION

Filago vulgaris Lam. [syn. *F. germanica* (L.) Huds.] (Asteraceae), commonly known as common cudweed or common cottonrose, is an annual herbaceous plant mostly distributed in Eurasia, from Spain to Western Russia, Iran and North Africa and introduced in North America (Andrés-Sánchez *et al*., 2011). Floral water of *F. vulgaris* has been used traditionally against cancer (Hartwell, 1968). Reports on chemical constituents of *Filago vulgaris* have not been reported previously. In a previous study three Egyptian *Filago* species were investigated and the presence of isoquercitrin was reported from *F. desertorum* Pomel, *F. prolifera* Pomel and *F. mareotica* Del., and luteolin-7-*O*-glucoside from *F. desertorum* and *F. prolifera* (Saleh *et al*., 1988). In addition, hyperoside, quercetin, lupeol and stigmasterol were identified from *F. desertorum* by Amer *et al*. (1989). In this study, we report for the first time the isolation and characterisation of secondary metabolites of *F. vulgaris* and the evaluation of the antiproliferative activity of its extracts and a main flavonoid (**1**) against different human tumor cell lines.

MATERIALS AND METHODS

General experimental procedures

NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C). The peak of the residual solvents were taken as reference. Two-dimensional data were acquired and processed with standard Bruker software. In the COSY, HSQC and HMBC experiments, gradient-enhanced versions were used. Low-resolution APCI- and ESIMS were performed on API 2000 Applied Biosystems instrument. For vacuum liquid chromatography (VLC) silica gel G (15 mm, Merck), for preparative thin-layer chromatography (preparative TLC) silica gel 60 F_{254} plates (Merck), and for rotation planar chromatography (RPC), applying Chromatotron instrument (Model 8924, Harrison Research) silica gel 60 GF_{254} were used.

Plant material

Whole plant specimens of *F. vulgaris* were collected in July 2013 by G. Király in Csapod (NW Hungary) for the antiproliferative assays and by Gy. Pinke in Öskü (Central Hungary) for the analytical experiments. Voucher specimens (Nos. 874 and 875, respectively) were deposited at the Herbarium of the Institute of Pharmacognosy, University of Szeged.

Preparation of the extracts for antiproliferative assay

The methanol extract was prepared from 10 g frozen, fresh plant material with 3x100 ml solvent using an ultrasonic bath (3 x 15 min). After filtration, the solution was evaporated to dryness under reduced pressure, and dissolved in 50 ml of 50% aqueous methanol. This solution was subjected to solvent–solvent partitions against *n*-hexane (3×50 ml) and then chloroform (3×50 ml). The organic phases and the residual 50% methanol phase were evaporated *in vacuo*, yielding 0.1353 g (*n*-

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hexane), 0.0391 g (chloroform) and 0.4838 g (50% methanol) for each phase.

Extraction and isolation of compounds

Whole plants (890 g) were extracted with 13.5 l of 70% MeOH in a percolator, followed by concentration *in vacuo* to yield 139 g residue. This extract was dissolved in 450 ml methanol-H_2O (1:6) and extracted with ethyl acetate (10 × 400 ml). After evaporation, the 23.97 g dry extract was adsorbed on polyamide and the column (P1) was eluted with methanol-H_2O mixtures (2:8, 4:6, 6:4, 8:2, 10:0). The fraction eluted with 40% methanol was evaporated, taken up with 100 ml water and extracted with chloroform (3×100 ml) yielding 1.92 g lipophilic material after concentration. The extract obtained was separated by VLC on silica gel using dichloromethane-methanol mixtures of increasing polarity. Fractions with a similar composition were combined based on TLC monitoring.

Combined fractions eluted with dichloromethane-methanol 98:2 were rechromatographed by RPC on silica gel as stationary phase and a benzene–ethyl-acetate gradient system as mobile phase. Three combined fractions, obtained by this step, were chromatographed by preparative TLC using silica gel with dichloromethane-methanol 9:1 as mobile phase, resulting in isolation of pinoresinol (2) (3.2 mg), syringaresinol (3) (2.7 mg) and scopoletin (4) (3 mg).

The fraction eluted with dichloromethane–methanol 19:1 was subjected to RPC using silica gel as stationary phase and *n*-hexane–acetone mixtures as eluents. Fractions provided by this separation were further purified by preparative TLC, to afford *p*-hydroxybenzaldehyde (5) (1.9 mg), vanillin (6) (0.7 mg) and vanillic acid (7) (4.4 mg)

Fractions eluted with 60% aqueous methanol from column (P1) were subjected to silica gel column chromatography under vacuum eluting with dichloromethane–methanol mixtures of increasing polarity. Fractions eluted with dichloromethane–methanol 19:1 followed by preparative chromatography on silica gel using methanol–H_2O 7:3 as developing system, yielded araneol (**1**) (4.6 mg).

Spectroscopic data of the isolated compounds

Araneol (=5,7-dihydroxy-3,6,8-trimethoxyflavone) (1): ESIMS positive: *m/z* 345 $[M+H]^+$, 367 $[M+Na]^+$; ESIMS negative *m/z* 343 $[M-H]^-$; NMR data are in good agreement with data published for 1 in $CDCl_3$ (Torrenegra *et al*., 1980; Liendro *et al*., 2007). NMR assignments for CD_3OD solution are first published here. ^{1}H-NMR (500 MHz, CD_3OD) δ (ppm) 8.11 (2H, m, H-2',6'), 7.55 (3H, m, H-3'–H-5'), 3.91 (3H, s, 8-OCH_3), 3.90 (3H, s, 6-OCH_3), 3.82 (3H, s, 3-OCH_3); ^{13}C-NMR from ^{1}H,^{13}C correlations in the HSQC and HMBC spectra (125 MHz, CD_3OD) δ (ppm) 132.3 (C-4'), 129.8 (C-2', C-6'), 129.4 (C-3', C-5'), 138.8 (C-3), 131.0 (C-6), 128.0 (C-8), 62.1 (3-OCH_3), 61.3 (6-OCH_3), 60.9 (8-OCH_3).

Pinoresinol (2): amorphous powder; APCIMS positive: *m/z* 359 $[M+H]^+$, 341 $[M+H-H_2O]^+$; ^{1}H- and ^{13}C-NMR data identical with those previously published (Lajter *et al*., 2015).

Syringaresinol (3) (syn. lirioresinol B): colourless needles; mp. 172–174 °C; APCIMS positive: *m/z* 419 $[M+H]^+$; NMR data were in agreement with those published earlier [Briggs *et al*., 1968; Vermes *et al*., 1991], but the ^{1}H- and ^{13}C-NMR assignments in CD_3OD are published here for the first time. ^{1}H-NMR (500 MHz, CD_3OD) δ (ppm) 3.15 (2H, m, H-1, H-5), 4.72 (2H, d, *J* =4.1 Hz, H-2, H-6), 3.88 (2H, dd, *J*=9.3, 3.5 Hz, H-4a, H-8a), 4.27 (2H, dd, *J*=9.3, 7.0 Hz, H-4b, H-8b), 6.65 (4H, s, H-2', H-2", H-6', H-6"), 3.85 (12H, s, 4×OCH_3); ^{13}C-NMR (125 MHz, CD_3OD) δ (ppm) 56.8 (C-1, C-5), 87.6 (C-2, C-6), 72.8 (C-4, C-8), 133.1 (C-1', C-1"), 104.5 (C-2', C-2", C-6', C-6"), 152.9 (C-3', C-3", C-5', C-5"), 134.9 (C-4', C-4"), 57.5 (4×OCH_3).

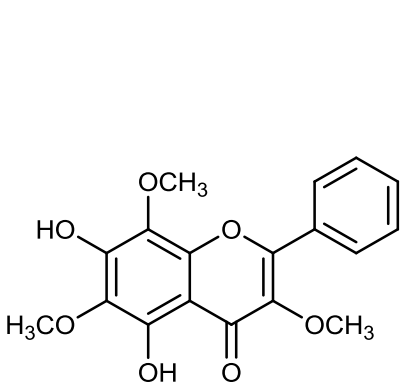
Scopoletin (4), *p-hydroxybenzaldehyde* (5), *vanillin* (6) and *vanillic acid* (7) were identified based on their ESIMS and NMR spectra and co-chromatography with authentic samples.

Antiproliferative assay

Antiproliferative effects of the isolated compounds were determined in vitro by means of a MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay, as described previously (Mossmann, 1983), using cervical (HeLa, SiHa), breast (MCF-7, T47D) and ovarian (A2780) cancer cell lines purchased from the European Collection of Cell Cultures (Salisbury, UK). In the experiments cisplatin (Ebewe Pharma GmbH, Unterach, Austria) was used as a reference active compound, and untreated cells were taken as the negative control. Stock solutions of the tested compound (**1**) was prepared with DMSO. The cell lines were treated with extracts at concentrations of 10 µg/ml and 30 µg/ml. The highest DMSO concentration (0.3%) of the medium did not have any substantial effect on the cell proliferation. All *in vitro* experiments were carried out on two 96-well dishes with at least five parallel wells.

STATISTICAL ANALYSIS

In case of compound 1 sigmoidal dose–response curve was fitted to the measured points, and the IC_{50} value was calculated by means of GraphPad Prism 4.0 (GraphPad Software; San Diego, CA, USA) (Lajter *et al*., 2014).



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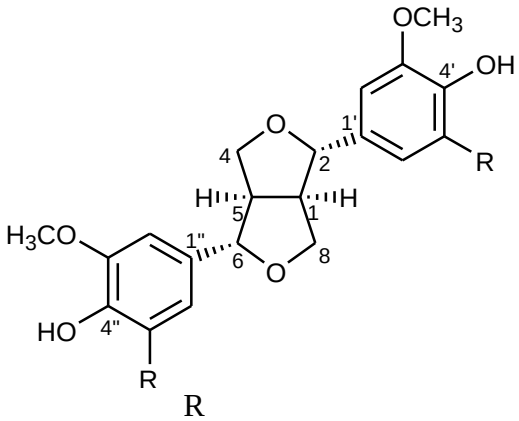

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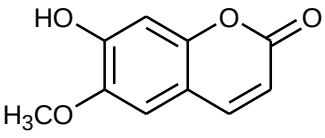

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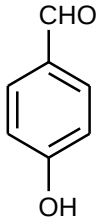

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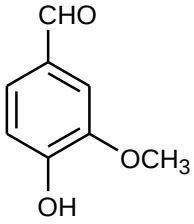

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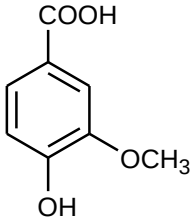

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RESULTS

In the course of the above experiment seven compounds, araneol (1), pinoresinol (2), syringaresinol (3), scopoletin (4), *p*-hydroxybenzaldehyde (5), vanillin (6) and vanillic acid (7), were isolated from the whole plant of *F. vulgaris*. All isolated compounds were obtained for the first time from the *Filago* genus. Araneol (1) is a rarely occurring methoxylated flavonol, fully substituted on rings A and C and unsubstituted on ring B. This compound was described earlier as a lipophilic surface flavonoid in the leaves of *Anaphalis*, *Gnaphalium* and *Achyrocline* species (Ali *et al*., 1979; Cuandra and Harborne, 1996; Liendro *et al*., 2007).

Antiproliferative activities of the extracts and araneol (1) were evaluated against cervical (HeLa, SiHa), breast (MCF-7, T47D) and ovarian (A2780) cancer cell lines by MTT cell viability assays (Réthy *et al*., 2007). First the *n*-hexane, chloroform and remnant aqueous methanol fractions, prepared by solvent-solvent fractionation from a methanol extract of *F. vulgaris* were investigated. The chloroform extract exhibited antiproliferative activity against HeLa, MCF-7 and A2780 cells with 62.1±0.9%, 52.9±0.6% and 28.5±1.8% cell growth inhibition (±SEM), respectively, at a 30 µg/ml concentration. Among the isolated compounds araneol (1) was studied for antiprolifertive activity. The highest potency of 1 was observed against HeLa cells with calculated IC_{50} of 8.36 µM. No substantial effect was exerted up to 30 µM against SiHa, MCF-7, T47D and A2780 cells.

DISCUSSION

Chemotaxonomic significance

The *Filago* group (Asteraceae, Asteroideae, tribe Inuleae, subtribe Gnaphaliinae) comprises eleven genera (including the genus *Filago* with approximately 50 species) mainly distributed in Eurasia, northern Africa and northern America (Wagenitz, 1979; Galbany-Casals *et al*., 2010). Sixteen *Filago* species are native to Europe, most of them grow in dry, open habitats (cultivated fields, open grasslands, roadsides and sand-dunes) (Tutin *et al*., 1976). A literature survey showed that the chemical characterisation of the genus *Filago* is insufficient; only some widespread flavonoids (quercetin, isoquercitrin, hyperoside, luteolin-7-*O*-glucoside) have been previously identified from three *Filago* species, besides lupeol and stigmasterol (Saleh *et al*., 1988; Amer *et al*., 1989). Our results demonstrate that lignans, flavonoids and coumarins are the representatives of phenolic compounds of the *Filago* genus. Interestingly, a methoxylated flavonol (1) lacking substitution on ring B, is a constituent of *F. vulgaris*; this finding supported the previous

observation that this specific type of flavonoids is regarded as a taxonomic marker of the tribe Inuleae (Ali *et al*., 1979; Torrenegra *et al*., 1980; Cuandra and Harborne, 1996; Liendro *et al*., 2007). Moreover, two tetrahydrofurofuranolignans, pinoresinol (2) and syringaresinol (3), were also isolated from *F. vulgaris*; this is the first report of the isolation of lignans in the genus *Filago*, however the presence of lignans is not unprecedented in the subtribe Gnaphaliinae. Although lignans have not been found previously in the *Antennaria* and *Gnaphalium* genera, in *Leontopodium* and *Helichrysum* species some lignan-type compounds have been identified. A lariciresinol-type lignan was isolated from *Leontopodium alpinum* by Dobner *et al*. (2003) and, for the *Helichrysum* genus, the occurrence of lignans is documented, since a lariciresinol-type lignan, acuminatin, has been reported from *H. acuminatum* DC (Jakupovic *et al*., 1987), tetrahydrofurofuranolignans, (+)-piperitol and its glucoside were isolated from *H. bracteatum* (Kisiel, 1980), and pinoresinol was detected in *H. melaleucum* using the HPLC-UV-MS method (Gouveia and Castilho, 2010). In the latter case, pinoresinol (2) was found to be a useful geographical marker. For *H. italicum* the presence of neolignans and oxyneolignans have been reported (D'Abrosca *et al*., 2013). In conclusion, the identification of lignans in *Filago* genus has taxonomical value for evaluation of relationships in the tribe Inulae. Considering the other isolated compounds [scopoletin (4), *p*-hydroxybenzaldehyde (5), vanillin (6) and vanillic acid (7)], they are poor chemical markers, since they have been isolated from many genera of angiosperms.

Antiproliferative activity

Many taxons of Asteraceae family can be characterize with antitumor activity, and sesquiterpenes, flavonoids and lignans have been identified as the main active compounds responsible for the antitumor potency. In case of *Filago* genus this is the first time when this activity could be detected. The chloroform extract of *F. vulgaris* exhibited antiproliferative activity against HeLa, MCF-7 and A2780 cells at a 30 µg/ml concentration. The flavonol araneol was isolated from the chloroform extract and was identified as one of the active compounds. The activity of araneol was investigated against different gynaecological cancer cell lines: cervical (HeLa, SiHa), breast (MCF-7, T47D) and ovarian (A2780) cells, and a cell line specific antiproliferative property could be observed. Our results are in agreement with those found by Thomas *et al*. (2012) describing the cell line specific antiproliferative property of this flavonoid. Araneol (1) was previously reported to exert pronounced activity against more differentiated carcinomas of the colon (Caco-2), and pancreas (Panc28), but was not active against less tumorigenic cell lines: breast cancer MCF-7 cells, androgen-responsive LNCaP human prostate cancer line, and androgen unresponsive PC3 prostate cancer cells (Thomas *et al*., 2012).

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