Short communication

Vasoprotective activity of standardized Achillea millefolium extract

Stefano Dall’Acqua, Chiara Bolego, Andrea Cignarella, Rosa Maria Gaion, Gabriele Innocenti

Achillea millefolium L. (Asteraceae) is widely used in Europe as a herbal remedy for the treatment of spasms, such as digestive complaints, as an emmenagogue, and for irregular menses (Schultz et al. 2001). Because A. millefolium L. comprises several species, it should be defined more precisely as A. millefolium L. sensu latiore (s.l.) (Montsko et al. 2008). Several A. millefolium constituents including monoterpene, sesquiterpene, flavonoid and caffeoylquinic acid derivatives have been reported (Nemeth and Bernath 2008). Each class of phytoconstituents can in least part account for specific medicinal properties of A. millefolium.

A number of studies document multiple biological effects of A. millefolium (reviewed in Nemeth and Bernath 2008). Among others, this plant is used in traditional medicine for the treatment of menstrual pain, as a contraceptive or as a remedy of menopausal complaints. This is consistent with our previous report that A. millefolium is endowed with estrogenic activity in vitro (Innocenti et al. 2007). However, the biological implications of this finding were not investigated in further detail.

Although A. millefolium extracts have been tested in numerous experimental settings, surprisingly no studies were performed in endothelial or vascular smooth muscle cells, which represent major cellular targets for estrogen action in the vessel wall (Xing et al. 2009). Based on previous studies showing anti-proliferative effects of A. millefolium components (Csupor-Läffler et al. 2009), we tested the present extract for the ability to modulate the growth of primary rat vascular smooth muscle cells (VSMCs) in culture as well as the potential involvement of estrogen receptors (ERs) in this process. In addition, the ability of A. millefolium extract to modulate the NF-κB pathway was tested in human umbilical vein endothelial cells (HUVECs). The fingerprinting of the extract was carried out by HPLC-DAD and LC–MS analysis and main constituents were flavonoids (10%) and dicaffeoylquinic acid derivatives (12%). The extract enhanced VSMC growth at least in part by acting through ERs and impaired NF-κB signaling in HUVECs. The various compounds may act with different modes of actions thus contributing to the final effect of the extract. Our findings support some of the traditional uses of A. millefolium, and suggest potential modes of actions as related to its effects on vascular inflammation. Therefore, A. millefolium may induce novel potential actions in the cardiovascular system.

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Keywords:
Achillea millefolium
Phenolic derivatives
Anti-inflammatory activity
Vascular cells

Abstract

We investigated the effects of Achillea millefolium extract in vitro on the growth of primary rat vascular smooth muscle cells (VSMCs) as well as the potential involvement of estrogen receptors (ERs) in this process. In addition, the ability of A. millefolium extract to modulate the NF-κB pathway was tested in human umbilical vein endothelial cells (HUVECs). The fingerprinting of the extract was carried out by HPLC-DAD and LC–MS analysis and main constituents were flavonoids (10%) and dicaffeoylquinic acid derivatives (12%). The extract enhanced VSMC growth at least in part by acting through ERs and impaired NF-κB signaling in HUVECs. The various compounds may act with different modes of actions thus contributing to the final effect of the extract. Our findings support some of the traditional uses of A. millefolium, and suggest potential modes of actions as related to its effects on vascular inflammation. Therefore, A. millefolium may induce novel potential actions in the cardiovascular system.

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Introduction

Achillea millefolium L. (Asteraceae) is widely used in Europe as a herbal remedy for the treatment of spasms, such as digestive complaints, as an emmenagogue, and for irregular menses (Schultz et al. 2001). Because A. millefolium L. comprises several species, it should be defined more precisely as A. millefolium L. sensu latiore (s.l.) (Montsko et al. 2008). Several A. millefolium constituents including monoterpene, sesquiterpene, flavonoid and caffeoylquinic acid derivatives have been reported (Nemeth and Bernath 2008). Each class of phytoconstituents can at least part account for specific medicinal properties of A. millefolium.

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Materials and methods

Plant material extraction

Aerial parts of A. millefolium L. (Asteraceae) were collected in August 2008 at Tonezza del Cimone (province of Vicenza, Italy). The plant was identified by Prof. N. Tornatore of the Department of Biology, University of Padova, and a voucher specimen was deposited at the Department of Pharmaceutical Sciences...
Table 1
Identified compounds in the A. millefolium extract.

<table>
<thead>
<tr>
<th>Compound</th>
<th>[M–H]$^-$</th>
<th>MS$^2$</th>
<th>MS$^3$</th>
<th>% in the extract (w/w)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Chlorogenic acid</td>
<td>353</td>
<td>191-178</td>
<td>191-178-134</td>
<td>1.43</td>
</tr>
<tr>
<td>2 3,5-Dicaffeoyl quinic acid</td>
<td>515</td>
<td>353-335-299-255-191</td>
<td>191-178-134</td>
<td>7.76</td>
</tr>
<tr>
<td>3 3,4-Dicaffeoyl quinic acid</td>
<td>515</td>
<td>353-191-178</td>
<td>1.80</td>
<td></td>
</tr>
<tr>
<td>4 4,5-Dicaffeoyl quinic acid</td>
<td>515</td>
<td>353-191-178-173</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>5 Apigenin</td>
<td>269</td>
<td>241-225-201-183-159-149-117</td>
<td>197-181-117</td>
<td>5.33</td>
</tr>
<tr>
<td>6 Apigenin-7-O-glucoside</td>
<td>431</td>
<td>269</td>
<td>240-225-201-183-117</td>
<td>0.17</td>
</tr>
<tr>
<td>7 Apigenin-4-O-glucoside</td>
<td>431</td>
<td>269</td>
<td>257-241-217-199-175-151-133</td>
<td>1.25</td>
</tr>
<tr>
<td>8 Luteolin</td>
<td>285</td>
<td>257-241-217-199-175-151-133</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>9 Luteolin-7-O-glucoside</td>
<td>447</td>
<td>285</td>
<td>257-241-217-199-175-151-133</td>
<td>0.33</td>
</tr>
<tr>
<td>10 Luteolin-4-O-glucoside</td>
<td>447</td>
<td>285</td>
<td>283-255-229-201-165-135</td>
<td>0.09</td>
</tr>
<tr>
<td>11 Rutin</td>
<td>609</td>
<td>301</td>
<td>283-255-229-201-165-135</td>
<td>0.50</td>
</tr>
<tr>
<td>12 Quercetin-3-O-glucoside</td>
<td>463</td>
<td>301</td>
<td>257-241-217-199-175-151-133</td>
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<tr>
<td>13 Luteolin-7-O-glucuronoside</td>
<td>461</td>
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<td>257-241-217-199-175-151-133</td>
<td>0.23</td>
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<tr>
<td>14 Luteolin-di-glucoside</td>
<td>609</td>
<td>447-285</td>
<td></td>
<td></td>
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</table>

$^a$ The amount was obtained by HPLC-DAD analysis.

(no. AMT0808). Air-dried powdered aerial parts of A. millefolium (50 g) were exhaustively extracted in a Buchi B811 apparatus with 150 ml of methanol for 24 h. The solvent was removed under vacuum yielding a semi-solid residue which was further freeze-dried obtaining a green hygroscopic powder (21% dry yield, w/w).

High performance liquid chromatography analysis and LC–MS$^n$ measurements

LC–MS equipment (Varian inc.) comprised a chromatographic system (Varian LC-212) coupled with a Varian 500-MS (ion trap) mass spectrometer fitted with an ESI source (Varian). MS conditions were the following: needle potential -5.0 kV, shield 600 V, spray chamber temperature 50°C, drying gas pressure 25 psi, drying gas temperature 310°C, capillary voltage 110 V, RF loading 75, MS range 50–1000 Da. MS$^n$ spectra were both obtained in direct injection mode as well as during chromatography run by use of the turbo-dds (tdds) utility of the instrument.

HPLC-DAD analysis was carried out by an Agilent 1100 series liquid chromatograph equipped with an Agilent 1100 Diode Array (DAD). An Agilent XDB-C-8 column (4.6 µm, 25 mm × 4.6 mm ID)

Table 2
Chemical structure of the identified compounds in the A. millefolium extract. 

<table>
<thead>
<tr>
<th>Compound</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>R$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Caff</td>
<td>H</td>
<td>H</td>
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</tr>
<tr>
<td>2 Caff</td>
<td>Caff</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>3 Caff</td>
<td>Caff</td>
<td>Caff</td>
<td>H</td>
</tr>
<tr>
<td>4 H</td>
<td>Caff</td>
<td>H</td>
<td>Caff</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>R$_3$</th>
<th>R$_4$</th>
<th>R$_5$</th>
<th>R$_6$</th>
<th>R$_7$</th>
<th>R$_8$</th>
<th>R$_9$</th>
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</thead>
<tbody>
<tr>
<td>5 H</td>
<td>H</td>
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<td>OH</td>
<td>OH</td>
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</tr>
<tr>
<td>6 H</td>
<td>H</td>
<td>H</td>
<td>O-Glc</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>OH</td>
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<td>OH</td>
</tr>
<tr>
<td>7 H</td>
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<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
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<td>OH</td>
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<td>OH</td>
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<tr>
<td>8 H</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>9 H</td>
<td>H</td>
<td>H</td>
<td>O-Glc</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
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<td>H</td>
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<td>OH</td>
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<tr>
<td>11 O-Glc-Rha</td>
<td>H</td>
<td>H</td>
<td>OH</td>
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<td>H</td>
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<tr>
<td>12 O-Glc</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
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</tr>
<tr>
<td>13 H</td>
<td>H</td>
<td>H</td>
<td>Glucuronic acid</td>
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</table>
Fig. 1. Direct injection mass spectrum (DI-MS) of *Achillea millefolium* extract. Identification of compounds was achieved on the basis of MS² fragmentation (see Table 1).

was used. Mobile phase consisted of (A) aqueous formic acid (0.1%) and (B) acetonitrile. Gradient condition was: 0–8.5 min, linear gradient from 12% to 26% of B; 8.5–11 min, isocratic conditions at 26% of B; 11–16 min, linear gradient from 26% to 40% of B; 16–45 min, linear gradient from 40% to 50% of B; 45–50 min, linear gradient from 50% to 100% of B. Flow rate: 1 ml/min. Calibration curves were obtained by standard solutions of rutin for flavonoid derivatives (at 350 nm), and chlorogenic acid for caffeoyl quinic derivatives (at 330 nm). The concentration ranges were 3.96–396 μg/ml and 1.0–53 μg/ml for chlorogenic acid and rutin, respectively. The limits of detection (LOD) and quantification (LOQ) were 1.5 and 4.0 μg/ml and 0.5 and 1.5 μg/ml for chlorogenic acid and rutin, respectively.

**Identification of compounds**

The following compounds were identified in the extract by comparing their retention times and UV spectra with authentic standards previously isolated from *A. millefolium* extracts (Innocenti et al. 2007) or authentic standards in the collection of the Department of Pharmaceutical Sciences: chlorogenic acid (1), 3,5-dicaffeoyl quinic acid (2), 3,4-dicaffeoyl quinic acid (3), 4,5-dicaffeoyl quinic acid (4), apigenin (5), apigenin-7-O-β-D-glucoside (6), apigenin-4-O-β-D-glucoside (7), luteolin (8), luteolin-7-O-β-D-glucoside (9), luteolin-4-O-β-D-glucoside (10), and rutin (11) (Tables 1 and 2). Quercetin 3-O-glucoside (12), luteolin-7-O-glucuronide (13) and luteolin diglucoside were identified on the

![Graph A](image)

**Fig. 2.** Concentration–response curves of *A. millefolium* extract (*Achillea*) on VSMC viability as measured by MTS assay. Data are expressed as % of viability relative to vehicle-treated cells. VSMCs were seeded at 2000 or 8000 cells/wells, synchronized in serum-free medium for 48 h and treated with increasing concentrations of *A. millefolium* extract for 24 h (A) or 48 h (B). Each point is mean ± SEM of 3 experiments each performed in 6 replicates.
basis of their MS spectra and by comparison with literature data (Tuberoso et al. 2009).

**Cell culture**

VSMCs obtained from aortic intimal-medial layers of male Sprague-Dawley rats express ER (Maggi et al. 2003). VSMCs were seeded for experiments in 96-well plates (3 × 10^3 cells/well) in phenol red-free M199 with 10% FCS for 24 h, then synchronized in serum-free medium for 48 h and incubated for the indicated time in the presence or absence of increasing concentrations of A. milfoilum extract. In selected experiments VSMCs were pretreated with the ER antagonist ICI 182,780 (Tocris).

HUVECs were grown as previously described (Bolego et al. 2006). For experiments, cells were seeded in 6-well plates (3 × 10^5/well) and incubated in M199 supplemented with 5% FCS. Experiments were carried out in the presence or absence of interleukin (IL)-1β (2 ng/ml) added for 5 min or Achillea extract as indicated.

**Cell viability assay**

VSMC viability was assessed as described elsewhere (Takahashi et al. 2003) by recording the absorbance at 490 nm generated by the bioreduction of the MTS tetrazolium compound into a colored soluble formazan product (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega).

**Western blot analysis**

HUVECs were harvested in lysis buffer and equal amounts of cell protein were loaded onto 10% SDS-acrylamide gels. At the end of the run, proteins were transferred to a nitrocellulose membrane and incubated overnight with a polyclonal antibody against IκBα (1:1000, Cell Signaling) and a monoclonal antibody against phospho (p)-IκBα (1:1000, Cell Signaling), then with suitable peroxidase-conjugated secondary antibodies (Vector) for 1 h. Proteins were detected by chemiluminescence (Amersham Biosciences). Loading control was performed using actin immunodetection.

**Immunocytochemistry**

HUVECs were cultured on coverslips in 24-well plates and after fixation with ethanol (30 min), were permeabilized with 0.1% Triton in PBS for 1 min, incubated with a 1:50 dilution of rabbit serum for 30 min, and incubated with polyclonal antibody for NF-κB p65 for 2 h. Cells were then incubated with biotinylated secondary antibody (1:1000, 30 min, Vector) and with streptavidin-FITC (1:1000 30 min, Vector) at room temperature. The coverslips were analyzed using the confocal microscope D-Eclipse C1 (Nikon). Several fields of view for each experiment were selected. Data were obtained from 3 independent experiments.

**Statistics**

Values are presented as mean ± SEM of 3–6 independent experiments. Two-way analysis of variance and unpaired t-tests were used to test for differences between groups. Differences were considered statistically significant when P < 0.05.

**Results and discussion**

**Phytochemical investigations**

The extract fingerprint was obtained by direct ESI-MS analysis. The extract under negative ESI-MS conditions showed several
major ionic species (Fig. 1); the most abundant of these ions were isolated and their collisionally induced dissociation (CID) product ions spectra were obtained. Fragmentation of the major peaks was used for the identification of the compounds (Table 1). The quantitative determination of phenol constituents was obtained on HPLC-DAD. Chlorogenic acid and rutin were used as reference standards for caffeic acid derivatives (CQAs) and flavonoids, respectively. The A. millefolium extract used in the present study is rich in phenolic constituents and does not almost contain sesquiterpene lactones. The content of flavonoids and caffeoyl quinic derivatives was 9.7 and 11.8%, with an amount of apigenin-7-O-glucoside and 3,5-dicaffeyl quinic acid of 5.3 and 7.8%, respectively.

VSMC growth

The effect of A. millefolium extract (20–200 μg/ml, corresponding to 1.94–19.4 μg/ml flavonoids and 2.36–23.6 μg/ml CQAs) on VSMC growth was tested under different conditions of cell seeding density (2000 or 8000 SMC/well) and incubation time (24 or 48 h) using the MTS assay. At variance with tumor cells (Csutor-Löffler et al. 2009), the yarrow extract at concentrations below 60 μg/ml enhanced VSMC growth by about 30–40% with respect to baseline at both time points (Fig. 2). By contrast, the highest concentration tested of 200 μg/ml induced inconsistent effects on VSMC growth probably due to toxicity of specific Achillea constituents. Such variability at the highest test concentration was not related to seeding density (data not shown).

Achillea extract interferes with the NF-κB pathway in HUVECs

In IL-1β-stimulated HUVECs, levels of IκBα were significantly reduced and p-IκBα became detectable as compared with baseline within 5 min incubation (Fig. 3), thus promoting NF-κB activation. Short-term preincubation (10 min) with 100 μg/ml A. millefolium extract, corresponding to 9.73 μg/ml of flavonoids and 11.78 μg/ml of CQAs, attenuated the effect of IL-1β, partially restoring the amount of IκBα in stimulated HUVECs without affecting the amount of p-IκBα. The lower Achillea extract concentration was ineffective on the formation of both proteins.

The nuclear translocation of NF-κB was monitored using a fluorescent anti-p65 antibody (Fig. 4). In unstimulated HUVECs p65 was detectable in the cytoplasm, indicating that the NF-κB pathway was turned off, whereas stimulation with IL-1β treated cells for 30 min led to nuclear translocation of p65. Treatment with 100 and 50 μg/ml Achillea extract redistributed extranuclear p65 labeling to a detectable extent in IL-1β-stimulated HUVECs.
Involvement of estrogen receptors in the proliferation-enhancing action of A. millefolium extract

To determine whether the observed increase in VSMC growth in response to treatment with the Achillea extract was mediated by ERs, VSMCs were treated with the extract in the presence or absence of the nonselective ER antagonist ICI 182,780. The extract concentrations of 5 and 20 μg/ml were chosen as a potential effective range. As shown in Fig. 4, a significant increase in VSMC viability was observed only in cells treated with 20 μg/ml (corresponding to 1.94 μg/ml flavonoids and 2.36 μg/ml CAQs) for 24 h. This effect was abolished by 1 and 10 μM ICI 182,780.

The present findings are consistent with previous reports of in vitro estrogenic activity of A. millefolium (Innocenti et al. 2007). In fact, the long-term enhancement of VSMC proliferation by Achillea extract was abolished in the presence of the nonselective ER antagonist ICI 182,780 (Fig. 5). Although we did not determine if this compound could reverse the impaired NF-κB activation mediated by the Achillea extract, ER activation is known to trigger rapid signaling events in vascular cells (Meyer et al. 2009). The relative contribution of ERα vs ERβ to these biological actions remains to be determined. It is also conceivable that different Achillea constituents mediate the proliferative and anti-inflammatory action in vascular cells. For instance, previous studies report on the inhibition of NF-κB by compounds that are present in Achillea (Rios et al. 2009). The impairment of NF-κB signaling as described in the present study might be therefore due to multiple extract constituents.

In conclusion, we here show that the test A. millefolium extract, containing flavonoid glycosides and CAQ derivatives, is able to enhance VSMC growth at least in part by acting through ERs and impair NF-κB signaling in endothelial cells. These findings support at least in part the traditional use of the plant, explaining some of its activity through the modulation of the inflammation pathway. Therefore, A. millefolium may induce novel potential actions in the cardiovascular system.

References


