Inhibitory effect of aqueous extracts of some herbs on human platelet aggregation in vitro

SAULNIER PIERRE, LYNN CROSBIE, & ASIM K. DUTTAROY

1 ENSBANA, Dijon, France, 2 Provexis Limited, Aberdeen, Scotland, UK, and 3 Department of Nutrition, Faculty of Medicine, University of Oslo, Norway

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Abstract
Effect of aqueous extract of several herbs on human platelet aggregation in vitro was investigated. Out of 28 herbs/nutriceuticals investigated, camomile, nettle alfalfa, garlic and onion exhibited most significant anti-platelet activity (>45% inhibition). Aqueous extracts of alfalfa, fresh nettle, and camomile inhibited ADP induced-platelet aggregation by 73, 65 and 60%, respectively, compared with control (P<0.05). Camomile and alfalfa inhibited collagen-induced platelet aggregation by 84 and 65%, respectively, but nettle could not inhibit collagen-induced aggregation. In contrast, nettle was the most potent inhibitor (66%) of whole blood aggregation induced by collagen, followed by alfalfa (52%), and camomile (30%) compared with control (P<0.05). None of these three herbs however could inhibit arachidonic acid or thrombin induced platelet aggregation. Camomile and alfalfa strongly inhibited thromboxane B2 synthesis induced by ADP or collagen, but nettle had no effect. Alfalfa and nettle increased cGMP levels in platelets by 50 and 35%, respectively, compared with the control (1.85±0.23 nM) (P<0.005). All these data indicate that camomile, nettle and alfalfa have potent anti-platelet properties, and their inhibitory actions are mediated via different mechanisms.

Keywords: Platelets, herbs, Platelet aggregation, camomile, nettle, alfalfa, human, cGMP, thromboxane B2, nutriceuticals, whole blood aggregation, herbal extract

Introduction
Activation of blood platelets plays a crucial role not only in haemostasis but also in pathological development of several arterial disorders, including strokes, and myocardial infarction [1–3]. There is increasing evidence that acute clinical manifestations of coronary atherosclerotic disease are caused by plaque disruption and subsequent platelet-thrombus formation [4–6]. It is documented that platelets also participate in tumor progression, allergic inflammation, and non-allergic responses by producing inflammatory substances like cytokines and eicosanoids. Thus platelet activity may play a major role in the development of several diseases as well as in the stability of atherosclerotic plaques [4]. In support of the pathophysiological role of platelets, platelet inhibitory drugs such as aspirin reduce the incidence of myocardial infarction, stroke and death from cardiovascular disease (CVD) in secondary prevention trials [7–10]. Therefore, today, the research is oriented toward the discovery of new compounds that can inhibit platelet aggregation reversibly and without any side effect.

The anti-thrombotic property of dietary nutrients and non-nutrients of plants and vegetables origin including tomatoes and kiwifruits have been described [2, 11, 12]. Herbs have been used as medical treatments since the beginning of civilization and some derivatives (e.g., aspirin, reserpine, and digitalis) have become mainstays of human pharmacotherapy [13, 14]. For cardiovascular diseases, herbal treatments have been used in patients with congestive heart failure, systolic hypertension, angina pectoris, atherosclerosis, cerebral insufficiency, venous insufficiency, and arrhythmia [14, 15]. With the high prevalence of herbal use in the world today, further research is necessary to elucidate the pharmacological activities of the many herbal remedies now being used to treat CVD. Very little information
is available on the anti platelet property of herbs. The purpose of this study is to investigate systematically the anti-platelet activity of several herbs using in vitro aggregation methods (platelet-rich plasma and whole blood aggregation). The herbs were chosen in this study on the basis of their reported beneficial effects in CVD and other diseases [13, 14]. In this paper we report anti-aggregatory activity of aqueous extracts of several herbs.

Materials and methods

Materials

ADP, collagen, and arachidonic acid were obtained from Helena Biosciences (USA). Bovine serum albumin fraction V fatty acid free was purchased from ICN Biomedical (UK). Human fibrinogen, and all other reagents used were purchased from Sigma Chemical (UK). cGMP and thromboxane B_2 assay kits were purchased from Amersham Pharmacia Biotech (UK).

Herbs

The following herbs were purchased from Grampian Health Foods (Aberdeen, UK). Lime flower, peppermint, nettle, lemon grass, camomile were the products of Heath & Heather (UK). Feverfew, hawthorn berry, passiflora, ginseng, liquorice root, mistletoe, yarrow, motherwort, prickly ash, lily of the valley, devils claw, horse chestnut, rosemary, marigold, ginger, and garlic were from Herbs of the valley, devils claw, horse chestnut, rosemary, marigold, ginger, and garlic were from Herbs of Grace (Newmarket, UK). Dandelion and black tea were produced by Salus (Warrington, UK) and Tetley (UK), respectively. In some cases, fresh plants (alfalfa, origan, mint, thyme, sage, and nettle) were also used.

Preparation of herbal extracts

Herbs (2 g) were boiled in 20 ml distilled water for 3 min for isolation of water soluble fraction. The boiled suspension was then clarified by centrifugation at 2000 × g for 10 min. Concentration of the aqueous extract was then determined by weighing the 5-ml freeze dried sample. The extract was then stored at −20°C. The dried extracts were then dissolved in an appropriate volume of phosphate-buffered saline (PBS) to produce final concentration of 10 mg/ml. The pH of these herbal extracts was adjusted to 7.4 immediately prior to the aggregation studies. In some cases, fresh plants such fresh plants alfalfa, origan, mint, thyme, sage, and nettle were also used. These plants (2 g) were homogenized and then the aqueous extract was prepared as mentioned above. Extracts of onions, garlic, and ginger were prepared as above without boiling.

Subjects

Healthy subjects (aged 20–35 years) were recruited after assessment of their medical and dietary history. The study protocol was approved by the Joint Ethical Committee of the Grampian Health Board, and the University of Aberdeen, Scotland, UK. Exclusion criteria were the presence of overt vascular, haematological or respiratory disease, hypertension, infection, frequent consumption of drugs which affect platelet function (e.g., aspirin, paracetamol, ibuprofen, steroids), habitual consumption of omega-3 fatty acid supplements.

Platelet aggregation in platelet-rich plasma

Overnight fasted venous blood was collected from volunteers who had not taken any medication for at least 14 days before donation. Blood was collected through siliconized needles into plastic syringes: coagulation was prevented by mixing 9 vol of blood with 1 vol sodium citrate (final concentration, 13 mM). Platelet-rich plasma (PRP) was obtained by centrifugation of samples for 15 min at 200 × g. The supernatant platelet-rich plasma (2–3 × 10^8 cells/ml) was collected. Platelet counts were performed using a Baker cell Counter.

Platelet aggregation was monitored on a Packs-4 aggregometer (Helena Biosciences, USA) at a constant stirring speed of 1000 rpm at 37°C, as described before [16]. Since maximal amplitude of aggregation of PRP was obtained with either of 2 μg/ml collagen, 10 μM ADP, 500 μg/ml arachidonic acid, these concentrations of agonists were used and aggregation was followed over 10 min. PRP (450 μl) was incubated with 50 μl of pH 7.4 adjusted extracts for 15 min at 37°C prior to the addition of an aggregating agent. Controls were run in parallel replacing extract with 50 μl of PBS. Inhibition of platelet aggregation is expressed as the decrease in the area under the curve compared with the control.

Effect of herbal extracts on thrombin-induced platelet aggregation was investigated in washed platelet suspensions. Their preparation from PRP was carried out as described previously [16].

Whole blood aggregation

Blood taken from healthy subjects and mixed with sodium citrate, was incubated at 37°C in a water bath during the course of the experiment. A Chronolog whole blood aggregometer was used for these experiments [17]. Typically, 500 μl of blood and 500 μl of PBS were placed in a plastic cuvette kept at 37°C. Herbal extract (final concentration, 1 mg/ml) was added and pre-incubated for 15 min before the addition of collagen (1 μg/ml). The average of several aggregations in the absence and presence of herbal extract was determined so that the
percentage inhibition could be calculated, as described before [17].

**Thromboxane B₂ (TxB₂) assay**

TxB₂, the stable metabolite of TxA₂ was estimated using the Amersham TxB₂ EIA assay kit, as described before [16, 17]. Briefly, at the end of platelet aggregation in the absence and presence of extracts (1 mg/ml), 400 µl post-aggregation plasma were acidified to pH 3 and then applied to a conditioned Amprep C-2 column. The TxB₂ was eluted using methylformate and then assayed according to the manufacturer’s instructions, as described [16, 17].

**cGMP assay**

cGMP was measured both in plasma and platelets, since cGMP produced in the platelets can be released into the plasma. Aliquots (450 µl) of PRP were incubated with extracts (1 mg/ml) for 15 min at 37°C. A control was also incubated with 50 µl of saline. The samples were then centrifuged at 8000 × g for 5 min. Supernatant plasma (150 µl) was kept in order to measure the plasma cGMP. The platelet pellet was washed in saline. The pellet was resuspended in 300 µl of ice-cold trichloroacetic acid and kept on ice for 1 h to lyse. After this time, samples were centrifuged at 8000 × g for 5 min and the platelet pellet was discarded. PPP and platelet extracts were washed four times with five volumes of water saturated with diethyl ether and then dried, using a freeze dryer. After having resuspended samples in assay buffer, the EIA procedure was performed in the 96-coated wells plate using protocol supplied with the kit.

**Statistical analysis**

Results are presented as the mean ± SEM. Results were analysed by the Student’s t-test. Values for the area under the curve for platelet aggregation in the absence and in the presence of herbal extract were evaluated by a computer assisted program. Other statistical analyses were performed using ANOVA where appropriate, values were considered to be significantly different when P<0.05.

**Results**

**Effect of extracts on platelet aggregation in PRP**

Since the amounts of the material extracted from these herbs were highly variable, the dried extracted material was dissolved in an appropriate volume of PBS to produce final concentration of 10 mg/ml for each herb. Table I shows the inhibitory effect of aqueous extracts (final concentration, 1 mg/ml) of 28 herbs/nutriceuticals on human platelet aggregation in vitro. Results were expressed as the percent inhibition of aggregation in response to 10 µM ADP compared with control. Among these extracts used in this study, camomile, nettle (fresh and powdered), alfalfa, garlic, and onion were the most effective against ADP induced platelet aggregation, inhibiting platelet aggregation by more than 45% (P<0.05). The maximum inhibitory effect (90%) was observed with garlic, followed by alfalfa, (73%), onion (71%), fresh nettle (65%), and camomile (60%), whereas peppermint, and lime flower inhibited platelet aggregation by 25 and 20%, respectively. Other herbs, such as feverfew, hawthorn, dandelion leaves, devil’s claw, ginseng, licorice root, lily of the valley, mint, marigold, mistletoe, motherwort, origan, passiflora, prickly ash, rosemary, thyme, and black tea had little or no effect.

Since several studies have been already published on the anti-platelet properties of garlic and onions [2, 18, 19], in this study we concentrated only on alfalfa, camomile, and nettle. Table II shows the dose dependant inhibition of ADP induced platelet aggregation by aqueous extracts of these three herbs. At 0.66 mg/ml, alfalfa, and fresh nettle inhibited 54–61% platelet aggregation, whereas camomile inhibited platelet aggregation by only 29%. Effects of these
Table II. Dose-dependent inhibition of ADP (10 μM)-induced platelet aggregation in PRP by three herbal extracts.

<table>
<thead>
<tr>
<th>Herbs</th>
<th>Concentration (mg/ml)</th>
<th>% Inhibition of aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa</td>
<td>0.30</td>
<td>23.2 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>0.66</td>
<td>61.4 ± 8.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>73.1 ± 10.4</td>
</tr>
<tr>
<td>Camomile</td>
<td>0.30</td>
<td>7.3 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>0.66</td>
<td>29.1 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>60.5 ± 10.7</td>
</tr>
<tr>
<td></td>
<td>1.44</td>
<td>65.3 ± 8.5</td>
</tr>
<tr>
<td>Nettle</td>
<td>0.30</td>
<td>39.1 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>0.66</td>
<td>54.5 ± 6.2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>65.1 ± 11.2</td>
</tr>
<tr>
<td></td>
<td>1.44</td>
<td>67.4 ± 9.8</td>
</tr>
</tbody>
</table>

Experiments were performed as described in the Methods section. PRP was aggregated by agonists as described in the presence and absence of varying concentrations of herbal extracts. Each experiment was done in triplicate, on each of 10 volunteers.

Table III. Effects of herbal extracts on TxB₂ synthesis in platelets in response to ADP and collagen.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Herbs</th>
<th>TxB₂ synthesis (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (10 μM)</td>
<td>–</td>
<td>28.0 ± 3.5</td>
</tr>
<tr>
<td>ADP (10 μM)</td>
<td>Alfalfa</td>
<td>16.6 ± 4.1*</td>
</tr>
<tr>
<td>ADP (10 μM)</td>
<td>Camomile</td>
<td>19.8 ± 2.8*</td>
</tr>
<tr>
<td>ADP (10 μM)</td>
<td>Nettle</td>
<td>27.5 ± 2.1</td>
</tr>
<tr>
<td>Collagen (2 μg/ml)</td>
<td>–</td>
<td>79.3 ± 8.5</td>
</tr>
<tr>
<td>Collagen (2 μg/ml)</td>
<td>Alfalfa</td>
<td>37.3 ± 7.5*</td>
</tr>
<tr>
<td>Collagen (2 μg/ml)</td>
<td>Camomile</td>
<td>41.0 ± 2.6*</td>
</tr>
<tr>
<td>Collagen (2 μg/ml)</td>
<td>Nettle</td>
<td>82.0 ± 3.4</td>
</tr>
</tbody>
</table>

Experiments were performed as described in the Methods section. TxB₂ was measured after PRP was aggregated by agonists as described. Each experiment was done in triplicate, on each of 10 volunteers. *P<0.05 compared with the respective control.

Collagen-induced TxB₂ synthesis (79.3 ± 8.5 ng/ml) was also inhibited by 53 and 48% by alfalfa (37.3 ± 7.5 ng/ml) and camomile (41.0 ± 6.2 ng/ml), respectively. However, nettle did not inhibit thromboxane synthesis either induced by ADP or collagen.

Effects of herbs on cGMP synthesis

cGMP levels in platelets and plasma were determined following incubation of extracts (1 mg/ml) with PRP (0.5 ml). Alfalfa and nettle increased synthesis of cGMP by 50 ± 9.4 and 32 ± 5.3% significantly compared with the control (1.85 ± 0.23 nM) (P<0.005, n = 4), whereas camomile had no inhibitory effect.

Discussion

Of the extracts investigated, garlic, onions, camomile, nettle and alfalfa proved to be the most potent inhibitors of platelet aggregation in vitro, after incubation with PRP for 15 min. Alfalfa, and camomile, and nettle inhibited ADP-induced platelet aggregation in PRP in a dose-dependent manner. In contrast to alfalfa and camomile, nettle could not inhibit collagen-induced platelet aggregation, indicating that nettle may have different mechanism of action. Despite its inability to inhibit collagen-induced platelet aggregation in PRP, nettle had a greater anti-aggregating activity in whole blood induced by collagen. These differences in activity and effectiveness of nettle in PRP and whole blood are not known at present but may be attributed to differences in their mode of action or metabolism in different blood cell types and platelets. This was clearly observed by the fact that thromboxane synthesis in platelets was inhibited by camomile and alfalfa but not by nettle. Nettle inhibited ADP induced platelet aggregation but not by collagen, indicating its mechanism of action is independent of thromboxane pathway. However, when using arachidonic acid as the agonist, very little or no inhibition

Effects of herbs on whole blood aggregation

Nettle, alfalfa, and camomile at 1 mg/ml inhibited collagen-induced whole blood aggregation by 60 ± 12.4, 50 ± 8.6, and 30 ± 8.4%, respectively, (P<0.05, n = 5) compared with a corresponding control.

Effects of herbs on TxB₂ synthesis in Platelets

To determine whether the inhibitory effect extracts on platelet aggregation was due to the decreased synthesis of TxA₂, levels of TxB₂, the stable breakdown product of TxA₂, were measured in post aggregated plasma. Table III shows effects of these herbs on TxB₂ synthesis. TxB₂ was measured in the plasma after platelet aggregation induced by ADP or collagen in the presence and absence of these extracts. Both camomile and alfalfa inhibited TxB₂ synthesis in response to ADP and collagen, with a great inhibitory effect observed for collagen induced thromboxane synthesis (48–53%) compared with ADP (30–41%), P<0.05. ADP-induced TxB₂ synthesis was inhibited by 41 and 30% by alfalfa (16.6 ± 4.1 ng/ml) and camomile (19.8 ± 2.8 ng/ml), respectively, compared with control (28.0 ± 3.5 ng/ml).
was observed by any of these three herbs, suggesting that platelet cyclooxygenase pre se is not inhibited by these extracts. Inhibition at upstream of cyclooxygenase at the release of arachidonic acid from membrane phospholipids is therefore more likely the possibility. However, further investigations are required for better understanding the mode of actions of these extracts.

Both nettle and alfalfa appear to inhibit platelets via the modulation of cyclic nucleotides. Both these herbs increased cGMP levels in platelets. cGMP is known to inhibit platelet aggregation [20]. Taken together, these data indicate that alfalfa, camomile, and nettle inhibited platelet aggregation via different mechanisms. It is important to note that the aggregation of platelets was performed in vitro, in a different environment than the blood stream. Studies on human volunteers would be required to ascertain their effects in vivo. Nevertheless, our study shows that consuming these herbs may be beneficial in CVD. Alfalfa is commonly used as diuretic, is rich in minerals has anti-inflammatory properties, and may reduce cholesterol levels [13, 14]. Nettle also acts as diuretic and has anti-arthritis and anti-inflammatory effects, while camomile is used as a sedative, and has anti hypertensive and anti-bacterial properties [13, 14]. In the current study, hitherto unknown we demonstrate that camomile, nettle and alfalfa have anti-platelet property.

In conclusion, camomile, nettle and alfalfa are equally effective at inhibiting platelet aggregation in vitro. Our data for the first time thus provide in vitro evidence that these three herbs may have potential for inhibiting platelet activation and platelet mediated events in CVD. Further investigation of the effects of individual herbs on in vivo platelet function, is required in order to elucidate their role in the relationship between herbs and the risk of CVD.

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