Effects of Achillea Santolina extracts and fractions on human platelet aggregation in vitro and on rat arteriovenous shunt thrombosis in vivo

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**ABSTRACT:**

**Background:** Achillea santolina (As), a plant grown in Irak, has been used in herbal medicine for thousands of years. It has anti-inflammatory properties, but its antiplatelet or antithrombotic activities have not been explored.

**Methods:** The effects of As leaf crude extracts and fractions extracted with chloroform (F1), diethyl ether (F2), ethyl acetate (F3) and water (F4) were assessed *in vitro* on platelet human aggregation induced by ADP and collagen and on a rat arteriovenous shunt thrombosis model *in vivo* after 10 days oral administration (10 mg/kg/d).

**Results:** A. santolina crude extract dose-dependently inhibited *in vitro* ADP and collagen-induced human platelet aggregation (maximal inhibition respectively 34.4 ± 2.9% and 78.3 ± 2.5 %). This effect was mostly contained in the diethylester (F2) fraction; F1 and F3 had about half the effect, and F4 was devoid of antiaggregant effect. Achillea Santolina extracts given orally daily for 10 days at the dose of 10mg/kg/day decreased thrombus weight but not significantly.

The discrepancy between clear dose-dependent *in vitro* effects on ADP and Collagen-induced human platelet aggregation and unclear *in vivo* effects in rats raises questions as to active concentrations, intestinal resorption and/or metabolisation of active compounds.

**Key words:** Achillea Santolina; Platelet aggregation; Arterial Thrombosis; Rat;

**INTRODUCTION:**

Achillea Santolina is a plant found around the southern mediterranean and the Middle East, where it is traditionally used for its anti-inflammatory(1), anti-microbial (2, 3) and vermifugal (4) properties (5). A. santolina contains flavones, particularly flavonoids and sesquiterpene lactone (2). Because anti-inflammatory properties are often associated with antiplatelet effects, such as is evident for another plant-derived product, aspirin (6, 7), we thought it might be instructive to test the effects of Achillea santolina on human platelet aggregation *in vitro*, and on *in-vivo* thrombosis using a rat arteriovenous shunt thrombosis model we previously used to show antithrombotic effects of armagnac and other plant extracts (8-10)
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**METHOD:**

### Crude Achillea Santolina extract

A. santolina was collected in Irak and dried at room temperature. The aerial parts of A. santolina, essentially dried leaves, were powdered and infused in boiling water. After cooling, the resultant decoction was then filtered and dissolved in water before freeze-drying to yield the crude extract. Analysis of the crude extract found 3.47 mg/L Caftanic acid, 1.52 g/L Caffeic acid, 5.06 g/L Rutin, 9.11 g/L Isoquercetin.

### Extract fractionation

0.5 g of the crude extract was dissolved in water with 5% ethanol (v/v) to obtain an aqueous phase of 2.5 g/l. Several liquid/liquid separations (v/v) were successively performed with chloroform (Acros Organic, ref 268320010), diethyl ether (Prolabo, ref 23811.361) and ethyl acetate (Prolabo, ref 23878.326). For each solvent, the extraction was realized three times. The three resulting organic phases were mixed and evaporated under vacuum before freeze-drying. The remaining aqueous phase was also freeze-dried. The fractions obtained were named F1, F2, F3 and F4 for chloroform, diethyl ether, ethyl acetate and water respectively. Achillea Santolina crude extract (AS), F1, F2, F3 and F4 fractions were dissolved in 0.9% saline with 0.5% ethanol (v/v) to obtain primary solutions of 0.2 g/l. These were then serially diluted with 0.9% saline to obtain concentrations of 2.10^{-2} to 2.10^{-7} g/l. The control was 0.9% saline solution with 0.5% ethanol.

### Preparation of platelet-rich plasma (PRP)

Samples were prepared according to standard practice (11-14): 4.5 ml of whole blood was obtained during platelet donation from healthy medication-free donors, after informed consent, in 0.5 ml of 3.8% sodium citrate solution. Samples were centrifuged at 1200 rpm for 10 min at room temperature, the supernatant platelet-rich plasma (PRP) was isolated. The residue was centrifuged at 3000 rpm for 10 min at room temperature to obtain platelet-poor plasma (PPP). The PRP was adjusted with PPP so as to obtain platelet counts of 2.5.10^{11} Pl/L.

### Platelet aggregation

Adenosine diphosphate (ADP, AMAX, St. Louis, MO, USA) Collagen (AMAX, St. Louis, MO, USA) were used to induce platelet aggregation at final concentration of 2 µmol/l and 5 µmol/l, respectively. Platelet aggregation was determined by Born’s method with modifications using a four-channel aggregometer (BIO/DATA, Horsham, PA, USA). PRP 350 µl and 50 µl of 0.9% saline with 0.5% ethanol (control), AS or fractions were added into the microcuvette and incubated at room temperature for 30 min. After pre-incubation at 37°C for 3 min. in the aggregometer, 20 µl of ADP or collagen were added to the cell and the aggregation curves were recorded for 6 min. Final concentrations of Achillea Santolina extracts therefore ranged from 2.38.10^{-2} to 2.38.10^{-7} g/l. Maximum aggregation was recorded for the control (CA) and the different tests (TA). The inhibition of aggregation (IA) was calculated as a measure of A. Santolina crude extract or fractions (10):

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IA = \frac{CA - TA}{CA} \times 100
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All tests were done in triplicate using different platelet donors.
**In vivo experiments**

**Animals:**
Male Wistar rats weighing 350–380 g were purchased from Charles River (Le Vaudreuil, France). They were kept in a temperature-controlled environment (20±1 °C) with a 12h:12h light–dark cycle, and fed with standard chow, for at least 1 week before any manipulations. Rats were kept and treated in accordance with the French regulations concerning animal care, in an approved environment.

**Treatments:**
Six groups of six Wistar rats were given orally A. Santolina crude extracts or one of the four fractions at the dosage of 10 mg/kg b.w. during 10 consecutive days, or saline with 0.5% ethanol for the control group. The arteriovenous shunt thrombosis model in rat was tested 2 h after the last administration. For each test, different batches of six or seven rats were used.

**Thrombosis model:**
The model tested was a rat arteriovenous shunt thrombosis model (6, 8-10, 15-18). After anesthesia with sodium pentobarbital (50 mg/kg i.p), an 8-cm polyethylene tube was inserted between the left jugular vein and the right carotid artery. The saline-filled shunt was assembled by connecting two cannulae with a slightly curved 6-cm-long tygon tubing (internal diameter 2 mm) containing a 5-cm-long cotton thread (diameter 0.25 mm) which had been scraped with a scalpel blade to render it more thrombogenic. The extracorporeal circulation was maintained for 15 min, during which time a thrombus adheres to the cotton thread. The shunt was then removed and the thread with its associated thrombus was withdrawn and immediately weighed. The thrombus wet weight was determined by subtracting from the value obtained the weight of the dry 5 cm cotton thread determined previously.

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**Statistical analysis**

ANOVA was used to test for differences between the effects of different preparation of A. Santolina (crude extract or fractions) and the different concentrations tested on the aggregation inhibition. If the ANOVA found significant effects, the Tukey test was used to identify significant differences between different concentrations (p<0.05).

**RESULTS:**

**In vitro tests**

**Effect of the whole extract**
Collagen-induced platelet aggregation was inhibited in a dose-dependent manner by concentrations of A. Santolina crude extracts ranging from 2.38x10⁻⁶ to 2.38x10⁻² g/l, after a 30 min incubation, from 1.3 ±1.53 % at 2.38 10⁻⁶ g/l to 78.3 ± 2.52 for 2.38 10⁻¹ g/l. (figure 1)ADP-induced human platelet aggregation in vitro was dose-dependently inhibited by concentrations of A. Santolina crude extracts ranging from 2.38x10⁻⁶ to 2.38x10⁻² g/l, after a 30 min incubation with an inhibitory effect of 1.01±2.67% to 34.4 ± 2.9% (Fig. 2). There was no inhibitory effect at the concentration of 2.38x10⁻⁷ g/l on either ADP or collagen-induced aggregation. At higher concentrations, Achillea santolina was more inhibitory of collagen than ADP-induced aggregation.

**Effect of the different fractions**
For collagen-induced aggregation, the maximal effect was found with the crude extract and with fraction 2 (Fig. 1). F1 and F3 fractions were mostly the same, and had about half the potency of crude extract or fraction F2. Fraction 4 was essentially devoid of effect. The most potent inhibitory effects on ADP-induced platelet aggregation were obtained with fractions 2 and 3 (Fig. 2). The effect on
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the platelet aggregation with fractions F2 and F3 was stronger than that observed with crude extract. With the highest concentration the aggregation inhibition was 45.2 ± 2.2% and 40.8 ± 1.6% for F2 and F3 respectively. The effect of F1 and F4 fractions which correspond to chloroform and aqueous fractions was very low with an inhibiting effect of 13.0 ± 1.8% and 9.7 ± 1.1% respectively, for a concentration of 2.38 .10^{-2} g/l. Overall fraction 2 appeared to inhibit about equally both collagen and ADP-induced aggregation, whereas fraction 3 was relatively more potent on ADP than on collagen-induced aggregation. \textbf{In vivo effect of A. Santolina extracts on a rat model of arteriovenous shunt thrombosis.}

A ten-day oral treatment with A. Santolina extract and fractions decreased thrombus weight in the rat model of arteriovenous shunt thrombosis compared to vehicle. Compared to a control thrombus weight of (42.8 ± 2.3 mg) the reduction was significant for extracts 1, 2, and 4 (respectively 31.9 ± 3.4, 32.8 ± 2.0, and 32.4 ± 2.0 mg) but not for the crude extract or fraction 3 (respectively 36.5 ± 3.3 and 36.6 ± 3.1 mg).

\textbf{DISCUSSION :}

In the present study, we have tested the \textit{in vitro} anti-aggregant and \textit{in vivo} anti-thrombotic effect of extracts and fractions of A. Santolina. Crude extract and some fractions dose-dependently inhibited platelet aggregation induced by Collagen and ADP in vitro, and seemingly more that induced by collagen than by ADP. In vivo, however, the inhibition of experimental thrombosis was more modest, compared to other product of plant origin we tested (8-10), or NSAIDs including aspirin, also a plant-derived product initially (6). In vitro, the most active fractions were fraction 2 on both collagen and ADP, fraction 3 on ADP. In vivo, at the dose used, the active fractions were 2, 3 and 4, the latter being mostly inactive in vitro. In previous experiments using the same fractionation methods with Armagnac, we found no effect on collagen, and the maximal effect on ADP induced aggregation or in vivo thrombosis resided in fraction 1 (19). Other studies have found anti-aggregant effects for onions (Allium cepa)(20), garlic (Allium Sativum) (21-23), tomatoes (Lycopersicum esculentum)(24, 25) and various herbs such as sweet basil (Ocimum Basilicum)(8) or other herbs (26). The practical consequences of these effects on human health is not ascertained, though it has been proposed they may be involved in the low cardiovascular disease rates associated with the use of the cretan or mediterranean diet, which is rich in herbs, garlic, onions and tomatoes (27), and of course red wine. Concerning Achillea santolina, it is regularly used in Iraqi medicine for its anti-inflammatory(1) and other properties. The anti-aggregant properties we found are consistent with the inflammatory ascribed to the plant and its constituent compounds(1).The anti-aggregant property of the plant could be attributed to flavones, particularly flavonoids and sesquiterpene lactone (2, 3, 5, 28-31) that are present in this plant. Several studies have shown that flavonoids significantly inhibit platelet adhesion, aggregation, and secretion (32). Chocolate drinks, rich in flavonoids, also inhibit the activity of platelets (33, 34). Therefore, we can suggest that the antiplatelet effect obtained here may be due in part to polyphenolic compounds. It is not excluded that other classes of fractions would be also
implied, such as the phenylpropanoid found in many essential oils from plants with antiplatelet activity(35). However, the contrast between clear activity in vitro, and a more diffuse effect in vivo with less differences between the fractions than in vitro suggests that bioavailability may not be optimal, or that significant metabolism occurs.(36-38) Phytochemical analyses are needed to characterize the active fractions that are responsible for the anti-aggregant effect, and determine their mechanism of action.

**CONCLUSION:**
We found evidence of an antiplatelet effect of achillea santolina extracts and some fractions in vitro, but the modesty of their effects in vivo raises questions as to their clinical efficacy.

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**Figure 1:** Effect of increasing concentrations of Achillea santolina crude extract and fractions on collagen (2μmol)-induced aggregation of human platelets (mean inhibition of control aggregation ± SD).
Figure 2: Effect of increasing concentrations of A. Santolina fractions and the whole extract on ADP-induced human platelet aggregation in vitro, (% inhibition of control ± SD).
Figure 3: Effects of A. Santolina 10 mg/kg/day on thrombus weight in arteriovenous shunt model in rats (mean (SD)). Animals were orally administered vehicle (Control), crude extract, F1, F2, F3, or F4 fractions for 10 consecutive days. Bars marked with the same letter are not significantly different (p<0.05)

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تأتي مستخلصات الأشيليا سانتولينا على تجمع الصفائح الدموية خارج الجسم والتخثر الدموي داخل جسم الفئران

*د.نجم عباس جابر العوادي

الخلاصة
الأشيليا سانتولينا نبات ينمو في العراق، استخدم في طب الأعشاب منذ الآلاف السنين. ويمتلك التأثير المضاد للالتهابات، ولكن فعالية المضادة لتجمع الصفائح الدموية أو التخثر الدموي غير مدوية لحد الآن.

طريقة العمل:
تم تضييق مستخلصاتها باستعمال الكلورو فوام كمستخلص (1) والاستير ثنائي الأيثر كمستخلص (2) واستناداً إلى التأثير المضاد ضد الاستير (1) واستناداً إلى التأثير ضد الاستير (2) على التآكل، هذا التأثير كان واضح جداً باستخدام المستخلص (1) الاستير ثنائي الأيثر. اما المستخلصين (1) و(3) ظهرت تأثير تنازلي نصف تأثير المستخلص (2) في حين ظهر المستخلص (4) كفاءة واضحة ونقيضية معنوية (P<0.05)

نتائج:
أوضح النتائج أن المستخلص الأخام لأوراق الأسيليا سانتولينا ثبتت وبفارق معنوي واضح تجمعات الصفائح الدموية المحترقة بواسطة الإدينوسين ثنائي الفوسفات والكولاجين. ومع ذلك، وجدنا هذا التأثير كان واضح جداً باستخدام المستخلص (1) الاستير ثنائي الأيثر. كما أوضح النتائج أن اعتناء الأسيليا سانتولينا بجرعة (10 ملغم/كغم/يوم) عن طريق الفم، الخثرات الدموية المتكونة داخل جسم الفئران بفرق معنوي واضح (P<0.05).

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