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In vitro immunomodulatory activity of plants used by the Tacana ethnic group in Bolivia

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Abstract

One hundred and seventy-eight ethanolic plant extracts from the pharmacopoeia of the Tacana, an ethnic group from Bolivia, were screened for immunomodulatory activity using complement cascade inhibition and ADP-induced platelet aggregation inhibition assays. Six impaired both complement pathways (classical and alternative): stem bark from *Astronium urundeuvea* (Anacardiaceae), *Cochlospermum vitifolium* (Cochlospermaceae), *Terminalia amazonica* (Combretaceae), *Triplaris americana* (Polygonaceae), *Uncaria tomentosa* (Rubiaceae) and *Euterpe precatoria* (Arecaceae) roots. Inhibition of complement cascade was independent of essential ion complexation, and was not due to direct hemolytic activity on target red blood cells. For *A. urundeuvea*, *C. vitifolium*, and *T. amazonica*, anti-inflammatory activity relied on cyclo-oxygenase inhibition. Four of these species (*A. urundeuva*, *T. americana*, *U. tomentosa* and *E. precatoria*) are used traditionally to treat inflammatory processes.

Keywords: Immunomodulation; Anti-inflammatory activity; Human complement system; Platelet aggregation; Traditional medicine; Bolivia; Tacana

Introduction

The activation of the complement cascade plays a significant part in the initiation and amplification of inflammation processes, phagocytosis and lysis of microorganisms.

But its activation may also degenerate into excessive reactions in a variety of inflammatory or degenerative diseases. In such cases, drugs inhibiting the complement system could be valuable therapeutic agents. Some extracts of medicinal plants display anti-inflammatory activity in vitro, as a consequence of the inhibition of the complement pathways (Halkes et al., 1997; Sharma et al., 1996). This is the reason we studied possible interference with the complement cascade by plants from the pharmacopoeia of Tacana Indians.

The Tacana dwell in amazonian Bolivia (Iturralde Province, La Paz Department) at the base of the last foothills of the eastern Andes. Tacana people still maintain a great knowledge related to heir environment. In previous studies, Bourdy (1999); Bourdy et al. (2000) and Dewalt et al. (1999) noted the high number of species still in use by the Tacana, of which more than 32% are used for medicinal purposes. To carry out the investigation, 178 plant parts were collected, botanically identified, and dried, and ethanolic extracts were prepared in order to determine their activity in the two complement pathways.

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The complement is a complex enzymatic system that can be activated mainly through IgM and IgG antibodies in the classical pathway (CPW) or by direct contact in the alternative pathway (APW), resulting in the formation of a lytic complex. During screening procedures, the detection of the complement activity, based on its hemolytic properties on specially prepared red blood cells, is highlighted by the released hemoglobin detected by spectrophotometric measurement at 405 or 542 nm (Huang et al., 1998; Benencia and Coulombié, 1998).

In order to complete our research, a second test, aimed at indirectly detecting the anti-inflammatory activity, was performed on extracts impairing the complement cascade. This test, based on ADP-induced platelet aggregation inhibition, is performed using Giemsa-stained smears of suspension of human platelets (Yun-Choi et al., 1985). The inhibition of ADP-induced platelet aggregation is linked with the inhibition of the cyclo-oxygenase pathway also involved in the inflammatory mechanism, through the formation of arachidonic acid and thromboxane A2. Other mechanisms can, of course, cause platelet aggregation inhibitions, which are not related by any means to inflammation.

Materials and methods

Methodologies employed for the ethnobotanical–ethnopharmacological survey and the vegetal samples treatment have been described previously (Bourdy, 1999; Bourdy et al., 2000; Muñoz et al., 2000). Briefly, voucher specimens were deposited in the Herbario Nacional de Bolivia in La Paz (voucher numbers and plant part tested are listed in Table 1). The ethnobotanists of the team, with the help of specialists from the herbarium, performed a preliminary identification of the specimens. Duplicate specimens were sent to specialists on the plant families of interest.

All parts of the plant (20–25 g) selected for biological study were dried, ground and submitted to a maceration process using ethanol-water (70–30%) for 48 h at 25°C, protected from sunlight. The aqueous-ethanolic solution obtained was evaporated in vacuo and the residue was directly assayed.

We did retain alcohol as solvent, because it is a good all-purpose solvent for preliminary extraction in a standardized screening program. Methyl or ethyl alcohol in particular has the ability to extract a broad spectrum of chemical substances. Nevertheless, water

Table 1. Results: Inhibition activity against the human complement system (classical (CPW) and alternative (APW) pathways) and anti-ADP-induced aggregation score of six ethanolic plant extracts

Voucher number	Scientific name (Family) Part of plant tested	CPW IC_{50} (μg/ml)	APW IC_{50} (μg/ml)	Score ADP 5 mg/ml
GB1559	*Astronium urundeuvea* (Allem.) Engl. (Anacardiaceae) Bark	64	111	1
GB1481	*Cochlospermum vitifolium* Willd. Spreng. (Cochlospermaceae) Bark	104	135	1
SD325	*Euterpe precatoria* C. Martius (Arecaceae) Roots	105	147	2
SD78	*Terminalia amazonica* (J. F. Gmelin) Exell. (Combretaceae) Bark	83	110	3
SD124	*Triplaris americana* L. (Polygonaceae) Bark	74	89	1
SD112	*Uncaria tomentosa* (Willdenow ex Roemer & Schultes) DC. (Rubiaceae) Bark	124	151	3
	Heparin	74	558	
	Aspirin (1 mg/ml)			1.5

extraction is generally the preferred form for the traditional preparation of remedies. Because of the difficulties encountered in developing a suitable work-up procedure with aqueous extracts, we avoided the use of water, but the traditional mode of preparation of remedies, as recorded from the Tacana, is also described herein.

Hemolytic assay for human complement activity

The stock solution of CPW buffer is a 5× concentrated veronal saline buffer (VBS, pH 7.3) containing 0.9 M NaCl, 10 mM diethylbarbituric acid, 9 mM sodium barbiturate, 250 mM Ca^{2+} and 250 mM Mg^{2+}. The stock solution of APW buffer has the same composition as the CPW buffer, without Ca^{2+} and with 8 mM ethylenglycol-bis (2-aminoethyl) tetra-acetic acid. Human pooled serum (HPS) was used as complement source.

In the CPW assay, sheep erythrocytes (SSRBC) were sensitized by incubating 4×10^8 cells/ml with an equal volume of VBS-EDTA (13 mM) containing a 1:2000 dilution of rabbit anti-sheep serum (BioMerieux France, ref. 72202) for 20 min at 37°C and for 20 min at 4°C. The excess of antibodies was removed by subsequent washings of the sensitized SSRBC with phosphate-buffered saline. In the APW assay, 1% of uncoated washed rabbit erythrocytes (RRBC) were diluted in APW buffer.

Ethanolic plant extracts were dissolved in a CPW-ethanol (1%) or APW-ethanol (1%) solution. The initial crude plant extract concentration was 5 mg/ml in respective buffers. Plant extracts were further vortexed, sonicated (for maximum 5 min) and heated (45°C), until complete dissolution.

The complement assay was based on the technique described by Demey et al. (1993), and modified as follows. All vegetal samples were diluted in a F-96 well microtiter plate (seven consecutive logarithmic dilutions, from 250 to 3.9 μg/ml with the appropriate CPW or APW buffer). The final volume in each well was 100 μl. Fifty μl of CPW-HPS (95:5) or 30 μl of APW-HPS (75:25) solution were then added to each well. After a preincubation of 30 min at 37°C, 50 μl of a 1% SSRBC-CPW or RRBC-APW suspension were added. All plates were furthermore incubated at 37°C for 60 min, slowly shaken and read at 690 nm with a micro-ELISA reader (Titertek Multiskan MCC/340).

As a positive control for both CPW and APW, a serial dilution (from 1000 to 3.9 μg/ml) of heparin, a sulfated polysaccharide with known anti-complement activity, was used (Klerx et al., 1985; Benencia et al., 1996). The buffer without plant extract served as a negative control. No influence of a 1% ethanol solution could be detected.

Because Ca^{2+} and Mg^{2+} chelating can impair the complement cascade, the ratio between the results obtained under standard conditions and the results obtained by increasing 4 times the concentrations of Mg^{2+} (for APW) and Ca^{2+} and Mg^{2+} (for CPW) was calculated for each positive extract (Simons et al., 1990).

All extracts were also subjected to a preincubation with the target cells, in order to detect a spontaneous lysis of erythrocytes.

IC_{50} values were calculated with the help of Excel 98 software tendance function.

Blood platelet aggregation assay

Nine volumes of blood were mixed gently with one volume of trisodium citrate (3.8% g/v). The samples were centrifuged (200*g* for 10 min) and platelet rich plasma (PRP) was collected. Platelet poor plasma (PPP) was prepared after further centrifugation of blood samples at 1000*g* for 10 min. PRP was then diluted with PPP up to a final concentration of 250.000 thrombocytes/μl. Platelets were used within 3 h. Each test was repeated in triplicate with two different samples of PRP, prepared on different days. A modified smear method, as described by Yun-Choi et al. (1985), was used for platelet aggregation screening.

Ethanolic extracts of plants were dissolved in an 8% saline–ethanolic solution, up to a concentration of 25 mg/ml. This previous dilution was then vortexed, sonicated (5 min), heated (45°C), diluted in physiological saline solution up to a final concentration of 5 mg/ml and centrifuged (20 s at 12000*g*). The final concentration of ethanol during the test did not exceed 1%.

A stock solution (2×10^{-4} mol/l) of ADP (adenosine-5-diphosphate-dicyclohexylammonium salt from the Sigma platelet aggregation screening test kit) was used as aggregation inductor. ADP, a mediator of aggregation released by the first stimulated platelets, helps to recruit other platelets and stabilizes the formation of the aggregate. Acetylsalicylic acid (used as positive control agent at 1 mg/ml saline) inhibits the platelet function by acetylation of the platelet cyclo-oxygenase. This prevents the access of the arachidonic acid to the catalytic site of the enzyme and results in an irreversible inhibition of platelet-dependent thromboxane formation (Schror, 1997; Samama and Elalamy, 2000).

The assay was performed as follows: in each well of a 96-well U-form microtiter plate, 160 μl of PRP were added to 20 μl of saline solution (negative control) or extract of plant or positive control, and preincubated for 2 min. Additionally, 20 μl of ADP were added. The plate was submitted to vortex for 10 s and incubated for 2 min at room temperature. Smears were immediately prepared and stained with Giemsa. The degree of

aggregation was determined using a light microscope and classified as follows: 0: complete inhibition of platelet aggregation; 0.5: slight aggregation of the platelets; 1: less aggregation than positive control; 2 and 3: equal or more aggregation than the positive control. We considered an extract active in this test when the score was <1.5, the mean score obtained with aspirin.

Results and discussion

Heparin was more efficient on CPW (IC_{50} value = 74 μg/ml) than on APW (IC_{50} value = 558 μg/ml) as described previously (Benencia and Coulombié, 1998). None of the selected plant extracts presented chelating effects or spontaneous lysis activity when incubated with the target cells. Thus, the mode of action of these extracts is based on true interference with the complement cascade, rather than on direct interaction with target cells or essential bivalent cation chelation. Of the 178 extracts tested, only six (*Astronium urundeuvea*, *Cochlospermum vitifolium*, *Terminalia amazonica*, *Triplaris americana*, *Uncaria tomentosa* stem barks and *Euterpe praecatoria* roots) were able to impair both complement pathways. Moreover, *A. urundeuvea*, *C. vitifolium* and *T. Americana* stem barks also displayed activity in the ADP inhibition test (Table 1), thus suggesting a possible anti-inflammatory activity related to the cyclo-oxygenase inhibition.

Astronium urundeuvea (Allem.) Engl. (Anacardiaceae)

The ethanolic stem bark extract of *A. urundeuvea* displayed the highest inhibitory activity in CPW with an IC_{50} value of 64 μg/ml, equivalent to heparin. It also showed inhibition of the APW (IC_{50} value = 111 μg/ml). In the ADP aggregation test, this extract also displayed very good activity, even stronger than that of the positive control, aspirin.

A. urundeuvea is a tree common to the eastern part of Bolivia, where it has a great reputation for efficacy, and is consequently widely used. *A. urundeuvea* stem bark is used mainly for healing in cases of wounds, broken limbs, inflamed sores and gastric ulcers.

Among the Tacana, the trunk bark is prepared in the form of a thick gelatinous substance, by boiling it in water for a long time. This jelly is applied directly on the affected area and, in the case of broken limbs, is spread generously over a piece of cloth, which is kept fixed. Also, pieces of trunk bark are prepared as a light tea, which is drunk regularly, in order to cure gastric ulcers. Finally, in the same group, a strong concentrated decoction of trunk bark absorbed on a piece of cotton wool introduced in the vagina is used as an emergency remedy to stop uterine hemorrhages, or metrorragias.

Among the Isoceño-guarani people, another ethnic group of south-east Bolivia, the "jelly" obtained from the boiled trunk bark is also used as an ointment, which is heated over live charcoal, with the resulting smoke being breathed in order to stop nasal hemorrhages. The trunk bark also is grated to a fine powder and mixed with some animal grease to help healing and reduce inflammation of wounds (Bourdy, 1999).

Similar uses have been reported in Brazil where a decoction of dried bark is used to cure cervicitis and gastric ulcers (Menezes et al., 1986). The same study demonstrated that 250 mg/kg body wt. of an ethanolic extract of dried bark administered orally is able to prevent histamine-induced ulcer in Guinea pigs and rats.

Therefore, the effective anti-inflammatory effect of *A. urundeuvea* bark seems to be well established, corroborating the traditional use of this plant. The traditional anti-hemorragic use of *A. urundeuvea* bark, which at first glance seems to conflict with its positive results in the APD inhibition test, can be explained by its strong positive action in the collagen aggregation pathway, at the onset of the aggregation process.

Cochlospermum vitifolium (Willd.) Spreng. (Cochlospermaceae)

The ethanolic bark extract of this species was two times less active than heparin in APW (IC_{50} value = 135 μg/ml), has an IC_{50} value of 104 μg/ml in the CPW pathway, and was more active than aspirin in the ADP aggregation test. The Tacana do not use this plant for any therapeutic purposes, but in many countries of Central and South America, the orally administered aqueous bark extract of *C. vitifolium* is used traditionally to treat liver (including hepatitis) and kidney ailments and also as a wash for ulcers (Esposito-Avella et al., 1985; Zamora-Martinez and Pola, 1992).

As far as we know, no specific hepato-protective activity has been reported in *C. vitifolium* bark directly corroborating its uses in liver disorders. But the anti-inflammatory-immunomodulatory properties presently highlighted suggest that the use of this bark could induce a significant reduction in any inflammatory processes concomitant with the pathologies described, hence reducing pain.

Euterpe precatoria C. Martius (Arecaceae)

The ethanolic root extract of *Euterpe precatoria* showed activity in APW (IC_{50} value = 147 μg/ml) and in CPW (IC_{50} value = 105 μg/ml) but was inactive in the ADP aggregation test. This palm tree (besides being widely known for its palatable "heart" and the delicious

drink made of its fruits) has medicinal roots, used almost everywhere it grows. Among the Tacana, roots are administered in the form of a decoction or syrup to alleviate strong muscular back or sciatic pains or liver pain, and also as a general tonic for weak people prone to illness (Bourdy, 1999). Also, in the Amazonian part of Ecuador, roots are used to calm muscular pain (Lescure et al., 1987). In Iquitos (Peru), root decoction is used to treat kidney and liver disease and is famous for being effective in all healing and skin ulceration problems (Vasquez, 1992). The preparation has been proved useful to decrease edema (Van den Berg, 1987, 1988).

Aqueous and methanolic extracts of the dried root of *E. praecatoria* collected in Bolivia were shown, via quenching of luminol-enhanced chemiluminescence, to have anti-oxidant activities (Desmarchelier et al., 1997). This is another biological effect, additive to the inhibition of the complement cascade in the anti-inflammatory process, as reactive oxygen species also contribute to aggravate tissue injuries observed in inflammatory disorders. Therefore, the traditional anti-inflammatory activity reported for *E. praecatoria* roots seems to be verified, although the cyclo-oxygenase metabolic pathway does not seem to be involved. Still, the mechanism of action and the active principles require elucidation.

Terminalia amazonica (J.F. Gmelin) Exell. (Combretaceae)

T. amazonica ethanolic bark extract displayed anti-complement activity in APW (IC_{50} value = 110 μg/ml U/ml) and in CPW (IC_{50} value = 83 μg/ml) and the extract enhanced ADP-induced aggregation. This species has no medicinal uses among the Tacana. We also found no literature references to ethnopharmacological, biological or chemical data on the possible anti-inflammatory activity of this species. This is the first report of anti-complement activity from the species.

Because *Terminalia* barks are well known to contain a high degree of tannins (Tacana people use this species to dye funerary cloth black) we do suspect that the enhancement of activity observed in the ADP test might be due to the presence of this kind of compound. This would be in accordance with the reports of Rohrbach et al. (1984, 1990) and Cloutier and Rohrbach (1989), who showed that tannins isolated from aqueous extracts of cotton bracts promoted platelet aggregation dose- and molecular weight-dependently.

Triplaris americana L. (Polygonaceae)

The ethanolic bark extract of *T. americana* showed the highest inhibitory activity (89μg/ml) in the APW (CPW, IC_{50} value = 74 μg/ml). Good activity was also observed in the ADP test. *T. americana*, a common species from the Bolivian lowlands, is used as a "cure-all" in Peru (Desmarchelier et al., 1997). More specifically, in Bolivia, the decoction of the bark is given to parturifacient women, in case of metrorragias, against diarrhea and stomach ache, and to cure intestinal worms. The powdered bark is also applied as a poultice for skin lesions induced by leishmaniasis. Although the Tacana recognize that this treatment helps reduce inflammation of the lesion, they also understand that it is not usually sufficient to completely cure the "ulcer" (Bourdy, 1999). This observation suggests such activity relies on the anti-inflammatory effect of this species, a hypothesis corroborated by the total lack of activity of the very same extract against *Leishmania* (data not shown). Also, *T. americana* bark methanolic extract showed anti-oxidant activity as measured by quenching of luminol-enhanced chemiluminescence (Desmarchelier et al., 1997); therefore, the same conclusion as for *E. praecatoria* can be made.

Uncaria tomentosa (Willdenow ex Roemer & Schultes) DC. (Rubiaceae)

The ethanolic bark extract of *U. tomentosa* displayed anti-complement activity in APW (IC_{50} value = 151 μg/ml) and CPW (IC_{50} value = 124 μg/ml), but was less active than the other extracts.

U. tomentosa, so-called "Uña de gato", is perhaps one of South America's most investigated species, and the list of traditional uses is almost endless. Most reported indications are concomitant with inflammatory processes, i.e.: gastritis, dermic and uro-genital inflammations, asthma, rheumatic pains, etc. This wine is also claimed to be a tonic for convalescent people (Obregon Vilches, 1996). Among the Tacana, a strongly concentrated bark decoction is administered against rheumatism, irregular menstruation, and in case of digestive, liver and kidney troubles (Bourdy, 1999).

Numerous scientific studies showed this plant to have anti-viral, anti-inflammatory, immunostimulating, anti-mutagenic and anti-oxidant properties (Obregon Vilches, 1996). Therefore, the anti-complement activity displayed through our test is not surprising, but the anti-inflammatory activity of *U. tomentosa* seems unrelated to the cyclo-oxygenase pathway.

Conclusion

The activation of the complement cascade, generally leading to an inflammatory process is normal and beneficial for the organism, as a defensive reaction against pathogens. But under certain circumstances,

however, depending on the site of action and the immunological background of the patient, a prolonged activation may contribute to an increase or set permanently an inflammatory process, leading to pathological conditions such as asthma, allergy, dermatitis, arthritis, auto-immune and degenerative diseases. Control over complement cascade can therefore play an important role in anti-inflammatory therapy: several reports have made it clear that extracts from medicinal plants display anti-inflammatory activity in vitro and/or in vivo as a consequence of their high inhibitory capacity against human and murine hemolytic complement (Benencia et al., 1994, 1995, 1996; Labadie et al., 1989). In this paper, we have demonstrated that six Amazonian plant extracts displayed anti-complement properties, making these extracts valuable for further anti-inflammatory/immunomodulation research.

Four of these species (*A. urundeuva*, *T. americana*, *U. tomentosa* and *E. precatoria)* are well-known medicinal plants used in diseases associated with inflammatory processes. Moreover, three of these extracts also displayed strong ADP inhibition activity, indicating that the traditional anti-inflammatory activity claimed is probably not only due to the inhibition of the complement cascade, but also to cyclo-oxygenase pathway inactivation.

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