Anti-inflammatory and antioxidant activity of a medicinal tincture from *Pedilanthus tithymaloides*

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Received 5 April 2005; accepted 18 July 2005

Abstract

*Pedilanthus tithymaloides* (L.) Poit. (Euphorbiaceae) is a low tropical American shrub with a reported wide range of healing properties such as emetic, anti-inflammatory, antibiotic, antiseptic, antihemorrhagic, antiviral, antitumoral, and abortive. In the present study, a tincture from *P. tithymaloides* collected in Cuba was evaluated for its in vivo anti-inflammatory activity, using the rat paw oedema assay, and for its in vitro scavenging effects on reactive oxygen species (ROS) (HO·, O2·, ROO· and H2O2), reactive nitrogen species (RNS) (ONOO− and NO), and DPPH radical. The protein, free amino acid, and phenolic contents of the tincture were also determined. Pertaining to the anti-inflammatory activity, the intraperitoneal administration of the tincture inhibited carrageenan-induced rat paw oedema, whereas in the scavenging assays the tincture showed to be effective against all the assayed ROS and RNS, specially for HO· (IC50 = 345 ± 7 μg/mL), O2· (IC50 = 143 ± 7 μg/mL), HOCl (IC50 = 113 ± 20 μg/mL), ONOO− (IC50 = 44 ± 3 μg/mL), and NO (IC50 = 54 ± 4 μg/mL), but displayed weak activity in the DPPH assay. The protein content of the tincture was 0.70%, and twenty free amino acids were identified and quantified. The content of total phenolics was 17.4 ± 0.15 mg of gallic acid equivalents (GAE)/g dry material. These results provide scientific support for the empirical use of *P. tithymaloides* tincture as an anti-inflammatory medicine.

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Keywords: *Pedilanthus tithymaloides*; Anti-inflammatory activity; Antioxidant activity; Reactive oxygen species; Reactive nitrogen species; Phenolic content; Amino acids content; Protein content

Introduction

*Pedilanthus tithymaloides* (L.) Poit. (Euphorbiaceae) is a low tropical American shrub with a reported wide range of healing properties, namely emetic, anti-inflammatory, antibiotic, antiseptic, antihemorrhagic, antiviral, antitumoral, and abortive (Roig, 1974; Correa, 1984; Cáceres et al., 1995; Renne, 1996). In the search for the bioactive principles, a new cancer cell growth inhibitor designated pedilastain was isolated from a Republic of Maldives *Pedilanthus* sp., possibly *tithymaloides* (Pettit et al., 2002), whereas the discovery of an antiprotozoa compound from plant material of Brazilian origin has been reported, although its structure remains unknown (Luize et al., 2003). A new proteolytic enzyme with oral anti-inflammatory activity, designated pedilanthain (Dutta and Dhar, 1984; Dhar et al., 1988), and a galatose-specific lectin possessing mitogenic activity with murine spleen lymphocytes (Seshagirirao, 1995), were isolated from the latex. The lectin was also assayed in biomedical research, to study the hemagglutination pattern in patients with diabetes mellitus (Nagda and Deshmukh, 1998), and tuberculosis (Ankush et al., 2003). Peroxidases from leaf tissues and calli were extracted, and characterized in terms of their specific activities, isoenzyme properties and physiochemical properties (Bricage, 1982, 1984). The isolation of known terpenoids and long-chain alcohols was reported from *P. tithymaloides* collected in India (Misra and Khastgir, 1969; Upadhyay and Hecker, 1974; Mukherjee et al., 1989, 1992; Mohamed et al., 1996).

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0024-3205/$ - see front matter © 2005 Elsevier Inc. All rights reserved. doi:10.1016/j.lfs.2005.07.037
In Cuba, this species, known as Itamorreal, Gallito, and Zapatitos, is traditionally used as a tincture in the treatment of stomatological infections, as stomatitis and gingivitis, but so far, its empirical use was not yet supported by scientific studies.

It is well known that periodontal invading microorganisms are a major cause in the development of gingivitis and stomatitis (Moran and Annuk, 2003). Among the host’s responses against these pathogens, neutrophils (polymorphonuclear leukocytes; PMNs) serve as the initial defense. During this response, PMNs and other phagocytic cells are mobilized to sites of injury or infection where they ingest and kill invading microorganisms (Scully and Langley-Evans, 2002; Swain et al., 2002). The combination of bacterial phagocytosis, and secretion of proteolytic enzymes and immuno-modulatory compounds that assist in the killing and digestion of bacteria, is accompanied by a “respiratory burst”—the sudden increase in non-mitochondrial oxidative metabolism, producing the superoxide radical (O$_2^-$) catalysed by NADPH oxidase and a battery of other reactive oxygen species (ROS), namely hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO$^-$), peroxy radical (ROO$^-$), and hypohalous acid (HOCl) (Barnes, 1990; Hampton et al., 1998; McCord, 2000). Reactive nitrogen species (RNS), like nitric oxide (NO) and peroxynitrite (ONOO$^-$) are also produced during inflammatory processes. NO is produced by a family of isoenzymes, named nitric oxide synthases (NOS), which may be induced during inflammation (Cuzzocrea et al., 2001). Possible pro-inflammatory effects of NO include augmentation of vascular permeability of inflamed tissues, the generation of other destructive reactive species (namely ONOO$^-$ by reaction with O$_2^-$), the induction of cycloxygenase as well as of angiogenic and inflammatory cytokines (Miyasaka and Hirata, 1997). Unfortunately, during the course of this upregulated neutrophil activity, overproduction of ROS and RNS may cause excessive and indiscriminate “collateral” host-tissue damage, contributing this way for the aggravation of the inflammatory process (Ritchie and Kinane, 2003).

Taking into account the above mentioned rationale, besides the importance of a direct anti-inflammatory effect, a putative scavenging activity against these reactive species may be of great importance in the treatment of stomatological affections by Pedilanthus tithymaloides tincture. Thus the aim of the present study was to evaluate the in vivo anti-inflammatory effect of a P. tithymaloides tincture, as well as its in vitro scavenging effects on ROS (HO$^-$, O$_2^-$, HOCl, ROO$^-$ and H$_2$O$_2$), RNS (ONOO$^-$ and NO), and DPPH radical. Furthermore, investigation about the presence of bioactive components, such as proteins and phenolic compounds was also pursued.

**Materials and methods**

**Plant material**

Plant material was collected in February 2002, in the region of II Frente Frank País at Santiago de Cuba, and identified at the herbarium of Museum Tomás Romay (Centro Oriental de Ecosistemas y Biodiversidad), Santiago de Cuba, where the voucher specimen no. 2726 has been deposited.

**Chemicals**

All the chemicals and reagents were of analytical grade. α,α-Diphenyl-β-picrylhydrazyl (DPPH), α-tocopherol, amino acid standards, phenyl isothiocyanate, carrageenan, indomethacin, Bradford dye reagent, bovine serum albumin standard, Folin–Ciocalteu phenol reagent, gallic acid, ninyhydrin, α-naphtol, phosphomolybdate acid, dihydrodorhadamine 123 (DHR 123), 4,5-diaminofluorescein (DAF-2), 30% hydrogen peroxide, β-nicotinamide adenine dinucleotide (NADH), phe- nazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), ethylenediaminetetraacetic acid (EDTA) disodium salt, ascorbic acid, sodium hypochlorite solution with 4% available chlorine (NaOCl), lipoic acid, diethyltriaminepentaacetic acid (DTPA), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), rutin, lucigenin, luminol, deferoxamine, and ebseben were purchased from Sigma-Aldrich (St. Louis, USA). α,α’-Azodisobutyramidine dihydrochloride (AAPH) and trolox were obtained from Fluka Chemie GmbH (Stein- heim, Germany). Fluorescein sodium salt was obtained from Aldrich (Milwaukee, USA). Reversed phase silica gel for flash chromatography, RP-18, cellulose, and NH$_2$ TLC plates were purchased from Merck (Darmstadt, Germany), ion-exchange resin Amberlite IR-120 from Sigma (St. Louis, USA), and normal phase silica gel TLC plates from Macherey-Nagel (Düren, Germany).

**Animals**

Male Sprague Dawley rats, 150–250 g body weight (LABEX, Camaguey, Cuba) were used for the anti-inflammato- ry study, after acclimatization for 15 days to the laboratory conditions (Laboratorio de Anticuerpos y Biomodelos Experimentales, Santiago de Cuba), and fed with ratonin (CENPA- LAB) and water, ad libitum, according to veterinary standard procedures.

**Tincture preparation**

Air dried stems and leaves (20 g) of P. tithymaloides were powdered with a blender and macerated with 30% EtOH. The obtained suspension was decanted, washed with the extraction solvent, filtered, and the final volume gauged to 100 mL to obtain a tincture of pH 7.8, refraction index 1.35, density 0.98, and total solids 2.2% w/v.

**Chromatographic fractionation of the tincture**

A portion (1 g) of the dried tincture of P. tithymaloides was submitted to RP-18 flash chromatography using H$_2$O (100 mL), H$_2$O/MeOH (1:1, 100 mL), MeOH (150 mL), and MeOH/CH$_2$Cl$_2$ (1:1, 100 mL), as eluents. The resulting
fractions were analyzed for their composition on normal- and reversed-phase silica TLC, and cellulose supports, using ninhydrin, α-naphthol, H₂SO₄, and phosphomolybdic acid as spray reagents.

**Rat paw oedema assay**

A total of 54 rats were randomly distributed into 9 groups (I–IX) of 6 animals. To groups I–III the tincture (500 mg/kg), indomethacin (10 mg/kg) as positive control, and 30% EtOH as negative control were administered, respectively. Groups IV–VI received a tincture dose of 750 mg/kg, indomethacin (10 mg/kg), and 30% EtOH, respectively, and groups VII–IX received a tincture dose of 1000 mg/kg, indomethacin (10 mg/kg), and 30% EtOH, respectively.

Paw inflammation was induced 30 min later by injecting 0.1 mL of 1% (w/v) carrageenan in 0.9% NaCl into the plantar surface of the right hind-paw. Paw volumes were measured using a plethysmometer, 4 h after carrageenan injection. The anti-inflammatory activity is expressed as the average percent inhibition of oedema in each group, which is calculated according to the general formula: % inhibition = 100 – (100 × Vᵢ/Vₑ), where Vᵢ and Vₑ represent the increase in paw volumes of rats treated with drug and control, respectively. Statistic treatment of data was performed using STAT Program (Faculty of Biology, University of La Habana), ANOVA-1 one-way analysis of variance, and Duncan test for multiple comparison.

**Assessment of scavenging activity against ROS and RNS**

For assessment of scavenging activity against ROS and RNS, the tincture (100 mL) was evaporated at room temperature under nitrogen flow, to yield 3.9 g of a solid residue. A portion of this solid was dissolved in the buffer solution applied in each assay in order to achieve concentration values up to 10 mg of dried solid/mL. All determinations were performed in a microplate reader (Synergy HT, BIO-TEK), using spectrophotometric, fluorimetric or chemiluminescence detection. Each study corresponds to four experiments, performed in triplicate at 37 °C. The conditions applied in each assay are summarised in Table 1.

<table>
<thead>
<tr>
<th>Scavenging assay</th>
<th>Analytical signal measurement</th>
<th>Positive control</th>
<th>Detection method</th>
<th>Expression of results</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂⁻ scavenging assay</td>
<td>The O₂⁻ scavenging activity was measured by monitoring the O₂⁻-induced reduction of nitroblue tetrazolium chloride (NBT) to the blue chromogen diformazan (Fernandes et al., 2003). O₂⁻ were generated by the phenazine methosulphate (PMS)/NADH system. The reaction mixtures in the sample wells contained, in a final volume of 300 μL, the following reagents at the indicated final concentrations: NBT (43 μM), NADH (166 μM), extract at various concentrations (0–10 mg/mL) and PMS (2.7 μM). All reagents and extract were dissolved in 19 mM phosphate buffer, pH 7.4.</td>
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<tr>
<td>HO scavenging assay</td>
<td>The HO scavenging activity was measured by monitoring the HO-induced oxidation of luminol (Oosthuizen and Greyl-ing, 2001) with modifications. HO was generated by a Fenton system (FeCl₂/EDTA/H₂O₂). Reaction mixtures in the sample wells contained, in a final volume of 250 μL, the following reagents at the indicated final concentrations: 0.5 M Na₂CO₃ buffer, pH 10, luminol (20 μM), FeCl₂ – EDTA (25 μM, 100 μM), extract at various concentrations (0–2.0 mg/mL) and H₂O₂ (3.5 mM). The iron salt was premixed with the chelator dissolved in water before addition to the reaction mixture.</td>
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<tr>
<td>H₂O₂ scavenging assay</td>
<td>The H₂O₂ scavenging activity was measured by monitoring the H₂O₂-induced oxidation of lucigenin, accordingly to a described procedure (Costa et al., 2005). Reaction mixtures in the sample wells contained, in a final volume of 250 μL, the following reagents at the indicated final concentrations: 50 mM Tris buffer, pH 7.4, lucigenin (3 mM), extract at various concentrations (0–10.0 mg/mL) and H₂O₂ (2%).</td>
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<tr>
<td>HOCl scavenging assay</td>
<td>The HOCl scavenging activity was measured by monitoring the HOCl-induced oxidation of luminol, accordingly to a described procedure (Yildiz et al., 1998) with modifications. HOCl was prepared immediately before use by adjusting the pH of a 1% (v/v) solution of NaOCl to 6.2 with diluted...</td>
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</table>
sulphuric acid. The concentration of HOCl was further determined spectrophotometrically at 235 nm using the molar absorption coefficient of 100 $M^{-1} \cdot cm^{-1}$ (Aruoma, 1997).

Reaction mixtures in the sample wells contained, in a final volume of 250 $\mu$L, the following reagents at the indicated final concentrations: 50 mM Na$_2$HPO$_4$ buffer, pH 12, luminol (250 $\mu$M), extract at various concentrations (0–400 $\mu$g/mL), and HOCl (25 $\mu$M).

**ROO scavenging assay**

The ROO scavenging activity was measured by monitoring the decay in fluorescence due to the oxidation of fluorescein, accordingly to a described procedure known by oxygen radical absorbance capacity (ORAC) assay (Fernandes et al., 2004). ROO was generated by thermodecomposition of $\alpha$,$\alpha'$-azodibutyramidine dihydrochloride (AAPH). Reaction mixtures in the sample wells contained, in a final volume of 200 $\mu$L, the following reagents dissolved in 75 mM potassium phosphate buffer, pH 7.4, at the indicated final concentrations: fluorescein (DAF-2) at various concentrations (0–0.01 mg/mL) and AAPH (19 mM). The scavenging effects are expressed as the relative trolox equivalent ORAC value, which is calculated by the following equation, where $AUC$ represents the area under curve:

$$\text{Relative ORAC value} = \frac{(AUC_{\text{sample}} - AUC_{\text{blank}})}{(AUC_{\text{trolox}} - AUC_{\text{blank}})} \times \left( \frac{\text{mass of trolox}}{\text{mass of tincture}} \right).$$

**NO scavenging assay**

The NO scavenging activity was measured by monitoring the NO-induced oxidation of non fluorescent 4,5-diamino-fluorescein (DAF-2) to the fluorescent triazolofluorescein, according to the described method (Nagata et al., 1999), with modifications. NO was generated by thermodecomposition of $\alpha$,$\alpha'$-azodibutyramidine dihydrochloride (AAPH). Reaction mixtures in the sample wells contained, in a final volume of 200 $\mu$L, the following reagents dissolved in 75 mM potassium phosphate buffer, pH 7.4, at the indicated final concentrations: fluorescein (DAF-2) to the fluorescent triazolofluorescein, with modifications. Briefly, each sample stock solution of 100 $\mu$L (0.06–1 mg/mL in ethanol) was added to 1.5 mL of 0.1 mM ethanolic solution of DPPH. The mixture was shaken vigorously and the absorbance was monitored at 515 nm after 45 min of incubation, when the reaction reached a steady state. Tocoferol was used as reference compound. The inhibition percentage (%) of radical scavenging activity was calculated as $(1 - A_s/A_0) \times 100$, where $A_0$ and $A_s$ are the absorbances of the control and sample, respectively.

**DPPH radical scavenging assay**

The tincture was evaporated at room temperature under nitrogen flow, and the resulting solid residue dissolved in EtOH so as to obtain a stock solution of 1 mg/mL. The scavenging activity of the tincture was measured using the stable radical DPPH, according to the method of Blois (Blois, 1958) with minor modifications. Briefly, each sample stock solution of 100 $\mu$L (0.06–1 mg/mL in ethanol) was added to 1.5 mL of 0.1 mM ethanolic solution of DPPH. The mixture was shaken vigorously and the absorbance was monitored at 515 nm after 45 min of incubation, when the reaction reached a steady state. Tocoferol was used as reference compound. The inhibition percentage (%) of radical scavenging activity was calculated as $(1 - A_s/A_0) \times 100$, where $A_0$ and $A_s$ are the absorbances of the control and sample, respectively.

**Isolation and HPLC determination of amino acids**

The tincture (100 mL) was evaporated at room temperature under nitrogen flow, to yield 3.9 g of a solid residue. A portion (1.5 g) of this residue was eluted on a reversed phase flash chromatography column, using mixtures of H$_2$O/MeCN (1:0, 400 mL; 90:10, 100 mL; 75:25, 100 mL; 50:50, 100 mL; 25:75, 100 mL; 0:1, 100 mL), to afford six fractions (F$_1$ to F$_6$). Chromatographic fractions were monitored for their composition by TLC on cellulose plates using butanol:acetic acid:water (1:1:1) as eluent. Fraction F1 (1.09 g) was mainly composed by amino acids and carbohydrates, as indicated by the revelation with ninhydrin and $\alpha$-naphthyl spray reagents. After passing through an Amberlite IR-120 ion exchange column with 10% ammonia elution, F$_1$ afforded an amino acid fraction (529 mg). Fractions containing amino acids were analyzed on silica gel TLC using MeOH:KH$_2$PO$_4$ [(0.01 M), pH 6.7], 9:1 as eluent, and ninhydrin as spray reagent. The composition of the amino acid fraction was determined by HPLC using the Pico-Tag method in a Waters Pico-Tag Aminoacid Analysis System (Hancock and Reeder, 1993). For phenyl isothiocyanate (PITC) derivatization, a stock solution containing 6.25 mM of each standard amino acid in water was used. A volume of 100 $\mu$L of this solution was dried
under vacuum, and the residue mixed with 20 µL of freshly prepared reagent (EtOH/H₂O/TEA/PITC, 7:2:1:1) for 15 min at 20–25 °C, then evaporated at constant pressure of 7 Pa to remove excess derivatizing reagent. A portion (1 mg) of the lyophilized amino acid fraction was dissolved in 500 mL of water, and purified on a RP-18 Sep-Pack column before derivatization. Norleucine was used as internal standard. Phenylthiocarbamyl (PTC) derivatives of the amino acids were derivatized. Norleucine was used as internal standard.

Determination of protein content

The Bradford reagent was used to determine the concentration of protein in solution (Bradford, 1976). As described above the tincture was evaporated at room temperature under nitrogen flow, and 20 mg of the resulting solid residue were dissolved in 1 mL of water. 0.1 mL of this solution and 3 mL of the Bradford reagent were mixed in a disposable cuvette. Samples were incubated at room temperature for 5 to 45 min after thorough mixing. The absorbance of the samples were recorded at 595 nm. Standard bovine serum albumin with concentrations (0.1–1.4 mg/mL) were used to plot a standard curve.

Determination of total phenolics

The total soluble phenolic content of the tincture was determined using the Folin–Ciocalteu colorimetric method (Singleton and Rossi, 1965; Singleton et al., 1999). 1 mg of the evaporated tincture was dissolved in 1 mL of MeOH:H₂O (1:1), and this solution (0.2 mL) was then pipetted to 1 mL of 10 times diluted Folin–Ciocalteu. After 3 min of incubation, 0.8 mL of 7.5% (w/v) sodium carbonate solution was added, vortexed and the pH adjusted to 10. For a control sample, 0.2 mL of distilled water was used instead. The absorbance was measured spectrophotometrically at 760 nm after 30 min of incubation at room temperature. A calibration curve using gallic acid as standard was established between 3 and 300 µg/mL. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams of GAE per gram of dried tincture.

Results

The administration of P. tithymaloides tincture at doses of 500, 750 and 1000 mg/kg, caused 83%, 94% and 92% inhibition of the rat paw oedema, respectively (Table 2). The tincture presented a remarkable capacity for scavenging all ROS and RNS tested as indicated by the corresponding IC₅₀ values, included in Table 3. With exception of H₂O₂, whose IC₅₀ subsists in the mg/ml range (2.5 ± 0.2 mg/mL), the IC₅₀ values obtained for all the other reactive species were found at the µg/mL range. IC₅₀ for O₂⁻, HO, HOCl and NO were 143 ± 7, 345 ± 77, 113 ± 20, and 54 ± 4 μg of dried tincture/mL, respectively. The tincture also showed a potent scavenging activity against ONOO⁻ (IC₅₀ = 44 ± 3 μg of dried tincture/mL), which was reduced in the presence of NaHCO₃ (IC₅₀ = 82 ± 12 μg of dried tincture/mL). The ORAC value obtained was 0.051 ± 0.002, indicating an effective capacity for scavenging ROO species when compared to the trolox value (1). The scavenging activity of the tincture towards DPPH at extract concentrations of 0.125, 0.250, 0.500, and 1 mg/mL, was 8.5 ± 2, 16.1 ± 5, 19.6 ± 1 and 37 ± 4%, respectively, which was indicative of weak activity against this radical.

The analysis of free amino acid content from the tincture by HPLC with PTIC precolumn derivatization, allowed the identification of aspartic acid (5.13 µM), glutamic acid (3.43 µM), hydroxyproline (0.15 µM), serine (3.52 µM), asparagine (0.72 µM), glycine (0.98 µM), glutamine (0.82 µM), histidine (0.06 µM), γ-aminobutyric acid (3.96 µM), citrulline (0.61 µM), threonine (1.91 µM), alanine (3.64 µM), arginine (0.06 µM), proline (1.87 µM), tyrosine (0.69 µM), valine (3.07 µM), isoleucine (1.68 µM), leucine (1.61 µM), phenylalanine (0.44 µM) and lysine (0.47 µM). Identical qualitative composition was found after acid hydrolysis of the tincture, without significant quantitative differences. The protein content of the tincture, as determined by the Bradford protein assay, was 0.070 ± 0.05 g/100 g of dried tincture.

<table>
<thead>
<tr>
<th>Target species</th>
<th>Extract (µg of dried tincture/mL)</th>
<th>Positive control (µg/mL)</th>
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<tbody>
<tr>
<td>O₂⁻</td>
<td>143 ± 7</td>
<td>519 ± 24 (trolox)</td>
</tr>
<tr>
<td>HO</td>
<td>345 ± 7</td>
<td>5.0 ± 0.6 (deferoxamine)</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>(2.5 ± 0.2)×10³</td>
<td>(0.18±0.01)×10³ (ascorbic acid)</td>
</tr>
<tr>
<td>HOCl</td>
<td>113 ± 20</td>
<td>1.2 ± 0.1 (lipoic acid)</td>
</tr>
<tr>
<td>NO</td>
<td>54 ± 4</td>
<td>0.32 ± 0.01 (rutilin)</td>
</tr>
<tr>
<td>ONOO⁻ (absence of NaHCO₃)</td>
<td>44 ± 3</td>
<td>0.69 ± 0.03 (ebselen)</td>
</tr>
<tr>
<td>ONOO⁻ (presence of NaHCO₃)</td>
<td>82 ± 12</td>
<td>4.4 ± 0.3 (ebselen)</td>
</tr>
</tbody>
</table>

Table 2

Anti-inflammatory effect of P. tithymaloides tincture in the rat paw oedema assay

<table>
<thead>
<tr>
<th>Sample dose</th>
<th>Increase in paw oedema volume (mm Hg) after carrageenan administration</th>
<th>% inhibition</th>
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<tbody>
<tr>
<td>Tincture, 500 mg/kg</td>
<td>4.66 ± 1.86</td>
<td>82.29</td>
</tr>
<tr>
<td>Tincture, 750 mg/kg</td>
<td>1.50 ± 0.54</td>
<td>93.85</td>
</tr>
<tr>
<td>Tincture, 1000 mg/kg</td>
<td>2.50 ± 1.22</td>
<td>91.79</td>
</tr>
<tr>
<td>Indomethacin, 10 mg/kg</td>
<td>6.16 ± 3.16</td>
<td>70.74</td>
</tr>
<tr>
<td>30% ethanol</td>
<td>29.0 ± 9.23</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3

IC₅₀ values (mean±SEM) calculated from the scavenging activity of P. tithymaloides tincture against ROS and RNS
Ciocalteu method to determine the total phenolics, it was shown that the tincture contains 17.4 ± 0.15 mg GAE/g dry material.

**Discussion**

The results observed in the rat paw oedema assay showed a significant inhibitory activity of the tincture in carrageenan-induced paw inflammation, for the administrated doses of 500, 750 and 1000 mg/kg (Table 2). This test model basically reflects the action of prostaglandins involved in the inflammation process induced by carrageenan (Guillén et al., 1997; Mujumdar and Misar, 2004). Oedema formation in paw is the result of a synergism between various inflammatory mediators that increase vascular permeability and/or mediators that increase blood flow (Ialenti et al., 1995). The development that increase vascular permeability and/or mediators that result of a synergism between various inflammatory mediators (Salvemini et al., 1996; Boughton-Smith et al., 1999).

Besides, TLC analysis of the tincture on normal and reversed phase silica, cellulose, and NH2 supports, indicated a high importance for the plant anti-inflammatory effect.

The present results showed for the first time that the tincture of *P. tithymaloides* exhibit scavenging activity against all ROS and RNS tested (Table 3). As mentioned before, patients with periodontal disease display increased PMN number and activity, as they are the predominant host immune response to oral bacterial infection (Sculley and Langley-Evans, 2003; Chapple et al., 2002; Čimen et al., 2003), the scavenging effects of the tincture on ROS (HO·, O₂⁻, HOCl, ROO· and H₂O₂), and RNS (ONOO⁻ and NO) could be considered of great importance for the plant anti-inflammatory effect.

The higher inhibition percentage was 37% at a concentration of 1 mg/mL, which is a lower value than the one observed (IC₅₀ 251 μg/mL) by Ramos et al. (2003) in a 70% hydroalcoholic stem extract of *P. tithymaloides*.

In a previous communication Dutta and Dhar (1984) reported the isolation of a protease from the latex of *P. tithymaloides*, whose oral anti-inflammatory activity was demonstrated in the rat paw oedema assay (Dhar et al., 1988). This enzyme, designated pedilanthain, was easily extracted with water followed by precipitation with cold acetone, without any pH control. It is well known that Euphorbiaceae are sources of proteases, phosphatases, lectins, and lysozymes with recognised biological properties (Lynn and Clevette-Radford, 1984a,b, 1985a,b, 1986, 1987; Harley and Beevers, 1985; Vattuone et al., 1991; Abdullaev and de Meija, 1997; Wititsuwannakul et al., 1998; O’Keefe, 2001), including scavenging activities on DPPH radical, hydroxyl radical, and superoxide radical, as it is the case for a glycoprotein isolated from *Solanum nigrum* (Heo et al., 2004). The anti-inflammatory activity of several plant proteases is also recognized (Rogers and Ransom, 1976). So, we could not discard the presence of proteins in the 30% hydroalcoholic extract of stem and leaves of *P. tithymaloides*, which also contains latex. Besides, TLC analysis of the tincture on normal and reversed phase silica, cellulose, and NH2 supports, indicated a high content of polar water soluble metabolites, as amino acids and carbohydrates, which are constituents of plant lectins. Following reversed-phase fractionation of the tincture, these two classes of compounds were concentrated in the aqueous fraction, whereas the H₂O/MeOH and MeOH fractions revealed complex mixtures of phenolic compounds, as indicated by TLC, UV, and NMR analysis. In what concern amino acids, peptides, and carbohydrates it is also known that these can be responsible for anti-inflammatory and antioxidative properties in plant extracts (Darshan and Doreswamy, 2004; Saito et al., 2003; Hattori et al., 1998; Shahidi and Amaroawicz, 1996; Chen et al., 1995; Kawashima et al., 1979; Yamaguchi, 1971). Nevertheless, this is not the case for the amino acids we have identified in the tincture, which include hydroxyproline, γ-aminobutyric acid, and citrulline, three nonprotein amino acids usually found in plant seeds (Harborne, 1988; Kuo et al., 2003). The fact that the qualitative and quantitative composition of the tincture was identical before and after mild acid hydrolysis, indicates the absence of peptides. The low protein content (0.70%) we found in the tincture, does not support neither proteins as responsible for the observed anti-inflammatory and antioxidant effects. Although a moderate content of total phenolics has been determined in the tincture (17.4 mg GAE/g dry material), we cannot exclude that the scavenging activity could result from their presence, namely, on the basis of a synergistic effect with other metabolites (Saito et al., 2003; Bishov and Henick, 1975).

The results of the present investigation suggest that the anti-inflammatory activity of the tincture of *P. tithymaloides* used in Cuban traditional medicine, and recognized by the Ministry of Health (MINSAP), could be explained, at least in part, by their antioxidative properties. The bio guided isolation of the active constituents of *P. tithymaloides* is being conducted, aiming the formulation of a safer and efficient drug.
Acknowledgements

The authors acknowledge REQUIMTE for financial support for this work, and Fundação para a Ciência e Tecnologia and Fundo Social Europeu (III Quadro Comunitário de Apoio) for post-doc (S. Matthew, SFRH/BPD/8570/2002) and Ph.D. grants (D. Costa, SFRH/BD/10483/2002). This work was also supported by the Programme Alþan, the European Union Programme of High Level Scholarships for Latin America, scholarship (No. E0D033475CU).

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