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# Free radical scavenging activities and inhibition of inflammatory enzymes of phenolics isolated from *Tripodanthus acutifolius*

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ABSTRACT

*Ethnopharmacological relevance:* Leaf extracts from *Tripodanthus acutifolius* (Ruiz and Pavón) Van Tieghem have long been used in argentinean traditional medicine as anti-inflammatory, however, there is no scientific evidence which supports this use in the literature.
*Aim of the study:* The present study was conducted to evaluate the ability of five phenolic compounds purified from infusion prepared from *Tripodanthus acutifolius* leaves to inhibit key enzymes in inflammatory processes. As anti-inflammatory compounds frequently possess free radical scavenging activities, purified substances were comparatively evaluated to asses their free radical scavenging properties. Genotoxic effects were also evaluated.
*Materials and Methods:* Compounds were evaluated on their ability to inhibit hyaluronidase and cyclooxygenase-2 (COX-2) activities to assess their anti-inflammatory capacities. Free radical scavenging activity was assessed by: 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH), superoxide anion assay and the inhibition on lipid peroxidation. Genotoxicity was evaluated by *Bacillus subtilis* rec assay.
*Results:* Fractionation of *Tripodanthus acutifolius* infusion yielded a novel phenylbutanoid derivative (tripodantoside) and four known flavonoid glycosides (rutin, nicotiflorin, hyperoside and isoquercitrin). Flavonoids produced higher inhibition on hyluronidase activity ($IC_{50} \approx 1.7$ mM) than tripodantoside ($IC_{50} = 27.90$ mM). A similar COX-2 inhibition activity was exerted by tripodantoside and monoglycosilated flavonoids ($IC_{50} \sim 50$ μM). Compounds were strong radical scavengers, with effective concentration 50 ($EC_{50}$) values for DPPH in the range of 2.7–6.3 μg/mL, and for superoxide anion in the range of 3.9–8.7 μg/mL. All compounds scavenged peroxyl radicals in the lipid peroxidation assay. The substances showed no genotoxic effects.
*Conclusions:* The anti-inflammatory effects, free radical scavenging activities and lack of genotoxicity of purified compounds may support the folk use of infusion from *Tripodanthus acutifolius* leaves as anti-inflammatory.



## 1. Introduction

Free radicals are high reactive compounds implicated in the aetiology of multiple diseases including cardiovascular and neurological diseases, or inflammatory process, therefore there has been increasing interest in free radical scavenging substances derived from fruits, vegetables and herbs in recent years (Katsube et al., 2006). Several of the reported biological activities of phenols, such as free radical scavenging activities or anti-inflammatory potential have been also associated with cancer chemopreventive potential (Parys et al., 2010). *Tripodanthus acutifolius* (Ruiz and Pavón) Van Tieghem (Loranthaceae) is an endemic shrub that grows in various arid and semiarid regions of South America (between 1800 and 2700 m on sea level), including northwestern and northeastern Argentina (Abbiatti, 1943; Daud et al., 2005), bolivian andean region (Macía et al., 2005), and southern of Brazil (Alice et al., 1991). Extracts obtained from *Tripodanthus acutifolius* leaves have long been used in argentinean traditional medicine as haemostatic, hypoglycaemic and anti-inflammatory (Saggese, 1949), though no scientific reports on anti-inflammatory studies could be found in the literature. A novel phenylbutanoid

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glycoside called tripodantoside [4-(3′,4′-dihydroxyphenyl)-2-R-butanol-2-*O*-$\beta$-D-glucopyranoside] and four flavonoid glycosides (rutin, nicotiflorin, hyperoside and isoquercitrin) have been previously isolated and identified from *Tripodanthus acutifolius* infusion prepared from leaves (Soberón et al., 2010). There is a significant correlation between free radicals proliferation *in vivo* and chronic inflammation (Schemppa et al., 2006), processes which contributes to tumor promotion through the action of prostaglandins and other inflammatory mediators. Taking this into consideration, the compounds were studied on their abilities to inhibit enzymes related to inflammatory process, such as cyclooxygenase-2 (COX-2) and hyaluronidase. Since active plant extracts and the purified substances may exert toxic effects at genomic levels, genotoxicity evaluation assays were performed on purified compounds, and the results were compared with those of reference compounds.

## 2. Materials and methods

### 2.1. Chemicals

Analytical grade solvents were from Cicarelli Labs. (San Lorenzo, Santa Fe, Argentina) and HPLC solvents were from Sintorgan Labs (Vicente López, Buenos Aires, Argentina). Cyclooxygenase-2 (human recombinant), arachidonic acid, hemin, hyaluronidase (testicular from bovine), NaCl, 2-aminoethyl diphenylborate, Tris, phenol, EDTA, 2-thiobarbituric acid, p-dimethylaminebenzaldehyde, sodium hyaluronate, $Na_2HPO_4$ and $NaH_2PO_4$ were from Sigma-Aldrich (Saint Louis, Missouri, USA). KOH, NaOH, sodium acetate, $CaCl_2$, $K_2B_2O_7{\cdot}4H_2O$, silica Gel 60 $F_{254}$ plates, trichloroacetic acid, and vanillin were from Merck (Darmstadt, Hesse, Germany). Flavonoid standards (HPLC quality) were from Indofine Chemical Company Inc. (Belle Mead, New Jersey, USA). Membrane filters (pore size 0.22 μm) were from Pall Life Sciences (Ann Arbor, Michigan, USA). Sephadex LH20 was from Amersham Biosciences (Uppsala, Sweden).

### 2.2. Extract preparation

Infusion was prepared and the extraction yield was calculated as described elsewhere (Soberón et al., 2007). The dried material obtained represented the extracted material (EM).

### 2.3. Activity guided fractionation of Tripodanthus acutifolius infusion

Activity guided fractionation of *Tripodanthus acutifolius* infusion was performed as reported elsewhere (Soberón et al., 2010). Briefly: lyophilized infusion (150 g) was successively extracted with hexane, ethyl ether, ethyl acetate and methanol in an order of increasing solvent polarity till reach 1 L of hexane (HX), ethyl ether ($Et_2O$), ethyl acetate (AcOEt) and methanol (MeOH) extracts. Each of the obtained extracts were evaporated under reduced pressure (at 45 °C) yielding dried residues which were quantified and then dissolved (in the same solvents used for extraction) to perform free radical scavenging activity experiments (DPPH assays on polystyrene 96-well plates and direct staining on TLC plates). MeOH extract was selected for further purification steps. An aliquot of MeOH extract containing 500 mg of EM was chromatographed (methanol as mobile phase) on Sephadex LH-20 (230 mL bed volume). The 160 aliquots (2 mL each one) resulting from column elution were analyzed by TLC, joined according to their chemical composition into ten groups (L1–L10), evaporated under reduced pressure (at 45 °C) to yield EM residues which were dissolved in methanol for further experiments. Aliquots were taken from L1 to L10 groups for free radical scavenging activity assays and chromatographic experiments.

### 2.4. Identification of active compounds

L2, L4 and L8 groups yielded active compounds which were purified, identified by spectroscopic methods, and quantified as described elsewhere (Soberón et al., 2010).

### 2.5. Anti-inflammatory activity

Anti-inflammatory activity was assayed by measuring the inhibitory effect of substances on cyclooxygenase-2 (COX-2) and hyaluronidase catalyzed reactions. The inhibitory effect on COX-2 reaction was assayed as described elsewhere (Sud'ina et al., 2008). Malondialdehyde generated from COX-2 reaction products is proportional to the COX-2 activity (Sharma et al., 2001), and could be detected at 532 nm as a 2-thiobarbituric acid reactive substance (Halliwell et al., 1987). The inhibitory effect on hyaluronidase catalyzed reaction was evaluated as described elsewhere (Takahashi et al., 2003) and the fragments containing N-acetylglucosamine were quantified (Reissig et al., 1955). Acetyl salicylic acid was employed as reference. The concentrations inhibiting the enzymatic activity by 50% ($IC_{50}$s) were calculated by graphic interpolation of the concentration–enzyme activity curves (Soberón et al., 2009b).

### 2.6. Free radical scavenging assays

DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging activity was performed in polystyrene 96-well plates according to Ley and Bertram (2003), quercetin, kaempferol and BHT were used as reference scavenging substances. DPPH staining method (Brem et al., 2004) was performed for samples over TLC plates after each purification step. Superoxide anion scavenging activity was evaluated by the nitroblue tetrazolium (NBT) assay, according to Soberón et al. (2009a). The lipid peroxidation inhibitory activity assay was performed as described by Kang et al. (2003) on human red blood cell ghosts. Samples were assayed at 0.1–1000 μg/mL. The measurements were made in triplicates. Arithmetic means of the absorbance measurements were calculated for each experiment. The concentrations inhibiting the radical activity by 50% ($EC_{50}$s) were calculated by graphic interpolation of the concentration-radical activity curves (Soberón et al., 2009a).

### 2.7. Genotoxic activity

Gentoxicity response was assessed by using *Bacillus subtilis* rec assay after quantification and Probit transformation of the differential growth plots of *Bacillus subtilis* rec strains exposed to samples (Soberón et al., 2009a). Samples were assayed between 0.1–5000 μg/mL. The results, referred to as S-Probit (Takigami et al., 2002) were compared with reference values described elsewhere (Matsui, 1988). $K_2Cr_2O_7$ and kanamycin were used as genotoxic and non-genotoxic reference drugs respectively.

### 2.8. Statistic analysis

Data were analyzed by either Student's *t*-test or one-way ANOVA, considering a probability level lower than 0.05 ($p < 0.05$) as statistically significant.

## 3. Results

### 3.1. Extraction and purification

A flow chart of the purification procedure is shown in Fig. 1. The percentage inhibition of DPPH reduction exerted by AcOEt and MeOH extracts was 74.2%/50 μg of sample and 70.9%/50 μg of sample for AcOEt and MeOH extracts respectively. Since MeOH

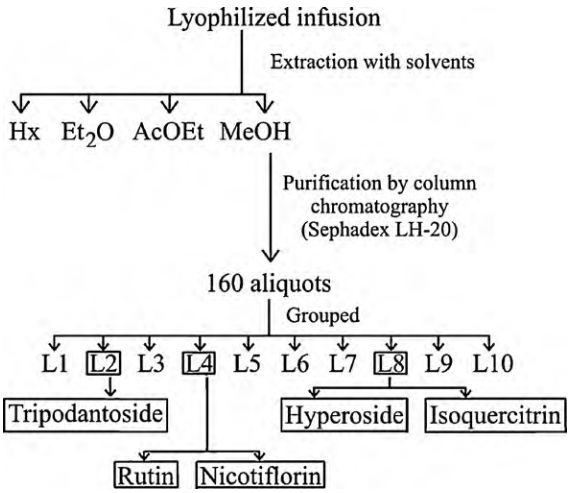


**Fig. 1.** Flow chart of the purification procedure.

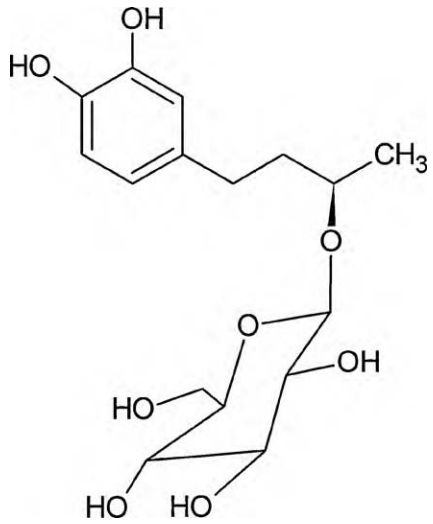


**Fig. 2.** Chemical structure of 4-(3′,4′-dihydroxyphenyl)-2-R-butanol-2-*O*-*β*-D-glucopyranoside (Tripodantoside).

extract posses almost 270-fold more EM than AcOEt extract, and the DPPH scavenging activity of MeOH extract was only 3.7% lower than AcOEt extract, MeOH extract was selected for large-scale extraction and isolation of bioactive compounds (Choudhary et al., 2008).

Column chromatography of MeOH extract yielded groups L2, L4 and L8 with DPPH scavenging activity evaluated by DPPH staining method (data not shown). HPLC experiments yielded the flavonoids rutin and nicotiflorin from L4, isoquercitrin and hyperoside from L8 whereas L2 yielded a new phenylbutanoid glycoside called tripodantoside [4-(3′,4′-dihydroxyphenyl)-2-R-butanol-2-*O*-*β*-D-glucopyranoside], which structure is shown in Fig. 2.

### 3.2. Anti-inflammatory activity

Quercetin glycosides (rutin, hyperoside and isoquercitrin) showed higher hyaluronidase inhibitory activity than nicotiflorin (a kaempferol glycoside) and tripodantoside. There were significant differences between isolated compounds $IC_{50}$ values and that for acetyl salicylic acid ($p < 0.05$) (Table 1).

Tripodantoside COX-2 inhibitory activity was significant higher than those for flavonoids ($p < 0.05$), though that inhibition was almost four-fold lower than acetyl salicylic acid (Table 1). Tripodantoside aglycone was more active against COX-2 than the glycoside ($p < 0.05$). Flavonoids with a bulky sugar moiety, such as nicotiflorin and rutin, showed lower COX-2 inhibitory activity than those with only one sugar bounded to the aglycone (hyperoside and isoquercitrin).

### 3.3. Free radical scavenging activity

With all five assayed substances there were dose-dependent free radical scavenging activities by the three assayed systems (Table 1). There were no significant differences among tripodantoside and quercetin glycosides free radical scavenging effects ($p > 0.05$) (Table 1), though the differences between these compounds and nicotiflorin (a kaempferol glycoside) were statistically significant ($p < 0.05$). A similar scavenging profile was observed when the aglycone moieties of purified compounds (tripodantoside aglycone, quercetin and kaempferol) were analyzed. All of the isolated compounds showed higher scavenging activity than butylated hydroxytoluene (BHT), a synthetic food additive with a questioned safety (Ames, 1983) and L-ascorbic acid.

### 3.4. Genotoxic activity

S-Probit values and the interpretations obtained with samples on *Bacillus subtilis* rec assay are shown in Table 2. The genotoxicity diminished as purification procedures were carried out, deduced from the decrease of S-Probit values (Matsui, 1988). None of the isolated compounds were genotoxic on *Bacillus subtilis* rec strains ($-0.123 >$ S-Probit $> 0.199$). $K_2Cr_2O_7$ is a well known genotic agent (S-Probit $> 0.593$), and kanamycin is an antibiotic that does not cause DNA damages ($-0.123 >$ S-Probit $> 0.199$).

**Table 1**
Anti-inflammatory and free radical scavenging activities of isolated compounds, infusion and reference substances.

| Substance | Hyaluronidase assay | | COX-2 assay | | DPPH assay | | Superoxide anion assay | | Lipid peroxidation assay | |
|---|---|---|---|---|---|---|---|---|---|---|
| | mM | mg/mL | mM | mg/mL | μg/mL | μmol/mL | μg/mL | μmol/mL | μg/mL | μmol/mL |
| Infusion | – | – | – | – | 15.8 | – | 18.7 | – | 355.0 | – |
| Tripodantoside | 27.90 | 9.60 | 50 | 17.2 | 2.7 | $8.0 \times 10^{-3}$ | 3.9 | $1.1 \times 10^{-2}$ | 120 | 0.35 |
| Rutin | 1.70 | 0.95 | 110 | 73.1 | 4.9 | $8.0 \times 10^{-3}$ | 7.4 | $1.2 \times 10^{-2}$ | 200 | 0.33 |
| Nicotiflorin | 3.70 | 2.20 | >160 | >95.1 | 6.3 | $1.0 \times 10^{-2}$ | 8.7 | $1.4 \times 10^{-2}$ | 254 | 0.43 |
| Hyperoside | 1.70 | 0.79 | 46 | 21.4 | 4.2 | $9.0 \times 10^{-3}$ | 6.8 | $1.5 \times 10^{-2}$ | 165 | 0.35 |
| Isoquercitrin | 1.67 | 0.78 | 52 | 24.1 | 4.2 | $9.0 \times 10^{-3}$ | 6.9 | $1.5 \times 10^{-2}$ | 162 | 0.35 |
| Tripodantoside aglycone | 43.91 | 8.00 | 42 | 7.6 | 1.8 | $9.0 \times 10^{-3}$ | 2.9 | $1.6 \times 10^{-2}$ | 67 | 0.36 |
| Quercetin | – | – | – | – | 2.4 | $8.0 \times 10^{-3}$ | 6.0 | $2.0 \times 10^{-2}$ | 40.0 | 0.13 |
| Kaempferol | – | – | – | – | 3.6 | $1.2 \times 10^{-2}$ | 9.1 | $3.2 \times 10^{-2}$ | 95 | 0.33 |
| BHT | – | – | – | – | 48 | $2.18 \times 10^{-1}$ | 36.3 | $16.5 \times 10^{-2}$ | – | – |
| L-Ascorbic acid | – | – | – | – | – | – | 19.0 | $10.8 \times 10^{-2}$ | – | – |
| Acetyl salicilic acid | 0.47 | 0.08 | 12.5 | 2.2 | – | – | – | – | – | – |

Data are expressed as $IC_{50}$ values (anti-inflammatory activities) and $EC_{50}$ values (free radical scavenging activities).

**Table 2**
Genotoxic activity of *Tripodanthus acutifolius* raw samples, isolated compounds and reference substances.

| Sample | S-Probit | Result |
|---|---|---|
| Infusion | 0.80 | Strong genotoxic (++) |
| MeOH | 0.67 | Strong genotoxic (++) |
| L2 group | 0.18 | Non-genotoxic (−) |
| Tripodantoside | 0.106 | Non-genotoxic (−) |
| Tripodantoside aglycone | 0.123 | Non-genotoxic (−) |
| Rutin | 0.086 | Non-genotoxic (−) |
| Nicotiflorin | 0.097 | Non-genotoxic (−) |
| Hyperoside | 0.124 | Non-genotoxic (−) |
| Isoquercitrin | 0.120 | Non-genotoxic (−) |
| $K_2Cr_2O_7$ | 2.95 | Strong genotoxic (++) |
| Kanamycin | 0.137 | Non-genotoxic (−) |

## 4. Discussion

Reactive oxygen species play a key role in the initiation and promotion phase of carcinogenesis (Parys et al., 2010). To detect radical-scavenging potential of purified compounds, we used three different free radical systems. The $EC_{50}$ values obtained for purified substances by DPPH assay were in a concentration range of 2.7–4.2 μg/mL ($8 \times 10^{-3}$–$1 \times 10^{-2}$ μmol/mL), which is in agreement with earlier studies (Luis et al., 2006). Flavonoids from plant sources are well known free radical scavenging substances (Pérez-Jiménez and Saura-Calixto, 2008). Though the four purified flavonoids had been previously referred as free radical scavenging substances (Onodera et al., 2006), this is the first report on free radical scavenging activities of molecules isolated from *Tripodanthus acutifolius* leaves. *Tripodanthus acutifolius* is the first angiosperm species reported to contain tripodantoside (Soberón et al., 2010). Tripodantoside is the main constituent purified from *Tripodanthus acutifolius* infusion. This substance showed similar free radical scavenging profile than flavonoids. The activity is attributed to the catechol group, present in both tripodantoside and tripodantoside aglycone, which may justify the absence of differences between the glycoside and the aglycone activity profiles.

Free radicals are also relevant during inflammatory reactions *in vivo* (Schemppa et al., 2006), because these substances may act as cell messengers along inflammatory process (Wiseman and Halliwell, 1996). Chronic inflammation contributes to tumor promotion through the action of prostaglandins as inflammatory mediators and has been estimated to be associated to 15% of malignancies (Parys et al., 2010). COX, the key enzyme in prostaglandin biosynthesis (Michaux et al., 2005), and hyaluronidase, an enzymatic activity increased during chronic inflammation, are targets of chemoprevention and could be used as marker systems for antitumor-promoting potential. COX-2 inhibitory substances are intensively studied not only for anti-inflammatory aims, but also for the COX-2 implication in other process, such as carcinogenesis or cell apoptosis (Goodsell, 2000). All of the purified compounds were identified as potent radical scavengers which moderately inhibited COX-2 activity, as an indication of anti-inflammatory potential. According to Kuppusamy et al. (1990), the inhibitory activity exerted by flavonoids on hyaluronidase is improved when a C2–C3 double bound, a carbonyl group at C4 and free hydroxyl groups at C5, C7 and C4′ are present. Though the purified flavonoids posses these structures, their hyaluronidase inhibitory activities were lower than the other assayed compounds.

The *Bacillus subtilis* rec assay was used to detect and characterize chemical mediation of DNA damage exerted by *Tripodanthus acutifolius* infusion, the fractionated extracts and purified compounds due to the high sensitivity of *Bacillus subtilis* rec spores to genotoxic compounds (McCarroll et al., 1981). Plant extracts are complex mixtures of various substances, consequently their genotoxic effects are often non-predictable. The genotoxic response of the raw extract (infusion) was diminished and eventually abolished as purification of active compounds was achieved.

*Tripodanthus acutifolius* is traditionally employed by northwestern argentinean people to attain extracts for topical anti-inflammatory uses (Saggese, 1949), an application that is in agreement with other authors (Macía et al., 2005). They described this species is commercialized in specific stalls for medicinal plants and small markets in Bolivia, where it is recommended for the treatment of inflammations caused by luxations and sprains. To the best of our knowledge, this is the first report on anti-inflammatory capacities, free radical scavenging activities and lack of genotoxicity of compounds purified from *Tripodanthus acutifolius* leaves. This information provides scientific evidence on the pharmacological activity described for this plant, and contributes to the validation of folk medicine applications of infusions prepared from *Tripodanthus acutifolius* leaves. These findings turn this species an interesting source of substances for investigations regarding pharmacological purposes.

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