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ORIGINAL ARTICLE

Purification and identification of antibacterial phenolics from *Tripodanthus acutifolius* leaves

J.R. Soberón[1]*, M.A. Sgariglia[1]†, D.A. Sampietro[1]‡, E.N. Quiroga[1]¶, M.G. Sierra[2]** and M.A. Vattuone[1]††‡‡

1 Cátedra de Fitoquímica. Instituto de Estudios Vegetales ''*Dr. A.R. Sampietro*'', Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán, Ayacucho, San Miguel de Tucumán, Tucumán, Argentina

2 Instituto de Química Rosario (IQUIR) – CONICET, Suipacha, Rosario, Santa Fé, Argentina

Keywords

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Correspondence

Marta A. Vattuone, Cátedra de Fitoquímica. Instituto de Estudios Vegetales ''Dr. A.R. Sampietro'', Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán, Ayacucho 471 (4000), San Miguel de Tucumán, Tucumán, Argentina.
E-mails: sampietro@tucbbs.com.ar; mavattuone@fbqf.unt.edu.ar

*,†Fellows from the CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina).

*,‡,¶,††Investigators from the UNT (Universidad Nacional de Tucumán, Argentina).

‡,**,‡‡Investigators from CONICET.

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Abstract

Aims: To perform an activity-guided purification, identification and quantification of antibacterial compounds from *Tripodanthus acutifolius* infusion. To validate the antibacterial activity of purified substances.

Methods and Results: Bioautographic methods were employed as screening assays for purifying bioactive substances. Purification procedures included sephadex LH-20 column chromatography and reverse phase HPLC. Identification was achieved by spectroscopic methods (UV-Vis, MS, NMR and polarimetry) and chromatographic assays (paper chromatography and HPLC). Antibacterial activity was studied by microdilution, colony count and photometric assays, Sytox green stain and transmission electron microscopy (TEM). Four glycoflavonoids (rutin, nicotiflorin, hyperoside and isoquercitrin) and an unusual phenylbutanoid glycoside (tripodantoside) were purified and identified. Tripodantoside was found at 6·59 ± 0·82 g per 100 g of dry leaves. The flavonoids showed bactericidal effect at a concentration of 4 mg ml^{-1} against *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains from *American Type Culture Collection*, while tripodantoside was almost four times more active than those compounds, with a minimum bactericidal concentration = 1·024 mg ml^{-1} against these strains. Tripodantoside aglycone showed bacteriolytic effects on the assayed strains, causing evident damages on cell wall and membrane, while tripodantoside did not exhibit those effects.

Conclusions: The antibacterial activity of *T. acutifolius* infusion would be partially attributed to the purified glycoflavonoids and mainly to tripodantoside.

Significance and Impact: The high extraction yield and the antibacterial activity exhibited by tripodantoside makes this chemical structure of interest to support further studies dealing with chemical modifications to increase the antibacterial activity or to seek another activities.

Introduction

Tripodanthus acutifolius (Ruiz & Pavón) Van Tieghem (Loranthaceae) (Abbiatti 1943) [formerly *Phrygilanthus acutifolius* (Ruiz & Pavón) Eichler] is an endemic shrub that spontaneously grows in the arid and semi-arid region of northwestern Argentina between 1800 and 2700 m above the sea level. It is extensively used in traditional medicine as anti-inflammatory, a haemostatic, hypoglycaemic (Alice *et al.* 1991) and vulnerary.

Previously, we found that infusion obtained from *T. acutifolius* dried leaves possess antibacterial effects against Gram positive and Gram negative strains, and some of the substances responsible for those effects were preliminary identified (Soberón *et al.* 2007), which represents the only one documented attempt to study the chemical composition of this plant.

This article describes the antibacterial-guided purification, structural elucidation and the antibacterial activity analysis of five phenolic substances obtained from *T. acutifolius* infusion.

The antibacterial assays included Gram positive and Gram negative strains from *American Type Culture Collection* (ATCC). Photometric assays were performed to study the mode of action of the main isolated substance and its hydrolysis product. Sytox green and transmission electron microscopy (TEM) assays provided detailed information on cellular damages and alterations caused by the assayed compounds.

Materials and methods

Chemicals

Analytical grade solvents were from Cicarelli Labs, (San Lorenzo, Santa Fe, Argentina) and HPLC solvents were from Sintorgan Labs (Vicente Lopez, Buenos Aires, Argentina). NaCl, 2-aminoethyl diphenylborate, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), Na_2HPO_4 and NaH_2PO_4 were from Sigma-Aldrich (Saint Louis, MO, USA). DMSO, glycerol, silica gel 60 F_{254} plates and vanillin were from Merck (Darmstadt, Hesse, Germany). Brain heart infusion medium (BHI), Müller-Hinton agar (MHA) and MH broth (MHB) mediums were from Britania S.A. Labs (Ciudad Autonoma de Buenos Aires, Buenos Aires, Argentina). Bacto agar was from Difco (Detroit, Michigan, MI, USA). Flavonoids standards (HPLC quality) were from Indofine Chemical Company Inc. (Belle Mead, NJ, USA). Membrane filters (pore size 0·22 μm) were from Pall Life Sciences (Ann Arbor, MI, USA). Sytox green and horseradish peroxidase were from Invitrogen Corporation (Carlsbad, CA, USA). Sephadex LH-20 was from Amersham Biosciences (Uppsala, Uppland, Sweden).

Reactives and media

MTT was prepared at 0·8% w/v in sterile sodium phosphate buffer (0·1 mol l^{-1}, pH 7·4). MHA medium contained 3·7 g of MHA per 1 l of water, soft MHA (sMHA) medium contained 2·2 g of MHB and 0·5 g of bacto agar per 1 l of water. Sytox green was prepared at 500 μmol l^{-1} in DMSO.

Micro-organisms

The micro-organisms used were bacterial strains from ATCC: *Pseudomonas aeruginosa* ATCC 27853 (Gram negative strain) and *Staphylococcus aureus* ATCC 25923 (Gram positive strain). These wild type strains are largely used (Karlowsky *et al.* 1997; Ba *et al.* 2006; Tam *et al.* 2007; Robertson *et al.* 2008), and their inclusion is recommended for antibacterial sensitivity tests (Andrews 2001; Sgariglia *et al.* 2009). *Staphylococcus aureus* strains were stored in BHI medium supplemented with 0·3% w/v agar and 1·5% v/v glycerol at −20°C. *Pseudomonas aeruginosa* strains were stored in sterile water (Liao and Shollenberger 2003) supplemented with 1% v/v glycerol at room temperature.

Inoculum preparation

Stock bacterium cultures were maintained at room temperature for 2 h. Each strain was streaked on MHA plate and incubated at 37°C for 24 h. The inoculum was prepared by emulsifying a minimum of three colonies from those plates in sterile 0·9% NaCl (w/v) till reach 10^8 colony forming units (CFU) ml^{-1} (0·5 McFarland scale). Working bacterial suspensions were prepared by diluting (1 : 10) the suspensions using sterile 0·9% NaCl (w/v) until they reach 10^7 CFU ml^{-1} before performing the antibacterial assays. The use of laminar flux equipment assured the sterile conditions of the procedures.

Extract preparation

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

Activity-guided fractionation of *T. acutifolius* infusion

An antibacterial activity-guided fractionation of *T. acutifolius* infusion was conducted as follows: 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 (EtOEt), ethyl acetate (AcOEt) and methanol (MeOH) extracts. Each of the obtained extracts was evaporated under reduced pressure (at 45°C) yielding dried residues which were dissolved in methanol to perform the bioautography assays. 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 cm^3 bed volume). The 160 aliquots (2 ml each one) resulting from column elution were analysed by TLC,

joined according to their chemical composition into ten groups (L1-L10) and evaporated under reduced pressure (at 45°C) to yield EM residues which were dissolved in methanol for further experiments. Aliquots were taken from L1-L10 groups for bioautography, TLC and HPLC experiments.

HPLC experiments

L2, L4 and L8 groups were selected for HPLC analysis. The analytical experiments were performed on a Gilson HPLC (Villiers Le Bel, Val d'Oise, France) using an IB-SIL 5 C18 column (5 μm, 250 × 4·6 mm ID) from Phenomenex (Torrance, CA, USA), an IB-SIL RP 18 pre-column (5 μm, 30 × 4·6 mm ID, Phenomenex), a 118 UV-Vis detector from Gilson and a Rheodyne injector fitted with a 20-μl loop. A gradient elution was performed with solvent A: 2% (v/v) formic acid in water and solvent B: 0·5% formic acid in water and acetonitrile (50 : 50 v/v) (Schieber *et al.* 2002). The gradient was 0% B to 39·8% B, 12 min; 39·8% B to 40·8% B, 13 min; 40·8% B to 41·2% B, 5 min; 41·2% B to 41·4% B, 5 min; 41·4% B to 41·8% B, 2 min; 41·8% B to 97·5% B, 8 min; 97·5% B to 100% B, 12 min and 100% B, 8 min. Re-equilibration was obtained at 100% B, 10 min; 100 to 0% B, 10 min and 0% B, 10 min. Compounds were detected at 254 nm at a flow rate of 0·5 ml min^{-1}. The retention times (*Rt*s) were registered. The detected compounds were collected, dried by lyophilization and then dissolved in methanol for further experiments. Multiple injections (40 μg of EM at a time) were carried out to obtain adequate quantity of material. The semipreparative experiments employed an IB-SIL 5 C18 column (5 μm, 250 × 10 mm ID) from Phenomenex, and the same pre-column and detector employed on analytical conditions. A Rheodyne injector fitted with a 500-μl loop was used. A gradient elution was performed with the solvents A and B employed on analytical experiments. The gradient was 0% B to 42% B, 8 min; 42% B to 49% B, 21 min; 49% B to 100% B, 6 min and 100% B, 6 min. Re-equilibration was obtained at 100% B, 10 min; 100 to 0% B, 8 min and 0% B, 6 min. Compounds were detected at 254 nm at a flow rate of 3 ml min^{-1}. The detected compounds were collected, dried by lyophilization and dissolved in methanol for further experiments. Multiple injections (*c.* 5 mg of EM at a time) were carried out to obtain adequate quantity of material. The purity of all the isolated compounds was verified by analytical HPLC experiments.

Hydrolysis

The compounds purified from L2, L4 and L8 groups were separately dried under reduced pressure (at 45°C) and then hydrolysed by standard procedures (Markham 1982). The procedure yielded ethyl acetate extracts (containing aglycone moieties) and aqueous extracts (containing sugar moieties), which were separately analysed as described by Markham (1982).

Phytochemical analysis

Samples were analysed by TLC on silica gel 60 F_{254} plates. The solvent systems were ethyl acetate:formic acid:acetic acid:water (100 : 11 : 11 : 27, v/v) and toluene:ethyl acetate:formic acid (50 : 40 : 10, v/v). Visualization was performed under visible and UV light (254 and 366 nm, UV Lamp Model UV 5L-58 Mineralight Lamp) before and after staining with 1% methanolic 2-aminoethyl diphenylborate reagent (flavonoids detection) and vanillin/sulfuric acid reagent (phenolics detection) as described by Wagner *et al.* (1984).

Identification of active compounds

Compounds purified from L4 and L8 groups and their aglycones were identified by the comparison of HPLC *Rt*s, mass spectrometry, polarimetric studies and UV-Vis absorption spectra (Mabry *et al.* 1970) with literature data and analytical grade standards and then confirmed by internal standard HPLC method (Sampietro *et al.* 2007). The purified substances were analysed by high-resolution mass spectrometry (HRMS) employing a direct sample introduction technique on a Thermo Finnigan Polaris Q ion trap mass spectrometer (Austin, Texas, USA). Positive ion chemical ionization mode, a soft ionization technique, was selected for the assays using isobutane as a reagent gas to generate the molecular ion $[MH]^+$. Electronic energy was 40 eV at 200°C. The heating was between 50°C and 40°C at 100°C min^{-1}. The mass spectrometer was scanned over an *m/z* range of 100–630 Da. The data processing was performed using Xcalibur 1.3 software (Austin, TX, USA).

A compound purified from L2 was derivatized by peracetylation according to Markham (1982). Fifteen milligrams of derivatized and underivatized compounds was separately analysed by one-dimensional NMR techniques (^{1}H NMR, ^{13}C NMR and ^{13}C DEPT) and bidimensional NMR techniques (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC and ^{1}H-NOE). NMR spectra were recorded on a Bruker Avance instrument (300 MHz for ^{1}H NMR and 75·14 MHz for ^{13}C NMR) and were referenced to the non-deuterated impurities of the used solvents as internal standard ($CDCl_3$ and D_2O for the derivatized and underivatized compounds, respectively) using standard Bruker Topspin software. An aliquot of the derivatized compound was analysed by gas chromatography-mass

spectrometry technique (GC-MS) on a Perkin-Elmer Autosystem XL mass spectrometry (Norwalk, CT, USA) equipped with a Turbomass detector. Positive ion electronic impact mode was selected for the assay to generate the molecular ion $[M]^+$. Electronic energy was 70 eV at 260°C. The heating was between 180°C and 310°C at 10°C min^{-1}. The mass spectrometer was scanned over an *m/z* range of 50–610 Da. The sugar moieties present in posthydrolysis aqueous extracts were identified by paper chromatography (Hussein *et al.* 2003) and polarimetric studies (Lee *et al.* 1970). Optical rotations were measured in a 0·1-dm cell at 25°C with a Horiba Sepa-300 polarimeter (Horiba Ltd., Kyoto, Japan) at 589 nm. The results were compared with literature data and analytical grade standards. The aglycone of the compound purified from L2 was also analysed by polarimetric studies (Das *et al.* 1993).

Quantitative analysis

The purified compounds were quantified by analytical HPLC using external standards (Sampietro *et al.* 2007). Compounds purified from L4 and L8 groups were analysed at 254 nm. The compound purified from L2 was analysed at 283 nm and quantified by using a calibration curve obtained with known quantities of the purified substance.

Antibacterial analysis

Bioautography assays

The antibacterial activity of samples was screened to assess their ability to inhibit the bacterial growth, as described by Sgariglia *et al.* (2009). Briefly, sterile samples aliquots (sterilized through a 0·22-μm membrane filter) containing 50 μg of EM were punctually placed on the surface of sterile silica gel F_{254} TLC plates and leaved till solvent evaporation. The plates were covered with 5 ml of sMHA containing 10^6 CFU ml^{-1} of *Staph. aureus* ATCC 25923 and incubated at 37°C for 24 h. Afterwards, the plates were covered with MTT solution and incubated at 37°C for 1 h. The presence of a yellow inhibition zone against a dark blue background indicated the antibacterial activity of the evaluated sample. The inhibition zones diameters were proportional to the antibacterial power of the assayed samples.

Broth microdilution and colony count assays

The assays were performed to assess the bacteriostatic (broth microdilution assays) and bactericidal (colony count assay) capacity of samples (Soberón *et al.* 2007). Broth microdilution assays were carried out in sterile polystyrene 96-well microtitre plates. Flavonoids were assayed at 0·03–16 mg ml^{-1}, while the compound isolated from L2 and its aglycone were assayed at 0·001–1·5 mg ml^{-1}. Final volumes were adjusted to 100 μl with MHB. Colony count assays were performed in sterile petri plates using MHA (Soberón *et al.* 2007). Experiments were conducted eight times for each sample concentration and repeated twice. The bacterial strains assayed were *Staph. aureus* (ATCC 25923) and *Ps. aeruginosa* (ATCC 27853). The arithmetic means of the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were calculated and reported (Hili *et al.* 1997).

Photometric assays

The assays were performed to differentiate bacteriolytic (cell lysis) from nonbacteriolytic effects of samples, as described byLehtinen *et al.* (2006). Briefly, bacteria cultures (5×10^6 CFU ml^{-1}) were prepared in sterile polystyrene 96-well microtitre plates containing the compound isolated from L2 (20 mg ml^{-1}) or its aglycone (10·5 mg ml^{-1}). The bacterial strains assayed were *Staph. aureus* (ATCC 25923) and *Ps. aeruginosa* (ATCC 27853). Final volumes were adjusted to 200 μl using MHB. Plates were covered, carefully mixed and incubated at 37°C for 24 h. Optical densities (OD) were measured in a Bio-Rad 550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) using a 620-nm emission filter at 0, 1, 2, 4, 6, 12 and 24 h of incubation. Growth controls replaced samples with sterile water, samples colour wells replaced bacterial inoculums with MHB (this avoided the interference of the samples colour in the results), and sterility controls only included MHB. Experiments were conducted eight times for each sample concentration and repeated twice. The arithmetic means of the OD lectures were corrected for each substance (by the subtraction of colour wells OD from samples OD values for each incubation time), and percent OD values (OD%) were calculated as $OD\% = [(ODt–ODo)/ODo] \times 100$, where ODt was the corrected OD value for each incubation time, and ODo was the corrected OD value at 0 h of incubation. OD% against incubation time was plotted for each substance and each strain.

Statistical analysis

Data were analysed by either Student's *t* test or one-way ANOVA, considering a probability level lower than 0·05 as statistically significant.

Sytox green assays

The assays were performed in Eppendorff tubes in similar conditions as microdilution assays (final volumes of 0·5 ml). The compound purified from L2 and its aglycone were assayed at 4·10 mg ml^{-1} and 2·05 mg ml^{-1}, respectively, corresponding to 4. MIC. Growth controls replaced

samples with sterile water. Cefotaxime (80–2400 μg ml^{-1}) was assayed as a reference substance which causes cell cover damage (Adu and Armour 1995). The tubes were added with 5 μl of Sytox green solution and incubated at 37°C for 1 h. Fifty microlitres of samples was placed on glass slides, covered and observed with incident light fluorescence of a Nikon fluorescence microscope (Nikon Fluophot) equipped with Osram HBO 200 W/2 mercury vapour lamp; exciter filter IF 420–490 was used. Photomicrographs were taken using Kodak Tri-X pan film.

Transmission electron microscopy

Working bacteria cultures (1×10^6 UFC ml^{-1}) were prepared in sterile Eppendorff tubes containing either the compound purified from L2 (512 μg ml^{-1}) or its aglycone (216 μg ml^{-1}) at sub-MIC concentrations, and final volumes were adjusted to 0·5 ml with MHB. Growth controls replaced samples with sterile water. Cefotaxime (20-600 μg ml^{-1}) was used as reference antibiotic. Samples were centrifuged (600 ***g*** for 5 min) after incubation at 37°C for 18 h, and the pellets were washed three times with PBS (10 mmol l^{-1}, pH 7·4) at 600 ***g*** for 5 min. Cells were suspended in 2 mg ml^{-1} horseradish peroxidase solution till reach 0·5 ml of final volume (Dahlen *et al.* 1978). After incubation at 37°C for 1 h, cells were three time washed and then fixed in sodium phosphate buffer (10 mmol l^{-1}, pH 7·4) added with 2·7% w/v glutaraldehyde and $CaCl_2$ (0·01 mol l^{-1}) at 48°C for 1 h. Cells were postfixed with osmium tetroxide 1% w/v (in PBS 10 mmol l^{-1}, pH 7·4) at 25°C for 2 h. Cells were rinsed in water (3–5 min) and dehydrated with a series of graded ethanol solutions (50%, 70%, 95% and 100%). Cells were embedded in Spurr's resin, and a series of thin sections (70–90 nm) were cut and collected on uncoated copper grids (200 mesh). Following staining with uranyl acetate (saturated in 50% ethanol, 5 min) and lead citrate (5 min), the sections were examined by TEM (Zeiss EM) at 50 000× magnification.

Results

Extraction and purification

The extraction yield of *T. acutifolius* leaves obtained from hot water (infusion) was 47·6 ± 4·0 g of EM per 100 g of dry leaves.

A complete flow chart of the total purification and identification procedure is shown in Fig. 1. The EM obtained in Hx, EtOEt, AcOEt and MeOH was 0·50 mg, 243·19 mg, 205·47 mg and 55·45 g, respectively. AcOEt and MeOH showed better inhibitory activity against *Staph. aureus* than the other samples, deduced from the inhibition halo diameters of 1 cm produced by both extracts using bioautography assay (Fig. 2). Because MeOH yielded almost 270 times more EM than AcOEt, and their antibacterial activities were similar, MeOH was selected for large-scale extraction and purification of bioactive compounds (Choudhary *et al.* 2008).

Column chromatography of MeOH yielded groups L2, L4 and L8 with antibacterial activity against *Staph. aureus* determined by bioautography assay (data not shown). HPLC analysis of L4 and L8 yielded four compounds preliminarily identified as flavonoid glycosides, and L2 yielded a nonflavonoid phenolic substance.

Identification of purified compounds

The flavonoids rutin and nicotiflorin were purified from L4, whereas isoquercitrin and hyperoside were purified from L8. Acid hydrolysis of purified rutin, isoquercitrin and hyperoside yielded ethyl acetate extracts containing quercetin, while kaempferol was identified from nicotiflorin ethyl acetate extract. Acid hydrolysis also yielded aqueous extracts containing D-galactose (the sugar moiety of hyperoside) and D-glucose (the sugar moieties of both isoquercitrin and the compound purified from L2), while equimolar quantities of D-glucose and L-rhamnose were obtained from both rutin and nicotiflorin aqueous extracts.

The compound purified from L2 was subjected to a standard set of NMR experiments that allowed to determine the structure of the aglycone and the point of attachment of the sugar moiety. Although the ^{13}C data suggested a glucose unit as the sugar moiety, the ^{1}H coupling constants in D_2O were difficult to measure because of overlapping. Therefore, the peracetylated compound was prepared, and the 1D and 2D experiments were performed again using $CDCl_3$ as solvent. From the new set of spectra (see Fig. S1), the coupling constants of the sugar protons were now clearly determined, and they confirmed that the glucose in its β-D-pyranose form was present. A selective NOE (nuclear overhauser effect) experiment on the anomeric proton is in agreement with the proposed β-glucopyranose structure (Perlin *et al.* 1970; Roberts and Dorman 1971) and also confirms the attachment point to the aglycone. All of these data indicated that the chemical structure of the compound is 4-(3′,4′-dihydroxyphenyl)-2-R-butanol-2-*O*-β-D-glucopyranoside (tripodantoside), shown in Fig. 3. To assign an unequivocal absolute configuration for the aglycone chiral centre (C2), a set of selective NOE experiments were performed on the peracetylated derivative, which demonstrated the NOE between H1 protons (attached to C1) and He protons (attached to the methyl carbon Ce from the acetyl group at C2″ from sugar moiety), which proves the spatial

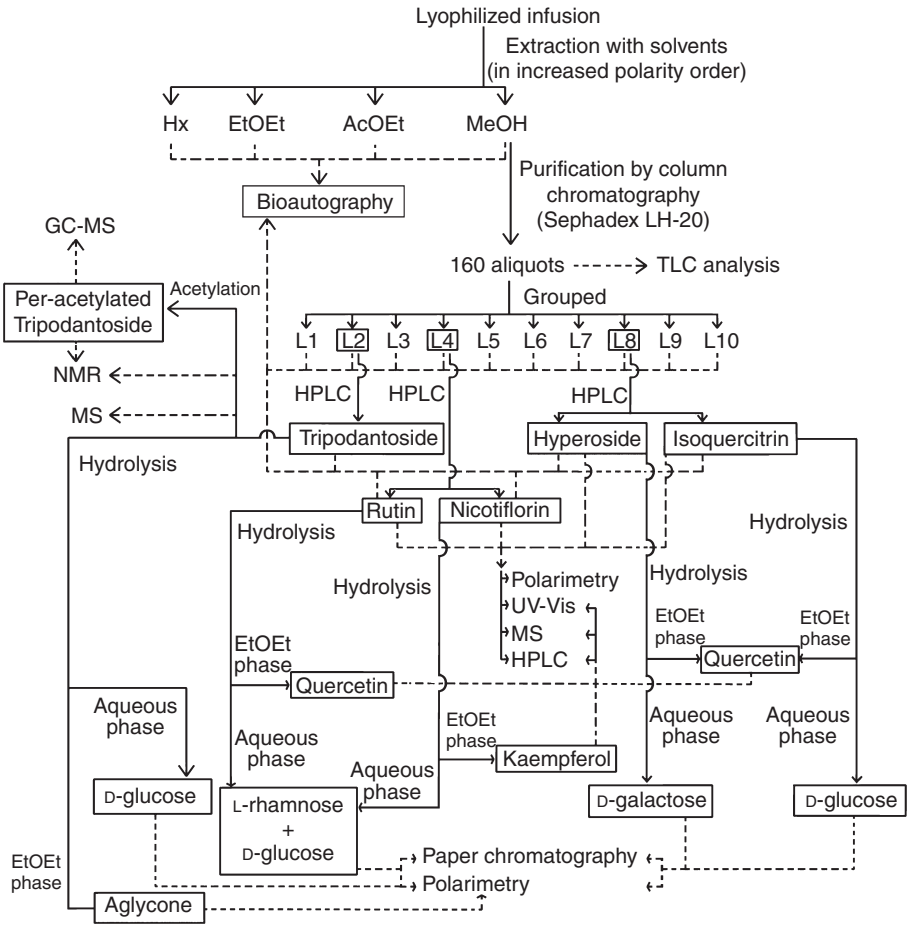

Figure 1 Flow chart of the total purification and identification procedure. Full lines: purification steps. Dotted lines: antibacterial activity screening and identification experiments.

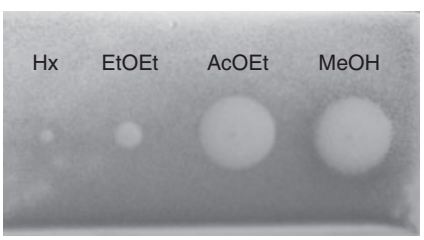

Figure 2 Bioautography assay performed with the extracts obtained from *Tripodanthus acutifolius* infusion.

proximity (2·0–3·4 Å) among them, consistent with the R configuration for C2. The optical activity of tripodantoside aglycone was $[\alpha]_D^{25} = -18{\cdot}1°$ (EtOH, c 0·3124), thus confirming the R configuration (Das *et al.* 1993). Spectral data and assignations are given in Table 1A,B. HRMS data (*m/z* = 344·1479 $[M]^+$, calc. 344·1471) were in agreement with the calculated formula $C_{16}H_{24}O_8$. Tripodantoside peracetylated derivative was analysed by GC-MS, and the main ions obtained were consistent with the chemical structure proposed for the underivatized compound. Signal at *m/z* (relative abundance) 596 (0·03) was attributed to the molecular ion $[M^+]$, while signals at *m/z* 554 (4·42) and 512 (7·88) were generated by ketene and vinyl acetate loss from $[M^+]$, respectively. Signals at *m/z* 331 (21·80), 271(9·30), 229 (3·53), 211 (5·90), 169 (96·40) and 81 (9·88) are typically generated by peracetylated glucopyranose moieties (Hussain and Rama 2002; Jerković and Mastelić 2004). Signal at *m/z* 207 (39·70) is proposed to be generated by the loss of sugar moiety and a three carbons group from $[M^+]$. Signal at 165 (39·04) is proposed to be generated by hydrogen atom subtraction from ion at *m/z* 207, and a subsequent loss of another hydrogen atom from *m/z* 165 generates the ion at *m/z* 164 (63·00). The base peak at *m/z* 123 is generated by an oxygenated tropylium ion obtained from o-diacetylated aglycone moiety. A full fragmentation pathway proposed, and the chemical structures can be found as Fig. S1.

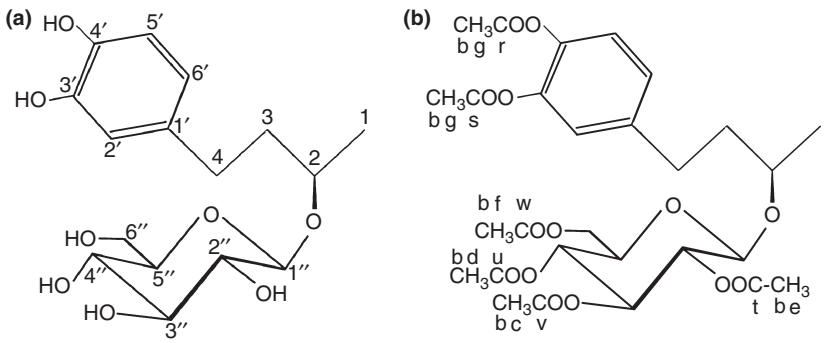

Figure 3 Chemical structure of 4-(3′,4′-dihydroxyphenyl)-2-R-butanol-2-*O*-*β*-d-glucopyranoside (a) Tripodantoside (isolated form). (b) Tripodantoside (peracetylated form).

Quantitative analysis

Tripodantoside was found at 6·59 ± 0·82 g per 100 g of dry leaves, while flavonoids were found at lower quantities: rutin (36·9 ± 0·12 mg), nicotiflorin (6·3 ± 0·14 mg), isoquercitrin (7·4 ± 0·35 mg) and hyperoside (15·1 ± 0·67 mg) per 100 g of dry leaves.

Antibacterial analysis

Microdilution and colony count assays

The purified substances showed inhibitory and bactericidal activities against the assayed strains (Table 2). Their MICs were higher than that obtained for infusion (502 μg of EM ml^{-1} for both strains) (Soberón *et al.* 2007),

Table 1 ^{1}H and ^{13}C NMR data (δ in ppm) for 4-(3′,4′-dihydroxyphenyl)-2-R-butanol-2-*O*-*β*-D-glucopyranoside (tripodantoside) in D_2O and the peracetylated derivative in $CDCl_3$. (A) Aglycone portion, (B) Sugar portion and acetyl groups

Assignations	H	^{1}H mult in D_2O	^{1}H mult (*J* in Hz) in $CDCl_3$	C	^{13}C in D_2O	^{13}C (mult) in $CDCl_3$
(A) Aglycone portion						
Aliphatics	H1	0·99 d	1·12 d (6·21)	1	18·2	19·9 (q)
	H2	3·55 m	3·77 ddq (6·21; 3·97; 3·97, 1H)	2	75·0	74·6 (d)
	H3	1·65 m	1·73 m	3	37·2	38·1 (t)
			1·82 m			
	H4	2·35 m	2·69 m	4	29·2	30·6 (t)
Aromatics	–	–	–	1′	143·0	141·1 (s)
	H2′	6·52 m	7·02 brd (1·78)	2′	115·3	123·4 (d)
	–	–	–	3′	141·0	141·8 (s)
	–	–	–	4′	134·5	140·0 (s)
	H5′	6·62 m	7·06 m	5′	115·5	123·0 (d)
	H6′	6·65 m	7·06 m	6′	119·9	126·7 (d)
(B) Sugar portion and acetyl groups						
Glucopyranose	H1′	4·27 m	4·53 d (7·86)	1′	101·8	99·2 (d)
	H2′	3·09 m	4·98 dd (7·86; 9·45)	2′	72·3	71·5 (d)
	H3′	3·28 m	5·21 t (9,45)	3′	75·1	72·9 (d)
	H4′	3·19 m	5·10 t (9·45)	4′	74·9	68·4 (d)
	H5′	3·15 m	3·66 ddd (9·45; 4·58; 2·52)	5′	68·8	71·7 (d)
	H6′	3·50 m	4·13 dd (2·53; 12·28)	6′	61·8	61·9 (t)
	H6′	3·68 m	4·25 dd (4·69; 12·28)			
Acetyl groups				C=O		168·4 (s); 168·4 (s); 169·3 (s); 169·4 (s); 170·3 (s); 170·7 (s)
	CH_3–		2·00 s; 2·02 s; 2·04 s, 2·06 s; 2 x 2,27 s.	CH_3–		20·6 (q)

s, singlet; d, doublet; dd, doublet of doublet; ddd, doublet of doublet of doublet; ddq, doublet of doublet of quartet; brd, broad doublet; t, triplet; q, quartet; m, multiplet.

Table 2 MIC/MBC values for compounds purified from *T. acutifolius* infusion (determined by microdilution and colony count assays)

	Staphylococcus aureus (MIC/MBC)		*Pseudomonas aeruginosa* (MIC/MBC)	
Substance	μg ml^{-1}	μmol ml^{-1}	μg ml^{-1}	μmol ml^{-1}
Tripodantoside	1024/1024	2·98/2·98	1024/1024	2·98/2·98
Tripodantoside aglycone	512/512	2·81/2·81	512/512	2·81/2·81
Rutin	4000/4000	6·02/6·02	4000/4000	6·02/6·02
Nicotiflorin	4000/8000	6·73/13·46	4000/8000	6·73/13·46
Hyperoside	4000/4000	8·61/8·61	4000/4000	8·61/8·61
Isoquercitrin	4000/4000	8·61/8·61	4000/4000	8·61/8·61

MIC/MBC, minimum inhibitory concentrations/minimum bactericidal concentrations.

however, the MBCs obtained against *Ps. aeruginosa* were lower than that for infusion (2010 μg of EM ml^{-1} for both strains) (Soberón *et al.* 2007). Flavonoid MICs and MBCs did not present significant differences among them ($P > 0{\cdot}05$), although all flavonoids exhibited lower antibacterial activities than tripodantoside ($P < 0{\cdot}05$).

Photometric assays

There was a significant decrease in the bacterial growth after 24 h incubation when strains were exposed to tripodantoside aglycone ($P < 0{\cdot}05$) (Fig. 4), this was interpreted as bacteriolytic action, while there was no significant decrease in the bacterial growth after the same incubation period of the same strains exposed to tripodantoside ($P > 0{\cdot}05$), which was interpreted as no bacteriolytic action.

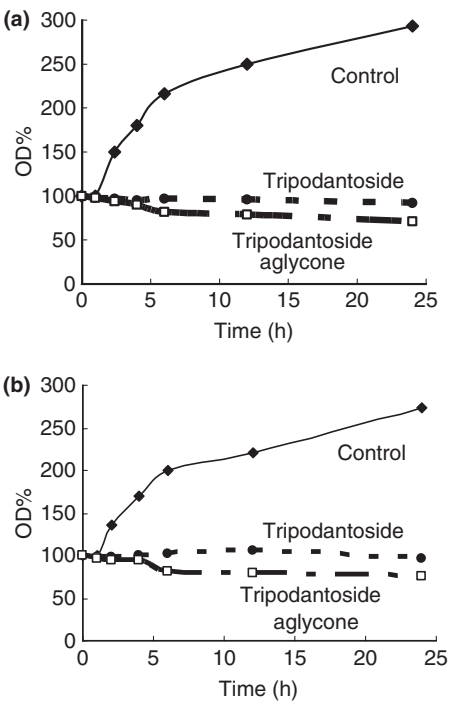

Figure 4 Growth curves obtained by photometric assays with bacteria exposed to tripodantoside and tripodantoside aglycone. (a) *Staphylococcus aureus*. (b) *Pseudomonas aeruginosa*.

Sytox green assays

Bacteria exposed to tripodantoside aglycone showed intense fluorescence after Sytox green stain in contrast to tripodantoside effect or control experiments (Fig. 5). These findings suggested that tripodantoside aglycone exerts the antibacterial effects by damage on cell envelopes, while tripodantoside does not act in the same manner at the assayed concentrations.

Transmission electron microscopy

Pseudomonas aeruginosa strains incubated with tripodantoside aglycone showed damages on cell wall and plasma membrane along with cytoplasm loss areas. *Staphylococcus aureus* exhibited a noteworthy amount of external vesicles emerging from cell surface, which could be interpreted as toxicity on cell envelope (Rolinson *et al.* 1977) (Fig. 6). There were no detectable changes on cell morphology when tripodantoside was assayed.

Discussion

Five bacteriostatic and bactericidal phenolic compounds were isolated and identified from *T. acutifolius* infusion. Flavonoids from plant sources are well known to possess antibacterial activity (Andersen and Markham 2006). Although the four isolated flavonoids had been previously referred as antibacterial substances (Hidalgo Báez *et al.* 1998; Basile *et al.* 2000; Nijveldt *et al.* 2001), they were not previously detected or characterized in *T. acutifolius*. Tripodantoside is the main antibacterial compound found in *T. acutifolius* infusion. This substance has an unusual phenylbutanoid structure (Chu *et al.* 1993). To the best of our knowledge, this is the first report on the purification and identification of this compound in an angiosperm species and the first report on its antibacterial activity. Significant differences ($P < 0{\cdot}05$) among the MICs and MBCs of tripodantoside and its aglycone were

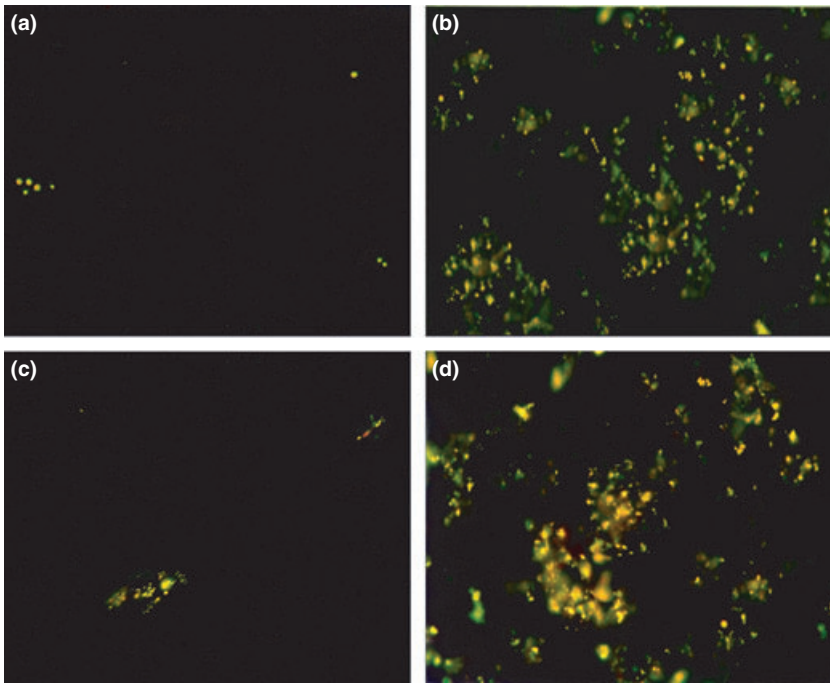

Figure 5 Sytox green assay. (a) *Staphylococcus aureus* (growth control). (b) *Staph. aureus* (exposed to tripodantoside aglycone). (c) *Pseudomonas aeruginosa* (growth control). (d) *Ps. aeruginosa* (exposed to tripodantoside aglycone).

found when the weight/volume unit (μg ml^{-1}) were compared. Nevertheless, these differences were not significant ($P > 0{\cdot}05$) when the amount of substance is expressed in μmol ml^{-1} (concentration units) of each compound, suggesting that the sugar moiety does not contribute to the antibacterial effect. Bacteriostatic, bactericidal or bacteriolytic effects of antibacterial substances on target cells depend on incubation period and bacterial inoculum concentration (Tornatore *et al.* 1997). The inoculation size in photometric assay was 20-fold greater (because of photometric measurements) than that proposed by the *National Committee for Clinical Laboratory Standards* (NCCLS 1999), that is the reason why the substances were assayed at concentrations nearly 20-fold MICs (Hartzen *et al.* 1997). Our results suggest that tripodantoside aglycone may act with a bacteriolytic mechanism against the

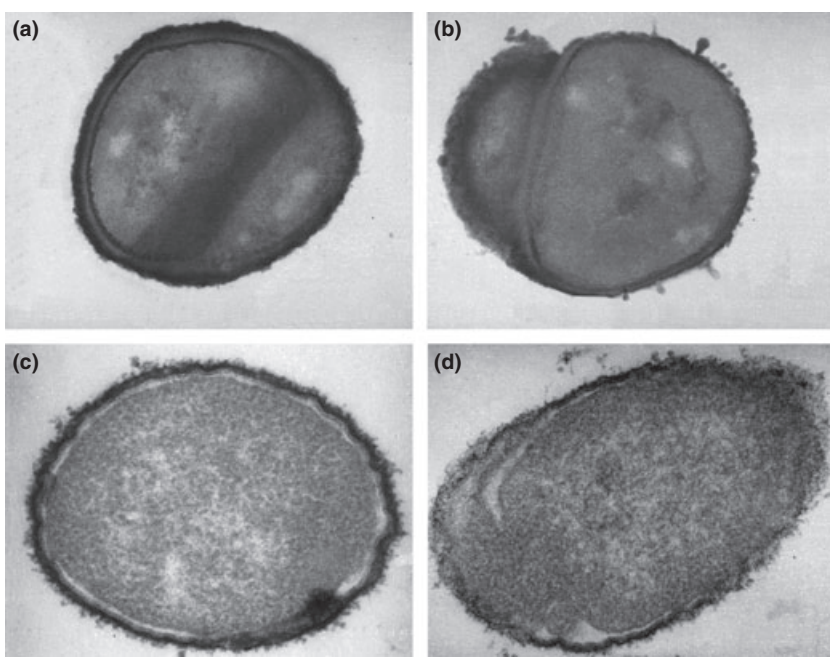

Figure 6 Transmission electron microscopy assay. (a) *Staphylococcus aureus* (growth control). (b) *Staph. aureus* (exposed to tripodantoside aglycone). (c) *Pseudomonas aeruginosa* (growth control). (d) *Ps. aeruginosa* (exposed to tripodantoside aglycone).

assayed strains according to the strain growth decreasing curves. Sytox green is a cationic molecule which can easily enter into cells with external cover damages and bound to nucleic acids, thus emitting intense green fluorescence after excitation between 420 and 490 nm radiations, while cannot enter into those cells with intact covers. These properties make Sytox green a suitable indicator for the visualization of both Gram positive and Gram negative bacteria with damages in cell walls or cell membranes (Langsrud and Sundheim 1996), as those exposed to tripodantoside aglycone. Horseradish peroxidase was used as cell cytochemical marker, because its reaction products enhance the thin section contrast to TEM (Gross *et al.* 1986). The bacteriolytic effect of tripodantoside aglycone was clearly observed by TEM for both of the assayed strains. Alcohols (like tripodantoside aglycone) are known to possess bactericidal rather than bacteriostatic activity against vegetative cells, probably acting as either protein denaturing agents (Pelczar *et al.* 1988), solvents or dehydrating agents (Dorman and Deans 2000), which may originate cell lysis. Only two reports on phenylbutanol derivatives with antibacterial activity were found (Lang and Rye 1972; Wilson *et al.* 1981). Wilson *et al.* (1981) suggested that the biological activity would be attributed to the fact that the hydrocarbon portion of aryl-alcohols (as phenylbutanols) may enter the cell and bound within the phospholipid region of the cell by hydrophobic interactions, and conversely the hydroxyl polar groups associate with the protein layer in Gram negative cells, all of this could lead to interferences with the membrane-bound enzymes and lead to physicochemical changes. As tripodantoside aglycone has a free aliphatic hydroxyl polar group on C2, this sort of action could be possible, while this effect would not be possible with tripodantoside, which lacks this group. Aryl-alcohols are ascribed to possess the ability to cause cell lysis by damages on bacterial cell membrane ultra structure (Silva *et al.* 1976). Taking this into account, it would seem reasonable to predict that tripodantoside aglycone would react in a similar manner. Phenolic compounds with vicinal hydroxyl groups (as catechol) possess antimicrobial activity (Suortti 1983). Alkyl high polar substituents on catechol rings are known to confer less antibacterial potency than that because of simpler phenolic compounds (Suortti 1983). All these facts lead us to conclude that the polarity increase by glycosilation or the bulky sugar moiety may be the reason of the absence of bacteriolytic effect for tripodantoside.

The antibacterial activity exhibited by all of the isolated compounds justifies, at least partially, the antibacterial effect of *T. acutifolius* infusion. Because tripodantoside has better antibacterial skills and is present at a significant higher amount than flavonoids in the dry leaves, it would be possible that tripodantoside be one of the main compounds responsible for *T. acutifolius* antibacterial effects. The high extraction yield of tripodantoside turns it an interesting substance to perform chemical modifications to improve its biological activities.

*4-(3′,4′-dihydroxyphenyl)-2-R-butanol-2-*O*-β-D-glucopyranoside (tripodantoside)*

Yellow amorphous powder; $[\alpha]_D^{25} = -41{\cdot}7^{o}$ ($CHCl_3$, c 0·2320); $\lambda_{max(\log \varepsilon)} = 283$ (4·05) nm; HRMS: $m/z = 344{\cdot}1479$ $[M]^+$ (calc. for $C_{16}H_{24}O_8$ 344·1471). Peracetylated derivative in $CDCl_3$, ^{1}H NMR: δ 1·12 (d, J = 6·21 Hz, 3H), 1,73 (m, 1H), 1·82 (m, 1H), 2·00 (s, 3H), 2·02(s, 3H), 2·04 (s, 3H), 2,06 (s, 3H), 2,27 (s, 6H), 2,69 (m, 2H), 3,66 (ddd, J = 9·79 Hz; 4·58 Hz; 2·52 Hz, 1H)**, 3·77 (ddq, J = 6·21 Hz; 3·97 Hz; 3·97 Hz, 1H), 4·13 (dd, J = 2·53 Hz; 12·28 Hz, 1H), 4·25 (dd, J = 4·69 Hz; 12·28 Hz, 1H), 4·53 (d, J = 7·86 Hz, 1H), 4·98 (dd, J = 7·86 Hz; 9·45 Hz, 1H), 5·10 (t, J = 9·45 Hz, 1H), 5·21 (t, J = 9·45 Hz, 1H), 7·02 (brd, J = 1·78 Hz), 7·06 (Overlapping m, 2H). ^{13}C NMR δ: 19·9 (q), 20·6 (q), 30·6 (t), 38·1 (t), 61·9 (t), 68·4 (d), 71·5 (d), 71·7 (d), 72·9 (d), 74·6 (d), 99·2 (d), 123·0 (d), 123·4 (d), 126·7 (d), 140·0 (s), 141·1 (s), 141·8 (s), 168·4 (s), 168·4 (s), 169·3 (s), 169·4 (s), 170·3 (s), 170·7 (s). See Fig. S1 for the NMR spectra. **J values estimated using J VISUALIZER ver. 2.5 Java software (Schimanski 2002).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 NMR spectra and GC-MS fragmentation pathways obtained for tripodantoside and peracetylated tripodantoside.

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