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Communication

Arjunolic Acid in the Ethanolic Extract of *Combretum leprosum* Root and its Use as a Potential Multi-Functional Phytomedicine and Drug for Neurodegenerative Disorders: Anti-Inflammatory and Anticholinesterasic Activities

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Os extratos etanólicos das folhas e raízes de *Combretum leprosum* Mart. & Eicher (Combretaceae) foram investigados por CGAR-EM, após derivatização com BSTFA/ TMCS. Mono e oligossacarídeos, ácidos graxos e triterpenos foram identificados como componentes majoritários. Análise quantitativa por padronização externa revelou a presença de 65% de ácido arjunólico (**1**) no extrato etanólico das raízes secas. Foram observadas atividades anti-inflamatória, antinociceptiva e anticolinesterásicas (AChE e BuChE) para o extrato das raízes e para o ácido arjunólico, descortinando uma nova classe de produtos naturais no tratamento da doença de Alzheimer através de drogas multi-funcionais.

Combretum leprosum Mart. & Eicher (Combretaceae) leaves and roots ethanolic extracts were investigated by HRGC-MS and showed mono- and oligosaccharides, fatty acids and triterpenes as major compounds after derivatization with BSTFA/ TMCS. Arjunolic acid (**1**) was quantified on dried roots ethanolic extract (65%) by external standard. Anti-inflammatory, antinociceptive and anticholinesterasic (AChE and BuChE) activities were observed for roots ethanolic extract of *C. leprosum* and arjunolic acid, suggesting both as promising targets for the development of innovative multi-functional medicines for Alzheimer desease treatment.

Keywords: *Combretum leprosum*, Combretaceae, HRGC-MS, arjunolic acid, anti-inflammatory, cholinesterase inhibitor

Introduction

Combretum leprosum Mart. & Eicher (Combretaceae), native from Brazil, is a widely distributed shrub in Brazilian Caatinga from Piauí to Bahia states and is commonly known as "mofumbo".[1] It is used by local people on the cicatrisation of wounds, in the treatment of haemorrhages or as a sedative,[2] and the analgesic property of the ethanolic stem barks extract was recently described.[3]

Braz-Filho *et al.*[4] studied two genera of Combretaceae. From *Thiloa glaucocarpa* they isolated arjunolic acid (**1**) and from *Combretum* sp., 3,3',4,5-tetra-*O*-methylflavellagic acid, ethyl β-D-glucopyranoside and arjunglucoside.[4] *C. leprosum* roots and leaves led to the isolation of arjunolic and mollic acids, 3β,6β,16β-trihydroxylup-20(29)-ene (**2**), together with the glycosilated flavonoids 3-*O*-methylquercetin and quercetrin.[5]

Arjunolic acid (2α, 3β, 23-trihydroxyolean-12-en-28-oic acid, **1**) was first isolated from *Terminalia arjuna* (Combretaceae), which has been used as a cardiac tonic by native Ayurvedic Indian people for centuries.[6] A patent

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based on the hormonal, wound healing and bactericide (particularly *Mycobacterium tuberculosis* and *M. leprae*) properties of arjunolic acid and its isomer, asiatic acid (2α, 3β, 23-trihydroxyursan-12-en-28-oic acid, **3**) was deposited[7] and, since then, a series of other biological activities of arjunolic acid were described.[8]

Continuing our search for anti-inflammatory and anticholinesterasic agents from natural sources,[9] we found *Combretum leprosum* as an interesting investigation target. It merged from the relatively few reported information about pharmacological profile of *C. leprosum* and the structural similarity of arjunolic and asiatic acids.

Analysis of the leaf ethanolic extract of *C. leprosum* by HRGC-MS,[10,11] after derivatization with BSTFA/TMCS (1%) in pyridine,[12] led to the identification of monosaccharides as major compounds (80%), followed by minor oligosaccharides (5%), fatty acids (3%) and triterpenes (10%). The known pentacyclic triterpenes 3β,6β,16β-trihydroxylup-20(29)-ene (**2**) and arjunolic acid (**1**) were also derivatized and co-injected with the leaf extract, confirming their presence in very low amounts (Figure 1).

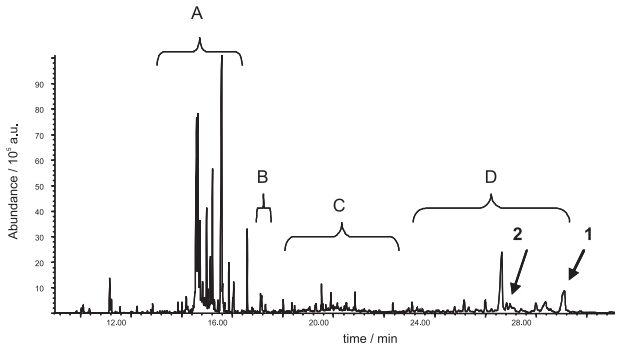


Figure 1. Fragmentogram of TMS leaf ethanolic extract of *C. leprosum.* A(monosaccharides), B (fatty acids), C (oligosaccharides), D (triterpenes), arjunolic acid (**1**) and 3β,6β,16β-trihydroxylup-20(29)-ene (**2**).

HRGC-MS of roots ethanolic extract of *C. leprosum* derivatized with BSTA/TMS revealed a high amount of arjunolic acid (**1**) besides minor triterpene (Figure 2, D), all them with two characteristic fragments at *m/z* 203 and 320 due to a retro Diels-Alder rearrangement at C-ring double bond.[13] Carbohydrates, mainly represented by monosaccharides (confirmed by co-injection of derivatized glucose and fructose) and fatty acids (oleic, linoleic and linolenic acids), were also observed. Co-injection with derivatized arjunolic acid and external standard calibration revealed the presence of **1** in 65% on the ethanolic extract obtained from dried roots.

Three sets of assays were used to investigate the anti-inflammatory activity of roots and leaves extracts and of pure arjunolic acid (**1**).[14] Unlike leaves, roots extract (200

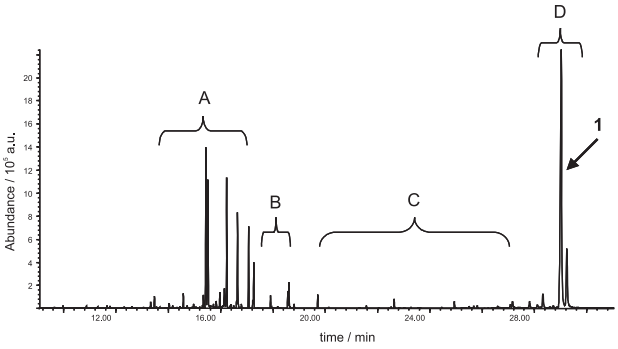


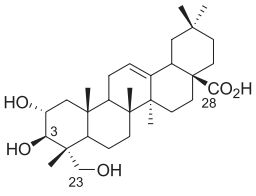
Figure 2. Fragmentogram of TMS roots ethanolic extract of *C. leprosum.* A (monosaccharides), B (fatty acids), C (oligosaccharides), D (triterpenes) and arjunolic acid (**1**).

mg kg^{-1}) and arjunolic acid (100 mg kg^{-1}) were able to inhibit significantly the carrageenan-induced paw edema with 37.6% and 80.8% of inhibition, respectively. At this dose, arjunolic acid was very gastrotoxic inducing a hemorrhagic damage of the gastric mucosa.[15] Meanwhile, at a lower dose of 10 mg kg^{-1}, toxicity was not observed and arjunolic acid (**1**) stayed inhibiting significantly the edema by 37.6%. Root extract (200 mg kg^{-1}) and arjunolic acid (10 mg kg^{-1}) inhibited the acetic acid-induced constrictions by 33% and 30.3% respectively.[14]

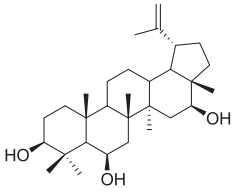
The arachidonic acid (AA) and 12-*O*-tetradecanophorbol-13-acetate (TPA) induced ear edemas are widely used to evaluate the anti-inflammatory activity of cyclooxygenase (COX) and lipoxygenase (LOX) inhibitors. Arjunolic acid showed a similar profile of indomethacin, inhibiting the AA-induced ear edema by 55.5% without interfere with the TPA-induced one.[16] Taken together, the overall results suggested that arjunolic acid affect the AA metabolism by cyclooxygenase, thus exerting its anti-inflammatory and antinociceptive activities.

Similar to anti-inflammatory results, only the root extract inhibited acetyl and butyril cholinesterase enzymes. Using the modified Ellman's method in TLC,[17] arjunolic acid proved to be the responsible for these activities. The acid **1** inhibited both cholinesterases at the same reference compound concentration limit (physostigmine, 0.01 mmol L^{-1}). No cholinesterase activity was observed for 3β,6β,16β-trihydroxylup-20(29)-ene (**2**) and nor to 3β-hydroxylup-20(29)-ene (lupeol). Associated to the negative cholinesterase activity observed for oleanolic acid (3β-hydroxyolean-12-en-28-oic acid),[18] these results suggest an important biological role in C(2)- and C(23)-OH groups added to topological differences on these triterpenes. Studies on the neuroprotective action of asiatic acid (2α, 3β, 23-trihydroxyursan-12-en-28-oic acid, **3**) and derivatives showed that free C(28)-CO_2H and the triol groups are very important against (Aβ)-induced neurotoxicity.[19]

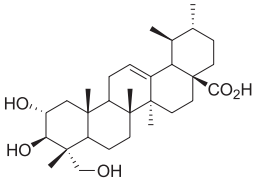
The small structural difference between arjunolic (**1**) and asiatic (**3**) acids, together with literature data on the oxidative stress reduction property of both acids [20] and our anti-inflammatory and anticholinesterasic results of arjunolic acid, suggest these compounds as promising models for the development of innovative multi-functional drugs for AD treatment.[21] As "Mofumbo" root major compound is arjunolic acid, followed by small amounts of monosaccharide and fatty acids, it can be a powerful candidate for the development of a new phytomedicine for neurodegenerative disorders.



arjunolic acid (**1**)



3β-6β-16β-trihydroxylup-20(29)-ene (**2**)



asiatic acid (**3**)

Supplementary Information

Supplementary data are available free of charge as PDF file at http://jbcs.sbq.org.br.

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10. *Crude extracts of C. leprosum and arjunolic acid (**1**) purification. C. leprosum* leaves and roots were collected in 2001 at Cocalzinho-Viçosa, Ceará, Brazil, and a voucher specimen (no. 12446) is deposited at the Herbarium Prisco Bezerra of the Biology Department, UFC, Ceará, Brazil. Dried and finely powdered leaves and roots of *C. leprosum,* 3β,6β,16β-trihydroxylup-20(29)-ene and arjunolic acid were extracted as described in reference 5.
11. *HRGC and HRGC-MS analyses.* HRGC (FID at 280 ºC) and HRGC-MS (ionization energy at 70 eV, ion source at 200 ºC, transfer line at 280 ºC) were performed on an Agilent 6890 GC with a FID and coupled to an Agilent mass selective detector 5973. Analyses were carried out on a HP-5 (17 m X 0.11 mm X 0.2 mm) capillary column. Helium was used as carrier gas (1.0 mL min^{-1}) and the injection port was set in the splitless mode at 280 ºC (0.5 min). Oven temperature was programmed from 100 ºC (10 ºC min^{-1}) to 310 ºC (15 min). Quantification of the dried roots ethanolic extract was performed by the external standard method and all the TMS derivative samples were run in triplicate. The calibration graph was constructed at concentrations of 0.5 to 5 mg per mL of arjunolic acid and presented a good linearity (r = 0.9930 to 0.9995).
12. *Derivatization.* TMS ether derivatives of the roots and leaves ethanolic extracts of *C. leprosum,* arjunolic acid, 3β,6β,16β-trihydroxylup-20(29)-ene, glucose and fructose were prepared by adding 100 μL of 1% TMCS/BSTFA reagent plus 100 μL

of pyridine to 15-100 mg of material following by heat at 60 °C for 60 min. Both ethanolic plant extracts were filtered before derivatization.

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14. The anti-inflammatory and analgesic properties of the extracts and arjunolic acid were evaluated in the carrageenan-induced rat paw edema (CIRPE) (a), acetic acid-induced mice abdominal constrictions (b), as previously described in: Ribeiro, I.G.; Silva, K. C. M.; Parrini, S. C.; Miranda, A. L. P.; Fraga, C. A. M.; Barreiro, E. J.; *Eur. J. Med. Chem.* **1998**, *33*, 225, and in the arachidonic acid (AA)- and 12-*O*-tetra-decanoylphorbol 13-acetate (TPA)-induced mice ear edema (c) as described in: Sanchez, T.; Moreno, J. J.; *Prostag. Oth. Lipid M.* **1999,** *57*, 119. Wistar rats (120 – 200 g) and Swiss albino mice (18 – 25 g) of both sexes were obtained from LASSBio (Faculty of Pharmacy, UFRJ, Brazil) breeding unit. Extracts (200 mg kg^{-1}) and arjunolic acid (10 and 100 mg kg^{-1}) were administered orally (0.1 mL *per* 20 g) as a suspension in EtOH/Tween 80/H_2O (2:2:20, v/v/v) (vehicle) one hour before the algogenic stimulus. A positive control group received indomethacin (10 or 37.5 mg kg^{-1}). Control animals received an equal volume of vehicle. In experiment (a), rats were injected subplantarly with either 0.1 mL of 1% carrageenan solution in saline (1 mg *per* paw) or sterile saline (NaCl 0.9%) into one of the hind paws, respectively. Paw volumes were measured 3h after carrageenan injection and edema calculated as the volume difference between the carrageenan and saline-treated paw. In experiment (b), mice were injected intraperitoneally with acetic acid 0.6% (0.1 mL *per* 10 g) and ten minutes after the number of constrictions *per* animal was recorded for 20 minutes. In experiment (c), 20 μL of TPA (2 μg *per* ear) or AA (500 μg *per* ear), dissolved in acetone, were applied to the inner and outer surface of the right ear of the mice. The left ear received acetone. The right and left ears were cute (6h after TPA- and 1h after AA-challenge), weighed and ear edema was measured as the difference in weight between the challenged and the unchallenged ear.
15. In the CIRPE model ulcerogenic effect in rats was investigated as described in: Chan, C. C.; Boice, S.; Brideau, C.; Fordhutchinson, A. W.; Gordon, R.; Guay, D.; Hill, R. G.; Li, C. S.; Mancini, J.; Penneton, M.; Prasit, P.; Rasori, R.; Riendeau, D.; Roy, P.; Tagari, P.; Vickers, P.; Wong, E.; Rodger, I. W.; *J. Pharmacol. Exper. Ther.* **1995***, 274,* 1531. Briefly, animals were euthanized and the stomachs excised along its greater curvature for visualization of gastric lesions with a stereomicroscope.
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