ACTA
AMAZONICA

Bioactive dihydroxyfuranonaphthoquinones from the bark of *Tabebuia incana* A.H. Gentry (Bignoniaceae) and HPLC analysis of commercial *pau d'arco* and certified *T.incana* bark infusions[†]

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ABSTRACT

Tabebuia incana A.H. Gentry (Bignoniaceae) is a tree from the Brazilian Amazon having medicinal uses and is one several *Tabebuia* spp. known as *pau d'arco* or *palo de arco* in this region. Fractionation of the bark ethanolic extract afforded a mixture of 5 and 8-hydroxy-2-(1-hydroxyethyl)naphtho[2,3-b]furan-4,9-diones (1 and 2, respectively) identified on the basis of nuclear magnetic resonance (NMR), infrared (IR) and mass (MS) spectra, whose *in vitro* antimalarial and antitumor activity have been shown previously. This is the first study on *T. incana* bark, and 2 are described in this species for the first time. Also, high performance liquid chromatography (HPLC) analysis of *T. incana* bark tea revealed the presence of the 1 + 2 mixture peak corresponding to a concentration in the range 10^{-6}-10^{-5} M. The chromatograms of teas prepared from commercial *pau d´arco* and *T. incana* bark were also studied and the presence of the 1 + 2 peak has potential for quality control of commercial plant materials.

KEYWORDS

Artemia franciscana Leach, 5-hydroxy-2-(1-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione, 8-hydroxy-2-(1-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione.

Diidroxifuranonaftoquinonas bioativas das cascas de Tabebuia incana *A.H. Gentry (Bignoniaceae) e análise por CLAE de infusões de cascas de pau d´arco comercial e* T. incana *certificada*

RESUMO

Tabebuia incana *A.H. Gentry (Bignoniaceae) é uma árvore da Amazônia brasileira com usos medicinais. É uma de várias espécies de* Tabebuia *conhecidas como* pau d'arco *ou* palo de arco *nesta região. O fracionamento do extrato etanólico das cascas resultou no isolamento da mistura de 5 e 8-hidróxi-2-(1-hidroxietil)nafto[2,3-b]furano-4,9-dionas (1 e 2, respectivamente), identificadas com base em seus espectros de ressonância magnética nuclear (RMN), infravermelho (IV) e massa (EM), e cujas atividades antimalárica e antitumoral* in vitro *foram mostradas previamente. Este é o primeiro estudo das cascas de* T. incana *e a primeira vez que o composto* **2** *é descrito nesta espécie. Análises por cromatografia liquida de alto empenho (CLAE) do chá das cascas de* T. incana *revelaram a presença de um pico correspondente à mistura de 1 + 2, permitindo inferir uma concentração na faixa de 10^{-6}-10^{-5} M desses componentes no chá. Os cromatogramas de chás (infusões) preparados a partir das cascas de* pau d´arco *commercial and* T. incana *certificada também foram estudados. A verficação da presença do pico das substâncias 1 + 2 nos cromatogramas tem potencial contribuição para o controle de qualidade de material vegetal comercial.*

PALAVRAS-CHAVE

Artemia franciscana *Leach, 5-hydroxy-2-(1-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione, 8-hydroxy-2-(1-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione.*

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INTRODUCTION

Tabebuia incana is an Amazonian tree, popularly known as "ipê amarelo" and "pau d'arco" (Silva *et al.*, 1977; Oliveira *et al.*, 1993). Pau d'arco bark tea is used by the local population for the treatment of inflammation, malaria, cancer, kidney and liver disorders (Silva *et al.*, 1977). In previous reports, only the trunkwood has been studied and prenylnaphthoquinones predominate among the isolated compounds. Thus, furanonaphthoquinones, such as (-)-**1** (Figure 1), the dehydrofuranonaphthoquinones dehydro-5-hydroxy-iso-a-lapachone and dehydro-iso-a-lapachone, together with lapachol, lapachenol, tecomaquinone I, and the lignans cycloolivil and pawlownin are all known components of the trunkwood (Oliveira *et al.*, 1990; Oliveira *et al.*, 1993).

In our previously published screenings on the cytotoxicity of Amazonian plant extracts towards the brine shrimp larva species *Artemia franciscana*, bark water extracts of *T. incana* were found to be inactive and methanol extracts showed moderate lethality (Quignard *et al.*, 2003; Quignard *et al.*, 2004). Also, bark methanol extracts were completely inactive to the larvae of the insect species *Aedes aegypti*, the hemorrhagic dengue fever vector (Pohlit *et al.*, 2004). In this report, a mixture of furanonaphthoquinones **1** and **2** was isolated from the bark of *T. incana* and these compounds were detected in bark teas using High Performance Liquid Chromatography (HPLC).

MATERIAL AND METHODS

PHYTOCHEMICAL STUDY

T. incana bark was collected from a previously catalogued individual at INPA´s Ducke Forest Reserve (Manaus) in January, 2001. A voucher specimen is deposited in the INPA Herbarium under the number 23866 and was identified by A. H. Gentry. Dried, milled bark (1.3 kg) was macerated in ethanol (2 - 7 days) and the extract (46.8 g, 3.6 %) was obtained after filtration and total evaporation. A portion of this extract (44.3 g) was suspended in methanol-water and partitioned in increasingly polar solvents, yielding, after total evaporation, to hexane (4.4 g), chloroform (11.1 g) and methanol-water (27.2 g) fractions. The chloroform fraction (8.85 g) was chromatographed on a column of silica gel 60 (63-210 mm) using a chloroform-methanol gradient as eluent, providing 4 sub-fractions. Sub-fractions 2 (82 mg) and 3 (2.2 g) were separately fractionated by normal phase preparative thin-layer chromatography (TLC, eluents: hexane-ethyl acetate) and by two sequential, normal phase column chromatographic separations, respectively. Sub-fractions obtained from these procedures were combined (53.1 mg) based on TLC and submitted to reverse-phase column chromatography (acetonitrile-acetic acid-water), affording a mixture of compounds **1** and **2** (6.5 mg, 0.015 % based on extract).

Mixtures of **1** and **2** are known to be unseparable on silica gel. The only known separation was performed by Fujimoto *et al.* (1991) who synthesized a mixture of (±)-**1** and (±)-**2** and achieved separation by first derivatizing as the myristoyl diesters, followed by normal phase chromatographic separation, and finally by saponification. Such separation was beyond the scope of this work due to, among other reasons, the low yield of the **1** + **2** mixture isolated from *T. incana* bark.

5- AND 8-HYDROXY-2-(1-HYDROXYETHYL)NAPHTHO[2,3-B]FURAN-4,9-DIONES (**1** AND **2**)

This mixture was obtained as a yellow amorphous powder which presented the following spectral data: IR (KBr) n_{max} cm^{-1} 3424, 3123, 2925, 1673, 1641, 1596, 1534; MS (70 eV) *m/z* (rel. int.) 258 $[M]^{+\bullet}$ (79), 243 (100), 215 (45), 221 (25), 187 (23), 63 (26), 43 (33); ^1H NMR (200 MHz, $CDCl_3$, TMS) ä 12.18 (s, 1H, 5-OH), 12.03 (s, 1H, 8-OH), 7.75 (m, 2H), 7.61 (m, 2H), 7.29 (m, 2H), 6.85 (bs, 1H), 6.84 (bs, 1H), 5.05 (q, *J* = 6.6 Hz, 2H), 1.8 (bs, 2H, 1'-OH), 1.66 (d, *J* = 6.6 Hz, 6H).

BIOLOGICAL ASSAY

Extract, fractions obtained from partition, chromatographic fractions and the isolated mixture were tested for lethality in the brine shrimp assay using *Artemia franciscana* larvae (McLaughlin *et al.* 1991; Meyer *et al.*, 1982). The dose-response curve was generated by submitting log concentration and lethality data to *probit* analysis, from which median lethal concentrations (LC_{50})

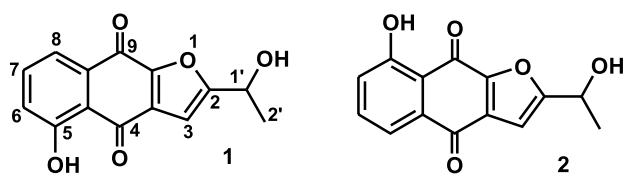

Figure 1 - Structures of the furanonaphthoquinones isolated as a mixture from *Tabebuia incana* bark ethanol extract.

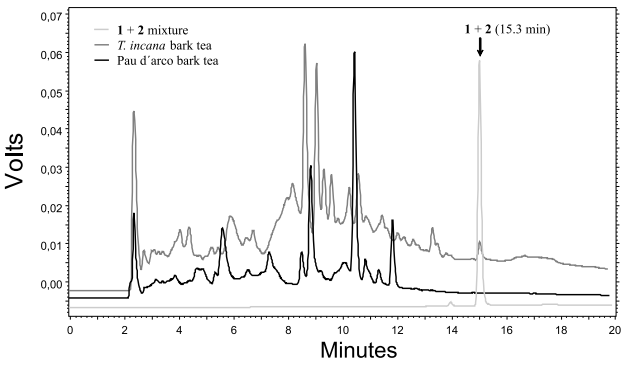

Figure 2 - HPLC chromatograms of *pau d´arco* bark teas and **1** + **2** sample.

and standard deviation were obtained, according to the method described by Litchfield and Wilcoxon (1949).

PREPARATION OF *T. incana* AND PAU D´ARCO BARK TEA (INFUSION).

Medicinal herb sellers at the Adolpho Lisboa Municipal Market in Manaus´s downtown provided information on the preparation (proportion of water to plant material, infusion method) of *pau d´arco* bark tea: milled bark (17 g) was infused in de-ionized water (100 ºC, 500 ml, 20 min) and after filtration the filtrate (tea) was stored in a freezer. Thus, bark teas were prepared using commercial *pau d´arco* samples as well as *T. incana* collected and identified in our study.

HPLC ANALYSIS OF *T. incana* AND PAU D´ARCO BARK TEAS.

HPLC was performed using a Shimadzu LC-10AT Liquid Chromatograph (SPD-10A UV-vis Detector, DGU-14A Degasser, FCV-10L Mixer, SCL System Controller, Rheodyne (manual) injector valve, 10 ml sample loop, and Shim-Pack CLC-ODS column (250 × 4.6 mm, 5 ìm particle size). Chromatographic analysis was performed under the following conditions: flow rate: 1.0 ml×min^{-1}, mobile phase: MeOH / H_2O 45:55 (0 min), linear gradient to 80:20 (10 min), then isocratic until end of run (20 min), detection at l = 254 nm. 30 min equilibration time was used. *T. incana* and *pau d´arco* bark teas, as well as the **1** + **2** mixture, were analyzed and the resulting chromatograms are presented in Figure 2.

In an attempt to provide an estimate for the quantity of the mixture in *T. incana* tea, and given that pure **1** and **2** were not obtainable, the *T. incana* bark tea and the 2:1 mixture of **1** and **2** (at concentrations of 1, 2 and 4 mg.l^{-1}) were analyzed in triplicate (Table 1) under the same chromatographic conditions as used to generate the chromatograms in Figure 2. A calibration curve was established from which the approximate concentration of the **1** + **2** mixture in the bark tea was established using linear regression analysis (Figure 3). It was assumed based on Fujimoto *et al*. (1991) UV data (which included molar extinction coefficients for both **1** and **2**) that the molar extinctions at 254 nm (detector wavelength) of **1** and **2** differ by an order of magnitude more or less, so that the *T. incana* tea prepared can be presumed to be 10^{-6}-10^{-5} M in **1** and / or **2**.

Table 1 - Calibration and tea peak areas corresponding to **1** + **2** mixture.

Standard Solution (mg / L)	Peak Areas	Avg. Peak Areas	SD
1	38507 37382 34237	36709	2213
2	79265 69553 63604	70807	'
4	157603 151355 141755	150238	7983
Bark tea	34321 34280 34044	34215	150

Peak areas refer to peak with t_R = 15.3 min (3 repetitions).

RESULTS AND DISCUSSION

The existence of isomers in the isolated mixture was evidenced by the chemical shifts of the *peri* hydroxyl groups of **1** (d 12.18) and **2** (12.03) in the ^{1}H NMR spectrum. As pointed out by Wagner *et al.* (1989), this is the only conspicuous spectroscopic difference between these isomers. Moreover, the ^{1}H NMR spectrum presented signals in pairs, with different intensities, indicating the presence of constitutional isomers. The relative intensity / integrals of signals suggested the proportion of **1** and **2** to be *ca.* 2:1. The IR spectrum exhibited absorptions at 1673 and 1641 cm^{-1} due to carbonyl stretching characteristic of naphthoquinones, while the mass spectrum presented a $[M]^{+\bullet}$ peak with *m/z* 258, compatible with the molecular formula $C_{14}H_{10}O_5$ of **1** and **2**. ^{1}H NMR data for **1** and **2** have been reported (Fujimoto *et al.*, 1991). Our ^{1}H NMR data for the **1** + **2** mixture were in general consistent with the previously reported data.

Mixtures of these two compounds have been isolated from *T. ochracea* ssp. *neochrysantha* (Pérez *et al.*, 1997) and *T. avellanedae* (Fujimoto *et al.*, 1991) bark and have been shown to possess important activity against rodent and human malaria parasites *Plasmodium berghei* and *P. falciparum* (Pérez *et al.*, 1997), respectively. Also, compound **1**, extracted from dry *T. avellanedae* bark, is active *in vitro* against several types of malignant tumor cells and was patented in Japan in 1994 (Derwent, 2006). Furanonaphthoquinone **2**, isolated from *T. barbata* bark, was significantly cytotoxic against A-549 human lung adenocarcinoma, MCF-7 human breast carcinoma, and HT-29 human colon carcinoma cells (Saizarbitoria *et al.,* 1997).

Lethality in the brine shrimp assay has been correlated with *in vitro* antitumor activity in general and this assay is considered an important pre-screen for biomonitored isolation of cytotoxic compounds and anticancer drug research (McLaughlin *et al.*, 1991). The ethanol extract and its chloroform fraction showed significant lethality (LC_{50} 167 ± 39 and 12 ± 4 mg.ml^{-1}, respectively) towards brine shrimp larvae, however, hexane and water-methanol fractions were inactive. These results show that during partition, cytotoxicity was concentrated solely in the

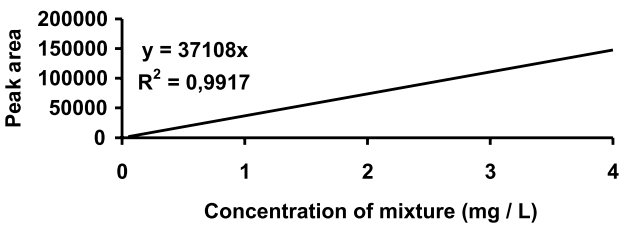

Figure 3 - Linear regression analysis of peak area *vs*. [mixture **1** + **2**]

fraction of intermediate polarity. The isolated mixture of **1** and **2** was about as active (LC_{50} 15 ± 10 mg.ml^{-1}) as the chloroform fraction (from which this mixture was isolated) towards brine shrimp consistent with the existence of other cytotoxic components in the chloroform fraction.

Despite published studies on more than 20 *Tabebuia* spp., furanonaphthoquinone **1** has so far only been reported in *T. avellanadae*, *T. chrysotricha*, *T. incana*, *T. ochracea* and *T. rosea*, while **2** has been found in *T. barbata*, *T. cassinoides, T. ochracea* and (given the results presented above) *T. incana* (Morais, 2003).

Under the HPLC conditions developed, *pau d´arco* bark teas presenting **1** + **2** are readily distinguished from those in which these compounds are absent. The HPLC chromatogram of the *T. incana* bark tea (Figure 2) reveals the **1** + **2** mixture peak at t_R 15.3 min. Given that *pau d´arco* (*palo de arco*) is among the most important Amazonian plants sold in European markets, it is conceivable that HPLC analysis using **1** and **2** as (bio)markers may be important for quality control and the distinction of *T. incana* from other species which are also commercialized using the name *pau d´arco*, such as *T. serratifolia.* Also, the presence of antitumor and antimalarial compounds **1** and **2** in *T. incana* bark teas lends support to the traditional use of this plant in the treatment of tumors and malaria.

CONCLUSION

The isolated compounds have potential in quality control as marker substances for the *pau d´arco* species *T. incana* and may have use in the detection of adulterated or false plant materials.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support of CNPq/PNOPG (grant numbers 520354/99-0 and 550260/01-3) and the Bioamazônia/BASA/FEPAD contract, as well as scholarships from FAPEAM (SKRM) and CNPq (SGS, SMN, ELJQ).

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Recebido em 30/07/2004
Aceito em 02/01/2007