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Artigo

Secondary metabolites isolated from *Richardia brasiliensis* Gomes (Rubiaceae)

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RESUMO: "Metabólitos secundários isolados de *Richardia brasiliensis* Gomes (Rubiaceae)". A família Rubiaceae compreende cerca de 637 gêneros e aproximadamente 10700 espécies, ocorrendo essencialmente nas regiões tropicais do Brasil. *Richardia brasiliensis* Gomes, popularmente conhecida por "poaia branca", é uma planta nativa da região sul do Brasil, utilizada na medicina popular como anti-emética e no tratamento de diabetes. Este trabalho reporta o isolamento e identificação estrutural de um flavonóide glicosilado, um triterpeno, uma cumarina e dois derivados de ácido benzóico, objetivando contribuir para quimiotaxonomia do gênero *Richardia.* Através deste estudo foi possível isolar e identificar os metabólitos isorametina-3-*O*-rutinosídeo, ácido oleanólico, a cumarina escopoletina e os ácidos *p*-hidroxi-benzóico e *m*-metoxi-*p*-hidroxi-benzóico, todos isolados pela primeira vez no gênero, exceto o último, apresentando, portanto, relevante importância quimiotaxonômica para o mesmo. As estruturas foram identificadas com o uso de técnicas espectroscópicas de IV, RMN ^{1}H e ^{13}C uni e bidimensionais e comparação com dados da literatura.

Unitermos: Rubiaceae, *Richardia brasiliensis*, isorametina-3-*O*-rutinosídeo, ácido oleanólico, escopoletina.

ABSTRACT: The family Rubiaceae comprises around 637 genera and approximately 10,700 species, occurring essentially in tropical regions of Brazil. *Richardia brasiliensis* Gomes, known popularly as "poaia branca", is native to Brazil south region, used in folk medicine as anti-emetic and in the treatment of diabetes. This work reports the isolation and structural identification of a flavonoid glycoside, a triterpene, a coumarin and two benzoic acid derivatives, aiming at contributing to the chemotaxonomy of the genus *Richardia*, through a phytochemical study of *Richardia brasiliensis*. By means of this study the metabolites isorhamnetin-3-*O*-rutinoside, oleanolic acid, the coumarin scopoletin and *p*-hydroxy-benzoic and *m*-methoxy-*p*-hydroxy-benzoic acids were isolated and identified. All of them, but the latter, were isolated for the first time in the genus, thereby presenting relevant chemotaxonomic importance to it. The structures were identified using spectroscopic techniques such as IR, one and two-dimensional ^{1}H and ^{13}C NMR besides comparison with literature data.

Keywords: Rubiaceae, *Richardia brasiliensis*, isorhamnetin-3-*O*-rutinoside, oleanolic acid, scopoletin.

INTRODUCTION

Since ancient times the world population has been using medicinal plants as raw material in the informal treatment, healing and prevention of their illnesses. That use dates the beginning of the civilization, when man took conscience of the need to fight against the diseases that afflicted the body and has arrived nowadays, being often the only effective therapeutic recourse available in many communities (Di Stasi, 1996).

Despite its distant history, a great part of the world flora is unknown because among the 250,000 to 500,000 plant species existing in nature not more than 10% were examined under chemical, biological and pharmacological aspects (Verpoorte, 1998). Brazil has a great diversity on plants that possess non-researched

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medicinal potential and are promising sources of therapeutic and pharmacological innovations to the most diverse areas of human health (Almeida et al., 2001; Souza et al., 2003; Rocha et al., 2005; Amaral et al., 2006; Barbosa-Filho et al., 2006; Barbosa-Filho et al., 2007; Oliveira et al., 2007; Barbosa-Filho et al., 2008).

Knowledge increasingly deepened on medicinal plants, developed through integrated studies in the fields of botany, chemistry, pharmacology, and other related sciences is vital to give support and greater longevity to the use of the floristic potential still existing on the planet.

Continuing the research on medicinal plants, the species *Richardia brasiliensis* Gomes ("poaia-branca") was taken as object of study. This species is used in folk medicine as anti-emetic and in the treatment of diabetes and belongs to the Rubiaceae family which is considered one of the biggest families among the Angiosperms, comprising around 637 genera and approximately 10,700 species, occurring essentially in tropical regions of Brazil and presenting great importance to food, ornamental and pharmaceutical industries (Mongrand et al., 2005; Adolpho et al., 2006; Coelho et al., 2006; Agra et al., 2007).

Rubiaceae species are known as bioproducers of alkaloids, tannins, saponins, steroids, terpenes and flavonoids, besides the report that some species are important to traditional medicine (Adolpho et al., 2006; Carbonezi et al., 2004; Hamerski et al., 2005; Silva et al., 2006; Alam et al., 2008; Cardoso et al., 2008).

Considering the wealth of metabolites of the family Rubiaceae, the phytochemical study of species that represent the genera of this family, especially of the genus *Richardia* whose chemistry is still little known (Tomaz et al., 2008), can lead to the discovery of new sources of naturally active substances. This work related to the phytochemical study of a specie of the genus *Richardia* shows the latter as a bioproducer of different classes of metabolites like terpenes and flavonoid glycoside.

The terpenoids comprise a great family of secondary metabolites that are known by their biological and physiological functions, and for this reason they are used in the pharmaceutical area. Maybe, triterpenes constitute the most important group of terpenoids. They present a lot of medicinal properties, specially the anti-inflammatory, analgesic, cardiovascular and anti-tumor effects (Niero and Malheiros, 2007). On the other hand, the flavonoids are part of the group of phenolic compounds and are known to possess antioxidant, anti-ulcer, cardiovascular, among other activities.

The presence of these classes of compounds in the species *Richardia brasiliensis* shows the potential of this genus to be investigated through pharmacognostic studies.

MATERIAL AND METHODS

Plant material

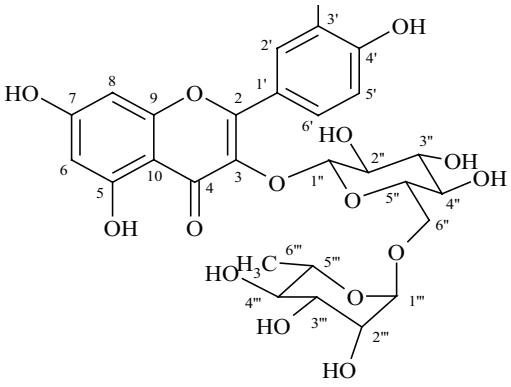
The plant material of *Richardia brasiliensis* Gomes was collected in August 2006, in the city of Santa Rita, State of Paraíba, and was identified by Prof. Maria de Fátima Agra, from the Setor de Botânica of Laboratório de Tecnologia Farmacêutica "Prof. Delby Fernandes de Medeiros" (LTF/UFPB). A voucher specimen is deposited in the Herbário Prof. Lauro Pires Xavier (JPB), from Universidade Federal da Paraíba under the code Agra et al. 3195.

Obtaining and partitioning of the extracts

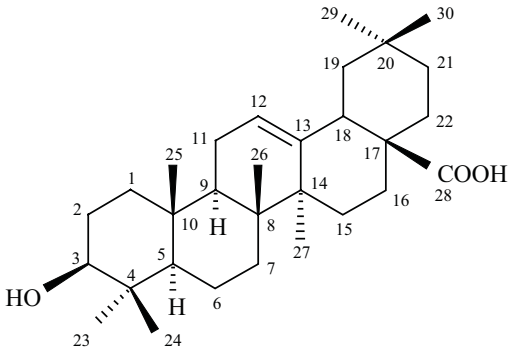
Dried and powdered plant material (2.280 kg) was subjected to exhaustive maceration with ethanol (EtOH) 95% in a stainless steel for three consecutive days. This process was repeated until the maximum extraction of the chemical constituents. The obtained ethanol solution was filtered and then concentrated in rotatory evaporator under average temperature of 50 ^{o}C yielding the crude ethanol extract (CEE) which weighed 177 g with an yield of 7.76 %. 2 g of this extract were separated to preliminary phytochemical evaluation, 25 g were reserved and the rest (150 g) was solubilized in a mixture of MeOH : H_2O (3:7 v/v) under mechanical agitation for 60 minutes, obtaining a hydroalcoholic solution I which was submitted to liquid/liquid partition in a separation funnel, under exhaustive manual agitation, using the solvents hexane, chloroform and ethyl acetate. The obtained solutions were treated with anhydrous sodium sulphate (Na_2SO_4) and submitted to filtration under reduced pressure. After this process, the solvents were evaporated in rotatory evaporator under average temperature of 50 ^{o}C, yielding the following fractions: hexane (23.3 g), chloroform (15 g) and ethyl acetate (6.9 g).

Isolation of the chemical constituents

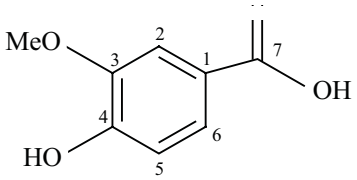
The ethyl acetate fraction (2 g) was subjected to chromatography on a column packed with Sephadex LH 20 and eluted with chloroform and methanol (50% : 50%). 14 fractions were obtained and joined according to their Rf's after being analysed through analytical thin-layer chromatography (TLC). The group of fractions 12-13 was chromatographed on column with Sephadex LH 20, eluted with chloroform and methanol (50% : 50%) to give 9 sub-fractions that were joined according to their Rf's after analysis on analytical TLC. The fraction 12-13.(2) was subjected to preparative TLC using chloroform and ethyl acetate (83% : 17%) as eluents, obtaing 3 sub-fractions. The sub-fraction 12-13.(2).(1) was subjected to successive chromatographic purification, resulting on a yellow solid that was coded



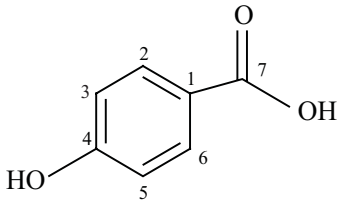
Isorhamnetin-3-*O*-rutinoside



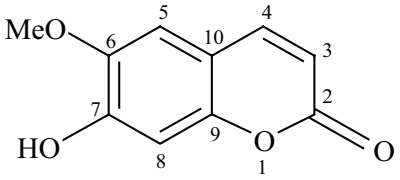
Oleanolic acid



m-Methoxy-*p*-hydroxy-benzoic acid



acid *p*-Hydroxy-benzoic



Scopoletin

Figure 1. Metabolites isolated from *Richardia brasiliensis* Gomes.

as substance 1 (26 mg) and submitted to spectral analysis.

The fraction 14-16 was purified through preparative TLC, using chloroform and ethyl acetate (50% : 50%) as eluents, showing itself as amorphous solid which resulted in the substance 2 (19 mg), that was subjected to spectral analysis.

An aliquot of 10 g of the chloroform fraction was subjected to CC, using 200 g of silica gel 60 (Art. 7734 MERCK) and eluting with hexane, chloroform and methanol pure or in binary mixtures, in increasing degree of polarity yielding more than 400 fractions of 100 mL that were concentrated in rotatory evaporator and joined by analytical TLC according to their Rfs.

The fractions 284-322, obtained from the column with pure chloroform or with chloroform and methanol in binary mixtures (0.5% to 2% of methanol), were recrystallized with methanol giving a white precipitate, coded as substance 3 (685 mg) which showed itself as a single spot through analytical TLC. It was subjected, then, to spectral analysis.

The fractions 196-210 were submitted to preparative TLC, using hexane and ethyl acetate (70% : 30%) as eluents, yielding 5 sub-fractions coded as: (196-210) 1; (196-210) 2; (196-210) 3; (196-210) 4; (196-210) 5. The sub-fraction (196-210) 5, after preparative TLC, using hexane and ethyl acetate (75% : 25%) as eluents, resulted in crystals and showed itself as a single spot through analytical TLC, being coded as substance 4 (67 mg) and subjected to spectral analysis.

RESULTS AND DISCUSSION

Structure determination of 1

The substance coded as 1 showed itself as a yellow solid, soluble in methanol, with yield of 0.0011%.

The spectrum of 1 in the region of IR revealed the presence of a strong absorption band in 3404 cm^{-1}, suggesting the presence of hydroxyl groups in the molecule. Absorptions between 1601 and 1429 cm^{-1} were verified and attributed to C=C of aromatic ring. It can be observed a signal characteristic of chelated ketone carbonyl in 1655 cm^{-1}, being possible to infer the presence of this functional group in the structure of the substance in question.

The ^{13}C NMR spectrum (125 MHz, CD_3OD) utilizing the techniques Broad Brand and DEPT-135 revealed the presence of 28 spectral lines. According to the observed chemical shifts, it was possible to infer that among the twenty-eight carbon atoms, two are referable to methyl carbons (δ_C 57.10 and δ_C 18.11); one is referable to methylene carbon (δ_C 67.56); five are referable to aromatic methyne carbons (δ_C 100.20; 95.07; 114.78; 116.13; 123.91); ten to aliphatic oxymethyne carbons (δ_C 105.12; 73.24; 75.66; 72.43; 75.16; 102.08; 70.18; 72.22; 73.98; 69.86) and the other ten signals

refer to non-hydrogen carbons (δ_C 158.99; 135.63; 179.60; 163.15; 166.51; 158.66; 105.75; 123.12; 148.55; 151.03).

The signal in δ_C 179.60 was suggested to be absorption of a flavone carbonyl, corroborating the IR spectrum which showed an absorption band to chelated carbonyl (155.4 cm^{-1}). The signals in δ_C 158.66 and δ_C 135.63 were attributed, after comparison with literature (Pizzolatti et al., 2003; Rastrelli et al., 1995), to the carbons C-2 and C-3 respectively, fact that strengthened the suggestion of 1 being a flavone.

The ^{1}H NMR spectrum (500 MHz, CD_3OD) showed signals in δ_H 8.03 (d, *J*=2 Hz), δ_H 7.60 (dd, *J*=2 Hz and 8.5 Hz) and δ_H 6.91 (d, *J*=8.5 Hz) that were attributed, respectively, to the hydrogens H-2', H-6' and H-5'. Thus, the presence of a trisubstituted flavonoid B-ring could be inferred, characterizing an ABX system with substituents in 3' and 4' in 1. The presence of a methoxyl was evidenced by a characteristic signal in δ_H 3.97 (s, 3H) and corroborated by the ^{13}C NMR spectrum which presented a signal in δ_C 57.10 that is characteristic of sterically unimpeded methoxyl of aromatic ring.

The ^{1}H NMR spectrum showed two doublets in δ_H 6.21 (1H, d, *J*=2 Hz) and δ_H 6.41 (1H, d, *J*=2 Hz) that are referable, respectively, to H-6 and H-8 of a flavonoid A-ring.

The diglucoside nature of the substance was evidenced by the number of oxymethyne signals observed in the ^{1}H and ^{13}C NMR spectra. The two glycoside units were identified as glucose and rhamnose based on ^{1}H and ^{13}C NMR spectral data. The doublets in δ_H 5.23 (d, *J*=7.8 Hz) and δ_H 4.54 (d, *J*=1.1 Hz) suggested themselves to be two anomeric hydrogens of the glucose and rhamnose molecules, respectively. This suggestion was strengthened by the ^{13}C RMN spectrum which showed absorptions in δ_C 105.12 attributed to the anomeric carbon of the glucose unit and in δ_C 18.11 and δ_C 102.08, that are referable to the methyl and to the anomeric carbon of the rhamnose unit, respectively.

The characterization and the location of the diglucoside unit (rutinoside) in C-3 was based on the following observations: a) the chemical shift of C-2 (δ_C 158.99), revealed by the ^{13}C NMR spectrum, suggested the presence of glycoside in C-3; b) the long-distance correlation between the glucose anomeric hydrogen H-1'' (δ_H 5.23, d, *J*=7.8 Hz) and the carbon C-3 (δ_C 133.53; $^3J_{CH}$), observed in the HMBC spectrum, permitted to define the bond -*O* rutinoside; c) the chemical shift of the glucose methylene carbon (δ_C 67.56) permitted the location of the rhamnose unit in this carbon atom, since the signal of hydroxymethylene group free of a glycoside unit appears around δ_C 62.12 (Pizzolatti et al., 2003); d) the HMBC spectrum revealed the correlation between the rhamnose anomeric hydrogen H-1''' (δ_H 4.54, d, *J*=1.1) and the glucose methylene carbon C-6''.

Through the two-bond correlations ($^2J_{CH}$) of H-6 (δ_H 6.21) with C-7 (δ_C 166.51) and H-6 (δ_H 6.21) with C-5 (δ_C 163.15) it was possible to determine that C-5 and C-7 sustain hydroxyl. Literature data for C-3' and C-4' (Rastrelli et al., 1995) were suggested to be inverted. The three-bond correlation ($^3J_{CH}$) between H-6' (δ_H 7.60) and C-4' (δ_C 151.03) and the $^2J_{CH}$ of H-2' (δ_H 8.03) with C-3' (δ_C 148.55) unambiguously defined those values.

The observation of the homonuclear correlation spectrum NOESY showed the spatial coupling between the aromatic hydrogen H-2' (δ_H 8.03) and the methoxyl hydrogens, thus suggesting that the methoxyl would be in the position C-3', corroborating the HMBC spectrum that presented three-bond correlation of the methoxyl hydrogens (δ_H 3.97) with C-3' (δ_C 148.55).

The analysis of the one and two-dimensional ^{1}H and ^{13}C NMR data of 1 and the comparison with values found in literature (Rastrelli, et al., 1995) permitted to identify the substance 1 as the glycoside flavonoid isorhamnetin-3-*O*-rutinoside, being the first report about it in the genus *Richardia*.

Structure determination of 3

The substance coded as 3 showed itself as a white powder, soluble in chloroform, with yield of 0.03%.

The ^{13}C NMR spectrum (50 MHz, CD_3OD) utilizing the APT technique revealed the presence of thirty signals, referable to one carbonyl carbon (δ_C 183.2), seven non-hydrogen carbons (δ_C 38.7; δ_C 39.2; δ_C 37.0; δ_C 143.5; δ_C 46.4; δ_C 30.6; δ_C 41.5), five methyne carbons (δ_C 55.1; δ_C 47.5; δ_C 122.6; δ_C 79.0; δ_C 40.9), ten methylene carbons (δ_C 38.3; δ_C 27.1; δ_C 18.2; δ_C 32.6; δ_C 22.9; δ_C 27.6; δ_C 23.3; δ_C 45.8; δ_C 33.7; δ_C 32.4) and seven methyl carbons (δ_C 28.0; δ_C 15.5; δ_C 15.2; δ_C 17.5; δ_C 25.9; δ_C 33.0; δ_C 23.5), suggesting a pentacyclic triterpene skeleton to 3.

The signals to non-hydrogen carbon in δ_C 143.5 and to methyne carbon in δ_C 122.6 suggested the presence of a double bond in the molecule and the signal in δ_C 79.0 indicated the presence of oxymethyne carbon.

The ^{1}H NRM spectrum (200 MHz, $CDCl_3$) of 3 showed an envelope of simple signals in the region between 1.89 e 0.73 ppm, which are characteristic of methyl hydrogens of pentacyclic triterpenes. The presence of a large singlet in δ_H 5.25 (1H) and a double doublet in 2.80 (1H) is compatible with the hydrogens 12 and 18 of the $^{12}\Delta$ oleanane skeleton.

The analysis of the ^{1}H e ^{13}C NMR spectral data of 3 and its comparison with models from literature (Mahato and Kundu, 1994; Pauletti et al., 2006) permitted to identify the substance 3 as the 3 β-hydroxyolean-12-en-28-oic, known as oleanolic acid, reported for the first time in the genus *Richardia*.

Other constituents

By means of analysis of the spectral data of one and two-dimensional NMR ^{1}H e ^{13}C and its comparison with literature data the coumarin 7-hydroxy-6-methoxy-2H-1-benzopyran-2-one, known as scopoletin (substance 4) (Vasconcelos et al., 1998; Carvalho et al., 2006; Razavi et al., 2008) and the mixture of the *m*-methoxy-*p*-hydroxy-benzoic and *p*-hydroxy-benzoic acids (substance 2) (Silva, 2002; Silveira and Pessoa, 2005) were also identified.

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