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Artigo

# Chemotaxonomic significance of flavonoids, coumarins and triterpenes of *Augusta longifolia* (Spreng.) Rehder, Rubiaceae-Ixoroideae, with new insights about its systematic position within the family

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**RESUMO: "Significância quimiotaxômica de flavonoides, cumarinas e triterpenos de *Augusta longifolia* (Spreng.) Rehder, Rubiaceae- Ixoroideae, com novos entendimentos sobre a posição sistemática dentro da família".** *Augusta* tem sido tradicionalmente colocada na tribo Rondeletieae, Cinchonoideae subfamília. No entanto, recentes filogenias moleculares posicionou-a perto de Wendlandia, porém localizando *A. longifolia* perto do clado Ixoroidinae II. O estudo de *A. longifolia* resultou em duas cumarinas, cinco flavonoides, três triterpenoides e um derivado do ácido benzóico. Estes metabolitos reforçam a separação da *Augusta* como um gênero monoespecífico, e *Lindenia* como um gênero de três espécies, intimamente relacionada com *Wendlandia.*

**Unitermos:** Rubiaceae, *Augusta, Wendlandia, Lindenia*, quimiotaxonomia.

**ABSTRACT:** *Augusta* has traditionally been placed in the tribe Rondeletieae, subfamily Cinchonoideae. However, recent molecular phylogenies positioned it near to *Wendlandia* (Ixoroideae), but locate *A. longifoli*a near to the clade Ixoroidinae II. The study of *A. longifolia* afforded two coumarins, five flavonoids, three triterpenoids and one benzoic acid derivative. These metabolites reinforce the separation of *Augusta* as a monospecific genus, and *Lindenia* as a genus of three species, closely related to *Wendlandia.*

**Keywords:** Rubiaceae*, Augusta, Wendlandia, Lindenia,* Chemotaxonomy.

## INTRODUCTION

The family Rubiaceae is composed by about 650 genera and 13,000 species, and represented by herbs, shrubs, trees, and lianas, mostly of tropical and subtropical distribution (Delprete, 2004). The family is currently divided into three subfamilies: Rubioideae, Cinchonoideae, and Rubioideae (Bremer et al., 1995; Rova et al., 2002; Delprete, 2004).

According to the delimitations by Kirkbride (1997) and Delprete (1997), *Augusta* (including *Lindenia*) is a genus of four species of rheophytic shrubs of puzzling geographic distribution: 1) *A. longifolia* Pohl, endemic to Brazil, 2) *A. rivalis* (Benth.) J.H. Kirkbr., endemic to Central America, 3) *A. austrocaledonica* (Brongn.) J.H. Kirkbr., endemic to New Caledonia, and 4) *A. vitiensis* (Seem.) J.H. Kirkbr., endemic to the Fiji Islands. Also, Kirkbride divided *Augusta* into two subgenera: Subgen. *Augusta*, containing only *A. longifolia*, with narrowly-campanulate, red flowers, and subgen. *Lindenia*, containing the other three species, with long-tubular, white flowers.

*Augusta* has traditionally been placed in the tribe Rondeletieae, in the subfamily Cinchonoideae (Robbrecht, 1988; Delprete, 1997). However recent molecular phylogenies have positioned it in subfamily Ixoroideae. Aside from its systematic position within the family, the phylogenies of Rova et al. (2002) supported the delimitation of *Augusta* proposed by Kirkbride and Delprete, even though only two species of *Augusta* and one of *Wendlandia* were included in the study (Delprete, 1997). *Wendlandia* is a genus of about 50-70 species occurring in the Indo-Malaysian Region, that has never been subject of a taxonomic revision, and its monophyly has never been tested. Both have capsular fruits with minute, wind-dispersed seeds, facilitating dispersals among distant areas, and supporting the unusual geographic distribution of these genera.

Recently, Robbrecht & Manen (2006) presented molecular phylogenies using DNA sequences of four regions of the plastid genome, rbcL, rps16, trnL-F and

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atpB-rbcL, with the intent of producing a new family classification. In their study, the general tree topology resembled that of the three-subfamily system (*e.g.*, Bremer et al., 1995; Rova et al., 2002), but the authors preferred to divide the Rubiaceae into two subfamilies and several supertribes. In the phylogenies of Robbrecht & Manen (2006), the position and delimitation of *Augusta* was not the same as those of Rova et al. (2002): "*Lindenia* was found as sister taxon to *Wendlandia*, based on the trnL-F source, while *Augusta* is sister taxon to the clade Ixoroidinae II in the atpB-rbcL spacer tree (Robbrecht & Manen, 2006). These results contrast with the reduction of *Lindenia* as a subgenus of *Augusta* proposed by Kirkbride (1997), and the authors placed the *Wendlandia* and *Lindenia* as part of the *Wendlandia/Augusta* informal group, while they kept *Augusta* as tentatively included in it.

Saad et al.(1988) were able to isolate two iridoid alkaloids, lindenialine and lindeniamine, from *Lindenia austrocaledonica* Brongn. (=*Augusta austrocaledonic*a), this being the only report of compounds isolated from *Augusta* as broadly delimited. Glycosidic iridoids were isolated from the leaves of *Wendlandia formosana* Cowan by Takeda et al. (1977) in the form of gardenoside, methyl deacetylasperulosidate, tarennoside and geniposidic acid, as well as 10-*O*-caffeoyl scandoside methyl, 10-*O*-caffeoyl daphylloside and 6-methoxy scandoside methyl ester isolated by Raju et al. (2004). Scandoside methyl ester was isolated from the wood of *W. bicuspidata* Wight & Arn. by De Silva et al. (1986), and geniposidic acid from the stem of *W. tinctoria* (Roxb.) DC. by Dinda et al. (2004).

The main objective of the present study was isolate compounds that might be used as taxonomic markers to help elucidate the systematic position and generic delimitation of the *Augusta-Lindenia-Wendlandia* complex.

## MATERIALS AND METHODS

### General procedures

Chromatographic separations in adsorption column were carried out using Merck silica gel (70-230 and 230-400 mesh ASTM, Merck), and Sephadex LH-20 (Aldrich). TLC were produced using Merck silica gel 60 $F_{254}$, and revealed by ultraviolet radiation at λ=254 nm and 366 nm, followed by nebulization with $H_2SO_4$/anisaldehyde/acetic acid (1:0.5:50) solution, and subsequently heated.

The $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker DRX 400 and on a Varian Unit Plus spectrometers at 400 and 100 MHz, respectively, using the appropriate deutered solvent and TMS as internal reference. The GC/MS analysis was performed on Shimadzu QP5050A instrument employing the following conditions: column DB-5 (Shimadzu), fused silica capillary column, 30 m x 0.25 mm, 0.25 μm film thickness, temperature gradient of 4 °C/min from 60 °C to 295 °C. The carrier gas was helium at 8 psi pressure; injector port and detector temperature were 250 °C and 200 °C, respectively. Samples were injected by splitting and the split ratio 1:50. The infrared spectroscopy were obtained with a Bomem FTIR, model MB-100, using KBr tablets.

### Plant material

Delprete (1997) recognized two varieties of *Augusta longifolia*: var. *longifolia*, occurring in the cerrado biome and var. *parvifolia* (Pohl.) Delprete, restricted to the Atlantic forest of the Rio de Janeiro State.

Plant material of *Augusta longifolia* var. *longifolia* was collected by P.G. Delprete and R. Choze in Mossâmedes, Goiás State, Brazil (S 16°04´, W 50°11´, 500 m), from natural populations growing at the margins and among rocks inside the Córrego Piçarrão, a small creek at the base of the Serra Dourada reserve, in December 2005. The material was identified by Delprete, and voucher specimens (collection N. 9442-A) were deposited at the herbarium of the Universidade Federal de Goiás, Goiânia, Brazil.

## EXTRACTION AND ISOLATION OF CONSTITUENTS

Dried and powdered stem barks of *A. longifolia* (400 g) were extracted with EtOH. The resulting EtOH extract was filtered and concentrated *in vacuo* to afford a brown gum (20.1 g), which was submitted to liquid-liquid partitioning. The *n*-hexane, ethyl acetate and butanol soluble parts of the EtOH extract were concentrated *in vacuo* affording 4.8, 5.2 and 6.1 g, respectively. The ethyl acetate fraction was submitted to a silica gel column (70-230 mesh) eluted with a $CHCl_3$/EtOAc/MeOH gradient, yielding three fractions. The chloroform fraction was submitted to a silica gel column (70-230 mesh) eluted with $CHCl_3$/EtOAc (65/35) yielding the esterified triterpenes lup-20(29)-en-3β-*O*-decanoate and lup-20(29)-en-3β-*O*-dodecanoate (25 mg). The EtOAc fraction was submitted to a flash silica gel CC (230-400 mesh), eluted with a $CHCl_3$/MeOH. The fraction 2 was then purified by preparative TLC (silica gel, $CH_2Cl_2$/MeOH, 70/30) yielding coumarin (6.5 mg). Fraction 3 was also purified by preparative TLC (silica gel, $CH_2Cl_2$/acetone/MeOH, 60/30/10), affording the coumarin scopoletin (4.0 mg) and 2-methoxy-4-hydroxy-benzoic acid (5.0 mg). The methanolic fraction was silica gel chromatographed (70-230 mesh) using acetone/MeOH in gradient form resulting nine fractions. The fraction 5 afforded the flavonoid naringenin (2.5 mg). Fraction 7 was then chromatographed by a flash silica gel CC (230-400 mesh), eluted with acetone/MeOH (15/85), yielding the flavonoids kaempferol (6.0 mg) and quercetin (4.5 mg). The butanol fraction was first chromatographed by sephadex LH-20, eluted with $H_2O$/MeOH (1/1) and then submitted to silica gel CC (70-230 mesh), eluted with

organic phase of $CHCl_3$/MeOH/$H_2O$ (5/5/3) + 5% MeOH, affording the flavonol myricitrin (155.0 mg).

Dried and powdered leaves of *A. longifolia* (800 g) were extracted with EtOH. The resulting EtOH extract was filtered and concentrated *in vacuo* to afford a 14.1 g of crude extract. This extract was silica gel chromatographed affording the hexane (9.7 g), ethyl acetate (2.1 g) and methanol (0.4 g) fractions. The methanol fraction was submitted to a silica gel (70-230 mesh), using $CHCl_3$/MeOH/$H_2O$ (5/5/3) + 10% MeOH, affording seven fractions. The fraction 3 was then flash chromatographed on silica gel (230-400 mesh), eluting with organic phase of $CHCl_3$/MeOH/butanol/$H_2O$ (6/5/1/3) + 6% MeOH, affording the flavonol rutin (7.0 mg). The hexanic fraction after silica gel CC (70-230 mesh), eluted with $CHCl_3$/acetone afforded the trieterpene ursolic acid (20.0 mg).

Dried and powdered woods of *A. longifolia* (150 g) were extracted with EtOH. The resulting EtOH extract was filtered and concentrated *in vacuo* to afford a 5.0 g of crude extract. This extract was silica gel chromatographed (70-230 mesh) using hexane, acetone and methanol in gradient form. The fraction acetone was submitted to a silica gel CC (70-230 mesh), eluted with a $CH_2Cl_2$/acetone in gradient form, resulting eight fractions. The fraction 2 was then flash chromatographed on silica gel (230-400 mesh), eluted with $CHCl_3$/MeOH (15/85), affording 2-methoxy-4-hydroxy-benzoic acid (7 mg). The methanolic fraction was chromatographed on a silica gel column (230-400 mesh), eluted with organic phase of $CHCl_3$/MeOH/$H_2O$ (2/5/1) + 20% MeOH, obtaining large quantities of myricitrin.

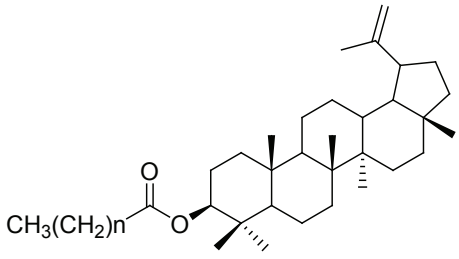
## RESULTS

In this paper we report the isolation of eleven known compounds from *Augusta longifolia* (Spreng.) Rehder var. *longifolia*. The compounds were identified by IR, GC/MS, $^1H$ and $^{13}C$ NMR one and two-dimensional techniques, and their structural propose were confirmed by literature data. From stem bark were isolated two pentacyclic triterpenes acyl lupeols (1 and 2, 25 mg, Brum et al., 1998); two coumarins, coumarin (3, 6.5 mg, Tonin & Tavares, 2002) and scopoletin (4, 4.0 mg, Silva et al., 2002); four flavonoids, naringenin (5, 2.5 mg, Almeida et al., 2005), kaempferol (6, 6 mg, Oliveira et al., 1999), quercetin (7, 4.5 mg, Barberá, 1986; Agrawal & Bansal, 1989) and myricitrin (9, 155.0 mg, Timbola et al., 2002), besides 2-methoxy-4-hydroxy-benzoic acid (11, 12 mg, Scott, 1972). From the leaves were isolated the flavonoid rutin (8, 7 mg, Buszewski et al., 1993; Agrawal & Bansal, 1989) and a pentacyclic triterpene ursolic acid (10, 20 mg, Tkachev et al., 2004). The compounds 9 and 11 were also isolated from the woods. No iridoids were found in the leaves and steam barks of *A. longifolia.*

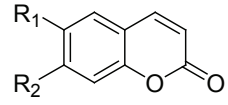
## DISCUSSION

### Terpenes

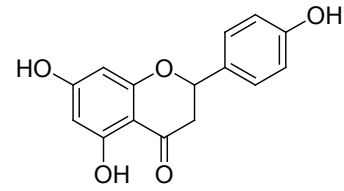
In this specie were isolated the pentacyclic triterpenes ursolic acid (**10**) from the leaves, and two acyl lupeols (**1** and **2**) from the stem bark. The abundance and



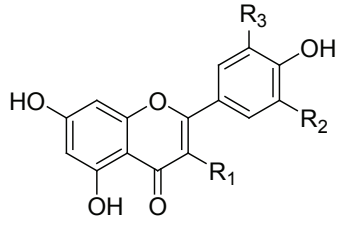

**1** n = 8
**2** n = 10



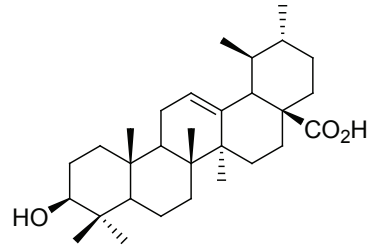

**3** $R_1 = R_2 = H$
**4** $R_1$ OMe; $R_2$ = OH



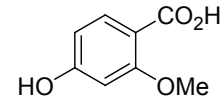

**5**




**6** $R_1$ = OH; $R_2$ = H; $R_3$ = H
**7** $R_1$ = OH; $R_2$ = H; $R_3$ = OH
**8** $R_1$ = OGly-Ram; $R_2$ = H; $R_3$ = OH
**9** $R_1$ = ORam; $R_2$ = OH; $R_3$ = OH




**10**




**11**

frequency of these compounds in Rubiaceae has been reported from many species of the three subfamilies (Delprete et al., 2006). The ursolic acid triterpene and the diterpene phytol were also isolated from *Wendlandia formosana* Cowan (Raju et al., 2004); however the universal presence of this compound in the family shows no taxonomic significance.

**Coumarins**

The compound coumarin (**3**), a simple coumarin, was the first time reported in Rubiaceae. Its biosynthesis is simple and rather rare in the Angiosperms. This compound has been commonly isolated in species of Fabaceae and Asteraceae, as in *Amburana cearensis* (Allemão) A.C. Sm. (Fabaceae; Canuto & Silveira, 2006), and in *Mikania* Willd. (Asteraceae; Dos Santos, 2005). A study focusing on the evolutionary trends in coumarin-producing *Angiosperm* families, reports the occurrence of 91 simple coumarins in the order Gentianales, of which 59 in the Rubiaceae, 21 in the Apocynaceae, 4 in the Menyanthaceae, 2 in the Gentianaceae, 3 in the Asclepiadaceae, and 2 in the Loganiaceae (Ribeiro & Kaplan, 2002). *A. longifolia* also afforded scopoletin (**4**), which belongs to the subclass of hydroxy-coumarins. This compound was isolated in many genera of the Rubiaceae, mainly in the subfamily Ixoroideae, as this studied specie.

**Flavonoids**

In *A. longifolia* were isolated the flavonoids: naringenin (**5**), kaempferol (**6**), quercetin (**7**) from stem bark, myricitrin from steam bark and woods (**9**) and rutin (**8**) from the leaves. Myrictrin was also isolated from the wood. These findings are in agreement with the biochemical survey conducted by Delprete et al. (2006), where the subfamily Ixoroideae was characterized by the moderate presence of flavonoids, where flavonols are in majority. Special attention should be given to the flavonol myricitrin (**9**), which is also reported the first time in the Rubiaceae. This glycosilated flavonol was also found in the fruits of *Pouteria* Aubl. (Sapotaceae; Ma et al., 2004) and of *Manilkara zapota* (L.) P. Royen (Sapotaceae; Ma et al., 2003). In addition, it was also isolated in the leaves *Eugenia* L. (Myrtaceae; Schmeda-Hirschmannet et al., 1987) and in the latex of *Croton draco* Schltdl. (Euphorbiaceae; Tsacheva et al., 1987).

**Benzoic acid derivates**

2-Methoxy-4-hydroxy-benzoic acid (**11**) was isolated, and it is present in many taxa of the three subfamilies of the Rubiaceae. This compound has a great structural similarity with salicylic acid, a benzoic acid derivate with pharmacologic importance (Berretta, 2007). Therefore, for possessing a similar bioactive nucleus, this benzoic acid derivate might also have similar biological properties; however, this remains to be tested.

**Iridoids**

Despite many attempts, no iridoids were found in *A. longifolia*. On the other hand, in *Wendlandia* and *Lindenia* were isolated glycosidic and alkaloidic iridoids, respectively. From the leaves of *Wendlandia formosana* were isolated gardenoside, 10-*O*-caffeoyl scandoside methyl ester, 10-*O*-caffeoyl daphylloside, 6-methoxy scandoside methyl ester, methyl deacetylasperulosidate, tarennoside and geniposidic acid (all glycosidic iridoids; Takeda et al., 1977; Raju et al., 2004); while scandoside methyl ester was found in the wood of *W. bicuspidata* Wight & Arn. (De Silva et al., 1986), and geniposidic acid in the stem of *W. tinctoria* (Roxb.) DC. (Dinda et al., 2004). In addition, two alkaloidic iridoids, lindenialine and lindeniamine, were isolated from the leaves of *Lindenia austrocaledonica* Brongn. (Saad et al., 1988).

**Taxonomic significance**

The presence or absence of certain compounds in *Augusta longifolia* supplied several taxonomic markers with valuable information about the systematic position of this species within the Rubiaceae. The isolation of flavonoids and coumarins in *Augusta*, not isolated at the moment in *Lindenia* or *Wendlandia*, suggests the chemical uniqueness of this species in the family and in the order Gentianales. On the other hand, glycosidic and alkaloidic iridoids were obtained in *Wendlandia* and *Lindenia*, and no iridoids were yet found in *Augusta*, suggesting that *Augusta*, could be treated as a monospecific genus, and is not closely related to *Lindenia* (as traditionally defined) and *Wendlandia*. Therefore, the results of this study reinforce the systematic position and the generic circumscriptions of the three genera as indicated in the atpB-rbcL spacer phylogeny of Robbrecht & Manen (2006, fig. 3).

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