Secondary Metabolites from Leaves of *Manilkara subsericea* (Mart.) Dubard

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**ABSTRACT**

**Background:** *Manilkara subsericea* (Sapotaceae) is a species widely spread in the sandbanks of Restinga de Jurubatiba National Park (Rio de Janeiro, Brazil). It is commonly known as “maçaranduba,” “macandrubinha” and “guracica,” being used in this locality as food, and timber. However, *M. subsericea* remains almost unexplored regarding its chemical constituents, including secondary metabolites from the leaves. **Objective:** Identify the chemical constituents from the leaves of *M. subsericea*. **Materials and Methods:** Leaves were macerated with ethanol (96% v/v), and dried crude ethanolic extract was sequentially washed with the organic solvents in order to obtain an ethyl acetate fraction. Substances from this fraction were identified by different techniques, such as negative-ion electrospray ionization Fourier and 1H and 13C nuclear magnetic resonance (NMR). Fresh leaves from *M. subsericea* were also submitted to hydrodistillation in order to obtain volatile substances, which were identified by gas chromatograph coupled to mass spectrometer. **Results:** NMR and 2D NMR spectra allowed for the identification of the compounds myricetin, quercetin, and kaempferol from the ethyl acetate fraction. The negative-ion electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry mass spectrum also revealed the presence in this fraction of a polyhydroxytriterpene acid (pomolic acid), and some flavonoids, such as quercetin, and myricitrin. In all 34 volatile compounds were identified by gas chromatography-mass spectrometry, including monoterprenes, sesquiterpenes, and long chain hydrocarbons. **Conclusion:** This study describes the first reports concerning the phytochemical information about leaves from *M. subsericea*. **Key words:** Essential oil, flavonoids, *Manilkara subsericea*, mass spectrometry, pomolic acid, sapotaceae

**SUMMARY**

- *Manilkara subsericea* fruits proved to be a rich source of triterpenes. However, no phytochemical studies were carried out with leaves. Thus, we described identification of volatile substances from its essential oils, in addition to non-reported triterpene and flavonoids from this species.

**INTRODUCTION**

*Sapotaceae* family contains 58 genus, and approximately 1250 species with morphological variation, ranging from shrubs to medium, and giant trees.¹ Brazil comprises of 11 genera, and 231 species, including 1 endemic genus, and 104 endemic species.² This family has the following synapomorphies, well-developed, elonget laticifers with white latex; 2-branched hairs, brownish, T-shaped; berry fruits, seeds usually with a hard shiny testa, and large hilum.³ Due to this genus circumscription, some species of the genera *Achras*, and *Mimusops* were included in *Manilkara*.⁴

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Manilkara subsericea (Mart.) Dubard is an endemic species from Brazilian Atlantic Rain Forest, commonly known as “maçaranduba”, “maçarandubinha”, and “guracica”. It is widely distributed at Restinga de Jurubatiba National Park (Rio de Janeiro State, Brazil), being used in this locality as food, and timber. This species also develops a main role in the ecology of Restinga de Jurubatiba, being an important host plant for some Lepidoptera species, and for porcupine (Chaetomys subspinosus) feeding. Despite some biological and chemical investigations were carried out with M. subsericea, to our knowledge, there is no information concerning secondary metabolites from leaves of this species. Thus, the aim of the present study was to perform phytochemical characterization of leaves from M. subsericea using spectroscopy methods such as nuclear magnetic resonance (NMR), high-resolution nuclear magnetic resonance (HR-NMR), negative-ion electrospray ionization fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), and gas chromatography (GC/MS) mass spectrum.

MATERIALS AND METHODS

Plant material

Leaves of M. subsericea were collected at Restinga de Jurubatiba National Park, Rio de Janeiro State, Brazil (22°14'46''S–41°34'56''W), October 2010 by Dr. Caio Pinho Fernandes. Identification was performed by the botanist Dr. Marcelo Guerra Santos, and voucher specimen of M. subsericea was deposited at the herbarium of the Faculdade de Formação de Professores (Universidade do Estado do Rio de Janeiro, Brazil) under the register number RFFP 15316. The nomenclatural update was realized in Lista de Espécies da Flora do Brasil (http://floradobrasil.brd.br/jabot/listaBrasil/PrincipalUC/PrincipalUC.do), and the plant list: A working list of all plant species (http://www.theplantlist.org/).

Preparation of extracts

Leaves (0.84 kg) were dried at 40°C for 2 days, and then extensively crushed, and macerated (15 L) in ethanol (EtOH) 96% (v/v) at room temperature. After filtration, the ethanolic extract was concentrated under vacuum using a rotary evaporator equipped with the water bath (35°C) (Fisatom, SP) in order to obtain 109.5 g of the ethanolic crude extract from leaves. This dried extract was sequentially washed with n-hexane (5 × 2000 mL), and dichloromethane (5 × 2000) in order to remove the less polar constituents from the extract. The insoluble fraction was washed with ethyl acetate (5 × 2000 mL). The ethyl acetate-soluble fraction was filtered, and concentrated under the vacuum using the rotary evaporator, affording 17.3 g of the ethyl acetate fraction from leaves (EAL).

Isolation of substances

The ethyl acetate-soluble fraction from leaves was fractionated through column chromatography using the Amberlite XAD-2 resin (Sigma-Aldrich, St. Louis). Elution was performed with water, methanol/water mixtures (5:95 → 9:1), methanol, and acetone. Fractions 28–40 were pooled together according to the thin layer chromatography (TLC) profile, and purified on Sephadex LH-20 using methanol as mobile phase, affording 1 (40.2 mg), 2 (35.7 mg), and 3 (11.9 mg). Fractions 8–19 were pooled together according to the TLC profile, and chromatographed in C-18 reversed phase silica gel (Sigma-Aldrich, St. Louis) using gradient of mobile phase constituted by methanol solutions (v/v) in water (60%→63%). Final purification on Sephadex LH-20 using methanol as mobile phase afforded a fraction (7.4 mg) containing 4 and 5. Fractions 41–52 was submitted to silica gel chromatography column using an isocratic system of mobile phase (n-hexane: ethyl acetate: methanol, 5:5:1), affording a fraction (9.7 mg) constituted by 6.

Essential oil extraction

Fresh leaves (340 g) from M. subsericea were ground with distilled water using an automatic blender (Etica Equipamentos Científicos SA, Brazil). Hydrodistillation method was employed for 3 h using Cleveger apparatus, and plant material was placed in a 5 L flask. At the end of the extraction, the essential oil was collected with n-hexane, dried over a hydrous sodium sulfate, and stored at 4°C for further analyzes.

Chemical analysis

1H and 13C NMR spectra of 1, 2, 3, and 6 were recorded at 500, and 125 MHz, respectively, on a Varian VNMRS 500 MHz spectrometer. Deuterated methanol was used for solubilization of flavonoids while deuterated DMSO was used for solubilization of triterpene fraction. The solvents were obtained from Cambridge Isotope Laboratories (USA), and TMS peak was used as an internal standard.

Substances 4, 5, and 6 were analyzed by negative-ion electrospray ionization fourier transform ion cyclotron resonance mass MS, ESI(−)-FT-ICR-MS. Briefly, each sample was diluted to 5 × 1.0 mg/mL in acetonitrile (containing 0.1% w/v of NH₄OH). The resulting solution was directly infused at a flow rate of 5 μl/min into the ESI source. The mass spectra were acquired over a mass range of m/z 200–2000. The ESI source conditions were as follows: nebulizer gas pressure of 0.5–1.0 bar, the capillary voltage of 2.5–3.5 kV, and transfer capillary temperature of 250°C. All mass spectra were externally calibrated using a NaTFA (m/z from 200 to 1200). A resolving power (m/Δm₉₀₀ = 530,000, in which Δm₉₀₀ is the full peak width at half-maximum peak height) of m/z400 and a mass accuracy (mass error) of <1 ppm provided unambiguous molecular formula assignments for singly charged molecular ions. Mass spectra were acquired and processed using the software package, Compass Data Analysis (Bruker Daltonics, Bremen, Germany). ESI(−)-MS/MS experiments were collected after 4–40 eV collision-induced dissociations (CID) with argon. Selection of ion was performed by quadrupole, using a unitary m/z window, and collisions were performed in the Ry-only hexapole collision cell, followed by mass analysis of product ions by the ultra-high resolution ICR analyzer.

The essential oil was analyzed by a GC/MS-QP2010 (SHIMADZU) gas chromatograph equipped with a mass spectrometer using electron ionization. The GC conditions were as follows: Essential oil: Injector temperature, 260°C; detector temperature, 290°C; carrier gas (Helium), flow rate 1 mL/min, and split injection with split ratio 1:40. Oven temperature was initially 60°C and then raised to 290°C at a rate of 3°C/min. The sample was diluted with n-hexane (1:100, v/v) and injected at RTX-5 column (i.d. =0.25 mm, length 30 m, film thickness = 0.25 μm). The MS conditions were voltage, 70 eV, and scan rate; 1 scan/s. The retention indices were calculated by the interpolation of retention times of the substances to the retention times of a mixture of aliphatic hydrocarbons (C7-C40) (Sigma) analyzed in the same conditions. The identification of substances was performed by comparison of their retention indices and mass spectra with those reported in the literature. MS fragmentation pattern of compounds was also checked with NIST mass spectra libraries. Quantitative analysis of the chemical constituents was performed by GC-flame ionization detector (GC/FID), under same conditions of GC/MS analysis and percentages obtained by FID peak area normalization method.

RESULTS AND DISCUSSION

Chemical structures from the substances identified after fractionation of ethyl acetate-soluble fraction from leaves of M. subsericea are presented in Figure 1. 1H NMR spectrum of 1 (CD3OD, 500 MHz) showed the two
doublets at $\delta$ 6.18 ($J = 1.45$ Hz) and $\delta$ 6.38 ($J = 1.45$ Hz) attributable, respectively, to the protons H-8 and H-6, and a two-proton singlet observed at $\delta$ 7.34, corresponding to the B-ring aromatic protons H-2' and H-6' of a flavonol. The signals observed in the $^1$H and $^{13}$C NMR spectrum are in accordance with the literature data for myricetin.$^{[16]}$ The substances 2 and 3 had their $^1$H and $^{13}$C NMR spectra compared with the literature data$^{[17,18]}$ and, therefore, were respectively, identified as quercetin, and kaempferol.

The remaining flavonoids, 4 and 5, were analyzed by ESI(−)-FT-ICR MS [Figure 2a-d]. For FL4 [Figure 2a], ESI(−)-FT-ICR mass spectrum, detected the presence of the O-glycosideo-flavonol-myricitrin ($M = C_{21}H_{20}O_{12}$), ions of m/z 463.0882 ($[M - H]^{-}$), 499.0651 ($[M + Cl]^{-}$) and 927.1844 ($[2M - H]^{-}$) as deprotonated molecule, chlorine adduct and dimer, respectively. A double bound equivalents (DBE) of 12 found for ions $[M - H]^{-}$ and $[M + Cl]^{-}$ agrees with the chemical structure of myricitrin, that presents two aromatic rings (DBE = 8), one rhamnose (DBE = 1), and a ring containing one double bond, and a ketone group (DBE = 3). To confirm the myricitrin detection, ESI(−)-MS/MS was performed for the ion of m/z 463 [Figure 2c]. The CID of $[M - H]^{-}$ of m/z 463 agrees well with its structure and connectivity, producing fragments with m/z 316, corresponding to the neutral loss of $C_6H_11O_4$. For FL5 [Figure 2b], ESI(−)-FT-ICR mass spectrum identified, simultaneously, ion $[M - H]^{-}$ of m/z463.0882, and ions $[N - H]^{-}$ and $[N + Cl]^{-}$ of m/z 447.0933 and 483.0703, respectively, where $N = C_{21}H_{20}O_{11}$, corresponding to the O-glycoside flavonol quercitrin. CID experiment of the ion of m/z 447 [Figure 2d], produces fragments of m/z 301, and 284 corresponding to the neutral losses of $C_6H_10O_4$ and one rhamnose molecule.

Some flavonoids are isolated and identified on the present study, have also been found in this genus. Quercetin was identified on Mimusops manilkara G. Don (Manilkara kauki (L.) Dubard), and Mimusops littoralis Kurz (M. littoralis (Kurz) Dubard)$^{[19,20]}$, while myricetin was identified on Achras zapota L. (Manilkara zapota (L.) P. Royen).$^{[21]}$ The glycosylated flavonoids quercitrin and myricitrin were isolated from M. zapota.$^{[22]}$ Chromatographic fractionation of the EAL also afforded a white powder. ESI(−) FT-ICR mass spectrum of the fraction revealed the presence of precursor ions $[C_{30}H_{48}O_4 - H]^-$, $[C_{30}H_{48}O_5 - H]^-$ and $[C_{30}H_{48}O_6 - H]^-$ of m/z 471, 487 and 503, being attributed to dihydroxy, trihydroxy, and tetrahydroxylidendroproline acids [Figure 3], which is in accordance with the derivatives from ursolic, and oleanolic acids. $^{13}$C NMR spectra of
Table 1: Relative abundance of essential oil constituents from leaves of Manilkara subsericea

| Substance                                      | RI     | Relative abundance (%) | Substance                                      | RI     | Relative abundance (%) |
|------------------------------------------------|--------|------------------------|------------------------------------------------|--------|------------------------|
| 3-hexen-1-ol                                    | 850    | 0.2                    | Trans-2-decenal                                | 1262   | 0.2                    |
| Hexanol                                         | 862    | 0.2                    | Beta-damascone                                 | 1388   | 0.2                    |
| 2-heptanone                                     | 889    | 0.1                    | Beta-damascene                                 | 1418   | 0.3                    |
| Heptanal                                        | 902    | 0.2                    | Beta-caryophyllene                             | 1423   | 0.3                    |
| Heptanol                                        | 966    | 0.3                    | Farnesene                                      | 1510   | 0.3                    |
| Octanal                                         | 1003   | 0.2                    | Caryophyllene oxide                            | 1588   | 0.7                    |
| (E)-3-hexenyl acetate                          | 1006   | 0.6                    | Hexadecanoic acid                              | 1969   | 8.6                    |
| Beta-caryophyllene                              | 1047   | 7.3                    | Eicosene                                       | 1994   | 0.2                    |
| Octanol                                         | 1069   | 1.3                    | Heneicosane                                    | 2100   | 0.3                    |
| Linalool oxide                                  | 1089   | 0.5                    | Phytol                                         | 2116   | 15.6                   |
| 2-nonanone                                      | 1092   | 0.2                    | Docosane                                       | 2194   | 0.4                    |
| Linalool                                        | 1100   | 27.6                   | Tricosane                                      | 2300   | 0.6                    |
| Terpineol                                       | 1193   | 0.9                    | Pentacosane                                    | 2500   | 0.4                    |
| Methyl salicilate                               | 1197   | 1.9                    | Heptacosane                                    | 2700   | 0.9                    |
| Safranal                                        | 1202   | 0.3                    | Squalene                                       | 2832   | 2.6                    |
| Beta-cyclocitrinal                              | 1223   | 0.3                    | Nonacosane                                     | 2900   | 8.9                    |
| Geraniol                                        | 1256   | 0.3                    | Untriacontane                                  | 3100   | 3.4                    |
| Total identified                                |        |                        |                                                |        | 86.5                   |

RI: Retention indices calculated by interpolation of retention times of the substances to the retention times of a mixture of aliphatic hydrocarbons. GC conditions: Injector temperature, 260°C; detector temperature, 290°C; carrier gas, Helium; flow rate, 1 mL/min; split ratio, 1:40. Initial temperature, 60°C; final temperature, 290°C; rate of 3°C/min. RTX-5 column (i.d.=0.25 mm, length 30 m, film thickness=0.25 µm). MS conditions: Voltage, 70 eV; scan rate; 1 scan/s. GC: Gas chromatographic; MS: Mass spectrometry.

**CONCLUSION**

Despite the ecological significance, great abundance, used as food, and literature data concerning its biological activities, *M. subsericea* remains almost chemically unexplored. To our knowledge, only secondary metabolites from fruits of this species were previously reported. As part of our ongoing studies, the present study allowed the identification of substances from the leaves of this species, being all substances identified for the first time on *M. subsericea*.

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**Conflicts of interest**

There are no conflicts of interest.

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