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Artigo

CHEMICAL COMPOSITION OF BIOLOGICAL ACTIVE EXTRACTS OF *Tapirira guianensis* (ANACARDIACEAE)

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This work describes the chemical composition of MeOH seeds and flowers extracts of *Tapirira guianensis*, a known tree that occurs in the Brazilian Atlantic forest. The CH_2Cl_2 soluble fraction of seeds extract was submitted to chromatographic procedures to obtain a mixture of new alkenyl dihydrobenzofuranoids which were identified as 2-[(10'*Z*)-dodec-10'-enyl]-dihydro-1-benzofuran-5-ol, 2-[(10'*Z*)-tridec-10'-enyl]-dihydro-1-benzofuran-5-ol e 2-[(10'*Z*)-pentadec-10'-enyl]-dihydro-1-benzofuran-5-ol (**1** – **3**) besides β-sitosterol. From the EtOAc soluble fraction of flowers MeOH extract quercetin, quercitrin and gallic acid were obtained by chromatographic techniques. The fatty acid composition of oils from leaves and seeds were also determinate and the leaves' oil is composed by 63.94% of saturated and 36.04% of unsaturated fatty acids while in the seeds the oil present 42.13% of saturated and 57.87% of unsaturated. All compounds and derivatives were identified by their spectrometric data analysis. The brine shrimp test of the extracts showed the seed CH_2Cl_2 and the EtOAc and BuOH soluble fractions of the flowers were actives and, the alkyl phenols are the responsible for this moderate activity. The antioxidant tests of the extracts indicated EtOAc soluble fraction of MeOH extract of flowers showed better results possibly due the presence of flavonoids and gallic acid.

Keywords: *Tapirira guianensis*; alkenyl dihydrobenzofuranoids; cytotoxic activity; quercetrin; quercetin; fatty acid composition.

INTRODUTION

Anacardiaceae is a family comprising 800 species distributed in 81 genera and these species are predominantly found in tropical and subtropical areas.[1] In Brazil, the family Anacardiaceae is represented by 14 genera and 54 species, being 13 of them endemics.[1] Several species of this family show significant economic importance due to their woods for carpentry, fruits, and edible seeds, all of them with expressive commercial value. For example, from *Anacardium occidentale* L. ("cajú" or cashew) is obtained the worldwide known cashew nut, besides juices, jams and other processed food.[2]

Besides the economic importance, Anacardiaceae is a family well known due to the existence of some toxic species. Studies indicate that about 25% of the genera of this family have species that are characterized as toxic and they are responsible for severe allergic contact dermatitis.[3] It is more likely this toxic effect of some species of Anacardiaceae is due to the presence of secondary metabolites known as non-isoprene phenolic lipids or alkyl/alkenyl phenols, present in the leaf surface or are secreted from the resin of these plants.[3]

The genus *Tapirira* consists of 35 known species found from the south of Mexico to all South America.[4] Brazil contributes with four endemic species of this genus, *Tapirira obtusa* (Benth.) J.D.Mitch., *T. pilosa* Sprague, *T. retusa* Ducke and *T. guianensis* Aubl..[5] Between them, *T. guianensis* is the most well-known native species occurring in all Brazilian biomes and popularly named as "pau-pombo"or"peito-de-pombo".[6] *Tapirira guianensis* is a 8-14 m tree present in humid soils of Brazil, especially in Atlantic forest areas.[7] The leaves of this species are employed in Brazilian folk medicine in the treatment of leprosy, diarrhea and syphilis.[8] Previous chemical studies dealing with this species related the presence of cytotoxic alkylphenols from the seeds besides alkyl ester ferulates, triterpenes, steroids, *nor*isoprenoids and flavonoids isolated from the barks and leaves.[8,9]

This paper describes the reexamination of organic extracts of seeds and flowers a specimen of *T. guianensis* as well as, the fatty acid composition, evaluation of cytotoxic and antioxidant activities.

EXPERIMENTAL

General procedures

The solvents (MeOH, EtOAc, CH_2Cl_2, $CHCl_3$ and hexane) employed in extraction and chromatographic techniques were all PA purchased from Quimex. In the CC were used Sigel 60 (0.063-0.200 and 0.040-0.063 mm) from Merck and Acros and, in TLC 60 F_{254} (Merck) precoated plates. The TLC monitoring were revealed by UV (254 and 365 nm) lamps, iodine fumes and Lieberman-Burchard reagent, prepared by the mixture of 10 mL sulfuric acid, 10 mL acetic anhydride in 50 mL of cold EtOH. The IR spectra were registered in the FT Shimadzu spectrophotometer mod IRAffinity-1 employing dry KBr plates or pellets. The ^1H and ^{13}C NMR spectra (mono and bidimensional) were carried out in the Varian® (GEMINI 2000) and Bruker (DRX 500) spectrometer using traditional pulse sequences for the homonuclear and heteronuclear correlation experiments. Optical rotations were performed using a Perkin-Elmer model 341 Polarimeter employing a microcell. The mass spectra were obtained on a negative ESI LCMS Shimadzu spectrometer mod. 2010A and in the EI (70 eV) HP mass detector mod. 5973. The UV spectra were recorded in a CARY I Varian spectrophotometer. The HPLC/DAD analysis were recorded in a Dionex equipment (mod. UltiMate 3000) with an injection volume of 10 µL. A DIONEX C18 column (120 Å 3.0 x 75 mm) with 5µm particles diameter was used. As eluent system, gradient mixtures MeOH (**B**) and 0.2% of aqueous acid formic (**A**): 30-50% of **B** for 15 min and 50-100% B from 15 to 20 min of running time at 0.5 mL min^{-1} flow rate. The detection was registered in 254, 265, 290 and 330 nm UV region.

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Plant material

The seeds and flowers of *T. guianensis* were collected Universidade Federal da Bahia campus (13°00'02.0"S, 38°30'26.1"W), Salvador (BA), Brazil and a plant voucher (# 032912) is deposited in the Herbarium Alexandre Leal Costa of Instituto de Biologia da UFBA. The access to the species was registered in the Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado (SisGen) under number A56BAD6.

Extraction and isolation of the constituents of *T. guianensis* seed extracts

The seeds of *T. guianensis* were previously dried in a forced circulating oven for 48 h at 40 °C, and then ground in a blender. The powdered seeds (316.15 g) were submitted to extraction by maceration by 3x employing hexane for 48 h each one. The extract was concentrated in rotaevaporator furnishing 6.62 g of hexane extract and, sequentially it was submitted to liquid-liquid partition between hexane and MeOH:H_2O (95:5). This procedure permitted to obtain the hexane soluble fraction (2.23 g) and the hydroalcoholic one (3.62 g). The hydromethanolic fraction was dissolved in MeOH:H_2O (1:1) and also submitted to partition between CH_2Cl_2, furnishing 3.15 g of the soluble fraction in CH_2Cl_2.

The CH_2Cl_2 soluble fraction was chromatographed over silica gel 60 (0.063-0.200 mm) and eluted with pure CH_2Cl_2 and gradient of mixtures of CH_2Cl_2:MeOH. The CC furnished 19 fractions of 20 mL each and they are monitored and grouped by silica gel TLC (UV lamps and iodine fumes). The first fractions eluted with CH_2Cl_2 (158.35 g) was then submitted to flash CC, also employing silica gel (0.040-0.063 mm). The fractions eluted with mixtures of CH_2Cl_2:MeOH furnished the mixture of the new compound identified as 2-[(10'*Z*)-dodec-10'-enyl]-dihydro-1-benzofuran-5-ol, 2-[(10'*Z*)-tridec-10'-enyl]-dihydro-1-benzofuran-5-ol e 2-[(10'*Z*)-pentadec-10'-enyl]-dihydro-1-benzofuran-5-ol (**1** – **3**, 20 mg) besides β-sitosterol (**4**, 25 mg).

Extraction and isolation of the constituents of the extracts of the flowers of *T. guianensis*

The flowers were previously dried at room temperature, and then ground in a blender and the powered material (344.04 g) was submitted to extraction 3x by MeOH, with about 48 hours for each extraction. The MeOH extract was diluted with H_2O to furnished a MeOH:H_2O (6:4) solution and sequentially it was partitioned with CH_2Cl_2, obtaining 6 g of material and, then, EtOAc furnishing 2.50 g of the soluble fraction of this solvent.

The CH_2Cl_2 soluble fraction was submitted to a silica gel 60 (0,063-0,200 mm) CC and eluted with mixtures of CH_2Cl_2:MeOH, obtaining 20 fractions of 100 mL each. The fraction eluted with 20% of MeOH (479 mg) was submitted to another CC and the fraction eluted with hex:EtOAc (3:2) furnished quercetin (**5**, 10 mg).

The silica gel 60 CC of the soluble fraction of EtOAc (2.50g) eluted with mixtures of CH_2Cl_2:MeOH, furnished 16 fractions of 100 mL each and the fractions eluted with 30% MeOH (203 mg) was subjected to gel permeation in Sephadex LH 20 eluted with MeOH. This procedure permitted to obtain quercitrin (**6**, 20 mg) and gallic acid (**7**, 25 mg).

2-[(10'Z)-Dodec-10'-enyl]-dihydro-1-benzofuran-5-ol, 2-[(10'Z)-tridec-10'-enyl]-dihydro-1-benzofuran-5-ol e 2-[(10'Z)-pentadec-10'-enyl]-dihydro-1-benzofuran-5-ol, (**1** – **3**). Oil. ESIMS(negative mode) *m/z* 301, 315 and, 343 ($C_{20}H_{30}O_2$, $C_{21}H_{32}O_2$ e $C_{23}H_{36}O_2$). $[\alpha]^{25}_D$ +13° (c.1.5, CH_3OH). $IR_{vmax\ film}$ (cm^{-1}): 3347; 3005; 2919; 2850; 1492; 1468; 1199; 862; 814. ^1H NMR (400 MHz, $CDCl_3$, δ_H) and ^{13}C NMR (75 MHz, $CDCl_3$, δ_C): Table 1.

Thioalkylation of 1-3

The derivatives were prepared based on a previously described methodology.[8] Briefly, the mixture of **1**-**3** (3.0 mg) was dissolved in dimethyl disulfide (DMDS 0.2 mL), and iodine (1.0 mg) was added to the solution. The resulting mixture was kept at room temperature for 24 h. Subsequently, the reaction was quenched with aqueous $Na_2S_2O_3$ (5%), and the mixture was extracted with n-hexane; the hexane layer was concentrated to provide a mixture of DMDS derivatives.

Analysis of fatty acids from leaves and seeds of *T. guianensis*

The hexane soluble fraction of seeds and the hexane extract of leaves (prepared employing 35 g of dried and ground *T. guianensis* leaves and 100 mL of hexane furnishing 1.19 g of extract) for *T. guianensis* were submitted to transesterification with a solution sodium methoxide (0.5 mol L^{-1}) in 2.0 mL of MeOH and 25 mg of the triglycerides/fatty acids mixture and the reaction was stirred during 30 min. In sequence, H_2O was added, and the methyl esters were extracted by partition between hexane.

The methyl ester derivatives were analyzed injecting a solution of the hexane extracts in a GC-MS system (Shimadzu CG-2010/ QP-2010 high efficiency, coupled with quadrupole mass detector and a Rtx®-1 MS (Crossbond® 100% dimethyl polisiloxane) column of 30 m, Φ 0.25 mm and 0.25 µm of thickness). The speed of carrier gas was set to 40 cm s^{-1}; the carrier gas flow in the column to 0.8 mL min^{-1}; the injection mode was split with a rate of 1:30; the temperature of the injector was set to 280 °C, interface temperature of 260 °C and the oven temperature gradient set to 80 °C (at 2.0 min), rising at 1.5 °C min^{-1} then 10 °C min^{-1} to 140 °C, 3 °C min^{-1} to 200 °C (10 min) and 5 °C min^{-1} to 250 °C; with the transfer line set to 250 °C; the ion source to 250 °C and an impact energy of 70 eV and voltage detector of 1.3 KV. The methyl ester constituents were identified by molecular ion and analyzing the fragmentation pattern in the mass spectra and comparison with NIST 147 and WYLEY 8 databases.

Brine shrimp test

For an initial screening on the bioactivity, the adapted Brine Shrimp Lethality assay described by Meyer and co-worker[10] was employed to study the general cytotoxicity of the compounds **1**-**6**. Alive brine shrimp (*Artemia salina* Leach) cysts (500 mg) were transferred to a conical flask containing 3500 mL of artificial seawater. The flasks were aerated with the aid of an air pump and kept at 28 ºC under a bright light and the nauplii hatched after 48 hours approximately. The compounds and extracts were separately dissolved artificial seawater and DMSO to reach the final concentration of 1 mg mL^{-1}. Solutions of 50, 100, 150, 200, 300 e 400 µg mL^{-1} of extracts and for the **1**-**3** each compound (25, 50, 75, 100, and 150 µg mL^{-1}) were transferred to vials, which already contained seawater (5 mL) and 10 nauplii. Controls containing DMSO, seawater, and 10 nauplii also performed. The assays were carried out in triplicate. After 24 hours of incubation, the number of live nauplii was counted. The mortality was defined as the absence of controlled forward motion during 30 seconds of observation. The lethal concentration doses for 50% of the brine shrimp (LC_{50}) and the respective 95% confidence intervals were determined by using the GraphPad Prism version 5.0 (GraphPad, La Jolla, CA) software.

DPPH scavenging activities

Radical scavenging activities of plant extracts were determined through spectrophotometry using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging radical assay (MeOH, 40 μg mL^{-1}) employing 3 mL of DPPH solution and 1 mL of the samples. The radical scavenging ability was calculated by the formula, % I = [(AbsB − AbsA) / AbsB] × 100, and the IC_{50} was determined from the linear decrease in the inhibition percentage at λ = 517 nm immediately and after 30 min of incubation at room temperature and the solutions protected from light. Gallic acid was employed as positive control and the extracts were evalueted at different concentrations (50, 100, 150, 200 e 250 μg mL^{-1}).

RESULTS AND DISCUSSION

From the CH_2Cl_2 soluble fraction of the hexane extract of the *T. guianensis* seeds were isolated by CC an inseparable mixture of three new alkyl dihydrobenzofuranoid (**1**-**3**, Figure 1) by usual chromatographic techniques. Besides them, β-sitosterol, quercetin, quercitrin and, gallic acid were also obtained. The TLC analysis of this mixture showed just one spot however the negative ESIMS showed [M-H] molecular ions at *m/z* 301, 315, and 343. These data combined with the peaks observed in the IR, ^1H, and ^{13}C spectra, including hydrogen integration and carbon counts, permitted to assign $C_{20}H_{30}O_2$, $C_{21}H_{32}O_2$, and $C_{23}H_{36}O_2$ as the molecular formula. The IR spectrum of **1**-**3** showed characteristic stretching bands of OH groups of phenolics (υ 3347 and 1199 cm^{-1}) besides aromatic and aliphatic C-H (3005, 2919 and 2850 cm^{-1}), C=C aromatic stretchings between 1492 and 1468 cm^{-1} and C-H out-of-plan angular deformation of trisubstituted aromatic ring at 862 and 814 cm^{-1}. These findings were corroborated with ^1H NMR spectra data, the coupling constants and integration of the peaks at δ 6.68, 6.62, and 6.58 confirmed the substitution pattern of the aromatic ring. The AMX spin coupling system could be confirmed by ^1H-^1H-COSY experiment, besides H-4 is registered as a broad singlet, the COSY spectrum showed a correlation with δ 6.58 (H-6). The multiplet at δ 5.36 (2H) is characteristic of hydrogens of aliphatic double bond carbons, similar to olefinic hydrogens of unsaturated fatty acids with *Z* configuration. The presence of an alkenyl chain was also confirmed by the hydrogen peaks at δ_H 0.91-2.03 ppm. The ^{13}CNMR spectra confirmed all these structural patterns of the compounds. Besides, the peak at δ_C 83.65 (C-5) was considered as oxymethine carbon once the nature of the hydroxyl group present was determined as phenolic. Thus, the carbon bearing the phenolic OH group is a non-hydrogenated one displayed at δ_C 153.62 (C-7a). This spectrum also confirmed the *Z* configuration of the double bond (δ_C 129.89 and 129.90) of the alkenyl chain by the observed deshielded chemical shifts of the allylic carbons at δ_C 27.23 and δ_C 26.95 (C-10'/C-11').[11]

The position groups attached in the structure were partially determined by HMBC. The correlations of methylene hydrogens (H-3) at δ_H 2.83 and 3.22 and the C-4 (δ_C 112.40), C-3a (δ_C 128.22), C-7a (δ_C 153.62) and the oxymethine carbon (C-2) are indicative both ring junction (Table 1).

The localization of the OH group at C-5 was just allocated by empirical calculations using the effect of the substituent groups on the aromatic ring described in the literature.[12] The HMBC correlations were useless for this kind of assignment. The obtained theoretical values indicated the position of OH in C-5 were closer than the experimental data.

The location of the double bond in each compound was established through the mass spectral analyses of the dimethyl disulfide (DMDS) derivatives. The EIMS of the DMDS derivatives showed ion peak fragments at *m/z* 321 ($C_{19}H_{29}O_2S$) due to the bond cleavage between the carbon bearing the methylthio groups of C-10' and C-11'. These evidences permitted to identify the mixture **1**, **2** and **3** as being 2-[(10'*Z*)-dodec-10'-enyl]-dihydro-1-benzofuran-5-ol, 2-[(10'*Z*)-tridec-10'-enyl]-dihydro-1-benzofuran-5-ol and 2-[(10'*Z*)-pentadec-10'-enyl]-dihydro-1-benzofuran-5-ol, respectively. These compounds present optical activity which indicates they are not artifacts but the absolute configuration of C-2 of these compounds could not be determinate by NMR and remains unknown.

Table 1. ^1H (400 MHz) and ^{13}C (75 MHz) NMR data and long-range correlations (HMBC) of **1**- **3**[$CDCl_3$, J (Hz), δ (ppm)]

position	δ_C	δ_H	HMBC
2	83.7	4.74 (m)	
3	36.1	3.22 (*dd*, 8.8, 8.8)	
2.83 (*dd*, 8.0, 8.0)	153.6, 128.2, 83.7, 35.9		
3a	128.2	-	-
4	112.4	6.68 (*brs*)*	153.6, 149.4, 114.1, 36.1
5	149.4	-	-
6	114.1	6.58 (*d*, 8.4)	153.6, 149.4, 112.4,
7	109.1	6.62 (*d*, 8.4)	153.6, 149.4, 128.2
7[a]	153.6	-	-
1'	35.9	1.82 (*m*)	
1.63 (*m*)	-		
2'- 8'	29.8-29.6	1.32 (*m*)	
9'	26.9	2.04 (*m*)	129.9
10'/11'	129.9	5.36 (*m*)	27.2
12'	27.2	2.04 (*m*)	129.9
13'	32.0	1.32 (*m*)	
14'	22.4	1.32 (*m*)	
15'	14.2	0.91 (*t*, 6.4)	22.4, 32.0

**brs* broad singlet.

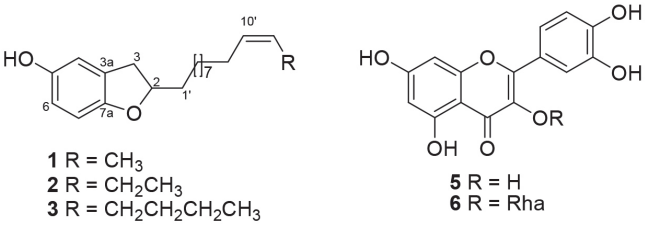

Figure 1. *Isolated compounds from* T. guianensis *seeds*

The know isolated compounds sitosterol, quercetin (**5**), quercitrin or quercetin-3-*O*-rhaminosyl (**6**) and gallic acid were identified by NMR, UV spectrometry and comparison with literature data and direct comparison with standard by HPLC (Supplementary Material).[13]

Methanolysis of the mixture of triglycerides from hexane extracts of leaves and seeds yielded a mixture of two fatty acid methyl ester mixtures which were identified by GC-MS and FID GC comparison with a commercial F.A.M.E standard (Table 2). The fatty acid profile of the oil's seeds indicated it is rich of unsaturated while the leaves' profile indicates a predominance of saturated fatty acid.

Table 2. Fatty acid composition isolated from *T. guianensis* leaves and seeds

Fatty acid derivatives	% ± SD (leaves)	% ± SD (seeds)
Myristic (14:0)	9.22 ± 0.63	n.d.
Isopalmitic (i-16:0)	5.48 ± 0.01	n.d.
Palmitic (16:0)	24.71 ± 2.48	17.64 ± 0.75
Linoleic (18:2)	12.26 ± 0.50	29.88 ± 0.39
Linolenic (18:3)	3.53 ± 0.38	n.d.
Oleic (18:1)	20.25 ± 1.25	27.99 ± 1.08
Stearic (18:0)	24.53 ± 1.06	24.49 ± 1.06
Saturated fatty acids	63.94	42.13
Unsaturated fatty acids	36.04	57.87
Total identified	100	100

SD: standard deviation; n.d. not detected.

The Brine Shrimp Test has been employed as a screening of cytotoxic compounds or extracts. The LD_{50} of CH_2Cl_2 soluble fraction of seeds (> 1000 μg mL^{-1}) and flowers (615.46 μg mL^{-1}) and, EtOAc (166.36 μg mL^{-1}) and BuOH (249.37 μg/mL) indicated that except the seed CH_2Cl_2 of *T. guianensis* all of them are moderately active (LD_{50} = 100-1000 μg mL^{-1}).[14] However, the mixture **1**-**3** (LD_{50} = 174.06 μg mL^{-1}) showed considerable activity. These compounds are present in minor concentrations in the seed oils, which justifies the absence of activity in the CH_2Cl_2 soluble fraction of seeds.

The most common and practical methodology to determine antioxidant is quenching of free radical DPPH (2.2-diphenyl-1-picryl-hydrazil).[15] The organic extracts of the flowers of *T. guianensis* were evaluated and the EtOAc soluble fraction (EC_{50} = 69.22 μg mL^{-1}) is more active than the BuOH (EC_{50} = 116.99 μg mL^{-1}) comparing with the positive standard (EC_{50} =26.45 μg mL^{-1}). This activity probably in accordance with the presence of flavonoids and gallic acid in the EtOAc soluble fraction.

CONCLUSIONS

The chemical study of the organic extracts of seeds of *T. guianensis* permitted to isolate known compounds and three new alkenyl dihydrobenzofuranoids (**1**-**3**) presenting cytotoxic activity in *Artemia salina* nauppli. Besides the fatty acid profile of hexane extracts of seeds and leaves indicated the oil from leaves is predominantly saturated and the seed oils were composed predominantly by unsaturated fatty acids. The presence of toxic alkylphenols in nonpolar fractions of the seed extracts could not indicate the *T. guianensis* seed oils for food purposes. However, more investigations must be performed.

SUPPLEMENTARY INFORMATION

Some spectra, spectrometric data, chromatograms are available as supplementary material free of charge at http://quimicanova.sbq.org.br

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