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Chemical constituents and evaluation of the toxic and antioxidant activities of *Averrhoa carambola* leaves

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Abstract: The liquid-liquid partitioning of a crude hydroalcoholic extract of *Averrhoa carambola* L., Oxalidaceae, leaves led to the isolation of a sterol and three flavone *C*-glycosides. From the *n*-hexane fraction β-sitosterol was isolated and from the ethyl acetate fraction apigenin-6-*C*-β-L-fucopyranoside (**1**) and apigenin-6-*C*-(2"-*O*-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**2**) were obtained. Apigenin-6-*C*-(2"-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside (**3**) was isolated from the *n*-butanol fraction. Compound **3** is new, while **1** and **2** have been previously isolated from *A. carambola*. The antioxidant activity was measured using the DPPH radical scavenging assay and reducing power of iron (III) to iron (II) ions. The ethyl acetate and *n*-butanol fractions showed the most antioxidant activity. As evaluated by ability of the sample to scavenge DPPH the IC50 values were 90.92 and 124.48 µg/mL, respectively. In the assay of reducing power these fractions presented 135.64 and 125.12 of ascorbic acid equivalents, respectively. The antioxidant activity exhibited a significant relationship with the phenolic content ($r^2 = 0.997$), but a poor relationship with the flavonoids content ($r^2 = 0.424$). The *n*-hexane fraction was the only fraction to present good toxicity using *A. salina* with LC50 800.2 µg/mL.

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Introduction

The family Oxalidaceae comprises more than 900 species belonging to seven genera, namely, *Oxalis, Biophytum, Sarcotheca, Dapania, Eichleria, Hypseocharis*, and *Averrhoa*. The genus *Averrhoa* includes two of Oxalidaceae species, *A. bilimbi* and *A. carambola* (Zonlefer, 1994). *Averrhoa carambola* L., commonly known as star fruit or carambola, is a tree originally from Asia which has become acclimatized in many tropical countries, including Brazil. This plant is a small bush that can grow to 4-6 m in height (Pio Corrêa, 1926-1975; Panizza, 1998).

The leaves and fruits of *A. carambola* have been used in folk medicine as an appetite stimulant, a diuretic, an antidiarrheal, and a febrifugal agent, as well as in the treatment of eczemas (Pio Corrêa, 1984). Also, decoction of the leaves has been used in diabetes treatment (Provasi et al., 2001). Previous investigations on *A. carambola* have revealed the presence of steroids and triterpenes (Ranganayaki et al., 1980; Tadros & Sleen, 2004), cyanidin glycosides (Gunasegaran, 1992), *O*-glycosyl flavonoids (Tiwari et al., 1979), and *C*-glycosyl flavones (Araho et al., 2005).

The species *A. carambola* presents hypoglycemic (Chau et al., 2004a; Ferreira et al., 2008), hypocholesterolemic (Chau et al., 2004b), antimicrobial (Hsu et al., 2007), antioxidant (Shui & Leong, 2006), and anti-inflammatory (Cabrini et al., 2011) effects. An alcoholic extract of the stems of *A. carambola* has been shown to exhibit selective activity against brain tumor cells while that of the leaves was effective against liver carcinoma cells (Tadros & Sleem, 2004). The aqueous leaf extract of *A. carambola* depresses atrial inotropism in the guinea pig (Vasconcelos et al., 2008).

In this paper we describe the isolation of one sterol and three glycosyl flavones from the leaves of *A. carambola*. A further aim of this study was to test the crude hydroalcoholic extract and *n*-hexane, ethyl acetate and *n*-butanol fractions in order to determine their toxicity towards *Artemia salina* and investigate their antioxidant activity using DPPH free radical scavenging and reducing potential assays.

Materials and Methods

General procedures

Column chromatography was performed over silica gel 230-400 mesh (Merck).Thin-Layer

chromatography (TLC) was performed on a pre-coated silica gel type 60 plates (Merck), spots were located by spraying with ferric chloride. The IR spectrum was recorded on a Perkin Elmer FTIR 16 PC spectrophotometer; NMR spectra were recorded on a Varian AS-400 spectrometer using TMS as internal standard. The absorbance of the assays were measured (each measurement was repeated three-times) in a Hitachi UV-Vis U 3000 spectrophotometer (Tokyo, Japan). *Artemia salina* were obtained from dehydrated eggs purchased in a specialized store.

Plant material

The leaves of *Averrhoa carambola* L., Oxalidaceae, were collected in Santo Amaro da Imperatriz-SC (10º 53' 57.55"S, 37º 07' 14.30'W, altitude 10 m) from a healthy and agrotoxic-free plant. Botanical identification was performed by Dr. Daniel de Barcellos Falkenberg. A voucher specimen (FLOR 24.144) has been deposited in the Herbarium FLOR, Universidade Federal de Santa Catarina, Santa Catarina, Brazil.

Extraction and isolation

The air-dried leaves of *A. carambola* (281.0 g) were extracted with 80% ethanol at room temperature for fifteen days. The solvent was removed by rotatory evaporation (<55ºC). The crude hydroalcoholic extract (41.3 g) was resuspended in EtOH:H_2O 20:80 v/v and partitioned with *n*-hexane, ethyl acetate (EtOAc) and *n*-butanol (n-BuOH) yielding the *n*-hexane (6.23 %), EtOAc (16.70 %) and *n*-BuOH (24.70 %) fractions, respectively.

The *n*-hexane fraction (2.6 g) was subjected to silica gel chromatographic column, using *n*-hexane, EtOAc and EtOH in mixtures of increasing polarity. Recrystallization from *n*-hexane of one fraction resulted in a compound obtained as white crystals (hex/EtOAc 90%; 15 mg; mp 120.1 - 121.5 °C), identified as β-sitosterol (Garg & Nes, 1984; De-Eknamkul & Potduang, 2003).

The EtOAc fraction (6.9 g) chromatographed on silica gel using EtOAc/EtOH mixtures with increasing polarity yielded 36 fractions (100 mL), which were combined in sub-fractions according to TLC analysis. The sub-fraction 6-8 (EtOAc/EtOH 98%) analyzed by TLC (R_f 0.65, EtOAc/MeOH 90%), yielded a yellow amorphous powder, and after successive recristallizations under methanol yield 150 mg of apigenin-6-*C*-β-L-fucopyranoside (**1**). The sub-fraction 10-13 (EtOAc/EtOH 95%) was analyzed by TLC (R_f 0.37, EtOAc/MeOH 90%), yielded a yellow solid. After recristallizations under methanol yielded 200 mg of apigenin-6-*C*-(2"-*O*-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**2**). Identification of these flavonoids were carried out by interpretation of their spectral data as well as by comparison with data reported in the literature (Cazarolli et al., 2009b; Suzuki et al., 2003; Araho et al., 2005). Analysis of the *n*-BuOH fraction (10.2 g) followed the same chromatographic procedure. Sub-fraction 11-13 (EtOAc/EtOH 70%) after observations on TLC (R_f 0.45, EtOAc/MeOH 80%) led to the isolation of 8.6 mg of apigenin-6-*C*-(-2"-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside (**3**) as a white solid.

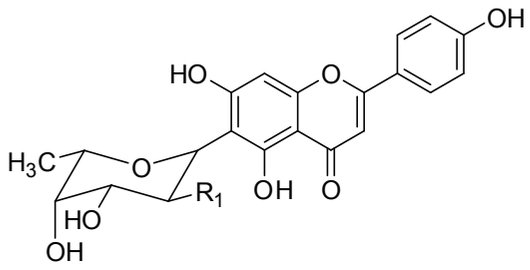
Apigenin-6-*C*-(-2"-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside (**3**): mp 253-256 ºC; IR bands (KBr): 3377, 2909, 1607, 1648, 1453, 1349, 1248, 1183, 1077, 835. NMR ^{1}H (400 MHz, DMSO-d6): 6.97 (s, H-3), 6.90 (s, H-8), 7.96 (d; 8.8 Hz, H-2', 6'), 6.94 (d; 8.8 Hz, H-3', 5'), 5.19 (d; 9.9 Hz, H-1"), 4.55 (m, H-2"), 3.83 (m, H-3"), 3.61 (m, H-4"), 3.50 (m, H-5"), 4.35 (m, H-6"), 5.11 (sl, H-1"'), 3.59 (m, H-2"), 3.23 (m, H-3"'), 2.95 (m, H-4"'), 2.49 (m, H-5"'), 1.09 (d; 6.4 Hz, H-6"'). NMR^{13}C (100 MHz, DMSO-d6): 164.86 (C-2), 103.81 (C-3), 182.84 (C-4), 162.05 (C-5), 111.86 (C-6), 163.64 (C-7), 95.32 (C-8), 159.92 (C-9), 102.82 (C-10), 121.59 (C-1'), 129.32 (C-2' and C-6'), 116.72 (C-3' and C-5'), 157.08 (C-4'), 73.72 (C-1"), 75.71 (C-2"), 76.38 (C-3"), 70.53 (C-4"), 78.06 (C-5"), 61.47 (C-6"), 105.69 (C-1"'), 74.56 (C-2"'), 74.15 (C-3"'), 72.57 (C-4"'), 68.24 (C-5"'), 17.64 (C-6"'). The NMR H^1 and C^{13} data are in agreement with the literature data (Rayyan et al., 2005).

Biological activity

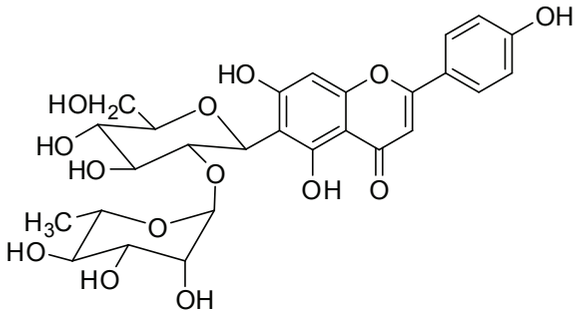
The crude hydroalcoholic extract and the fractions of the liquid-liquid partition (*n*-hexane, EtOAc and *n*-BuOH) were subjected to different assays. The results were subjected to analysis of variance (ANOVA) and Fisher's test for multiple comparisons, setting $p<0.05$ for statistical significance indices.

Determination of total phenolic

The amount of total phenolics was determined using the Folin-Ciocalteu method (Gutfinger, 1981), with modifications. In this method, the reaction mixture was composed of 0.5 mL of extract (1.0 mg/mL), 5.0 mL of distilled water, and 0.5 mL of the Folin-Ciocalteu reagent. After a period of 3 min, 1.0 mL of saturated sodium carbonate solution was added. These mixtures were shaken and allowed to stand for 1 h. The absorbance was measured at 725 nm. A calibration curve of gallic acid was prepared ($y=5.51x+1.77$; $r^2=0.999$), and the results were expressed as gallic acid (GA) equivalents (mg gallic acid/g dry extract).



1 R_1=H
2 R_1=Rham



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Determination of total flavonoid content

The total flavonoid content was determined spectrophotometrically according to Lamaison and Carnat (Quettier-Deleu et al., 2000) with modifications. Briefly, 0.5 mL of 2% aluminum chloride ($AlCl_3$) in ethanol was mixed with the same volume of vegetal extracts (1.0 mg/mL). Absorption readings at 415 nm were taken after 1 h against a blank (ethanol). The total flavonoid content was determined using a standard curve with quercetin ($y=8.776x+0.869$; $r^2=0.999$). The mean of three readings was used and expressed as quercetin (QE) equivalents (mg quercetin/g of dry extract).

DPPH radical scavenging assay

The free radical scavenging activity of the vegetal sample was measured using the method described by Brand Williams et al. (1995) with some modifications. One milliliter of the ethanol extracts of the plants and compounds (5-200 µg/mL) was added to 2 mL of a solution of DPPH radicals in methanol (0.004%). The mixture was shaken vigorously and allowed to stand for 30 min at room temperature. The absorbance (Abs_{sample}) of the resulting solution was measured at 517 nm and converted into percentage of antioxidant activity (AA) using the following formula: $AA\% = 100-\{[(Abs_{sample}-Abs_{blank})\times100]/Abs_{control}\}$. A ethanol (2.0 mL) and plant extract/compound (1.0 mL) solution was used as the blank (Abs_{blank}). A DPPH (2.0 mL) and ethanol (1.0 mL) solution was used as the control ($Abs_{control}$). Ascorbic and gallic acids were used as standards. The radical scavenger activity was expressed in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (IC50). The IC50 value for each sample was determined graphically by plotting the percentage disappearance of DPPH as a function of the sample concentration.

Determination of reducing power

This assay is based on the method of Price & Butler, proposed by Waterman & Mole (1994), with modifications. 100 mL of test solutions (crude extracts and fractions, diluted in methanol at a concentration of 1000 ppm) were added to 8.5 mL of deionized water. It was then added 1.0 mL of 0.1 M $FeCl_3$, and after 3 min, 1.0 mL of 0.08 M potassium ferricyanide. After 15 min, the absorbance was measured at 720 nm. For the blank, was used a solution prepared as above without the addition of the sample. The reducing power was determined using a standard curve with ascorbic acid ($y=0.0015x + 0.124$; $r^2=0.999$). The results were expressed as ascorbic acid (AA) equivalents (mg ascorbic acid/ g dry extract).

Toxicity in Artemia salina

The extracts, fractions and pure isolated compounds were evaluated in a test for toxic activity against larvae of *Artemia salina* (Meyer et al., 1982), with minor modifications. Toxicities of compounds were tested at 10-1000 ppm in 1 mL sea-water solutions with 1% DMSO (v/v). Ten, one-day nauplii were used in each test and survivors counted after 24 h. Three replications were used for each concentration. A parallel series of tests with the standard potassium dichromate solution (LC50 20-40 ppm) and the blank control were always conduced. After 24 h of exposure, the results were expressed as LC50 value, concentration able to kill 50% of the larvae.

Results and Discussion

The liquid-liquid partitioning of a crude hydroalcoholic extract of *A. carambola* leaves resulted in the isolation of β-sitosterol (Garg & Nes, 1984; De-Eknamkul & Potduang, 2003) from the *n*-hexane fraction and two *C*-glycosyl flavones, apigenin-6-*C*-β-L-fucopyranoside (**1**) and apigenin-6-*C*-(2”-*O*-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**2**), from the EtOAc fraction. These compounds had previously been isolated from *A. carambola* leaves (Ranganayaki et al., 1980; Araho et al., 2005). Recently, studies have shown that these flavones reduce blood glucose levels in diabetic rats, stimulate insulin secretion and potentiate

glucose-induced insulin secretion in hyperglycemic rats. In addition, these flavonoids can stimulate glucose uptake in rat soleus muscle through the involvement of the well-known mechanism of insulin signal transduction (Cazarolli et al., 2009a,b). Apigenin-6-C-(-2"-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside (**3**) was isolated from the *n*-BuOH fraction and is reported for the first time in *A. carambola.* Compound **3** has previously been found in *Oxalis triangularis* of the Oxalidaceae family (Rayyan et al., 2005). The low yield of this compound prevented subsequent biological studies.

The crude extract of the leaves of *Averrhoa carambola,* and the fractions obtained from the liquid-liquid partitioning, were evaluated for antioxidant activity. The antioxidant activity in plants is associated with phenolic compounds, mainly flavonoids. According to Table 1, the highest phenolic contents were found in the EtOAc and *n*-BuOH fractions. In these fractions the most accentuated antioxidant activity was observed, as evaluated through of the ability of the extract or fraction to scavenge DPPH and to reduce iron(III) to iron(II) ions. This suggests that the high antioxidant activity of the more polar fractions is probably due to the substances with a hydroxyl group, capable of donating a hydrogen radical to the oxidant molecule (Mensor et al., 2001).

The flavonoids are a class of vegetal secondary metabolites of phenolic nature, which have remarkable antioxidant properties. According to the results shown in Table 1, it can be seen that the highest concentration of flavonoids is present in the EtOAc fraction, with 35.25 mg QE/g dry fraction. This result is in agreement with the data obtained in the phytochemical analysis of the fraction which yielded flavonoids **1** and **2**. The low activity observed for the *n*-BuOH fraction was probably due to the low concentration of flavonoid **3**. A direct correlation was observed between the ability to capture the DPPH with the phenolic content, with a correlation coefficient of r^2=0.997. This good correlation confirms the data presented in several other studies, which have shown that the antioxidant capacity is strongly correlated with the content of phenolic compounds (Kaur & Kapoor, 2002; Thaipong et al., 2005; Abdille et al., 2005; Makris et al., 2007). However, no relationship is maintained when correlating the ability to capture the DPPH with the amount of flavonoids in the extract and fractions of *A. carambola* (r^2=0.424). Flavonoids **1**-**3** were also tested through observing the capture of free radicals and these compounds showed IC50 values greater than 200 µg/mL, considered high values for isolated compounds. So, it shows that these flavonoids are not responsible for the antioxidant activity of the EtOAc and *n*-BuOH fractions.

Determination of the toxicity to *Artemia salina* has been used efficiently to analyze the biological potential of plant extracts (Colegate & Molyneux, 1993). Several natural compounds, particularly substances with antitumoral, antimicrobial, insecticidal and anti-trypanosomicidal activity, have been tested through these bioassays, showing a significant correlation (Meyer et al., 1982; McLaughlin et al., 1991; Alves et al., 2000). According to Meyer et al., (1982) who classified crude extracts and pure substances as toxic (LC50 value <1000 µg/mL) or non-toxic (LC50 value >1000 µg/mL), *n*-hexane was the only fraction to present good toxicity against *A. salina* (LC50 800.2 µg/mL). This activity can be explained by the presence of β-sitosterol the activity of which is described in the literature, with an LC50 value of 79 µg/mL (Adoum, 2009). Toxic activity was observed for compounds **1** and **2** with LC50 values of 134.3 µg/mL and 95.5 µg/mL, respectively. However, no activity was observed for the EtOAc and *n*-BuOH fractions. This is probably due to the low concentration of compounds **1, 2** and **3** in these fractions.

Conclusions

This paper reports on a phytochemical investigation involving *Averrhoa carambola* leaves and the evaluation of toxicity and antioxidant effect. The glycosyl flavone **3** is reported for the first time in *A. carambola*. The compounds isolated from *A. carambola* showed low antioxidant activity but high toxicity against *Artemia salina.* This indicates that the toxic activity displayed by the isolated compounds is not associated with the antioxidant activity observed for the extracts.

Table 1. Antioxidant activity of crude extract and fractions of *Averrhoa carambola.*

Extract/Fraction	Phenolics (mgGA)/g	Flavonoids mgQE/g	Reducing potential mgAA/g	DPPH IC50 µg/mL
Crude extract	33.39+0.35	14.28+1.31(*)	98.02+0.23	212.59+0.30
n-hexane	29.44+0.36	26.70+0.74	86.22+1.52	223.67+0.98
EtOAc	79.07+0.63	35.25+0.05	135.64+1.63	90.92+0.10
n-BuOH	59.66+1.00	14.23+0.23(*)	125.12+1.63	124.48+1.21
Ascorbic acid	-	-	-	8.4+0.26
Gallic acid	-	-	-	2.6+0.01

All means, except those marked by (*), showed significant differences in the Fisher test (p<0.05).

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