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Isolation of scopoletin and biological activities from *Warszewiczia coccinea* (Rubiaceae)

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Abstract

Rubiaceae is a plant family that produces several bioactive metabolites, including: indole alkaloids, triterpenes, iridoids, anthraquinones, among other. Among these species is *Warszewiczia coccinea* (Vahl) Klotzsch for which there are few chemical studies in the literature consulted. Considering this gap, the present study intended to deepen the phytochemical study and carry out evaluations of antimicrobial and antiangiogenic activities (chorioallantoic membrane assay). For phytochemical investigation, thin layer chromatography (TLC) and open column chromatography (CC) techniques were used and for chemical analyses, nuclear magnetic resonance (NMR). The results showed that methanolic extracts from the leaves presented antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*. The hexane extract of the branches showed antiangiogenic activity. Furthermore, hexane and methanolic leaf extracts caused toxicity at all concentrations evaluated in the angiogenesis assay. From the methanolic extract of leaves, it was possible to isolate the coumarin scopoletin. Furthermore, this extract showed an indication of the presence of other metabolites in ^{1}H NMR analysis, such as terpenes and indole alkaloids. This is the first report of antimicrobial and antiangiogenic activity for *W. coccinea* and isolation of a coumarin for the genus *Warszewiczia*.

Keywords: Antimicrobial. Antiangiogenic. Scopoletin. NMR. Alkaloids.

Introduction

Plant metabolites still can offer compounds with important biological activities. The chemodiversity of these Natural Products may provide important molecular structures, some of them may be useful against microbial infections, resistant microorganisms, or human tumor vascularization[1,2]. In this context, phytochemical investigations may lead to the isolation and chemical characterization of these metabolites with biological activities[3].

Brazilian plants have an important contribution to phytochemistry investigations[4]. Among these plants, species of the Rubiaceae family have some importance to Natural Products Chemistry. In the Amazon rainforest, this family ranks as the second-largest botanical family in the number of species [5]. The main metabolites that occur in Rubiaceae species are alkaloids, triterpenes, iridoids, anthraquinones, and other classes. These metabolites have some biological activities and contributions to Rubiaceae's chemosystematics[6,7]. Most alkaloids from this family have an indolic skeleton[7,8], like monoterpenes indolic alkaloids found in *Duroia macrophylla*[9-11]. Additionally, other metabolites can be found like flavonoids and other classes[12]. Despite the chemodiversity of Rubiaceae, some species still lack chemical studies.

One of Rubiaceae genus is *Warszewiczia*, where there are few reports in the literature consulted. Only *Warszewiczia coccinea*, *W. cordata,* and *W. schwackei* showed chemical or biological studies[13-17]. For *W. coccinea* and *W. cordata*, there are results for antileishmanial activities, but the metabolites responsible for this activity remain unknown[13,16]. There's been one report on the isolation of metabolites from *W. coccinea*: Calderon[15] reports the isolation of two triterpenes: 6*β*,19*α*-dihydroxyursolic acid, and sumaresinolic acid which demonstrate activities on acetylcholinesterase inhibition. In *W. schwackei* can be found indolic alkaloids, highlighting the *β*-carboline alkaloids. Previously, we reported the antioxidant, antimycobacterial, antimalarial, and cytotoxicity activities of this species[17]. However, the few reports demonstrate a gap in the literature for the chemical characterization of bioactive metabolites from the *Warszewiczia* genus.

Aiming to increase the chemical knowledge and isolate bioactive metabolites from *W. coccinea* (Rubiaceae) we performed this phytochemical study. The present study is the first report of antimicrobial, antiangiogenic activity, and isolation of coumarin from leaves.

Material and Methods

List of reagents and equipment

Deuterated solvents ($CDCl_3$ and DMSO-d_6) were obtained from Cambridge Isotope Laboratories, Andover, USA. For chromatography experiments, was used Silica gel 60 (230-240 Mesh), Florisil (100 – 200 Mesh), and Thin-Layer Chromatography (20x20 cm commercial plates) were obtained from MERCK®, Germany. In antimicrobial assays, was used a Spectrophotometer for 96-well plate reading: Thermo Scientific, Multiskan GO. In angiogenic assays, an Egg incubator was used: Juli, Chocmaster®. NMR experiments were analyzed on a Bruker spectrometer (300 MHz), equipped with an EASYPROBE S1 5mm Z-GRADIENT. The Mass spectra were obtained in a Bruker Amazon Speed, Ion trap, and ESI ionization font. The Mass spectrometer is hyphenated on a liquid chromatographer Model Prominence UFLC (Shimadzu), luna C18 column, equipped with an LC-20AT binary pump, SPDM-20A diode array detector (DAD), and SIL-20A automatic injector.

Collection and Botanical identification

Leaves, branches, and bracts were collected from *Warszewiczia coccinea,* the plant material was collected in Campus I: Instituto Nacional de Pesquisas da Amazônia, INPA (3°05'46''S 59°59'17''W on March 30, 2019). A voucher with fertile botanical material is available at INPA´s Herbarium (numbers 283507 and 285835). The plant collection has been registered on SISGEN (A85ED6B).

Plant extraction

The plant material was separated into leaves, branches, and bracts from the inflorescences, which were dried in a controlled temperature room. After drying, the samples were powdered in a knife mill. The plant parts were extracted with organic solvents in increasing order of polarity: hexane (Hex) and methanol (MeOH). Using maceration assisted on ultrasonic bath (40 MHz) for 20 min, adopting at least 3 extractions and obeying the proportion of 1 g of plant material to 3 mL of solvent (1:3, m/v). The extracts were concentrated in a rotary evaporator at a temperature < 50 °C.

Phytochemistry analysis by TLC and ^{1}H NMR spectroscopic experiments

The crude extracts were analyzed by Hydrogen Nuclear Magnetic Resonance (^{1}H NMR) operating at 300 MHz for ^{1}H nucleus. For sample preparation, 20 mg of each crude extract was used and dissolved in 550 µL of deuterated solvents containing TMS for internal reference. $CDCl_3$ was used for the hexanic extracts and DMSO-d_6 for the methanolic extracts. The spectra obtained were processed and analyzed using Bruker® TopSpin software (v3.6.4). To aid in the interpretation of the spectra, data from the literature on the family's chemosystematics were used for the chemical characterization of the crude extracts.

In addition, the crude extracts were analyzed through Thin Layer Chromatography (TLC) to previously detect the chemical classes present and evaluate the separation profile. The samples were eluted in appropriate solvent systems. UV light (λ: 254 and 365 nm) and chemical reagents like sulfuric anisaldehyde, ceric sulfate, ferric chloride, and Dragendorff reagent were used to indicate the classes of secondary metabolites in these extracts.

Isolation of scopoletin

Initially, the methanolic extract of leaves was submitted to liquid-liquid partition. Sample (19.72 g) was solubilized in a hydromethanolic mixture (MeOH/H_2O 1:1, v/v), obeying the proportion of 1 g of sample to 50 mL of MeOH/H_2O. Subsequently, the hydromethanolic phase was partitioned with Hexane (Hex), Dichloromethane (DCM), and ethyl acetate (EtOAc), respectively.

The DCM phase (FDCM, 1.5 g) was fractionated by column chromatography (h x ø: 5 x 8 cm) using silica gel, and as mobile phase Hex, DCM, EtOAc, and MeOH with an increasing gradient of polarity, starting with a mixture of Hex/DCM 1:1, yielding 12 fractions. The fractions 5-6 (36 mg) were refractionated by column chromatography (h x ø: 22 x 1,3 cm) using Florisil and as mobile phase DCM, EtOAc, and MeOH with an increasing gradient of polarity, starting with DCM 100%, yielding 39 fractions. The subfractions 30-32 (15 mg) were refractionated by preparative thin-layer chromatography using silica gel and as mobile phase DCM/EtOAc 95:05. After the elution, the plate was placed under UV light (λ: 254 and 365 nm), the band with Rf: 0,5 and intense blue fluorescence was delimited and removed from the plate. The resulting sample (3 mg) was

analyzed by mass spectrometry coupled with liquid chromatography (LC-MS) and ^{1}H, ^{13}C, and 2D NMR, which allowed the identification of the scopoletin. In LC-MS the sample was analyzed in positive and negative mode with Electrospray source (ESI), with H_2O (0.1% Formic Acid)/ACN (71.25: 28.75) elution system.

Antimicrobial assay

The microorganism strains used in this assay were: *Pseudomonas aeruginosa* (PA – Strain ATCC 10145), *Candida albicans* (CA – Strain ATCC 10231), *Escherichia coli* (EC – Strain ATCC 11775), *Pseudomonas fluorescens* (PF – Strain ATCC 13525), *Aeromonas hydrophila* (AH – Strain ATCC 7966), *Klebsiella pneumoniae* (KP – Strain ATCC 13883), *Salmonella enterica* (SE – Strain ATCC 13076), SM *Serratia marcensces* (SM – Strain ATCC 13880), *Citrobacter freundi* (CF – Strain ATCC 8090), *Staphylococcus aureus* (SA – Strain ATCC 12600). For this assay with natural products, the adapted guidelines that are recommended by the Clinical and Laboratory Standards Institute[18] for *in vitro* antimicrobial sensitivity assays were used.

To determine the antimicrobial activity of the extracts, the microorganism inocula were standardized at 0.5 McFarland, then the standardized inocula were diluted (1:20) to be aliquoted for the assay. In a 96-well plate, 90 µL of each aliquot of extracts (1000 $\mu g.mL^{-1}$ in Müeller-Hinton broth with DMSO 5%) was evaluated in triplicate. For negative control, the culture media Müeller-Hinton broth with DMSO at 5% was used. For positive control, 90 µL the antibiotic oxytetracycline was used (125 $\mu g.mL^{-1}$ in Müeller-Hinton broth). In each well, 10 µL of the aliquot of the standard microorganism inoculum was added.

Then, the 96-well plates were incubated at ± 37°C for 16 to 24 hours. The prepared plates were read in a spectrophotometer (λ: 625 nm) before and after the appropriate incubation. In the end, they were revealed with 40 µL of 2,3,5-triphenyl tetrazolium chloride solution in 2% (v/v), to verify cell viability. The results were submitted to analysis by one-way ANOVA followed by Dunnett post-test, comparing the samples with the negative control (CI of 95%, P value < 0.05). The data obtained were processed and analyzed with GraphPadPrism (v7.0) and Excel® software.

Angiogenic assay

The evaluation of antiangiogenic activity was performed based on the methodology described by Nguyen, Shing, and Folkman[19] performed in the chicken egg (*Gallus domesticus*) embryo chorioallantoic membrane model (CAM). Using concentrations at 1000, 500, and 100 $\mu g.mL^{-1}$ of the extracts diluted with ethyl alcohol, were implanted in methylcellulose discs, following the methodology described by Falcão-Bücker[20].

Fertilized eggs were placed in an incubator, in the horizontal position (temperature 37.5°C and under 33% relative humidity). After 48 h of incubation, a small window of 5 mm diameter was opened in the shell, in the region of the air chamber of the egg, to aspirate 3 mL of egg white. The number of test eggs was in triplicate for each treatment and negative control. Another window of 15 mm in diameter was also opened in the region of the egg positioned above the region of the chorioallantoic membrane of the embryos and closed with black tape to minimize loss of humidity. The embryos remained under incubation, for another 72 h until the embryonic age of 6 days. At this moment, a methylcellulose disk (1.5%) embedded with extract samples, was placed over the chorioallantoic membrane, and implanted over the blood vessels in the external third of the chorioallantoic membrane. The orifice was again closed with the same tape. Incubation continued for a

further 48 h, until the embryonic age of 8 days. For negative control, the methylcellulose disk was embedded in ethyl alcohol and dried.

For the analysis of angiogenic activity, the tape was removed to visualize the embryonic and vascular development around the disk implantation and was recorded a photograph for subsequent counting of blood vessels that were intercepted and near the disk (area of 0.9 cm^2). The results were expressed in percentage of vessels ± standard deviation and compared to the negative control, the values were represented with the aid of GraphPad Prism software (v7.0).

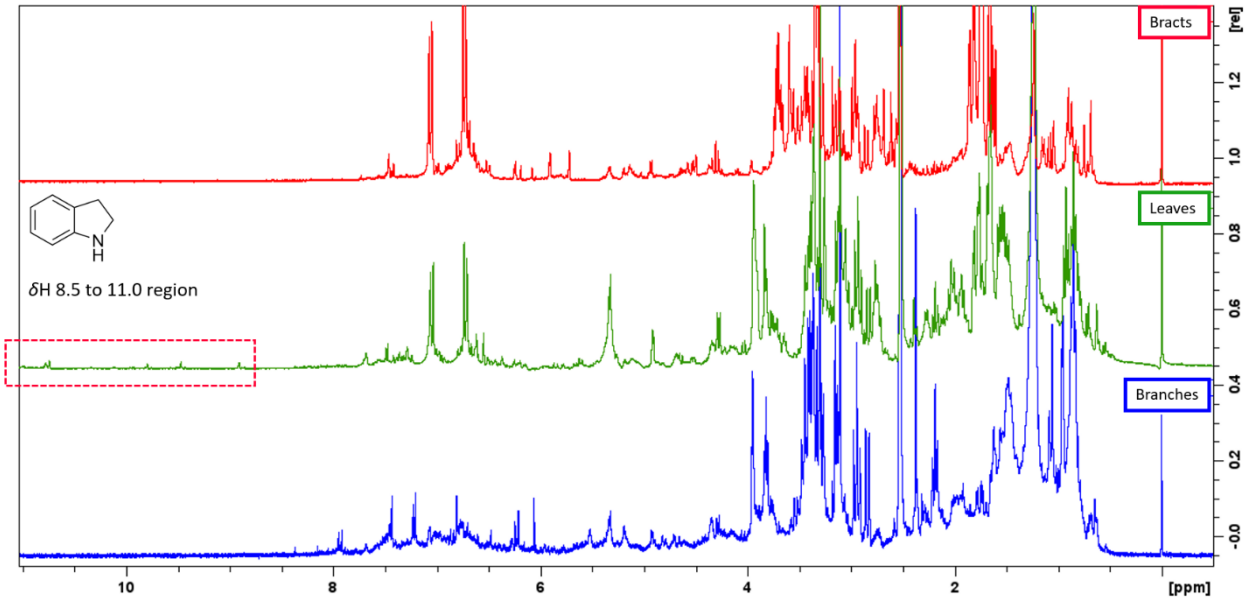
Results and Discussion

Phytochemical analysis of crude extracts

The ^1H NMR spectra of hexanic extracts of leaves and branches showed signals in d_H 0.3 and 1.5, which are characteristic of methyl hydrogens that are present in the terpenes' structure. For both extracts, it is observed the presence of olefinic hydrogens in d_H 5.36, 5.31, and 5.11, which are indicatives of the presence of the common plant steroids: b-sitosterol and stigmasterol[21,22]. In the aromatic region between d_H 6.0 and 8.0, both extracts showed low-intensity signals, demonstrating that in the crude hexane extracts the phenolic or aromatic metabolites were not observable by ^1H NMR analysis.

The methanolic extracts of leaves, branches, and bracts showed some spectral similarities among the signals. Signals in d_H 0.3 to 1.5 are characteristic of methyl hydrogens that are present in terpenes. Observed multiplets signals in d_H 3.0 to 4.0 may be related to free sugars or linked to other metabolites, and in d_H 4.0 to 5.5 were observed indications of anomeric hydrogens. In the aromatic region between d_H 6.0 and 8.0, were observed signals that suggest the presence of a phenolic, aromatic ring, or metabolites with conjugated double bonds, like flavonoids, coumarin, and other aromatics metabolites. However, only methanolic extracts of leaves showed hydrogens in d_H 10.77, 10.73, 9.79, 9.46, 8.90, which is also indicative of chelated hydroxyls, aldehydes, or nitrogen-bonded hydrogens of indolic alkaloids. In TLC analysis, the crude extract of leaves showed non-reactive with Dragendorff, although the FDCM from this extract showed orange reactive spots with Dragendorff reagent, supporting the indication of alkaloids in the extracts. There is only one report of indolic alkaloids in the *Warszewiczia* genus[17], some indolic alkaloids in Rubiaceae species can exhibit signals of the nitrogen-bonded hydrogens between d_H 8.5 to 11.0 region on ^1H NMR spectra[7,10,11,17]. This suggests that this chemical class may be present in the methanolic extracts of *Warszewiczia coccinea* leaves but at lower concentrations (**FIGURE 1**).

FIGURE 1: ^{1}H NMR (300 MHz, DMSO-d_6) spectra of methanolic extracts of leaves, branches, and bracts of *W. coccinea*.

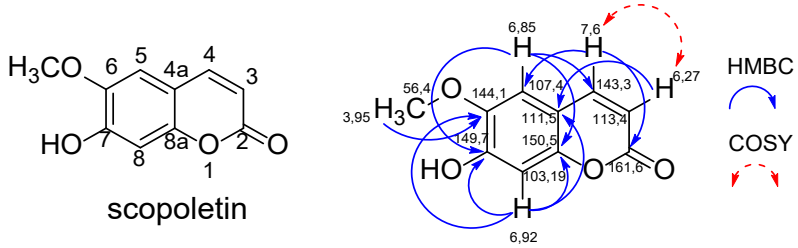


Identification of scopoletin

Scopoletin was obtained in the form of yellow crystals, showing an intense blue fluorescence under UV 365 nm light in TLC. Its structural characterization was identified by ^{1}H and ^{13}C NMR experiments and mass spectrometry (LC-MS) analyses, data were compared with the literature[23,24]. In HMBC, the methoxyl hydrogens in d_H 3.95, showed only one correlation with the carbon in d_C 144.1, nor even with the carbon in d_C 149.7, characterizing that the methoxyl is in the C-6 position (**FIGURE 2**). This demonstrated that the structure corresponded to scopoletin and not to its isomer, isoescopoletin.

Data observed: ^{1}H NMR (300 MHz, $CDCl_3$) d 3.95 (*s*, 3H, -OCH_3), 6.92 (*s*, 1H, H-8), 6.85 (*s*, 1H, H-5), 6.27 (*d*, 1H, *J* 9.57 Hz, H-3), 7.60 (*d*, 1H, *J* 9.57 Hz, H-4); ^{13}C NMR (75 MHz, $CDCl_3$) d 161.6 (C-2); 150.5 (C-8a), 149.7 (C-7); 144.1 (C-6); 143.3 (C-4); 113.4 (C-3); 107.4 (C-5); 103.2 (C-8); 56.4 (-OCH_3); LC-MS (ESI) *m/z*, $[M + H]^+$: 193.57; $[M - H]^-$: 191.57; mass: 192 u [$C_{10}H_8O_4$].

FIGURE 2: Molecular structure and 2D NMR correlation of scopoletin.



This coumarin has been already isolated from some plants like *Cordia insignis* (Boraginaceae)[25]. In the Rubiaceae family, it may be found in the leaves of *Morinda citrifolia*[26,27]. As far as we know, this is the first report of coumarin in *Warszewiczia* genus.

Antimicrobial activity

Only the methanolic extract of leaves showed significant antimicrobial activity, against *Pseudomonas aeruginosa*, *Candida albicans*, *Escherichia coli*, and *Staphylococcus aureus* in the concentration of 1000 μg.mL^{-1} (**TABLE 1**).

TABLE 1: Antimicrobial activity of methanolic extracts of leaves from *W. coccinea* extracts.

Microorganisms / Extracts	AH	CF	EC	KP	PA	PF	SA	SE	SM	CA
Leaves-MeOH	*	*	61,62 ± 0,08^{b}	*	95,9 ± 0,10^{a}	*	95,96 ± 0,22^{a}	*	*	77,75 ± 0,15^{c}
Leaves- Hex	*	*	*	*	*	*	*	*	*	*
Branches-MeOH	*	*	*	*	*	*	*	*	*	*
Branches-Hex	*	*	*	*	*	*	*	*	*	*
Bracts-MeOH	*	*	*	*	*	*	*	*	*	*

Legend: Data expressed as inhibition percentage (%) and standard deviation ($n = 3$). (*) samples that showed no activity, with inhibition values lower than 20%; $^{a}p < 0,001$, $^{b}p < 0,01$, $^{c}p < 0,5$.

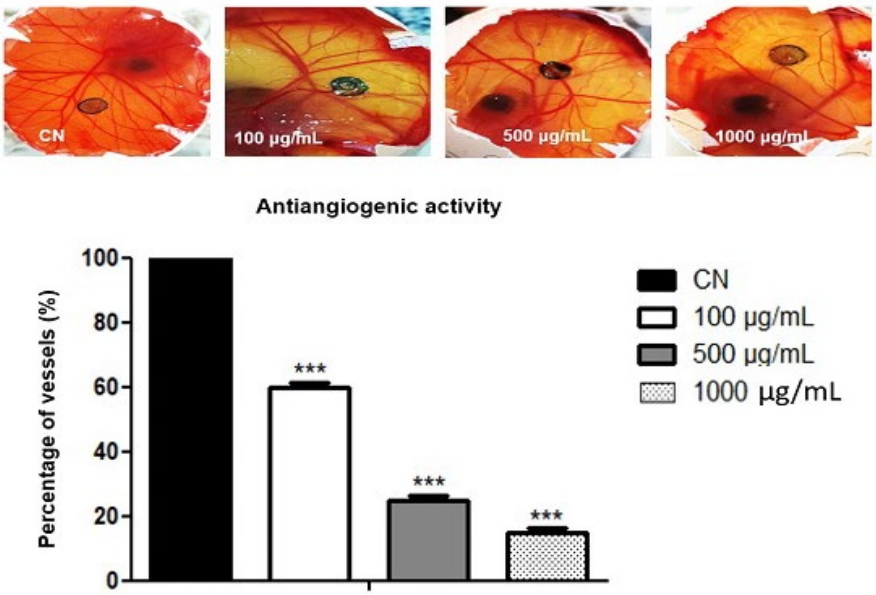
According to the literature, Rahgozar and collaborators[28] describe in their chemoinformatics-based study that in *Warszewiczia coccinea*, the triterpene sumaresinolic acid may show antimycobacterial activity because it has structural similarity with oleanolic acid, which has strong antimycobacterial synergism against *Mycobacterium Bovis*. However, so far there are no studies for the antimycobacterial activity of extracts, fractions, or metabolites isolated from *W. coccinea*.

Our phytochemical investigation may lead to the isolation of scopoletin. This coumarin demonstrates antimicrobial activity in the front of biofilms of *Candida tropicalis* and other pharmacological receptors[29]. However, other metabolites could be responsible for antimicrobial activity.

Antiangiogenic activity

Through the angiogenic assay using the CAM models, it was possible to reveal the antiangiogenic and toxicity of the evaluated extracts. Both methanolic and hexanic extracts of leaves showed toxicity at all concentrations tested, with the viability of the negative control group. The hexanic extracts of branches demonstrate dose-dependent antiangiogenic activity was found. In this extract, vessel inhibition was observed with 40%, 70%, and 80%, to 100, 500, and 1000 μg.mL^{-1}, respectively (**FIGURE 3**).

FIGURE 3: Antiangiogenic activity of hexanic extract of branches. (*n*=3 ***) compared to Negative control (CN).



Despite the toxicity for *Gallus domesticus* embryos of methanolic and hexanic extracts of leaves, in these extracts because of the high toxicity, it was not possible to observe the angiogenic activity, being necessary to evaluate lower concentrations than 100 µg.mL^{-1}. According to the recommendations of Nguyen, Shing, and Folkman[19], the viability of embryos is necessary to describe the angiogenic activity, as well as the viability of the negative control for assay validation. However, containing such toxic substances is a very promising result in the search for antitumor substances. From these extracts, the ^{1}H NMR and TLC showed terpenes, aromatics, and alkaloids evidence that might be the metabolites mainly responsible for the biological activity.

After the chromatography fractionation of the methanolic extract of leaves, it was possible to isolate the scopoletin, which has expressive antiangiogenic activity. This coumarin is present in extracts of leaves from *Morinda citrifolia* (Rubiaceae) and has been shown as a potential agent with an angiogenic suppressor, with no toxicity in healthy strains under cytotoxicity assays[27]. Furthermore, the scopoletin indicates promising interaction *in silico* of tumor vascularization factors, like VEGF-A, FGF-2, and ERK-1. In murine models, this metabolite shows inhibition of vascular formation in rat aorta and tumor growth[30]. The literature on the antiangiogenesis activity of scopoletin demonstrates the main role of this substance in front of tumoral vascularization, which may be useful in the inhibition of tumoral growth.

Conclusions

In the present study, we report the antimicrobial and antiangiogenic activity of leaves and branches from *Warszewiczia coccinea* (Vahl) Klotzsch. Among the active extracts, the methanolic extracts of the leaves showed antimicrobial activity including for a yeast strain: *Candida albicans*, the same extract showed toxicity in the angiogenesis assay (CAM). In addition, through TLC and ^{1}H NMR analyses, we observe evidence of terpenes, aromatic substances, and alkaloids in leaves. After chromatography fractionation of the methanolic extracts of the leaves, it may be possible to isolate a bioactive substance: scopoletin. This is the first report of coumarin in the *Warszewiczia* genus.

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Conflict of interest

There is no conflict of interest.

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Contributions

Study design: GSC; CVN.
Data curation: GSC; CVN.
Data collection: GSC; MTFE.
Data analysis: GSC; MTFE; NCFB; DRS; CVN.
Original manuscript writing: GSC.
Review writing and editing: GSC; CVN.

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