© 2017
Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 16 (1): 14 - 25
ISSN 0717 7917
www.blacpma.usach.cl

Artículo Original | Original Article

Antifungal activity against *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* of the major constituents from wood sawdust of *Platymiscium gracile* Benth

[Actividad antifúngica contra *Colletotrichum acutatum* y *Colletotrichum gloeosporioides* de los constituyentes mayoritarios del aserrín de madera de *Platymiscium gracile* Benth]

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Abstract: The tree tomato (*Solanum betaceum* Cav., Solanaceae) anthracnose, caused by the fungi *Colletotrichum acutatum* and *Colletotrichum gloeosporioides*, is the most important disease of this crop in Colombia for its wide distribution and the losses it causes. In the present work, the in vitro antifungal activity of the soluble fractions in n-hexane, dichloromethane, and ethyl acetate, and their major constituents from the sawdust of timber specie *Platymiscium gracile* Benth. (Fabaceae) against both fungi was evaluated. The n-hexane-soluble fraction exhibited the greatest inhibitory effect. The metabolites homopterocarpin (a pterocarpan, 0.39% dry weight), calycosin (an isoflavone, 2.01%) and scoparone (a coumarin, 1.48%) were isolated for the first time from wood sawdust of *P. gracile*. The structure of these compounds was determined by ^{1}H and ^{13}C NMR analyses. The three compounds tested showed significant antifungal activity.

Keywords: *P. gracile, C. gloeosporioides, C. acutatum,* homopterocarpin, calycosin, scoparone.

Resumen: La antracnosis del tomate de árbol (*Solanum betaceum* Cav., Solanaceae), ocasionada por los hongos *Colletotrichum acutatum* y *Colletotrichum gloeosporioides*, es la enfermedad más importante de este cultivo en Colombia por su amplia distribución y las pérdidas que ocasiona. En el presente trabajo se evaluó la actividad antifúngica in vitro de las fracciones solubles en n-hexano, diclorometano y acetato de etilo, y sus componentes mayoritarios, del aserrín de la especie maderable *Platymiscium gracile* Benth. (Fabaceae), contra ambos hongos. La fracción en n-hexano exhibió el mayor efecto inhibitorio. Los metabolitos homopterocarpina (un pterocarpano; 0.39% del peso seco de aserrín), calicosin (una isoflavona; 2.01%) y escoparona (una cumarina; 1.48%) se aislaron por primera vez desde el aserrín de madera de *P. gracile* empleando técnicas cromatográficas. La estructura de los compuestos se determinó por análisis de RMN de ^{1}H y ^{13}C. Los tres metabolitos mostraron una actividad antifúngica significativa contra ambos hongos.

Palabras clave: *P. gracile*, *C. gloeosporioides*, *C. acutatum*, homopterocarpina, calicosin, escoparona.

Recibido | Received: March 4, 2016
Aceptado | Accepted: June 26, 2016
Aceptado en versión corregida | Accepted in revised form: July 27, 2016
Publicado en línea | Published online: January 30, 2017
Este artículo puede ser citado como / This article must be cited as: J Martinez, C García, D Durango. 2017. Antifungal activity against *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* of the major constituents from wood sawdust of *Platymiscium gracile* Benth **Bol Latinoam Caribe Plant Med Aromat** 16 (1): 14 – 25.

INTRODUCTION

Anthracnose, caused by the fungi *Colletotrichum acutatum* J.H. Simmonds, and *C. gloeosporioides* (Penz.) Penz. y Sacc., is the most important disease of tamarillo crop (*Solanum betaceum* Cav.*;* syn.: *Cyphomandra betacea* (Cav.) Sendtn.; Solanaceae) in Colombia (Afanador-Kafuri *et al*., 2003; Saldarriaga-Cardona *et al.*, 2008). Traditionally, the control of the disease has been carried out by applying synthetic fungicides, which have played an important role in increasing agricultural production (Echeverri *et al.*, 2007). However, the growing resistance developed by these microorganisms to the substances used for its control has forced the use of higher amounts and frequencies of application in the field. This excessive use has led to increase the production costs, the presence of traces of fungicides in food, and greater risks to human health and the environment (Zhou *et al.*, 2015; Lozowicka, 2015). For these reasons, a growing interest in the search for safer alternatives to increase agricultural productivity and mitigate the negative impact of synthetic fungicides has been generated. Among those alternatives, the use of essential oils and plant extracts, or their major components has caused the highest expectations. Such natural substances have a greater acceptance by the consumer who considered safer for human health and the environment (Tripathi & Dubey, 2004; Tripathi & Shukla, 2007).

On the other hand, the genus *Platymiscium* contains 33 species with restricted distribution in America. In Colombia, *Platymiscium sp.* (Granadillo), has been detected in the departments of Amazonas, Guainía, Caqueta and Putumayo (López & Cárdenas, 2002). The wood from *Platymiscium sp.* achieved great economic value in national and international markets thanks to its use in the manufacture of floor and wall coverings with high durability and resistance to termites and fungi (López & Cárdenas, 2002; Gómez & Toro, 2007). It highlights that during these manufacturing processes, high amounts of wood sawdust are generated (approximately 15 to 35%), which is generally wasted.

In the present work, the phytochemical study and inhibitory effect of the major constituents from wood sawdust of *P. gracile* against the fungi *C. acutatum* and *C. gloeosporioides* were carried out.

MATERIALS Y METHODS

Plant material

The wood sawdust from *P. gracile* (783 g) was collected in the municipality of Puerto Asís (0°30’0’’N, 76°28’59’’O, altitude of 239 m), department of Putumayo (Colombia). A voucher specimen (identified by Dr. Jorge Mario Vélez) has been deposited in the Herbarium Gabriel Gutiérrez Villegas of the National University of Colombia-Medellín (MEDEL#64111).

General

A thin layer chromatography (TLC) was performed on precoated plates (Si 60 F_{254}, 0.25 mm, Merk). Mixtures of *n*-hexane:EtOAc were used as mobile phase. Compounds were visualized under UV radiation at 254 and 365 nm, and by aspersion with AcOH-H_2SO_4-H_2O (143:28:30) followed by brief heating. Column chromatography (CC) was performed using silica gel 60 (0.040 - 0.063 mm; Merck) or Sephadex LH-20 (Sigma-Aldrich). High-performance liquid chromatography (HPLC) was carried out on a Gilson chromatograph equipped with a Gilson model 170 diode array detector, using a Phenomenex Security Guard cartridge C18 (4.0 x 3.0 mm) followed by a Phenomenex Luna 5µ C18 (2) reverse-phase column (150 mm x 4.6 mm i.d., 5µm) (Torrance, USA). The compounds were eluted at a flow rate of 0.7 mL/min with the solvents A = methanol, and B = 0.05% acetic acid in water, as follows: from 10% A to 70% A in 40 min, then 70% A to 90% A in 20 min, and subsequently by holding for 8 min to reequilibrate the column, for the next injection. Injection volume was 20 µl. Compounds were monitored at 254 nm. NMR spectra were measured on a Bruker AMX 300 NMR spectrometer (^{1}H NMR, 300.12 MHz; ^{13}C NMR, 75.42 MHz). Chemical shifts, δ, are expressed in ppm units downfield from TMS and coupling constants *J* in Hertz (Hz). Mass spectrometry analysis was carried out using a Hewlett-Packard 6890 (Agilent Technologies) gas chromatograph coupled with a HP 5973 MSD (Mass selective detector-Quadrupole type). FTIR spectra were carried out using $CHCl_3$ on a Perkin-Elmer RXI. Optical rotations were measured in $CHCl_3$ solution at 25° C with a JASCO P-2000 digital polarimeter.

Sample extraction

The ground wood sawdust dried (783 g) of *P. gracile* was extracted at room temperature for 48 h using successively *n*-hexane, CH_2Cl_2, and EtOAc by percolation until exhaustion. Then, the solvents were removed under reduced pressure to dryness with a rotary evaporator R210 (Buchi) at 35° C to yield respectively 15.6, 25.1, and 68.3 g with *n*-hexane, CH_2Cl_2, and EtOAc.

Isolation and identification

Examination of the *n*-hexane, CH_2Cl_2, EtOAc-soluble material by HPLC showed the presence of the three major compounds. Thus, all fractions were subjected to CC on silica gel using as mobile phase *n*-hexane-EtOAc mixtures with increasing polarity (10:0, 9:1, 8:2, 7:3, 6:4 y 5:5). Subfractions that appeared to be similar based on the TLC chromatogram were combined. Those subfractions containing the major metabolites were again subjected to CC using Sephadex LH-20 as stationary phase and *n*-hexane-CH_2Cl_2-MeOH (50:25:25) as eluent. Then, similar subfractions were combined and further purified by preparative TLC using Si gel 60 and $CHCl_3$. Three compounds were isolated in sufficient amounts for their identification by UV spectroscopy, mass spectrometry, ^{1}H- and ^{13}C-NMR.

Quantification

Quantification of metabolites was performed using standard calibration curves (peak areas vs. compound concentration for different concentrations). Four working solutions were prepared for each standard in methanol containing scoparone, calycosin, and homopterocarpin at 12.5, 25, 50, and 100 mg/L. All calibration curves presented high linearity (correlation coefficient $r^2 > 0.96$). Data for each peak were collected using the wavelength that provides a maximum response. The results were expressed as mg of compound/per gram of extract (% w/w dry weight).

Antifungal activity

Fungi *C. gloeosporioides* and *C. acutatum* were isolated from infected tamarillo fruits (*Solanum betaceum*). Fungi were maintained on potato dextrose agar medium (PDA; Merck, Darmstadt, Germany) at 25±2°C. Inhibition of mycelial growth was determined by the poison food technique (Grover and Moore, 1962). Different concentrations (10-200 μg/mL) of all three compounds dissolved in ethanol were diluted in Petri dishes (9 cm) with PDA. Subsequently, fungi were inoculated immediately by placing in the center of each plate a 5 mm diameter of the mycelial mass with the culture of the fungi to be tested, which were cut with a sterile cork borer from the periphery of growing cultures on PDA plates. The final ethanol concentration was identical in both control and treated cultures (i.e. 2 μL/L). Petri dishes were incubated at room temperature and the diameter of the mycelial growth was measured every 24 hours during 7 days. Carbendazim (methylbenzimidazol-2-ylcarbamate) at 50 μg/mL was used as positive control. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the negative control. All concentrations were tested in triplicate. The results are shown as mean values of colony diameters (± SD). Inhibition percentages of radial growth was calculated by the formula:

Inhibition (%) = {1 – [radial growth of treatment (mm)/radial growth of control (mm)]} x 100.

Statistical Analysis

The data about the effect of the treatments on the growth of phytopathogens considered analysis of variance (ANOVA), and treatment means were compared by Fishers least significant difference test (LSD) at P = 0.05.

RESULTS

Extracted yields obtained from dried wood sawdust from *P. gracile* were 2.0, 3.2, and 8.7 g/100 g of material dry weight for the *n*-hexane, CH_2Cl_2, and EtOAc fractions, respectively. The highest yield was obtained from EtOAc-fraction, while the yield from *n*-hexane was much lower. As a first approach, all fractions from *P. gracile* were assayed against the phytopathogenic fungus *C. gloeosporioides*, using the poisoned food technique. In general, the three fractions showed significant antifungal activity against the fungus (Figure 1), being the *n*-hexane-soluble fraction the most active. Inhibition percentages for *n*-hexane, CH_2Cl_2, and EtOAc-soluble fractions at 50 μg/mL during 76 to 144 h, ranged between 18.2-28.6, 13.6-18.5 and 16.6-24.4%, respectively. These results suggest that the antifungal compounds of *P. gracile* may belong to less polar

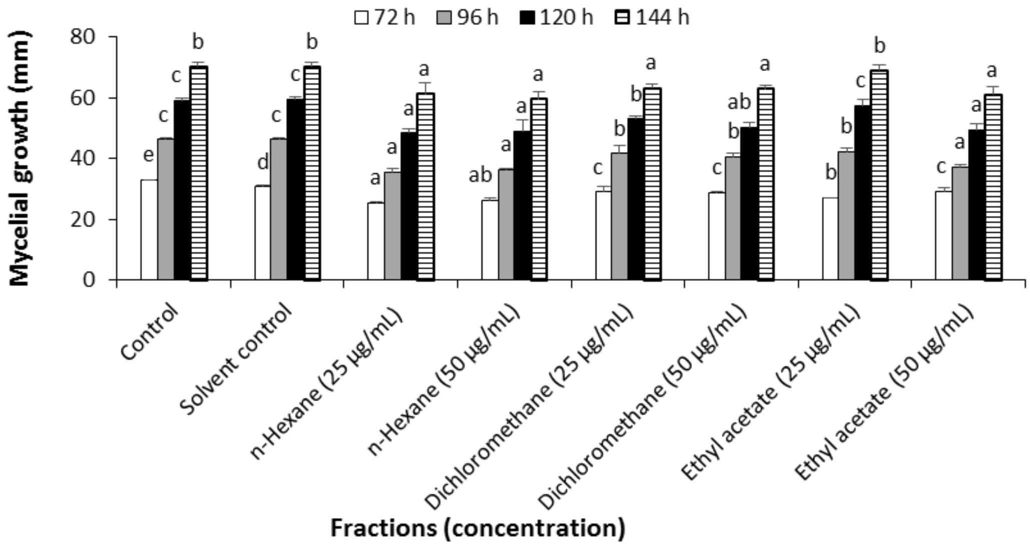


Figure 1
Mycelial growth of *C. gloeosporioides* tested with *n*-hexane, dichloromethane, and EtOAc-soluble fractions from *P. gracile*. Data are shown as mean ± SD of three different experiments. For each time, means with the same letter are not significantly different at 5% level by LSD.

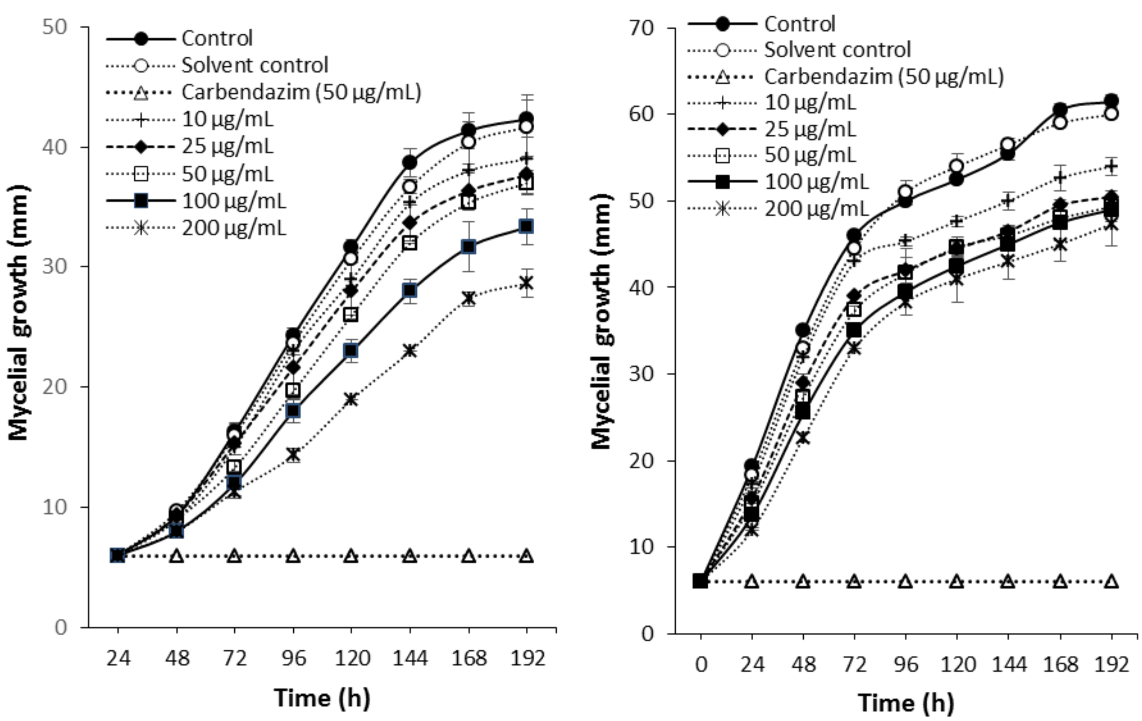


Figure 2
Mycelial growth of *C. acutatum* (left) and *C. gloeosporioides* (right) treated with *n*-hexane-soluble fraction from *P. gracile*. Data are shown as mean ± SD of three different experiments.

chemical compounds, although all fractions displayed fungistatic properties. Then, the most active fraction was evaluated against both fungi at 10, 25, 50, 100 and 200 μg/mL during 192 h. As can be seen in Figure 2, *n*-hexane-soluble fraction reduced the mycelial growth in a dose-dependent manner. After 72 h, the mycelial growth of *C. gloeosporioides* was significantly inhibited by *n*-hexane-soluble fraction at 10 μg/mL and above, as compared to control (P = 0.05). Overall, *n*-hexane fraction exhibited an effective activity. For instance, the inhibition percentages of *C. acutatum* and *C. gloeosporioides* at 200 μg/mL ranged between 37-54% and 25-55%, respectively.

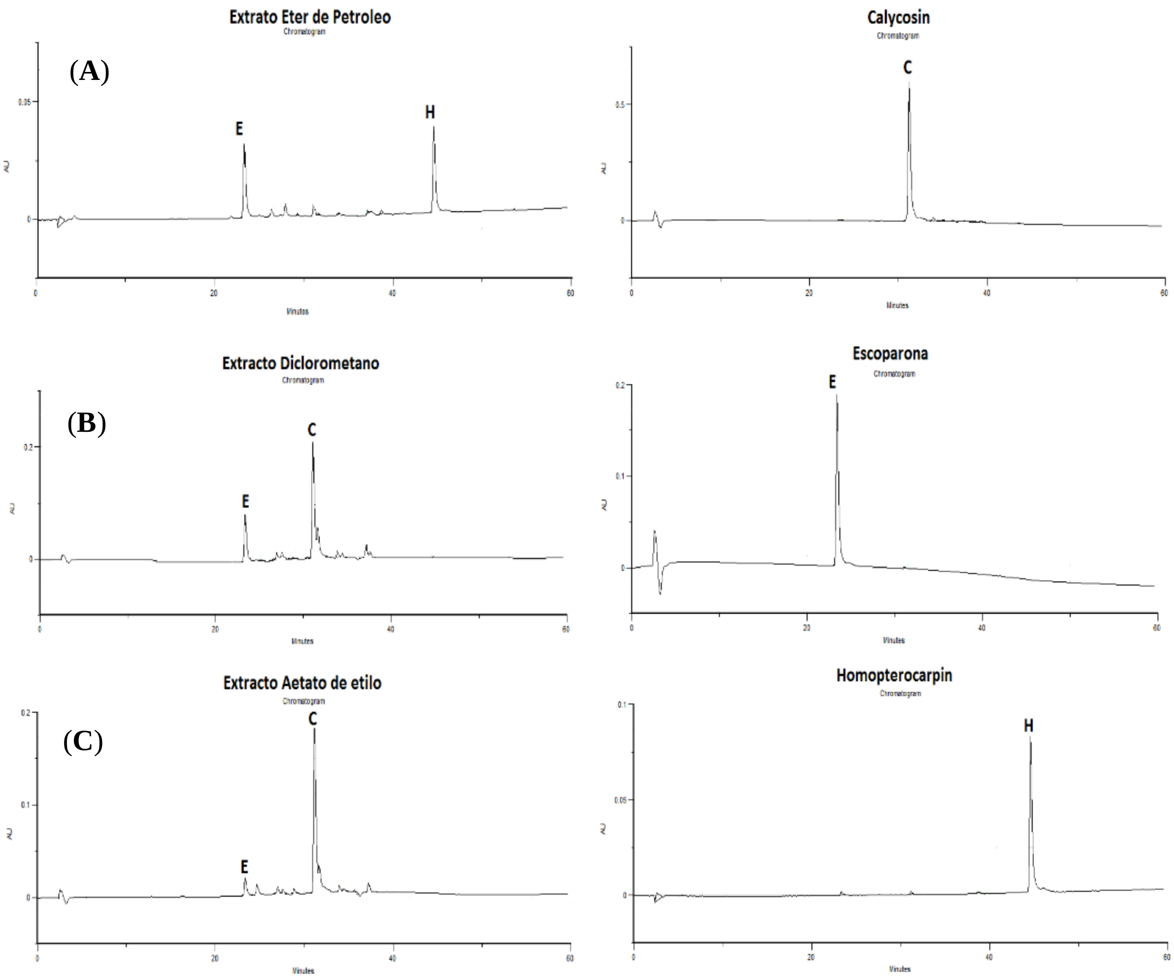


Figure 3
HPLC chromatograms of *n*-hexane (A), CH_2Cl_2 (B), EtOAc-soluble (C) fractions from *P. gracile*, and their major constituents (E: scoparone; H: homopterocarpin; C: calycosin). UV detector: 254 nm.

The higher antifungal activity occurred during the first 24 h for *C. gloeosporioides*, and between 48 and 72 h for *C. acutatum*. Next, the inhibitory effect was gradually decreased at all the evaluated concentrations. Indeed, the growth inhibition percentage after 192 h was only 25% at 200 μg/mL (highest concentration tested). Then, the first step to isolate the active compounds was evaluate the HPLC-DAD-chromatographic profile of each fraction. As shown in Figure 3A, there were two major peaks (i.e. E and H) of different retention times in the *n*-hexane extract at 23.41 and 44.59 min, respectively. HPLC profiles of the CH_2Cl_2 and EtOAc-soluble fractions (Figures 3B and 3C) were slightly similar. Thus, the major peaks E and C were detected at retention times of 23.41 and 31.19 min, respectively. Accordingly, the *n*-hexane, CH_2Cl_2 and EtOAc-soluble fractions were further subjected to open column chromatography using sequentially silica gel and Sephadex LH-20, and finally three components were obtained (Figure 3).

The structural identification of the three components was carried out by MS-EI, UV, ^{1}H and ^{13}C-NMR spectra.

Peak H: The compound was isolated as a white solid [yield: 60.4 mg; m.p. 89-90° C; lit. m.p. 87.6-87.8° C (Gadelha Militão *et al.*, 2005)]. EI-MS m/z: 284(100)$[M]^+$, 285(18), 283(44), 270 (7), 269(37), 161(13), 148(17). IRν_{max} cm^{-1}: 1620, 1580, 1490, 1465, 1350, 1275, 1145, 1120, 1025. UV (CH_3CN) λ_{max} nm (log ε): 285 (3.9). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.48 (1H, d, *J* = 8.4, H-1), 7.18 (1H, d, *J* = 8.7, H-7), 6.70 (1H, dd, *J* = 8.4, 2.4, H-2), 6.53-6.49 (3H, m, H-10, H-8, H-4), 5.56 (1H, d, H-11a), 4.28 (1H, dd, *J* = 7.0, 3.0, H-6_{ec}), 3.84 (3H, s, -OCH_3), 3.82 (3H, s, -OCH_3), 3.62-3.55 (2H, m, H-6_{ax}, H-6a). ^{13}C NMR (75 MHz, $CDCl_3$): δ 39.57 (C-6a), 55.40 (-OCH_3), 55.52 (-OCH_3), 66.62 (C-6), 78.61 (C-11a), 96.93 (C-8), 101.66 (C-4), 106.38 (C-10), 109.19 (C-2), 112.40 (C-11b), 119.18 (C-6b), 124.78 (C-7), 131.88 (C-1), 156.66 (C-4a), 160.76 (C-3), 161.07 (C-10a), 161.16 (C-9). $[\alpha]_D$ = +146° (*c* 0.001, $CHCl_3$); [lit. $[\alpha]_D$ = +205° (*c* 0.021, $CHCl_3$) (McMurry *et al.*, 1972)]. This compound was obtained as colorless crystals (yield 23.7 mg). After comparing the data with spectral information from literature (Maekawa & Kitao, 1970), the first component was confirmed as (6a*S*, 11a*S*) – 3, 9 - dimethoxypterocarpan (homopterocarpin).

Peak E: The compound was isolated as a white crystalline solid [yield: 73.4 mg; m.p. 144-145°C, lit. m.p. (Gao *et al.*, 2013)]. IRν_{max} cm^{-1}: 1700, 1660, 1560, 1500, 1420, 1380, 1280, 1135, 870. EI-MS m/z: 206(100)$[M]^+$, 191(37), 163(30), 135(19), 79(16). UV (CH_3CN) λ_{max} nm (log ε): 227 (4.1), 294 (3.6), 341 (3.9). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.61 (1H, d, *J* = 9.5, H-4), 6.85 (1H, s, H-5), 6.82 (1H, s, H-8), 6.27 (1H, d, *J* = 9.5, H-3), 3.94 (3H, s, -OCH_3), 3.91 (3H, s, -OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 161.82 (C-2), 153.28 (C-7), 150.45 (C-6), 146.78 (C-9), 143.75 (C-4), 113.94 (C-10), 111.87 (C-3), 108.45 (C-5), 100.42 (C-8), 56.80 (2x-OCH_3). Compared with the reported information, the spectra data of the second component was in agreement with those of scoparone (Soares *et al*., 2013).

Peak C: This compound was isolated as a pale yellow crystalline solid [yield: 37.9 mg; m.p. 248-250°C, lit. m.p. 230-232°C (Zhao *et al.*, 2009)]. EI-MS m/z: 284(100)$[M]^+$, 269(37), 285(18), 241(12), 283(9). IRν_{max} cm^{-1}: 3420, 3160, 1615, 1570, 1505, 1230, 1190, 1130. UV (CH_3CN) λ_{max} nm (log ε): 226 (4.0), 294 (3.5), 342 (4.0). ^{1}H NMR (300 MHz, $(CD_3)_2CO$): δ 8.03 (1H, s, H-2), 7.94 (1H, d, *J* = 8.8, H-5), 7.04 (1H, d, *J* = 2.0, H-2’), 6.99 (1H, dd, *J* = 8.8, 2.0, H-6’), 6.89-6.84 (2H, m, H-6, H-5’), 6.77 (1H, d, *J* = 2.2, H-8), 3.80 (3H, s, -OCH_3). ^{13}C NMR (75 MHz, $(CD_3)_2CO$): δ 180.37 (C-4), 168.01 (C-7), 163.50 (C-9), 158.22 (C-2), 153.03 (C-4’), 151.81 (C-3’), 133.24 (C-5), 131.04 (C-1’), 129.80 (C-3), 125.84 (C-6’), 123.31 (C-10), 121.66 (C-2’), 120.43 (C-6), 116.89 (C-5’), 107.90 (C-8), 61.03 (-OCH_3). After comparing the data with spectral information from literature (Zhao *et al.*, 2009; Tolleson *et al.*, 2002), this component was confirmed as calycosin (7,3’-dihydroxy-4’-methoxyisoflavone). Structures of isolated compounds are presented in Figure 4.

Quantitative determinations of the homopterocarpin, scoparone, and calycosin were carried out by HPLC analyses on a C18 reversed-phase column and by the calibration curve method. The regression equations were: scoparone, $y = 5.0x10^{-6}x – 7.65$ (r^2=0.970)*;* homopterocarpin, $y = 1.2x10^{-6}x – 1.58$ (r^2=0.997), and calycosin, $y = 5.0x10^{-7}x – 3.99$ (r^2=0.993). The quantification results of compounds showed that the *n*-hexane-soluble fraction contained a higher level of homopterocarpin, while little amount were detected in CH_2Cl_2 and EtOAc-soluble fractions. Thus, in the *n*-hexane fraction, a concentration of 191.2, 85.5 and 5.65 mg/g was found for homopterocarpin, scoparone and calycosin, respectively. For CH_2Cl_2 and EtOAc fractions, concentrations of 4.96 and 0.52, 255.2 and 55.8, 165.8 and 142.3 mg/g were established in that order for homopterocarpin, scoparone, and calycosin. Consequently, percentages (w/w dry weight) of each compound in the wood sawdust of *P. gracile* were 0.39% (homopterocarpin), 1.48% (escoparone), and 2.01% (calycosin). Thus, it can be seen that *P. gracile* accumulate very high levels of these compounds.

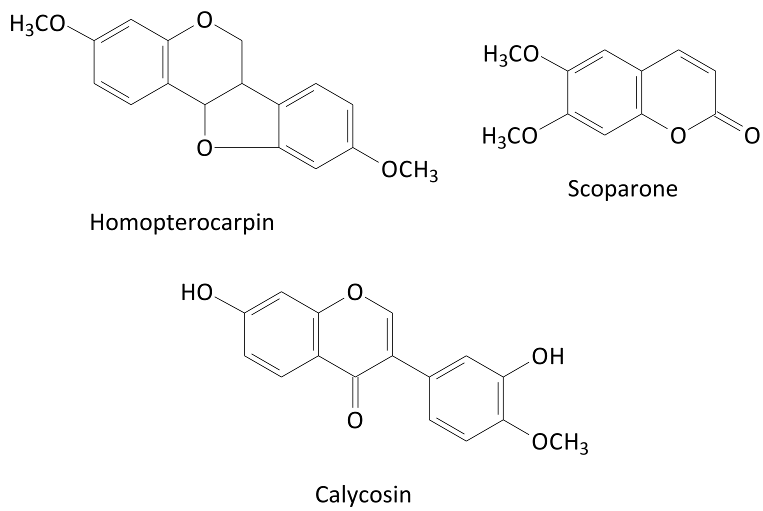


Figure 4
Isolated compounds from *Platymiscium gracile*

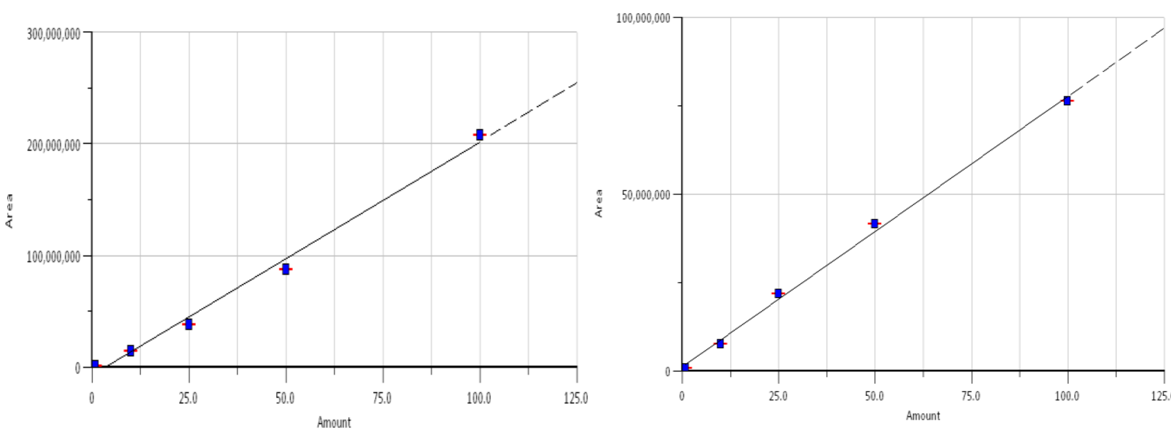
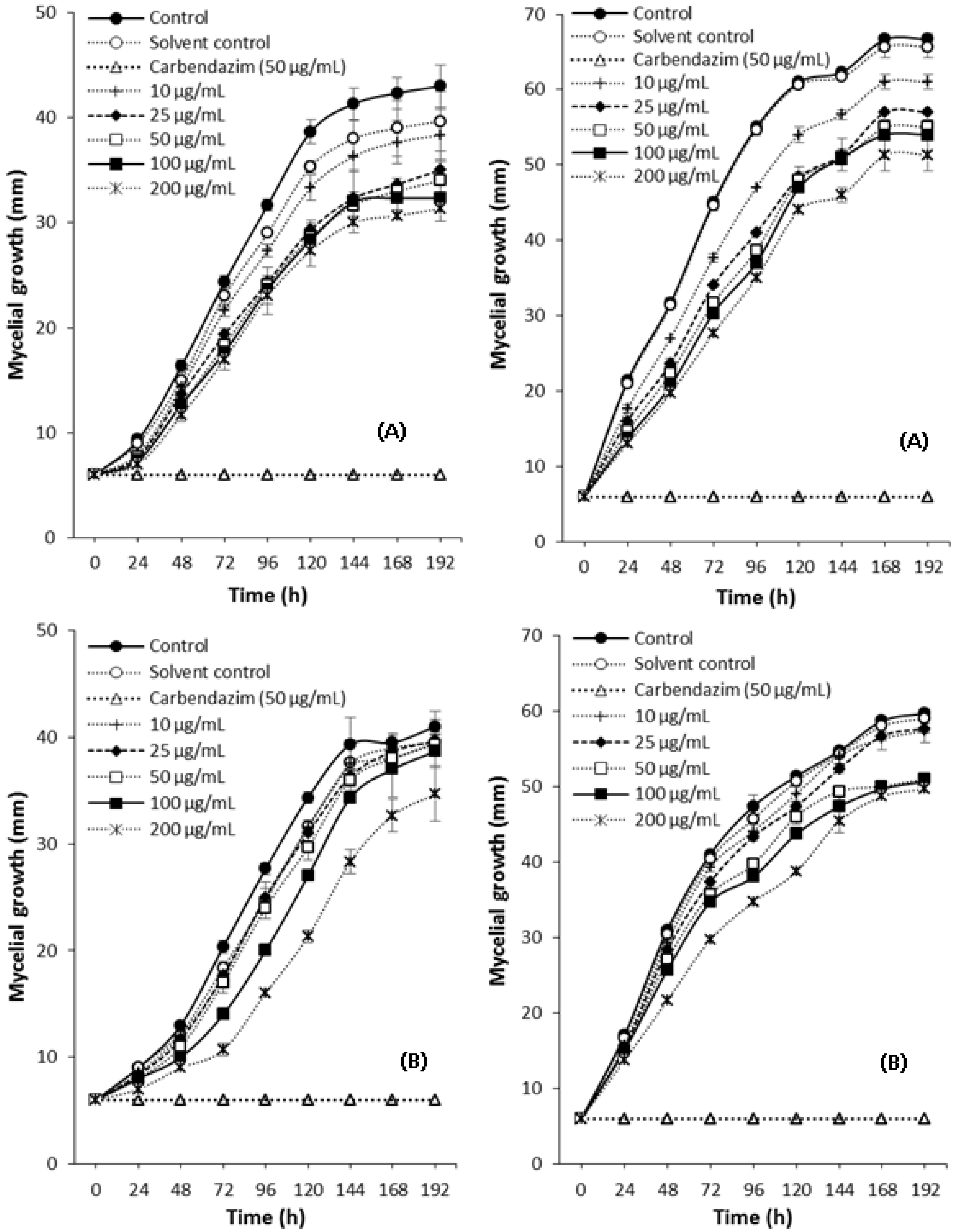


Figure 5
Calibration curve of calycosin (left) and homopterocarpin (right)

Antifungal activity
The effect of homopterocarpin, calycosin, and scoparone on *in vitro* mycelia growth of *C. acutatum* and *C. gloeosporioides* was determined during 192 h of incubation (Figure 6). The pure compounds exhibited a significant activity against the fungi in a dose-dependent manner. Generally, the fungal growth was highly inhibited after 24 h, and thereafter decreases. Homopterocarpin, a pterocarpan isolated from *n*-hexane extract, possess a strong antifungal effect against *C. acutatum* and *C. gloeosporioides* with inhibition percentages at 24 h ranging from 40 to 70% and 24 to 54%, respectively. Then, the inhibitory effect of homopterocarpin was strongly decreased for *C. acutatum* (from 70 to 45% at 200 µg/mL after 48 h) and slowly decreased for *C. gloeosporioides* (from 54 to 41 at 200 µg/mL after 96 h). Overall, homopterocarpin showed a significant inhibitory effect in the mycelial growth of *C. acutatum* (at 10 µg/mL and above) and *C. gloeosporioides* (at 25 µg/mL and above) in relation to the control and solvent control (P = 0.05). For its part, calycosin (an isoflavone) exhibited the highest antifungal activity against *C. acutatum*. For the interval of 24 to 72 h at 200 µg/mL, the inhibition of the mycelial growth with calycosin varied throughout a range of 58-68%. Under the same conditions (i.e. 24 to 72 h, and 200 µg/mL calycosin), the growth of


Control
Solvent control
Carbendazim (50 µg/mL)
10 µg/mL
25 µg/mL
50 µg/mL
100 µg/mL
200 µg/mL
Mycelial growth (mm)
Time (h)
(A)
Control
Solvent control
Carbendazim (50 µg/mL)
10 µg/mL
25 µg/mL
50 µg/mL
100 µg/mL
200 µg/mL
Mycelial growth (mm)
Time (h)
(A)
Control
Solvent control
Carbendazim (50 µg/mL)
10 µg/mL
25 µg/mL
50 µg/mL
100 µg/mL
200 µg/mL
Mycelial growth (mm)
Time (h)
(B)
Control
Solvent control
Carbendazim (50 µg/mL)
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100 µg/mL
200 µg/mL
Mycelial growth (mm)
Time (h)
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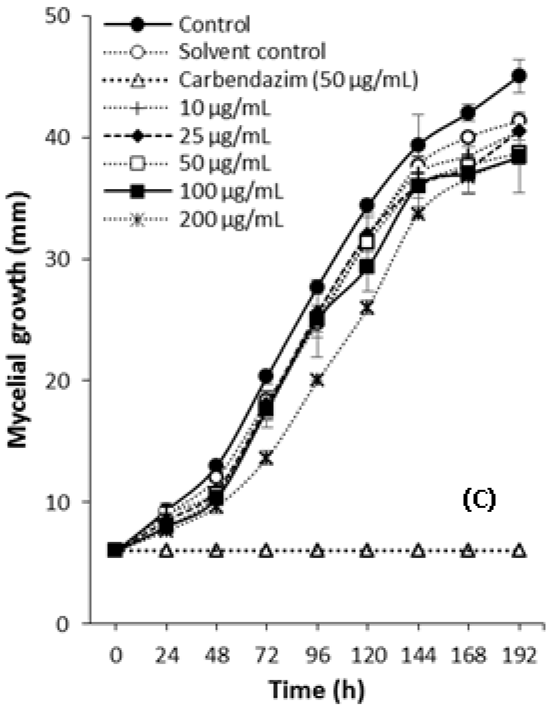

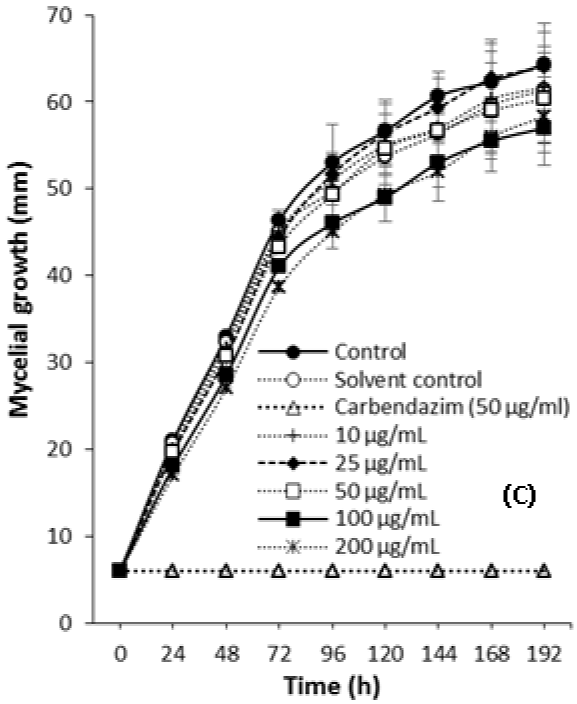

Figure 6
Mycelial growth of *C. acutatum* (left) and *C. gloeosporioides* (right) tested with homopterocarpin (A), calycosin (B), and scoparone (C) from *P. gracile*. Data are shown as mean ± SD of three different experiments.

C. gloeosporioides was inhibited between 30-35%. Furthermore, at all concentrations tested, the mycelial growth inhibitions were decreased gradually while increase the incubation time, as well as was observed with homopterocarpin. Evaluations made at higher concentrations than 25 and 50 µg/mL of calycosin inhibited significantly the growth of *C. gloeosporioides* and *C. acutatum*, respectively, in relation to the control (P = 0.05). Finally, the coumarin scoparone was more active against *C. acutatum* than *C. gloeosporioides*. Scoparone slightly retarded the growth of *C. gloeosporioides*; at all concentrations, the inhibitions displayed were less than 30% and they remained almost constant throughout the evaluation period. In addition, scoparone showed values ranging between 10 and 50% as mycelial growth inhibition percentage against *C. acutatum*, which could be considered as a modest response. In general, *C. acutatum* and *C. gloeosporioides* were significantly inhibited at 50 and 100 µg/mL, respectively, as compared to the control. The highest concentration used in the present study (i.e. 200 µg/mL) can be still considered a low concentration for a natural antifungal drug.

These results showed that homopterocarpin and calycosin exhibit an appreciable inhibitory activity toward the assayed phytopathogens, depending on the fungal strain, while scoparone showed moderate ability to inhibit mycelial growth.

DISCUSSION

Several studies have shown that some secondary metabolites present in heartwood are toxic or deterrent for termites, bacteria and fungi (Schultz & Nicholas, 2000; Santana *et al.*, 2010). In the present study, the antifungal effect against *C. gloeosporioides* and *C. acutatum* of fractions, and their major constituents from wood sawdust of *P. gracile* were evaluated. In general, the mycelial growth of both fungi was significantly inhibited using the three fractions (*n*-hexane, CH_2Cl_2, and EtOAc). However, the most pronounced *in vitro* antifungal activity was exhibited at 200 µg/mL of the *n*-hexane-soluble fraction, with inhibition values ranging from 37 to

54% and 25 to 55% for *C. acutatum* and *C. gloeosporioides*, respectively. Therefore, the antifungal effect could be attributed to low polarity substances, although all fractions displayed fungistatic properties. From the active organic fractions, we have isolated three major metabolites, scoparone, homopterocarpin and calycosin. These compounds are present in high amounts in the wood sawdust of *P. gracile*, being 0.39% dry weight for homopterocarpin, 2.01% for calycosin, and 1.48% for scoparone. Scoparone has been recognized as an antimicrobial secondary metabolite formed *de novo* as a result of physical, chemical, or biological stress (phytoalexin) on citrus (Kuniga *et al.*, 2005; Ortuño *et al.*, 2011). Actually, scoparone is considered as the main phytoalexin involved in the resistance of citrus against pathogens (Arras *et al.*, 2006; Sanzani *et al.*, 2014). This coumarin has strong antifungal effect; *Trichophyton mentagrophytes* and *Rhizoctonia solani* were totally inhibited by scoparone at 125 and 250 μg/mL, respectively (Cespedes *et al.*, 2006). Moreover, the increased concentration of scoparone in fruits closely correlated with the enhanced antifungal activity of the fruit extract. *Citrus aurantium*, *C. paradisi*, *C. limon*, *C. sinensis* accumulated scoparone as a resistance mechanism against *Phytophthora parasitica*, *P. citrophthora*, *Botrytis cinerea*, *Penicillium digitatum*, among others (Kuniga & Matsumoto, 2006; Ballester *et al*., 2010; Ballester *et al.*, 2013). Scoparone was also reported from *P. trinitatis* Bth., *P. praecox* Mart., *P. yucatanum* and *P. floribundum* (Braga de Oliveira *et al*., 1972; Craveiro & Gottlieb, 1974; Reyes-Chilpa *et al*., 1998; Soares *et al*., 2013). In this study, scoparone showed moderated inhibitions (ranging between 10 and 50%) against *C. gloeosporioides* and *C. acutatum*. However, the fungistatic effect on *C. acutatum* was kept almost constant throughout the evaluation. Meanwhile, the isoflavone calycosin has been reported to possess antimicrobial activities against *Bacillus subtilis*, *Staphylococcus aureus* and *Candida mycoderma* (Chacha *et al*., 2005; Kuete, *et al*., 2011). Here was presented that 200 μg/mL calycosin showed moderated inhibitions with values ranging from 58 to 68%, and 30 to 35% against *C. acutatum* and *C. gloeosporioides*, respectively.

According to biogenetical considerations, the B/C rings junction of all natural pterocarpans is *cis*, leading to only two enantiomeric forms. In addition, polarimetric measurements has shown that (-) optical rotation can be associated with the α,α configuration (6a*R*, 11a*R*), while the (+) optical rotation with β,β configuration (6a*S*, 11a*S*) (Jiménez-González *et al.*, 2008; Veloso *et al.*, 2012). From the (+) optical rotation of homopterocarpin, it could be supposed an (6a*S*, 11a*S*) absolute configuration. Thus, the compound corresponds to the (6a*S*, 11a*S*)-3,9-dimethoxypterocarpan. This compound has also been isolated from *P. floribundum* (Gadelha Militão *et al.*, 2005) and *P. yucatanum* (Reyes-Chilpa *et al.*, 1998). Homopterocarpin has been reported to be an active insect antifeedant against the common cutworm *Spodoptera litura* F. and the subterranean termite *Reticulitermes speratus* (Kolbe) (Morimoto *et al.*, 2006). In general, pterocarpans play an important role as antimicrobial compounds synthesized *de novo* by plants in respond to microbial attack (phytoalexins). They have been reported to inhibit the growth and sporulation of fungal pathogens (Jiménez-González *et al.*, 2008). Our study showed that homopterocarpin displays significant inhibitory effect against *C. acutatum* and *C. gloeosporioides*.

CONCLUSIONS

The results of the present study indicate that *n*-hexane, dichloromethane, and ethyl acetate fractions from sawdust of *P. gracile* possess significant antifungal properties. The highest inhibition of mycelial growth of *C. gloeosporioides* and *C. acutatum* was achieved by *n*-hexane-soluble fraction, suggesting that low polarity compounds could be responsible for the antifungal activity. Each fraction was analyzed by HPLC and the major metabolites were isolated, identified and quantified. (+)-Homopterocarpin and scoparone were found in the *n*-hexane fraction while calycosin and scoparone were detected in the rest of fractions. Overall, these secondary metabolites are present in high levels in wood sawdust of *P. gracile*. Growth of *C. acutatum* was significantly inhibited at a concentration of 25, 200, and 50 μg/mL and above for (+)-homopterocarpin, calycosin, and scoparone respectively. Meanwhile, significant inhibitions were found for *C. gloeosporioides* at 10, 50 and 100 μg/mL and above, for homopterocarpin, calycosin, and scoparone respectively. Thus, wood sawdust of *P. gracile* could be a good source of antifungal extracts, and their major compounds.

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