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Controlling anthracnose by means of extracts, and their major constituents, from *Brosimum rubescens* Taub

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ABSTRACT

Anthracnose, caused by the fungus *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., is the most limiting fungal disease of mango and papaya crops in Colombia. The *in vivo* and *in vitro* activity against *C. gloeosporioides* of the extracts from sawdust of *Brosimum rubescens* Taub. (Moraceae) was evaluated. The extracts of less polarity (*n*-hexane and dichloromethane) displayed the greatest inhibitory effects. Then, the coumarins xanthyletin (2.74 % d.w.) and 7-demethylsuberosin (2.19 % d.w.) were isolated from these extracts. The compound 7-demethylsuberosin displayed a strong *in vivo* and *in vitro* antifungal activity. Furthermore, the metabolism of 7-demethylsuberosin by the fungus *C. gloeosporioides* was analyzed. The fungus slowly detoxified 7-demethylsuberosin to marmesin and decursinol. Therefore, the high antifungal activity and low level of detoxification make 7-demethylsuberosin, and the extracts that contain it, promising candidates for controlling *C. gloeosporioides*. Sawdust of *B. rubescens* may be considered a valuable source of extracts and coumarins with antifungal activity.

1. Introduction

Anthracnose caused by the fungus *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., affects a wide range of susceptible plants from temperate, tropical and subtropical zones, producing significant fruit losses in the field and postharvest [1]. In Colombia, anthracnose is the most important constraint to mango (*Mangifera indica* L., Anacardiaceae) and papaya (*Carica papaya* L., Caricaceae) production, causing losses that exceed 50 % and forcing, in most cases, to the abandonment or replacement of the crop [2]. The application of synthetic fungicides has been used as a primary control method of the disease, although deleterious effects on human health and the environment may be derived. In addition, the development of resistance in pathogenic fungi, through the detoxification of current fungicides, has required the use of higher doses in crops [3,4]. This fact has notably increased the production costs and the presence of toxic residues in foods. Therefore, it has become necessary to search for new and better antifungal agents to control plant diseases, including anthracnose [5]. Some alternatives that have attracted attention in recent years are the use of UV—C radiation, hot water, plant extracts and essential oils, or their major components, which are effective treatments to phytopathogenic microorganisms control. In addition, these treatments are perceived by consumers as safer for human health and the environment [6–9].

Plant extracts with fungistatic properties, particularly those that come from abundant forest residues and without apparent use, could be used directly and at low cost for disease control in crops, especially in organic agriculture. These bioactive extracts would also allow identify compounds as valuable structural templates that may be subsequently used to design new antifungal agents. In addition, metabolic studies on potential antifungal agents can indicate the structural modifications used by the microorganisms as detoxification mechanism and suggest possible metabolic targets to control the anthracnose. These studies are also necessary for the subsequent safe and effective use of the antifungal agent.

On the other hand, the Moraceae family comprises 38 genera and 1180 species widely distributed in the tropical and subtropical regions [10]. Among the species is *Brosimum rubescens* Taub., known as “palosangre”, a large tree that produces wood for the production of handicrafts, frames, musical instruments, billiard cues, drum sticks, and veneers for flooring in the Amazonian region of Colombia, Peru, Brazil, Suriname and Guyana [11]. During these manufacturing processes, large quantities of sawdust are produced and generally discarded. This waste could be converted by different technologies into usable products or it could even be a source of highly bioactive extractives. A phytochemical study of *B. rubescens*

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reported the presence of coumarins, such as xanthyletin, suberosin and 7-demethylsuberosin, and triterpenes [12–15]. Xanthyletin has been recognized as a phytoalexin (an antimicrobial secondary metabolite produced in response to infections) in citrus fruits [16], having strong antifungal and herbicide activity [17] and inhibitory effects over the symbiotic fungus of leaf-cutting ants [18]. In the present study, the antifungal activity of extracts and the major constituents from wood sawdust of *B. Rubescens* against *Colletotrichum gloeosporioides* were evaluated.

2. Materials and methods

2.1. Equipment and conditions for the chemical analysis

High-performance liquid chromatography (HPLC) was made on a Shimadzu chromatograph equipped with a diode array detector (Shimadzu prominence model SPD-M20A), using an Agilent Zorbax Eclipse plus C18 (150 mm × 4.6 mm i.d., 5 μm) (USA). The compounds were eluted with the solvents A = acetonitrile, and B = 1 % acetic acid in water, as follows: from 40 to 95 % A in 15 min, then held A to 95 % for 5 min. Injection volume and flow rate were 10 μL and 1 mL/min, respectively. Nuclear magnetic resonance (NMR) spectrometer used here was a Bruker AMX 300 NMR. Chemical shifts (δ) and coupling constants (J) are expressed in ppm units and Hertz (Hz), respectively. A JASCO P-2000 digital polarimeter was used for optical rotation measurements.

2.2. Extraction

Brosimum Rubescens Taub. was identified by macroscopic comparison of the specimens in the MEDELw xylotheque (voucher No. MEDELWM1-190218 by Dr. Angela María Vásquez C., Curator of the xylotheque) of the National University of Colombia, Medellín, according to the technical specifications of IAWA committee [19,20]. The dry and ground sawdust (250 g) of *B. Rubescens* was extracted by percolation until exhaustion at room temperature using successively *n*-hexane, dichloromethane, ethyl acetate, and methanol. Then, the solvents were removed under reduced to yield the respective extracts.

2.3. Isolation and identification

HPLC analysis of the *n*-hexane, dichloromethane, and ethyl acetate-soluble extracts showed the presence of two major compounds. *n*-Hexane and dichloromethane extracts were subjected to column chromatography (CC) on silica gel (0.040-0.063 mm; Merck) using mixtures of *n*-hexane-ethyl acetate with increasing polarity, as mobile phase. Fractions containing the major metabolites were again subjected to CC using as stationary phase Sephadex LH-20 (Sigma-Aldrich) and *n*-hexane-dichloromethane-methanol (50:25:25) as eluent. Two compounds were isolated and identified by spectroscopic methods. *Compound A* was obtained as light yellow crystals with m.p. 129−132 °C. The UV absorption spectrum in acetonitrile showed three bands at 265, 303 and, 347 nm; and the proton and carbon NMR spectra exhibited the following signals: ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.45 (s, 6H, 2xMe), 6.19 (d, 1H, J =9.3 Hz, H-3), 7.58 (d,1H, J =9.3 Hz, H-4), 5.68 (d, 1H, J =9.9 Hz, H-4′), 6.33 (d, 1H, J =9.9 Hz, H-3′), 6.68 (s, 1H, H-8), 7.04 (s, 1H, H-5). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 28.3 (C-Me), 104.3 (C-8), 112.9 (C-3), 120.8 (C-3′), 124.9 (C-5), 131.2 (C-4′), 143.5 (C-4), 77.2 (C-2), 118.5 (C-6), 156.8 (C-7), 161.2 (C-2), 155.4 (C-9), 112.7 (C-10). *Compound B* was isolated as light yellow crystals with m.p. 130−133 °C. The UV spectrum (CH_3CN) showed two peaks at wavelengths of 211 and 332 nm. The proton and carbon NMR spectra showed the following signals: ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.79 (s, 3H, Me), 1.82 (s, 3H, Me), 6.28 (d, 1H, J =9.6 Hz, H-3), 7.71 (d, 1H, J =9.6 Hz, H-4), 7.10 (s, 1H, H-8), 7.25 (s, 1H, H-5), 7.56 (s,1H, OH), 3.42 (d, 2H, J = 7.2, H-1′), 5.36 (t, 1H, J = 7.2, H-2′). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 17.9 (C-Me), 25.9 (C-Me), 28.4 (C-1′), 126.7 (C-2), 158.7 (C-7), 154.1 (C-9), 135.0 (C-6), 126.0 (C-10), 112.2 (C-3′), 103.2 (C-8), 112.1 (C-3), 121.1 (C-2′), 128.3 (C-5), 144.5 (C-4).

2.4. Quantification

Quantification of metabolites was performed by HPLC using calibration curves (peak areas vs. compound concentration). Five working solutions were prepared in methanol containing xanthyletin (1.1, 2.8, 5.5, 13.6 and 21.4 mg/L) and 7-demethylsuberosin (11.4, 22.9, 40.7, 61.1 and 106.9 mg/L). The wavelengths, λ_{max} = 265 and 332 nm were used for quantification of xanthyletin and 7-demethylsuberosin, respectively. The results were expressed as mg compound/g extract, and % w/w dry weight wood sawdust.

2.5. Antifungal activity

2.5.1. Mycelial growth inhibition

The fungus *C. gloeosporioides* was isolated from infected papaya fruits (*Carica papaya* L.). Inhibition of mycelial growth was established by the poison food technique [21], according to the methodology described in [22] with some modifications. Different concentrations (10, 25, 50, 100 and 200 μg/mL) of extracts and the major compounds dissolved in ethanol (2 μL/mL) were used. Inhibition (%) of radial growth relative to the negative control was calculated. All assays were tested in triplicate. The results are expressed as mean values of colony diameters (±SD). For bioactive extracts and their major constituents, the concentration that inhibits 50 % fungal mycelial growth (IC_{50}) after 72 h was determined according to the method described in [23].

2.5.2. Inhibition of the spore germination

In addition, antifungal activity against *C. gloeosporioides* was evaluated using the spore germination inhibition technique. The compounds were dissolved in DMSO at 1 %. Then, compounds were evaluated at 200 μg/mL in a 3 % PDA solution with a spore concentration of 2×10^5 spores per mL. The tests were performed in Eppendorf tubes of 1.5 mL capacity. Finally, the evaluation was carried out by measuring in a Neubauer chamber counting the number of spores that develop germ tube greater than twice the radius, at 8 and 24 h [24]. The results were presented as percentage of inhibition of germination (GI%) and were determined by comparing the germinated spores in the treatments with those in the solvent controls, according to the formula: GI% = {1–[T/C]} x100; where T: percentage of germinated spores on the treatments and C: percentage of germinated spores in controls (germinated spores/total spores x 100).

2.5.3. In vivo *assay on natural infected papaya and mango fruits*

The *in vivo* assay was made as described previously in [25] with some modifications. Briefly, papaya (*C. papaya* L. cv. Hawaiian) and mango (*M. indica* L. cv. Hilacha) fruits with uniform size and color (maturity index 3 for mango and papaya: green color with yellow traces in 25 % of the total area and free from defects [26]) were washed, disinfected with sodium hypochlorite solution (0.05 %), rinsed with distilled water, and air-dried. Then, an incision of 6 mm in diameter and 2 mm deep was made with a sterile steel punch in the equatorial zone. In this hole, a mycelial mass of *C. gloeosporioides* from a culture of seven days old, with the same dimensions was located. Three fruits per treatment were stored in sealed plastic containers at room temperature (24 ± 2 °C) with a relative humidity of 75 % (obtained with saturated NaCl solution). After 12 h, the fruits were impregnated with a dose of the most promising materials and positive controls (Carbendazim) at 1 % in

ethanol, using a fine brush. Fruits were stored again in plastic containers under the same conditions. The incidence was evaluated by measuring the diameter (mm) of the infection caused by the fungus at 7 days for papaya and 15 days for mango. All experiments were performed with five replicates per treatment and untreated fruits were used as controls.

2.5.4. Metabolism

C. gloeosporioides was grown at 120 rpm for 15 days into three 250 mL Erlenmeyer flasks containing 125 mL of the Czapek-Dox medium and 200 μg/mL of the most active compound. The culture medium was taken from the flasks after 3, 7, and 15 days, extracted with CH_2Cl_2, and analyzed by HPLC. The results were expressed as relative abundances. A control experiment (without substrate) was performed. The metabolic products C and D were isolated by chromatographic techniques and identified by spectroscopic methods. *Compound C;* UV (CH_3CN) λ_{max}: 223, 247, and 335 nm. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.28 (s, 3H, Me), 1.42 (s, 3H, Me), 3.26 (dd, 2H, *J* = 8.1, 6.0, H-1'), 4.78 (t, 1H, *J* = 8.1, H-2'), 6.25 (d, 1H, *J* = 9.6, H-3), 6.78 (s,1H, H-8), 7.27 (s, 1H, H-5), 7.64 (d, 1H, *J* =9.6 Hz, H-4). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 24.3 (Me), 26.5 (Me), 29.5 (C-1'), 71.7 (C-3'), 91.2 (C-2'), 98.0 (C-8), 112.3 (C-3), 112.8 (C-4a), 123.5 (C-5), 125.1 (C-6), 143.8 (C-4), 155.7 (C-8a), 161.5 (C-3), 163.2 (C-7). ($[\alpha]_D^{25} = +20.6^\circ$ in $CHCl_3$). *Compound D;* UV (CH_3CN) λ_{max}: 202, 224, and 329 nm. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.36 (s, 3H, Me), 1.40 (s, 3H, Me), 2.72-2.90 (dd, 1H, *J* = 16.6, 5.9, H-1'), 3.00-3.10 (dd, 1H, *J* = 16.6, 5.9, H-1'), 3.87 (br t, 1H, *J* = 5.9, H-2'), 6.21 (d, 1H, *J* = 9.5, H-3), 6.78 (s, 1H, H-8), 7.19 (s, 1H, H-5), 7.58 (d, 1H, *J* = 9.5, H-4).

2.6. Statistical analysis

The data about antifungal activity were considered by analysis of variance (ANOVA). Mean values were compared by Fishers least significant difference test (LSD) at p=0.05.

3. Results and discussion

3.1. Antifungal activity of extracts

Yields obtained from *B. rubescens* dried sawdust (250 g) were 3.08, 1.45, 19.35 and 23.00 g dry weight for the *n*-hexane, dichloromethane, ethyl acetate, and methanol extracts, respectively. The highest and lowest yields were obtained from methanol and dichloromethane extracts, respectively. As can be seen in Fig. 1, all extracts reduced significantly the mycelial growth of *C. gloeosporioides* and in a dose-dependent manner, being the nonpolar extracts (*n*-hexane and dichloromethane) the most actives (Fig. 1, A and B). Inhibition percentages for *n*-hexane and dichloromethane extracts at 200 μg/mL from 24 to 192 h, ranged between 77-73% and 71-64%, respectively. Although all extracts displayed fungistatic properties, the results suggest that the nonpolar chemical compounds of *B. rubescens* could be associated with the antifungal activity; it is known that nonpolar compounds (with lipophilic character) can kill the microorganisms by disruption of the cell membrane [27]. IC_{50} values for the *n*-hexane and dichloromethane extracts were 49.6 and 50.0 μg/mL, respectively. For the ethyl acetate and methanol extracts, IC_{50} values were higher than 200 μg/mL.

3.2. Isolated compounds

Two compounds were obtained by CC from the most active extracts (*n*-hexane and dichloromethane) and their structural identification was carried out by NMR spectra (^{1}H and ^{13}C). The compounds A and B were confirmed as xanthyletin and 7-demethylsuberosin, respectively, according to spectroscopic data reported in [12,28]. The chemical structure of the isolated compounds is shown in Fig. 2.

3.3. Quantification of isolated compounds

Quantitative analyses of xanthyletin and 7-demethylsuberosin were carried out by HPLC. The regression equations were: $y = 153108x - 38420$ (Fig. 3A, $R^2 = 0.9996$) for xanthyletin (retention time, Rt, 10.63), and $y = 14636x - 7700.8$ (Fig. 3B, $R^2 = 0.9994$) for 7-demethylsuberosin (Rt: 9.78 min). The results of the quantification show that xanthyletin was found in a higher amount in *n*-hexane (852.0 mg/g) and dichloromethane (600.8 mg/g) extracts than in the ethyl acetate (119.8 mg/g) and methanol (44.7 mg/g) extracts. The dichloromethane extract contained the highest level of 7-demethylsuberosin (345.4 mg/g), followed by ethyl acetate (175.8 mg/g) and methanol (68.2 mg/g) extracts. The percentages (% w/w dry weight) of 7-demethylsuberosin and xanthyletin in *B. rubescens* sawdust were 2.19 and 2.74 %, respectively. Thus, it can be inferred that both compounds are found in very high levels on *B. rubescens* sawdust.

3.4. Antifungal activity of isolated compounds

3.4.1. Mycelial growth inhibition

The effect of 7-demethylsuberosin and xanthyletin on *in vitro* mycelia growth of *C. gloeosporioides* was analyzed during 192 h of incubation. The inhibitory effects are shown in Fig. 4. As can be seen, both coumarins displayed a significant inhibitory effect on the mycelial growth of *C. gloeosporioides* (at 10 μg/mL and above) compared to the control experiment and solvent control experiment ($p < 0.05$). Overall, the mycelial growth of the fungus was dependent on the concentration of xanthyletin (Fig. 4A) and 7-demethylsuberosin (Fig. 4B) in the culture medium. Inhibition percentages of *C. gloeosporioides* at 100 μg/mL ranged from 100 (day 1) to 53% (day 8) for 7-demethylsuberosin and 100 (day 1) and 43% (day 8) for xanthyletin. So, a higher antifungal activity of 7-demethylsuberosin in comparison to xanthyletin was found. As can be seen, growth inhibition percentages of *C. gloeosporioides* achieved by 7-demethylsuberosin and xanthyletin decreased with time, a fact that suggests that the fungus possesses a detoxification mechanism. IC_{50} values for 7-demethylsuberosin and xanthyletin were 30.1 and 84.0 μg/mL, respectively.

It is noteworthy that, although the structural difference between 7-demethylsuberosin and xanthyletin is only seen in the cyclization of the prenyl group on the aromatic ring, 7-demethylsuberosin was significantly more active than xanthyletin against the fungus. The cyclization between C-3' of prenyl group (in C-6) and 7-OH of 7-demethylsuberosin provide the 2,2-dimethylchromene system on xanthyletin. These results indicate that electronic and/or steric factors in the coumarins might be important for antifungal activity. According to [29], osthenol, a coumarin with prenylation at C-8 and hydroxylated at C-7, showed the highest antifungal activity in a series of di-substituted coumarins, and concluded that the pattern of substitution and the characteristics of the substituting groups are important for antifungal activity of prenylated coumarins.

3.4.2. Inhibition of spore germination

In order to investigate the relationship between the ring substitution and the antifungal activity of these compounds, the coumarin and 7-hydroxycoumarin (umbelliferone) were also tested against the fungus *C. gloeosporioides* using the technique of spore germination inhibition. The inhibitory effects of spore germination of coumarins 7-hydroxycoumarin, 7-demethylsuberosin and xanthyletin at 200 μg/mL against *C. gloeosporioides* are

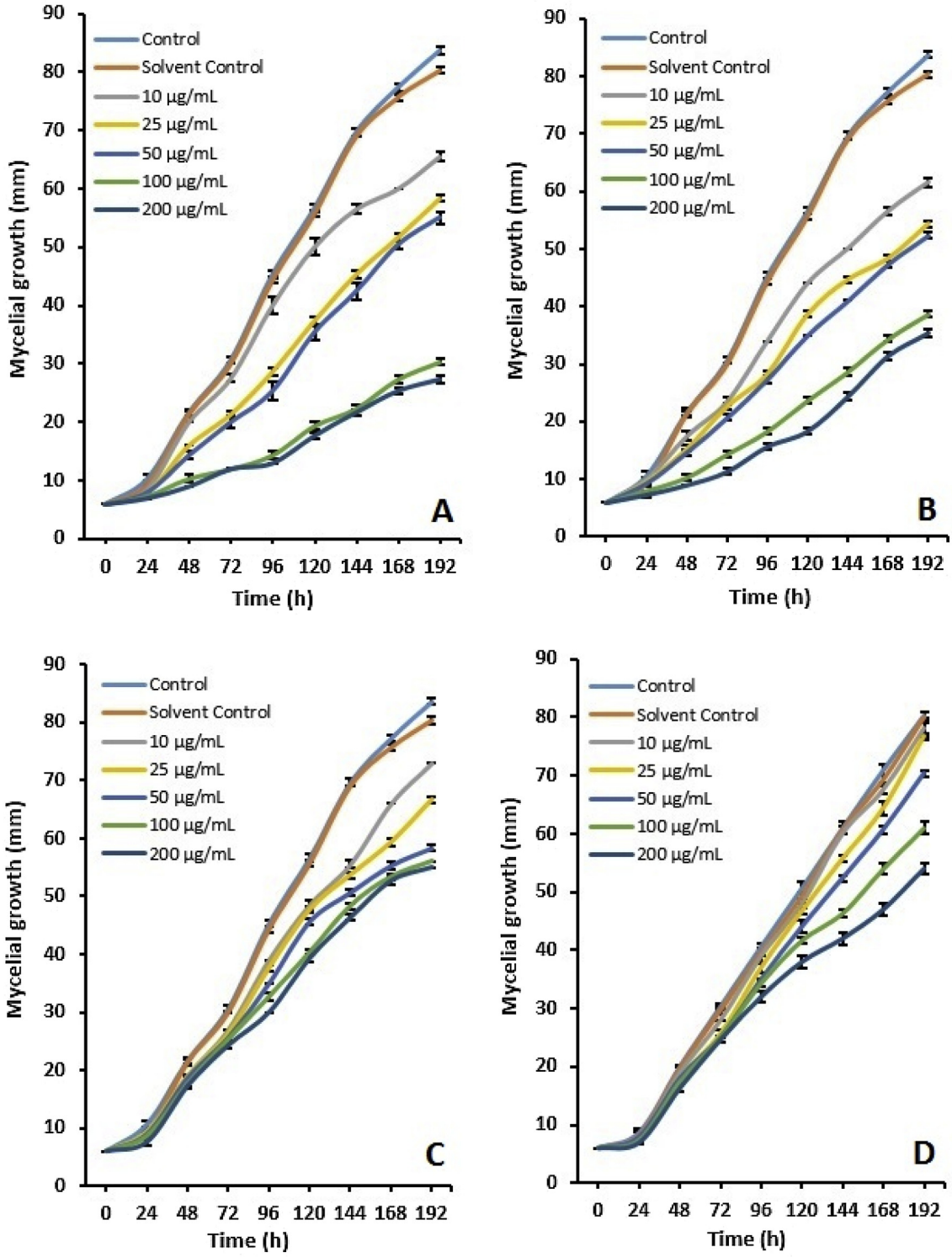


Fig. 1. Mycelial growth of *C. gloeosporioides* treated with *n*-hexane (A), dichloromethane (B), ethyl acetate (C), and methanol (D) extracts from *B. rubescens*.

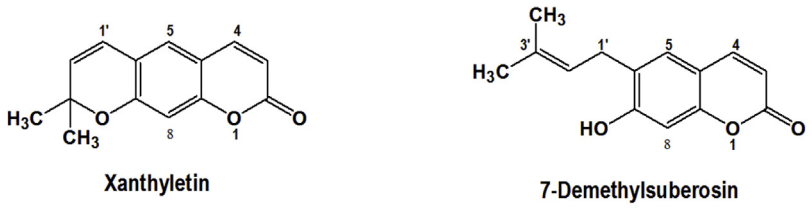


Fig. 2. Structure of isolated compounds from *B. rubescens*.

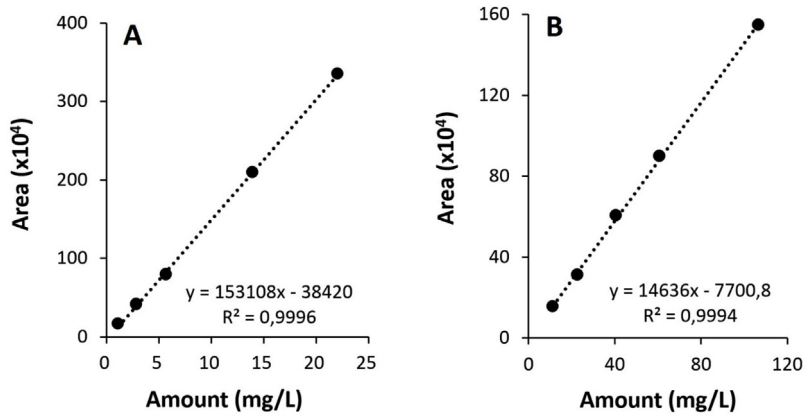


Fig. 3. Calibration curve of xanthyletin (A) and 7-demethylsuberosin (B).

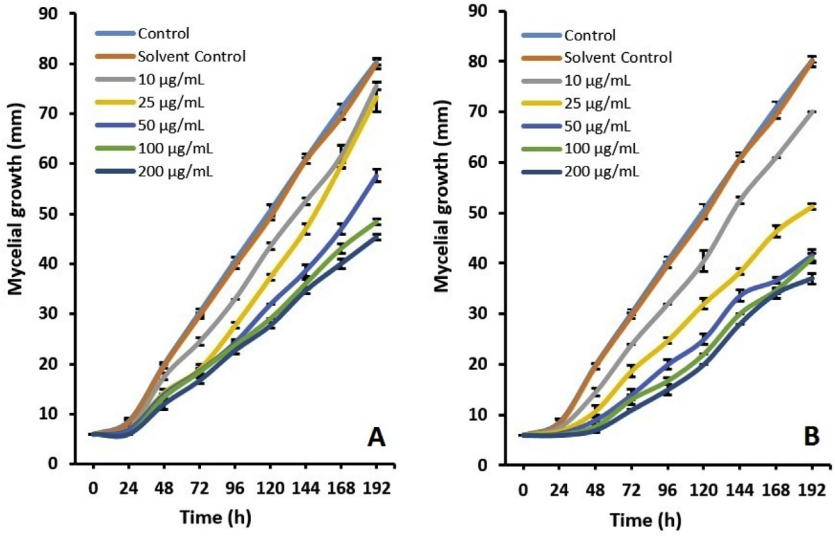


Fig. 4. Mycelial growth of *C. gloeosporioides* tested with xanthyletin (A) and 7-demethylsuberosin (B).

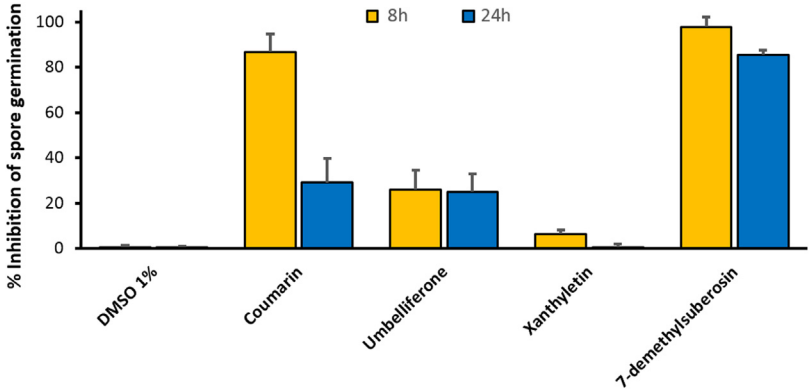


Fig. 5. Inhibition of spore germination of *C. gloeosporioides* tested with coumarin, umbelliferone, xanthyletin and 7-demethylsuberosin.

shown in Fig. 5. As can be seen, the highest spore germination inhibition was found with 7-demethylsuberosin, 97.8 and 85.2 %, after 8 and 24 h, respectively. Xanthyletin had a weak spore germination inhibition of 6.7 and 0.5 %, after 8 and 24 h, respectively. Coumarin displayed a high spore germination inhibition after 8 h (87.2 %), and then, the activity decreased rapidly after 24 h (29.4 %). Umbelliferone exhibited a moderated antifungal activity, which remained stable for 24 h.

The higher antifungal activity of 7-demethylsuberosin against *C. gloeosporioides* (mycelial growth and spore germination inhibition) is related to both the presence of the free hydroxyl group and the 3,3-dimethylallyl substituent [29,30]. A comparison between 7-demethylsuberosin and umbelliferone (coumarin hydroxylated in C-7) shows that the inclusion of the 3,3-dimethylallyl group in the aromatic ring produced a strong increase in the inhibitory activity of spore germination. The presence of the 3,3-

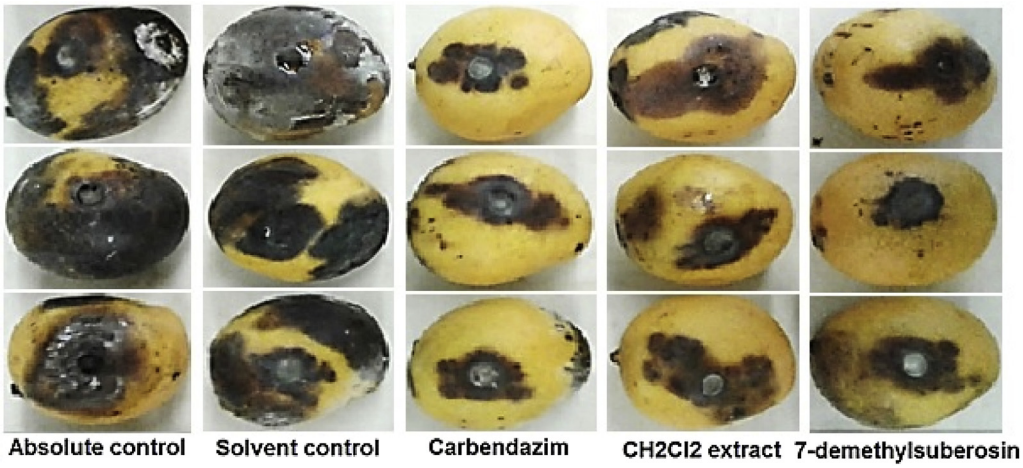


Fig. 6. Effect of the impregnation of Carbendazim, dichloromethane extract from *B. rubescens*, and 7-demethylsuberosin in mango fruits, 15 days post-inoculation with *C. gloeosporioides*.

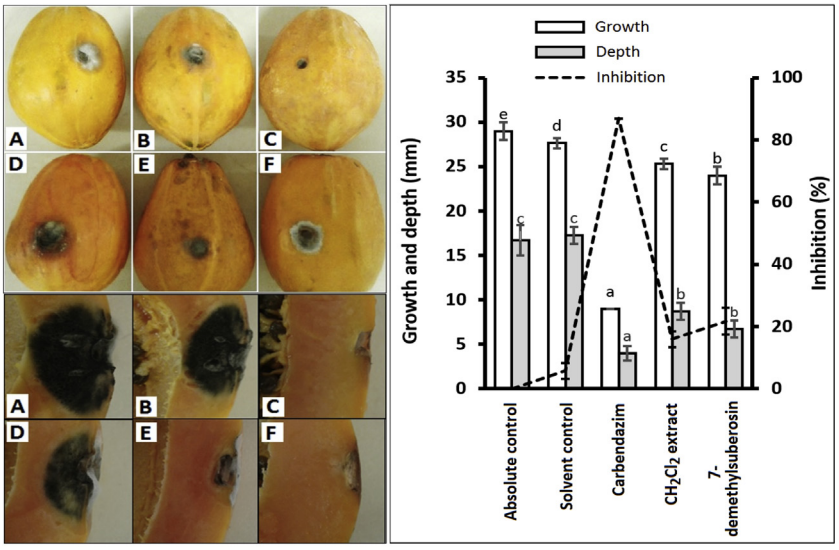


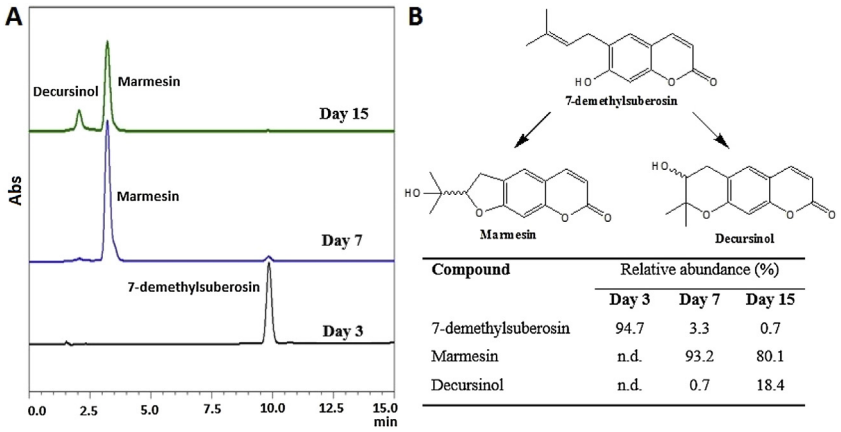
Fig. 7. Antifungal activity of Carbendazim, dichloromethane and methanol extracts from *B. rubescens*, and 7-demethylsuberosin in papaya cv. Hawaiian, 7 d post-inoculation with *C. gloeosporioides*. (A) and (B) are the untreated and solvent controls, respectively; (C) fruits treated with Carbendazim; (D), (E) and (F) fruits treated with the methanol extract, 7-demethylsuberosin and dichloromethane extract, respectively. Different lowercase letters indicate significant differences between groups.

dimethylallyl substituent increases the lipophilicity of the 7-demethylsuberosin and its permeability in the fungal cell membrane, which consequently might result in membrane disruption and releasing of the cellular contents. Similar considerations have been used to explain the influence of hydrocarbon chains on the antifungal properties of compounds like thymol, carvacrol, and eugenol [31,32]. However, lipophilicity alone does not ensure toxicity against *C. gloeosporioides* since xanthyletin, a derivative of 7-demethylsuberosin with the dimethylallyl group as a six-membered ring coupled to a neighboring hydroxyl group, was not fungitoxic against spores. The presence of the free hydroxyl group is a very important structural requirement for the antifungal activity, as seen by the lack of inhibitory effects on spore germination of xanthyletin. The hydroxyl group allows the cytoplasmic membrane to destabilize and act as a proton exchanger, reducing the pH gradient across the cell membrane. This mechanism has been suggested to explain the high antifungal activity of terpenoid phenols such as thymol and carvacrol [31,32].

3.4.3. In vivo *antifungal activity*

Mango and papaya fruits inoculated on a wound with *C. gloeosporioides* were treated with the most promising materials according to *in vitro* assay: CH_2Cl_2 extract and 7-demethylsuberosin. The results of the experiments are shown in Figs. 6 and 7. In general, a compact white mycelial mass surrounded by small water-soaked spots was observed on the surface of the fruits. It can be observed in Fig. 7 that both dichloromethane-soluble extract and 7-demethylsuberosin suppressed lesion development on the fruits and showed protective efficacy after 15 days. It is noteworthy that the compounds Carbendazim and 7-demethylsuberosin in mango fruits exhibited an almost similar protective effect. In papaya fruits after 7 days of storage, significant differences in the fungal growth were found between all treatments and controls. The decreasing order of growth inhibitory effect was Carbendazim, 7-demethylsuberosin and dichloromethane extract.

The progress of the lesion into the parenchyma (depth) of fruit was significantly lower in the treatments with Carbendazim (4.0 ± 0.8 cm), 7-demethylsuberosin (6.8 ± 1.0 cm) and



Compound	Relative abundance (%)		
	Day 3	Day 7	Day 15
7-demethylsuberosin	94.7	3.3	0.7
Marmesin	n.d.	93.2	80.1
Decursinol	n.d.	0.7	18.4

Fig. 8. Metabolism in the course of time of 7-demethylsuberosin by *C. gloeosporioides*. A. HPLC profiles; B. Metabolic pathways.

dichloromethane extract from *B. rubescens* (8.8 ± 1.0 cm) compared to controls (near 17.0 ± 1.7 cm). No significant differences were found between 7-demethylsuberosin and dichloromethane extract from *B. rubescens*; it could be explained either by the high amount of 7-demethylsuberosin in this extract or because there are synergistic effects with the other components of the extract. In general, there was a 15.9, 21.7 and 87.0 % reduction of the lesion diameter on treatments with dichloromethane extract, 7-demethylsuberosin, and Carbendazim, respectively, when compared to the absolute control (untreated fruits).

3.4.4. Microbial conversion

Microbial conversion provides information about the detoxification mechanism used by the pathogenic microorganism and the structural requirements necessary for the antifungal activity. HPLC chromatograms obtained from microbial conversion and the control showed that *C. gloeosporioides* transformed 7-demethylsuberosin into two major metabolites (compounds C and D) (Fig. 8A) not found in the control (data not shown). The structure of both metabolites was determined from the interpretation of spectral data. The spectroscopic data of compounds C and D agree with those reported in [33,34] for the *S*-(+)-marmesin, and [35] for the decursinol, respectively.

C. gloeosporioides consumed the 7-demethylsuberosin slowly, but only transformed about 5 % after 3 days (Fig. 8B). However, the starting substrate was almost completely modified after 7 days (Fig. 8, A and B), being mainly converted to marmesin (a dihydrofuranocoumarin). Marmesin was found to be dextrorotatory ($[\alpha]_D^{25} = +20,6^\circ$), a feature indicative of the *S*-configuration at C-2' in the side-attachment [34]. Then, a decrease in the relative abundance of marmesin coincided with a slight increase in the concentration of decursinol (a dihydropyranocoumarin), after 15 days (Fig. 8). These results suggest that under the conditions used, the fungus can slowly modify the 7-demethylsuberosin. Both metabolic products lack the phenolic OH group, which as mentioned above is an important structural requirement for the antifungal activity exhibited by 7-demethylsuberosin because it destabilizes the cytoplasmic membrane and acts as a proton exchanger. In addition, the lipophilic 3,3-dimethylallyl side-chain was hydroxylated, which increases the hydrophilic character of the product and reduces its ability to penetrate the lipid membrane. As can be seen in Fig. 8A, both metabolic products possess retention times (Rt: 2.06 and 3.23 min for marmesin and decursinol, respectively) lower than 7-demethylsuberosin (Rt: 9.90 min). This means that marmesin and decursinol have higher polarity and hydrophilic character than 7-demethylsuberosin and consequently, less capacity to cross the lipid membrane. So, these transformations may be the result of a process of detoxification of 7-demethylsuberosin by *C. gloeosporioides*.

The conversion of 7-demethylsuberosin by *C. gloeosporioides* to afford derivatives possessing dihydrofurano and dihydropyrano side-attachments is presumed to be formed via epoxidation of the unsaturated side-chain and subsequent nucleophilic attack of the adjacent phenolic OH group. This oxidation process was found to be the major pathway of the prenyl group metabolism in 6-prenylnaringenin by *Aspergillus flavus* [36], bavachin (a prenylated flavanone) by *A. coerulea* [37] and 2,3-dehydrokievitone (a prenylated isoflavone) by *A. flavus* and *Botrytis cinerea* [38]. A microsomal cytochrome P_{450}-dependent monooxygenase-type enzyme has been suggested to catalyze the epoxidation of the double bond [37,38] and it could be successfully exploited as a molecular target for the development of antifungal agents.

In order to determine if the transformation of 7-demethylsuberosin by *C. gloeosporioides* corresponds to a mechanism of detoxification, the antifungal activity of marmesin was evaluated. Decursinol was not considered in the assay for its limited amount. Results show that 200 μg/mL marmesin exhibited mycelial growth inhibitions of *C. gloeosporioides* between 71 (day 1) and 10 % (day 8). Since marmesin was less inhibitory to *C. gloeosporioides* than 7-demethylsuberosin, the biotransformation was considered a detoxification process. Interestingly, the formation of marmesin also provides a toxic medium for the microorganism, which would force the fungus to a new metabolic conversion. So, the fungistatic action of 7-demethylsuberosin could remain for a longer time.

4. Conclusions

The antifungal effect of extracts and major compounds from wood sawdust of *B. rubescens* against *C. gloeosporioides* was evaluated. The mycelial growth of *C. gloeosporioides* was strongly inhibited using the *n*-hexane and dichloromethane-soluble extracts. Therefore, the antifungal effect could be attributed to the less polar substances. From the more fungistatic extracts, two major metabolites were isolated and identified corresponding to xanthyletin and 7-demethylsuberosin. The compound 7-demethylsuberosin displayed a significant antifungal activity (mycelial growth and spore germination inhibition) against *C. gloeosporioides*. Also, 7-demethylsuberosin was slowly transformed by *C. gloeosporioides* to two oxidized metabolites. The presence of the phenolic hydroxyl group and the 3,3-dimethylallyl substituent are two important structural requirements for the antifungal properties seen in this compound. Thus, wood sawdust of *B. rubescens* could be a source of antifungal extracts and compounds.

Declaration of Competing Interest

We have no conflict of interest to declare.

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