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Article

Extracts of the Native Brazilian Tree *Garcinia gardneriana* Inhibit Urediniospore Germination of Coffee Leaf Rust Fungus

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The fungal *Hemileia vastatrix* is the causal agent of coffee leaf rust, one of the worst and devastating disease in coffee cultures worldwide. As a result of our research on natural products for the development of novel agrochemicals, we found that the hexane extract from leaves of the Brazilian medicinal plant *Garcinia gardneriana*, at 500 μg mL^{-1}, inhibited in 98% the germination of *H. vastatrix* urediniospores. This extract showed no phytotoxicity when tested for seed germination and seedling growth inhibitory activity using sensible plant species. Also, the hexane extract from leaves was tested for anti-acetylcholinesterase activity, which constitutes a mechanism of action of major commercial insecticides used in agriculture, and showed low activity even at concentrations about two times higher than the half maximal inhibitory concentration (IC_{50}) found in the antifungal assays. Gas chromatography-mass spectrometry (GC-MS) analysis showed that the hexane extract is constituted mainly by the pentacyclic triterpene lupeol, together with a series of sesquiterpenes as minor components. This is the first report on the investigation of antifungal, phytotoxic and acetylcholinesterase activities of extracts from leaves of *G. gardneriana*. These findings indicate that *G. gardneriana* may constitute a promising source of natural products for controlling the coffee leaf rust fungus.

Keywords: coffee rust, triterpenes, sesquiterpenes, *Rheedia gardneriana*, fungicides

Introduction

Garcinia gardneriana (Planch. & Triana) Zappi, popularly known as bacupari, is an herb native to the Amazon region and disseminated throughout the Brazilian territory. This species is used in Brazilian traditional medicine for the treatment of a series of affections, including pain relief, arthritis, and inflammation of the urinary tract, hepatitis, and gastritis.[1-5] Such traditional knowledge has been supported by studies[6-12] on pharmacological properties of *G. gardneriana*, whose results have shown that extracts from different parts of this plant display antioxidant, cytotoxic, anti-inflammatory, antifungal, antimicrobial, and antiprotozoal activities.

Despite the known antimicrobial activity of *G. gardneriana* against medicinal relevant microorganisms, no study on the activity of extracts from this plant against phytopathogenic fungi has been carried out so far. Our research group has focused on the search for fungal and plant extracts, secondary metabolites, and synthetic analogues of natural products with potential agrochemical applications. In a survey for biological activities of agricultural interest of plant extracts, we found that extracts metabolites from the pericarp of *G. gardneriana* showed nematostatic activity against *Meloidogyne incognita*, a parasite responsible for high agricultural losses in important crops worldwide.[13] More recently, we reported[14] that organic extracts from seeds of *G. gardneriana* show phytotoxic activity. Now, a wide range of extracts from plant and phytopathogenic fungi have been subjected to screening in search of those that are capable of inhibiting the sporulation of *Hemileia vastatrix*.

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The search for novel antifungal agents against *H. vastatrix* has recently become a center of interest for our research group. *H. vastatrix* is the causal agent of coffee rust, one of the worst fungal diseases of coffee plants and responsible for severe agricultural losses in coffee crops.[15,16] In Central America, *H. vastatrix* epidemics have been enhanced mainly due to climate changes that have made weather conditions more favorable to a faster spread of the fungus. In these countries, coffee is often the sole source of income for many small farmers, so the decrease in coffee production caused by *H. vastatrix* has resulted in economic losses and a huge social impact.[17] In this context, several research groups have been focused on natural products, such as botanical extracts and essential oils, as sources of effective and with increased selective toxicity agents for the *H. vastatrix* control.[18-21]

In the present work, we show that extracts from leaves of *G. gardneriana* strongly inhibit the germination of urediniospores of *H. vastatrix*. The chemical constitution of such bioactive extracts was assessed by gas chromatography-mass spectrometry (GC-MS). Besides, to investigate their possible phytotoxic effects, the extracts were tested for inhibition of germination and seedling growth of some sensitive plant species used as indicators. The extracts also were tested for anti-acetylcholinesterase (AChE) activities to preliminarily investigate their possible effects on non-target organisms. This is the first report on the bioactivity of *G. gardneriana* against agricultural relevant pathogens.

Experimental

Collection and preparation of plant material

Naturally growing plants of *G. gardneriana* were collected in the municipality of Viçosa, state of Minas Gerais, Brazil (20°47'48.6" S and 42°51'35.6" W) in February 2017. The Genetic Patrimony/CTA of the *G. gardneriana* was registered in SisGen No. A1DE111. Aerial parts were separated, dried at 40 °C, triturated, and subjected to solvent extraction.

Extraction procedures

Leaves of *G. gardneriana* were dried at room temperature and then triturated in an industrial blender. The plant material (100 g) was subjected to Soxhlet extraction for 4 h using hexane, ethyl acetate, and ethanol (Exodo Científica, Sumaré, Brazil), successively (2 × 1.4 L each). The organic layer was collected, filtered, and concentrated under vacuum, affording the extract from leaves in hexane (3.0 g), ethyl acetate (4.9 g), and ethanol (10.1 g).

Antifungal assay

Urediniospores of *H. vastatrix* were collected from fresh pustules formed on infected *Coffea arabica* cv. Catuaí Vermelho, previously inoculated with urediniospore suspensions, as described by Salcedo-Sarmiento *et al.*,[22] and kept in a growth chamber at 22 ± 1 °C. The collection of urediniospores was performed a few hours before the tests were performed. Freshly collected *H. vastatrix* urediniospores were suspended in a 1% (m/v) Tween 20 (Sigma, St. Louis, USA) solution (prepared with sterile distilled water) and the urediniospore concentration was adjusted to 1×10^6 spores mL^{-1} (concentration calibrated with a hemocytometer). An aliquot of 10 μL of the resulting suspension was transferred to the center of each of a series of sterile glass slides. Each of the slides then received, separately, 10 μL of solutions of each extract at 500 μg mL^{-1} and lupeol at 250 μg mL^{-1}, prepared in aqueous Tween 20 1% (m/v). A droplet of each product was added to the drop of spore suspension immediately afterward. The slides were placed inside transparent polystyrene boxes (gearbox type) previously lined with a moistened paper towel to generate a humid chamber and preventing the evaporation of the drops of spore suspensions. The boxes were left in the dark at 22 ± 1 °C for 6 h. After that period, the germination of urediniospores was interrupted by adding a 10 μL drop of lactophenol onto each drop. Slides on which only the urediniospore suspension was placed served as a negative control whereas drops of urediniospore suspension to which a 10 μL drop of a solution of the fungicide copper oxychloride at 125 μg mL^{-1} in aqueous Tween 20 1% (m/v) served as the positive control. Germination of urediniospores (in percentage) was determined by observation of individual slides with an Olympus BX51 microscope (Tokyo, Japan) adapted with differential interference contrast lighting and digital image capture system (Olympus Q-Color 3 ™, Tokyo, Japan). Each of the first hundred urediniospores visualized under the microscope was ranked as germinated or non-germinated based on the length of germ tubes. Urediniospores bearing germ tubes with a length equal to or greater than its largest diameter were considered as germinated. Germination percentage was calculated concerning the average number of urediniospores found to be germinated in the negative control. Dose-response curves were constructed by assaying the active extracts at 50, 100, 200, 300, 400, and 500 μg mL^{-1}, and the half maximal inhibitory concentration (IC_{50}) values were calculated from non-linear regression using GraphPad Prism.[23] The experiments were conducted in four replicates.

Phytotoxicity assays

The phytotoxic activity of extracts was assessed employing a germination and seedling growth inhibitory assay, according to the methodology reported in da Silva *et al*.[24] Experiments were carried out in triplicate. Seeds of *Cucumis sativus* L. and *Sorghum bicolor* L. were placed on Germintest™ paper in Petri dishes (20 seeds *per* plate). Extracts were prepared at concentration ranging from 0.1 to 2 mg mL^{-1} in Tween 80 (Merck, Hohenbrunn, Germany) at 0.5% (m/v). A volume of 5 mL from this solution was added to the plates and the germination was conducted under a 12 h photoperiod in a germination chamber at 25 °C. After 5 days, germinated seeds (radicle > 2 mm) were counted, and shoot and root lengths were measured. Tween 80 at 0.5% (m/v) and pure distilled water were used as a negative control.

Phytochemical screening of plant extracts

The detection of the main classes of secondary metabolites present in the extracts was carried out by preliminary phytochemical analysis: alkaloids (Dragendorff and Wagner tests), triterpenes (Liebermann-Burchard and Salkowski tests), quinone (ammonium hydroxide reagent), phenolics (Shinoda test, ferric chloride reagent and sodium hydroxide reagent), carbohydrates (Keller Killiani test), and proteins (Biuret test).[25-28]

AChE inhibition assays

Extracts were evaluated for inhibition of acetylcholinesterase (*Electrophorus electricus*, type VI, Sigma-Aldrich, St. Louis, Missouri, USA) by spectrophotometric assay in a 96-well microplate, according to the methodology reported by de Sousa *et al*.[29] Stock solutions of extracts (20 mg mL^{-1} in ethanol) were prepared and kept at –5 °C. These stocks were used to prepare intermediate solutions on three consecutive days. On each day, the stock solutions were sonicated using an ultrasound bath (Sanders©, Soniclean model 2, Santa Rita do Sapucaí, Brazil) at 40 kHz for 5 min and subsequently diluted to 4 mg mL^{-1} in Tris-HCl buffer (50 mmol L^{-1}, pH 8.0). The 200 µL of bovine serum albumin solution (0.1% BSA in Tris-HCl buffer), 100 µL of acetylthiocholine iodide solution in ultrapure water (14.5 mmol L^{-1}), 500 µL of 5,5'-dithiobis 2-nitrobenzoic acid solution (3 mmol L^{-1} of DTNB in Tris-HCl buffer containing 10 mmol L^{-1} of NaCl and 20 mmol L^{-1} of $MgCl_2$) and 100 µL of the intermediate solutions (4 mg mL^{-1} of the extracts) were transferred to microtubes, thus producing reading solutions containing the extracts at the final concentration of 400 µg mL^{-1}. An aliquot of 225 µL was taken from each microtube, added into microplate wells and subjected to spectrophotometric readings on a Thermoplate reader (TP-reader model, Schaffhausen, Switzerland) at 405 nm. After background reading (without enzyme), 25 µL of AChE (0.2 U mL^{-1}, 0.1% BSA in Tris-HCl buffer) was added to each well. Spectrophotometric readings were performed after 20 min and inhibition enzyme (in percentage) was calculated using equation 1. Ethanol dissolved in Tris-HCl buffer and galantamine (17 µmol L^{-1}) was used as negative control and standard inhibitor, respectively. All tests were performed in three biological replicates and triplicate.

$$\text{Inhibition (\%)} = 100 - \frac{[(\text{Ab sample} - \text{Ab background}) \times 100]}{\text{Ab control}} \quad (1)$$

where Ab is the absorbance value.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed in a Shimadzu GCMS-QP5050A apparatus (Shimadzu Europe, Duisburg, Germany) under the following operational conditions: capillary column RTx5 (30 m × 0.25 mm, 0.25 µm); carrier gas (He) flow 1 mL min^{-1}; split ratio 1:5; injector and detector temperature 300 °C; sample injection volume 1.0 µL; electron impact method (70 eV); scan mode *m/z* 30.00 to 700.00; oven temperature 80 °C for 5 min, following a gradient of 4 °C min^{-1} until 300 °C. Hexane extracts were dissolved in dichloromethane (3 mg mL^{-1}) and directly subjected to GC-MS analysis, operating initially at 40 °C for 2 min, gradient 20 °C min^{-1} up to 300 °C. The ethyl acetate and ethanol extracts were subjected to hydrolysis and derivatization protocols before GC-MS analysis. Hydrolysis was performed according to the methodology previously described by Freire *et al*.[30] The derivatization was performed according to the methodology reported by Isidorov *et al*.[31] and Silvério *et al*.[32] Only compounds with mass spectra showing at least 90% similarity with spectrometry library spectra were considered as identified.

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) and nuclear magnetic resonance (NMR) analysis

ATR spectra were obtained using a Varian 660-IR (Varian, Palo Alto, CA, USA) spectrometer. ^{1}H and ^{13}C NMR experiments were recorded on a Varian Mercury 300 instrument (Varian, Palo Alto, California, USA) at 300 and 75 MHz, respectively, using $CDCl_3$ as the solvent and the chemical shift of the solvent as reference. Coupling constants (*J*) are given in hertz.

Isolation of lupeol from hexane extracts

The dried and crushed leaves (1.1 kg) of *G. gardneriana* were subjected to extraction by maceration in hexane (6 × 3 L) for 48 h. The suspension was filtered, and the solvent was removed under vacuum using a rotary evaporator, affording the hexane extract (22.8 g). The extract was subjected to vacuum column chromatography on silica gel 60 (60-230 mesh, Macherey-Nagel, Düren, Germany) using a gradient of hexane-ethyl acetate 1:1 to 8:2 (v/v). Fractions were grouped based on thin-layer chromatography (TLC) profiles using silica gel 60 G F254 TLC plates (Macherey-Nagel, 0.25 mm, Düren, Germany). The obtained fraction (12.4 g) was subjected to vacuum column chromatography using hexane-ethyl acetate 95:5 (v/v) as mobile phase. The lupeol-rich fraction (5.62 g), as determined by GC-MS analyses, was dissolved in hot hexane (6.0 L) followed by cooling to room temperature and then keeping at –18 °C for 72 h. The obtained precipitate was washed with cold hexane and concentrated under vacuum, affording a white solid (2.96 g) identified as lupeol. The melting point of the compound was determined using a Microchemical MQAPF-302 apparatus (Microquímica Equipamentos, Palhoça, Santa Catarina, Brazil) and the identity was confirmed by spectrometric and spectroscopic data.

Lupeol

White solid; mp 195-197 °C; IR (ATR) ν / cm^{-1} 3309, 3061, 2922, 2849, 1637, 1455, 1382, 1031, 878; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.68 (d, 1H, *J* 2.5 Hz, H-29), 4.56 (dd, 1H, *J* 2.6, 1.4 Hz, H-29), 3.18 (dd, 1H, *J* 10.5, 5.7 Hz, H-3), 2.37 (td, 1H, *J* 11.0, 5.6 Hz), 1.92 (m, 1H), 1.68 (s, 3H), 1.03 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.83 (s, 3H), 0.79 (s, 3H), 0.76 (s, 3H), 0.68 (d, *J* 8.8 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.0, 109.4, 79.0, 55.4, 50.5, 48.4, 48.0, 43.1, 42.9, 40.9, 40.1, 38.9, 38.8, 38.1, 37.2, 35.6, 34.3, 29.9, 29.8, 28.0, 27.5, 27.5, 25.2, 21.0, 19.4, 18.4, 18.1, 16.0, 15.4, 14.6; MS (EI, 70 eV), *m/z* (%): 426 (10), 408 (4), 365 (8), 218 (31), 207 (40), 203 (26), 190 (20), 189 (47), 121 (68), 109 (63), 55 (100).

Results and Discussion

Inhibition of *Hemileia vastatrix* conidia germination

The ethyl acetate extract produced moderate inhibition of *H. vastatrix* spore germination, and ethanol extract was not active (Table 1). On the other hand, at 500 μg mL^{-1}, the hexane extract produced high inhibition, which did not differ from that produced by the positive control copper oxychloride ($p < 0.001$). Considering that the hexane extract of leaves showed higher antifungal activity, its IC_{50} values were determined using dose-response curves. Even though the IC_{50} value found for hexane extract is much higher than that for the commercial fungicide copper oxychloride, the hexane extract was capable of inhibiting over 98% of *H. vastatrix* conidia germination, at a concentration as low as 500 μg mL^{-1}. This result allows considering the hexane extract from leaves of *G. gardneriana* as a potential natural product to the control of *H. vastatrix* and as a source of metabolites with antifungal activity of agricultural interest.

Table 1. Inhibition of *Hemileia vastatrix* urediniospores germination by extracts (500 μg mL^{-1}) from leaves of *G. gardneriana*

Extract	Inhibition / %	IC_{50} / (μg mL^{-1})
Hexane	98.3 ± 2.2	211.3 ± 13.7
Ethyl acetate	23.7 ± 3.4[a]	–
Ethanol	0	–
Copper oxychloride	100 ± 0.0	5.3 ± 0.5
Tween-20 at 1% (m/v)	0	–

[a]$p < 0.001$ according to *t*-test. Copper oxychloride and Tween-20 at 1% (m/v) were used as positive and negative controls, respectively. IC_{50}: dose capable of inhibiting 50% of germination.

Phytotoxic and acetylcholinesterase inhibitory activities

The development of novel fungicides with no or very low toxicity against non-target organisms is of great interest. In this sense, the extracts from leaves of *G. gardneriana* were tested for phytotoxicity and acetylcholinesterase inhibitory activities. Acetylcholinesterase is an enzyme that degrades acetylcholine (ACh), an essential neurotransmitter in the central nervous systems of insects. The major chemical classes of insecticides act by inhibiting AChE, decreasing the rate of acetylcholine hydrolysis and thus increasing ACh activity, which results in system excitation and death.[33-35] Exposure to acetylcholinesterase insecticides has contributed to the decline of pollinator insect populations, especially honeybees, constituting a threat for both wild biodiversity and agriculture.

The phytotoxic activity of the extracts from leaves of *G. gardneriana* was assessed by means of germination and seedling growth assay using *C. sativus* and *S. bicolor* as indicator species (Tables S1 and S2, Supplementary Information (SI) section). At the concentrations tested, none of the extracts affected germination of seeds of both species, and only low to moderate effects were observed for the root and shoot growth of both tested species (Figure 1). Hexane extract, which caused the highest inhibition of *H. vastatrix* spore germination, produced no phytotoxic

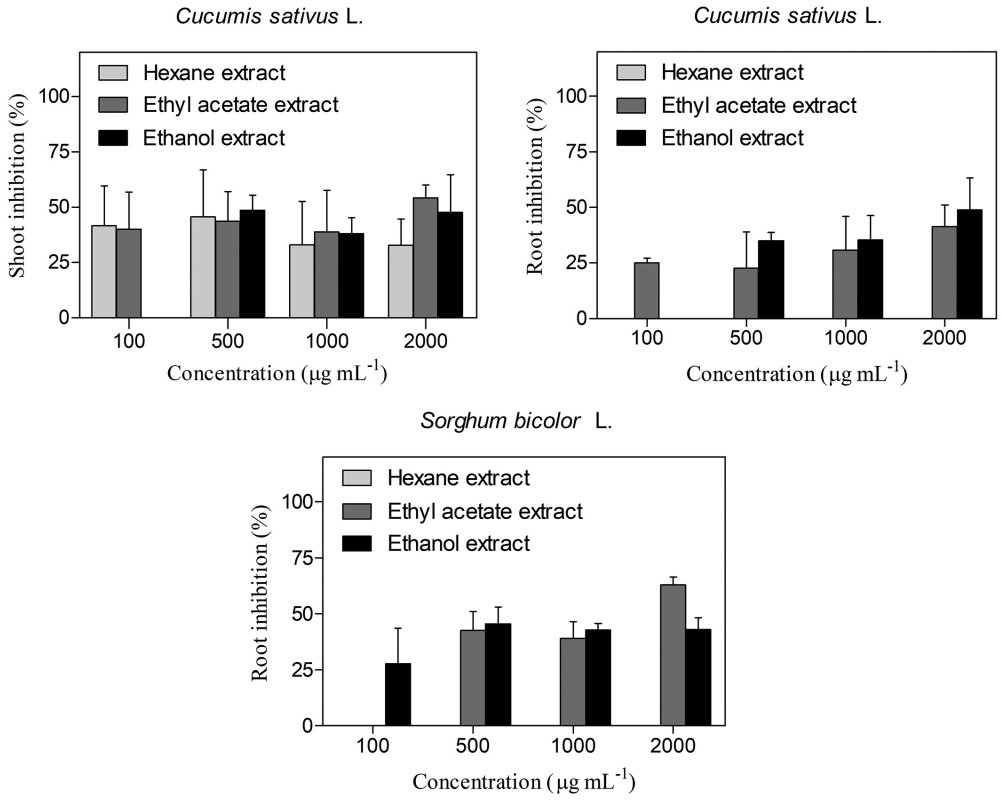

Figure 1. Effects of the extracts from leaves of *G. gardneriana* on the shoot and root growth of *Cucumis sativus* L. and *Sorghum bicolor* L.

effects on germination and root growth of both species. Even at the highest concentration tested (2,000 μg mL^{-1}), which was about 1,000 times higher than the IC_{50} found in the antifungal assay, the hexane extract only discretely affected the shoot growth of *C. sativus*.

The extracts were tested at 400 μg mL^{-1} for inhibition of AChE (Figure 2). The ethyl acetate extract showed the highest inhibitory activity (49.18 ± 2.52%). As also observed from the phytotoxicity assays, the hexane extract, which showed the highest target activity against *H. vastatrix*, produced low inhibition of AChE. Besides, the advantage of not having the phytotoxic and anti-AChE activities, it is worth mentioning that, to the best of our knowledge, this is the first study on the anti-AChE activity of extracts from leaves of *G. gardneriana*.

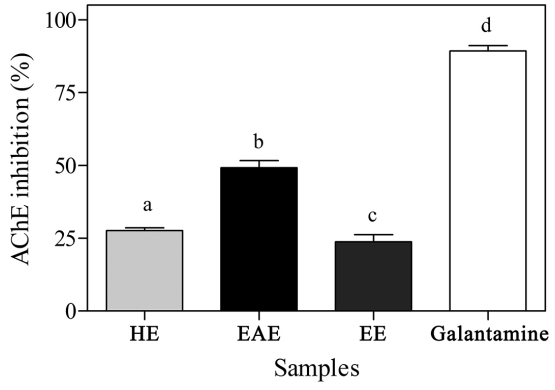

Figure 2. Acetylcholinesterase inhibitory activity (mean ± standard deviation) of extracts from *G. gardneriana* at 400 μg mL^{-1} ($p < 0.05$ according to ANOVA followed by Tukey's post-test). HE = hexane extract; EAE = ethyl acetate extract; EE = ethanol extract; Galantamine = positive control.

Chemical constitution of the extracts

A preliminary phytochemical screening was carried out to detect the main metabolites present in each extract. The obtained data, presented in Table 2, showed that triterpenes were identified in all extracts, and phenolic compounds were present in the ethyl acetate and ethanol extracts.

A series of pentacyclic triterpenes[13,36,37] and phenolic metabolites[7,8,11,38,39] have already been isolated from extracts of different parts of *G. gardneriana*. For instance, the bioflavonoids volkensiflavone, fukugetin, fukugeside, I3-naringenin-II8-eriodictyol (GB-2a) and naringenin-II8-4'-*O*Me-eriodictyol were isolated from ethanolic extracts from *G. gardneriana* leaves.[40,41] Besides, Verdi *et al.*[42] reported the isolation of GB-2a-I-7-*O*-glucoside, epicatechin, volkensiflavones, and fukugetins from the hydroalcoholic extracts leaves of this plant. Recently, a series of other phenolic compounds, including flavonoids, bioflavonoids, and xanthones were reported in the ethanolic extracts of the leaves and fruits of *G. gardneriana*.[8]

Table 2. Extraction yields and phytochemical screening of extracts from leaves of *G. gardneriana*

Extract		Hexane	Ethyl acetate	Ethanol
Yield / % (m/m)[a]		3.0	4.9	10.1
Alkaloids	Dragendorff's test	–	–	–
	Wagner's test	–	–	–
Triterpenes	Liebermann-Burchard test	+	+	+
	Salkawsk's test	+	+	+
Quinone	NH_4OH	–	–	–
Phenolics	Shinoda's test	–	+	+
	$FeCl_3$ test	–	+	+
	NaOH	–	+	+
Carbohydrate	Keller Killian test	–	+	+
Protein	Biuret reagent	–	–	–

[a]Relative to the dried weight of leaves.

Following the preliminary phytochemical screening, the chemical constitution of the extracts was examined through GC-MS analysis. Ethyl acetate and ethanol extracts were previously derivatized to convert more polar compounds into their corresponding silyl ethers, allowing detecting such substances by spectrometry analysis. Besides, aiming at breaking non-volatile long chain esters and glycosides into their constituting chemical entities, another aliquot of both the ethyl acetate and ethanol extracts was subjected to alkaline hydrolysis before derivatization. Results from GC-MS analysis for such samples are presented in Table 3. Apart from high amounts of carbohydrates, a series of long chain fatty acids, together with two phenolic acids (phloroglucinol and isovanillic acid), lupeol, and β-sitosterol were identified in the derivatized ethyl acetate extract, while only two metabolites could be identified in the ethanol extract. After subjecting the extracts to alkaline hydrolysis before derivatization, the GC-MS analysis showed an increase in the content of various chemical constituents of the ethyl acetate extract. Similarly, a series of alcohols and fatty acids not detected in the derivatized ethanol extracts were identified after hydrolysis. Altogether, these results indicate that such compounds are present in the extracts, mainly in the form of their corresponding esters or glycosides.

Unlike the extracts in ethyl acetate and ethanol, the hexane extract was subjected directly to GC-MS analysis, without derivatization or hydrolysis. The pentacyclic triterpene named lupeol was identified as the main metabolite in the hexane extract (Figure S1, SI section), together with a series of sesquiterpenes as minor constituents (Table 4). Muurolene and γ-cadinene have also already been reported[37] in the hexane extract of the bark of fruits from *G. gardneriana*.

Because GC-MS analysis showed a very high relative percentage of lupeol in the hexane extract, additional amounts of extracts from leaves of *G. gardneriana* were

Table 3. Relative percentage of the chemical constituents identified by GC-MS (detected as trimethylsilyl esters) from extracts of leaves of *G. gardneriana* after derivatization (D) or derivatization preceded by hydrolysis (H)

	Compound	Percent composition				Retention index	
		Ethyl acetate		Ethanol		Calculated	Literature
		D	H/D	D	H/D		
1	lactic acid	0.5	–	–	–	–	–
2	benzoic acid	–	6.3	–	–	1243	1250[43]
3	glycerol	–	–	0.9	–	1285	1292[24]
4	phloroglucinol	4.6	0.3	0.6	–	1654	1659[43]
5	isovanillic acid	1.4	1.1	–	2.4	1767	1775[43]
6	palmitic acid	–	19.0	–	38.3	2039	2050[31]
7	phytol	0.6	18.8	–	3.0	2169	2183[31]
8	oleic acid	0.6	7.5	–	17.3	2208	2219[43]
9	α-linolenic acid	–	5.0	–	10.8	2209	2217[24]
10	stearic acid	–	0.7	–	3.2	2234	2240[24]
11	β-sitosterol	0.9	3.3	–	–	–	–
12	lupeol	5.5	11.1	–	–	–	–
Carbohydrate		28.0	–	76.5	–		
Phenolics		6.0	1.4	0.6	2.4		
Terpenes		6.4	14.4	–	–		
Organic acids		2.5	39.6	–	72.0		

D: derivatized; H/D: hydrolyzed and derivatized.

Table 4. Chemical constituents identified by GC-MS in the hexane extract of leaves from *Garcinia gardneriana*

N	Retention time / min	Compound	Relative percentage / %	Retention index	
				Calculated	Literature
1	9.94	α-copaene	0.7	1417	1412[44]
2	10.03	β-elemene	2.6	1429	1421[45]
3	10.65	γ-muurolene	2.6	1512	1513[44]
4	10.79	β-selinene	2.0	1534	1520[46]
5	10.94	γ-cadinene	1.8	1556	1543[47]
6	11.47	(–)-caryophyllene oxide	3.4	1632	1619[47]
7	18.30	squalene	6.4	2841	2835[48]
8	18.71	nonacosane	1.8	2895	2900[49]
9	27.00	lupeol	51.8		

produced and subjected to vacuum column chromatography followed by precipitation of the fractions in hexane, allowing for obtaining 2.96 g of lupeol (13 and 0.26% relative to the hexane extract and the dry matter, respectively). After isolation, the identity of the lupeol was confirmed by IR, GC-MS, ^{1}H and ^{13}C NMR. All spectra are presented in SI section (Figures S1-S5), and all spectroscopic and spectrometric data are in agreement with data from the literature (Table S3, SI section).[24,50,51] This pentacyclic triterpene is a chemical constituent of several medicinal plants[52] and has been isolated as the main constituent of extracts from different parts from *G. gardneriana*.[36,37] In the work of Corrêa *et al*.,[53] lupeol was isolated in 5.25% yield from dichloromethane extract of leaves of *Garcinia brasiliensis*, which correspond to a yield 1.5 times less than the obtained in the present study.

The weak phytotoxicity and anti-AChE effects of the extracts can be related to the chemical constituents identified in the GC-MS analysis. A series of studies[24,54-56] has shown that some fatty acids, phloroglucinol, phytol and β-sitosterol, identified in the ethyl acetate and ethanol extracts of *G. gardneriana*, have phytotoxic activity and are related to the allelopathic effects of botanical extracts. Also, the weak anti-AChE activity of these extracts can be attributed to some of the fatty acids and phytol, which have also shown AChE inhibition.[57-60]

To verify if the inhibition of *H. vastatrix* urediniospores germination produced from the hexane extract was caused by its major metabolite, pure lupeol was also subjected to conidia germination assay. Tested at 250 μg mL^{-1} (half of the extract concentration used in the initial screening), lupeol inhibited only 11.1 ± 2.5% of conidia germination. This result indicates that the high activity of the hexane extract is due to synergistic action involving lupeol and other minor metabolites takes place. In fact, some terpene-rich essential oils and botanical extracts have been displayed inhibition of phytopathogenic fungi. For instance, β-elemene, β-selinene, and (–)-caryophyllene oxide, three sesquiterpene hydrocarbons constituents of the hexane extract of *G. gardneriana*, have already been related as responsible for the activities of essential oil and extracts against phytopathogenic fungi.[18,61-64] Among these works, Caetano *et al*.[18] found that essential oil of different *Eucalyptus* species, whose GC-MS analysis showed a series of terpenes as the main constituents, were capable of strongly inhibit the germination of *H. vastatrix* spores. Copper-based drugs are commonly used to treat fungal disease in plants. However, the potential toxicity of these compounds constitutes a great concern.[65] Thus, the discovery of natural products capable of efficiently control phytopathogenic fungi and presenting environmentally friend behavior is a great interest.

Conclusions

This is the first report on the investigation of antifungal, phytotoxic, and acetylcholinesterase inhibitory activities of extracts from leaves of *G. gardneriana*. The best results were found for the hexane extract, which inhibited in 98.3 ± 2.2% the *H. vastatrix* conidia germination when tested at a concentration as low as 500 μg mL^{-1}, and showed an IC_{50} of 211.3 ± 13.7 μg mL^{-1}. Also, the hexane extract showed no phytotoxic activity on germination and seedling growth of *C. sativus* and *S. bicolor*, two plants highly sensitive to phytotoxins and used as indicator species, and did not significantly inhibit acetylcholinesterase activity, a mechanism of action of the major chemical classes of insecticides. The absence of phytotoxicity and anti-AChE inhibition is of great interest in the search and development of novel antifungal agents for agricultural use. Finally, GC-MS analysis allowed to identify the pentacyclic triterpene lupeol as the main constituent of the hexane extract, together with a series of sesquiterpenes as minor constitutes. The identified compounds are also present in botanical extracts and essential oils of plant species

with antimicrobial activity against both human and plant pathogens, some of them being used in traditional medicine. Altogether, our finds indicate that *G. gardneriana* extracts may constitute natural products with potential direct application for controlling *H. vastatrix* or as sources of metabolites for the development of novel antifungal agents of agrochemical interest.

Supplementary Information

Supplementary information (phytotoxic assays data, IR, ^{1}H and ^{13}C NMR and HRMS spectra) is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Ueveton P. da Silva was responsible for conceptualization, methodology, investigation, resources, writing - original draft, writing - review and editing; Bruno W. Ferreira for methodology, resources, writing - review and editing; Bianca L. de Sousa for data curation, investigation, visualization, software, validation, writing - original draft; writing - review and editing; Gabriela M. Furlani for methodology, resources, writing - review and editing; Robert W. Barreto for data curation, investigation, visualization, software, validation, writing - original draft; writing - review and editing; Ana Paula Agrizzi for data curation, methodology, investigation, writing - review and editing; João Paulo V. Leite for data curation, methodology, investigation, writing - review and editing; Marcelo H. Santos was responsible for resources, investigation, writing - review and editing; Eduardo V. V. Varejão for project administration, funding acquisition, supervision, investigation, resources, writing - original draft, review and editing.

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