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Research article

Antifungal activity against anthracnose-causing species of homopterocarpin derivatives

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ABSTRACT

Derivatives of 3,9-dimethoxypterocarpan (**1**, homopterocarpin) were prepared by nitration, amination, and oxidation reactions, among others, and their antifungal activity was evaluated against the phytopathogenic fungi *Colletotrichum gloeosporioides* and *C. lindemuthianum*. Derivatives were purified by chromatographic techniques and identified by nuclear magnetic resonance spectroscopy. Eight derivatives were obtained from **1** corresponding to 3,9-dimethoxy-8-nitropterocarpan (**2**), 3,9-dimethoxy-2,8-dinitropterocarpan (**3**), 3,9-dimethoxy-2,8,10-trinitropterocarpan (**4**), 2,8-diamino-3,9-dimethoxypterocarpan (**5**), 3,9-dimethylcoumestan (**6**), medicarpin (**7**), 2′-hydroxy-4-(2-hydroxyethylsulfanyl)-7,4′-dimethoxyisoflavan (**8**), and 4-(2-hydroxyethylsulfanyl)-7,2′,4′-trimethoxyisoflavan (**9**). The *in vitro* antifungal activity of the derivatives was determined at concentrations between 35 and 704 μM. Compounds **7** and **8** at 704 μM, showed an inhibition of radial growth and spore germination close to 100%, exceeding that found for the starting compound **1**, which was 46%. Growth inhibition assays were also performed for the derivative **8** on papaya fruits (*Carica papaya* L. cv. Hawaiana) and mango (*Mangifera indica* L. cv. Hilacha) infected with *C. gloeosporioides*. Compound **8** showed fungal growth inhibition in fruits higher than that found for **1** and thymol (a recognized natural antifungal), under the same conditions. In general, derivatives that exhibited greater antifungal activity correspond to the compounds containing hydroxyl groups in the structure. Some of the compounds obtained could be considered promising for the control of phytopathogenic fungi.

1. Introduction

The control of fungal diseases with synthetic fungicides is currently the most widely used method in world agriculture, enabling sustainable food production and agricultural industry growth [1,2]. Unfortunately, many of the fungicides used today have adverse effects on human health and the environment. Some of the existing fungicides were discovered after random selection and optimization of the structure-activity relationship, have structural cores that are rare or non-existent in nature, and may be

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non-biodegradable substances or affect non-target species. Furthermore, due to the increasing resistance of microorganisms to fungicides, new, safer, and environmentally friendly compounds are needed. According to above facts, a recent focus of research for the control of fungal diseases, based on metabolic knowledge of the plant-pathogen interaction, have been explored. Antimicrobial compounds from plants, such as phytoanticipins (constitutive antimicrobial compounds) and phytoalexins (low molecular weight antimicrobial compounds induced by biotic or abiotic stress), can be used as chemical structural templates for the development of new antifungal agents [3]. It is even possible to prepare synthetic compounds structurally related to natural antimicrobials but with improved properties, such as higher antifungal activity and/or better water solubility. Thus, for instance, Caruso et al. [4] reported that some furyl compounds closely related to phytoalexin resveratrol, were more fungitoxic towards *in vitro* mycelial growth of *Botrytis cinerea*. Furthermore, because the chemical structural core is involved in the plant-pathogen interaction, it is possible to think that structurally related compounds may have a more selective antimicrobial action (without affecting the non-target species) or be more biodegradable antifungal agents than those with structural nuclei rare or absent in nature.

On the other hand, pterocarpans (a group of isoflavonoids) play an important role in plant resistance to diseases [5]. Some pterocarpans act as phytoalexins or phytoanticipins in plants and are mainly found in species belonging to the Leguminosae (Fabaceae) family. The antifungal properties of pterocarpans have been well documented and reported [6]; therefore, pterocarpans can be used as a structural template for the development of new antifungal agents. Homopterocarpin is a naturally occurring pterocarpan that has been isolated from *Platymiscium yucatanum* [7], *P. floribundum* [8], *P. gracile* Benth [9], *Pterocarpus macarocarpus* Kurtz heartwood [10], *Dalbergia saxatilis* [11] among other. Homopterocarpin offered gastroprotection against indomethacin-induced ulcers by antioxidative mechanism and the modulation of gastric homeostasis [12]; this compound has also been shown to exhibit hepatoprotective, antioxidant properties [13], and antiproliferative activity on HL-60 human leukemia cells [14]. Furthermore, homopterocarpin exhibits antimicrobial activity against Gram-positive and negative bacteria and against phytopathogenic fungi such as *Aspergillus niger*, *C. acutatum*, and *C. gleosporioides* [9,15,16]; consequently, there has recently been intense interest in the potential of pterocarpans. In the present study, a series of derivatives were obtained from the homopterocarpin, and then, the activity against the fungi *Colletotrichum gloeosporioides* and *Colletotrichum lindemuthianum* of derivatives was evaluated. These fungi are the causal agents of the disease named anthracnose, which affect important crops around the world.

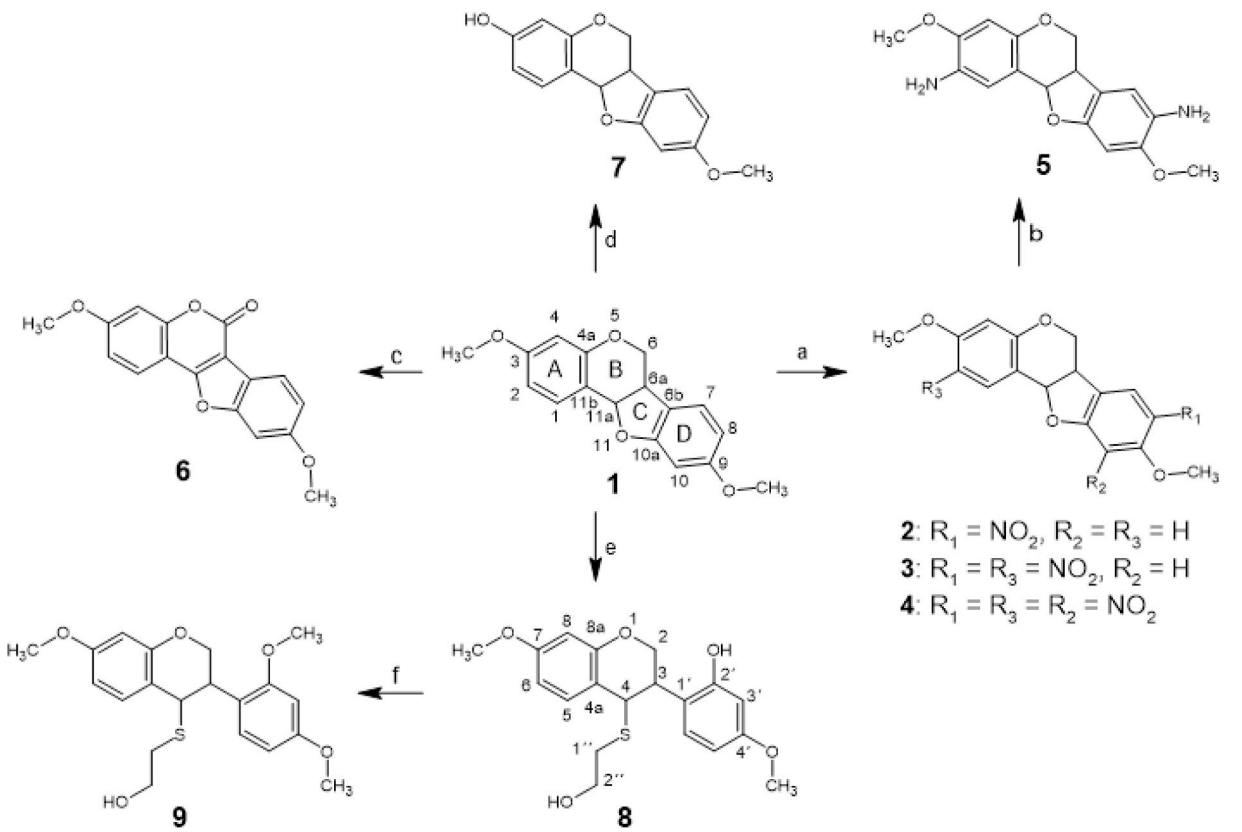

Fig. 1. Preparation of compounds 2–9 from homopterocarpin. Reagents and conditions: a) 80% HNO_3 in H_2SO_4, DCM, −15 °C, 30 min. b) 3, EtOH, $SnCl_2$, 45 °C, 12 h. c) DDQ, DCM, r.t., 12 h. d) HCl (33%), acetonitrile, −15 °C for 30 min. e) DCM:2-mercaptoethanol (1:1), $AlCl_3$, r.t., 24 h. f) K_2CO_3, acetone, 30 min; then, CH_3I, 24 h.

2. Materials and methods

2.1. General

Homopterocarpin was purified from *Platymiscium gracile* Benth [16]. Glacial acetic acid (AcOH) and sulfuric acid (H_2SO_4) were acquired from PanReac Applichem (Darmstadt, Germany). Acetone, acetonitrile, and 2-mercaptoethanol were obtained from Merck Co. (Darmstadt, Germany). Dimethylsulfoxide (DMSO) was from Mallinckrodt Baker Inc. (Kentucky, USA). Technical-grade solvents, *n*-hexane, ethyl acetate (EtOAc), dichloromethane (DCM), methanol (MeOH), and ethanol (EtOH) were from Protokimica S.A.S. and purified by distillation and subsequent drying with anhydrous sodium sulfate. 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was from Sigma-Aldrich Co. (Sigma-Aldrich Co., St. Louis, USA). Colizim® (500 g carbendazim/L) was from Colinagro (Bogotá, Colombia). Aluminum chloride anhydrous ($AlCl_3$) was from Alfa-Aesar Co. (Ward Hill, USA). The progress of the reactions was monitored by thin layer chromatography (TLC) on silica gel, using different mixtures of *n*-hexane/EtOAc as mobile phase. The visualization of the compounds was carried out with UV radiation (254 and 365 nm) and by spraying with AcOH:H_2SO_4:H_2O (143:28:30), followed by heating. Column chromatography was used to purify the compounds using silica gel 60 (Merck KGaA, Darmstadt, Germany) and/or Sephadex LH-20 (Sigma-Aldrich Co., St. Louis, USA). The synthetic compounds were characterized using mono- [^{1}H NMR, ^{13}C NMR, DEPT (Distortionless Enhancement by Polarization Transfer)] and two-dimensional nuclear magnetic resonance [^{1}H–^{1}H COSY (COrrelation SpectroscopY), ^{1}H–^{13}C HSQC (Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation)] in a Bruker AMX 300 spectrometer. The chemical shifts (δ) and spin-spin coupling constants (*J*) are expressed in parts per million (ppm) and Hertz, respectively. The following abbreviations are used: s = singlet, d = doublet, t = triplet, dd = doublet of doublets, m = multiplet. The antifungal activity tests against *C. gloeosporioides* and *C. lindemuthianum* were performed in a class II type laminar flow cabinet (CSB 180 A). The instruments and materials were sterilized in an automatic horizontal autoclave (Centricol AUA 80 L brand). For the spore count, a Carl Zeiss Primo Star microscope and a Neubauer camera (Deep 1/10 mm, Boeco) were used. The strain of *C. gloeosporioides* and *C. lindemuthianum* were obtained from tomato fruits (*Solanum betaceum* Cav.) and common bean pods (*Phaseolus vulgaris* L.) infected. The morphological characteristics and the identification of the fungi were carried out in the Laboratorio de Microbiologia Industrial of the Universidad Nacional de Colombia (Medellín).

2.2. Preparation of compounds 2–9 from homopterocarpin

Compounds 2 to 9 were obtained from 1 according to Fig. 1.

Compounds 2–4. They were obtained by nitration reaction. Compound 1 (200 mg, 0.704 mmol) was dissolved in DCM (4 mL) and 150, 1800, and 4600 μL of HNO_3 (80%) in H_2SO_4 was added to prepare 2, 3, and 4, respectively. The reactions were carried out at −15 °C for 30 min with constant stirring. The reactions were stopped with water (50 mL) and $NaHCO_3$ solution. Extractions were carried out with DCM (3 × 50 mL) and the organic phases were combined and dried with anhydrous Na_2SO_4. The solvent was removed by rotary evaporation under reduced pressure. The obtained residues were fractionated by column chromatography using silica gel (mobile phase: mixtures *n*-hexane-EtOAc) and Sephadex LH-20 (mobile phase: *n*-hexane-DCM-MeOH, 2:1:1). Finally, 45 (23.7%), 25 (9.5%) and 11 (3.9%) mg of compounds 2, 3 and 4, respectively, were obtained. *3,9-dimethoxy-8-nitropterocarpan* (2). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.44 (1H, d, J = 8.4, H1), 8.01 (1H, s, H7), 6.70 (1H, dd, J = 8.4, 2.1, H2), 6.57 (1H, s, H10), 6.52 (1H, d, J = 2.1, H4), 5.74 (1H, d, J = 5.7, H11a), 4.34 (1H, dd, J = 10.2, 4.3, H6ec), 3.97(3H, s, –OCH_3), 3.84(3H, s, –OCH_3), 3.81–3.75 (2H, m, H6ax, H6a). ^{13}C NMR (75 MHz, $CDCl_3$): δ 39.2 (C6a), 55.5 (-OCH_3), 56.8 (-OCH_3), 66.1 (C6), 80.4 (C11a), 95.5 (C10), 101.8 (C4), 109.7 (C2), 111.3 (C11b), 119.2 (C6b), 123.4 (C7), 131.7 (C1), 132.9 (C8), 156.8 (C4a), 157.0 (C9), 161.4 (C3), 165.0 (C10a). *3,9-dimethoxy-2,8-dinitropterocarpan* (3). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.27 (1H, s, H1), 8.03 (1H, s, H7), 6.64 (1H, s, H10), 6.60 (1H, s, H4), 5.75 (1H, d, J = 7.2, H11a), 4.59 (1H, dd, J = 10.8, 4.2, H6ec), 3.99 (3H, s, –OCH_3), 3.98 (3H, s, –OCH_3), 3.94–3.82 (2H, m, H6ax, H6a). ^{13}C NMR (75 MHz, $CDCl_3$): δ 38.9 (C6a), 56.8 (-OCH_3), 56.9 (-OCH_3), 66.2 (C6), 78.7 (C11a), 95.8 (C10), 102.0 (C4), 111.2 (C11b), 111.1 (C6b), 123.4 (C1), 130.0 (C7), 133.4 (C2), 155.7 (C9), 157.1 (C3), 160.6 (C4a), 164.5 (C10a). *3,9-dimethoxy-2,8,10-trinitropterocarpan* (4). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.27 (1H, s, H1), 8.14 (1H, s, H7), 6.66 (1H, s, H4), 5.99 (1H, d, J = 6.9, H11a), 4.51 (1H, dd, J = 9.7, 3.3, H6ec), 4.09 (3H, s, –OCH_3), 4.07 (3H, s, –OCH_3), 4.04–3.97 (2H, m, H6ax, H6a). ^{13}C NMR (75 MHz, $CDCl_3$): δ 39.3 (C6ec), 66.6 (C6), 66.9 (-OCH_3), 78.6 (C11a), 96.9 (C10), 103.7 (C4), 106.4 (C8), 109.8 (C2), 112.6 (C11b), 119.1 (C6ax), 124.8 (C7), 132.2 (C1), 156.7 (C4a), 158.1 (C3), 160.7 (C10a), 161.1 (C9).

Compound 5. It was obtained by a reduction reaction with $SnCl_2$. Compound 3 (200 mg, 0.704 mmol) dissolved in EtOH (4 mL) was mixed with $SnCl_2$ (2 g). The reaction was carried out at 45 °C for 12 h with constant stirring. Subsequently, water (50 mL) was added, and the reaction mixture was extracted with DCM (3 × 50 mL). The combined organic phases were dried over anhydrous Na_2SO_4; the supernatant was decanted, and the solvent was removed by rotary evaporation under reduced pressure. The resulting residue was fractionated by silica gel column chromatography and 5 mg (0.016 mmol, 2.3%) of a light brown amorphous solid was obtained. Compounds 2 and 4 were not reduced under these reaction conditions. *2,8-diamino-3,9-dimethoxypterocarpan* (5). ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.88 (1H, s, H1), 6.70 (1H, s, H7), 6.48 (1H, s, H10), 6.46 (1H, s, H4), 5.43 (1H, d, J = 6.9, H11a), 4.23 (1H, dd, J = 10.5, 4.8, H6ec), 3.87 (3H, s, –OCH_3), 3.85 (3H, s, –OCH_3), 3.66–3.52 (2H, m, H6ax, H6a). ^{13}C NMR (75 MHz, $CDCl_3$): δ 40.6 (C6a), 55.6 (-OCH_3), 55.8 (-OCH_3), 66.9 (C6), 78.1 (C11a), 94.7 (C10), 99.8 (C4), 111.5 (C1), 112.8 (C11b), 115.7 (C7), 117.8 (C6b), 129.5 (C8), 130.8 (C2), 146.7 (C9), 146.7 (C3), 153.1 (C4a), 156.6 (C10a).

Compound 6. Compound 1 (200 mg, 0.704 mmol) dissolved in DCM (25 mL) was treated with DDQ (60 mg) [17]. The mixture was left at room temperature for 12 h under constant stirring. Then, brine solution was added (50 mL) and the mixture extracted with DCM (3 × 50 mL). The combined organic phases were dried (anhydrous Na_2SO_4), filtered, and the solvent was evaporated under reduced

pressure on a rotary evaporator. The obtained residue was fractionated by column chromatography to provide the compound 6 (60 mg, 0.160 mmol, 22.7%) as a colorless amorphous solid. *3,9-dimethylcoumestan* (6). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.99 (1H, d, J = 8.4, H1), 7.92 (1H, dd, J = 8.1, 2.1, H7), 7.09 (1H, dd, J = 8.4, 2.2, H2), 7.21 (1H, d, J = 2.1, H4), 7.04–7.00 (2H, dd, J = 8.4, 2.2, H8), 7.04–6.73 (2H, dd, J = 8.4, 2.2, H10) 3.96 (3H, s, –OCH_3), 3.95 (3H, s, –OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 55.9 (2 x –OCH_3), 96.9 (C10), 101.4 (C4), 106.2 (C6a), 113.1 (C2), 113.2 (C8), 113.2 (C11b), 115.5 (C6b), 121.7 (C7), 122.5 (C1), 155.2 (C4a), 156.5 (C10a), 158.6 (C3), 159.3 (C11a), 160.1 (C9), 162.6 (C6).

Compound 7. Compound 1 (200 mg, 0.704 mmol), dissolved in 5 mL of acetonitrile, was treated with 100 μL of concentrated HCl (33%). The reaction was carried out at −15 °C for 30 min with constant stirring. Subsequently, a $NaHCO_3$ solution (1 M, 50 mL) was added until neutralization. The crude reaction was extracted with DCM (3 × 50 mL), and the combined organic phases were dried over anhydrous Na_2SO_4, filtered and the solvent was removed by rotary evaporation under reduced pressure. The resulting residue was fractionated by silica gel column chromatography to obtain 4 mg (0.015 mmol, 2.2%) of a colorless crystalline solid (Medicarpin). Compound 7.^{1}H NMR (300 MHz, $CDCl_3$): δ 7.44 (1H, d, J = 8.4, H1), 7.18 (1H, d, J = 8.7, H7), 6.59 (1H, dd, J = 8.4, 2.2, H2), 6.52–6.46 (3H, m, H-10, H8, H4), 5.55 (1H, d, J = 6.6, H11a), 4.29 (1H, dd, J = 10.0, 4.5, H6ec), 3.83 (3H, s, –OCH_3), 3.70–3.57 (2H, m, H6ax, H6a). ^{13}C NMR (75 MHz, $CDCl_3$): δ 39.5 (C6a), 55.6 (-OCH_3), 66.6 (C6), 78.6 (C11a), 96.9 (C10), 103.7 (C4), 106.4 (C8), 109.8 (C2), 112.6 (C11b), 119.1 (C6b), 124.8 (C7), 132.2 (C1), 156.7 (C4a), 158.1 (C3), 160.7 (C10a), 161.1 (C9).

Compound 8. Compound 1 (200 mg, 0.704 mmol), dissolved in 10 mL of a mixture of DCM:2-mercaptoethanol (1:1), was treated with aluminum chloride (60 mg). The reaction mixture was kept under stirring at room temperature for 24 h. Subsequently, water (50 mL) was added, and the resulting mixture was extracted with DCM (3 × 50 mL). The combined organic phases were dried (anhydrous Na_2SO_4) and the solvent was evaporated on a rotary evaporator. The resulting residue was fractionated by column chromatography, from which a colorless viscous oil (95 mg; 0.262 mmol, 37.2%) corresponding to compound 8 was obtained. *2′-hydroxy-4-(2-hydroxyethylsulfanyl)-7,4′-dimethoxyisoflavan* (8). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.33 (1H, d, J = 8.4, H5), 7.13 (1H, d, J = 8.1, H6′), 6.54 (1H, dd, J = 8.4, 2.5, H6), 6.45–6.41 (3H, m, H8, H5′,H3′), 4.63 (1H, dd, J = 11.4, 3.4, H2eq), 4.38 (1H, m, H2ax), 4.23 (1H, d, J = 5.1, H4), 3.81–3.77 (8H, m, 2x-OCH_3, H2″), 3.66 (1H, m, H3), 2.93–2.77 (1H, m, H1″). ^{13}C NMR (75 MHz, $CDCl_3$): δ 33.6 (C1″), 37.4 (C3), 44.3 (C4), 55.3 (2 x –OCH_3), 61.4 (C2″), 66.9 (C2), 101.7 (C8), 102.3 (C3′), 105.9 (C5′), 108.3 (C6), 113.3 (C4a), 119.7 (C1′), 128.5 (C6′), 131.8 (C5), 154.5 (C2′), 155.8 (C8a), 159.5 (C7), 159.8 (C4′).

Compound 9. A mixture of acetone (5 mL) and K_2CO_3 (0.4 g) was stirred for 30 min. Then, compound 8 (60 mg, 0.16 mmol) was added and stirred at room temperature for 30 min. Subsequently, CH_3I (180 μL) was added, and the reaction mixture was kept stirred for 24 h. The reaction was finished with water (50 mL) and extracted with DCM (3 × 50 mL). The combined organic phases were dried (anhydrous Na_2SO_4), filtered and the solvent was removed by rotary evaporation under reduced pressure. The resulting residue was fractionated by silica gel column chromatography to obtain 9 (17 mg, 0.045 mmol, 28.1%) as a colorless viscous oil. *4-(2-hydroxyethylsulfanyl)-7,4′,2′-trimethoxyisoflavan* (9): ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.29 (1H, d, J = 8.4, H5),7.16 (1H, d, J = 8.2, H6′), 6.53 (1H, dd, J = 8.5, 2.5, H6), 6.42–6.39 (3H, m, H8, H5′, H3′), 4.56 (1H, dd, J = 11.4, 3.4, H2eq), 4.40 (1H, m, H2ax), 4.19 (1H, d, J = 5.1, H4), 3.88–3.80 (11H, m, 3x-OCH_3, H2″), 3.64 (1H, m, H3), 3.06–2.79 (1H, m, H1″). ^{13}C NMR (75 MHz, $CDCl_3$): δ 34.3 (C1″), 37.1 (C3), 43.6 (C4), 55.3 (3x-OCH_3), 60.9 (C2″), 66.1 (C2), 98.8 (C8), 101.2 (C3′), 104.1 (C5′), 108.2 (C6), 113.2 (C4a), 121.1 (C1′), 128.3 (C6′), 131.9 (C5), 155.6 (C2′), 157.7 (C8a), 159.9 (C7), 159.9 (C4′).

2.3. Antifungal activity

2.3.1. Inhibition of mycelial growth

Inhibition of mycelial growth of *C. gloeosporioides* and *C. lindemuthianum* was determined by the poisoned food technique [16]. Different concentrations (35.2, 88.0, 176.0, 352.0, and 704.0 μM) of all compounds 1–9 dissolved in DMSO were obtained in Petri dishes (9 cm) with Potato Dextrose Agar (PDA). The mycelial growth was measured every 24 h for 7 days. For *C. lindemuthianum*, the oat-oat culture medium (2% oats, 1.8% Agar-Agar, Scharlau Chemie, Barcelona, Spain) was used and measurements were made every 48 h for 18 days. The plates prepared without compounds 1–9 or fungicides (thymol/carbendazim) served as negative control (alone pathogen); all treatments were performed in triplicate. Growth inhibition was calculated as inhibition percentage of radial growth relative to the negative control. The results are shown as mean values of colony diameters (±SD). The inhibition percentage of radial growth was calculated by the formula: I% = {1–[T/C]}x100, where, I% = Inhibition, C = average radial growth of control (mm) y T = average radial growth of treatment (mm). In addition, the concentration that inhibits 50% fungal mycelia growth (IC_{50}) was determined according to Rivillas-Acevedo and Soriano-García [18].

2.3.2. Inhibition of spore germination

The antifungal activity of compounds 1–9 against C. gloeosporioides was also evaluated through the inhibition percentage of spore germination. Initially, spores of C. gloeosporioides were collected from a Petri dish culture of 9.0 cm in diameter, PDA medium, and 7 days of incubation. Next, 20 mL of sterile water was added and a repetitive sweep with a sterile cotton swab was performed to solubilize the largest number of spores. The solution of mycelium and spores was filtered with sterile gauze and diluted with water type I until reaching a concentration of 3 × 105 spores/mL. Subsequently, 1 mL of the spore solution and 10.56 μmol of the compound to be tested (dissolved in 15 μL of DMSO and 0.5 mL of 9% PDA) were added to Eppendorf tubes (2 mL) and homogenized. The medium of germination reached a concentration of 3% PDA and 1% DMSO which did not inhibit the development of the spores. Finally, in a Neubauer chamber, the number of spores that developed a germinative tube greater than twice the radius was counted, in a range of 8–24 h [19]. The results were expressed as inhibition percentage of spore germination (IG%), which was calculated using the formula: IG% = (EG-CCo)/(EG) x100%, where EG: Percent of germinated spores on the control (germinated spores/total spores x 100) and CCo:

Percent of germinated spores on treatments (germinated spores/total spores x 100).

2.3.3. Inhibition of infection by C. gloeosporioides in postharvest fruits

The antifungal activity of the most promising compound was evaluated *in vivo* by the growth of *C. gloeosporioides* in the peel of papaya (*C. papaya* cv. Hawaiana) and mango (*Mangifera indica* L. cv. Hilacha) fruits. Fruits with maturity index 4 [20], size and uniform color, free from physical damage and fungal infections were acquired from a local market. The fruits (3 fruits per treatment) were washed with distilled water, disinfected with sodium hypochlorite (1%), and dried at room temperature. Then, in the middle part of the fruit, an incision of 6 mm in diameter and 2 mm deep was made with a sterile steel punch. In this hole was located the mycelium of *C. gloeosporioides* with the same dimensions and from a Petri dish culture in a PDA medium seven days old. The fruits were stored for 12 h in sealed plastic containers, 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 derivative and positive controls at 1% in EtOH, using a fine brush. The commercial fungicide carbendazim and the metabolite thymol were used as positive controls. All the experiments were performed in triplicate [21]. Finally, the fruits were stored in plastic containers at room temperature (24 ± 2 °C) and the incidence was evaluated by measuring the diameter (mm) of the infection caused by the fungus at 7 and 15 days for papaya and mango, respectively.

2.4. Statistical analysis

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

3. Results and discussion

3.1. Preparation of compounds 2–9 from homopterocarpin

Eight compounds, obtained from 1, were purified by column chromatography and characterized by NMR techniques. Three nitro derivatives with different substitution patterns, mono-, di- and trisubstituted, corresponding to derivatives 2, 3, and 4, respectively, were obtained by reaction between homopterocarpin and HNO_3/H_2SO_4. It was only possible to reduce compound 3 to obtain the diamine compound 5 using $SnCl_2$; compounds 2 and 4 were not reduced under the conditions evaluated. ^{1}H NMR of compounds 2, 3, 4, and 5 exhibited characteristic signals (a typical ABMX spin system) for H6ec (δ_H: approx. 4.5, dd, J = approx. 10.0 and 4.5), H6ax (δ_H: approx. 3.8, m), H-6a (δ_H: 3.7, m) and H11a (δ_H: 5.7, d, J = approx. 7.0) of a pterocarpan skeleton. Also, compound 2 showed an AMX system in the A ring [H1 (δ_H: 7.44, d, $J = 8.4$), H2 (δ_H: 6.70, dd, $J = 8.4, 2.1$), and H4 (δ_H: 6.52, d, $J = 2.1$)], and an AX system in the D ring (two singlets at δ_H: 8.01 and 6.57 for H7 and H10, respectively). For compound 3, two AX systems were observed for A (two singlet signals at δ_H: 8.27 and 6.60 for H1 and H4, respectively), and D (two singlet signals at δ_H: 8.03 and 6.64 for H7 and H10) rings. The ^{1}H and ^{13}C NMR spectra of 5 were close to those of 3 except that a nitro group was replaced by an amino group. Thus, the AX signals patron found in rings A and D of 3 were also observed for compound 5; although proton signals suffered a small upfield shift. Additionally, the oxidation of 1 with DDQ produced the coumestan 6 in a yield of 22.7%. The disappearance of the aliphatic proton signals and the appearance of an oxygenated carbon confirm the oxidation of (1). ^{13}C NMR of compound 6 exhibited signals to δ_C 162.6 (C6), 106.2 (C6a), and 159.3 (C11a) indicating this compound to be a coumestan derivative [22]. Treatment of compound 1 with concentrated hydrochloric acid resulted in the selective demethylation of aromatic methoxyl present in C3 to produce the mono-methoxylated pterocarpan 7. The deprotection of the methoxyl group in position 9 was not possible. Additionally, compound 1 was subjected to methoxy group deprotection reactions using $AlCl_3$ in the presence of 2-mercaptoethanol [23]; however, the substrate underwent opening of the furan ring and the addition of 2-mercaptoethanol to produce the isoflavan derivative 8. It was found a spin coupling system ABMX in the ^{1}H NMR spectrum; δ_H: 4.63 (dd, $J = 11.4, 3.4$, H2eq), 4.38 (m, H2ax), 4.23 (d, $J = 5.1$, H4), and 3.66 (1H, m, H3) assigned to the –SCHCH(Ar)CH_2O– moiety of C ring of a 4-S-alkylsustituted isoflavan skeleton. This skeleton was further supported by ^{13}C NMR and HMQC data, which showed peaks for C2, C3, and C4 at δ_C 66.9 (C2–O-substituted), 37.4, and 44.3 (C4–S), respectively. Also, a spin coupling system A_2X_2 was observed in the ^{1}H NMR spectrum; δ_H: 3.81–3.77 (H2″) and 2.93 (H1″). ^{1}H–^{13}C HSQC and DEPT-135 spectra indicated that the chemical shifts of the corresponding carbons were 61.4 (C2″, methylene group) and 33.6 (C1″, methylene group). HMBC correlations of the proton H4 at δ_H: 4.23 with δ_C: 33.6 (C1″) along with correlations of the carbon C4 (δ_C: 44.3) with δ_H: 2.93 (H1″) permitted the placement of the 2-hydroxyethylsulfanyl substituent at C4. Also, compound 8 showed two AMX systems. Compound 8 was therefore identified as 2′-hydroxy-4-(2-hydroxyethylsulfanyl)-7,4′-dimethoxyisoflavan. Thereafter, the phenolic hydroxyl group of the isoflavan derivative 8 was methylated (*O*-alkylation) with CH_3I/K_2CO_3 to obtain the trimethoxy isoflavan derivative 9. The ^{13}C and ^{1}H NMR spectra of 9 resembled those of 8, except that an additional methoxy group was evidenced. The structure of compound 9 was completely assigned based on COSY, HMQC, and HMBC correlations. To the best of our knowledge, compounds 8 and 9 have not been previously reported in the literature.

3.2. Antifungal activity of the compounds

3.2.1. Inhibition of mycelial growth

Thymol and carbendazim were used as a positive control. They showed a strong antifungal activity, which was concentration and time dependent. The positive control carbendazim, a commercial broad-spectrum carbamate fungicide, showed 100% growth inhibition even at the lowest concentration tested (35.2 μM) throughout the evaluation period (*Data not shown*). On the other hand, it is

observed that thymol at 352 and 704 μM, inhibits about 90–100% of the growth of *C. lindemuthianum* (Fig. 2A) and *C. gloeosporioides* (Fig. 2B) during the evaluation period. Thymol is a recognized antifungal compound against species of the genus *Colletotrichum* [24]. Some studies propose that the free hydroxyl group of thymol is essential for its antimicrobial activity; thymol inserts into the cytoplasmic membrane affecting bilayer stability, resulting in increased passive proton flux across the membrane [25,26].

The results of the antifungal activity of compounds 2–9 against *C. lindemuthianum* and *C. gloeosporioides* are presented in Fig. 3, Fig. 4, and Fig. 5, respectively. In general, antifungal activity depended on the concentration and structure of the derivative, and on the time; the antifungal activity decreased with increasing time, suggesting a possible mechanism of detoxification of the medium by the fungi. It was evidenced that the addition of nitro groups in the structure of 1 generates derivatives with less antifungal activity. The percentage of mycelial growth inhibition by the nitro-derivatives 2, 3, and 4 at 704 μM, presented values against *C. lindemuthianum* during 48–384 h of 82.0 to 25.2%, 44.1 to 24.1%, 47.2 to 15.3% respectively (Fig. 3B, C and D); these values were lower than those exhibited by 1, 67.0 to 35.0%, under the same conditions (Fig. 3A). In the same way, these derivatives showed a lower growth inhibition range against *C. gloeosporioides* (18.6–21.4 for 2, Fig. 4B; 28.2 and 21.1 for 3, Fig. 4C; and 33.1 to 16.2% for 4, Fig. 4D) compared to 1 (71.0–36.1%, Fig. 4A) in the period from 24 to 192 h. It should be noted that the decrease in antifungal activity was correlated with the increase in the number of nitro substituents in the structure of the pterocarpan derivative. Additionally, the diamino derivative 5, obtained by reduction of 3, also presented low activity with inhibitions at 704 μM ranging between 28.1 to 13.7% and 75.2 to 22.7% for C. *gloeosporioides* (Fig. 4E) and *C. lindemuthianum* (Fig. 3E), respectively. These values were lower than those presented by 1, which may suggest a decreasing effect of the biological activity by the nitrogen-containing substituents (both electron-donor as the $–NH_2$ group, and electron-withdrawing as the $–NO_2$ group). The presence of these substituents, which increase the hydrophilicity and reduce effective penetration of fungal membranes, added to the lack of free phenolic OH groups, could explain the low fungitoxicity of the compounds 2, 3, 4, and 5 [27].

The derivative 6, a coumestan generated by the oxidation of 1 with DDQ, showed the lowest percentage of activity against the two fungi evaluated. Between 24 and 192 h, compound 6 at 704 μM displayed inhibition ranges between 28.6 and 4.9% (from 24 to 192 h) against *C. gloeosporioides* (Fig. 4F); and between 43.7 and 15.9% (from 48 to 348 h) against *C. lindemuthianum* (Fig. 3F). For both fungi, derivative 6 showed a quick decrease in the inhibition of mycelial growth over time. In particular, compound 6 showed low solubility in ethanol and has a flat three-dimensional structure, different from 1 and the other pterocarpan and isoflavan derivatives, which could explain the low antifungal activity [28]. It is important to note that compound 7, which, unlike 1, has an aromatic hydroxyl group at the C3 position, has reached the highest inhibition values, mainly against *C. lindemuthianum* where the inhibition was nearly maintained over time between 100 and 85% (from 48 to 348 h) (Fig. 3G). This fact is very important since, in addition to the high antifungal activity of 7, there is a low detoxification by *C. lindemuthianum* towards 7 compared with the other derivatives. Remarkably, 7 also strongly affected the growth of *C. gloeosporioides* during the first 48 h, reaching 100% growth inhibitions (Fig. 4G). However, the activity drops rapidly to values of 48% after 192 h. Bandara et al. [29] reported that compound 7, known as medicarpin, presented greater activity than the commercial fungicide Benlate against *Cladosporium cladosporioides,* and established the influence of the hydroxyl group on the antifungal activity through its acetylation, where the 3-acetoxy-9-methoxy-pterocarpan derivative exhibited less inhibition of the fungus than medicarpin. The presence of the phenolic hydroxyl group could be related to damage to the integrity of the cytoplasmic membrane in fungi, in a similar way as has been proposed for thymol and related compounds [26]. It should be noted that medicarpin has been reported as an antimicrobial compound produced in response to an infection (a phytoalexin) of the Fabaceae family [30,31], particularly in plants attacked by *C. lindemuthianum* [32,33]. In addition, this compound inhibits the

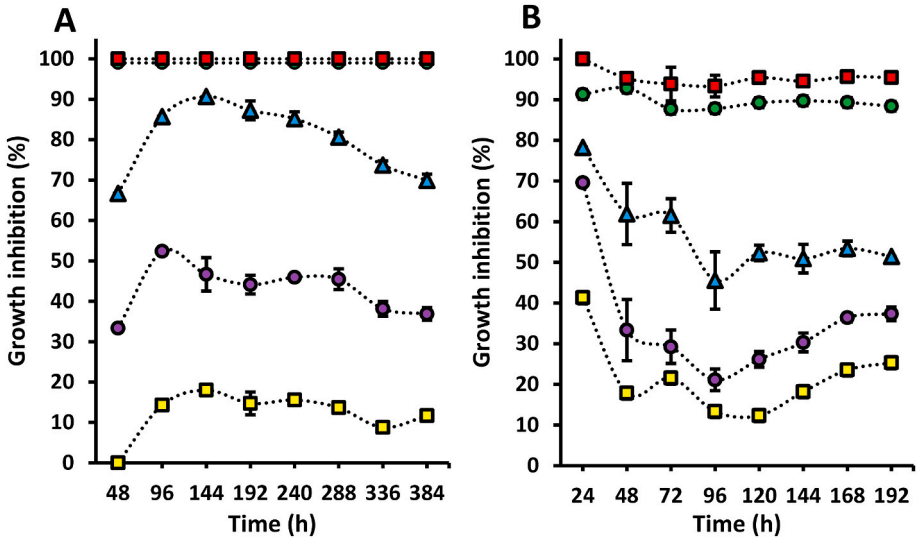

Fig. 2. Effect of thymol at 35.2 (···□···); 88.0 (···●···); 176.0 (···▲···); 352.0 (···●···) and 704.0 μM (···■···) on mycelial growth of *C. lindemuthianum* (A) and *C. gloeosporioides* (B).

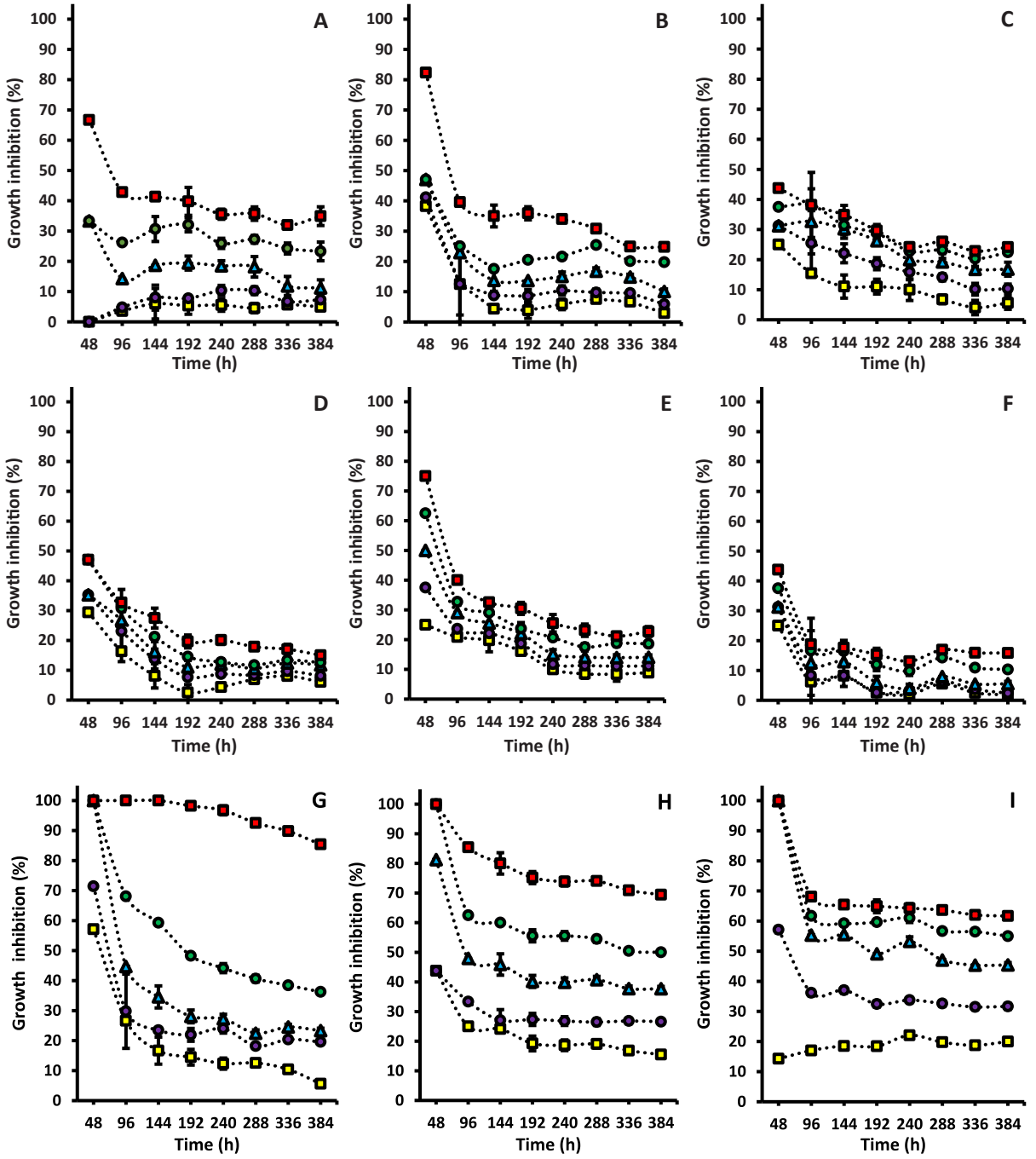

Fig. 3. Mycelial growth inhibition (%) of *C. lindemuthianum* with the compounds: 1 (panel A), 2 (panel B), 3 (panel C), 4 (panel D), 5 (panel E), 6 (panel F), 7 (panel G), 8 (panel H) and 9 (panel I) at 35.2 (···□···), 88.0 (···●···), 176.0 (···▲···), 352.0 (···●···), and 704.0 μM (···■···).

mycelium growth of *Lenzites trabea* and *Coriolus versicolor* [7] and the germination of spores of *F. oxysporum* in *C. arietium* (chickpeas) [34].

Compound 8, an isoflavan with a hydroxyl group at C2′ and a 2-hydroxyethylsulfanyl substituent at C4, exhibited better solubility in ethanol than 1 and considerable antifungal activity. Growth inhibitions of *C. gloeosporioides* and *C. lindemuthianum* were 75.1 to 63.2% (at 24 and 192 h, Figs. 4H) and 100 to 69.4% (at 48 and 384 h, Fig. 3H), respectively. Interestingly, antifungal activity displayed by 8 was like that found in medicarpin and higher than that exhibited by 1. *O*-alkylation (methylation) of 8 at the hydroxyl in C2' position yield 9; it was found that 9 showed growth inhibitions of *C. gloeosporioides* and *C. lindemuthianum* between 70.2 and 50.2% (at 24 and 192 h, Fig. 4I) and between 100.0 and 61.7% (at 48 and 384 h, Fig. 3I), respectively. The antifungal activity of 9 was slightly

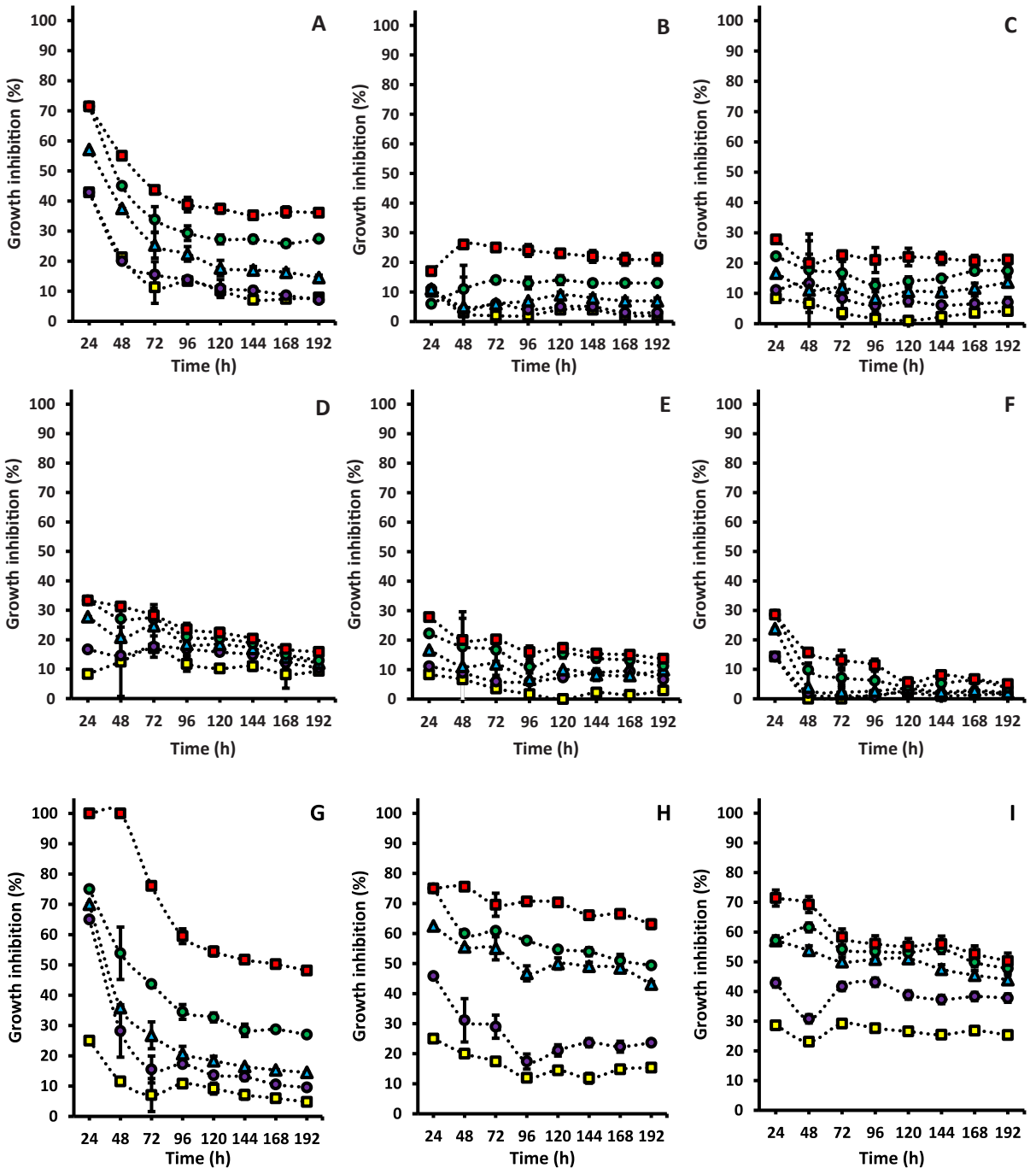

Fig. 4. Mycelial growth inhibition (%) of *C. gloeosporioides* with the compounds: 1 (panel A), 2 (panel B), 3 (panel C), 4 (panel D), 5 (panel E), 6 (panel F), 7 (panel G), 8 (panel H) and 9 (panel I) at 35.2 (···□···), 88.0 (···●···), 176.0 (···▲···), 352.0 (···●···), and 704.0 μM (···■···).

lower than that observed for the hydroxylated precursor 8; it appears that *O*-alkylation reduces the antifungal activity of the derivatives. In general, the fungistatic activity of 8 and 9 was almost constant between 96 and 384 h for *C. lindemuthianum*, and between 72 and 192 h for *C. gloeosporioides*, indicating weak detoxification of the compounds by the fungi.

The biological activity reported for homopterocarpin and derivatives is scarce; nevertheless, homopterocarpin and the structurally related compounds, pterocarpan, hydroxyhomopterocarpin, metoxyhomopterocarpin, and isomedicarpin showed good antifeedant activity against the subterranean termite *Reticulitermes speratus* [35]. Kakuda et al. [36] synthesized pterocarpan derivatives and evaluated the inhibitory effect on microbial growth and biofilms of various microorganisms; the authors found that azamedicarpin HCL salt had an inhibitory effect against microbial growth and Candida biofilm.

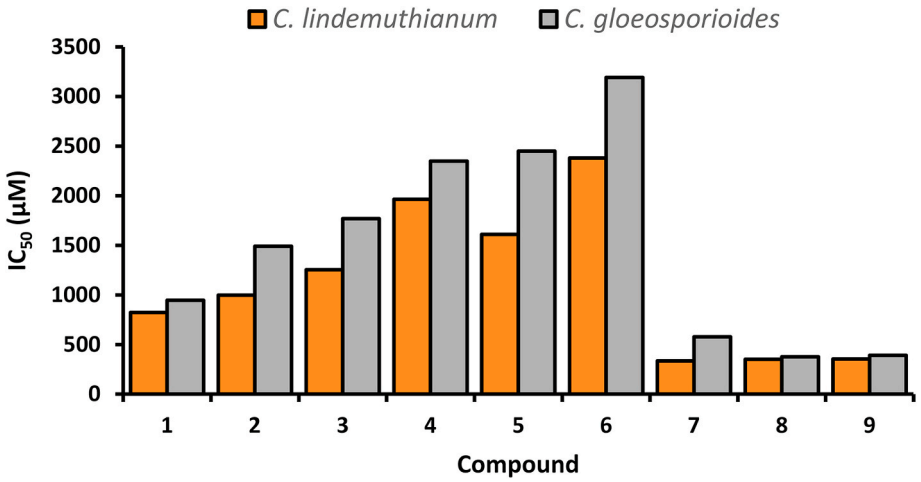

Fig. 5. IC_{50} values of the derivatives 1–9 against C. lindemuthianum and C. gloeosporioides at 120 and 96 h, respectively.

After analyzing the inhibition of both fungi over time, IC_{50} values at a fixed time (120 and 96 h for *C. lindemuthianum* and C. *gloeosporiodes*, respectively) were chosen to compare the antifungal activity of all derivatives (Fig. 5). IC_{50} values of compounds 2 and 3 were between 1000 and 1800 μM for the two fungi, indicating weak antifungal activity. Compounds 4, 5, and 6 exhibited IC_{50} values ranging between 1900 and 3200 μM, being almost inactive. For compound 1, IC_{50} values were 823.1 and 946.1 μM for *C. lindemuthianum* and *C. gloeosporioides*, respectively. Taking as reference the growth inhibition of the fungi by the starting compound 1, the derivatives 2, 3, 4, 5, and 6 presented lower antifungal activity. In general, the presence of the –nitro and -amine groups, and the oxidation of the pterocarpan 1 to the coumestan 6, had a negative effect on the inhibitory activity against fungi. In contrast, compounds 7, 8, and 9 displayed a higher mycelial growth inhibition of fungi than 1. IC_{50} values for compound 7 were 335.4 and 577.3 μM against *C. lindemuthianum* and *C. gloeosporioides*, respectively. It is important to highlight that compound 7 corresponds to the phytoalexin medicarpin, a pterocarpan with strong antifungal activity. Interestingly, IC_{50} values against *C. lindemuthianum* and *C. gloeosporioides* were respectively 352.1 and 375.9 μM for compound 8 and 354.5 and 392.0 μM for compound 9, being similar and even lower than that observed for medicarpin 7. On the other hand, derivatives having phenolic hydroxyl groups in the structure or the isoflavan core exhibited the highest antifungal activity and water solubility.

3.2.2. Inhibition of spore germination

The fungus C. lindemuthianum did not sporulate under laboratory conditions; consequently, it was only possible to evaluate the effect of compounds 1 to 9 on the sporulation of C. gloeosporioides. The spore germination inhibition percentages (% IG) obtained for C. gloeosporioides are shown in Fig. 6. During the first 8 h of evaluation, all spores germinated on the negative control and solvent control (DMSO). Percentages of inhibition of fungal spore germination (%IG) for the positive controls (thymol and carbendazim) were 100%. Remarkably, compounds 7 and 8 exhibited complete inhibition of spore germination of C. gloeosporioides. In addition, thymol, carbendazim, and the isoflavan compounds 7 y 8 retained the antifungal effect up to 24 h of evaluation. Furthermore, derivative 9 and compound 1 exhibited inhibition values of 72.8% and 41.9%, respectively; however, the inhibition capacity of 9 drastically decreased (1.4%) at 24 h. The other derivatives (2–6) showed low inhibitions of spore germination at 8 h (%IG between 32.7 and 8.4%) and subsequently decreased drastically (between 9.8% and 0.7%) at 24 h. It is noteworthy that the spore germination inhibitory activity of compounds 7 and 8 was comparable to that of thymol, a recognized natural antifungal, and carbendazim, a commercial synthetic fungicide. Thymol and carbendazim have been reported as highly toxic to C. gloeosporioides [24,37].

3.2.3. Inhibition of infection by C. gloeosporioides in postharvest fruits

Among the synthesized derivatives, compound 8 exhibited the highest *in vitro* antifungal properties against *C. gloeosporioides;* therefore, it was selected to evaluate the effectiveness to control the fungus in an *in vivo* model. Papaya (*Carica papaya* L. cv. Hawaiana) and mango (*Mangifera indica* L. cv. Hilacha) fruits were inoculated with the mycelium of *C. gloeosporioides*, and then, impregnated with the compounds 1, 8 and the positive controls (thymol and carbendazim) at 1% in EtOH, using a fine brush. The growth and depth of the lesion are presented in Figs. 7 and 8, respectively. In general, the progression of the lesion on the fruit surface was less in the treatment with carbendazim (Fig. 7C) followed by compound 8, thymol, and 1 (Fig. 7F, C, and E, respectively). It was observed that compound 8 decreased the diameter of the lesion by 55.4%, a value higher than that obtained for the antifungal compound thymol (34.1%) and 1 (14.6%). It is important to highlight the higher activity of compound 8 compared to thymol and compound 1. The commercial fungicide carbendazim was the most effective of the treatments, with a reduction in the growth of *C. gloeosporioides* of 82.9%.

On the other hand, the internal tissue in the infected fruit area showed a grayish-white discoloration that was deeper in the negative controls (absolute and solvent; Fig. 8A and B, respectively) than in the treatments, as shown in Fig. 8. Compared with controls, the progression of the lesion in the fruit parenchyma was lower when applying carbendazim (Fig. 8C) and compound 8 (Fig. 8F), with mean lesion depth values of 3 and 9 mm, respectively. The fruits treated with thymol (Fig. 8D) and compound 1 (Fig. 8E) presented

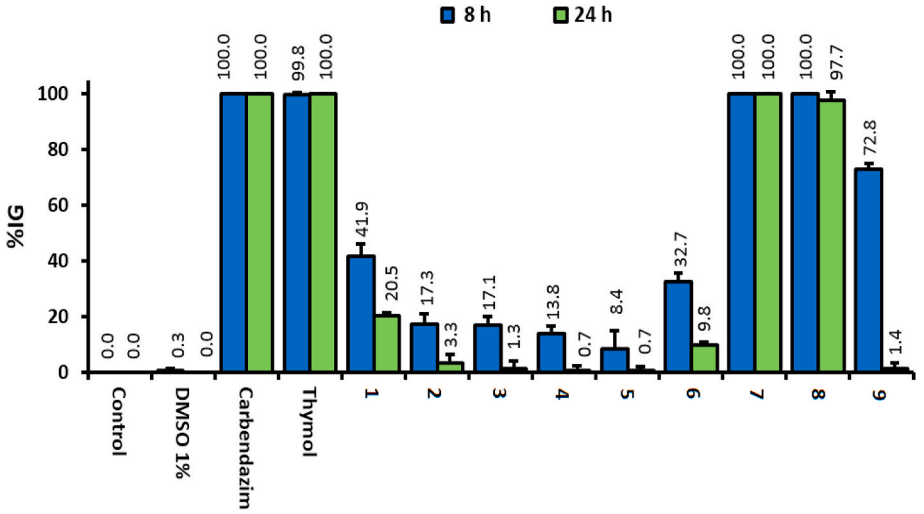

Fig. 6. Spore germination inhibition (%IG) of *C. gloeosporioides* by the compounds 1 to 9 at 704 μM.

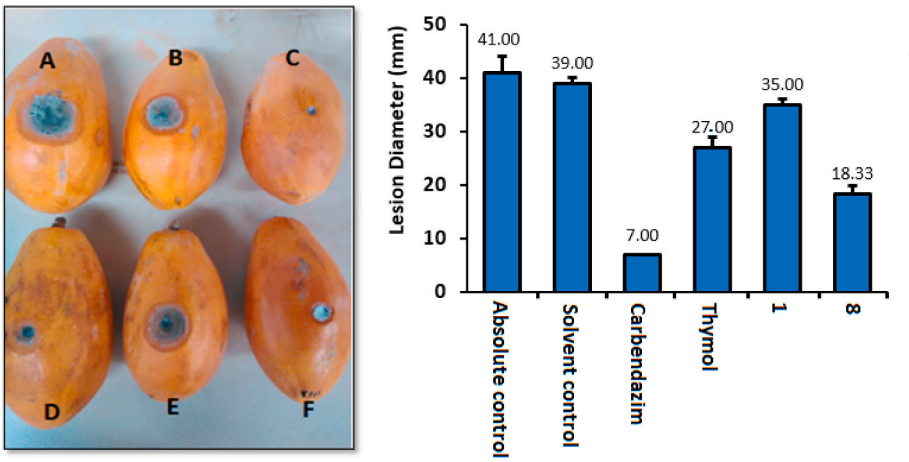

Fig. 7. Growth of the lesion caused by *C. gloeosporioides* in papaya (*C. papaya* L. cv. Hawaiian) fruits on day 7 after inoculation. Treatments: A, absolute control; B, solvent control; C, carbendazim; D, thymol; E, compound 1; and F, compound 8.

lesions of 12 and 17 mm. In general, compound 8 exhibited relatively high activity against *C. gloeosporioides* infecting papaya and low phytotoxicity on the fruit skin.

Finally, the results of the antifungal activity *in vivo*, against *C. gloesporioides* in mango (*M. indica* L. cv. Hilacha), did not show a homogeneous radial growth that would allow inhibition to be quantified based on the diameter of the lesion, but it was possible to qualitatively observe different levels of involvement by *C. gloeosporioides* (Fig. 9). Visually, the least damage was observed in mango fruits treated with carbendazim (Fig. 9D), followed by compound 8, thymol, and compound 1 (Fig. 9F, C, and E respectively).

4. Conclusions

Eight compounds were obtained from homopterocarpin (1): five pterocarpans (2, 3, 4, 5, and 7), one coumestan (6), and two isoflavans (7 and 8). The oxidation and introduction of nitro and amino groups in the aromatic rings of homopterocarpin (1) had negative effects on the antifungal activity against *C. gloeosporioides* and *C. lindemuthianum*; the increase in the number of nitro groups, compounds 3 and 4, decreased, even more, the antifungal activity compared to homopterocarpin (1). On the other hand, compounds 7, 8, and 9 showed greater inhibitory activity against *C. gloeosporioides* and *C. lindemuthianum* than homopterocarpin (1). Compound 7, a well-known antifungal compound called medicarpin, displayed higher fungistatic activity than 1, which demonstrates that the phenolic hydroxyl group has a positive influence on antifungal activity. It should be noted that the conversion of pterocarpan 1 to isoflavan 8 involved an important structural modification for the improvement of antifungal activity. As a result, compound 8 exhibited strong *in vitro* and *in vivo* activity against *C. lindemuthianum* and *C. gloeosporioides*, inhibiting mycelial growth and spore

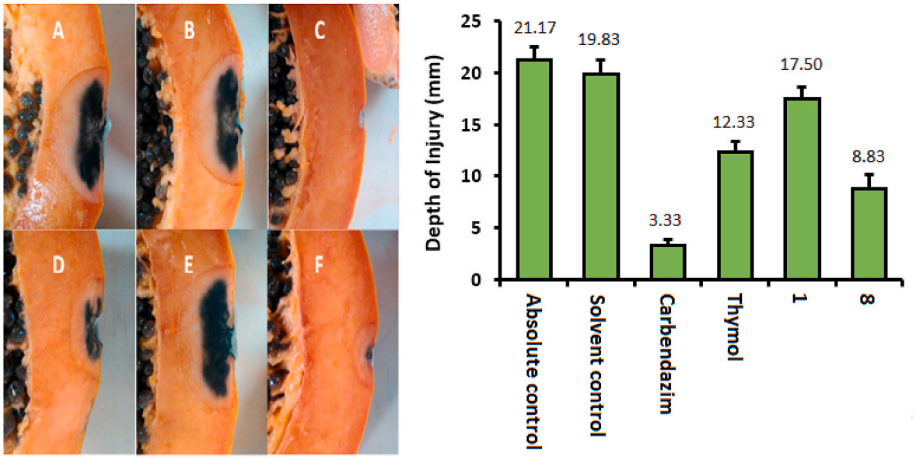

Fig. 8. Depth of lesion in *C. papaya* caused by *C. gloeosporioides* seven days after inoculation. Treatments: A, absolute control; B, solvent control; C, carbendazim; D, thymol; E, compound 1; and F, compound 8.

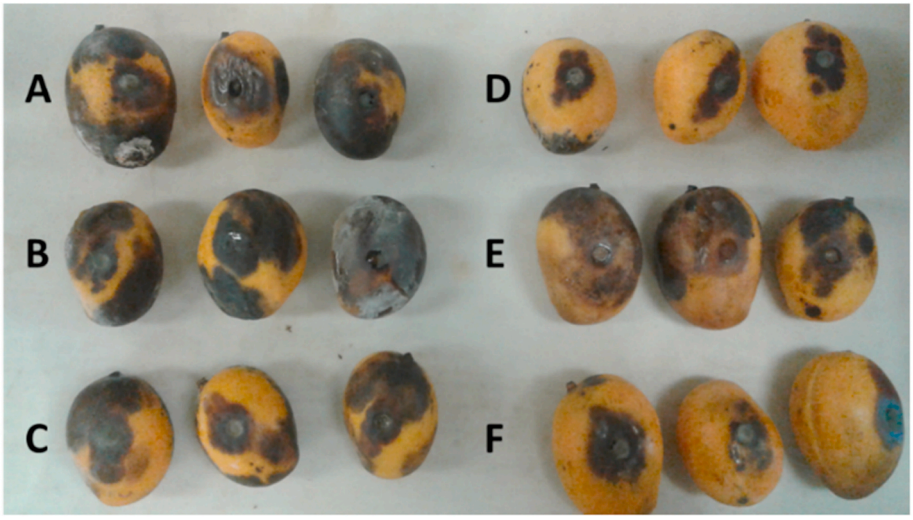

Fig. 9. Growth of the lesion in mango (*M. indica* L. cv. Hilacha) fruits caused by *C. gloeosporioides* 15 days after inoculation. Treatments: A, absolute control; B, solvent control; C, thymol; D, carbendazim; E, compound 1; and F, compound 8.

germination, and delaying the development of the fungus *C. gloeosporioides* in artificially inoculated papaya and mango fruits.

Author contribution statement

Janio Martinez: Performed the experiments; Wrote the paper.
Cesar Ramirez: Performed the experiments.
Jesús Gil, Diego Durango: Contributed reagents and materials; Analyzed and interpreted the data; Wrote the paper.
Winston Quiñones: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no competing interests.

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