1. Introduction

1-H-Phenalen-1-ones (perinaphthenones or phenalenones) are a class of compounds with a system of three fused, six-membered rings (Figure 1). Perinaphthenone is the basis of many antimicrobial secondary metabolites isolated from leaves, fruits, and rhizomes of banana and plantain (Musaceae), as well as related natural products from Strelitziaceae, Pontederiaceae, and Haemodoraceae (DellaGreca et al., 2008; Echeverri et al., 2012; Holscher and Schneider, 2000, 2005; Opitz and Schneider, 2002). Natural perinaphthenones, as well as their derivatives and structural analogs, have exhibited a wide array of biological properties, such as antifungal, antiparasitic, antiprotozoal and radical scavenging capacity (Duque et al., 2013; Freijo et al., 2018; Gutierrez et al., 2015; Rosquete et al., 2010). In addition, photophysical and singlet oxygen photosensitization properties of phenalenones have been widely recognized (Sandoval-Altamirano et al., 2018; Flors and Nonell, 2006). As a result, phenalenones have shown toxicity against larvae of *Aedes albopictus* and the nematode *Meloidogyne incognita* in the presence of light, while the toxic activity was reduced in the dark (Song et al., 2017). Besides, dental drugs based on phenalenone derivatives have been used for the photodynamic inactivation of oral bacteria (Spath et al., 2014).

In the Musaceae family, these compounds act as phytoalexins; antimicrobial protective phytochemicals that are synthesized in response to infections by pathogens (for example, *Mycosphaerella fijiensis*, a causative agent of the disease known as Black Sigatoka), nematodes (*Holscher et al.*, 2014, 2016), mechanical damage and chemical treatments (such as aminoglycosides) (Echeverri et al., 1986). For the above, phenalenones have attracted the attention of scientists from various fields, who are searching new potential agents for pest control. In this sense, it has been reported that 9-phenyl and 2-hydroxy substituted phenalenone analogs have potential activity against cowpea aphids (Zhang et al., 2017). In addition, studies on the relationship between structure and activity have shown that the compounds 2-hydroxy-1H-phenalen-1-one and...
2-methoxy-1H-phenalen-1-one exhibited significantly improved activity against *M. fijiensis* in comparison with the phenalenones containing the phenyl groups (Otalvaro et al., 2007), demonstrating that the fungistatic effect can be modulated by permutation of the substituents on the perinaphthenone moiety.

On the other hand, Citrus are one of the most important fruit crops in the world. Citrus includes sweet oranges, mandarins (including clementine and tangerine), grapefruit (including pummelo), and lemons/limes (Dugrand et al., 2013). In 2018/19, the global production of citrus fruits was 47.6 million metric tons (tons) of oranges, 31.4 million tons of tangerines/mandarins, 7.0 million tons of grapefruit, and 8.2 million tons of lemons/limes (USDA/FASS, 2020). Unfortunately, citrus production is largely limited by pathogenic fungi. *Fusarium* spp., *Botrytis* spp., and *Botryodiplodia* spp. cause vascular, foliar, fruit, trunk and root diseases in citrus crops resulting in massive economic losses. *Botrytis* and *Fusarium* were included in the top 10 globally most important genera of plant pathogenic fungi, based on perceived scientific and economic importance (Dean et al., 2012). Particularly, *Fusarium* species have been associated with the major diseases of citrus, such as dry root rot, root rot, feeder root rot, wilt, twig dieback and citrus decline (Spina et al., 2008). In addition, with the major diseases of citrus, such as dry root rot, root rot, feeder root rot, wilt, twig dieback and citrus decline (Spina et al., 2008). In addition, many species are mycoxenogenic and cause human and animal diseases (O’Donnell et al., 2016). In the present paper, the inhibitory effect of perinaphthenone (1) against these three fungi isolated from *Citrus latifolia* Tanaka, along with its metabolism, were studied. Additionally, the effect of some substituents in the perinaphthenone system on the fungistatic activity against *Botryodiplodia* spp. was evaluated.

2. Materials and methods

2.1. Biological and chemical materials

The pythiopathogenic fungi *Botryodiplodia* spp. (voucher L. Cañasto-CL01), *Botrytis* spp. (voucher L. Cañasto-CL12) and *Fusarium* spp. (voucher L. Cañasto-CL14) were isolated from Lima Tahiti (*Citrus latifolia* Tanaka ex Yu. Tanaka, Tanaka) showing stem-end rot, grey mold, and root rot disease symptoms. Isolates were morphologically identified and kindly donated by the Laboratory of Phytopathology-National University of Colombia (Medellín). Fungi were kept in Potato Dextrose Agar (PDA) medium (24 ± 2 °C) and subcultured monthly in Petri dishes.

In order to evaluate the antifungal activity, sterile Petri dishes of 9 cm in diameter were inoculated with a mycelial suspension of the fungi and incubated at 25 °C for 48 h. A 6 mm-diameter mycelial disc was used for the fungitoxicity test. 1H-phenalen-1-one (perinaphthenone, 1) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Media ingredients were acquired from Oxoid Ltd. (Hampshire, England), Merck KGaA (Darmstadt, Germany), and Sigma-Aldrich (St. Louis, MO, USA).

2.2. Analytical methods

Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F254 (0.25 mm thick) plates. Column chromatography (CC) was performed using silica gel 60 (0.040–0.063 mm; Merck) or Sephadex® LH-20 (Sigma-Aldrich). High performance liquid chromatography (HPLC-DAD) analysis was carried out on a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan), equipped with reciprocating pumps (Shimadzu LC-20AT), degasser (DGU 20A5), integrator (CBM 20A), diode array detector (SPD-M20A) and software (LC solution 1.22 SP1). A Phenomenex Security Guard cartridge C18 (4.0 × 3.0 mm) followed by a Zorbax Eclipse Plus C18 column (4.6 mm × 150 mm, 5 μm diameter particles, Agilent, USA) were used. The compounds were eluted at a flow rate of 1.0 mL/min with the solvents A = water, and B = acetonitrile, as follows: from 80% A to 60% A in 5 min, then 60% A to 40% A in 5 min, 40% A to 20% A in 5 min, and holding for 5 min 20% A. Finally, 5 min more of conditioning of the column at the initial proportion, A = 80%, for the next injection. The injection volume was 15 μL. The 1H (300 MHz) and 13C (75 MHz) nuclear magnetic resonance (NMR) and 2D-NMR spectra were recorded on Bruker AMX 300 NMR spectrometer in CDCl3. The chemical shifts (δ) and coupling constants (J) are given in ppm and Hertz, respectively. Notations s = singlet, d = doublet, m = multiplet, and dd = double doublet, were used.

2.3. Fungitoxicity bioassays of perinaphthenone (1) and derivatives

2.3.1. Mycelial growth inhibition

2.3.1.1. Fungistatic effect. The poisoned food technique was used to investigate the toxicity of (1) against *Botryodiplodia* spp., *Botrytis* spp. and *Fusarium* spp. (El Moussaoui et al., 2019; Velasco et al., 2010). Briefly, in Petri dishes (9 cm diameter), compound (1) was dissolved in ethanol (<0.2%, v/v) and then diluted with molten PDA medium to reach concentrations of 62.5, 125, 250, 500, and 1000 μM. After complete solidification of the medium, a mycelial disk (6-mm diameter) of *Fusarium*, *Botrytis*, and *Botryodiplodia*, was placed in the center of the agar plate. The Petri dishes with the mycelial disc but containing only PDA medium, and ethanol (0.2%, v/v) in the PDA, served as absolute control and solvent control, respectively. The commercial fungicide methyl benzimidazol-2-yl carbamate (Carbendazim®) along with thymol (2-isopropyl-5-methylphenol) were used as positive controls at 250 and 1000 μM, respectively. Similarly, derivative compounds at 1000 μM were evaluated against *Botryodiplodia* spp. The Petri dishes were incubated at room temperature in diffused light and the diameter of the mycelial growth was measured every 24 h. The incubation was stopped when the mycelial mass filled Petri dishes in the absolute control experiments (ca. 3.5 days for *Botryodiplodia* spp., 10 days for *Botrytis* spp., and 11 days for *Fusarium* spp.). The radial growth inhibition of perinaphthenone (and derivatives) was calculated using the formula: Inhibition (%) = (1 - (T/C)) × 100; where, C = average colony diameter (mm) in the absolute control and T = average colony diameter (mm) in the treatment. The results are shown as mean values of three replications of colony diameters [± standard deviation (SD)].

2.3.1.2. IC50 determination. For the compound (1), the concentration that inhibits 50% fungal mycelial growth (IC50) for all fungi was preliminarily determined according to the methodology of Rivillas-Acevedo and Soriano-Garcia (2007).

2.3.1.3. Light effect in the fungitoxicity. The effect of the darkness and direct light on the *in vitro* fungitoxicity of (1) against *Fusarium* spp. was similarly determined by the poisoned food method, as follows: a concentration of 250 μM of (1) was used and the diameter of the mycelial growth was measured every 24 h during a period of 144 h. Petri dishes (3 for each experiment) were kept in the dark in a cardboard box. For the assay exposed to direct light, the Petri dishes were placed 40 cm below from 4 × 4 white light tubes (Sylvania, ES standard, 18W, Daylight 154, luminous efficacy (Lm/W) 35). Inhibition percentages and control experiments were performed as described above.

2.3.2. Spore germination inhibition

The effect of (1) on *in vitro* spore germination inhibition was determined by the technique described by Cronin et al. (1996) with some modifications. 15 μL of the desired concentration (62.5, 125, 250, and 500 μM) of (1) in DMSO (1%) was mixed with sterile liquid PDA (9.0 g/100 mL water, 0.5 mL) and placed in an Eppendorf-tube. After 2 min, 1 mL of a suspension of freshly prepared spores of *Botrytis* spp. (controlled at 3 × 107/mL using a hemocytometer) was placed in the Eppendorf-tube, covered and maintained at 25 °C. The solvent control was an Eppendorf-tube containing DMSO (1%) mixed with sterile liquid PDA. After 8 and 24 h, the spore germination was examined under an optical microscope (40 magnifications). 200 spores of four microscopic fields, in three replicated plates, were counted to determine the number of germinated spores. A spore was considered germinated when the
lengt of the germ tube was greater than the spore itself. Results were expressed in terms of the percentage of spores germinated as compared to the control, according to the following formula: Spore germination inhibition (%) = (1 - (T/C)/x100; where C = average number of spore germinated in solvent control and T = average number of spore germinated in treatment.

2.4. Metabolism of perinaphthenone

2.4.1. Preculture

Fungi were inoculated into 1.0 L Erlenmeyer flasks, containing 500 mL of Czapek-Dox liquid medium. The Erlenmeyer flasks were shaken (reciprocating shaker, 120 rpm) at room temperature for 72 h. Mycelia were harvested by filtration, washed with H2O and employed in the preparative-scale metabolism and time-course experiments.

2.4.2. Time-course experiments

Precultured Botryodiplodia spp., Botrytis spp. and Fusarium spp. were separately transferred into 250 mL Erlenmeyer medium and 25 μg/mL of (I). The fungi were cultivated at 120 rpm for 12 days. The culture medium was taken from flasks after 1, 3, 6, 9, and 12 days, extracted with CH2Cl2 and the solvent was subsequently evaporated. These extracts were analyzed by means of TLC and HPLC. Controls (without substrate) were carried out to verify the presence of similar compounds in the cultures of the fungi. The evaluations were carried out at least twice.

2.4.3. Preparative-scale metabolism

Mycelia of Fusarium spp. from a 3-day-old culture were transferred into six 1.0 L Erlenmeyer flasks containing 0.5 L of a Czapek-Dox liquid medium and 500 μM of (1) dissolved in 96% ethanol (~0.2%, v/v). Cultivation was carried out through stirring (reciprocating shaker, 120 rpm) at room temperature for 9 days. After filtering, mycelia were discarded and the culture medium was used to isolate the metabolic products.

2.4.4. Isolation and identification of metabolic products

The culture medium was extracted with CH2Cl2 (3 × 2.0 L), dried over anhydrous Na2SO4, and concentrated in vacuo. The extract was chromatographed on a silica gel column. Extracts from the metabolism (1) of (I) were eluted into fractions grouped according to TLC profiles. The first (n-hexane-CH2Cl2, 10:1) fraction was re-chromatographed over SiO2 column using n-hexane-CH2Cl2 (15:1) as eluent to yield one metabolic compound (2). The fourth fraction (n-hexane-CH2Cl2: 8:2) was chromatographed over Sephadex LH-20 column using n-hexane-CH2Cl2-methanol (2:1:1) as eluent to afford (3). The identification of these metabolites was based on the interpretation of their spectroscopic data. Metabolic product (2): UV (MeOH-H2O, 1:1) λmax (nm): 222, 286. 1H NMR: δ 7.85 (d, J = 8.1 Hz, 1H, H-6), 7.76 (d, J = 8.4 Hz, 1H, H-1), 7.62 (d, J = 6.9 Hz, 1H, H-9), 7.53 (d, J = 7.1 and 8.1 Hz, 1H, H-5), 7.47 (d, J = 7.2 and 8.4 Hz, 1H, H-8), 7.35 (d, J = 7.1 Hz, 1H, H-4), 5.17 (d, J = 4.5 and 5.7 Hz, 1H, H-1), 3.42-3.34 (m, 1H, H-3ax), 3.20-3.10 (m, 1H, H-3ec), 2.26 (m, 2H, H-2). 13C NMR: δ 137.4 (C-9a), 135.65 (C-7), 132.52 (C-6), 132.34 (C-9), 132.20 (C-6a), 131.54 (C-5), 129.20 (C-12, C-9a), 129.15 (C-5a), 128.72 (C-9a), 127.95 (C-3a), 127.56 (C-4), 126.96 (C-8), 126.74 (C-9b), 125.90 (C-2).

2.4.5. Quantification

Standard calibration curves (peak areas vs. compound concentration for different levels) were used to quantify the metabolic products. Five working solutions were prepared for each compound in methanol containing (1), (2), or (3) at 20, 40, 60, 80, and 100 mg/L. High linearity (correlation coefficient R2 > 0.96) was found for all calibration curves (correlation coefficient R2 > 0.96). Data for each peak were collected using the wavelength that provides a maximum response: 247, 286, and 240 nm for compounds (1), (2) and (3), respectively.

2.5. Preparation of derivatives

Perinaphthenone derivatives were prepared by conventional organic reactions of nitrification, bromination, chlorination, hydroxylation, methoxylation, alkylation, and dimerization (Hidalgo et al., 2009; Misaki et al., 2008; Otalvaro et al., 2007). Briefly, the nitrification of (1, 100 mg) using a mixture of nitric acid (65%) and concentrated sulfuric acid (96%) at 25 °C for 4 h gave two compounds. Then, the reaction mixture was neutralized with 10% NaOH solution and extracted with CH2Cl2. The organic phase was dried with anhydrous Na2SO4, filtered, and evaporated to dryness. The residue was chromatographed by column using a mixture of nitric acid (65%) and concentrated sulfuric acid (96%) using a mixture of nitric acid (65%) and concentrated sulfuric acid (96%).

For hydroxylation of (1), 100 mg was dissolved in toluene in an ice bath. Subsequently, 50 μL of aqueous tert-butylhydroperoxide (70% in water, Alfa-Aesar, Heysham, Lancs) and 50 μL of Triton B (40% in methanol) were added. After 10 min, the mixture was allowed to reach room temperature and kept under constant stirring until then. A second addition of aqueous tert-butylhydroperoxide and Triton B was carried out. After 2 h, p-toluenesulfonic acid crystals were added. The reaction was stirred at room temperature for 24 h. The orange amorphous solid obtained (8 mg) was purified by chromatographic column using silica gel 60 (0.040–0.063 mm; Merck). Compound (7) was identified as 2-hydroxy-1H-phenalen-1-one. 1H NMR (CDCl3): δ 8.78 (d, J = 7.5 Hz, 1H,
To obtain (8), perinaphthenone (1, 50 mg) was dissolved in methanol and a bead of NaOH was added. The mixture was kept under constant stirring at room temperature for 4 h. The solution turned greenish-brown. Subsequently, the reaction was neutralized with 10% HCl solution. The resulting solution was subjected to liquid-liquid extraction with CH2Cl2. The organic phase was dried with anhydrous Na2SO4 and concentrated to afford (8), which was identified as 6-methoxy-1H-biphenalen-1-one. 1H NMR (CDCl3): δ 8.71 (dd, J = 7.5 and 0.9 Hz, 1H, H-9), 8.65 (dd, J = 0.9 and 8.1 Hz, 1H, H-7), 7.80 (dd, J = 7.5 and 8.1 Hz, 1H, H-8), 7.71 (d, J = 9.6 Hz, 1H, H-3), 7.70 (d, J = 8.1 Hz, 1H, H-4), 6.90 (d, J = 8.1 Hz, 1H, H-5), 6.67 (d, J = 9.6 Hz, 1H, H-2), 4.13 (s, -OCH3). 13C NMR (CDCl3): δ 185.8 (C-1), 138.5 (C-3), 136.2 (C-4), 133.5 (C-6a), 131.8 (C-8), 126.9 (C-9a), 126.4 (C-3a). Compound (9) was prepared when 20 mg of (1) was dissolved in isopropyl alcohol (10 mL) in a round-bottomed flask; the solution was heated to 70 °C. Subsequently, a few drops of concentrated sulfuric acid (96%) and aluminum trichloride (10 mg) were added and the solution was left under constant stirring for 48 h. Finally, the resulting solution was neutralized with NaHCO3 and subjected to extraction with CH2Cl2. The organic phase was dried with anhydrous Na2SO4, filtered, and evaporated to dryness in a rotary evaporator. The residue was passed through a silica gel chromatographic column and eluted with a gradient of n-hexane-CH2Cl2 to obtain a pale yellow amorphous solid (6 mg). Compound (9) was identified as 6-methoxy-1H-biphenalen-1-one, [1,2']biphenalenyl-3,1'-dione. 1H NMR (CDCl3): δ 8.75 (d, J = 6.3 Hz, 1H, H-9), 8.74 (d, J = 7.8 Hz, 1H, H-4), 8.37 (dd, J = 7.8 Hz, 1H, H-9), 8.29 (d, J = 8.1 Hz, 1H, H-4'), 8.20 (d, J = 8.1 Hz, 1H, H-7), 8.09 (d, J = 8.1 Hz, 1H, H-7), 8.02 (s, 1H, H-3), 7.95 (d, J = 7.2 Hz, 1H, H-6), 7.92 (d, J = 7.8 Hz, 1H, H-6'), 7.86 (dd, J = 7.5 and 7.8 Hz, 1H, H-5), 7.79 (dd, J = 6.9 and 7.8 Hz, 2H, H-8, H-5'), 7.56 (dd, J = 7.8 and 7.8 Hz, 1H, H-8), 6.86 (s, 1H, H-2). 13C NMR (CDCl3): 185.4 (C-1'), 183.6 (C-1), 152.3 (C-3'), 151.0 (C-3'a'), 150.2 (C-3a'), 141.7 (C-3), 138.0 (C-9b'), 135.5 (C-7'), 132.7 (C-6), 132.5 (C-6'), 132.3 (C-9), 132.4 (C-9'a), 132.2 (C-6a), 131.5 (C-9'), 131.0 (C-4'), 130.4 (C-5'), 129.6 (C-4'), 129.3 (C-9b), 127.9 (C-6a'), 127.8 (C-9a), 127.6 (C-5), 127.2 (C-2), 127.1 (C-8), 127.0 (C-8'), 126.5 (C-2').

Compound (10) was prepared by methylation of (7) (50 mg), which was dissolved in acetone (10 mL) in a round-bottomed flask. Potassium carbonate (50 mg) was added, and the resulting mixture was placed in an ice bath and stirred for 15 min. Methyl iodide (1.0 mL) was added. The resulting mixture was warmed gradually from 0 °C to reflux over 30 min, and then was refluxed for 1 h. The resulting mixture was allowed to cool to room temperature. Subsequently, the reaction was neutralized with 10% HCl solution. The resulting solution was separated with CH2Cl2. The organic phase was dried with anhydrous Na2SO4, filtered, and then concentrated on a rotary evaporator. The resulting oil was separated by column chromatography on SiO2 with n-hexane/ethyl acetate 10/1, to afford 10 mg of a yellow oil, which was identified as 2-methoxy-1H-biphenalen-1-one. 1H NMR (CDCl3): δ 8.76 (d, J = 7.5 Hz, 1H, H-9), 8.30 (d, J = 7.8 Hz, 1H, H-7), 7.96 (d, J = 7.8 Hz, 1H, H-5), 7.81 (dd, J = 6.9 and 7.8 Hz, 1H, H-4), 7.62 (dd, J = 7.5 and 7.8 Hz, 1H, H-8), 7.21 (s, 1H, H-3), 3.89 (s, -OCH3). 13C NMR (CDCl3): δ 180.5 (C-1), 154.5 (C-2), 136.5 (C-7), 135.5 (C-6a), 132.2 (C-6), 131.6 (C-9), 130.9 (C-5), 129.4 (C-9b), 128.4 (C-9a), 127.3 (C-4), 126.9 (C-8), 124.4 (C-3a), 113.9 (C-3), 60.4 (-OCH3).

Compound (11) was prepared by chlorination of (1) (50 mg) using hydrochloric acid (36%, 10 mL) in a round-bottomed flask at 25 °C. Nitric acid (65%) was slowly added (drop by drop, 1 mL) and the final solution was left under constant stirring for 12 h. Then, the reaction mixture was neutralized with 10% NaOH solution and extracted with CH2Cl2. The organic phase was dried with anhydrous Na2SO4, filtered, and evaporated to dryness. The residue was chromatographed by column using silica gel 60 as the stationary phase (0.040–0.063mm; Merck) and an n-hexane-CH2Cl2 gradient as the mobile phase; a yellow amorphous solid was obtained (6 mg). Compound (11) was identified as 2,4-dichloro-1H-biphenalen-1-one. 1H NMR (CDCl3): δ 8.82 (d, J = 7.8 Hz, 1H, H-9), 8.74 (d, J = 8.7 Hz, 1H, H-7), 7.97 (s, 1H, H-3), 7.96 (dd, J = 7.5 and 8.7 Hz, 1H, H-5), 7.74 (d, J = 7.5 Hz, 1H, H-6), 7.72 (d, J = 7.8 Hz, 1H, H-8). 13C NMR (CDCl3): 186.1 (C-1), 138.5 (C-3), 136.2 (C-4), 133.5 (C-9a), 132.8 (C-7), 132.3 (C-6), 131.1 (C-9), 130.0 (C-2), 129.0 (C-6a), 128.3 (C-8), 127.3 (C-9b), 126.4 (C-3a).

Figure 1. Chemical structures of some natural (from Musa spp.) and synthetic perinaphthenone derivatives.
Spectroscopic data were in agreement with those reported by Hidalgo et al. (2009), Misaki et al. (2008) (compounds 4, 5 and 6), and Otálvaro et al. (2007) (Compounds 7 and 10). The structures of compounds 8, 9, and 11 were elucidated by comprehensive spectra data analysis. The lipophility (CLogP) parameter was calculated virtually, using ChemDraw Pro 12.0.

2.6. Statistical analysis

Data were subject to analysis of variance (ANOVA). The means were compared using the Least Significant Difference test (LSD) at $P = 0.05$. 

Figure 2. Effect of perinaphthenone (1) on radial mycelial growth of Botrytis spp. (2a), Botryodiplodia spp. (2b) and Fusarium spp. (2c). Results are shown as average values of three replicates of mycelium diameter; bar = ±SD.
3. Results and discussion

3.1. Antifungal activity of perinaphthenone (1)

Antifungal activity of (1) was analyzed by its ability to inhibit the mycelial growth using the poisoned food technique. The inhibitory effects of (1) against *Fusarium* spp., *Botrytis* spp., and *Botryodiplodia* spp. are shown in Figure 2. In general, compound (1) showed significant antifungal activity against all fungi. As can be seen, fungal mycelial growth was dependent on the concentration of (1) in the culture medium. At 500 μM and above, the compound (1) inhibited the radial growth of *Botryodiplodia* spp. and *Fusarium* spp. completely and this effect remained, respectively, during the 3.5 and 11 days of the experiment. Furthermore, fungal colony development began only 4 days after inoculation when *Botrytis* spp. was treated with (1) at 1000 μM. However, a slight decrease in the antifungal activity of (1) was seen as time progressed.

In general, the results revealed that (1) exhibited a strong antifungal action toward *Botrytis* spp., *Fusarium* spp. and *Botryodiplodia* spp.: IC_{50} values were, respectively, 36.2 (at day 3), 13.3 (at day 3), and 39.0 μM (at 60 h). The inhibitory effects shown by (1) were higher than those found for thymol (98-87% inhibition at 1000 μM), a recognized natural antifungal; but slightly less than those of Carbendazim (100% inhibition at 250 μM), a strong synthetic antifungal. These results are in agreement with those previously obtained by Quinones et al. (2000), who reported that compound (1) has a very strong antifungal activity against *M. fijiensis*; a complete inhibition of mycelial growth (mycelial weight) at 50–100 μg/mL was found and its action continued over 15 days. According to the authors, this action was similar to the commercial fungicide Benomyl.

Furthermore, the effect of light on the antifungal properties of (1) against *Fusarium* spp. was evaluated. According to Figure 3, the inhibitory effect of compound (1) against *Fusarium* spp. was significantly higher in light than in the dark. Compound (1) at 250 μM and after 48 and 72 h, showed inhibition percentages of 100 and 92% for Petri dishes exposed to light and 94 and 84% in the dark. These results are in agreement with those of Flors and Nonell (2006) and Lazzaro et al. (2004), who demonstrated that the antifungal activity of the phenalenone derivatives is enhanced in the presence of light.

On the other hand, assays over the effect of (1) on the spore germination of *Botrytis* spp. for the 8 h observation period shows that there were inhibitions of 69.2, 100, and 100% at concentrations of 250, 500, and 1000 μM, respectively. Even after a period of 24 h, the compound (1) caused a total inhibition of spore germination of *Botrytis* spp. at concentrations of 500 and 1000 μM. According to Quinones et al. (2000), compound (1) at 50–100 μg/mL causes a higher inhibition of germination of *M. fijiensis* spores than the commercial fungicides Benomyl, Propiconazole, and Tridermorph. Those previous results, together with those reported here, support the fact that compound (1) could be used directly as an antifungal agent or as an interesting structural template for designing new compounds against fungal pathogens. Furthermore, since the compound (1) is structurally related to the molecules produced by plants as a result of their interaction with the pathogens, their application for the control of fungi could be less damaging to the environment.

Although not much is known about the mechanism of antifungal action of compound (1), studies of the structure-activity relationship on compounds having the α, β-unsaturated carbonyl group have suggested that it is an important structural feature for exhibiting strong antifungal activity (El Ouadi et al., 2017; Meepagala et al., 2003). It has been postulated that one possible mode of antifungal action of phenalenones may be due to their role as Michael-type acceptors for biological nucleophiles (Hidalgo et al., 2009). On the other hand, Lazzaro et al. (2004) have suggested that upon absorption of light, phenalenone derivatives sensitize the production of singlet oxygen and increase the antifungal activity towards *Fusarium* spp. The singlet oxygen could react with biomolecules essential for the development of the microorganism (DNA, proteins, fatty acids). In addition, since the compound (1) is a planar molecule, it could be intercalated into DNA, thus interrupting DNA functions (Quinones et al., 2000).

As previously mentioned, the inhibitory effect of compound (1) decreased with time, a fact that could suggest that these fungi possess a detoxification mechanism. In order to study this mechanism, *Botryodiplodia* spp., *Botrytis* spp. and *Fusarium* spp. were incubated with (1) at 62.5 μM.

3.2. Metabolism of perinaphthenone (1)

A comparison of the HPLC-DAD chromatographic profiles obtained from the metabolism of (1) is presented in Figure 4. The chromatographic
profiles of the metabolism of (1) by Fusarium spp. and Botrytis spp. were slightly similar, showing only two additional peaks with respect controls (Figures 4a and 4b). As shown in Figure 4c, Botryodiplodia spp. metabolized (1) into three major compounds (retention times, tR = 13.5, 13.8 and 16.0 min; tR of (1) = 13.3 min) not found in the control (data not shown). The compounds 2 and 3 were isolated and purified by means of preparative thin layer and column chromatography, and their structures were determined from the interpretation of spectroscopic data (1H and 13C NMR), and comparison with authentic samples. The metabolites were identified as 2,3-dihydro-1H-phenalen-1-ol (tR = 13.8 min, 2) and 2,3-dihydro-phenalen-1-one (tR = 16.0 min, 3). The third metabolic product (tR = 13.5 min), detected only in the microbial transformation of (1) using Botryodiplodia spp. could not be purified and identified. According to results, all fungi were able to reduce the double bond and carbonyl group to give the metabolic products. Both compounds were also found in the conversion of perinaphthenone by M. fi jiensis (data not shown). Because the α,β-unsaturated carbonyl group has largely been associated with the antifungal activity of many compounds, it is possible to think that the conversion of (1) to (2) and (3) by Botryodiplodia spp., Botrytis spp. and Fusarium spp. could be the result of a detoxification process. The reduction of the α,β-conjugated carbonyl group not only eliminates the capacity as Michael-type acceptor of (1) but also its photophysical and photochemical properties, and consequently its ability as a singlet oxygen sensitizer. The compound (2) was previously identified as a product of photodegradation of (1) using a model of leaf epicuticular waxes (Trivella et al., 2014).

Then, the quantitative determinations of (1), (2), and (3) in the course of time were carried out using calibration curves. The regression equations were: compound (1), y = 274372x − 445151 (R² = 0.9999); compound (2), y = 33045x − 217515 (R² = 0.9842), and compound (3), y = 32721x − 509185 (R² = 0.9853) (Figure 5).

The metabolism of compound (1) with Botryodiplodia spp., Botrytis spp. and Fusarium spp. was monitored for 12 days by taking samples each 1, 3, 6, 9 and 12 days. The ratios among the compound (1) and the metabolic products (2) and (3) in the course of time, for the biotransformations with the different fungi, are shown in Figure 6. In general, the amount of metabolic products varied with the species.

Figure 4. HPLC-DAD elution profile of metabolism of (1) by Fusarium spp. (4a), Botrytis spp. (4b) and Botryodiplodia spp. (4c). The outsets show the UV spectra of 1 (top) and its metabolites, 2 (middle) and 3 (down). Wavelength: 254 nm. Day: 6.
It is evident that during the first 72 h of the process, the compound (1) was consumed in approximately 90%. The metabolic product (2) reached the highest concentrations at day 6 (3.2 μg/mL for Fusarium spp.) and day 9 (2.6 μg/mL for Botrytis spp. and 14.3 μg/mL for Botryodiplodia spp.). For its part, compound (3) showed an increasing concentration during the time of analysis for the fungus Fusarium spp. until reaching 20.3 μg/mL on day 12. The other two fungi achieved lower concentrations, getting

![Figure 5. Calibration curves for perinaphthenone (1) (left), 2,3-dihydro-1H-phenalen-1-ol (2) (middle) and 2,3-dihydro-phenalen-1-one (3) (right).](image1)

![Figure 6. Concentration in the course of time for the microbial transformation of perinaphthenone (1) by the fungi Fusarium spp. (up-left), Botrytis spp. (up-right), and Botryodiplodia spp. (down). Compound (1), perinaphthenone; compound (2), 2,3-dihydro-1H-phenalen-1-ol; and compound (3), 2,3-dihydro-phenalen-1-one.](image2)
the highest level of the compound (3) at day 3 (4.1 μg/mL) for Botrytis spp. and day 1 (10.7 μg/mL) for Botryodiplodia spp.

The greatest formation of the compound (3) shows the high efficiency of Fusarium spp. to reduce the activated C-C double bond through the action, possibly, of an enzyme enoate reductase (ER). Botryodiplodia spp. has the ability not only to reduce the C-C double bond of (1), but also to convert the ketone (3) to the alcohol (2). The reduction of carbonyl to hydroxyl group can be attributed to the action of enzymes alcohol dehydrogenase (ADH) and coenzymes NADH and NADPH, from which a hydride ion is transferred to the carbonyl group of (3) to produce (2) (Hall et al., 2006).

The major production of (3) by Fusarium spp. is an indication that the hydrogenation rate of the carbon-carbon double bond is greater than the reduction of the carbonyl group (no allyl alcohol was detected). This selective conversion (chemoselectivity) is strange and interesting, as some authors have proposed that the bioreduction of α, β-unsaturated carbonyl system is frequently poor, due to the competence of the enzyme ER and the ADH enzymes, since both depend on the nicotinamide cofactor (Hall et al., 2006; Velasco et al., 2012). These detoxifying enzymes could be important metabolic targets for developing new control alternatives (for example, through inhibitors) of plant pathogenic fungi. In addition, it would be possible to modify the C-2 and C-3 positions of perinaphtenone to restrict or block the metabolic conversions and thus improve antifungal activity.

3.3. Antifungal activity of perinaphtenone derivatives

A series of perinaphtenone derivatives were prepared by conventional chemical methods and assessed for their antifungal activity at 1000 μM against the fungus Botryodiplodia spp. Among the derivatives, the metabolic products (2) and (3) were included. The percentages of inhibition of radial growth are shown in Figure 7.
Interestingly, there were no significant differences in the antifungal activity between the compounds (1) and (2) after 72 h. Mycelial growth of *Botryodiplodia* spp. was inhibited between 77 and 94%, and 67 and 89% for (1) and (2), respectively. The high inhibitory effect of (2) was also observed against *Fusarium* spp. (data not shown). This fact is fascinating since compound (2) lacks the α, β-conjugated carbonyl group, which has been proposed to be responsible for the capacity of (1) for acting as Michael-type acceptor and sensitizing the singlet oxygen, and consequently, exhibiting the fungistatic properties. It can be inferred that the α, β-unsaturated carbonyl system is not the only structural feature responsible for the antifungal activity of the compound (1).

On the other hand, inhibition of mycelial growth by the metabolic product (3) ranged between 48 and 66%; the inhibitory effect against *Botryodiplodia* spp. of (3) was significantly lower in comparison to (1). Therefore, the metabolic conversion of the compound (1) to (3) may be the result of a mechanism of detoxification by the fungi. In addition, the fact that the fungi metabolize compound (1) to (2) and (3), two products that also have significant antifungal activity, would explain the strong antifungal activity of (1). Although fungi convert compound (1) rapidly, the formation of (2) and (3) would continue to provide a toxic medium for the microorganism. This fact would force the fungi to a new metabolic conversion. So, the fungistatic action of compound (1) would remain for a longer time. The molecular similarity of the three compounds, along with their antifungal activity, allow us to suggest that steric factors apparently play an important role in antifungal properties.

According to Figure 7, it is clear that methoxylation in C-6 (compound 8) and nitration in C-5 (compound 4) of the naphthalene system significantly reduced the antifungal activity against *Botryodiplodia* spp. in comparison to (1). However, the presence of the nitro group (α conjugating substituent) caused inhibitory activity against *Botryodiplodia* spp. slightly greater than the methoxy (a donor-electron group) derivative. It seems that the electronic effects on the naphthalene system are somewhat important for the activity.

Taking into account that the microbial metabolism of compound (1) affected the α, β-unsaturated carbonyl system, a series of derivatives substituted in C-2 was prepared. The presence of substituents at the C-2 position (nitro-, 5; bromo-, 6; hydroxy-, 7; 1-oxophenalenyl, 9; methoxy-, 10) also significantly reduced the inhibitory effect on *Botryodiplodia* spp. in comparison to (1). Compounds 5 and 9 with α conjugating substituents (strong electron-withdrawing groups) in C-2 of the α, β-unsaturated carbonyl system, exhibited moderate inhibitory activity of almost 79.9 and 58.1% after 48 h, respectively. However, the fungistatic effect of (5) decreased rapidly after 72 h, while for (9) remained almost constant. Hidalgo et al. (2009) reported strong antifungal activity against *M. fijiiensis* of compound (5). Meanwhile, compound (6) with a weak electron-withdrawing group, showed poor mycelial growth inhibition (38.6% after 72 h). The higher antifungal effect of (9) and (5) with respect to (6) is in agreement with the fact that the former possess strong electron-withdrawing substituents in C-2, which increases the capacity as Michael acceptors. Nonetheless, compounds (7) and (10), having the electron-donor groups in C-2 (HO-, 7; CH3O-, 10), displayed mycelial inhibitions similar to or even greater than those observed for the compounds (5) and (9). Since compounds (7) and (10) would not be as good Michael-type acceptors as (5) and (9), a relationship between this factor and antifungal activity does not seem clear. Escobar (2003) reported that the conversion of nitro derivatives to amino derivatives at C-2 of perinaphthenone did not significantly affect the inhibitory activity of mycelial growth of *M. fijiiensis*, which is in accordance with our results and contradicts the relationship between the antifungal activity and the character as Michael-type acceptors of the perinaphthenone derivatives.

Mycelial growth inhibitions for the compound (7) and (10) ranged from 57.9 to 57.5, and 78.1–82.1% after 48 and 72 h, respectively. These findings are according to Hidalgo et al. (2009), who reported a higher inhibition of *M. fijiiensis* using the O-methyl (10) derivative in comparison to the hydroxyl derivative (7). Compounds (7) and (10) have been isolated from rhi-zomes of *Musa acuminata* var. “Yagambi km 5” and have shown to be significantly more active against *M. fijiiensis* than the natural phenylphenalenones (Otálvaro et al., 2007). According to Otálvaro et al. (2007), both compounds displayed a complete inhibition of mycelial growth at 50–100 ppm and their effect continued for 15 days. These findings demonstrate that the antifungal activity of perinaphthenone scaffold can strongly be modulated by the insertion of substituents at C-2.

On the other hand, the optimum balance between hydrophilic and lipophilic for traversing the cell barriers could also be responsible for the activity. The ClogP value of the compounds (Figure 7), which is the logarithm of its partition coefficient between n-octanol and water, is a well-established measure of the lipophilicity of a compound. Low lipophilicities and therefore low ClogP values cause poor absorption or permeation. In general, the most active compounds (1, 2, 3, 7, and 10) presented ClogP values ranging between 2.4 and 2.9. In contrast, compounds with ClogP values above or below that range showed low inhibitory effect (i. e. 4, 6, and 8), except for compounds (11) and (9) (ClogP > 4.3). The compounds (9) and (11) have a highly lipophilic nature, which gives them the ability to interact and penetrate biological membranes exerting different effects (Domagala, 1994). Therefore, the fungistatic effect could be the result of a strict lipophilic/hydrophilic balance, which may affect the initial interaction with the membrane.

The above shows that the antifungal properties of derivatives containing the perinaphthenone scaffold are slightly influenced by the electronic effects but strongly influenced by steric effects and the lipophilic nature of the substituents. Nonetheless, studies with more derivatives are necessary.

4. Conclusions

Perinaphthenone exhibited a significant fungistatic effect against the three fungi evaluated; 500 and 1000 μM of perinaphthenone showed total inhibition of *Botryodiplodia* spp. and *Fusarium* spp. Moreover, fungistatic properties were improved by exposure to light. However, a slight decrease in the inhibitory effect was observed over time, suggesting a possible mechanism of detoxification of perinaphthenone. Metabolic studies show that perinaphthenone is rapidly metabolized by the three pathogenic microorganisms, to form mainly 2,3-dihydro-1H-phenalen-1-ol and 2,3-dihydro-phenalen-1-one. Maximum levels of 2,3-dihydro-1H-phenalen-1-ol and of 2,3-dihydro-phenalen-1-one were reached at day 9 for *Botryodiplodia* spp. and day 12 for *Fusarium* spp. The above demonstrates the predilection of the three fungi to convert the α, β-unsaturated carbonyl system. An analysis of the antifungal activity of derivatives revealed that the formation of 2,3-dihydro-1H-phenalen-1-ol from perinaphthenone obeys a process of detoxification by the fungi. However, the simultaneous reduction of the double bonds C-C and C-O on the α, β-unsaturated carbonyl system does not significantly affect the antifungal activity. On the other hand, the inclusion of substituent groups in perinaphthenone scaffold, both in C-2 and C-6, significantly reduced the antifungal activity against *Botryodiplodia* spp. in comparison to perinaphthenone. The steric effects and lipophilic nature of the substituents attached to perinaphthenone scaffold influence strongly in the inhibitory effect. New antifungal agents and modes of action can be developed from the perinaphthenone scaffold.

Declarations

Author contribution statement

Luisa M. Castaño: Performed the experiments.
Andrés F. Gómez: Contributed reagents, materials, analysis tools or data.
Jesús Gil: Analyzed and interpreted the data.
Diego Durango: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
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Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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