Synthesis of Eugenol Derivatives and Evaluation of their Antifungal Activity Against Fusarium solani f. sp. piperis

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Abstract: Background: Fusarium solani f. sp. piperis is a phytopathogen that causes one of the most destructive diseases in black pepper crops, resulting in significant economic and crop production losses. Consequently, the control of this fungal disease is a matter of current and relevant interest in agriculture.

Objective: The objective was to synthesize eugenol derivatives with antifungal activity.

Methods: In this study, using bimolecular nucleophilic substitution and click chemistry approaches, four new and three known eugenol derivatives were obtained. The eugenol derivatives were characterized and their antifungal and cytotoxic effects were evaluated.

Results: Eugenol derivative 4 (2-(4-allyl-2-methoxyphenoxy)-3-chloronaphthalene-1,4-dione) was the most active against F. solani f. sp. piperis and showed acceptable cytotoxicity. Compound 4 was two-fold more effective than tebuconazole in an antifungal assay and presented similar cytotoxicity in macrophages. The in silico study of β-glucosidase suggests a potential interaction of 4 with amino acid residues by a cation-π interaction with residue Arg177 followed by a hydrogen bond with Glu188, indicating an important role in the interactions with 4, justifying the antifungal action of this compound. In addition, the cytotoxicity after metabolism was evaluated as a mimic assay with the S9 fraction in HepG2 cells. Compound 4 demonstrated maintenance of cytotoxicity, showing IC₅₀ values of 11.18 ± 0.5 and 9.04 ± 0.2 µg mL⁻¹ without and with the S9 fraction, respectively. In contrast, eugenol (257.9 ± 0.4 and 133.5 ± 0.8 µg mL⁻¹), tebuconazole (34.94 ± 0.2 and 26.76 ± 0.17 µg mL⁻¹) and especially carbendazim (251.0 ± 0.30 and 34.7 ± 0.10 µg mL⁻¹) showed greater cytotoxicity after hepatic biotransformation.

Conclusion: The results suggest that 4 is a potential candidate for use in the design of new and effective compounds that could control this pathogen.

Keywords: Antifungal, eugenol derivatives, cytotoxicity, Piper nigrum, Fusarium solani f. sp. piperis.

1. INTRODUCTION

The black pepper (Piper nigrum L.) is the most-consumed spice species in the world. It is mainly cultivated in Asian countries such as India, Indonesia, Thailand, Vietnam and China, as well as in South America, in Brazil [1-2]. Brazil is the third-largest producer of pepper in the world and the fifth largest exporter of this commodity [3]. The Brazilian national harvest reached almost 79 thousand tons in 2017 [4].

However, the phytopathogen Fusarium solani f. sp. piperis has been responsible for a decline in productivity and an increase in production costs for Brazilian black pepper crops [5-7]. The infection causes vessel obstruction leading to root rot, or Fusarium disease (fusariosis). The first symptoms start at the roots or branches and promote yellowing and falling of leaves, stem blight, root rot, and plant death. This fungus produces secondary metabolites, such as naphthoquinones and fusaric acid, which have toxicogenic properties capable of inducing root rot and rust [5-6, 8-10].

Control measures have been taken to increase black pepper resistance to fusariosis, including micrografting, micropropagation, and in-vitro plant regeneration. However, the narrow genetic variability and the homogeneous plantations of this crop have led to the spread of the fungus [11-12].

Strategies for F. solani f. sp. piperis control are limited because there is no officially approved fungicide in Brazil and information on resistant cultivars is scarce [8]. Nevertheless, synthetic fungicides, such as carbendazim and tebuconazole, have been widely used for control of this fungal disease. Their indiscriminate use has led to the appearance of resistant isolates that increase production costs [10,13].

Furthermore, the World Health Organization classifies some fungicides, including carbendazim and tebuconazole, as hazardous chemicals and as possible human carcinogens and teratogens, respectively. In addition, with respect to carbendazim, its repeated applications lead to accumulation in and contamination of various ecosystems [14-15]. These undesirable effects have induced a search for new and effective fungicide agents, mainly from natural resources [7,16-18].

Recently, plant-derived natural antifungals have been reported as alternatives to chemical fungicides. The application of essential oils has attracted much attention as a method of controlling fungal
infections due to their antimicrobial properties and low toxicity [5,19].

Eugenol (4-allyl-2-methoxyphenol) is the major constituent of essential oil from Syzygium aromaticum (clove). This molecule exhibits several applications in the pharmaceutical, cosmetic, food, and agricultural industries [20]. It has a wide range of biological activities, including antifungal characteristics [21]. Furthermore, eugenol has structural standards that enable the semi-synthesis of compounds with applicability in the pharmaceutical and agrochemical industries [22-23]. Various studies of the antifungal activity of eugenol and analogs against phytopathogenic fungi have been reported [16,19]. However, there are few reports on the antifungal activity of eugenol against F. solani f. sp. piperis.

Thus, we report here the synthesis of a series of eugenol derivatives and their antifungal activity and cytotoxic effects.

2. MATERIALS AND METHODS

2.1 General Procedures

Solvents with analytical grades and purity higher than 99.5% were purchased from Synth (São Paulo, SP, Brazil). Dimethylformamide, commercial azides, commercial halides, potassium carbonate, sodium ascorbate, copper sulfate, and deuterated solvents (CDCl₃ and DMSO-D₄) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Microwave reactions were conducted using a CEM Discover Synthesis Unit (CEM Corp., Matthews, USA). The machine consists of a continuous focused microwave power delivery system with operator-selectable power output from 0 to 300 W. Reactions were performed in glass vessels (capacity 10 mL) sealed with a septum. For the open layer chromatography, a 2.5 × 12 cm silica gel packed in hexane:ethyl acetate, 4:1, v/v) was used.

2.2. Synthesis of eugenol derivatives

Eugenol was isolated from clove essential oil according to the standard procedure [24], using hydrodistillation. The synthetic procedures used to obtain compounds 1-7 followed the methodology described by Brito et al. [25] with modifications. Synthetic of eugenol derivatives is shown in Scheme 1.

In a 25-mL round bottom flask containing eugenol (1 equivalent) in acetone (5 mL), under stirring and ice bath, potassium carbonate (1 equivalent) was added. The system was left 30 min under this condition. Commercial halides (1 equiv.) were then added and the system was kept at room temperature under stirring for over 12 h. The reaction solutions were concentrated under reduced pressure and the final products were purified by column chromatography using silica gel as the stationary phase and hexane:acetate (7:3, v/v) as mobile phase to obtain compounds 1-5.

In a microwave tube (10 mL capacity) containing compound 5 (1 equivalent) in 1.0 mL DMF, 4 equivalents of commercial azides, 0.1 equivalents of sodium ascorbate and 0.03 equivalents of a 0.1 mol L⁻¹ solution of CuSO₄ were added. Subsequently, the tube was sealed and microwave irradiated at 150 W and 70 °C for 20 min. After this process, the reaction solutions were concentrated under reduced pressure and the final products were purified by column chromatography using silica gel as the stationary phase and hexane:acetate (7:3, v/v) as mobile phase to obtain compounds 6 and 7.

**Scheme 1.** (i) potassium carbonate, acetone, and commercial halide, R.T.; (ii) sodium ascorbate, dimethylformamide, copper sulfate and commercial azides, MW, 70 °C.

Compounds 1 (4-allyl-2-methoxy-1-(4-nitrobenzyl)oxy benzene) was obtained as a yellow oil with a yield of 16.4% (0.015 g, 0.05 mmol). TLC: RF = 0.33 (hexane:ethyl ether, 4:1, v/v); FT-IR (Attenuated total reflection - ATR) νmax / cm⁻¹: 2920, 1604, 1509, 1229. ¹H NMR (400 MHz, CDCl₃) δ 8.22 (d, 2H, J 8.6 Hz, CH), 7.62 (d, 2H, J 8.6 Hz, CH), 6.77 (d, 1H, J 8.2 Hz, CH), 6.76 (b, 1H, CH), 6.67 (dd, 1H, J 1.9; 8.2 Hz, CH), 5.95 (m, 1H, CH), 5.21 (s, 2H, CH₃), 5.08 (m, 1H, CH), 5.06 (m, 1H, CH), 3.89 (s, 3H, CH₃), 3.34 (d, 2H, J 6.6 Hz, CH₂). ¹³C NMR (100 MHz, CDCl₃) δ 149.7 (CO), 147.5 (CN), 145.8 (CO), 145.0 (C), 137.4 (CH), 134.3 (C), 127.5 (2C), 123.8 (2H), 120.4 (CH), 115.8 (CH₂), 114.6 (CH), 112.5 (CH), 70.2 (CH₂), 56.0 (OCH₃), 39.8 (CH₃). These data are consistent with the literature [26]. HR-ESI-MS [M + H]⁺ Found: 300.1230, Calc. for C₁₅H₁₄NO₄ 300.1230; [M + Na]⁺ Found: 322.1051, Calc. for C₁₅H₁₄NO₄Na⁺ 322.1050; [2M + Na]⁺ Found: 621.2221, Calc. for C₃₄H₃₂N₂NaO₈ 621.2207.

Compounds 2 (4-(allyl-2-methoxyphenoxacyetonitrile) was also obtained as a yellow oil, with a yield of 62.6% (0.770 g, 3.7 mmol). TLC: RF = 0.25 (hexane:ethyl ether, 9:1, v/v); FT-IR (ATR) νmax / cm⁻¹: 2984, 2360, 1740, 1243, 1047. ¹H NMR (400 MHz, CDCl₃) δ 6.97 (d, 1H, J 7.8 Hz, CH), 6.75 (m, 2H, CH), 5.93 (m, 1H, CH), 5.10 (d, 1H, J 6.2 Hz, CH₂), 5.07 (s, 1H, CH), 3.85 (s, 3H, CH₂), 3.33 (d, 2H, J 5.7 Hz, CH₂). ¹³C NMR (100 MHz, CDCl₃) δ 150.4 (CO), 144.0 (CO), 137.1 (C), 137.0 (CH), 120.8 (CH), 117.9 (CH), 116.1 (CH₂), 115.4 (CN), 112.8 (CH), 55.8 (OCH₃), 39.9 (CH₂). HR-ESI-MS [M + Na]⁺ Found: 204.1019, Calc. for C₇H₇N₂NaO₂ 204.1019; [M + Na]⁺ Found: 226.0837, Calc. for C₇H₇N₂NaO₂ 226.0838; [M + K]⁺ Found: 243.0991, Calc. for C₇H₇KNO₂ 242.0578.

Compounds 3 (4-(allyl-2-methoxy-1-(2-(4-(4-(4-nitrobenzyl)oxy)benzene) was obtained as a white powder with a yield of 31.4% (0.039 g, 0.95 mmol). TLC: RF = 0.65 (hexane:ethyl acetate, 4:1, v/v); FT-IR (ATR) νmax / cm⁻¹: 2918, 1519, 1235, 1035, 688. ¹H NMR (400 MHz, CDCl₃) δ 7.65 (d, 2H, J 7.8 Hz, CH), 7.60 (t, 1H, J 7.8 Hz, CH), 7.44 (t, 2H, J 7.8 Hz, CH), 7.40 (d, 1H, J 7.4 Hz, CH), 7.32 (t, 1H, J 7.4 Hz, CH), 7.22 (t, 1H, J 7.4 Hz, CH), 7.07 (d, 1H, J 7.4 Hz, CH), 6.82 (d, 1H, J 8.2 Hz, CH), 6.73
Compounds 4 (2-(4-allyl-2-methoxyphenoxy)-3-chlorophenol-1,4-dione) was obtained as a red powder with a yield of 57.7% (0.124 g, 0.35 mmol). TLC: Rf = 0.24 (hexane:ethyl ether, 4:1, v/v); FT-IR (ATR) νmax / cm⁻¹ 3065, 1517, 1232, 837. ¹H NMR (400 MHz, DMSO-D6) δ 8.31 (s, 1H, CH), 8.24 (d, 2H, J 8.6 Hz, CH), 7.52 (d, 2H, J 8.6 Hz, CH), 7.01 (d, 1H, J 8.2 Hz, CH), 6.79 (brs, 1H, CH), 6.67 (brd, 1H, J 8.2 Hz, CH), 5.93 (m, 1H, CH), 5.80 (s, 2H, CH₂), 5.09 (s, 2H, CH₂), 5.04 (m, 2H, CH₂), 3.29 (d, 2H, J 6.6 Hz, CH₂). ¹³C NMR (100 MHz, DMSO-D6) δ 149.1 (CO), 147.2 (CN), 145.6 (CO), 143.4 (C), 143.3 (CN), 137.9 (CO), 133.0 (2CH), 125.1 (CN), 121.9 (CH), 112.8 (CH), 111.6 (CH), 113.2 (CH), 56.2 (OCH₃), 39.9 (CH₂). HR-ESI-MS [M + H]+ Found: 355.0730, Calc. for C₂₅H₂₄ClO₄: 355.0732; [M + Na]+ Found: 377.0545, Calc. for C₂₅H₂₅ClNaO₄: 377.0551; [M + Na]+ Found: 731.1199, Calc. for C₃₅H₃₃ClNaO₈: 731.1210.

Compound 7 (4-(4-allyl-2-methoxyphenoxy)methyl)-1-(2-(phenylsulfonyl)methylene)-1H-1,2,3-triazole) was obtained as a yellowish oil with a yield of 71.3% (0.067 g, 0.17 mmol). TLC: Rf = 0.75 (hexane:ethyl ether, 7:3, v/v); FT-IR (ATR) νmax / cm⁻¹ 2933, 1516, 1303, 1124, 691. ¹H NMR (400 MHz, CDCl₃) δ 8.15 (s, 1H, CH), 7.82 (d, 2H, J 7.4, CH), 7.76 (t, 1H, J 7.7 Hz, CH), 7.64 (t, 2H, J 7.4, CH), 7.32 (t, 1H, J 7.4 Hz, CH), 7.24 (t, 1H, J 7.4 Hz, CH), 7.10 (d, 1H, J 7.4 Hz, CH), 6.97 (d, 1H, J 7.4 Hz, CH), 6.76 (brs, 1H, CH), 6.65 (brd, 1H, J 7.7 Hz, CH), 5.91 (m, 1H, CH), 5.67 (s, 2H, NCH₂), 5.04 (s, 2H, OCH₂), 5.03 (m, 2H, CH₂), 4.91 (s, 2H, SCH₂), 3.83 (s, 3H, OCH₃), 3.26 (d, 2H, J 6.6 Hz, CH₂), 2.93 (s, 3H, CH₃), 2.46 (s, 3H, CH₃), 2.39 (s, 3H, CH₃), 2.01 (m, 1H, CH), 1.76 (m, 1H, CH). HR-ESI-MS [M + H]+ Found: 383.1557, Calc. for C₃₀H₂₇N₂O₄: 383.1557; [M + Na]+ Found: 783.2847, Calc. for C₃₀H₂₆Na₁N₂O₄: 783.2861.
provided by the Banco de Céulas do Rio de Janeiro, Brazil (BCRJ). Macrophages were maintained in Dulbecco's Modified Eagle Medium (DMEM) and HepG2 in DMEM/F-12, which were supplemented with 10% fetal bovine serum and incubated at 37 °C with a 5% CO₂ atmosphere until reaching the confluence about 70-90%. Macrophages were dissociated using a cell scraper, and HepG2 cells were trypsinized and counted in a Neubauer chamber to obtain the required concentration for the cytotoxicity assay.

2.5.2. Basal Cytotoxicity

Aliquots (0.2 mL) of the medium containing the RAW cell suspension were seeded into 96-well tissue-culture plates at 10⁵ cells mL⁻¹. Then, the plates were incubated at 37 °C under 5% CO₂ for 24 h for adherence. After this period, the medium was removed, and the cells were treated with medium modified with various concentrations of eugenol and eugenol derivatives (50-400 µg mL⁻¹) or unmodified medium (control) and incubated for 24 h under the same conditions. Afterwards, the medium was removed, and the plates were prepared for the MTT-tetrazolium assay [35]. Tebuconazole and carbendazim were used as controls to compare their cytotoxic effect. The assays were performed in triplicate and repeated at least three times.

2.5.3. Cytotoxicity in Metabolic Activation System (S9 fraction)

Aliquots (0.2 mL) of the medium containing HepG2 cells at the concentration 3x10⁴ cells mL⁻¹ were seeded into 96-well tissue-culture plates and incubated at 37 °C under 5% CO₂ for 24 h for adherence. After this time, the medium was removed, and the cells were treated with a modified medium with various concentrations of eugenol and its derivatives, with or without the S9 system. The system consisted of 10% S9 fraction, 1% 0.4 M MgCl₂, 1% 1.65 M KCl, 0.5%, 1 M d-glucose-6-phosphate disodium, 4% 0.1 M β-nicotinamide adenine dinucleotide, 54% 0.2 M phosphate buffer, and 29.5% sterile distilled water. After incubation for 24 hours at 37 °C under 5% CO₂, the medium was removed and the basal cytotoxicity and metabolism-mediated cytotoxicity were evaluated using an MTT-tetrazolium assay. Tebuconazole and carbendazim were used as benchmarks to evaluate the cytotoxicity, and cyclophosphamide was used as a positive metabolism control. The concentrations ranged from 12.5 to 1600 µg mL⁻¹ for cyclophosphamide and carbendazim, 6.25 to 400 µg mL⁻¹ for eugenol, 3.12 to 200 µg mL⁻¹ for compound 4 and 6.25 to 200 µg mL⁻¹ for tebuconazole. The assay was performed in triplicates and repeated at least two times [36-37].

2.6. Statistical Analysis

Linear regression analysis with 95% confidence limits was used to define a dose-response curve and to compute the inhibitory concentration, that is, the concentration needed to reduce absorbance of the system by 50% (IC50), the so-called cytotoxic midpoint, in both cytotoxic assays. All results were obtained from three independent experiments and expressed as mean ± SD (n=3). Statistically significant differences were determined by two-way analysis of variance with post-hoc Bonferroni test (using the GraphPad Prism 5.0 statistics package). Means were considered statistically significant at p < 0.05.

3. RESULTS AND DISCUSSION

Natural products have been considered as a source for obtaining agricultural fungicides; essential clove oil and eugenol have shown excellent antifungal activity against filamentous fungi, including Fusarium species [16,18-19].

Structural modifications were carried out on the phenol group of eugenol using bimolecular nucleophilic substitution and click chemistry approaches, which resulted in seven derivatives. The synthesis and spectroscopic characterization of compounds 1, 5, and 6 have been described previously [26-27]. Compounds 2-4 and 7 are new and are chemically described here for the first time. Their chemical structures were determined by spectrometric and spectroscopic methods, such as unid and bi-dimensional ¹H and ¹³C NMR, FT-IR, and HR-ESI-MS. For all obtained compounds, the signals observed in the NMR spectra (δ in ppm) related to eugenol moiety were similar.

Compounds 2-4 were obtained through bimolecular nucleophilic substitution reaction.

For compound 2, apart from the signals related to eugenol moiety, a hydrogen singlet (2H) was observed at δ 4.75 ppm bound to carbon at δ 55.8 ppm, indicating etherification with the methylene-cycano group. The carbon related to the cyano group was observed at δ 115.4 ppm. The structure was confirmed by HRESIMS Found: 204.1019, which is consistent with the formula C₁₂H₁₂NO₂.

For compound 3, the data obtained with HRESIMS established the molecular formula to be C₁₃H₁₄O₃S. Hydrogen singlets were observed at 4.68 ppm (2H) and 4.95 ppm (2H), related to benziloyl and methylenesulfonoxides groups, respectively. In the ¹³C NMR spectrum, two methylene groups were observed at δ 89.4 ppm and 70.5 ppm. Four aromatic hydrogens were also observed at 6.70 ppm (d, 7.4 Hz), 7.22 ppm (t, 7.4 Hz), 7.32 ppm (t, 7.4 Hz) and 7.40 ppm (d, 7.4 Hz), confirming the presence of a 1,2-disubstitute ring in the structure. Three additional aromatic hydrogen signals were observed at δ 7.44 ppm (t, 2H, 7.8 Hz), 7.60 ppm (t, 7.8 Hz), and 7.65 ppm (d, 2H, 7.8 Hz), indicating a monosubstituted ring in the structure and confirming the final structure of 3.

With respect to compound 4, the ¹³C NMR spectrum showed 10 signals related to naphthoquinone moiety. Signals observed at δ 178.6 ppm and 177.5 ppm were related to ketone groups. Four additional aromatic hydrogens were observed at 6.72 ppm (dt, 1.5; 7.4 Hz), 7.72 ppm (dd, 1.5; 7.0; 7.4 Hz), 8.01 ppm (dd, 1.5; 7.0 Hz) and 8.18 ppm (dd, 1.5; 7.4 Hz), confirming a 1,2-disubstitute aromatic ring in the structure. The molecular formula C₂₀H₂₂ClO₄ was confirmed by HRESIMS.

Compounds 6-7 were obtained through the click chemistry reaction.

The hydrogen related to the triazole ring was observed at δ 8.31 ppm (s) in compound 6 and at δ 8.15 ppm (s) in compound 7. The carbons of the triazole ring were observed at δ 125.1 ppm and 143.3 ppm and at δ 124.9 ppm and 143.4 ppm for compounds 6 and 7, respectively. The benzyl group was observed at δ 5.80 ppm (s, 2H) and 5.67 ppm (s, 2H) in compounds 6 and 7, respectively. A methylenesulfonoxide group was observed at δ 4.91 (s, 2H) and δ 5.77 ppm in compound 7.

For compound 6, four aromatic hydrogens were observed at δ 7.52 ppm (d, 2H, 8.6 Hz) and δ 8.24 ppm (d, 2H, 8.6 Hz). The molecular formula C₉₀H₃₃N₄O₂ was confirmed by HRESIMS.

For compound 7, four aromatic hydrogens were present at δ 7.04 ppm (d, 7.4 Hz), 7.10 ppm (d, 7.4 Hz), 7.24 ppm (t, 7.4 Hz) and 7.32 ppm (t, 7.4 Hz), confirming the presence of a 1,2-disubstituted ring in the structure. Three additional aromatic hydrogen signals were observed at δ 7.64 ppm (t, 2H, 7.4 Hz), 7.76 ppm (t, 7.4 Hz), and 7.82 ppm (d, 2H, 7.4 Hz), indicating a monosubstituted ring in the structure and confirming the final structure of 7. The molecular formula C₂₇H₂₇N₄O₄S was confirmed by HRESIMS.

3.1. Antifungal Activity

The Fusarium genus is one of the main economic problems in agricultural and food production, causing serious damage to the production and reducing product quality [16]. In black pepper (Piper nigrum L.) crops affected by fusariosis (caused by Fusarium solani f. sp. piperis), there is an annual reduction of 3% in production. This severe disease can reduce a pepper crop's productive cycle to 5 to 6 years, whereas a healthy pepper crop has a productive cycle of 12 years on average [10].
The antifungal activities of eugenol and its derivatives were evaluated using *Fusarium solani* f. sp. *piperis* native isolate. Antifungal activity was evaluated based on minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The fungicides tebuconazole and carbendazim, which are used in agriculture, were included as positive controls. The results, shown in Table 1, indicate that compound 4 was the most active eugenol derivative to affect fungus viability. Compounds with MICs above 400 µg mL⁻¹ were not evaluated for fungicidal activity. According to the results, eugenol exhibited fungistatic activity at 400 µg mL⁻¹.

In the literature, there are several reports of antifungal activity of eugenol or essential clove oil against filamentous fungi of *Fusarium* spp. [18,22,38-39] but only a few reports for *F. solani* f. sp. *piperis*.

Eugenol’s antifungal activity might be due to its high lipophilicity and the presence of functional groups. These characteristics allow this compound to move through the plasma membrane, causing alteration of lipids in the fungal membrane, increasing permeability and consequently, causing cell content extravasation [38-40].

Compound 4 exhibited antifungal activity superior to that of tebuconazole, an important yield since *Fusarium solani* f. sp. *piperis* has shown resistance to tebuconazole compared to other *Fusarium* species, such as *Fusarium oxysporum* [16].

The promising antifungal activity of compound 4 is probably due to the eugenol scaffold and its association with the naphthoquinone moiety in the structure. In the development of agrochemicals, naphthoquinones are extremely useful compounds due to their relatively non-toxic nature and their redox and acid–base properties, which can be synthetically modulated [41-42]. These compounds are recognized by their antimicrobial activity, including activity against filamentous fungi. The addition of halogen groups increases antifungal activity due to the ability to remove electrons and form free radicals [43-44].

**Table 1. Antifungal activity of eugenol derivatives against *F. solani* f. sp. *piperis*.**

<table>
<thead>
<tr>
<th>Compounds</th>
<th><em>Fusarium solani</em> f. sp. <em>piperis</em></th>
<th>MIC (µg mL⁻¹)</th>
<th>MFC (µg mL⁻¹)</th>
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<tr>
<td>Eugenol</td>
<td></td>
<td>400</td>
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<td>1</td>
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<td>&gt;400</td>
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<td>50</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>&gt;400</td>
<td>Nt</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>&gt;400</td>
<td>Nt</td>
</tr>
<tr>
<td>Tebuconazole</td>
<td></td>
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<tr>
<td>Carbendazim</td>
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</table>

Nt (no tested)

**3.2. Docking Studies on Eugenol Derivatives**

Many advances have been made in the study of phytopathogenic fungi at the molecular and cellular levels, enabling the understanding of the mechanisms of interaction between new phytochemicals and phytopathogenic species, such as *Fusarium solani* f. sp. *piperis* [5,45-46]. From this perspective, β-glucosidase proteins from fungi are attractive and promising targets for the design of new antifungal agents to control *Fusarium* disease in plants [5].

β-glucosidase enzyme plays an important role in various biological processes. It occurs in most living organisms and catalyses the hydrolysis of O-glycosidic bonds, releasing glucose units [47]. In the binding site, the glucose chemical structure is in its β-anomeric state, adopting a C1 chair conformation. The glucose molecule is stabilized in a region between the N-terminal domain and a cleft comprised of several loops. The chemical structure of glucose is well stabilized by several hydrogen bonds to the amino acids Asp¹⁷¹, Arg²³⁵, His²⁶⁹, Asp³⁵⁴, and Glu³⁶⁸ with a short distance of 2.6-2.9 Å (Fig. 1).

The docking accuracy was evaluated by redocking, i.e., extracting the co-crystal ligand (glucose) from the protein-ligand complex and performing its placement in the binding site through docking calculations. The resulting docking poses were then compared to the protein-ligand complex by measuring the Root Mean Square Deviation (RMSD), obtaining a value of 0.37 Å for the top docked pose and an average RMSD of 1.168 Å for the other docking poses from this calculation (Fig. 2).

![Fig. (1). Major amino acids involved in the β-glucosidase binding site of *F. Solani*: Asp¹⁷¹, Arg²³⁵, His²⁶⁹, Asp³⁵⁴, and Glu³⁶⁸. (A higher resolution / colour version of this figure is available in the electronic copy of the article).](image)

![Fig. (2). Redocking of glucose in the generated model of β-glucosidase from *F. solani*. RMSD deviation at 0.37 Å and average RMSD = 1.168 Å. (A higher resolution / colour version of this figure is available in the electronic copy of the article).](image)
Table 2. Docking results of selected compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Docking Score</th>
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<td>85.884</td>
</tr>
<tr>
<td>4</td>
<td>67.812</td>
</tr>
<tr>
<td>6</td>
<td>78.652</td>
</tr>
<tr>
<td>7</td>
<td>91.353</td>
</tr>
</tbody>
</table>

Fig. (3). Docking results of the evaluated compounds: a) eugenol; b) Compound 1; c) Compound 2; d) Compound 3; e) Compound 4; f) Compound 6; g) Compound 7. (A higher resolution / colour version of this figure is available in the electronic copy of the article).
The evaluated compounds placed properly at the β-glucosidase active site, extending its docking from the catalytic core to a channel comprised of aromatic residues, conferring an array of π-π interactions (Fig. 4). The docking score also indicated a potential for binding with score values ranging from 56 to 91 (Table 2), whereas the glucose top-ranked docking poses range from 57 to 62.

The docking results indicated the potential for eugenol to form hydrogen bonds with the amino acids Lys28 (3.3 Å), Ser170 (3.0 Å), and Asp171 (2.6 Å). Part of its backbone is also stabilized by a π-interaction with Trp175 (Fig. 4a). Compound 1 is well stabilized by two additional π-stacking interactions with the amino acids Tyr328 (3.2 Å) and Trp300 (3.4 Å). Compounds 2, 3, and 6 presented an allyl-methoxy benzene ring system stabilized by π-stacking interactions, mainly with the amino acids Tyr328 and Trp300, ranging from 3.2 to 5.9 Å. The polar groups are stabilized by hydrogen bonds with nearby histidine and arginine residues (Fig. 3).

The analysis of the docking poses at the β-glucosidase binding site of compound 4 indicates that its naphthoquinone ring system could be stabilized by a cation-π interaction with Arg177 residue followed by a hydrogen bond with Glu306. Compound 4 is also stabilized by π-stacking interactions in the allyl-methoxy benzene moiety with Trp300 and Tyr328 residues. These interactions could result in enhanced stability compared to the other evaluated compounds (Fig. 4f). The docking results suggest a potential for the evaluated compounds to bind to the β-glucosidase binding site, highlighting that the activity of compound 4 could be enhanced by the additional interactions it can produce at the active site.

### 3.3. Cytotoxic Effects of Eugenol Derivatives

The use of agrochemicals as fungicides may result in exposure of the environment and humans to these chemicals. Humans could be exposed during production or application, or through the consumption of food products contaminated with residues of these chemicals [48].

The toxic profile of tebuconazole has been investigated by the European Food Safety Authority. Studies have shown effects on the endocrine organs, including hypertrrophy and malformations of the adrenal glands, leading it to be classified as a developmental toxin [49].

Regarding carbendazim, this fungicide has been banned in the USA, most of the European Union and Australia due to its severe toxicity and persistent nature. Repeated applications of it lead to accumulation and contamination of various ecosystems, with long-lasting impacts on soil sustainability and human and animal health. However, developing countries, such as China, Brazil, and India, are still permitting its production and use [14].

Thus, in vitro models have been used to study the cytotoxicity pattern of new drugs or other foreign compounds [48].

In this study, the basal cytotoxicity of compounds that presented antifungal activity was evaluated on a macrophage lineage to investigate their safety for use. The IC50 values, i.e., the compound concentrations that inhibit 50% of cell growth, are shown in Table 3. Values mean ± standard deviation of two distinct experiments performed in triplicate. IC50 (concentration of tested compounds that reduce cell viability by 50%); Raw 264.7 (lineage of murine macrophages). Significantly different at level of \( p < 0.05 \) according to Bonferroni’s test.

Eugenol showed cytotoxicity to macrophages at the highest concentrations (200-400 µg mL-1) however, it was less cytotoxic than tebuconazole at these concentrations (\( p<0.05 \)). These results confirm that the cytotoxicity and antifungal activity of eugenol are related to the same structural patterns, mainly due to the presence of a double bond in the allyl chain [38].

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Raw 264.7 IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eugenol</td>
<td>100.8 ± 0.79**</td>
</tr>
<tr>
<td>4</td>
<td>21.61 ± 0.23'</td>
</tr>
<tr>
<td>Tebuconazol</td>
<td>23.83 ± 0.72'</td>
</tr>
<tr>
<td>Carbendazim</td>
<td>25.98 ± 0.07'</td>
</tr>
</tbody>
</table>

Compound 4 showed a cytotoxicity profile similar to that of tebuconazole. Most likely, this is due to the presence of electrophilic carbons and the quinone nucleus from the naphthalene ring, which are capable of inducing the formation of deleterious radicals through redox cycles [50-52].

The variation in substitution of the eugenol molecule and the change in structural patterns could explain the observed differences in cytotoxicity results, as observed by Bar et al. [53] and Xie et al. [16].

The xenobiotic-metabolizing capacity is a central point for in vitro/in vivo comparisons in toxicity evaluation. The bioactivation is an important consideration for in vitro cytotoxicity assays, since the compound test may be biotransformed in vivo [54].

Thus, in vitro methods using the S9 system have been used as an important tool to study the biotransformation patterns of chemicals [55-56]. The use of the S9 fraction from liver is the most appropriate screening system compared to hepatocytes and microsomes. The S9 fraction includes the phase I and II metabolic enzymes, and it is relatively inexpensive, easy to use, and amenable to automation. Microsomes report phase I metabolism only, whereas hepatocytes are not easily automated [56].

In this study, we evaluated the cytotoxicity of antifungal active compounds on hepatic cells (HepG2) in the presence and absence of the S9 metabolic system. Dose-response curves for compounds and standards are shown in Fig. 4.

The cyclophosphamide results confirm the assay efficiency in the cytotoxic evaluation post-metabolization. The dose-response curves obtained before and after the metabolic activation of tebuconazole were similar, while carbendazim was bioactivated in the presence of the S9 system, resulting in an IC50 value 7 times lower than that of carbendazim in the absence of S9. Eugenol presented changes in dose-response curves before and after metabolization, with an increase in cytotoxicity of about 2 times (Fig. 5). The IC50 values of compound 4 in the presence and absence of S9 system were not statistically significantly different (\( p<0.05 \)). Table 4 shows the IC50 values of the compounds in the absence and presence of the metabolizing system.

The results obtained with eugenol exposure to the S9 hepatic fraction were similar to those found by other authors. They reported increased eugenol cytotoxicity in the presence of the hepatic S9 fraction of rats or hamsters in hepatoma cells [57-58]. Babich et al. [57] reported that eugenol’s cytotoxicity after S9 exposure is related to depletion of the intracellular glutathione level. Maralhas et al. [59] also showed that eugenol, in the presence of S9, exhibited increases in cytotoxicity and genotoxicity.

Compound 4 metabolism may be associated with the detoxification of naphthoquinones by quinine oxidoreductase enzyme (QR). According to Cuendet et al. [60], the reduction of electrophilic quinones by the QR enzymes is an important detoxification...
route, converting quinines to hydroquinones. However, no significant difference in IC50 value was observed in compound 4 cytotoxicity in the presence and absence of the S9 system, indicating that either this compound was not metabolized or it was metabolized into another compound with the same cytotoxic profile.

In recent years, tebuconazole toxicity has been reported as having hepatotoxic effects, causing long-term hepatocellular tumors, liver weight increase, and centrilobular hypertrophy in rats and mice [49,61-62]. Carbendazim toxicity has also been reported. It is known to manifest embryotoxicity, germ cell apoptosis, teratogenesis, infertility, and developmental toxicity in different mammalian species [14].

In contrast with the toxicity reported for tebuconazole and carbendazim, demonstrating hazards to human health primarily due to hepatotoxic effects [14,49,61,63], compound 4 is a promising candidate for further studies and the development of a new agricultural fungicide against *Fusarium solani f. sp. piperis*.

Future investigations aimed at mechanisms of action and genotoxic activity are necessary for a better understanding of the cytotoxic effects and for the determination of the potential of the compounds reported in this work as future agricultural fungicides. Considering that other heteroaromatic ring-containing compounds displayed biomedical potential due to their ability to interact with nucleic acids [64-66], also eugenol derivatives are interesting candidates as therapeutics that deserve future exploration of their DNA and RNA-binding ability.
Table 4. Inhibitory concentration (IC50) values of samples on human hepatocarcinoma cell line (HepG2) in the presence and absence of the S9 hepatic system.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>HepG2 IC50 (μg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- S9</td>
</tr>
<tr>
<td>Eugenol</td>
<td>257.9 ± 0.4*</td>
</tr>
<tr>
<td>Compound 4</td>
<td>11.8 ± 0.5*</td>
</tr>
<tr>
<td>Tebuconazole</td>
<td>34.94 ± 0.20*</td>
</tr>
<tr>
<td>Carbendazim</td>
<td>251.0 ± 0.30*</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>412.7 ± 0.17</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SD of up to two separate experiments performed in triplicate. *p<0.05 (ANOVA) indicates significantly different results between cyclophosphamide (+ S9) and the samples. † p<0.05 (ANOVA) indicates significantly different results between the samples in the presence of the metabolizing system (+ S9) and in the absence (-S9). ‡ p<0.05 (ANOVA) indicates significantly different results between carbendazim and eugenol derivative in the presence of the metabolizing system (± S9). §p<0.05 (ANOVA) indicates significantly different results between tebuconazole and eugenol derivative in the presence of the metabolizing system (+ S9). <p>CONCLUSION

It was possible to demonstrate that structural modifications of the eugenol molecule resulted in one effective compound. Compound 4 showed promising fungicidal activity against Fusarium solani f. sp. piperis. In addition, compound 4’s cytotoxic activity was similar to that of tebuconazole and was more stable than that of carbendazim after metabolism. Thus, this compound is the most promising for further optimization due to its antifungal potency being greater than that of eugenol and tebuconazole and its acceptable cytotoxicity profile.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPORTIVE/SUPPLEMENTARY MATERIAL

Supplementary information (IR, NMR, and HRMS) is available as a PDF file.

RESEARCH INVOLVING PLANTS

The cloves used in this work were bought at a street market in the city of Alegre, Espírito Santo State, Brazil. As it is edible food, there was no need for authorizations for its purchase.

Experimental studies of the plant complied with institutional, national and international guidelines.

REFERENCES

[15] Di Renzo F, Bacchetta R, Bizzo A, Giavini E, Menegola E. Is the amphibian X. laevis WEC a good alternative method to rodent WEC teratogenicity assay? The example of the three triazole de-


