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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Antimicrobial and cytotoxic evaluation of eugenol derivatives

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Abstract Eugenol is the major phenolic component of clove essential oil and it has been used in medical and dental practice for its properties like analgesic, local anesthetic, and antioxidant. It is known that eugenol can denature proteins and react with cell membrane phospholipids changing their permeability and inhibiting a great number of Gram-negative and Gram-positive bacteria as well as different types of yeast. Eugenol has ever demonstrated antimicrobial properties; thus, the search for the optimization through structural changes appears to be interesting for the development of new antimicrobials. This study aimed to evaluate the antimicrobial activity and cytotoxic characteristics of eugenol analogs. From natural eugenol, 14 derivatives were obtained by typical acylation and alkylation. Their antimicrobial activity was evaluated by the broth microdilution method. The compounds were assessed against *Staphylococcus aureus* ATCC 19095, *Enterococcus faecalis* ATCC 4083, *Escherichia coli* ATCC29214, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 62342 and the following clinical isolates from the human oral cavity: *C. albicans* (**3**), *C. parapsilosis C. glabrata* *C. lipolytica*, and *C. famata*. Cytotoxicity against mouse embryonic fibroblast (NIH/3T3) cell line was evaluated by MTT colorimetric assay. The majority of compounds demonstrated significant antimicrobial activities. In general, the compounds presented very low or no cytotoxicity, with an inhibitory ratio lower than 50 % against NIH/3T3 cell line.

Keywords Eugenol · Antibacterial activity · Antifungal activity · Cytotoxic activity

Introduction

A matter of concern in public health in recent decades is the increasing resistance of bacteria and fungi to antimicrobial drugs, especially among immunocompromised hosts, having an enormous impact on morbidity and mortality. A matter of concern in the treatment of infections is the limited number of efficacious drugs and, in addition, they possess a certain degree of toxicity and develop quickly resistance due to the large-scale use (Katz et al., 2006; Palaniappan and Holley, 2010; Gündoğdu et al., 2010). Thus, the continuing need for novel therapeutic compounds is still of urgent concern in a number of new diseases and resistant strains of microorganisms (Palaniappan and Holley, 2010; Tekwu et al., 2012; Ritter et al., 2014; Ritter et al., 2015).

Nature is an amazing source of chemical diversity, and, as a consequence, naturally derived compounds have unique pharmacological properties. This is easily observed if we consider that nearly 60 % of all drugs introduced in therapy between 1981 and 2006 were first identified as natural products. (Newman and Cragg, 2012).

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Plants are among the most important sources of medicines. They are known to offer a large and attractive phytochemical repertoire for the discovery of novel microbial disease control agents (Phatthalung et al., 2012). The potential of these phytochemicals in managing infectious diseases has attracted considerable interest among the scientific community, and numerous research institutions around the world have conducted studies on the antimicrobial properties of medicinal plants (Duarte et al., 2005; More et al., 2008; Phatthalung et al., 2012; Tekwu et al. 2012; Chander et al., 2016; Shad et al., 2016).

The antimicrobial activities of several plant-derived essential oils have been demonstrated, and a variety of active components in these oils has been identified (Kavanaugh and Ribbeck, 2012). Eugenol (4-allyl-2-metoxyphenol), the major phenolic component of clove essential oil, has been long known in medical and dental practice for its analgesic, local anesthetic, anti-inflammatory, antioxidant, antibacterial, antifungal, and antiatherogenic properties (He et al., 2007; Thosar et al., 2013; D'Avila Farias et al., 2014; Hemalatha et al., 2015; Venkadeswaran et al., 2016). It is well known that eugenol can denature proteins and react with cell membrane phospholipids changing their permeability and inhibiting a great number of Gram-negative and Gram-positive bacteria as well as different types of yeast (Walsh et al., 2003; He et al., 2007; Chaieb et al., 2007; Nuñez and D'Aquino, 2012).

Eugenol has ever exhibited antimicrobial properties; thus, the search for optimization through structural changes appears to be interesting for the development of new antimicrobials. We report here the antimicrobial activity and cytotoxic characteristics of eugenol analogs.

Materials and methods

Chemical data on synthesized compounds

The structural elucidation of eugenol derivatives were performed by ^{1}H NMR, ^{13}CNMR and CG–MS spectral data (D'Avila Farias et al., 2014).

Synthesis of eugenol derivatives 11–18

Eugenol (10 mmol) was dissolved in the solution of NaOH (12 mmol). The solution was placed in a flat-bottomed flask, and the chloride derivative (10 mmol) was added. The mixture was stirred at room temperature on agitation for 30 min. After consumption of the starting material the reaction medium was extracted with dichloromethane (3 × 10 mL) and washed with Na_2CO_3 5 %. The solvent was dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The pure products were obtained by recrystallization from ethanol.

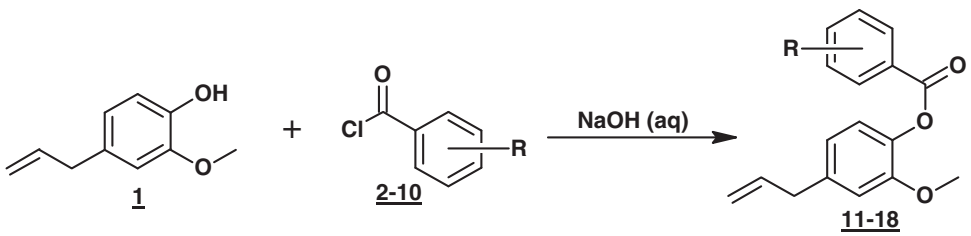
Synthesis of eugenol derivatives 26–31

In one flask, K_2CO_3 (20 mmol), dissolved dried acetone (40 mL) and eugenol (10 mmol) were added, followed by the addition of alkyl chloride (10 mmol), and stirred at reflux for 2 h. After complete consumption of the starting material (TLC), the reaction was cooled, filtered, and washed with acetone (20 mL). The organic solvent was evaporated under vacuum. The final product was resuspended in dichloromethane (50 mL) and washed with H_2O (2 × 25 mL). The organic extract was dried under anhydrous Na_2SO_4 and the residual solvent was evaporated in vacuum. Pure product was obtained by column (*n*-Hexane: AcOEt gradient). (Scheme 1, Scheme 2)

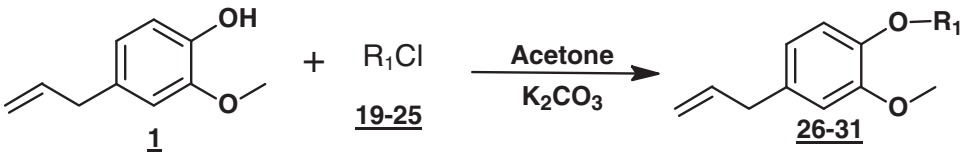
Antimicrobial screening

The in vitro antimicrobial activity of each compound and the standard drugs was determined against two representatives of Gram-positive bacteria, *Staphylococcus aureus* ATCC 19095 and *Enterococcus faecalis* ATCC 4083; two Gram-negative bacteria, *Escherichia coli* ATCC29214 and *Pseudomonas aeruginosa* ATCC 9027; and eight strains representing five different species of yeasts—*Candida albicans* ATCC 62342 and clinical strains from the human oral cavity of the *C. albicans* (3), *C. parapsilosis*, *C. glabrata*, *C. lipolytica*, and *C. famata*—by the broth microdilution method according to the guidelines of the National Committee for Clinical and Laboratory Standards for yeasts (M27-A3) and for bacteria (M7-A7).

Scheme 1 Reaction of obtaining derivatives **11–18**



Scheme 2 Reaction of obtaining derivatives **26–31**



Microbial strains were primarily inoculated for overnight growth. A number of colonies were directly suspended in saline solution until the turbidity matched the turbidity of 0.5 McFarland standard and adjusted to give a final microorganism of 5×10^5 CFU mL^{-1}. Mueller-Hinton broth (BD, Sparks, MD, USA) was used as a nutrient medium to grow and dilute the compound suspension for the test bacteria, and RPMI-1640 (Sigma, St Louis, MO, USA) buffered to pH 7.0 with MOPS was used for fungal nutrition. Dilutions of tetracycline and fluconazole were used as reference compounds for comparison of data between independent experiments and as indicators for assessing the relative level of inhibition of the samples tested. Dimethyl sulfoxide (DMSO) was used as diluents/vehicle to get the desired concentration of the compounds and standard drugs.

Diluted samples were serially transferred in duplicate to 96-well microplates and 100 µL microbial inoculum were added to achieve a final volume of 200 µL and concentrations ranging from 1 to 500 µg mL^{-1}. The final concentration of DMSO in the assay did not exceed 0.5 %. Controls of the microbial viability, sterility of the medium, and sterility the extract were also carried out.

The plates were incubated at 37 °C for 24 h for bacteria and 48 h for fungi. Plates were read at 630 nm for bacteria or 590 nm for fungi, prior to and after incubation and the results were expressed as the percentage of activity (%AE) with the formula $\%AE = 100-(AE-AEB/AC-ACB) \times 100$, where AE represents the "absorbance of the test plates after the incubation time"; AEB is the "absorbance of plates containing medium, sample and inoculum at t = 0 of incubation"; AC is the "absorbance of plates containing negative control (100 % of inoculum growth)"; and ACB is the "absorbance of plates containing culture medium" (Stein et al., 2011).

The antimicrobial effect was characterized by IC_{50} values, the concentration that affords 50 % inhibition of bacterial/fungal growth relative to the growth control; and MIC values, the minimal concentration of a substance that completely inhibits the bacterial and fungal growth. IC_{50} values were determined from logarithmic graphs of growth inhibition vs. concentration using Graph Pad prism software. After determining the MIC, the minimal microbicidal concentration (MMC) was done. Aliquots of 20 µL of the wells were plated on Mueller Hinton Agar for bacteria or Sauboraud Dextrose Agar for fungi and incubated at 37 °C for 24 h. MMC was defined as the lowest concentration of each compound that resulted in no cell growth on the surface of the plates.

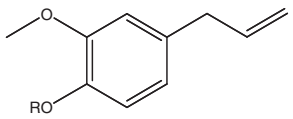
Cytotoxicity assay

The in vitro cytotoxicity of eugenol derivatives was determined against NIH/3T3 cells. The cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil) and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO, USA), supplemented with 10 % fetal bovine serum (FBS), purchased from Vitrocell Embriolife (Campinas, Brazil) and Gibco (Grand Island, NY, New York, USA), respectively. Cells were grown at 37 °C in an atmosphere of 95 % humidified air and 5 % CO_2, as described previously (Nedel et al., 2012; Santana et al., 2012). The experiments were performed with cells in the logarithmic phase of growth.

The viability of the NIH/3T3 cells was determined by measuring the reduction of soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to water-insoluble formazan (Henn et al., 2011). Briefly, the cells were seeded at a density of 2×10^4 cell per well in a volume of 100 µL in 96-well plates and grown at 37 °C in a humidified atmosphere of 5 % CO_2/95 % air for 24 h before being used in the MTT assay. Cells were incubated with different concentration of compounds for 24 and 48 h. These compounds were dissolved in DMSO and added to the DMEM supplemented with 10 % FBS to the desired concentrations. The final DMSO concentration in the culture medium never exceeded 0.5 % and a control group exposed to an equivalent concentration of DMSO was evaluated.

After incubation, the media was removed and 180 µL of DMEM and 20 µL MTT (5 mg MTT mL^{-1} solution) were added to each well. The plates were incubated for an additional 3 h, and the medium was discarded. 200 µL of DMSO was added to each well, and the formazan was solubilized on a shaker for 5 min at 100×g. The absorbance of each well was read on a microplate reader (MR-96A, Mindray Shenzhen, China) at a wavelength of 492 nm. Cell inhibitory growth was determinate as follows: inhibitory rate = $(1-Abs_{492\text{treated cells}}/Abs_{492\text{control cells}}) \times 100$ % (Nedel et al., 2012). All observations were validated by at least two independent experiments, and for each experiment the analyses were performed in triplicate.

Table 1 In vitro antimicrobial activities of compounds and reference substances against human pathogens



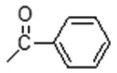
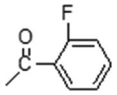
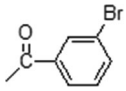
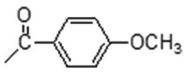
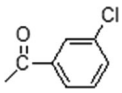
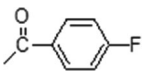
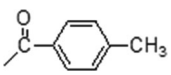
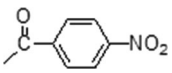
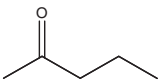
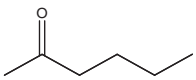
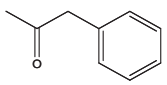
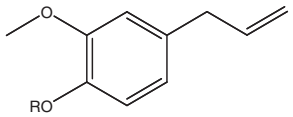
Compound	R	Assay	Microbial strains										
		(μg mL^{-1})	*C.a*	*C.a*1	*C.a*2	*C.a*3	*C.p*	*C.g*	*C.l*	*C.f*	*S.a*	*E.c*	*P.a*	*E.f*
11		IC_{50}	52,01	86,94	125.60	121.30	46.91	72.16	101.60	78.07	147.8	202.6	150.4	98.48
		MIC	_	_	_	_	_	_	_	_	250	500	250	250
		MMC	nd	nd	nd	nd	nd	nd	nd	nd	_	_	_	_
12		IC_{50}	75.15	80.06	165.60	84. 65	67.97	30.76	_	146.20	_	_	_	_
		MIC	_	_	_	_	125	500	_	250	_	_	_	_
		MMC	nd	nd	nd	nd	250	500	nd	250	nd	nd	nd	nd
13		IC_{50}	46.75	47.11	77.60	40.40	68.65	51.77	29.96	95.51	39.3	_	58.35	39.17
		MIC	500	250	500	500	125	250	125	250	_	_	_	_
		MMC	_	500	_	_	250	250	125	250	nd	nd	nd	nd
14		IC_{50}	398	_	401.40	398.42	_	156.30	_	_	_	_	_	_
		MIC	_	_	_	_	_	_	_	_	_	_	_	_
		MMC	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
15		IC_{50}	48.26	51.58	48.36	35.43	56.86	60.21	30.27	88.46	45.26	_	59.11	38.06
		MIC	500	250	500	500	125	125	62.5	125	_	_	_	_
		MMC	_	500	_	_	250	125	125	125	nd	nd	nd	nd
16		IC_{50}	_	_	_	_	_	_	_	_	_	_	_	_
		MIC	_	_	_	_	_	_	_	_	_	_	_	_
		MMC	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
17		IC_{50}	97.22	84.34	78.16	156.80	96.59	72.86	72.15	80.71	_	_	_	_
		MIC	_	_	500	500	500	500	125	250	_	_	_	_
		MMC	nd	nd	_	_	_	_	250	250	nd	nd	nd	nd
18		IC_{50}	_	_	_	_	_	_	_	_	1.92	_	_	25.84
		MIC	_	_	_	_	_	_	_	_	125	_	_	125
		MMC	nd	nd	nd	nd	nd	nd	nd	nd	125	nd	nd	125
26		IC_{50}	_	_	_	_	_	_	_	_	_	_	_	_
		MIC	_	_	_	_	_	_	_	_	_	_	_	_
		MMC	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
27		IC_{50}	_	_	_	_	_	_	_	_	21.84	_	_	27.73
		MIC	_	_	_	_	_	_	_	_	125	_	_	125
		MMC	nd	nd	nd	nd	nd	nd	nd	nd	125	nd	nd	125
28		IC_{50}	277.20	_	389	_	43.58	341	36.78	30.40	_	_	_	_
		MIC	_	_	_	_	500	_	125	125	_	_	_	_
		MMC	nd	nd	nd	nd	500	nd	125	125	nd	nd	nd	nd
29	$-CH_2CH_2CH_2Ph$	IC_{50}	_	_	_	_	205.10	_	14.58	13.98	_	_	_	_
		MIC	_	_	_	_	_	_	250	500	_	_	_	_
		MMC	nd	nd	nd	nd	nd	nd	500	500	nd	nd	nd	nd
30	$-CH_2CH_2CH_3$	IC_{50}	_	_	_	_	_	_	_	_	_	_	_	_
		MIC	_	_	_	_	_	_	_	_	_	_	_	_
		MMC	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
31	$-CH_2CH_2CH_2CH_3$	IC_{50}	_	_	_	_	225.90	63.24	78.16	211.80	_	_	_	_

Table 1 continued



Compound	R	Assay	Microbial strains										
		(μg mL^{-1})	*C.a*	*C.a*1	*C.a*2	*C.a*3	*C.p*	*C.g*	*C.l*	*C.f*	*S.a*	*E.c*	*P.a*	*E.f*
		MIC	_	_	_	_	500	500	500	500	_	_	_	_
		MMC	nd	nd	nd	nd	500	500	500	500	nd	nd	nd	nd
Reference Substances														
Eugenol	IC_{50}	134.8	437.5	334.3	11.4	20	222.8	139.9	149.1	295.1	422.9	294.8	_
	MIC	250	500	500	500	500	500	250	250	500	_	500	_
	MMC	500	500	500	_	500	_	500	250	_	nd	_	nd
Fluconazole	IC_{50}	5.3	21.2	<1	<1	<1	14.6	<1	<1	nt	nt	nt	nt
	MIC	_	_	1.9	31.25	3.9	125	3.9	3.9	nt	nt	nt	nt
	MMC	nd	nd	62.5	31.25	7.8	250	7.8	3.9	nt	nt	nt	nt
Chloramphenicol	IC_{50}	nt	nt	nt	nt	nt	nt	nt	nt	2.1	148.7	1.92	1.94
	MIC	nt	nt	nt	nt	nt	nt	nt	nt	3.9	250	3.9	3.9
	MMC	nt	nt	nt	nt	nt	nt	nt	nt	62.5	500	31.3	31.3
Tetracycline	IC_{50}	nt	nt	nt	nt	nt	nt	nt	nt	<1	36.4	<1	<1
	MIC	nt	nt	nt	nt	nt	nt	nt	nt	<1	62.5	<1	<1
	MMC	nt	nt	nt	nt	nt	nt	nt	nt	3.9	500	3.9	15.6

(–) > 500 μg mL^{-1}, *nd* not determined, as MIC was greater than 500 μg mL^{-1}, *nt* not tested

Microbial strains: *C.a Candida albicans ATCC 62342*, *C.a1 Candida albicans clinical isolate 1*, *C.a2 Candida albicans clinical isolate 2*, *C.a3 Candida albicans clinical isolate 3, C.p Candida parapsilosis clinical isolate*, *C.g Candida glabrata clinical isolate*, *C.l Candida lipolytica clinical isolate*, *C.f Candida famata clinical isolate*, *S.a Staphylococcus aureus ATCC 19095*, *E.c Escherichia coli ATCC29214*, *P.a Pseudomonas aeruginosa ATCC 9027*, *E.f Enterococcus faecalis ATCC 4083*

Results

Regarding the antibacterial activity, compounds **11**, **18**, and **27** were the most active compounds, with better results than those of eugenol (Table 1). The compound **11** have shown bacteriostatic activity against all the four bacterial strains, i.e., *S. aureus*, *E. coli*, *P. aeruginosa*, and *E. faecalis*. Compounds **18** and **27** exhibited bactericidal activities only against gram-positives strains.

The eugenol derivatives **13** and **15** exhibited antifungal activities against all tested strains and, in addition, they showed not only fungistatic but also fungicidal activity (Table 1). Those compounds were actives against the fluconazole-resistant strains *C. albicans* ATCC 62342 and *C. albicans* clinical isolate 1. Although the IC_{50} values were higher compared to those of fluconazole, the MIC was better, with values of 500 and 250 μg mL^{-1} against *C. albicans* ATCC 62342 and *C. albicans* clinical isolate 1, respectively. These values are >500 μg mL^{-1} for fluconazole. Compound **15** exhibited activity equal to fluconazole against *C. glabrata* (MIC and MMC 125 μg mL^{-1}). Furthermore, this compound showed better results than eugenol against all Candida non-albicans strains tested.

Compound **17** demonstrated activity against all tested fungal strains, except fluconazole-resistant strains *C. albicans*. Compounds **12**, **28**, **29**, and **31** have presented better activities against Candida non-albicans strains. In addition, compound **12** was better than eugenol against *C. parapsilosis,* and compound **28** was better than eugenol against *C. lipolytica* and *C. famata.* Compounds **14**, **16**, **26**, and **30** showed very low or no activity against microbial strains tested at concentrations up to 500 μg mL^{-1}.

The results from the cytotoxicity screening of the eugenol derivatives against the NIH/3T3 cell line are shown in Fig 1. In general, the compounds presented very low or no cytotoxicity against the cells.

Discussion

In this study, eugenol derivatives were synthesized attempting to find compounds with antimicrobial action.

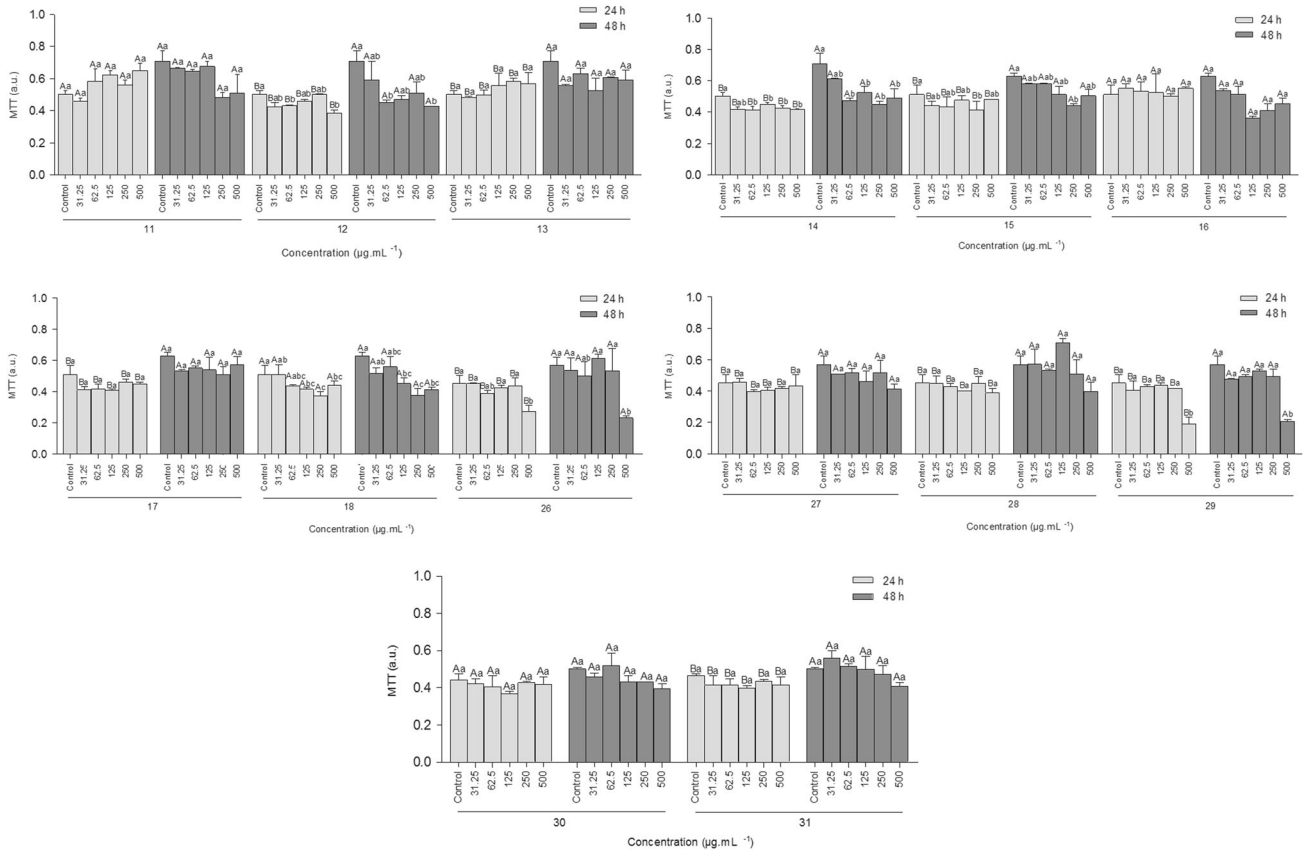


Fig. 1 Effect in the inhibition of NIH/3T3 cell line exposed to 24 and 48 h of different concentrations of Eugenol derivatives. Data are expressed as means ± SD. *Uppercase letters* indicate significant differences between times of exposure and *lowercase letters* indicate significant differences between the concentrations tested. A p-value < 0.05 was considered significant (Tukey test)

Some of these compounds have already been tested for other activities such as 15-lipoxygenase inhibitors, herbicidal (Cutler et al., 2002) and antioxidant agents (D'Avila Farias et al., 2014).

The compounds were synthetized with different modifications in the hydroxyl group at 1-position of eugenol. These modifications conferred different physical-chemical characteristics on the lateral chain of the compounds obtained. Differences in size and in the nature of the carbon chain (aliphatic or aromatic) cause stereo-electronic variations that help identify characteristics needed to increment the activities of these molecules.

Campaniello et al. (2010) found that eugenol at concentrations = 100–150 µg mL^{-1} is an effective antifungal compound against phytopathogenic *Aspergillus*, *Penicillium*, *Emericella*, and *Fusarium* spp., suggesting that this activity could be attributed, in part, to the presence of a phenolic group. Gill and Holley (2006) proposed that the antibacterial mechanism of action of eugenol is the disruption of the cytoplasmic membrane, which could be due to the fact that the phenolic hydroxyl group might increase the solubility of this molecule in aqueous suspensions improving the ability to pass through the hydrophilic portion of the cell envelope. In contradiction, Voda et al. (2004) found that the best antifungal activities were displayed by the most hydrophobic phenylpropanoid derivatives that possess a higher ability to penetrate the walls of fungal cells than the hydrophilic ones. In accord, we demonstrate in this work the phenolic group does not seem essential to both antifungal and antibacterial activities, since most active compounds hydroxyl group is replaced by aryl or alkyl moiety. Indeed, these substances have a better pathogen cell penetration leading to desirable accumulation in these cells, as shown by others researchers (Shin and Pyun, 2004; Fontenelle et al., 2011).

The derivatives that presented promising antifungal activities, in general, have aryl group substituting the hydroxyl group of eugenol. Furthermore, aryl group with halogen atom in meta-position appear to be particularly interesting, since that compounds **13** and **15** demonstrated activity against all the fungal strains tested, including the fluconazole-resistant samples. Presence of halogen atoms bonded to molecule provide an additional lipophilicity and polarizability. These features are important to improve

antimicrobial activities, as cited by different works (Fontenelle et al., 2011; Zengin, 2011). Moreover, although MIC/MMC values $> 69\,\mu g\ mL^{-1}$ are considered toxic for fluconazole (CLSI, 2008), those compounds did not display any cytotoxic effects at concentrations up to $500\,\mu g\ mL^{-1}$.

Regarding the antibacterial activities of compounds, the presence of aryl group substituting the hydroxyl group of eugenol also appear to favor the activity. In addition, a presence of NO_2 in para-position increased the specificity against gram-positive strains, with no activity against gram-negative strains at concentrations up to $500\,\mu g\ mL^{-1}$. The high antibacterial activity of compound **18** may be assigned to the presence of nitro functional group. These electron with drawing groups may decrease electron density on benzene ring resonance effect assisting to the higher effect (Saeed et al., 2010; Halim et al., 2012). Furthermore, in agreement with our results, Halim et al., 2012 have shown that nitrobenzene derivatives were active only in gram-positive bacteria.

In general, eugenol derivatives tested were found to have no cytotoxic effect on the normal cell line, demonstrating the potential to selectively kill microorganism cells but do little damage to normal cells. Although interesting, our results are still preliminary and further research is necessary to better understand their antimicrobial structure–activity relationship.

We have synthesized eugenol derivatives with potential antimicrobial activity. The compounds were tested for their antimicrobial activities and most of them showed significant activities. In addition, they presented very low or no cytotoxicity against NIH/3T3 cell line.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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