

Antifungal Activity and *in Silico* Toxicology of the *O*-Eugenol

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ABSTRACT

The frequent and indiscriminate use of antifungal drugs corroborates to the emergence of highly resistant micro-organisms, forcing the research and development of new compounds with fungicide activity. Among these are the phenylpropanoids, in particular the *o*-eugenol in, which its antifungal activity and *in silico* toxicology was evaluated in this study. The MIC and the MFC of the product against *C. albicans* LM 35 and ATCC 76645 were 64 and 128µg/mL respectively. However, the MIC and the MFC of the strain LM 45 was 64µg/mL. For the strains LM 102 and 233 the MIC, as well as the MFC was respectively 128µg/mL. For *C. tropicalis* LM 665 the MIC and the MFC were 128 and 256µg/mL respectively, and for the strain ATCC 13803 the MIC, as well as the MFC was 32µg/mL. In the *in silico* toxicology analysis was observed that the *o*-eugenol is similar to drugs with 27 possible activities with probability of being active superior to 70% (Pa > 70%), as well as the absence of tumorigenic effects and damage to the reproductive system. Therefore, the *o*-eugenol showed antifungal activity against the strains used in this study and presents low risk of theoretical toxicity.

Keywords: *O*-eugenol; Antifungal activity; *In silico* toxicity; *Candida albicans*; *Candida tropicalis*.

INTRODUCTION

The frequent and indiscriminate use of antimicrobial drugs has enabled the development of micro-organisms capable of developing auto-protective mechanisms, making them highly selective and/or resistant¹. The multiple drug resistance (MDR) is associated to the pathogens capacity to promote alterations in the absorption, elimination and in the enzymatic inactivation of the antifungal drugs. Considerably reducing their action spectrum, process which has boosted the emergence of new pharmacological perspectives^{2,3}. The use of plants for medicinal purposes began in the primitive civilizations and is related to the presence of bioactive metabolites, responsible for the triggering of pharmacological properties⁴. These compounds may act synergistically or solely during the therapeutic response, seen as the heterogeneity and chemical versatility of the molecules can enhance the biological action⁵. Among these bio-constituents is the eugenol, considered to be a phenylpropanoid isolated mainly from essential oils (EO) derived from aromatic plants, being commonly found in the *Eugenia caryophyllata* (clove) and *Pimenta dioica* (L.) Merr (Jamaican pepper)^{6,7}. It is used as anesthetic in dental treatments, aroma in cosmetics and food products, in addition to promote biological activity related to the

antioxidant and hemo-protection capacity⁸⁻¹⁰. The antibacterial and antifungal nature of the compound is related to the formation of biofilms, as well as of planktonic cells particularly in the strains of enterohemorrhagic *E. coli* (EHEC)¹¹, *S. aureus*¹² and *C. albicans*¹³. In the later the inhibition of the fungal aggregation occurs by means of the insertion of the molecule to β-ciclodextrin, promoting damage to the cell walls of the hyphae¹⁴. The reduction of the proliferation and the fungal survival caused by the eugenol, raises the interest in the development of studies with molecules derived from this compound, such as the *o*-eugenol. Therefore, the objective of this work was to evaluate the antifungal activity performed by the *o*-eugenol, as well as to compare its toxicological potential to standard drugs, by means of *in silico* analysis.

MATERIALS AND METHODS

In silico analysis

Pass online

The spectrum of biological activities of a chemical compound is a set of different types of biological activities which reflect the results of the interaction of the compound with various biological entities¹⁵. The biological activity is qualitatively defined ("Yes"/"No") suggesting that the

Table 1. Predicted activities of the *o*-eugenol at Pa >70% depicted through Pass online tool. Pa: probability "being active" and Pi: probability "of being idle"

N° activities	Pa	Pi	Activity
1	0.944	0.003	Aspulvinone dimethyl allyltransferase inhibitor
2	0.928	0.001	Carminative
3	0.896	0.001	Steroid N-acetylglucosaminyl transferase inhibitor
4	0.876	0.008	Chlordecone reductase inhibitor
5	0.870	0.019	Membrane integrity agonist
6	0.857	0.008	Beta-adrenergic receptor kinase inhibitor
7	0.857	0.008	G-protein-coupled receptor kinase inhibitor
8	0.848	0.004	Caspase 3 stimulant
9	0.851	0.009	Antieczematic
10	0.828	0.004	Antiseptic
11	0.830	0.006	JAK2 expression inhibitor
12	0.832	0.009	Ferulylesterase inhibitor
13	0.838	0.020	Ubiquinol-cytochrome-c reductase inhibitor
14	0.817	0.013	Gluconate 2-dehydrogenase (acceptor) inhibitor
15	0.800	0.004	Antimutagenic
16	0.798	0.030	CDP-glycerolglycerophospho transferase inhibitor
17	0.774	0.009	Apoptosis agonist
18	0.763	0.005	Anesthetic general
19	0.757	0.008	CYP2A substrate
20	0.756	0.019	5 Hydroxytryptamine release stimulant
21	0.741	0.006	CYP2E1 substrate
22	0.737	0.006	CYP2E substrate
23	0.759	0.032	Mucomembranous protector
24	0.724	0.010	Fatty-acyl-CoA synthase inhibitor
25	0.739	0.024	Membrane permeability inhibitor
26	0.716	0.005	MMP9 expression inhibitor
27	0.704	0.014	Respiratory analeptic

spectrum of biological activity represents the "intrinsic" property of a substance depending only on its structural physical and chemical characteristics^{16,17}.

Pass (Prediction of Activity Spectra for Substances) is a tool designed to evaluate the general biological potential of an organic molecule candidate to become a drug¹⁸. Pass provides simultaneous predictions of many types of biological activities based on the structure of the organic compounds. However, Pass can be used to estimate the profiles of the biological activities in relation to the virtual molecules, before their chemical synthesis and biological tests. Pa (probability of "being active") and Pi (probability of "being inactive"), estimate the categorization of the compounds potential to belong to the subclass of active or inactive compounds respectively^{19,20}.

Osiris

The software Osiris (<http://www.organic-chemistry.org/prog/peo/>) is used to verify the biological effects of the organic compounds by means of computational experiments of molecular fragments, generating toxicity predictions²¹ resulting from possible mutagenic, tumorigenic, irritating properties and harmful effects on the reproductive system, as well as the cLogP, druglikeness and drug-score parameters of the molecules^{22,23}.

For the evaluation of the possible pharmacological activities, as well as the oral administration of the drugs, it is necessary the use of Lipinski's rule of five, which

stipulates that the compound presents at least three of four requirements so as to have considerable theoretical oral bioavailability²⁴.

The established rule for the majority of the "drug-like" molecules have cLogP ≤ 5, molecular weight ≤ 500 Da, number of hydrogen acceptors ≤ 10 (nALH ≤ 10) and the number of hydrogen donors ≤ 5 (nDLH ≤ 5)^{25,26}.

Microbiological tests

Phytoconstituent

The substances used in this work were: *o*-eugenol [2-methoxy-6-prop-2-enylphenol] (purity ≥ 98%), dimethylsulfoxide acid (DMSO) and tween 80 (0.02%) (all obtained commercially from Sigma-Aldrich, São Paulo, SP, Brazil). The tween 80 and the DMSO were solubilized at a proportion which did not exceed 0.5% in tests, later it was diluted in sterile distilled water with the *o*-eugenol so as to obtain a doubly concentrated emulsion of 2048 µg/mL^{27,28}.

Fungal strains

The tests were carried out with seven fungal strains: *C. albicans* LM 35, 45, 102, 233, *C. tropicalis* LM 665 (clinical isolates), *C. albicans* ATCC 76645 and *C. tropicalis* ATCC 13803 (standard strains) obtained from the collection from the Mycology Laboratory (LM) of the Department of Pharmaceutical Sciences (DCF), Health Sciences Center (CCS) of the Federal University of Paraíba (UFPB). All the strains were maintained in SDA

Table 2: Osiris calculations of toxicity risks and drug-score of compound *o*-eugenol monoterpenes compared to the standard antibiotics drugs.

Compounds	Toxicity risk ^[a]					Drug score ^[b]					
	MUT	TUMO	IRRI	REP	CLP	S	D-L	D-S	nALH	nDLH	Da
<i>O</i> -eugenol	Highlytoxic	Nontoxic	Slightlytoxic	Nontoxic	2.27	-2.05	-4.64	0.22	2	1	164.2
Fluconazole	Nontoxic	Nontoxic	Nontoxic	Nontoxic	-0.10	-2.17	3.03	0.90	7	1	306.2
Nystatin	Nontoxic	Nontoxic	Nontoxic	Nontoxic	0.57	-5.30	-4.26	0.18	18	12	926.1
Amphotericin B	Nontoxic	Nontoxic	Nontoxic	Nontoxic	0.32	-5.07	-0.13	0.27	18	12	924.0

Legend: Nontoxic; Slightlytoxic; Highlytoxic; ^[a]MUT: Mutagenic; TUMO: Tumorigenic; IRRI: Irritant; REP: Reproductive effective. ^[b]CLP: cLogP; S: Solubility; DL: Drug-likeness; DS: Drug-Score; nALH: number of acceptors hydrogen bonding; nDLH: number of hydrogen bond donor groups; Da: Molecular Weight.

Table 3: MIC values ($\mu\text{g/mL}$) of the monoterpene *o*-eugenol against fungal strains.

Fungal strains / Treatment	<i>C. albicans</i> LM 35	<i>C. albicans</i> LM 45	<i>C. albicans</i> LM 102	<i>C. albicans</i> LPM 233	<i>C. albicans</i> ATCC 76645	<i>C. tropicalis</i> LM 665	<i>C. tropicalis</i> ATCC 13803
1024 $\mu\text{g/mL}$	+	+	+	+	+	+	+
512 $\mu\text{g/mL}$	+	+	+	+	+	+	+
256 $\mu\text{g/mL}$	+	+	+	+	+	+	+
128 $\mu\text{g/mL}$	+	+	+	+	+	+	+
64 $\mu\text{g/mL}$	+	+	-	-	+	-	+
32 $\mu\text{g/mL}$	-	-	-	-	-	-	+
16 $\mu\text{g/mL}$	-	-	-	-	-	-	-
Negative control	-	-	-	-	-	-	-
Positive control	+	+	+	+	+	+	+

(+) inhibition (-) no inhibition

at 4°C. Cultures of 24-48h incubated at 35±2°C were used in the tests.

Inoculum

The suspensions were prepared from recent fungal cultures sown in SDA and incubated at 35±2°C during 24-48h. After the incubation, around 4-5 yeast colonies were transferred (with a sterile microbiological handle) to test tubes containing 5.0mL of sterile saline solution (NaCl 0.85%). The resulting suspensions were agitated during 15 seconds with the aid of a vortex mixer (Fanem Ltd., Guarulhos, SP, Brazil). The turbidity of the final inoculum was normalized using a barium sulfate suspension (McFarland 0.5 standard tube). The final concentration obtained was of 1-5 × 10⁶ colony forming units per milliliters (CFU/mL)^{29,30}.

Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Fungicidal Concentration (MFC)

The MIC of the compound on the strains used was determined by means of the microdilution in broth technique^{31,32}. 100 μL of liquid medium RPMI-1640 were distributed doubly concentrated in the 96 holes of the microdilution plates. Then, 100 μL of the emulsion of the doubly concentrated product were dispensed in the cavities of the first horizontal line of the plate by means of serial dilution in which an aliquot of 100 μL was transferred from a more concentrated well to a less concentrated one, obtaining concentrations of 1024 $\mu\text{g/mL}$ up to 16 $\mu\text{g/mL}$. Later, 10 μL of the fungal suspension were added in the wells of the plate, in which each column was represented by a strain. At the same time, controls were made in order to verify the fungal viability and susceptibility with the

standard antifungal nystatin (100UI/mL), and 100 μL of the standard drug was injected in the control wells. The prepared and closed plates were submitted to incubation at a temperature of 35±2°C for 24-48 hours. After the established time the presence or absence of growth of fungal strains was visually verified, by means of cell clusters in the orifices of the plate. The MIC was defined as the lowest concentration of the product capable of producing visible inhibition of the fungal growth verified in the holes when in comparison with the control.

The effectiveness of the product was defined according to the MIC concentrations, based on the following criteria: good antifungal action (MIC<100 $\mu\text{g/mL}$), moderate action (MIC100-500 $\mu\text{g/mL}$), weak action (MIC 500-1000 $\mu\text{g/mL}$) and inactive product (MIC>1000 $\mu\text{g/mL}$)³³⁻³⁵. After the reading of the MIC, aliquots of 10 μL of the supernatant from the cavities where were observed complete inhibition of fungal growth (MIC, MIC × 2 and MIC × 4) in the microdilution plates were sown in Petri dishes containing SDA and subsequently incubated at 35±2°C for 24-48 hours. The MFC was considered as the lowest concentration in which there was no growth or that the fungal growth was inferior to three colonies (approximately 99 to 99.5% of death action)³⁶.

The biological activity tests were carried out in duplicate and the results were expressed as the arithmetic average of the MIC and the MFC.

RESULTS

The analysis of the possibilities of actions of the *o*-eugenol revealed that the molecule is "drug-like" with 27 possible

Table 4: MFC values ($\mu\text{g/mL}$) of the monoterpene *o*-eugenol against the fungal strains.

Fungal strains / Treatment	<i>C. albicans</i> LM 35	<i>C. albicans</i> LM 45	<i>C. albicans</i> LM 102	<i>C. albicans</i> LPM 233	<i>C. albicans</i> ATCC 76645	<i>C. tropicalis</i> LM 665	<i>C. tropicalis</i> ATCC 13803
1024 $\mu\text{g/mL}$	+	+	+	+	+	+	+
512 $\mu\text{g/mL}$	+	+	+	+	+	+	+
256 $\mu\text{g/mL}$	+	+	+	+	+	+	+
128 $\mu\text{g/mL}$	+	+	+	+	+	-	+
64 $\mu\text{g/mL}$	-	+	-	-	-	-	+
32 $\mu\text{g/mL}$	-	-	-	-	-	-	+
16 $\mu\text{g/mL}$	-	-	-	-	-	-	-
Negative control	-	-	-	-	-	-	-
Positive control	+	+	+	+	+	+	+

(+) inhibition; (-) no inhibition

actions in the $\text{Pa} > 70\%$ (Table 1), and numerous properties similar to drugs in the $\text{Pa} > 30\%$, for example: Antimutagenic (Pa : 0.800 and Pi : 0.004), Antiprotozoal (Leishmania) (Pa : 0.544 and Pi : 0.019), Insecticide (Pa : 0.507 and Pi : 0.004), Antifungal (Pa : 0.471 and Pi : 0.032), Antiparasitic (Pa : 0.471 and Pi : 0.019), Hepatoprotective (Pa : 0.443 and Pi : 0.026), Antioxidant (Pa : 0.422 and Pi : 0.010) and Antibacterial (Pa : 0.336 and Pi : 0.047).

The toxicological evaluation of the *o*-eugenol, as well as its pharmacological properties were analysed *in silico*, enabling the determination of mutagenic, tumorigenic, irritating characteristics and damage to the reproductive system, and its theoretical oral bioavailability²² (Table 2).

The values of the MIC and the MFC of the *o*-eugenol against *C. albicans* LM 35 and ATCC 76645 were of 64 $\mu\text{g/mL}$ and 128 $\mu\text{g/mL}$ respectively. However, the strain LM 45 presented MIC and MFC of 64 $\mu\text{g/mL}$. By contrast, the strains LM 102 and LPM 233 has MIC and MFC of 128 $\mu\text{g/mL}$. For *C. tropicalis* LM 665 and ATCC 13803 the MIC was of 128 and 32 $\mu\text{g/mL}$ respectively and the MFC was 256 and 32 $\mu\text{g/mL}$ (Tables 3 and 4).

DISCUSSION

The *in silico* models are being applied for the toxicity evaluation of organic compounds in metabolic environment of mammals simulated in computers. Its use in regulated environments has been encouraged by the recent legislation¹⁷. However, the main limitation of the toxicity evaluation in animal models is that they are efficient in the evaluation of organic molecules with low average molecular weight³⁷. Therefore, several statistical methods of efficient automatic learning have been used to develop *in silico* tools to predict the toxicological hazards of the molecular structures. This way, these tools are used to study hypothetical existing compounds, which are fast, reproducible and that are typically based in human biorregulators³⁸.

The pharmacological effects can be predicted by computational tools capable of producing reliable results, in addition to emerge as a viable alternative in the assessment of the bioavailability and theoretical toxicity of the compound³⁹. The software Osiris generates alerts about the mutagenicity, tumorigenicity, irritability and possible damage to the reproductive system. These results are

codified by colors, and the color red indicates risks of undesirable effects, yellow, moderate risk and green, absence of risk²³ (Table 2).

According to Lipinski *et al.* (2001)²⁵, the use of these tools assists in the pharmacokinetic analysis of the phytoconstituents, in which is established some physical and chemical parameters, so that the compound presents at least three of the four proposed requirements ($\text{nDLH} \leq 5$, $\text{nALH} \leq 10$, $\text{DA} \leq 500$ and $\text{cLogP} \leq 5$).

Therefore, the *o*-eugenol presents itself within the limitations proposed by Lipinski's rule of five, not obtaining problems with the theoretical bioavailability. However, it demonstrated an irritating and mutagenic potential as a possible toxic effect (Table 2). For the data "Drug-Likeness" nystatin, fluconazole and amphotericin B were used, drugs used in the treatment of fungal infections, in which were not theoretically observed toxic action, however some values of nDLH, nALH, Da and cLogP did not follow the standards pre-established by Lipinski. According to Ujikawa (2003)⁴⁰, these substances have little stability and solubility, being, therefore are toxic to the organism.

Researches carried out with fluconazole and amphotericin B, showed hepatotoxic action in both as a main collateral effect, however in the later may cause nephrotoxicity and myelotoxicity⁴¹⁻⁴³, results which may be associated to the discrepancy in the reference values of the *in silico* analysis verified in this research with these drugs.

The self-medication or even the indiscriminate prescription of antimicrobials contributes to the emergence of micro-organisms resistant to various pharmacological classes. In which, these drugs are capable of promoting intrinsic physiological alterations in the pathogens which over time will subdue the pharmacological selectivity⁴⁴. Therefore, the natural compounds which have pharmacological activity and low toxicity, among them, the essential oils constitute a promising alternative for possible treatment of bacterial and fungal infections⁴⁵.

Plants of the genus *Eugenia caryophyllus*, *Campylocentrum zehntneri* and *Croton nepetaefolius* have EO constituted of bioactive molecules such as the eugenol, considered to be a phenylpropanoid capable of exerting analgesic and anti-convulsant activity⁴⁶. Besides the effects on the nervous system, the molecule connects in the

fungal structure and conducts the release of its internal content causing its destruction⁴⁷.

The *Eugenia caryophyllus* presents fungicide action against species of *Fusarium oxysporum* in different concentrations, function which may be related to the presence of the eugenol which it contains⁴⁸. Similarly, it was observed in this study with the *o*-eugenol, a promising molecular prototype capable of reducing and/or eliminating fungal strains. These pharmacological effects may be related to the molecular structure of this phytoconstituent which basically differ in the position of some atoms linked to the benzene ring common to both molecules.

The hydroxyl present in the molecule promotes the compound's acid characteristic, in addition to forming a hydrogen bond with the micro-organism's enzymes, facilitating its destruction⁴⁹. Mechanism which predisposes the antifungal action of the eugenol, considering that the concentrations of 62.9µg/mL and 36.9µg/mL inhibit 50% of the *Laetiporus sulphureus* and *Lenzites betulina* mycelium respectively⁵⁰, conformity analyzed against *Fusarium verticillioides* which obtained MIC of 0.3µg/mL⁵¹.

Similarly, it was observed in this study that the dilutions of 64µg/mL and 128µg/mL of the *o*-eugenol proved to be fungicide against species of *C. albicans* suggesting that relatively low concentrations of the compound have similar actions to its analogue eugenol (Table 3 and 4).

The fungicity of the compound may be related to the broad hydrophobic spectrum which facilitates the penetration in the fungal cells and, consequently generates ionic disorders in these micro-organisms⁵². This mechanism correlates with the vulnerability presented by one of the species of *C. tropicalis* ATCC 13803, in which the MIC and the MFC were effectively 32µg/mL.

On the other hand, *C. tropicalis* LM 665 required higher concentrations of the *o*-eugenol (MIC 128µg/mL and MFC 256µg/mL) for the complete inhibition and death. According to Janssen *et al.* (1986)⁵³, the EO can suffer alterations during the laboratorial tests, and enables the micro-organisms belonging to the same species to present distinct MIC and MFC values.

The phytoconstituent with fungicide properties needs to present the relation between MFC/MIC between 1 and 2, being defined as the lowest concentration of the substance capable of causing the death of 99.9% of the pathogens⁵⁴. Therefore the *o*-eugenol presents fungicide effect, and has shown to be effective against all the species used in this study.

CONCLUSION

In conclusion, the compounds derived from plants constitute an effective alternative in the medicinal therapy, as observed in the broad spectrum of biological activities envisaged by the Pass online software, as they are constituted of bioactive molecules capable of exerting pharmacological effects. Based on the results of this study, the *o*-eugenol has fungicide effect against the strains *C. albicans* and *C. tropicalis*. Therefore, the molecule emerges as a promising prototype with antifungal effect,

with a good theoretical oral bioavailability, however with possible toxic effects, worthy of more robust pharmacological and toxicological evaluations with *in vitro* and *in vivo* tests.

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