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Geraniol: *In Silico* Pharmacological and Toxicological Profiles and *In Vitro* Antifungal Activity Studies

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ABSTRACT

Discovery and development of new drugs requires costly expenditures. However, application of computer technology reduces the number of laboratory trials for new drug selection and development, thus at this stage improving both costs and time. From this premise, we sought to evaluate the potency of the phytoconstituent geraniol as a possible drug using results from computational tools Molinspiration, Osiris, and PASS Online. In addition to in silico analysis we used broth microdilution technique to confirm the phytoconstituent's antifungal potential. The in silico geraniol study showed that it has several possible biological effects in humans, as well as theoretically good oral bioavailability with low risks of mutagenic, tumorigenic orreproductive system damage, however, high risk of being an irritant. The observed antifungal activity against multidrug-resistant strains of Candida makes this organic molecule an excellent candidate in the fight against increasing microbial resistance.

Keywords: Geraniol; *In-silico*; *Candida*

INTRODUCTION

The recent emergence of resistant fungal pathogens has driven the search for both more effective and less toxic drugs, than the antimicrobial agents currently available for treatment. For businesses, discovery and development of new drugs requires great capital. However, application of computer technology at this stagecan reduce the number of laboratory trials for selection and development of new drugs, thus improving both costs and time [1]. In the early stages of discovery and development, *in silico* computational tools enable pharmacokinetic, biological, and toxicological evaluations, enabling pharmaceutical scientists to select the best candidate compounds for development, and to reject those having low success probabilities [1].

Natural products and their derivatives have been recognized for many years as sources of both therapeutic agents and structural diversity [2]. Among such natural products is geraniol, a phytoconstituent studied by many researchers in the world for its biological activities [3]. Geraniol, an acyclic monoterpene, with a hydroxyl group and two double bonds is a major component of geranium oil [4]. This phytoconstituent is present in a large number of plant tissues normally being found together with geranial and neral;oxidation products of geraniol [5]. Seeking a new alternative antifungal therapy, this study aimed to verify the *in silico* potential of geraniol as a drug by evaluating its pharmacokinetic, biological, and toxicological parameters obtained through software used for this purpose, and then confirming any potential antifungal activity in the laboratory by determining its minimum inhibitory concentration - MIC in yeasts strains of *Candida*.

EXPERIMENTAL SECTION

In this study for geraniol *in silico* tests, the following software was used: Osiris, Molinspiration, and Pass online. Seeking to confirm the antifungal potential of geraniol obtained through *in silico* trials, minimum inhibitory concentration - MIC studies were conducted.

Osiris

Osiris is an online software tool (http://www.organic-chemistry.org/prog/peo/) which was able to predict the toxicity of geraniol through comparison of its chemical structure with molecular fragments whose toxicities are defined in a database. The results are expressed as mutagenic, and tumorigenic toxicity, irritability, and effects on the reproductive system [6,7].

In addition to reporting on the possible toxicity of a molecule, Osiris is able to inform important physicochemical parameters in predicting oral bioavailability of the drug under study. The parameters are: partition coefficient (water/oil) - ClogP, molecular weight, number of hydrogen acceptors - nALH, number of hydrogen-donors nDLH [6,7]. According to the Lipinski (2001) "rule of five" [8]; if the molecule presentsa score meeting at least 3 parameters of those required (CLP $\leq$ 5, molecular weight <500, nALH $\leq$ 10 nDLH $\leq$ 5), the molecule may well present good oral bioavailability.

Molinspiration

Molinspiration is web based software, widely used for *in silico* trials (www.molinspiration.com.). It is able to assess a molecule and provide several parameters such as: ion channel modulation– ICM, kinase inhibition– K, nuclear receptor bonding– NRL, protease inhibition– IP, and enzyme inhibition– EI, and thus,the ability of geraniol to act on certain pharmacological targets [9].

PASS Online

PASS Online is software that reports potential biological activity based on molecular arrangement [10]. By means of a consulting database, the program compares the organic molecule under study with other molecules with defined biological activity, and thus provides simultaneous predictions of many biological activities based solely on chemical structure [11, 12]. The results of this software are expressed as the probability of the molecule to be active for a particular biological activity (Pa), and the probability of molecule being inactive for a particular biological activity (Pi) [11, 12].

Determination of Minimum Inhibitory Concentration - MIC

Six (6) clinical *Candida* strains (*C. albicans*: 649, 271; *C. glabrata*: 46, 221 and *C. krusei*: 08, 656), all resistant to fluconazole were selected from the fungal collection of the Mycology Laboratory Clinic of the Federal University of Paraiba, Brazil. The antifungal activity assays for geraniol (Sigma-Aldricht/USA) were performed according to Cleeland and Squires (1991) [13],Hadacek and Greger (2000) [14], and Standards Institute Laboratory (CSLI) (2008) protocols [15]. Determination of the geraniol MIC, against *Candida* strains was performed by broth microdilution cell culture technique in 96 well plates. Initially, 100 µL of RPMI 1640 L-Glutamine(Sigma-Aldrich®/USA) without sodium bicarbonate and doubly concentrated,was distributed to the wells of the microdilution plates. Then 100 uL of doubly concentrated test product emulsion was dispensed to the wells of the first row of the plate. By means of a serial dilution at a ratio of two, concentrations of 1024 µg/ml to 2µg/ml were obtained. Finally, 10 uL of different *Candida*s train inoculates were added to the cavities, each plate column referred to a particular fungal strain. In parallel, a control of the inoculum with 100 µL RPMI-1640 medium, supplemented with 100 uL of geraniol dilutions, and 10 uL of yeast inoculates was performed. In addition as a sterility control, 100 uL of RPMI-1640 alone was added to selected plate wells. The MIC was defined as the lowest concentration of the product able to produce inhibition of visible fungal growth recorded in wells as compared to their controls. The assay was performed in duplicate and the results were expressed as the minimum inhibitory concentration – MIC, and the minimum inhibitory concentration for 90% of the strains - MIC_{90}.

RESULTS AND DISCUSSION

Assessing the pharmacokinetic and toxicological parameters of an organic substance using *in silico* tests is extremely important, since it allows laboratory time and cost economies [1]. This study is the first *in silico* study performed with the geraniol molecule using the Osiris, Molinspiration, and PASS Online software. Analyzing the results obtained in Osiris through the Lipinski"rule of five", it was found that geraniol has good theoretical oral bioavailability, since all the physicochemical parameters measured for this molecule were within the cutting point established by the Lipinski "rule of five" (Table 1).

Table 1: Theoretic analysis of geraniol's physicochemical properties for oral drug bioavailability in accordance with Lipinski's "Rule of Five" [8]

Substance	nDLH	nALH	Da	cLogP
Geraniol	1	1	154.25	3.2
Standard "Rule of Five" Lipinski	≤ 5	≤ 10	< 500	< 5

nALH: number hydrogen bonding acceptors; nDLH: number of hydrogen bond donor groups; Da: Molecular Weight; cLogP: Partition coefficient

Since it enables obtaining these and other parameters without having to sacrifice animals,the use of *in silico* models to evaluate a compound's toxicity in a mammalian metabolic environment is being stimulated by current legislation [16]. Compared to currently available drugs such as itraconazole, which has a high mutagenic and tumorigenic effect, geraniol was very promising since it showed no mutagenic, tumorigenic or reproductive systemdamage effects, although indicating high risk for irritability (Table 2). The results detected by the software are consistent with animal testing. Farhath,Vijaya and Vimal (2012) [17] in tests with mice reported the absence of sub-acute geranioltoxicity. As for the high irritability risk detected by Osiris software, this is possibly due to side effects of geraniol in persons sensitized to the substance. Hagvall, Karlberg and Christensson (2013) [18] observed no significant irritability reactions in their studies with pure and oxidized geraniol in topical formulations, at 4-11% concentrations, when applied to the skin. Currently, geraniol is widely used in the cosmetics and food industries, however,there are reports in the literature of people with sensitivity to this monoterpene, a patient sample that developed contact cheilitisto geraniol present in foods [19], and others having urticaria due to geraniol in cosmetics [20].

Table 2: Osiris calculations for toxicity risk, and drug-score of geraniol as compared to standard antifungal drugs

Compounds	Toxicity risk			
	MUT	TUMO	IRRI	REP
Geraniol				
Amphotericin B				
Fluconazole				
Itraconazole				

: Nontoxic; : Slightlytoxic; : Highlytoxic;
[a]MUT: Mutagenic; TUMO: Tumorigenic; IRRI: Irritant; REP: Reproductive effective

Knowing the pharmacological targets of a molecule helps to understand its mechanism of action and thus predict possible side effects and adverse reactions. The Molinspiration software informs scores representing the likelihood of a drug under study to interact with differing pharmacological targets. In this software, a ≥1 value indicates the high probability of the molecule to interact. As can be seen, geraniol behaved similarly to antifungals amphotericin B, fluconazole and itraconazole against GPCRL, ICM, KI, NRL, PI and EI. This shows the potentialof geraniol to act on specific pharmacological targets when in contact with the tested micro-organisms (table 3). Microorganisms usually possess various pharmacological targets, but studies show that geraniol acts neither by eliminating *Candida* yeasts through direct interaction with ergosterol forming pores in the cell membrane, nor through inhibition of cell wall synthesis [21].

Table 3: Molinspiration calculations of geraniol as compared to the standard antifungal drugs

Compounds[a]	Drug-likeness[b]					
	GPCRL	ICM	KI	NRL	PI	EI
Geraniol	-0.6	0.07	-1.32	-1.32	-1.03	0.28
AMB	-3.06	-3.53	-3.59	-3.45	-2.45	-2.95
FLUC	0.04	0.01	-0.09	-0.23	-0.09	0.03
ICZ	-0.4	-1.5	-1.3	-1.31	-0.66	-0.97

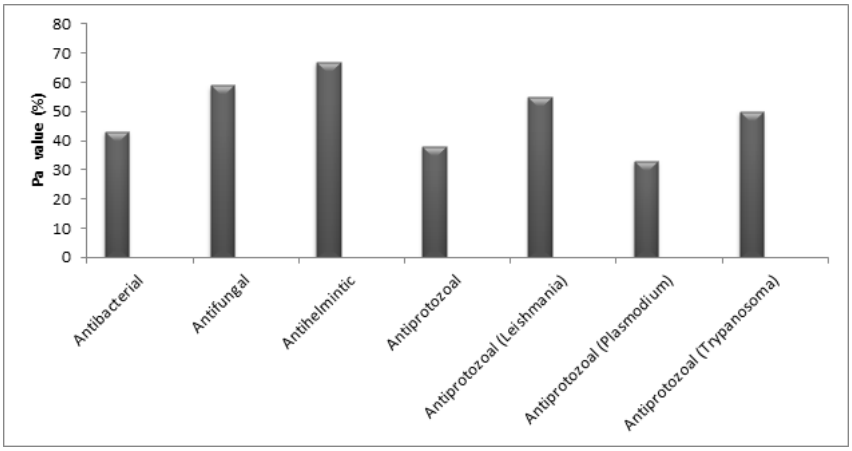
[a]AMB: amphotericin B; FLU: fluconazole; ICZ: itraconazole ; [b]GPCRL: protein G ligand; ICM: ion channel modulator; KI: kinase inhibitor; NRL: nuclear receptor ligand; PI: protease inhibitor; EI: enzyme inhibitor.

Known to possess insecticidal and repellent properties, geraniol is currently being used for pest control. Studies have suggested that geraniol represents a new class of chemo-preventive agents against cancer. Other biological activities such as antimicrobial, anti-inflammatory and vascular effects have also been investigated in non *in silico* trials [3]. In this study, through the computational tool PASS Online we found that geraniol has 74 possible activities for a Pa> 70% (Table 4) and numerous properties for a Pa> 30% (Figure 1), standing out among them antifungal (Pa: 59.6 and Pi: 0.019) and anthelmintic (Pa: 66.9 and Pi: 0.004). In vitro studies corroborate the accuracy of this software for activities detected [21, 22, 23].

Table 4: Predicted (> 70%) activities using the Pass online tool

N° Activities	Pa	Pi	Activity
1	0.712	0.001	2,3-Oxidosqualene-lanosterol cyclase inhibitor
2	0.787	0.009	5-O-(4-coumaroyl)-D-quinate 3'-monooxygenase inhibitor
3	0.744	0.033	Acrocylindropepsin inhibitor
4	0.792	0.001	Alcohol dehydrogenase substrate
5	0.84	0.012	Alkenylglycerophosphocholine hydrolase inhibitor
6	0.77	0.012	Alkylacetylglycerophosphatase inhibitor
7	0.843	0.004	All-trans-retinyl-palmitate hydrolase inhibitor
8	0.717	0.005	Allyl-alcohol dehydrogenase inhibitor
9	0.742	0.033	Antieczematic
10	0.75	0.018	Antineoplastic
11	0.71	0.009	Antisecretoric
12	0.77	0.004	Antiulcerative
13	0.766	0.001	Antiviral (Rhinovirus)
14	0.723	0.013	Apoptosis agonist
15	0.736	0.015	Arginine 2-monooxygenase inhibitor
16	0.886	0.011	Aspulvinone dimethylallyltransferase inhibitor
17	0.867	0.001	BRAF expression inhibitor
18	0.808	0.012	Beta-adrenergic receptor kinase inhibitor
19	0.866	0.002	Beta-carotene 15,15'-monooxygenase inhibitor
20	0.765	0.038	CDP-glycerol glycerophosphotransferase inhibitor
21	0.924	0.003	CYP2E1 inhibitor
22	0.847	0.011	CYP2J substrate
23	0.781	0.016	CYP2J2 substrate
24	0.807	0.007	Carboxypeptidase Taq inhibitor
25	0.724	0.035	Chlordecone reductase inhibitor
26	0.744	0.033	Chymosin inhibitor
27	0.885	0.001	Dolichyl-phosphatase inhibitor
28	0.816	0.003	Ecdysone 20-monooxygenase inhibitor
29	0.719	0.015	Exoribonuclease II inhibitor
30	0.804	0.001	Farnesyltranstransferase inhibitor
31	0.793	0.005	Fatty-acyl-CoA synthase inhibitor
32	0.8	0.012	Feruloyl esterase inhibitor
33	0.72	0.013	Fucosterol-epoxide lyase inhibitor
34	0.808	0.012	G-protein-coupled receptor kinase inhibitor
35	0.712	0.021	GST A substrate
36	0.717	0.004	Gastrin inhibitor
37	0.741	0.006	Glucan 1,4-alpha-maltotriohydrolase inhibitor
38	0.775	0.005	Gluconate 5-dehydrogenase inhibitor
39	0.744	0.019	Glucose oxidase inhibitor
40	0.721	0.023	Glutamyl endopeptidase II inhibitor
41	0.735	0.027	Glycosylphosphatidylinositol phospholipase D inhibitor
42	0.76	0.004	Lactase inhibitor
43	0.71	0.011	Linoleate diol synthase inhibitor
44	0.885	0.004	Lipid metabolism regulator
45	0.737	0.004	Long-chain-aldehyde dehydrogenase inhibitor
46	0.816	0.004	Macrophage colony stimulating factor agonist
47	0.704	0.027	Mannotetraose 2-alpha-N-acetylglucosaminyltransferase inhibitor
48	0.881	0.016	Membrane integrity agonist
49	0.71	0.001	Mevalonate kinase inhibitor
50	0.953	0.003	Mucomembranous protector
51	0.754	0.009	N-acetylneuraminate 7-O(or 9-O)-acetyltransferase inhibitor
52	0.716	0.024	NADPH peroxidase inhibitor
53	0.701	0.004	Nitrite reductase (NO-forming) inhibitor
54	0.713	0.007	Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase inhibitor
55	0.807	0.031	Phobic disorders treatment
56	0.864	0.004	Phosphatidylcholine-retinol O-acyltransferase inhibitor
57	0.724	0.005	Phosphatidylglycerophosphatase inhibitor
58	0.802	0.002	Plastoquinol-plastocyanin reductase inhibitor
59	0.966	0.001	Prenyl-diphosphatase inhibitor
60	0.823	0.007	Protein-disulfide reductase (glutathione) inhibitor
61	0.707	0.015	Pullulanase inhibitor
62	0.774	0.004	Reductant
63	0.951	0	Retinol dehydrogenase inhibitor
64	0.735	0.015	Ribulose-phosphate 3-epimerase inhibitor
65	0.744	0.033	Saccharopepsin inhibitor

66	0.783	0.015	Sphingosine kinase inhibitor
67	0.857	0.008	Sugar-phosphatase inhibitor
68	0.84	0.003	TNF expression inhibitor
69	0.801	0.025	Testosterone 17beta-dehydrogenase (NADP+) inhibitor
70	0.714	0.014	UDP-glucuronosyltransferase substrate
71	0.813	0.004	UGT1A9 substrate
72	0.881	0.009	Ubiquinol-cytochrome-c reductase inhibitor
73	0.952	0.001	Undecaprenyl-phosphate mannosyltransferase inhibitor
74	0.875	0.002	Vitamin-K-epoxide reductase (warfarin-insensitive) inhibitor



Graphic 1: Forecast of geraniol antimicrobial activity obtained using the PASS Online tool (Pa> 30%)

Knowing that the terpenoid under study does not have a 100% Pa as an antifungal, it is necessary to confirm its activity *in vitro*. Miron et al. (2014) [24], and Singh, Fatima and Hameed (2016) [25], and Leite et al. (2014) [21] confirmed geraniol's*in vitro* antifungal activity against several strains of *Candida*. In the present study clinical multi-resistant strains of *Candida* showed a geraniol MIC of 256-512 µg/ml, and an MIC_{90} of 512 µg/ml, (Table 5), which allows classifying geraniol as having strong antifungal activity [26].

Table 5: Antifungal Activity: determining geraniol's MIC

Fungal Strains	Geraniol (µg/mL)	Inoculum Control	Sterile Control
C. albicans 49	256	+	-
C. albicans 271	512	+	-
C. glabrata 46	512	+	-
C. glabrata 221	512	+	-
C. krusei 08	256	+	-
C. krusei 656	512	+	-

(+) Presence of microbial growth (-) absence of microbial growth

CONCLUSION

This *in silico* geraniol study revealed several possible biological effects in humans as well as a theoretically good oral bioavailability with low risks of being mutagenic, tumorigenic or of damaging the reproductive system, however, a high risk as an irritant was also revealed. The good antifungal activity observed against multidrug-resistant strains of *Candida* makes this organic molecule an excellent candidate in the fight against increasing microbial resistance.

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