Antiparasitic effect of Farnesol against *Leishmania major*: a rationale from *in vitro* and *in silico* investigations

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Abstract: Leishmaniasis is a vector-borne parasitic infection caused by the bite of female Phlebotomine sandflies. World Health Organization (WHO) estimates 100,000 cases to be reported annually on a global scale, moreover, 13 million people were infected between 2010 and 2015 worldwide. Treatment of leishmaniasis by conventional synthetic compounds is met by challenges pertaining to adverse effects which call for the discovery of newer anti-leishmanial molecules. This study was performed to evaluate the effect and modes of action of a sesquiterpene alcoholic molecule Farnesol on *Leishmania major*, the causative agent of Zoonotic Cutaneous leishmaniasis. The cytotoxic effect of Farnesol against *L. major* promastigotes, amastigotes and macrophages was assessed by MTT test and counting. The IC_{50} on promastigotes by Farnesol on *L. major* was evaluated by flow cytometry. In the findings, Promastigotes were reduced at 167 µM/mL & the mean numbers of *L. major* amastigotes in macrophages were significantly decreased in exposure to Farnesol at 172 µM/ml. In addition, Farnesol demonstrated no cytotoxicity on macrophages as the IC_{50} value was 945 µM/ml; it induced significant apoptosis dose-dependent on *L. major* promastigotes. *In silico* protein-ligand binding analyses indicated the effect of Farnesol in perturbation of the ergosterol synthesis pathway of Leishmania with attributes suggesting inhibition of Lanosterol-α-demethylase, the terminal enzyme of ergosterol synthesis machinery. Findings from flow cytometry reveal the role of farnesol in apoptosis-induced killing in promastigotes. Farnesol was effective at very lower concentrations when compared to Paromomycin. Further studies are crucial to evaluate the therapeutic potentials of Farnesol alone or in combination with other conventional drugs using clinical settings.

Keywords: Leishmania major; Sesquiterpene; in silico; in vitro; Flow Cytometry

Introduction

Leishmaniasis is a vector-borne parasitic infection caused by the bite of female Phlebotomine sandflies (1). World Health Organization (WHO) estimates 100,000 cases to be reported annually on a global scale, moreover, 13 million people were infected between 2010 and 2015 worldwide (2,3). There are six clinical types of leishmaniasis: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), visceral leishmaniasis (VL), post-kala-azar dermal leishmaniasis (PKDL), and leishmaniasis recidivans (LR). The symptoms might range from mild ulceration with self-healing skin sores to immune complications leading to death (4). The current CL treatment strategies include the administration of antimony-based drugs, Glucantime, Paromomycin, and Miltefosine. However, these compounds are associated with a number of adverse effects that limits their usage, like systemic side effects, toxicities, drug resistance, and painful injections which leads to a reduction in patient acceptance (5). Besides being expensive these are also long and tiring therapies. Patients can suffer damage to their hearts, livers, pancreas, hematopoietic tissues, and renal systems when these compounds fail to provide coverage against *Leishmania*. As a result, it is critical to introduce compounds with fewer complications for CL patients (6,7). Compounds with natural antibacterial, anticancer, anti-inflammatory properties, including anti-leishmanial properties, contribute to the popularity of alternative methods (8,9).

Farnesol is one such natural compound derived from a range of plants such as citronella, cyclamen, balsam, musk while it is also a constituent of many essential oils (10–13)**.** Farnesol shows anti-cancer effects on several forms of cancers such as prostate cancer and lung cancer etc. In addition, to being identified as a quorum-sensing molecule of *Candida albicans*, it induces cell death above physiological concentrations which were also observed against bacterial species such as *Staphylococcus aureus*, *Streptococcus mutans* and the plant pathogenic fungus *Fusarium graminearum* (14). Farnesol also regulates the anti-inflammatory responses which have been reported in asthma, edema, gliosis and skin tumors (15). It has been reported to exhibit significant antimicrobial properties against *Plasmodium* causing Malaria and *Toxoplasma* (16). Now, given these background investigations and the lack of data about anti-leishmanial effects of Farnesol, we designed the current study to screen the efficacy of Farnesol against an Indian standard strain of *Leishmania major* (MHOM/SU/73/5ASKH) both *in silico* and *in vitro.*

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1.0 Materials and Methods

1.1 Chemical Preparation

Farnesol (Catalog No. F203) and Paromomycin were purchased from Sigma-Aldrich Co. Germany (Catalog No. P-5057). To achieve desired concentrations (110-560 M/ml), farnesol and paromomycin were dissolved in absolute alcohol and sterile distilled water, respectively and then further dissolved in RPMI media for combination studies. For the assessment of any combinatory effect of Farnesol and Paromomycin, concentrations of 100+100, 165+350, 180+390, and 300+300 µM/ml were also prepared.

1.2 Promastigotes Culture & Promastigotes Assay

L. major promastigotes (strain MHOM/SU/73/5ASKH) were grown in RPMI 1640 supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco, New York, NY, USA) at 28 °C, 500 µl Pen-Strep containing 100 IU/ml penicillin and 100 µg/ml streptomycin was used **(17)**. Logarithmic growth-phase promastigotes of *L. major* (1×10^6 ml^{-1} cells) were cultured for 72 hours at 28 °C in 96-well plates (Thermofisher Scientific) in the presence of various doses of Farnesol (110-560 µM/ml). Promastigotes were counted directly in the Neubauer chamber under a light microscope after 24, 48, and 72 hours of incubation for counting assay and for MTT Assay after drug treatment for 72 hours at 28 °C, 20 µl MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] at 5 mg ml^{-1} concentration was added to each well and incubated for another 4 hours (Table 1). Centrifugation was used to remove the medium, and 100 µl dimethyl sulfoxide (DMSO) was added to each well. The MTT was measured using an ELISA reader at 540 nm. The study comprised both negative and positive controls using Paromomycin at 350 µM ml^{-1}. All experiments carried out as triplicate (18).

1.3 Flow Cytometry Analysis

The current work employed flow cytometry to determine the likely effective concentration of Farnesol on promastigotes using double labelling with annexin V-FLUOS and propidium iodide (PI). In brief,1×10^6 ml^{-1} promastigotes treated with Farnesol (164 µM) and those untreated were rinsed twice with cold PBS solution and centrifuged for 10 minutes at 1500 g. The promastigotes were then incubated for 15-20 minutes at 25 °C in the dark with 5 µl of annexin-V FLUOS in the presence of 5 µl PI plus 500 µl buffer. Finally, the samples were examined using the FACS Calibur flow cytometer (FACS Canto II). The data was analysed using Flow Jo software, and the percentages of necrotic, apoptotic, and normal cells were calculated (19).

1.4 Macrophage culture

THP-1 Cell line was obtained from Cell Repository, National Centre for Cell Culture (NCCS) Pune, India & cultured in RPMI-1640 supplemented with 10% Fetal Bovine Serum and 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin and incubated in a CO_2 incubator (37 °C, 5% CO_2, and 95% relative humidity).

1.5 Cytotoxicity Assay

The monocytic cells were cultured in 6 well plates for 72 h with RPMI 1640 and PMA treated for differentiation and placed in a CO_2 incubator (37 °C, 5% CO2, and 95% relative humidity). The cells were then trypsinized and seeded in 96 well plates containing Farnesol at concentrations of 100-1000 µM/ml. After 72 h of drug action, 100 µl of 5 mg/ml MTT dye was added. After a 4h incubation with MTT dye and breakdown of formazan crystals with DMSO, readings at 570 nm were obtained. For MTT test readings, a Tecan i-control, 2.0.10.0 reader was used(17).

1.6 Amastigotes Assay

The macrophage cells were initially grown in 4-well chamber slides with RPMI 1640 for 24 hours before being put in a CO_2 incubator (37 °C, 5% CO2, and 95% relative humidity). After that, the medium was removed from the culture and stationary phase promastigotes were inoculated at a 1:10 macrophage to promastigote ratio. The macrophages and promastigotes were cultured for another 2 hours at 32°C to allow for phagocytosis. After 2 hours, the excess parasites were washed away. The cells were then cultivated in new media with Farnesol at different doses and incubated at 32°C. Paromomycin (350 µg ml^{-1}) was used as a positive control. After 72 hours of drug treatment, the slides were methanol-fixed and stained with Giemsa (Figure1). Light microscopy was used to calculate the number of amastigotes per 100 macrophages (19).

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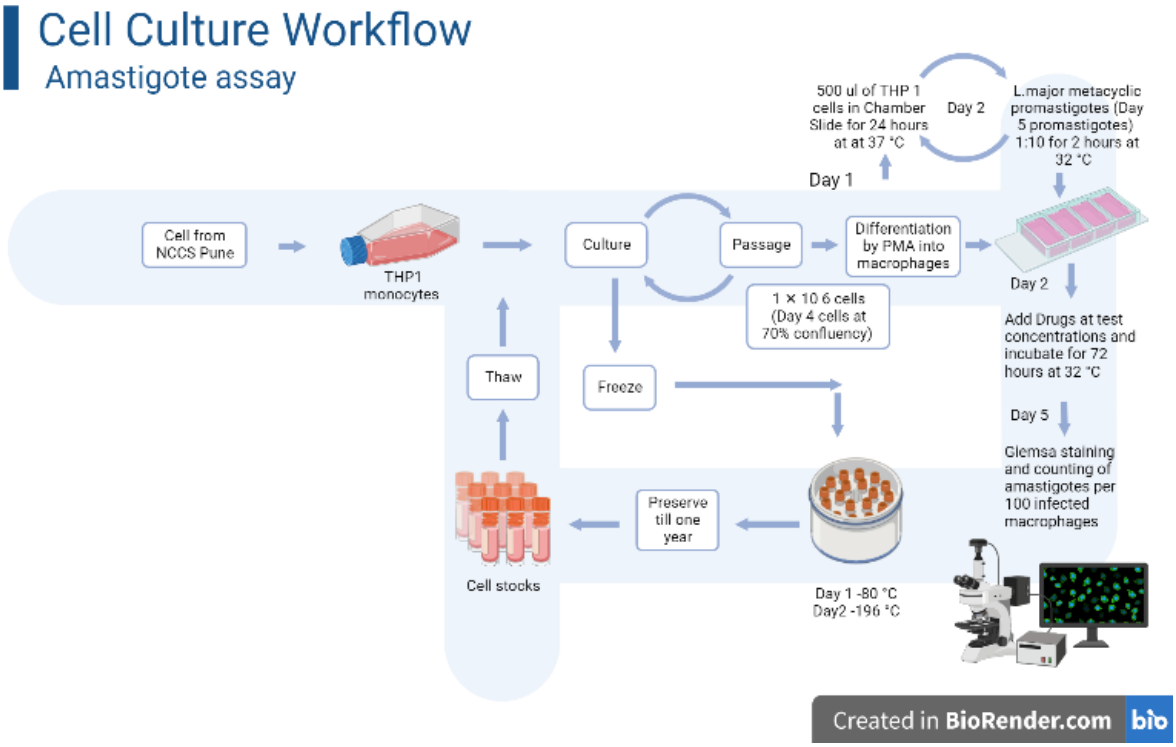

Figure 1- Cell culture and amastigote assay workflow

1.7 Combination Therapy

Different concentrations of Farnesol and Paromomycin (100+100, 165+350, and 300+300 µM/ml) were combined and cultured with *L. major* log phase promastigotes for 24 hours at 28°C for anti-promastigote & amastigote assays.

1.8 *In silico* drug-binding investigation

Upon relevant literature search, the binding affinity of farnesol was tested against the key enzymes of the ergosterol synthesis pathway i.e., Farnesol pyrophosphate synthase (FPPS) [PDBid: 4K10], Squalene synthase [modbase model], and Lanosterol 14-demethylase (CYP51) [modbase model]. The binding of farnesol was assessed based on outcomes generated from molecular docking, MM-GBSA-based ΔG_{bind} calculation and molecular dynamics (MD) analysis. All *in silico* operations were performed on Schrödinger Maestro and associated modules i.e., Glide for docking, Prime for MM-GBSA and Desmond for MD simulations and analysis.

1.9 Statistical Analysis

Data were analysed by Graph Pad Prism version 9.3.1.

2. Results

2.1 Promastigote Assay

The anti-leishmanial action of Farnesol was observed using light microscopy by assessing the number of parasites present upon treatment (Table 1). Farnesol's inhibitory effects on promastigotes are dosage and time dependent, which means that percentage killing increases depending on period of exposure and concentrations of Farnesol demonstrating anti-promastigote effects of Farnesol
with an IC50 value of 167.6 ± 4.5µM/ml and IC_{90} @ 273.10 ± 2.44 µM/ml by MTT assay and by counting assay- IC_{50} @ 170.5 ± 4.2 µM/ml and IC_{90} @ 287.69 ± 2.46 µM/ml whereas for Paromomycin - IC_{50} @ 332.0 ± 5.1 µM/ml & IC_{90} @ 510.79 ± 2.71 µM/ml for MTT & IC_{50} @ 353.2 ± 3.7 µM/ml & IC_{90} @ 639.62 ± 2.81 µM/ml for counting assay respectively (Table 1).

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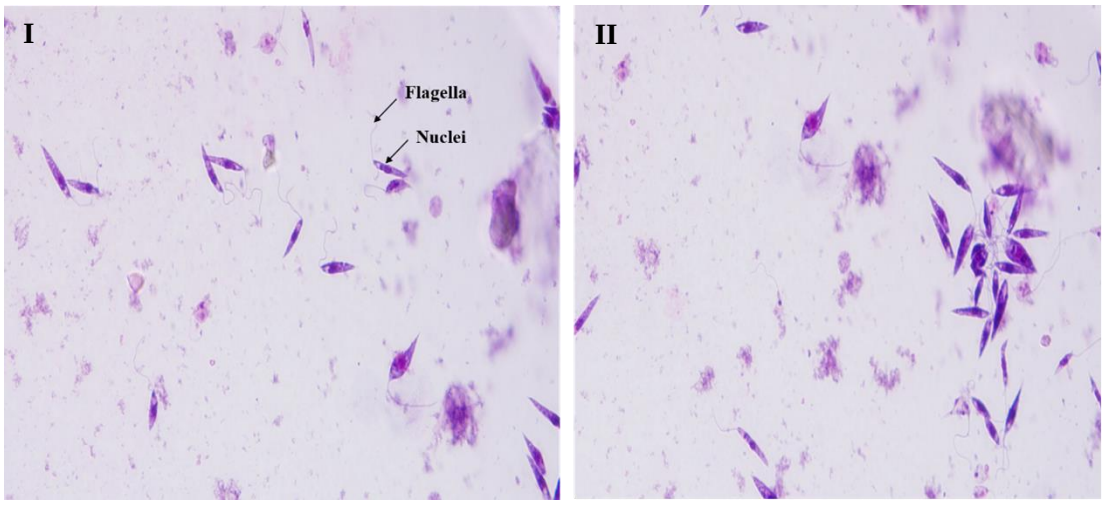
Table 1. Promastigote count after drug treatment at ('-'No live promastigotes observed)

Concentrations of Farnesol (µM)	(Mean± SD)
110	310±38.4
130	233±52.5
150	211±20.3
170	179±13.2
190	142.6±30.22
210	86.6±22.03
230	90.3±14.64
250	46±8.88
260	33.66±4.93
270	19.3±7.63
360	-
460	-
560	-
Positive Control	375.6±1.52
Paromomycin @ 332 µM	286±7



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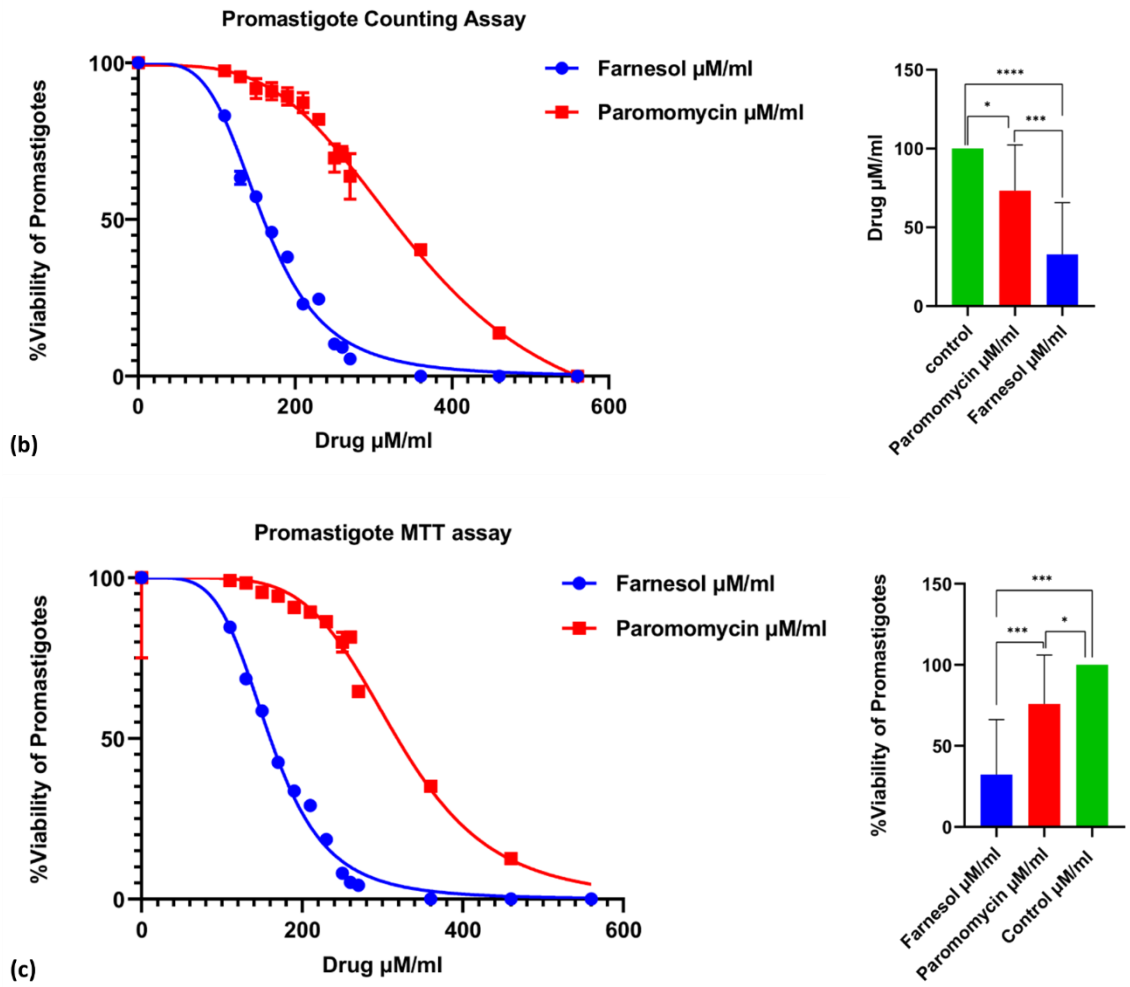
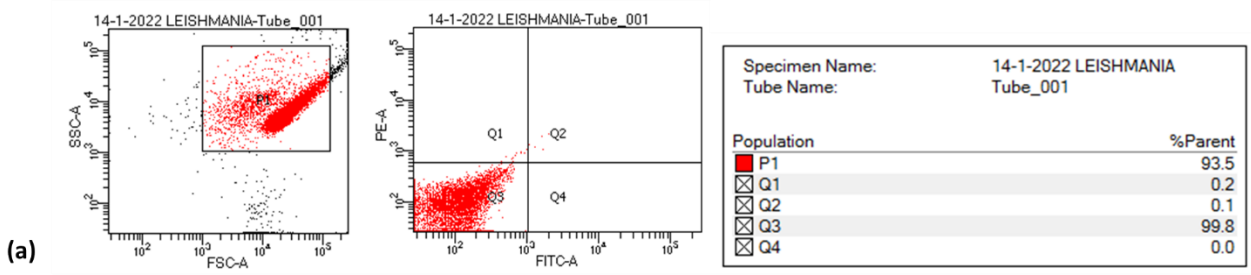


Figure 2- a. Promastigotes by Giemsa staining under light microscopy **b.** Graph with IC 50 curve by Promastigote counting assay **c.** IC 50 graph by Promastigote MTT assay. All statistical tests were two-tailed, with a significance level of p <0.05,* indicates p<0.05,** indicates p<0.01, *** indicates p <0.001 & **** indicates p<0.0001 was considered statistically significant

2.2 Flow Cytometry Assay

The purpose of this experiment was to determine the percentage of necrotic, apoptotic, and normal cells caused by the drug's activity. After 72 hours of incubation, farnesol was observed to decrease *L. major* promastigotes. At 175.7 ± 1.7 µM/ mL, normal, necrotic, and apoptotic promastigotes were estimated to account for 54%, 5.53%, and 40.43% of the total. The status of the control group was determined to be 99.2%, 0.021%, and 0.731%, respectively (Figure 3). Farnesol's treatment caused apoptosis in promastigotes.



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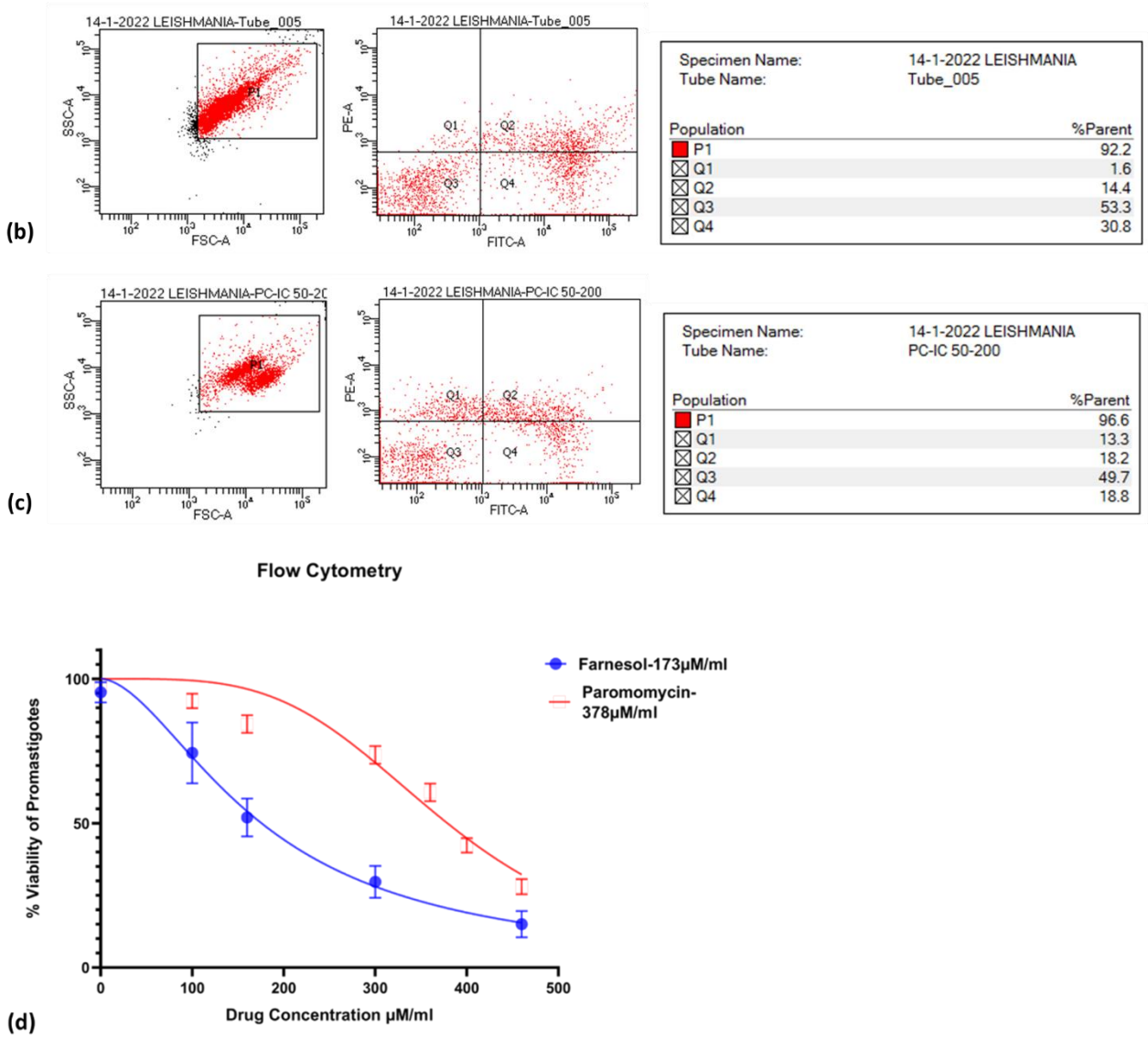

Figure 3-Flow cytometry analysis- A. control, B. 50 % inhibition of *L. major* promastigotes on addition of Farnesol @ 175.7µM/ml against C. Paromomycin @ 378.3 µM/ml D. IC_{50} analysis of Farnesol against promastigotes of *L. major* by flow cytometry

2.3 Cytotoxicity of Farnesol to Macrophages by MTT

After 72 hours of incubation, macrophages subjected to various dosages of farnesol showed 50 % killing at 945 µM ml^{-1}compared to Paromomycin at 362 µM ml^{-1} and macrophages were not cytotoxic to macrophages.

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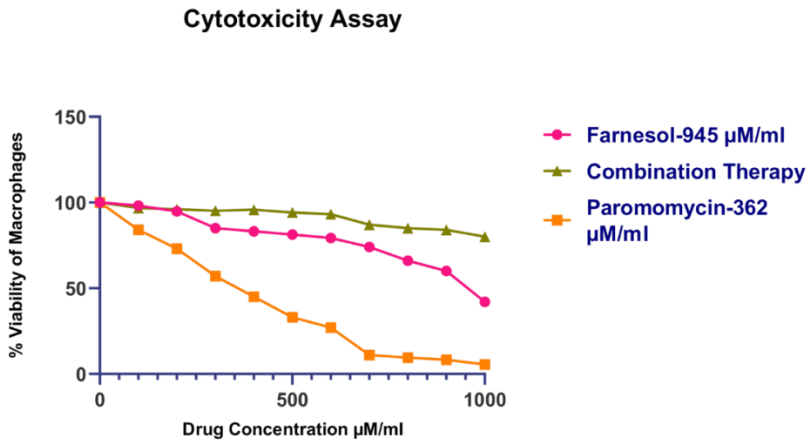

Figure 4- Farnesol Cytotoxicity Assay on THP 1 macrophages

2.4 Amastigote Assay

Light Microscopy was employed to evaluate the rate of infected macrophages based on the number of infected cells in the negative and positive control slides. 43% of macrophages were infected by Paromomycin, but infection rates in those treated with Farnesol at 160 and 200 µM ml^{-1} concentrations were 29% and 23%, respectively. In the positive control slides, however, 55% of the macrophages were infected. These findings demonstrate Farnesol's anti-amastigote properties, with an IC50 value of 172.3 ± 2.2 µM/ml & IC90 @ 328.80 ± 2.4 µM/ml & Paromomycin-IC50 @ 366.0 ± 2.5 µM/ml & IC90 780.78 ± 2.89 µM/ml (Table 2).

Table 2. Amastigote assay - Amastigote rate (per 100 macrophages) and % of Macrophages infected.

Conc. of Farnesol	Amastigote rate (per 100 macrophages)	% of Macrophages infected
100µM	130±3.29	44±3.11
160µM	110±5.27	29±3.66
200µM	72±1.39	23±2.28
260µM	31.33±2.178	15±2.8
300µM	17.66±1.17	7.66±5.94
360µM	7±2.33	3±2.64
460µM	2.33±3.30	2.3±3.26
560µM	1±0.33	2±1.8
Paromomycin @366 µM/ml	89.667±4.5	43.4±2
Positive Control	175±3.1	55.66±5.13

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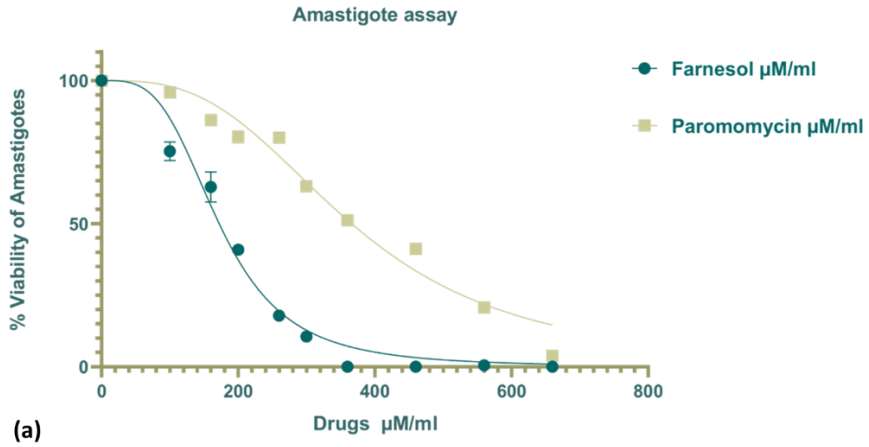

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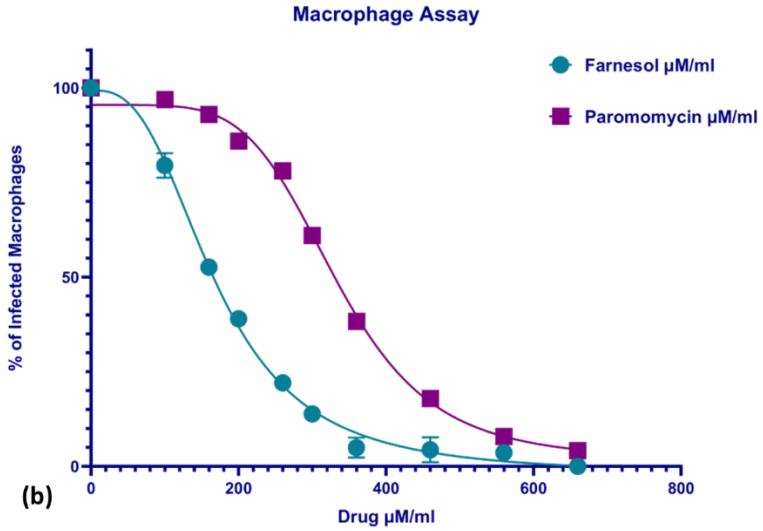

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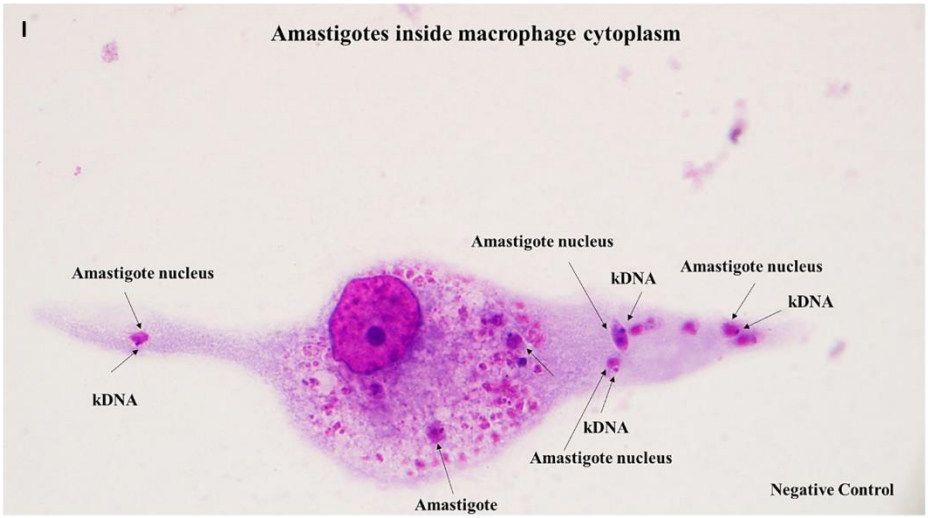

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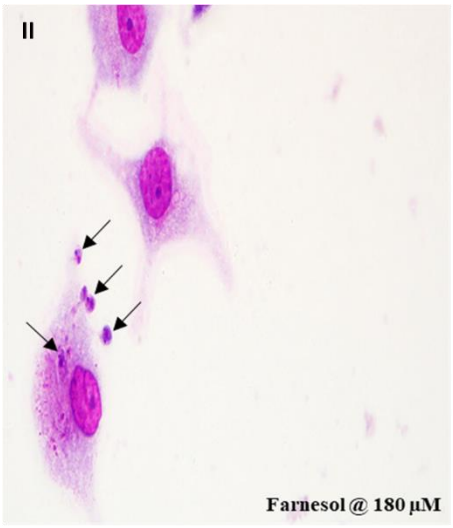

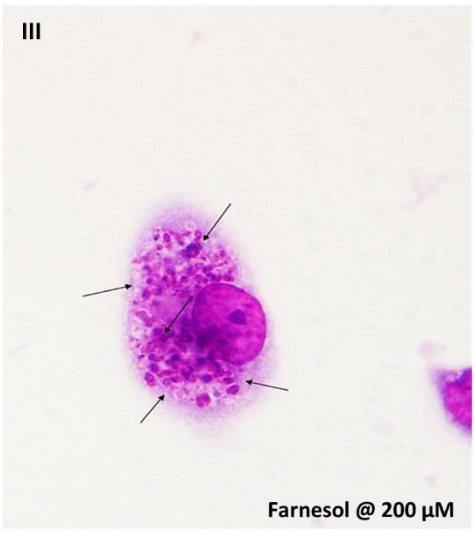

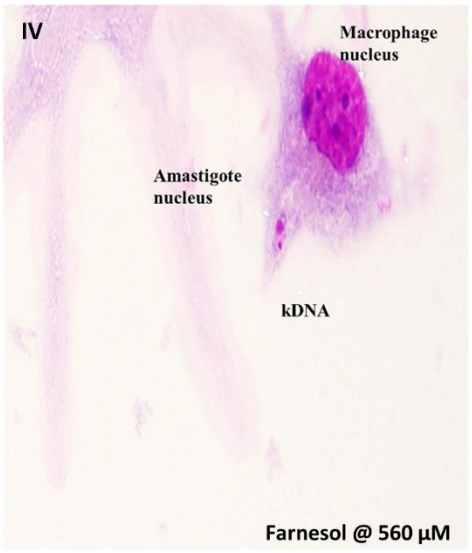

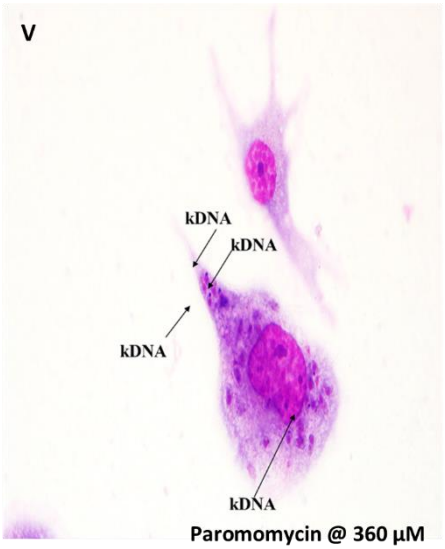

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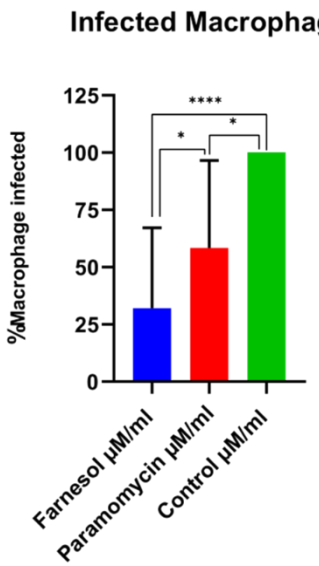

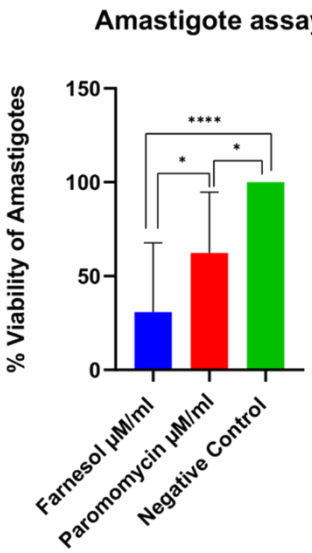

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Figure 5- a. IC 50 values by Amastigotes assay **b.** IC 50 values by Macrophage assay **c.** Macrophages infected by *L. major* amastigotes after treatment at different concentrations of Farnesol, **d.** All statistical tests were two-tailed, with a significance level of p<0.05,* indicates p<0.05,** indicates p<0.01, *** indicates p<0.001 & **** indicates p<0.0001 was considered statistically significant

Table 3. Promastigote and Amastigote assay by MTT and counting assays

DRUG ASSAYS		FARNESOL (TEST DRUG)		PAROMOMYCIN (CONTROL DRUG)	
		IC-50 µM/ml	IC-90 µM/ml	IC-50 µM/ml	IC-90 µM/ml
Promastigote Assay	MTT	167.6±4.5	273.10±2.44	332.0±5.1	510.79±2.71
	Counting	170.5±4.2	287.69±2.46	353.2±3.7	639.62±2.81
Amastigote assay	%Macrophage infected	169.1±2.8	370.63±2.57	334.3±5.1	514.33±2.71
	Amastigote /100 macrophages	172.3±2.2	328.80±2.4	366.0±2.5	780.78±2.89
Flow Cytometry		175.7±1.7	ND	378.8±3.8	ND

2.5 Combination Therapy

Both drugs had an antagonistic impact. Paromomycin and farnesol capped each other's killing activity, as they displayed normal killing effects when treated separately with Farnesol @ 167 µM/ml and Paromomycin @ 332µM/ml but the combination of both drugs reduced their combined ability to fight off infection, for both promastigotes or amastigotes assays.

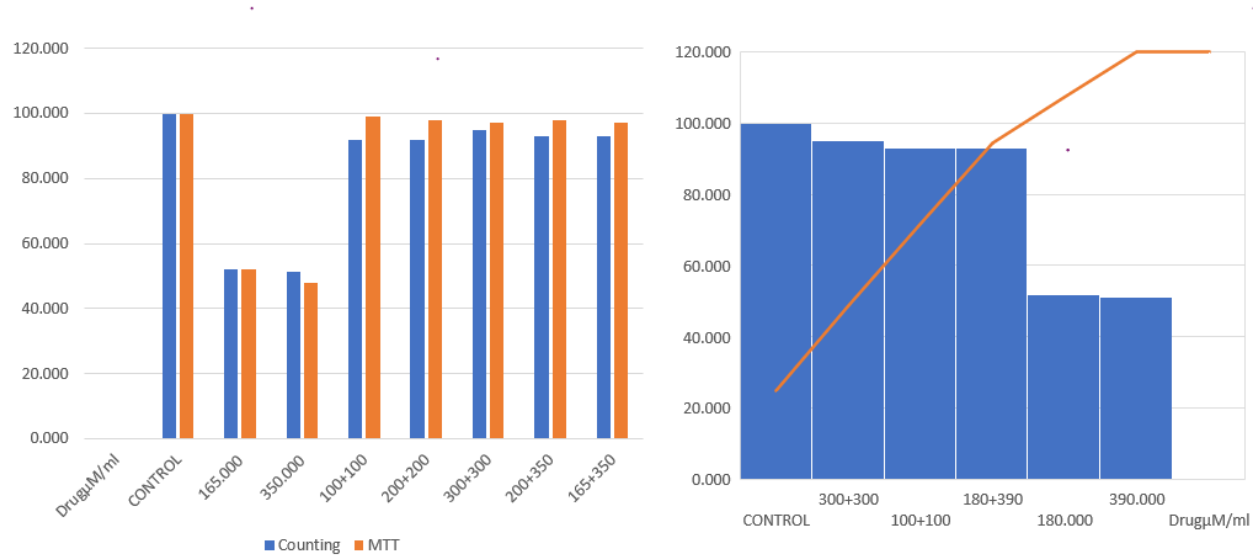

Figure 6-Combination Therapy of Farnesol & Paromomycin on *L. major* promastigotes

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2.6 *In silico* drug-binding Assay for tracing the inhibitory action of Farnesol

Farnesol accumulation has been linked to the inhibition of ergosterol synthesis in *Coccidiodes* spp., which ultimately resulted in antifungal activity (20). Farnesol pyrophosphate synthase (FPPS) [PDBid: 4K10], Squalene synthase [modbase model], and Lanosterol 14-demethylase (CYP51) [modbase model] - enzymes of the ergosterol synthesis pathway - were chosen to assess farnesol binding in comparison to respective inhibitors/substrates of the mentioned enzymes (Figure 7) (Table 4). The result from molecular docking suggests the affinity of farnesol is more inclined towards CYP51 in comparison to the other two target enzymes. Moreover, farnesol surpasses the intrinsic inhibitor, fluconazole in the binding scores. To further look into the efficacy of farnesol in engaging the active site of CYP51, the MD simulations of the respective protein-ligand (P-L)-complexes were performed for 100ns under the conditions- NPT ensembles, TIP3P water model, at 310°C and 1Pa pressure- to further examine the effective binding of farnesol with CYP51 over FPPS and Squalene synthase reported in docking and MM-GBSA scores. It was found that Farnesol displays contrastingly efficient interaction with the CYP51 receptor, i.e., stable binding with CYP51 in comparison to fluconazole, as seen in Figure 7. In the case of farnesol, the root mean square deviation (RMSD) of the P-L complexes may be shown to be stabilizing.

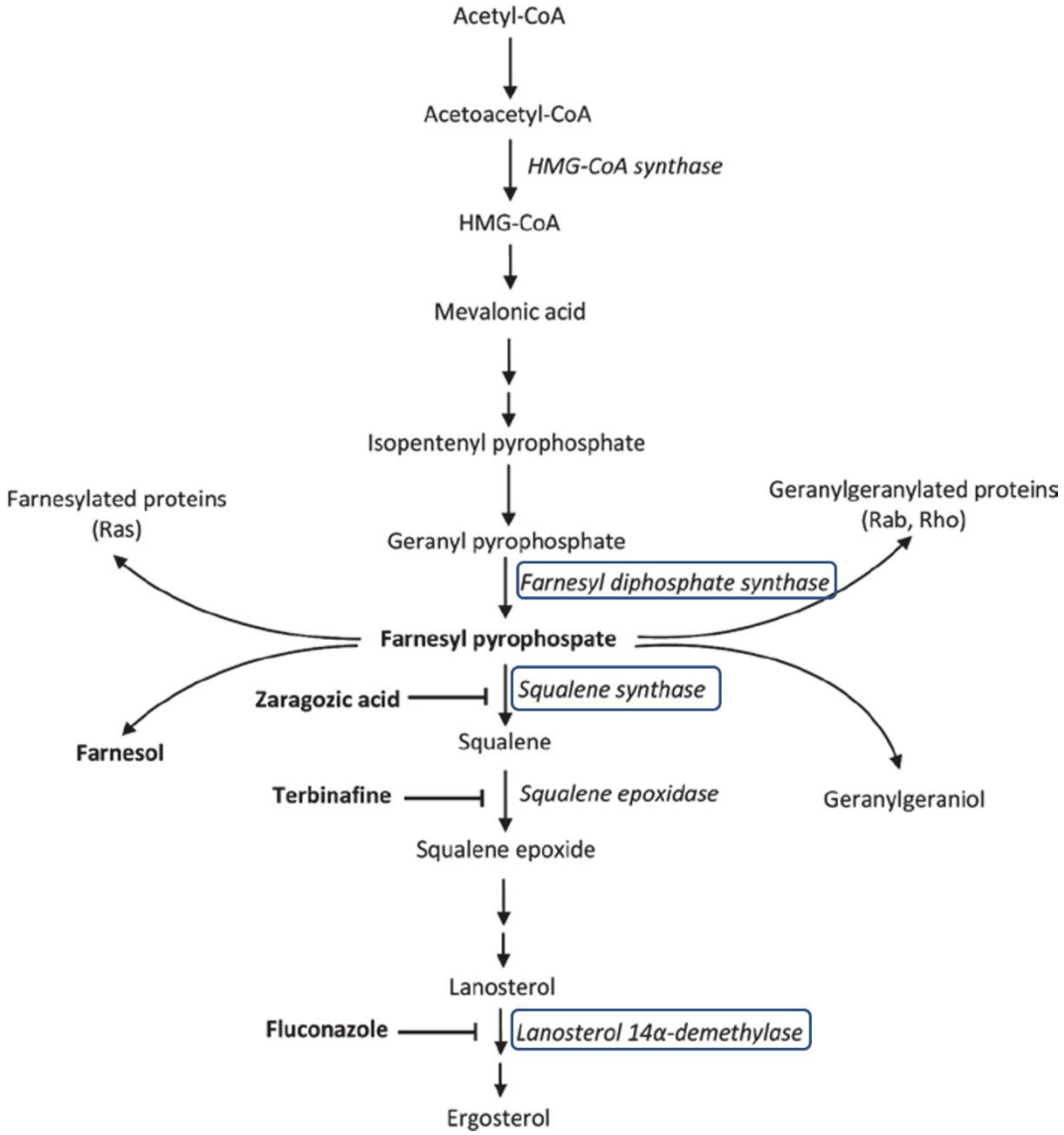

Figure 7. Ergosterol synthesis pathway and the 3 key inhibitors chosen for the study (22)

Furthermore, it has greater interaction percentages than fluconazole due to its better H-bond formation with Tyr115 and Ile423 (Figure 7c). Also, the binding free energy change of MD trajectory (Figure 7d) exhibit

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similar results, it can be seen further declining for the last 30ns of simulation up to -69.8 kcal/mol in comparison to fluconazole which only lies around -46 kcal/mol (21).

Table 4 . Docking score to assess farnesol binding in comparison to respective inhibitors/substrates of the mentioned enzymes.

Enzymes	Docking score	ΔG_{bind} (kcal/mol)	Docking score	ΔG_{bind} (kcal/mol)
FPPS	Farnesol		Farnesyl pyrophosphate	
	-3.51	-32.6	-6.24	-44.3
Squalene synthase	Farnesol		Farnesyl thiopyrophosphate	
	-3.04	-38.52	-9.38	-38.31
Lanosterol 14-α demethylase	Farnesol		Fluconazole	
	-5.10	-49.75	-6.9	-41.02

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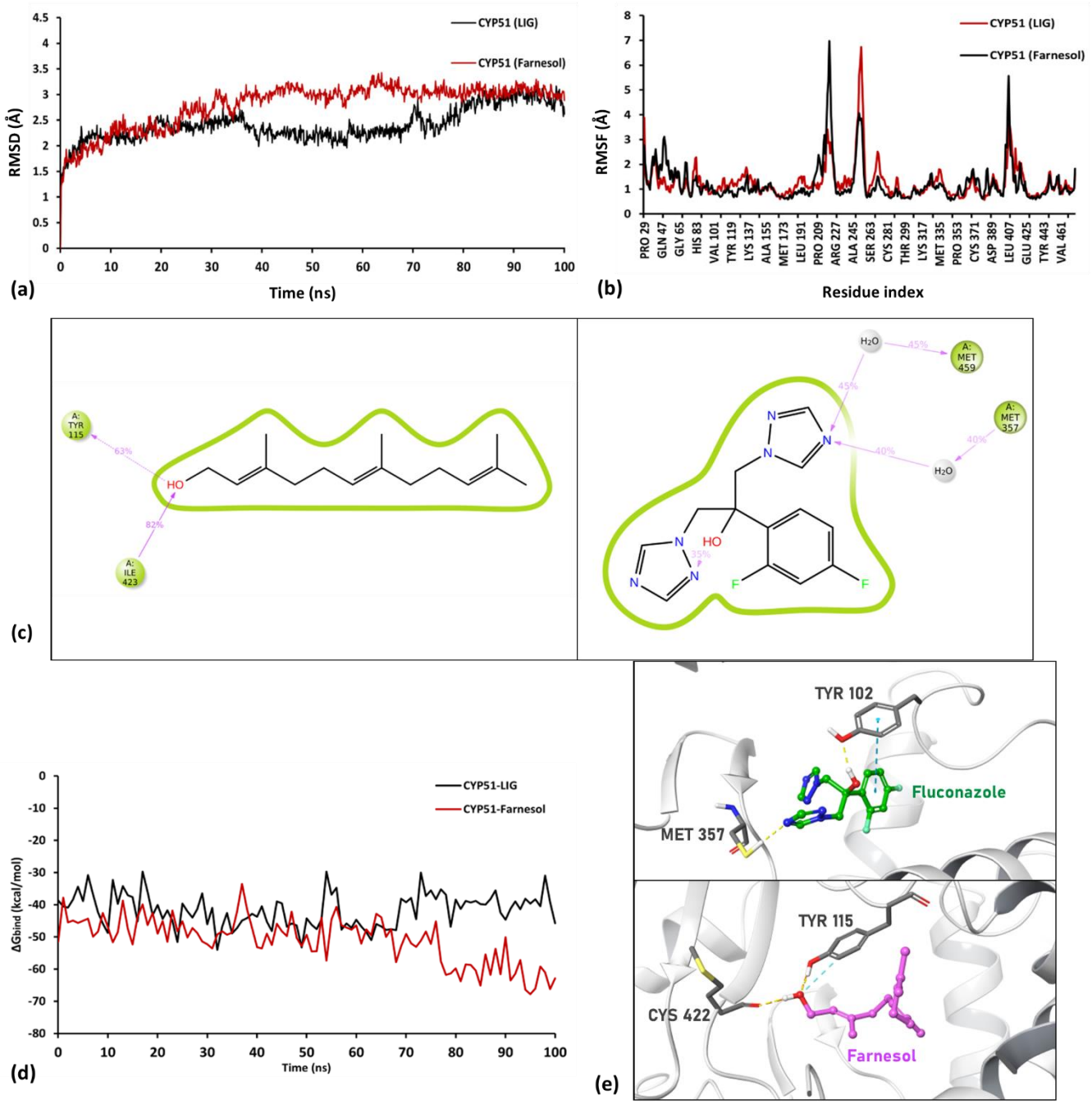

Figure 8: *In silico* binding profile; (a) Root mean square deviation (RMSD) of the 100ns molecular dynamics (MD) run of farnesol-CYP51(red) and fluconazole (or LIG)-CYP51 (black) protein-ligand (P-L) complexes, **(b)** Root mean square fluctuation of respective CYP51-ligand complexes in 100ns MD run, **(c)** Protein-ligand interaction summary of 100ns MD run where I shows farnesol and II shows fluconazole as ligand in centre, **(d)** ΔG_{bind} free energy change of respective CYP51-ligand complexes in 100ns MD run calculated using *thermal_mmgbsa.py* script provided by Schrodinger Inc., **(e)** Docked poses of fluconazole and farnesol with CYP51 active site with dotted lines representing different P-L interactions (yellow: H-bond, teal: aromatic interaction, orange: hydrophobic contacts)

Discussion

8.1.1 Farnesol better choice of drug due to high selectivity index and plant-based compound

 doi:10.20944/preprints202212.0458.v2

Leishmania is one of the greatest health concerns in infectious biology, prompting substantial research into newer treatments and drugs. Cutaneous leishmaniasis and visceral leishmaniasis clearly pose differing drug distributional challenges, with good dermal distribution through to more extensive tissue distribution requirements, respectively (23). One of the novel treatment strategies that has received significant attention recently is the use of plant-based compounds. Farnesol, a sesquiterpene has found increasing biological and therapeutic applications (24). Being a plant-based compound, it can also be distinguished by its low toxicity in comparison to its effects and to other chemical drugs with anti-leishmanial properties such as Gentamycin and Miltefosine.

8.1.2 ADME Profiling - Lipophilicity and feedback inhibition reveals probable leads of Farnesol's inhibition

Farnesol is a self-secreted quorum-sensing molecule that has also been found to have antiviral, anticancer, and anti-protozoan properties. These properties may cause apoptosis to be inhibited by farnesol. Farnesol has a Log k*p* value of -3.81 cm/s, which lies within the accepted range for a medicine to be used topically, according to the Swiss-ADME profiling of the drug. Furthermore, because of this drug's high lipophilicity (Log $P_{o/w}$ = 4.32), it is an indicative of it to pass through several membrane barriers and a significant pH barrier to reach the intracellular amastigotes in the phagolysosomes of most macrophages and stop their proliferation. Farnesol's inhibitory effect on Leishmania parasites may be partially attributed to the feedback inhibition caused by the fact that it is a by-product of the ergosterol production pathway.

The MTT assay used in the current investigation demonstrated Farnesol's anti-leishmanial activities against *L. major* promastigotes. The IC50 and IC90 values of this drug were found to be Farnesol - IC_{50} @ 167.6 ± 4.5 µM/ml and IC_{90} @ 273.10 ± 2.44 µM/ml by MTT assay, while Paromomycin's values were IC_{50} @ 332.0 ± 5.1 µM/ml & IC_{90} @ 510.79 ± 2.71 µM/ml. The findings of MTT assay were analogous with Promastigote counting assay results (Table 3) and the % killing of amastigotes was found to be dose-dependent. Farnesol at @ 175.7 ± 1.7 µM/ mL induced apoptosis in *L. major* promastigotes when compared to paromomycin which was at 378.8 ± 3.8 µM/ml. Paromomycin exhibited 50% killing of amastigotes at 366.0 ± 2.5 µM/ml and 90% killing at 780.78±2.89 µM/ml while, farnesol demonstrated 50% inhibition of amastigotes at a dose 172.3 ± 2.2 µM/ml [1] and 90% inhibition at 328.80 ± 2.4 µM/ml. We also employed the MTT assay to investigate the potential toxicity of Farnesol on macrophage cells. Farnesol killed 50% macrophages at the maximum concentration of 945 µM ml^{-1} (Figure 4). Farnesol had an IC50 & IC90 of approx. half that of the standard drug Paromomycin.

8.1.3 Relevant literature search led to an idea that the probable mechanism of inhibition might be through ergosterol synthesis pathway

Although the mechanism of action for farnesol's antiparasitic activity is unknown, published research on farnesol's antifungal effect points to its influence on sterol biosynthesis. Based on the previous studies, we chose three specific intermediate enzymes of the ergosterol pathway to investigate their interactions with the proposed drug *in silico*. The results from protein-ligand interaction studies in present study imply that farnesol may be exerting this action via blocking Lanosterol 14-demethylase, which is the terminal enzyme in the ergosterol production pathway. Moreover, the mechanism of action of antifungal drug fluconazole is attributed to the inhibition of ergosterol synthesis pathway by targeting Lanosterol 14-demethylase and farnesol has shown greater binding properties as compared to fluconazole-tested against cutaneous leishmaniasis by *L. braziliansis* (21).

This is, to the best of our knowledge, the first study to explore the action of Farnesol on cutaneous leishmaniasis. Our findings show that *L. major* promastigotes and amastigotes were susceptible to Farnesol in a dose dependent manner. Farnesol's IC50 and IC90 values were approximately half those of the current FDA-approved medication for CL therapy, Paromomycin, with no toxicity to macrophages. While the mechanism of action of the anti-leishmanial activity of Farnesol is not completely known, we discovered that Farnesol induces killing by apoptosis in *L. major* promastigotes. Moreover, the *in-silico* results indicate the inhibition of ergosterol synthesis pathway might be another route of farnesol's anti-leishmanial activity.

Conclusion

According to the above findings of *in vitro and in silico* assays, Farnesol has proven to be the preferable alternative having low toxicity, following all drug likeliness profiles according to ADMET profile like Veber, Lipinski's five rules, Ghose, Egan (25). The bioavailability score was below the allowed range of 0.55 which indicates that farnesol gets distributed evenly in the biological system, and suggests that it can be anticipated to produce excellent outcomes as a drug in an *in vivo* system. Hence, we tested the drug in BALB/c mice further on.

Author Contributions: "Conceptualization, Sehgal R; methodology, Sharma H; Sehgal R.; software, Medhi B; validation - Sharma. H, Sehgal. R. formal analysis, Sharma H; investigation, Sharma H; resources, Sehgal R; data curation, Sharma H; writing—Sharma H; writing—review and editing, Sharma H, Shekhar N, Kaur U; visualization,

Sharma H; supervision, Sehgal R; project administration, Sharma H; funding acquisition, Sehgal R. All authors have read and agreed to the published version of the manuscript."

Funding: This research received no external funding.

Institutional Review Board Statement: NA as no animals were used in the current study.
Informed Consent Statement: "Not applicable" as the study did not involve humans.

Data Availability Statement: The data presented in this study are available in the main text only, no supplementary file for the present study.

Acknowledgments: We thank PGIMER, for equipment support. We would like to extend our gratitude to Dr. Bhaskar Saha and Dr. Neelam Bhodale from NCCS, Pune, India for providing *L. major* strain for the study and kind support and guidance throughout the study.

Conflicts of Interest: There was no conflict of interests by any of the authors.

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