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Original Article

# Antiparasitic Effect of Leaf Extract and Major Metabolites of *Pelargonium quercetorum* Agnew. against *Leishmania Major*: *In Vitro* and *In Silico* Studies

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**Abstract**

**Introduction:** *Leishmania* is flagellate protozoa, the cause of leishmaniasis that has affected more than 12 million people in 88 countries throughout the world. The aim of this study was to evaluate the cytotoxic effect of the aqueous leaf extract and the main metabolites of *Pelargonium quercetorum* plant species on amastigotes and promastigotes forms of *Leishmania major* via *in vitro* and *in silico* surveys.

**Materials and Methods:** The cytotoxicity on the promastigotes and amastigotes was calculated by direct counting and MTT assay for 24, 48, and 72 h in different concentrations of the plant extract. In silico study, the interactions of four major metabolites of *P. quercetorum* including α-Pinene, α-Fenchyl acetate, limonene, and *trans*-β-Caryophyllene were evaluated towards the major virulence factor of *L. major* (GP63). After three days, samples exposed to 1, 5, and 10 mg/ml of plant extract showed a significant reduction compared to the control group.

**Results:** $IC_{50}$ values of *P. quercetorum* extract and amphotericin B were 1508.99 and 86.34 µg/ml, respectively, following 24 h of incubation. Surprisingly, *in vitro* results indicated that the *P. quercetorum* extract had a cytotoxic effect on amastigotes and promastigotes of *L. major* by non-dose-dependent effect.

**Conclusions:** Molecular docking illustrated that the highest binding affinity (-6.1 kcal/mol) was found for *trans*-β-Caryophyllene as bicyclic sesquiterpene. Docking studies showed a more efficient interaction of *trans*-β-Caryophyllene with GP63 compared to other metabolites.

**Keywords:** Antiparasitic Activity, *Pelargonium Quercetorum*, *Leishmania Major*, Docking Studies, Trans-β-Caryophyllene, GP63

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## Introduction

Clinical forms of leishmaniasis are cutaneous, muco-cutaneous, and visceral with two morphological forms including amastigote (non-flagellate) and promastigote (flagellate). Its transmission is through the bite of sandfly *Phlebotomus* (in the old world) and *Lotzumia* (in the new world).[1,2] *Leishmania major* as a zoonotic parasite is the cause of cutaneous leishmaniasis and is common in rural areas, in which gerbils and rodents are important reservoirs of this parasite.[3] Cutaneous leishmaniosis has been reported in 17 out of 31 provinces of Iran by the annual incidence (~20,000) of cutaneous leishmaniosis in Iran.[4,5] Some *Leishmania* compounds involving kinetoplastid membrane protein with 11 kDa (KMP-11), proteophosphoglycan (PPG), lipophosphoglycan (LPG), and glicoinositolphospholipids (GIPLs) were found as virulence factors that contribute to the parasite pathogenesis by invading and establishing the cellular infection.[6] In addition, several proteinases such as metalloproteinases (MP), cysteine-proteinases, and serine-proteinases can able cellular invasion and survival of parasites in macrophages.[7] Leishmanolysin (glycoprotein of 63 kDa: GP63), as the major virulence factor related to MP, is abundantly expressed on the surface of *Leishmania major*.[8-11] Therefore, the targeting of this glycoprotein may be an appropriate option to hinder these parasites in physiological conditions.

As the conventional therapy, pentavalent antimony compounds involving meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam) are the first line of treatment.[12] Clinical application of these drugs is limited due to the long duration of therapy, painful injections, toxicity, high cost, low solubility in water, and severe side effects.[13] Alternative therapies include amphotericin B, especially for muco-cutaneous leishmaniasis. However, there are side effects of acute toxicity (nausea, vomiting, fever, hypertension/hypotension, and hypoxia) and chronic toxicity (renal toxicity, anemia, hyperbilirubinemia, hypomagnesemia, hyperphosphatemia, hypokalemia, and cardiomyopathy) for amphotericin

B.[14,15] Therefore, providing more effective alternative therapies with fewer side effects is considered necessary. The World Health Organization (WHO) has also focused on treating leishmaniasis with new drugs.[16]

Applications of plant species in various biology, medicine, and nanotechnology fields have been clarified for a long time by numerous investigations.[17-19] Bio-medicinal application of the various extracts of medicinal plant species or herbal compounds as the natural sources of molecular diversity such as flavonoids, terpenoids, and alkaloids have become more widespread due to various reasons such as fewer side effects, availability, bioavailability, lower costs, and high biocompatibility with normal physiological conditions of the human body.[20-24] For examples, *Thymus migricus*, *Carum copticum heirm*, *Satureia hortensis*, *Caesalpinia gilliesii*, *Ferula assa-foetida*, *Glycyrrhiza glabra*, and *Eucalyptus globulus* have shown anti-leishmanial activity as medicinal plant species.[25-28] *Pelargonium* (purple *Geranium*) genus which belongs to the family of Geraniaceae has over 220 species in the world, which was first found in two provinces of Mosul and Erbil in Iraq.[29,30] The *P. quercetorum* species were collected and reported in 1992 in in the province of West Azerbaijan (Northwestern of Iran). Moreover, *P. quercetorum* was collected from Marivan region in Kurdistan province (western of Iran) in May 2005 followed by chemical composition of the essential oils in Kurdistan Natural Resources and Agricultural Research Center. The local name of *P. quercetorum* is "gela rivasi", which has been traditionally used as a medicinal plant in various regions of Kurdistan. Consumption of its aerial section in raw or cooked forms as an antiparasitic substance is common among local people. Twenty six natural compounds have been identified in the essential oil of this plant species, which α-Pinene (25.28%), α-Fenchyl acetate (20.63%), limonene (9.94%), and *trans*-β-Caryophyllene (8.20%) were the most abundant metabolites.[31] In addition, the antioxidant, antimicrobial, antiparasitic, and anticancer properties of *P. quercetorum* have been reported by various studies.[32-34]

According to the above explanations, we first used the aqueous extract of *P. quercetorum* leaves to evaluate its anti-leishmanial activity against *Leishmania major* through MTT and $IC_{50}$ assay *in vitro*. Next, the binding interaction (docking) of four main natural compounds α-Pinene, α-Fenchyl acetate, limonene, and *trans*-β-Caryophyllene of the medicinal plant with GP63 protease from *L. major* as its main virulence factor was investigated *in silico*.

## Materials and Methods

### *Materials*

Chloroform, alcohol (70%), Phosphate-Buffered Saline (PBS), trypan blue, Fetal Calf Serum (FCS), gentamicin, amphotericin B, and Roswell Park Memorial Institute (RPMI) 1640 Medium, were prepared from Sigma-Aldrich and Capricorn Scientific companies. For MTT assay, we applied the MTT cell viability assay kit (DNAbioTech, Iran). The experimental assays of this study were done in the Parasitology Laboratory of Kurdistan University Medical of Sciences in 2020.

### *Aqueous Leaf Extract of P. querceterum*

*P. querceterum* was collected from Marivan town in Kurdistan province in spring 2019. Identification of this plant species was carried out by the herbarium of Kurdistan Agricultural Research Center, Kurdistan, Iran. The plant leaves were completely washed in distilled water and kept in the dark at 25 °C for two weeks. Afterwards, 10 mg of dried leaves were ground in grinder and macerated in 250 ml of distilled water. This mixture was placed on a shaker at room temperature for 48 h, filtered by Whatman 40 filter paper for three times and then was stored in the refrigerator at 4 °C for further analyses.[35] For preparation of different concentrations of plant extracts, the samples were centrifuged at 1,000 rpm for 10 min at 4 °C followed by removing supernatants and drying the pellets at 60 °C for 48 h.[36] According to a previous study,[37] range of concentrations (1, 10, 100, and 1000 µg/ml) has been determined to evaluate anti-parasitic activity.

### *Promastigotes and Amastigotes Culture*

*L. major* promastigote Iranian strain (MRHO/IR/75/ER) was purchased from the Parasitology Department of Tehran University of Medical Sciences, Iran. Parasites were cultured in RPMI 1640 medium containing 10% Fetal Bovine Serum (FBS) and 10 µg/ml gentamicin. The flasks were incubated at 23 °C. For amastigotes culture, infected mice peritoneal macrophages with *L. major* amastigote were used to measure the cytotoxic effect of the different concentrations of the plant extract, *in vitro*.

### *Macrophages Culture*

Firstly, two mice were anesthetized with high doses of chloroform and were eventually killed. For sterilization, the mice were immersed in alcohol (70%) and then transferred to a biological hood and the skin of their abdomen was opened with sterile scissors and forceps. Cold sterile PBS (3 ml) was injected into the peritoneum and it was aspirated after several gentle blows to the mice abdomen. The fluid was transferred to a 15 ml falcon and centrifuged at 1500 rpm for 10 min, the supernatant was discarded and the precipitate containing macrophages was washed with sterile PBS. Using trypan blue the viability percent of macrophages was calculated, then RPMI 1640 medium containing 10% FCS and 10 µg/ml gentamicin was added to the macrophages.[38]

### *Infecting Macrophages*

Due to the adhesion of macrophages and their hard harvesting,

a round coverslip has been placed in the wells of a 12-cell culture plate. The RPMI 1640 medium (100 ml) containing $10^4$ cells/ml of macrophages was added to each well, and then the plate was incubated at 37 °C with 5% $CO_2$. To infect the macrophages, 100 ml of culture medium containing $10^5$ promastigotes in the stationary phase, was added to the wells. The plate was incubated at 37 °C with 5% $CO_2$. To remove non-adherent macrophages and promastigotes that did not enter the cell, the surface fluid of the well was discarded and fresh culture medium was added after 24 h. The plates were examined by the inverted microscope (Model: OLYMPUS CKX53) to confirm the infected macrophages.[39]

### *Treatment of Promastigotes by Leaf Extract*

When the parasites were in the log phase of growth, 100 µl of culture medium containing $10^6$ cell/ml was added to each well of 96 well plates. Then, 100 µl of plant extracts at concentrations of 0.1, 1, 10, 100, 500, 1000, 5000, and 10000 µg/ml were added to the wells. Plates were incubated at 23 °C. Three wells were considered for each concentration and three wells were considered as negative control (triple repetition). Amphotericin B was considered as positive control with 0.1, 1, 10, and 100 µg/ml concentrations. Each concentration was added to wells as the triple repeat. After 24, 48, and 72 h, cytotoxicity (%) was calculated by both direct counting (using haemocytometer slide) and colorimetry Methylthiazol Tetrazolium (MTT) methods.[39]

### *MTT Assay*

MTT is a quantitative non-radioactive spectrophotometry method used for determining cell proliferation, cell viability and drug toxicity. This is a colorimetric method in which MTT tetrazolium salt is converted to an insoluble formazan purple product. This reduction reaction is mediated by the parasite mitochondrial succinate dehydrogenase, which is used as an indicator of the growth and viability of promastigotes against the drug response, so that a decrease in the amount of reduced MTT indicates drug toxicity to the parasite.[40] For the MTT assay test, the MTT cell viability assay kit was used (DNAbioTech, Iran). According to the kit instructions, 1 ml of sterile PBS was added to each vial of reagent, then 100 µl was added to each tested well. After 3h, Optical Density (OD) was read at 570 nm by spectrophotometry. The cytotoxicity (%) was calculated using the following formula:

Cytotoxicity (%) = 1- (sample O.D – blank O.D) / (control O.D – blank O.D) × 100.

### *Treatment of Infected Macrophages by Leaf Extract*

Aqueous leaf extract of *P. quercetorum* with 1, 10, 100, and 1000 µg/ml concentrations was added to the wells. Amphotericin B (with three concentrations of 1, 10, and 100 µg/ml) was considered as the positive control. Following 24, 48, and 72 h, the cover slide inside wells were removed and stained with Giemsa. The mean of amastigotes was calculated by directly counting the number of amastigotes in 100 infected macrophages.[37]

### *Molecular Docking Study*

Surface proteinase leishmanolysin (GP63) with the crystal structure by resolution of 1.86 Å and total structure weight of 51.67 kDa, sequence length of 478 containing chain A as a single chain (Figure 3e) (Protein Data Bank (PDB) ID: 1LML) was extracted from the Research Collaboratory for Structural Bioinformatics (RCSB) (http://www.rcsb.org/). Molecular docking and interaction studies of four compounds of α-Pinene, α-Fenchyl acetate, limonene, and *trans*-β-Caryophyllene (obtained from the NCBI PubChem database) with the enzyme were carried out by two docking programs of Molegro Virtual Docker 2019 v7.0.0 (Molexus; MVD v7.0.0)[41] and AutoDock Vina (ADV) 1.1.2.[42] All the water molecules were removed and subsequently final structures were minimized by UCSF Chimera1.12 to investigate molecular docking. Five cavities by the volume of 110.592, 77.824, 64, 49.664, and 43.52 $Å^3$ as the potential binding site of GP63 were predicted via MVD and also five cavities with sizes 401, 237, 169, 701, and 299 Å3 were detected by CB-Dock (http://clab.labshare.cn/cb-dock/php/). Setting of the binding site was made inside a restriction sphere with the radius of 15 Å and the center X: 11.86, Y: 38.25, and Z: 17. Additionally, a grid resolution of 0.30 Å was applied for the MolDock grid score [GRID]. In addition, the default set up of tolerance and strength parameters were used for the side chain flexibility of the amino acid residues at the potential binding sites of GP63 proteinase. The parameter settings including the total of 10 runs with a maximum of 1500 iterations by a population of 50 individuals, the energy threshold of 100, and maximum steps of 100 were validated for the algorithm MolDock SE (Simplex Evolution). For comparing the molecular docking with MVD, the binding affinity of each ligand, type of their interaction and viewing of the docking interaction with GP63 were carried out by ADV program and Discovery Studio Visualizer 2016 academic free version. The DockThor (http://dockthor.lncc.br) program was also used as a receptor-ligand docking program in order to obtain the parameters of affinity, total energy, Van der Waals (vdW) energy, and electrostatic energy.[43] In order to analyze docking results, RMSD (root-mean-square deviation) to cluster conformers and number of binding modes were 2Å and 3Å, respectively. Grid settings were determined for soft docking as different values for center x, center y, center, total size x, total size y, and total size z for each bioactive compound. General algorithm settings were number of evaluations = 1000000, population size = 750, number of runs = 24, and seed at run #1 = -1985. Also, the

PatchDock program (https://bioinfo3d.cs.tau.ac.il/PatchDock/php.php) was used to evaluate the molecular docking scores, approximate interface area of each receptor-ligand complex, and Atomic Contact Energy (ACE)[44]

### *Statistical Analysis*

Statistical analysis was performed by SPSS software version 19 using descriptive and parametric methods (one-way ANOVA test). For all estimated values, a 95% confidence interval was also calculated.

## Results

### *Direct Counting of Promastigotes*

The anti-parasite effect of plant extract with concentrations of 0.1, 1, 10, 100, 500, 1000, and 10000 µg/ml as well as amphotericin B at amounts of 0.1, 1, 10, and 100 µg/ml on the number of promastigotes in control group and samples after 24, 48, and 72 h have been shown in Figure 1. Promastigotes in all groups showed a significant decrease following 24 h compared to the control group ($p$<0.05). After 48 h, all samples except the groups treated by amounts of 1 and 5000 µg/ml of plant extract demonstrated a prominent reduction compared to the control group ($p$<0.05). The groups exposed to concentrations of 1000, 5000, and 10000 µg/ml of plant extract and all concentrations of amphotericin B following the period of three days demonstrated a significant decrease at $p$<0.05 level compared to the control group.

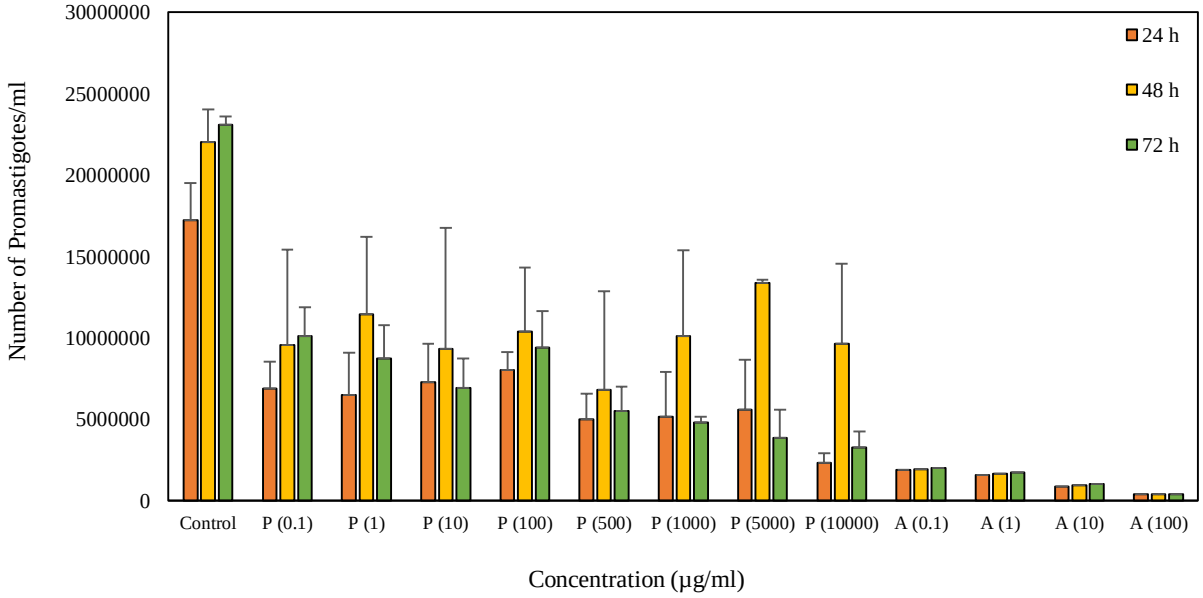


**Figure 1.** Exposure of Number of Promastigotes in Control Group and Groups Exposed to Herbal Extracts and Amphotericin B (P: Plant extract; A: amphotericin B).

**Table 1.** Cytotoxicity Percentage (%) in Groups Treated with Plant Extract (PE) and amphotericin B (A) after 24, 48, and 72 h (PR) Promastigote

| Groups | Time (h) | | |
|---|---|---|---|
| | 24 | 48 | 72 |
| PE ( 0.1 µg/ml) + PR | 53.32 | 42.87 | 30.24 |
| PE (1 µg/ml) + PR | 48.26 | 21.92 | 21.86 |
| PE ( 10 µg/ml) + PR | 45.26 | 39.32 | 21.32 |
| PE ( 100 µg/ml) + PR | 54.07 | 27.40 | 14.78 |
| PE ( 500 µg/ml) + PR | 52.16 | 31.97 | 23.38 |
| PE ( 1000 µg/ml) + PR | 52.57 | 29.20 | 21.10 |
| PE ( 5000 µg/ml) + PR | 61.31 | 58.92 | 52.55 |
| PE ( 10000 µg/ml) + PR | 54.21 | 74.37 | 68.81 |
| A ( 0.1 µg/ml) + PR | 29.90 | 22.30 | 11.32 |
| A (1 µg/ml) + PR | 31.50 | 25.84 | 20.17 |
| A ( 10 µg/ml) + PR | 54.51 | 50.44 | 45.84 |
| A ( 100 µg/ml) + PR | 74.33 | 74.15 | 76.10 |

### *$IC_{50}$ and MTT Results*

The $IC_{50}$ amounts after 24 h were 1508.99 and 86.34 µg/ml for *P. querceterum* leaf extract and amphotericin B, respectively. The cytotoxicity percentage in the groups exposed to plant extracts and amphotericin B following 24, 48, and 72 h has been shown in Table 1. Accordingly, over time the cytotoxicity percent decreases in all groups except the group exposed to plant extract (10000 µg/ml) and amphotericin B

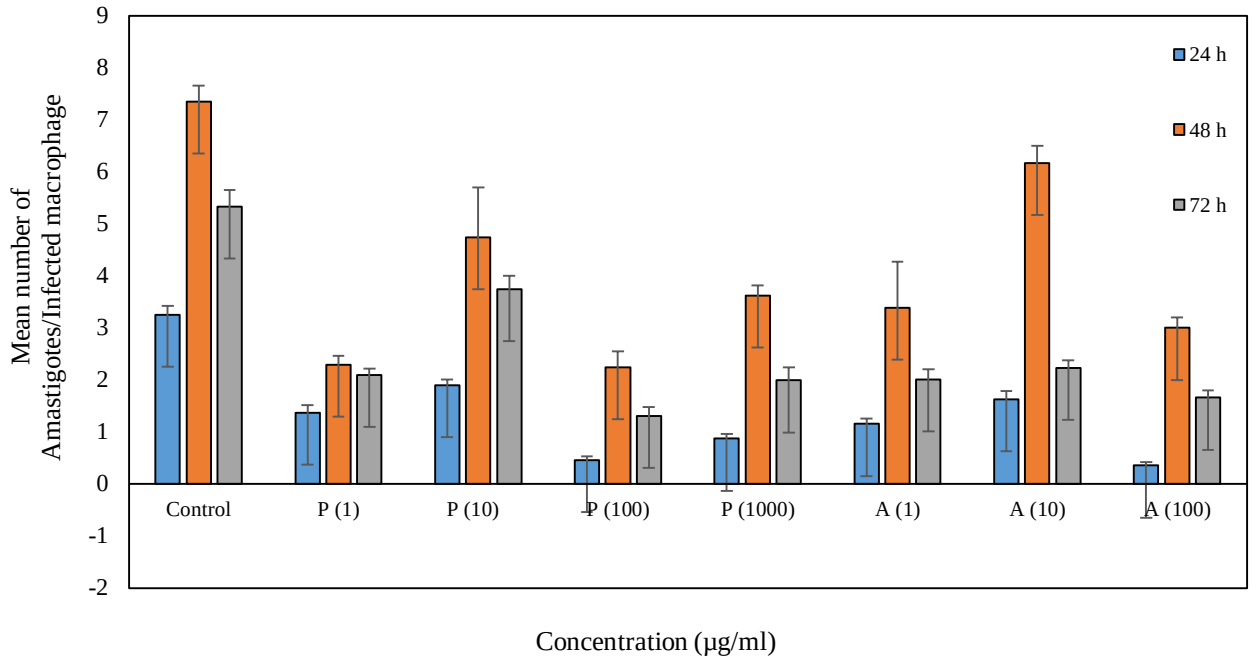


**Figure 2.** Mean Number of Amastigotes Per Infected Macrophage in Control Group and Groups Exposed to Different Concentrations of Plant Extract and Amphotericin B (P: plant extract; A: amphotericin B).

(100 µg/ml). The results of this study revealed that geranium leaf extract reduced the number of promastigotes and amastigotes, although this reduction was non-dose-dependent. At 24 h, the highest growth inhibitor was related to the amount of 10,000 µg/ml of the leaf extract. The effect of 1000 µg/ml was more than 5000 µg/ml concentration. The $IC_{50}$ of plant extract following 24 h was 1508.99 µg/ml, which is consistent with the count results.

The mean number of intra macrophage amastigotes in groups exposed to plant extract with concentrations 1, 10, 100, and 1000 µg/ml and amphotericin B (1, 10, and 100 µg/ml) following 24, 48, and 72 h has been shown in Figure 2. After 24 h, the number of amastigotes in all the samples under treatment with plant extracts and amphotericin B significantly reduced compared to the control group at the level of $p<0.05$. All groups after 48 h exposed to the plant extract showed a significant decrease, but only the group exposed to amphotericin B (100 µg/ml) had a significant difference compared to the control group. In addition, after 72 h there was a significant difference in all treated samples with plant extracts and amphotericin B compared to the control group (Figure 2).

The MTT test results demonstrated that the highest cytotoxicity percentage was in the group under stress of a concentration of 5000 µg/ml of plant extract, which is in contrast to the results of direct counting. After 48 h, the number of promastigotes increased due to the reduction of the inhibitory effect of the extract in the culture medium. Also, as the parasite was in the logarithmic phase of growth, the parasite began to grow again by reducing the inhibitory effect of the plant extract. This result in growth was also observed in the control group. The highest cytotoxicity percentage (74.37%) was observed in the group exposed to a concentration of 10000 µg/ml of plant extract. After 72 h, the parasite stopped growing again and actually its growth had even reduced. The highest reduction was observed in the group exposed to a concentration of 10000 µg/ml of plant extract. In the MTT assay test, the highest cytotoxicity value (68.81%) was observed in the group exposed to the amount of 10000 µg/ml of plant extract, which was completely consistent with direct counting. The results of direct counting showed that the groups treated by amphotericin B decreased the number of promastigotes at 24 h, but at 48 and 72 h this inhibitory effect decreased and the number of parasites increased again. The cytotoxicity percentage in the MTT assay test was completely consistent with the direct counting method and the cytotoxicity percentage decreased over time. The results of direct counting of promastigotes and the MTT assay test demonstrated that the parasite starts to grow again after 48 h and even this increment was seen in the groups exposed to the plant extract and amphotericin B. This increase in the number of promastigotes is due to the parasite being in the logarithmic phase of growth and reducing the inhibitory effect of plant extracts and amphotericin B. However, after 72 h, considerable decreases in the number of promastigotes was observed again, indicating that the parasite entered a static phase of growth. The results of direct counting of intra macrophage amastigotes showed that after 24 h, the greatest decrease in the number was observed in the group under stress of a concentration of 1000 µg/ml of

**Table 2.** Physicochemical Properties of Four Secondary Metabolites of of α-pinene, α-fenchyl acetate, Limonene, and *Trans*-β-caryophyllene

| Compound | Chemical Formula | Molecular Weight | Solubility |
|---|---|---|---|
| α-pinene | $C_{10}H_{16}$ | 136.23 g/mol | Soluble in oils with very low solubility in water |
| α-fenchyl acetate | $C_{12}H_{20}O_2$ | 196.29 g/mol | Soluble in oils and slightly soluble in water |
| Limonene | $C_{10}H_{16}$ | 136.23 g/mol | Miscible with alcohol and ether, very low soluble in water |
| *Trans*-β-caryophyllene | $C_{15}H_{24}$ | 204.35 g/mol | Soluble in oils and ethanol, insoluble in water |

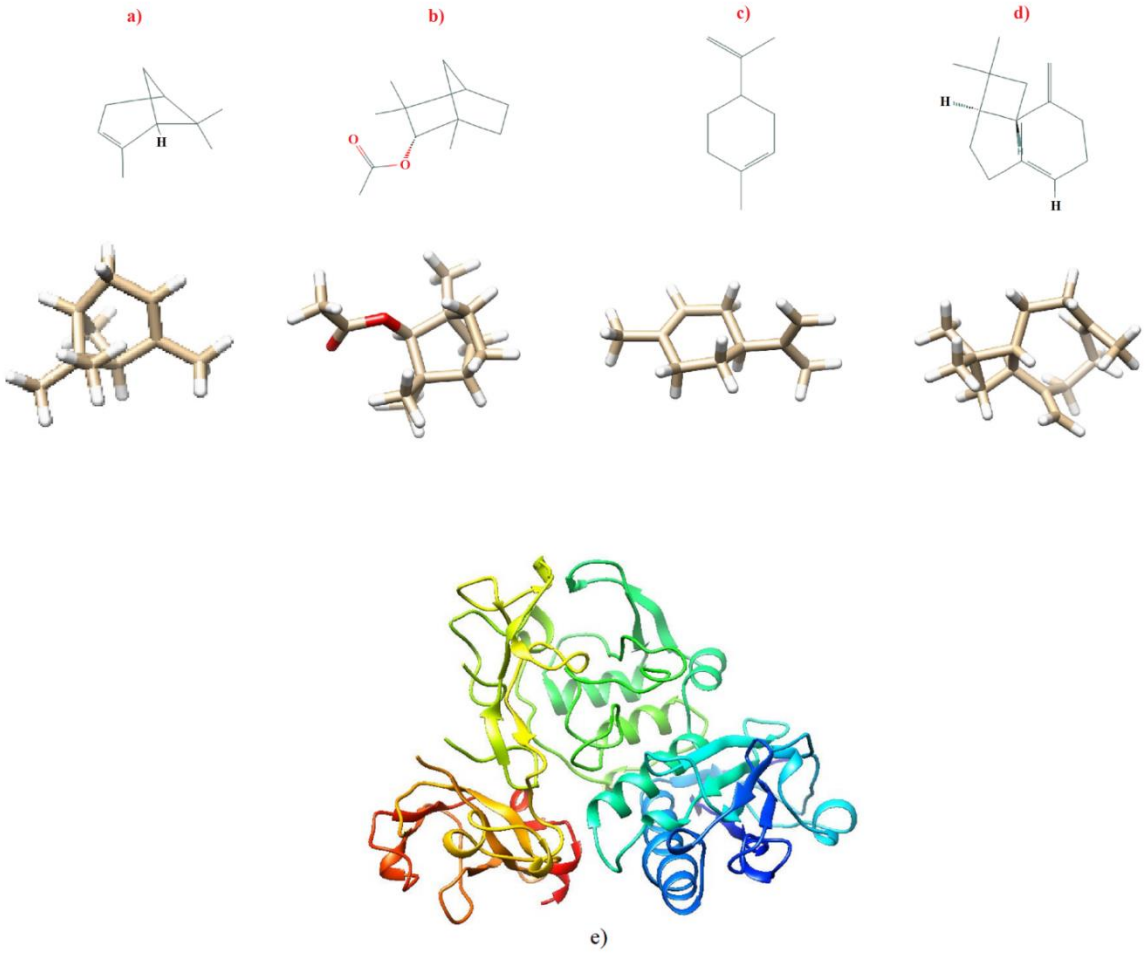


**Figure 3.** 2D and 3D Chemical Structures of α-pinene **(a)**, α-fenchyl acetate **(b)**, limonene **(c)**, *trans*-β-caryophyllene **(d)**, and 3D Structures of GP63 Proteinase (e) (PDB ID: 1LML).

plant extract. After two days, the number of amastigotes in the control sample and all the groups treated via plant extracts and amphotericin B were increased. This result is due to the reduced inhibitory effect of plant extracts and amphotericin B. However, after 72 h, the number of amastigotes decreased again. Moreover, this decrease was also observed in the control group, indicating that macrophages reduced the number of amastigotes during three days.

### *Molecular Docking*

The chemical formula and two main physicochemical properties (molecular weight and solubility) of four bioactive metabolites of α-Pinene, α-Fenchyl acetate, limonene, and *trans*-β-Caryophyllene have been presented in Table 2. All compounds are more lipophilic with very low solubility in aqueous media. Moreover, *trans*-β-Caryophyllene has higher molecular weight (204.35 g/mol) compared to the other compounds. These properties can determine a level of bioavailability and biodegradability properties of these compounds particularly at physiological conditions.[45] The two-Dimensional (2D) and three-Dimensional (3D) chemical structures of four herbal metabolites as well as 3D structures of leishmanolysin enzyme were prepared from PubChem and PDB databases, respectively (Figures 3a-d). Then, all 3D structures of bioactive materials were converted from .sdf format to .pdb by Open Babel version 3.1.1 program. Minimization of these structures was obtained by UCSF chimera 1.12 before the docking process. Five cavities of leishmanolysin enzyme as the main interaction sites were found by MVD, as shown in Figures 4a-c.

The ligand-protein interaction energy analysis was based on the best pose of each bioactive metabolite. Results of

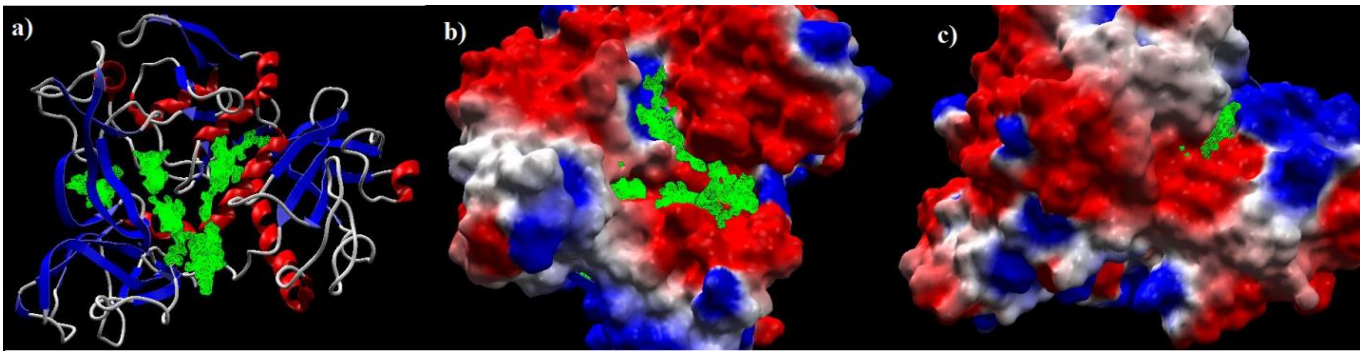


**Figure 4. a)** Secondary Structure and **b)** Surface Structure of the Five MVD-detected Cavities (green sections) in GP63 Enzyme.

**Table 3.** Docking Interaction Parameters Obtained from MVD Four Major Metabolites of *P. quercetorum* (α-Pinene, α-Fenchyl acetate, limonene, and *trans*-β-Caryophyllene) with Leishmanolysin of *L. major*.

| Ligands | Interacting Amino Acids | MolDock Score [GRID] | MolDock Score | Rerank Score |
|---|---|---|---|---|
| α-Pinene | Ala225, Ala346, Ala348, Ala349, Ala350, Arg260, Glu265, His264, Leu224, Leu344, Pro347, Val261 | -45.75 | -46.75 | -41.43 |
| α-Fenchyl acetate | Ala225, Ala346, Ala348, Ala349, Glu265, Gly222, His264, His334, Leu224, Leu344, Pro347, Val223, Val261 | -66.21 | -66.90 | -54.49 |
| Limonene | Ala346, Ala348, Ala349, Ala350, Arg260, Clu265, Glu343, Gly352, His264, Leu257, Leu344, Met345, Pro347, Tyr353, Val261 | -64.24 | -65.71 | -51.24 |
| *trans*-β-Caryophyllene | Ala348, Ala349, Ala350, Ala351, Arg419, Asp342, Gln341, Leu257, Leu420, Met337, Pro416, Pro460, Ser418, Ser421, Thr417, Val457, Val458, Val459 | -75.82 | -77.38 | -37.07 |

Ala: alanine; Glu: glutamine; Gly: glycine; His: histidine; Met: methionine; Pro: proline; Val: valine; Leu: leucine; Ser: serine; Tyr: tyrosine.

**Table 4.** Docking Results of ADV for Four Major Metabolites of *P. quercetorum* (α-Pinene, α-Fenchyl acetate, limonene, and *trans*-β-Caryophyllene) with Leishmanolysin of *L. major.* Only two best modes are presented for each compound.

| Ligands | Interacting Amino Acids | Binding Affinity (kcal/mol) | RMSD L.B | RMSD U.B |
|---|---|---|---|---|
| α-Pinene | Ile413, Tyr461, Asp463, Gly464, His474, Ser476, Leu477 | -5.1 | 0 | 0 |
| α-Fenchyl acetate | Lys307, Glu310, Gln311, Thr355, Ala356, Leu357, Ala375, Val377, Met378, Gln382 | -5.3 | 0 | 0 |
| Limonene | Ile413, Tyr461, Asp463, Gly464, His474, Ser476, Leu477 | -5.0 | 0 | 0 |
| *trans*-β-Caryophyllene | Lys307, Glu310, Gln311, Thy355, Ala356, Phe372, Ala375, Glu376, Val377, Met378, Gln382 | -6.1 | 0 | 0 |

RMSD/L.B: root-mean-square deviation/lower bound; RMSD/U.B: root-mean-square deviation/upper bound

docking studies (Table 3) showed lower MolDock Score [GRID] and MolDock Score values of -75.82 and -77.38 for *trans*-β-Caryophyllene compared to other metabolites, wherein there was a lower value for the better binding affinity of these secondary metabolites with GP63. The sequence of these scores for the four compounds was *trans*-β-Caryophyllene > α-Fenchyl acetate > limonene > α-Pinene. Similar results were observed by ADV program, the highest binding affinity as amount of -6.1 kcal/mol was found for *trans*-β-Caryophyllene as bicyclic sesquiterpene (Table 4). The binding affinity values for α-Fenchyl acetate as bicyclic terpenoide, α-Pinene (terpenoide), and limonene (cyclic monoterpene) were -5.3, -5.1, and -5 kcal/mol, respectively.

In order to obtain a clear understanding of the interaction amino acids at the binding cavity of GP63, a 3D interaction diagram obtained from MVD and Discovery Studio Visualizer have been presented in Figures 5a-d and Figures 7a-d, sequentially. In these figure, the electrostatic surface surrounding each compound shows the contribution of this interaction in the docking of the ligand-receptor complex.

Figures 5a-d and Figures 7-a-d show the interaction of amino acids of leishamnolysin with α-Pinene, α-Fenchyl acetate

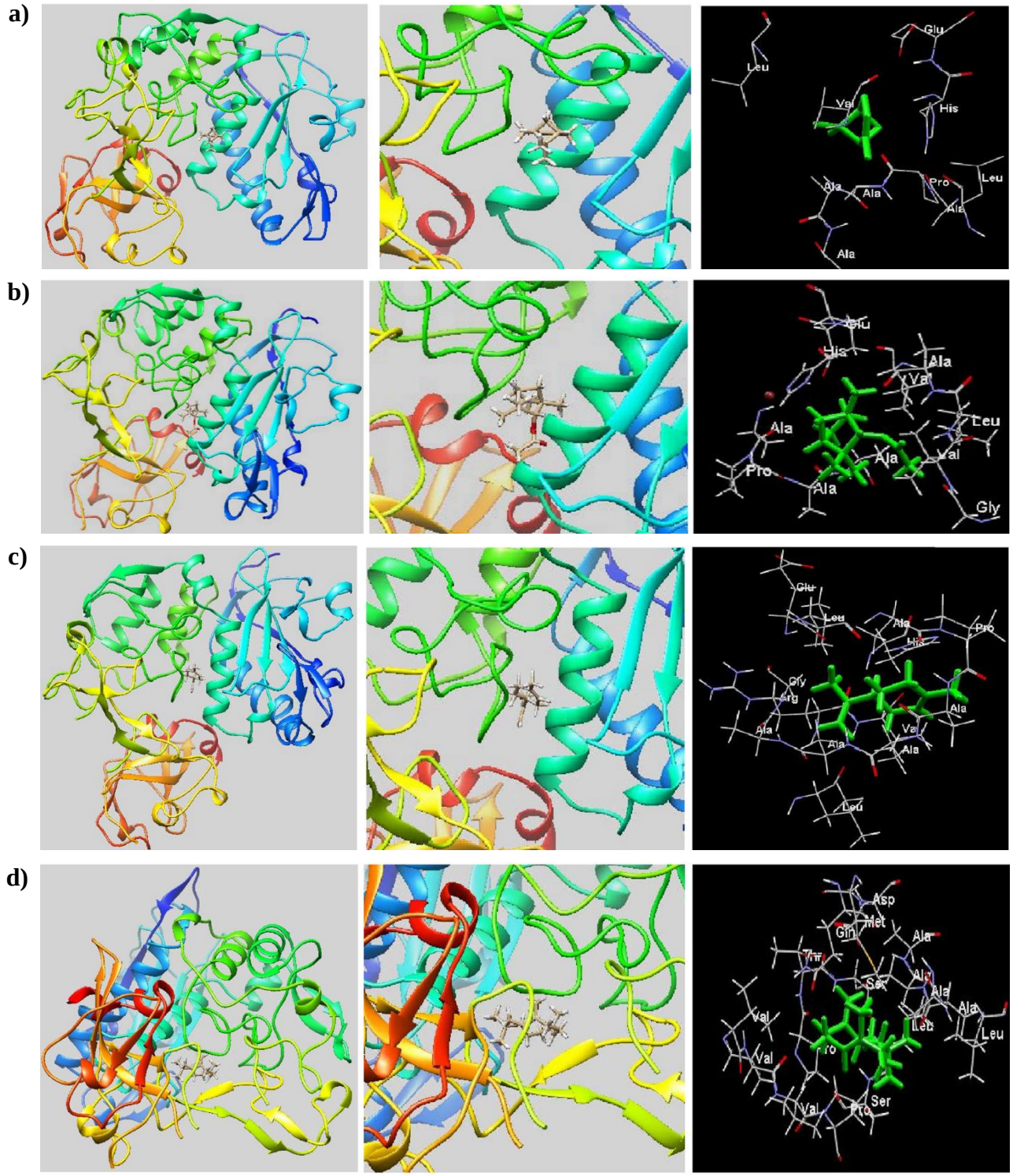


**Figure 5.** 3D Diagram Presenting α-pinene **(a)**, α-fenchyl acetate **(b)**, limonene **(c)**, and *trans*-β-caryophyllene **(d)** Docking Interactions with GP63.

limonene, and *trans*-β-Caryophyllene, separately. For α-Pinene, steric interactions were indicated for Ala349 and Glu265 (Table 3). Leu224 of leishmanolysin had hydrogen bonds with α-Fenchyl acetate. Steric interaction by amino acids of Leu224, Ala349, Glu265, Val261, and His264 was observed for α-Fenchyl acetate. Pro347, Ala346, Ala348, His264, Ala349, Val261, Leu344, Arg260, and Gly352 were indicated for limonene. In the case of *trans*-β-Caryophyllene, Ser418, Leu420, Ala349, Met337, Pro460, Gln341, and Val458 of leishmanolysin were contributed in steric interaction (Table 3). In addition to steric interaction, there was electrostatic interaction between each ligand at the binding cavity of leishmanolysin, which is observable in Figures 6 a-d. In order to compare our results with another docking program, we utilized DockThor as a protein-ligand docking program. As presented in Table 5, affinity values for α-pinene, α-fenchyl

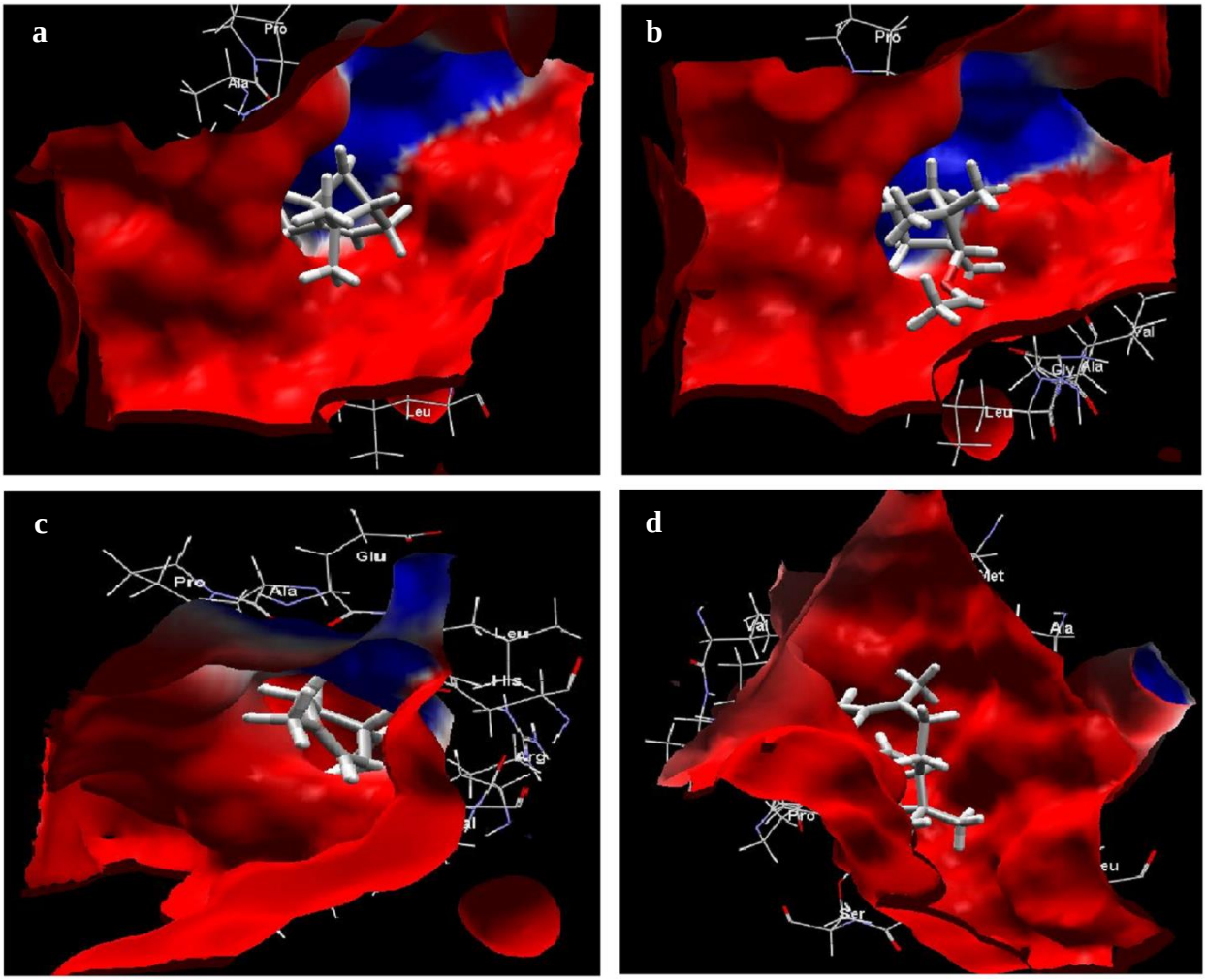


**Figure 6.** Electrostatic Interaction α-Pinene **(a)**, α-Fenchyl acetate **(b)**, limonene **(c)**, and *trans*-β-Caryophyllene **(d)** at the Binding Cavity of the Surface Proteinase GP63.

**Table 5.** Protein-ligand Docking Obtained from DockThor Server (The unit for all parameters is kcal/mol)

| Ligands | Affinity | Total Energy | Interaction Energy | vdW Energy | Electrostatic Energy |
|---|---|---|---|---|---|
| α-Pinene | -7.07 | 47.16 | -14.01 | -13.03 | -0.97 |
| α-Fenchyl acetate | -7.22 | -2.55 | -20.12 | -10.60 | -9.52 |
| Limonene | -6.33 | -14.50 | -10.90 | -9.30 | -1.60 |
| *trans*-β-Caryophyllene | -7.29 | 38.45 | -15.81 | -13.86 | -1.95 |

**Table 6.** Molecular Docking Scores and ACE Resulted from PatchDock Server

| Ligands | Score | Area | ACE |
|---|---|---|---|
| α-Pinene | 2946 | 329.20 | -108.10 |
| α-Fenchyl acetate | 3342 | 375.90 | -125.51 |
| Limonene | 3080 | 351.40 | -112.10 |
| *trans*-β-Caryophyllene | 3434 | 401.40 | -148.49 |

acetate, limonene, and *trans*-β-caryophyllene were -7.07, -7.22, -6.33, and -7.29 kcal/mol, respectively. Therefore, it can be concluded that *trans*-β-caryophyllene has a better docking energy relative to other metabolites suitable to inhibit leishmanolysin. According to the PathDock results, well docked structure and the stable were found for *trans*-β-caryophyllene by score, geometric shape complementarity area, and ACE of 3434, 401.40, -148.49, separately (Table 6).

## Discussion

The $IC_{50}$ amounts of 0.43 ± 0.23 and 0.60 ± 0.36 µg/ml were found for hirudin (the natural peptide available in the salivary glands of blood-sucking leeches) against axenic amastigote and promastigote forms via increasing membrane permeability and apoptosis.[46] The $LD_{50}$ values of 10.40 ± 0.09 and 14.11 ± 0.11 µg/ml were reported against *Leishmania tropica* promastigote for methyl 3,4-dihydroxybenzoate and

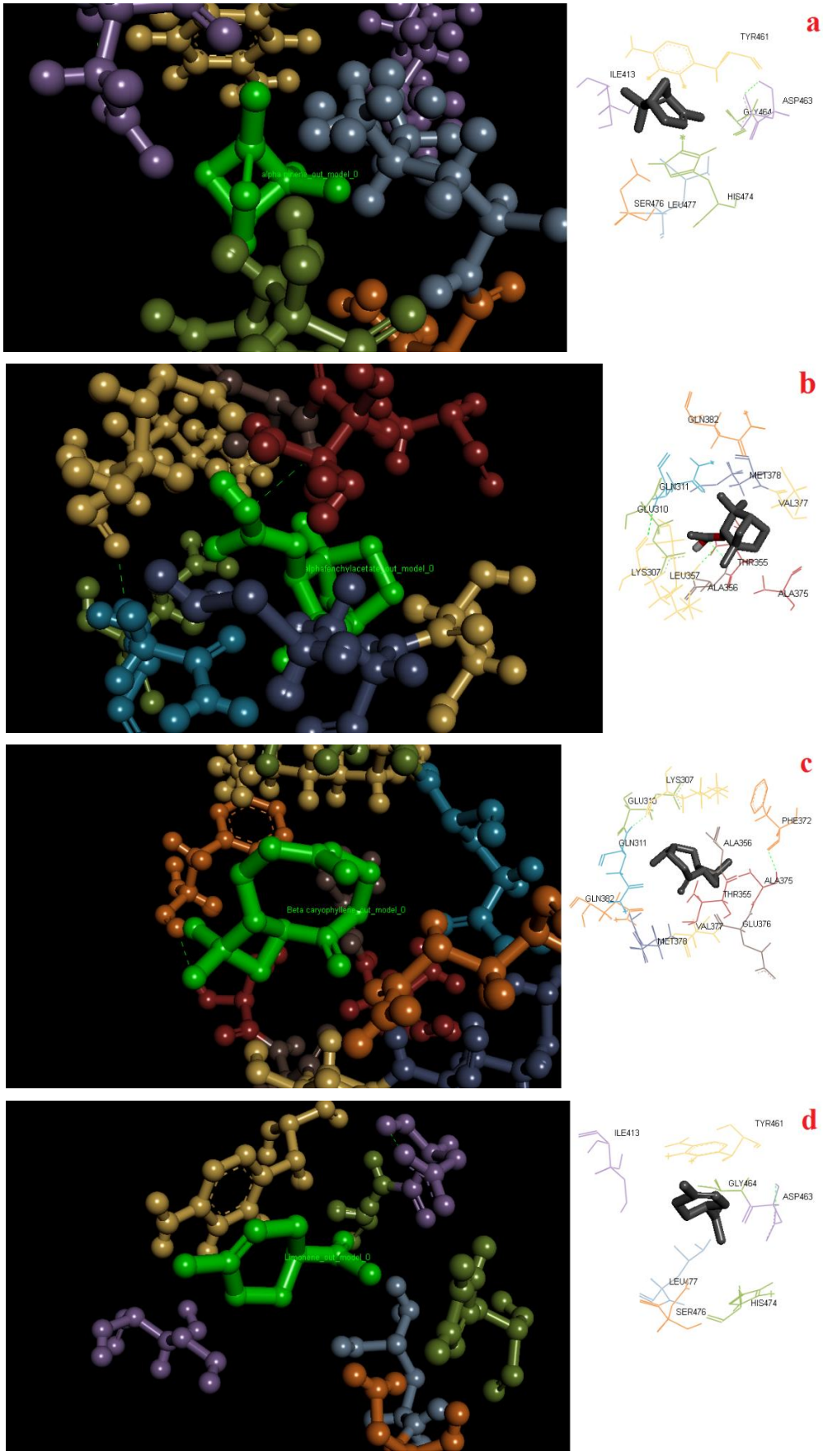


**Figure 7.** The Orientation and 3D Interaction of Target-ligand Diagram Showing Docking Interactions of GP63 with Amino Acids of α-pinene **(a)**, α-fenchyl acetate **(b)**, limonene **(c)**, and *trans*-β-caryophyllene **(d)**.

octadecyl benzoate obtained from *I. spicata* medicinal plant species, respectively.[47] There was $IC_{50}$ amount of 9.2 µg/ml as the significant anti-leishmanial potential toward leishmania promastigotes for β-sitosterol extracted from *I. spicata* compared to the glucantaime drug by $IC_{50}$ of 5.33 µg/ml.[48] Essential oils related to aerial parts of *Rosmarinus officinalis* and *Mentha pulegium* plant species had 2.6 and 1.3 µg/ml of $IC_{50}$ against *L. major*.[49]

The $IC_{50}$ value of 12.1 µg/ml was reported for ferruginol compound, abietane diterpenoid extracted from root extract of *Salvia hydrangea* plant species as the antileishmanial impact on the promastigotes of *L. major*.[50] So far, no data have been found on the effect of *P. quercetorum* on parasites. Only two cases of cytotoxic properties of this plant have been studied on cancer cells. Ari et al. (2017) studied the cyto-genotoxic activity of *P. quercetorum* methanolic extract (at six concentrations of 3.12 to 100 µg/ml for antioxidant effects and three concentrations of 50 to 100 µg/ml for cytotoxic effect) on human breast cancer cells (MCF-7 and MDA-MB-231). The results showed that this plant has many phenolic compounds that have antioxidant effect. Also, it has been shown that this plant has a dose-dependent growth inhibitory effect on cancer cells. The plant significantly killed cancer cells by inducing apoptosis. Extracts of this plant in low concentrations showed a significant genotoxic effect on human breast cancer cells,[51] which was consistent with the results of the present study. The results about the induction of apoptosis by *P. quercetorum* in the non-small cell lung cancer cell line showed that the methanolic extract of *P. quercetorum* has the ability to induce apoptosis in lung cancer cells in three types of cells (A549, PC3, H1299) in vitro,[32] which was similar to the results of our study.

In a similar study, amphotericin B, lanaroflavone, podocarpusflavone A, amentoflavone, and podocarpusflavone B had the binding affinity of -11.5, -10.5, -10.1,-9.9, and -9.6 kcal/mol, sequentially.[52] Based on the docking score evaluated by extra precision glide docking (Glide XP) program, the better binding affinity was as score of -7.834 for N-methyltyrosyl-Nmethyltyrosyl-leucylalanine isolated from *Streptomyces griseus* in comparison with hirsutide isolated from *Hirsutella* sp (-6.325), vignatic acid A extracted from *Vigna radiata* (-5.932), and Cyclo-(Phe-Tyr) by -6.402 value.[53]

Hydrogen bond at the active site of GP63 was reported for four natural flavone metabolites of podocarpusflavone B, podocarpusflavone A, amentoflavone, and lanoraflavone.[52] For all molecules, Ala348 and Ala349 contributed in docking interaction as common amino acids (Table 3 and Figures 5a-d). In addition, Ala349 participated in hydrogen bond interaction in the case of amentoflavone, lanaroflavone, and podocarpusflavone A similar to the terpenoides of the present result.[52] In another study, the binding energies of -5.6 and -5.3 kcal/mol were reported for octadecyl benzoate and 3,4-dihyroxybenzoate (isolated from *Ifloga spicata* medicinal plant species) towards the active site of GP63 by one and two hydrogen bonds related to Ser449 and Glu265/ Leu344 amino acids.[47] Furthermore, β-Sitosterol isolated from this medicinal plant exhibited -33.24 and -6.62 Kcal/mol for binding energy and binding affinity parameters by contribution of Trp226 residue at H-pi interaction, respectively.[48]

Coumarin ($C_9H_6O_2$) is the natural metabolite found in various plant species such as *Hierochloe odorata*, *Dipteryx odorata*, and *Cinnamomum cassia* acting as an anticoagulant and a chemical defense against predators.[54] Strong and stable hydrophobic π-interactions and hydrogen bonds were deciphered for coumarin-incorporated isatin hydrazones compound against the active binding cavity of GP63 as the target protein with an appropriate biocompatible effect on human erythrocytes.[55] In this formula, isatin hydrazones can induce cytotoxicity by inhibiting the Cyclin-Dependent Kinases (CDK2) and Receptor Tyrosine Kinases (RTKs).[56]

## Conclusion

For the first time, the interaction of herbal metabolites of α-Pinene, α-Fenchyl acetate, limonene, and *trans*-β-Caryophyllene were compared against the virulence factor of *L. major* (GP63). According to *in vitro* and *in silico* results, there was more efficent interaction of *trans*-β-Caryophyllene with GP63 enzyme reltive to α-Pinene, α-Fenchyl acetate, and limonene. As a serious challenge, the further surveys considering *in vivo* studies are required to disclose and rediscover the therapeutic behavior of other natural metabolites associated with other medicinal herbs or trees against *L. major*. To the best of our knowledge, the results of this investigation showed that the *P. quercetorum* leaf extract in different concentrations has a cytotoxic effect on amastigotes and promastigotes of *L. major in vitro*, which was not in a dose-dependent manner. Molecular docking studies by two main methods of MVD and ADV programs illustrated the contribution of the various amino acids in interaction of four secondary metabolites of α-Pinene, α-Fenchyl acetate, limonene, and *trans*-β-Caryophyllene with leishmanolysin as one of the important virulence factor of *L. major*. According to the results of these two programs, *trans*-β-Caryophyllene demonstrated a higher binding affinity with GP63 compared to other secondary metabolites of *P. quercetorum* medicinal plant species. The formulation of these secondary metabolites may be considered as a suitable option to treat the leishamaniasis disease in future. We hope that this investigation will open a new horizon in the treatment of leishmaniosis disease by biological compounds.

## Authors' Contributions

Research ideas were conceptualized and refined by YM and MA. YM, SRH, and MA performed the literature search and conducted the experiments. In silico studies were carried out by MA. MA and YM wrote and edited the manuscript. The final manuscript was read and approved by all the authors.

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## Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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