









## Antileishmanial Derivatives of Humulene from *Asteriscus hierochunticus* with *in silico* Tubulin Inhibition Potential

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**Abstract:** Two humulene derivatives (asteriscunolides A (**1**) and C (**2**)) were isolated from the methanolic extract of the whole plant of *Asteriscus hierochunticus*. Their structures were characterized by IR, 1D, and 2D NMR and HRESIMS data analysis, in addition to comparison with literature. Compounds **1** and **2** exhibited excellent *in vitro* antileishmanial activities against *Leishmania donovani* promastigotes with IC<sub>50</sub> values of 8.38 and 3.26 µg/mL and against *Leishmania donovani* axenic amastigotes with IC<sub>50</sub> values of 9.41 and 4.69 µg/mL, respectively while Pentamidine (the standard drug) exhibited IC<sub>50</sub> values of 4.40 and 29.36, respectively. The isolated compounds possessed good activities against *Plasmodium falciparum* (D6) and (W2) strains with IC<sub>50</sub> values ranging from 3.134 - 4.153 µg/mL. Furthermore, **1** and **2** showed no cytotoxic activity against the transformed human monocytic (THP1) cells. The chemical structures of **1** and **2** showed the essential pharmacophoric features of tubulin inhibitors that act through the colchicine binding site. Accordingly, molecular docking studies against the colchicine binding site of Leishmania tubulin have been preceded. Compounds **1** and **2** showed excellent binding mode with free energies of -5.62 and -5.54 kcal/mol, respectively. Further *in silico* studies were carried out and expected that **1** and **2** have the likeness to be drugs through exhibiting good ADMET results, no significant affinity against CYP3A4, and general low toxicity.

**Keywords:** Humulene derivatives; *Asteriscus hierochunticus*; antileishmanial; antimalarial; tubulin inhibitor  
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## 1. Introduction

Natural secondary metabolites are a significant source for the discovery of anti-infective agents from antiquity and till nowadays [1, 2]. These metabolites could be obtained from various sources such as plants [3, 4], marines [5], and microbes [6, 7] and they could be diversified following their chemical structure as saponins [8, 9], pyrones [10], isochromenes [11], flavonoids [12, 13], isoflavonoids [14], terpenoids [15, 16] and alkaloids [17].

Leishmaniasis and malaria were listed by WHO as neglected tropical diseases that affected more than one billion people worldwide. The worst affected countries are the poor without adequate sanitation and who are in close contact with infectious vectors, domestic animals, and livestock [18]. Leishmaniasis is caused by an obligate intracellular protozoan of the genus *Leishmania* (order Kinetoplastida) and infects both humans and animals through the bite of infected female phlebotomine sandflies [19]. Of the four main forms of the disease, Visceral leishmaniasis (VL) is the most fatal in 95 % of the cases if left untreated [20]. The current anti-leishmanial therapy (Sodium stibogluconate, paromomycin, miltefosine, and liposomal amphotericin B) are far from ideal, being not only toxic to sufferers but also expensive, lack efficacy, unavailable to a large population of affected patients, noncompliance to therapy due to mostly parenteral route of administration, and the alarming rate of development of resistance to these drugs, and in some cases, the activity of a particular agent varies with the parasite species, symptoms, and geographical regions and or locations necessitates the need for tolerable and effective agents [20-22].

Malaria on the other hand remains a global health problem and a foremost infectious disease worldwide, despite the huge financial investments (US\$ 3.1 billion in 2017) towards its control and elimination in the past few decades [23]. There are over 3.3 billion people worldwide at risk of the diseases and 90 % of these numbers are from sub-Saharan Africa [23]. Malaria is caused by a highly adaptable protozoan parasite of the genus *Plasmodium*, of which five species are known to cause the disease in humans: *Plasmodium falciparum* (PF), *P. vivax*, *P. ovale*, *P. malaria*, and *P. knowlesi*. *Plasmodium falciparum* is considered to be the most deadly, being responsible for most of the malaria cases amongst infants and young children in sub-Saharan Africa [24, 25]. The development of resistance to most drugs that are currently used in the treatment of malaria; cost; the resistance of the vector (mosquito) to the various insecticides and lack of compliance to therapeutic regimen necessitates the need for new and affordable agents with lower side effects.

*Asteriscus hierochunticus* (Michon) A. Wiklund, family Asteraceae, is a widely distributed plant in several parts of the world. The plant is found mostly in the tropics, Asia, Africa, the Mediterranean region, Europe, North and South America in addition to some islands in the Atlantic and Pacific oceans [26]. Plants and herbs that belong to the family Asteraceae have been used in folklore medicine for the treatment of different diseases such as fever, stomach pain, bronchitis, hemorrhoids, inflammations, and other disorders [27]. There is a lack of phytochemical and pharmacological researches on *A. hierochunticus*. On the other hand, several studies reported the presence of bioactive secondary metabolites such as diterpenes, sesquiterpenes, and phenolics in plants that belong to the family Asteraceae [28]. The trypanocidal, cytotoxic, antidiabetic, antiprotozoal, and antiparasitic activities of different plants belong to this family were also reported [29-31]. Interestingly, the isolation of bioactive humulene sesquiterpenes lactones such as asteriscunolides A-D, steriscanolide, and aquatolide has been reported before from some species of the genus *Asteriscus* [32, 33].

The current study was designed to investigate the phytochemical and biological properties of the whole plant of *A. hierochunticus* through chromatographic fractionation, spectroscopic characterization of isolated compounds, *in vitro* antileishmanial, and antimalarial screening, and determination of their mechanism of action at the cellular level through molecular docking and *in silico* ADMET studies.

Besides, Further docking studies against human cytochrome P450 3A4 (CYP3A4) have been carried out to investigate the ability of the isolated compounds to inhibit CYP3A4.

## 2. Materials and Methods

### 2.1. General

<sup>1</sup>D and <sup>2</sup>D NMR spectra acquisition were done on Bruker Avance III 500 MHz spectrometer, and samples were dissolved in DMSO-d<sub>6</sub> [<sup>13</sup>C and <sup>1</sup>H NMR data at 125 and 500 MHz, respectively]. Chemical shift values were reported in ppm and referenced to the residual protons of solvent (DMSO). Mass spectra were acquired on an Agilent Technologies 6200 series mass spectrometer. IR spectra were measured on Agilent Cary 630 FTIR spectrometer (Agilent Technologies, USA). Optical rotations were measured by AUTOPOL IV Automatic Polarimeter (Rudolph, Hackettstown, NJ, USA). Isolation and purification of all compounds were performed by column chromatography (CC), over normal silica gel (32–63 μ, Dynamic adsorbents Inc.), and reversed-phase C-18 silica (Polar bond, J. T. Baker). Analytical TLC was conducted on precoated silica gel F<sub>254</sub> aluminum sheet (0.25 mm, Sorbent Tech.) or Silica 60 RP-18 F<sub>254</sub> aluminum sheet (20 × 20 cm, Merck). Spots were visualized by observing under UV-254 and 365 nm light and by spraying with 1% vanillin (Sigma) in conc. H<sub>2</sub>SO<sub>4</sub>-EtOH (1:9) followed by heating with a heat gun. All isolation and purification procedures were done by using analytical grade solvents (Fischer chemicals). Pentamidine and amphotericin-B (Sigma-Aldrich, St Louis, MO) were used as standard antileishmanial agents. Chloroquine and artemisinin (Sigma-Aldrich, MO) were used as drug controls in the antimalarial assay.

### 2.2. Plant Material

The whole plant of *A. hierochunticus* was collected from the Mediterranean Coastal area of Egypt and identified in the Department of Botany, Faculty of Science, Mansoura University, Egypt. A voucher specimen (Taxonomic voucher no. AH-14-PD) was deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Mansoura Egypt.

### 2.2. Isolation of Bioactive Compounds

The dried whole plant of *A. hierochunticus* was pulverized to powder. The powdered plant material (500 g) was macerated with methanol (98 %) by percolation (4L × 4 × 20h) at room temperature. The solvent was evaporated *in vacuo* at 40 °C to give 35 g of crude extract (7% yield). The extract (33 g) was mixed with 30 g RP-18 silica gel and applied to a VLC over RP-18 silica (30 cm × 3.5 cm, 500 g) and eluted with gradients of H<sub>2</sub>O/MeOH (90:10 – 0:100) and acetone to give 29 fractions (1-29). Fraction 10 (1.8 g) was further subjected to CC [SiO<sub>2</sub>, EtOAc: CHCl<sub>3</sub>: MeOH: H<sub>2</sub>O (15:8:4:1; 10:6:4:1; 8:2:0.25; 7:3:0.5; MeOH 100%)]. Fractions with similar R<sub>f</sub> were pooled to get 7 fractions (10A-10G). Fraction 10F (200 mg) was processed over CC with normal silica gel (3 x 65cm) eluted with hexane:EtOAc (4:1, 7:3, 3:2) to give 8 fractions (10F1-10F8). Repeated purification of fraction 10F4 (40 mg) on CC with normal silica using hexane: EtOAc (4:1 -3:2) yielded compounds **1** (3 mg) a white solid, and **2** was purified from PTLC (20x20 cm, 500μm pore size), eluted with hexane: chloroform (1:4), 50 mL, and further washed with acetone (20 mL) to give 6.9 mg of white solid.

### 2.3. Antileishmanial Assay

The compounds (asteriscunolides A and C) and standard antileishmanial agents (positive controls) were evaluated against *L. donovani* promastigote, axenic amastigotes, and intracellular amastigotes according to the Alamar Blue colorimetric assay which measures quantitatively the proliferation of various cell lines (humans, animals, and bacteria) [34]. The assay was done on a culture of *L. donovani* promastigotes (2 × 10<sup>5</sup> cells/mL) in a 96-well microplate into which test sample solutions were added, diluted appropriately to a final concentration of 10 μg/mL. The plates were subsequently incubated for 72 hours at 26°C for and parasites growth determined. Included in the assay as positive control agents were Pentamidine and amphotericin B used as standard antileishmanial agents. The IC<sub>50</sub> values of test compounds and standard antileishmanial agents were computed from the growth-inhibition curve.

## Two antileishmanial asteriscunolides

### 2.4. *In vitro* Macrophage Amastigote Assay

This was done according to the parasite-rescue and transformation assay described before [35]. In this assay differentiated THP1 cells (human acute monocytic leukemia cell line), that obtained from NCNPR, are infected *in vitro* with *Leishmania donovani*, and the efficacy of the pure compounds and standard antileishmanial agents determined. The THP1 culture was prepared from a four-day-old cell culture (not more than  $10^6$  cells/ml) and diluted with 10% heat-inactivated fetal bovine serum (FBS) to give a cell count of  $2.5 \times 10^5$  cells/mL in RPMI-1640. The cells previously diluted with pure DMSO were seeded into a 96-well microplate to which phorbol 12-myristate 13-acetate (PMA) at a final concentration of 25 ng/ml was added. Cells suspension (200  $\mu$ L) with cells count of  $5 \times 10^4$  cells were dispensed into a clear flat-bottom 96-well plate and incubated overnight in a 37 °C, 5% CO<sub>2</sub> incubator for the differentiation of the cells. After the incubation period, PMA-treated THP1 cells were carefully washed twice with serum-free RPMI-1640 medium. The medium was replaced with 200  $\mu$ l ( $5 \times 10^5$  cells/mL) of the diluted *Leishmania donovani* promastigotes culture harvested at the stationary phase (metacyclic infective stage) and suspended in RPMI-1640 medium with 2% FBS at a density of  $2.5 \times 10^6$  cells/mL and further incubated in a 5% CO<sub>2</sub> incubator at 37°C for at least 24 hours to allow infection of macrophages with the Leishmania parasites. After 24 hours the non-adherent macrophages and unattached Leishmania promastigotes were washed off with serum-free RPMI-1640 medium. The infected macrophages were then incubated with different concentrations of standard antileishmanial drugs (Pentamidine and amphotericin B) or the test compounds for 48 hours at 37°C and 5% CO<sub>2</sub> in 200  $\mu$ L RPMI1640 medium and 2% FBS. The experiment was repeated for uninfected THP1 cells, infected cells without drugs or test compounds. After the incubation period, the cultures were washed off with serum-free RPMI-1640 and treated with 20  $\mu$ L of 0.05% sodium dodecyl sulfate in RPMI-1640 medium for 30 seconds to release amastigotes from the infected macrophages. Furthermore, 180  $\mu$ L of RPMI-1640 medium with 10% FBS was added to each well and incubated at 26°C for 48 hours to allow the transformation of the rescued amastigotes to promastigotes. Promastigotes can reduce the oxidized, blue, non-fluorescent Alamar Blue to a pink fluorescent dye [36]. Consequently, to each well containing transformed promastigotes, 20  $\mu$ L of Alamar blue was added and the plates incubated at 26°C for 24 hours and read on a Fluostar Galaxy fluorimeter (BMG Lab Technologies) at 544 nm excitation, 590nm emission wavelengths. Each compound was tested in duplicate at six concentrations and the dose-response curves (percent growth vs. concentration of the drug or test compound) were prepared with ExcelFit and IC<sub>50</sub>/IC<sub>90</sub> values computed.

### 2.5. Cytotoxicity Assay

The cytotoxicity of the compounds was also tested against transformed human monocytic (THP-1) cells. The use of THP-1 cells gives some advantages over human primary monocytes such as the homogeneity of its genetic background which minimizes the variability in the cell phenotype. Another advantage is the relative simplicity to down-regulate specific protein's expression [37]. The assay method previously described by Jain et al was adopted. In this experiment, a 4 days old culture of THP1 cells in the experimental phase diluted with RPMI medium to  $2.5 \times 10^5$  cells/mL was used. To achieve the parasite cells transformation to the adherent macrophages, Phorbol 12-myristate 13-acetate (PMA) was added to the culture at a concentration of 25 ng/mL. The THP1 cell culture treated with PMA was seeded into 96 well plates with 200  $\mu$ l culture ( $2.5 \times 10^5$  cells/mL) in each well and incubated overnight at 37 °C in a 5 % CO<sub>2</sub> incubator. The medium in plates with THP1 cells was replaced with a fresh medium. The test compounds and standards diluted with RPMI medium in separate plates were added to these plates and further incubated in a 5 % CO<sub>2</sub> incubator at 37 °C for 48 h. After the incubation period, 10  $\mu$ L of Alamar Blue solution (AbD Serotec, catalog number BUF012B) was added to each well and the plates were incubated further overnight. Again, standard fluorescence was measured on a Fluostar Galaxy fluorometer (BMG LabTechnologies) at 544 nm excitation, 590 nm emission wavelengths. The half-maximal concentration IC<sub>50</sub> and IC<sub>90</sub> values were computed from the dose-response growth inhibition curve by XLfit version 5.2.2 [38].

## 2.6. Antimalarial Assay

The *in vitro* antiplasmodial activity of asteriscunolides A and C was measured by a colorimetric assay that determines the parasite's lactate dehydrogenase (pLDH) activity [39, 40]. Included in this assay are two strains of *Plasmodium falciparum* (Sierra Leone D6 [chloroquine-sensitive] and Indochina W2 [chloroquine-resistant]) obtained from the Walter Reed Army Institute of Research, Silver Spring, MD. The effects of the test compounds on plasmodial LDH activity were determined using Malstat reagent (Flow Inc, Portland, OR). DMSO (0.25 %) and chloroquine/artemisinin were included in each assay which serves as a vehicle and positive control drugs, respectively. The selectivity indices (SI) were computed by measuring the cytotoxicity of the test compounds against Vero cell lines (monkey fibroblast).

## 2.7. In-silico Investigations

### 2.7.1. Docking Studies

The crystal structures of the tubulin heterodimer (PDB ID: 4o2b, resolution: 3.20 Å) and Cytochrome P450 3A4 (PDB ID: 4D7D, resolution: 2.76Å) were downloaded from Protein Data Bank (<http://www.pdb.org>). Molecular Operating Environment (MOE) version 2014 was used for the docking analysis [41]. In these studies, the free energies and binding modes of the examined molecules against target proteins were determined. The MMFF94X force field was applied. At first, the water molecules were removed from the crystal structures of the target proteins, retaining only the chains that are essential for binding. The co-crystallized ligands (colchicine and PKT) were used as reference ligands. Then, the protein structures were protonated and the hydrogen atoms were hidden. Next, the energy was minimized and the binding pockets of the colchicine binding site and CYP3A4 active pocket. were identified. The structures of the examined compounds and the co-crystallized ligands were drawn using ChemBioDraw Ultra 14.0 and saved as SDF formats. Then, the saved files were opened using MOE software and 3D structures were protonated. Next, the energy of the molecules was minimized. Validation processes were performed for each target receptor by running the docking process for only the co-crystallized ligands. Low RMSD values between docked and crystal conformations indicate valid performance [42, 43]. MOE docking parameters include Triangle Matcher Algorithm with two rescoring functions London dG and GBVI/WSA dG were utilized to generate 30 poses of each compound. As a result, mdb output files were generated enclosing all docking results with scoring and multiple conformations of ligands. Results were finally inspected to determine the most promising compound by visualizing various interactions of ligands within the binding pocket. The output from MOE was further analyzed and visualized using Discovery Studio 4.0 software [44-46].

### 2.7.2. ADMET

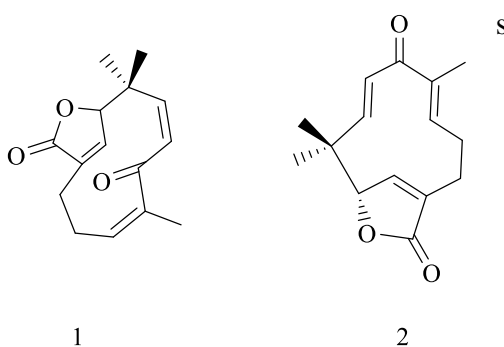
ADMET descriptors (absorption, distribution, metabolism, excretion, and toxicity) of the compounds were determined using Discovery studio 4.0. At first, the CHARMM force field was applied then the compounds were prepared and minimized according to the preparation of small molecule protocol. Then ADMET descriptors protocol was applied to carry out these studies [42, 43].

## 3. Results and Discussion

### 3.1. Isolation and Structure Elucidation

Two sesquiterpene lactones (**1** and **2**) were isolated from the methanolic extract of the whole plant of *Asteriscus hierochunticus*. They were identified as asteriscunolides A and C (Figure 1) by analysis of their IR, NMR, and mass spectral data (supplementary data) which were in agreement with those found in the literature [33]. To the best of our knowledge, compounds **1** and **2** were isolated from *Asteriscus hierochunticus* for the first time.

## Two antileishmanial asteriscunolides



**Figure 1.** Chemical structures of asteriscunolides A (**1**) and C (**2**)

### 3.2. Biological Activities

Compound **1** exhibited excellent activities against *L. donovani* promastigotes, and axenic amastigotes with  $IC_{50}$  values of 8.38 - 9.41  $\mu\text{g/mL}$ , respectively (Table 1). Interestingly, Compound **2** was 1.34-fold more potent than Pentamidine against *L. donovani* promastigotes and 6.62-fold than the axenic amastigotes with  $IC_{50}$  values of 3.26 and 4.69 as against pentamidine values of 4.40 and 29.36, respectively.

**Table 1.** Antileishmanial and cytotoxic activities of asteriscunolides A and C as  $IC_{50}$  in ( $\mu\text{g/mL}$ ) and their SI, test conc. (10 – 0.4  $\mu\text{g/mL}$ )

Compounds	<i>L. donovani</i> Promastigotes		<i>L. donovani</i> axenic amastigotes		<i>L. donovani</i> intracellular Amastigotes		THP1 cytotoxicity
	$IC_{50}$	SI	$IC_{50}$	SI	$IC_{50}$	SI	$IC_{50}$
<b>1</b>	8.38	>1.19	9.41	>1.06	>10	1	>10
<b>2</b>	3.26	>3.06	4.69	>2.13	>10	1	>10
<b>Pentamidine</b>	4.4004	NT	29.366	NT	9.303	NT	NT
<b>Amphotericin B</b>	0.2315	NT	1.233	NT	0.1937	NT	NT

NT=not tested.

The test of the compounds against THP1 cells was done to assess the cytotoxic effects of the compounds against host cells. From the study, asteriscunolides A and C were not cytotoxic against THP1 and hence may not show cytotoxicity against host cells. Asteriscunolides A and C were however less effective against intracellular amastigotes ( $IC_{50}$  >10  $\mu\text{g/mL}$ ) (Table 1). Several studies have shown the effectiveness of compounds isolated from medicinal plants in inhibiting the growth of *L. Donovanii* promastigotes, axenic amastigotes, and intracellular amastigotes *in vitro* cultures [47-49]. Asteriscunolides A and C showed significant inhibition of both chloroquine-sensitive (D6) and resistant (W2) strains of *plasmodium falciparum* in the pLDH assay with  $IC_{50}$  values of 3.1336 and 3.4929  $\mu\text{g/mL}$  and 4.1536 and >4.760  $\mu\text{g/mL}$ , respectively (Table 2). Asteriscunolide A was more effective against the two plasmodium strains compared to **2**. However, asteriscunolide C was more effective against the chloroquine-resistant strain compared to the sensitive strain. The standard antimalarial drugs (chloroquine and artemisinin) exhibited better antimalarial activity against the two plasmodium strains. Chea et al. demonstrated the *in vitro* antimalarial activity of sesquiterpene lactones isolated from *Vernonia cinerea* and show that the isolated compounds 8 $\alpha$ -tigloyloxyhirsutinolide-13-O-acetate) and 8 $\alpha$ -(4-hydroxymethacryloyloxy)hirsutinolide-13-O-acetate) exhibited a strong *in vitro* activity against chloroquine-resistant strain (W2) of *P. falciparum* with  $IC_{50}$  3.9 and 3.5 mM, respectively [50, 51]. In another study, some sesquiterpene lactones isolated from *Ambrosia tenuifolia*, peruvian, and psilostachyin, showed significant antiplasmodial activity against both resistant and sensitive strains of *P. falciparum* ( $IC_{50}$  values 0.3 – 1.8  $\mu\text{g/mL}$ ) [51]. Also, Rauter et al. reported the cytotoxic activities of compounds **1** and **2** against different cell lines: P388; A-549; HT-29, and MEL-28 with good  $IC_{50}$  values

range between 4-10  $\mu\text{M}$  [52]. Asteriscunolide A in particular was reported to induce significant apoptosis against human tumor cells with  $\text{IC}_{50}$  values of approximately 5  $\mu\text{M}$  [53]. The selectivity indices of the compounds (ratio of the  $\text{IC}_{50}$  values of the compounds against THP1 cells to that against the test organisms) are as shown in table 1. Sanon *et al.* reported the SI values of sixty crude plant extracts and showed that  $\text{SI} > 1$  possesses low levels of cytotoxicity risks [54]. As a result, compounds **1** and **2** could be classified as possessing a low risk of cytotoxicity with  $\text{SI} > 1$ .

**Table 2.** Antimalarial activity of compounds 1-3 as  $\text{IC}_{50}$  in ( $\mu\text{g}/\text{mL}$ ) and their SI values, test conc. (4.760 - 0.5288  $\mu\text{g}/\text{mL}$ )

Compounds	<i>P. falciparum</i> D6		<i>P. falciparum</i> W2		VERO
	$\text{IC}_{50}$	SI	$\text{IC}_{50}$	SI	$\text{IC}_{50}$
<b>1</b>	3.1336	>1.5	3.4929	>1.4	>4.760
<b>2</b>	>4.760	1	4.5136	>1.1	>4.760
<b>Artemisinin*</b>	<0.0264	>9.0	<0.0264	>9.0	>0.238
<b>Chloroquine*</b>	<0.0264	>9.0	0.2028	>1.2	>0.238

\* Artemisinin and Chloroquine test concentration (0.238 - 0.0264  $\mu\text{g}/\text{mL}$ )

### 3.3. In-silico Studies

#### 3.3.1. Docking Against Tubulin Protein

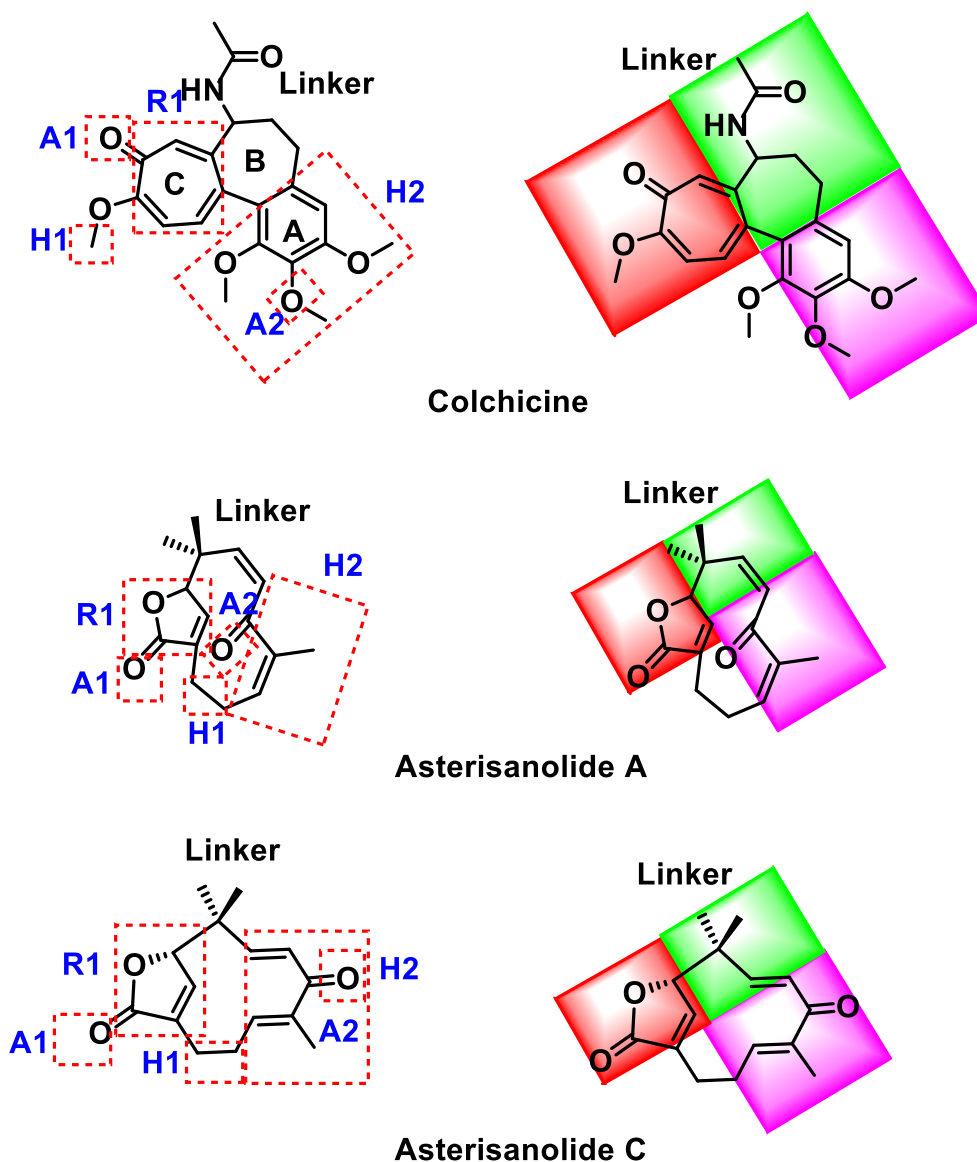
Tubulin is the known protein dimer consisting of  $\alpha$ - and  $\beta$ -subunits and is the principal constituent of microtubule filaments. The microtubule filaments play an essential role in the nuclear division of eukaryotic cells [55]. Consequently, the inhibition of tubulin is a major target of several antileishmanial [56, 57], antimalarial [58, 59], and of course anticancer drugs [60].

The colchicine binding site (CBS) is a major target for several tubulin inhibitors. The colchicine binding site is composed of  $\alpha$  and  $\beta$  subunits [61]. The reported pharmacophoric features shared by tubulin inhibitors are three hydrogen bond acceptors (A1, A2, and A3), a hydrogen bond donor (D1), two hydrophobic centers (H1 and H2), and a planar group (R1) [62, 63]. The presence of these features or most of them in a molecule makes it a probable CBSI.

Regarding colchicine, as the prototype of this category of active molecules, it is formed of three parts: "A" ring, "B" ring (linker), "C" and ring. Structure-activity study indicates the essentiality of A and C rings for high-affinity binding to tubulin [64]. Figure 2 clarifies that asteriscunolides A and C share the same pharmacophoric points with colchicine as CBSIs.

We encouraged to investigate the binding affinities of **1** and **2** as CBSIs against the tubulin enzyme because of three reasons; firstly, the promising antileishmanial and antimalarial activities of **1** and **2** drove us toward further investigations for their possible mechanism(s) of action on a molecular level by exploring their binding affinity against the enzyme which may be responsible for both activities. Secondly, the reported data mentioned the connection between anti-tubulin and antileishmanial [64, 65] as well as antimalarial [66] activities. Finally, the presence of essential pharmacophoric features of CBSIs in compounds **1** and **2**. In our molecular docking studies, compounds **1** and **2** were docked against tubulin heterodimers (PDB ID: 4o2b) using MOE2014 to determine the free energy and binding mode to select the most promising molecules ( $\Delta\text{G}$ ) [67]. (Table 3).

## Two antileishmanial asteriscunolides



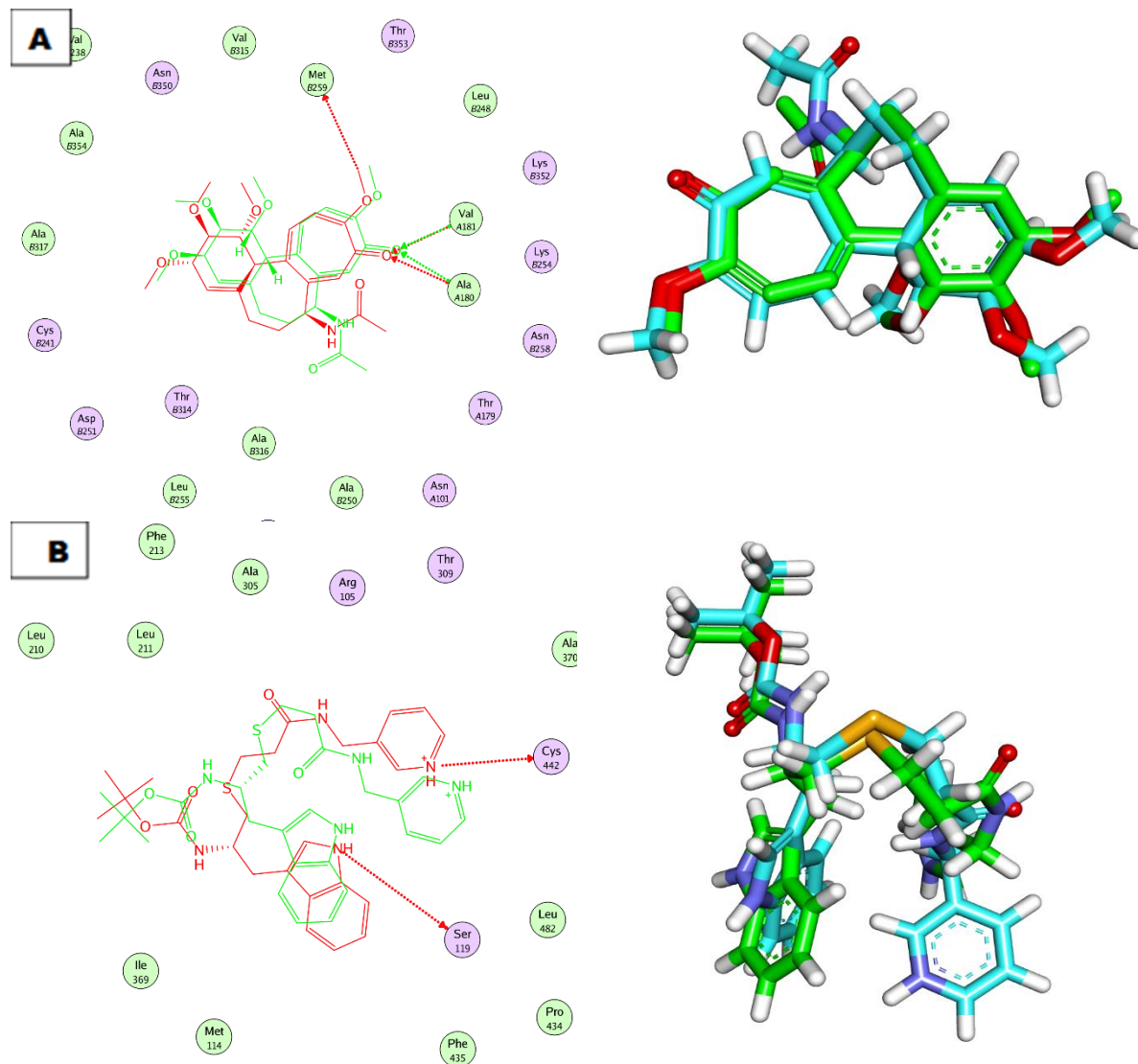
**Figure 2.** Asteriscunolides A and C as colchicine having almost the same essential pharmacophoric features of the reported CBSIs

**Table 3.** The docking binding free energies of compounds 2 and 3 and co-crystallized ligand (colchicine) against tubulin (PDB ID: 4o2b)

Compounds	Binding free energy (kcal/mol)	No. of hydrogen bonds	No. of hydrophobic interaction
1	-5.62	1	7
2	-5.54	2	3
Colchicine	-8.18	3	5



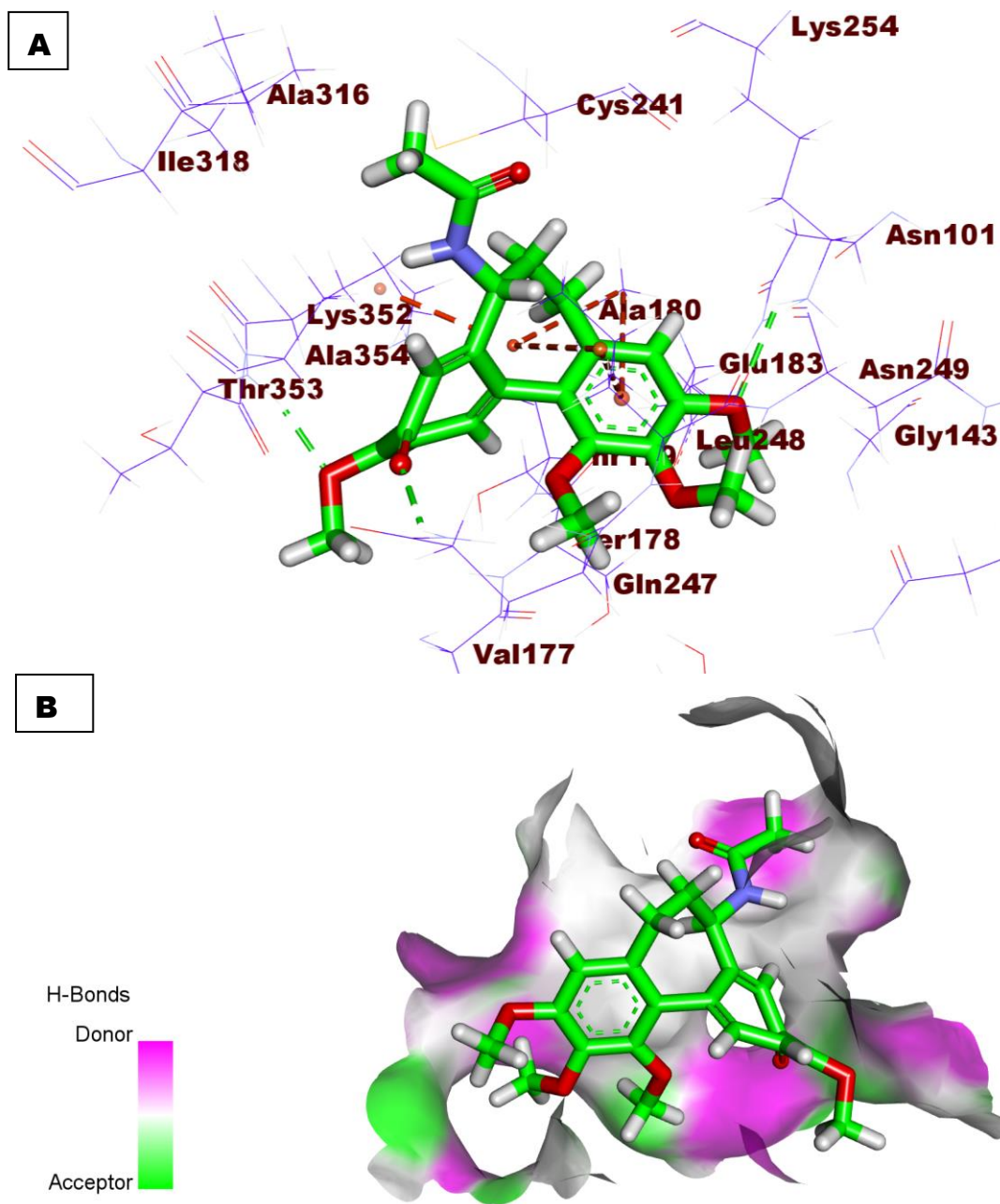
Validation of the docking procedures was achieved via re-docking of the co-crystallized ligands (colchicine and PKT) against the active pockets of colchicine binding site and CYP3A4), respectively. The calculated RMSD values between the re-docked pose and the co-crystallized one were 1.00 and 2.50 Å, respectively. Such values of RMSD indicated the efficiency and validity of the docking processes (Figure 3).



**Figure 3.** A) Superimposition of the co-crystallized pose and the docking pose of colchicine.  
B) Superimposition of the co-crystallized pose and the docking pose of PKT

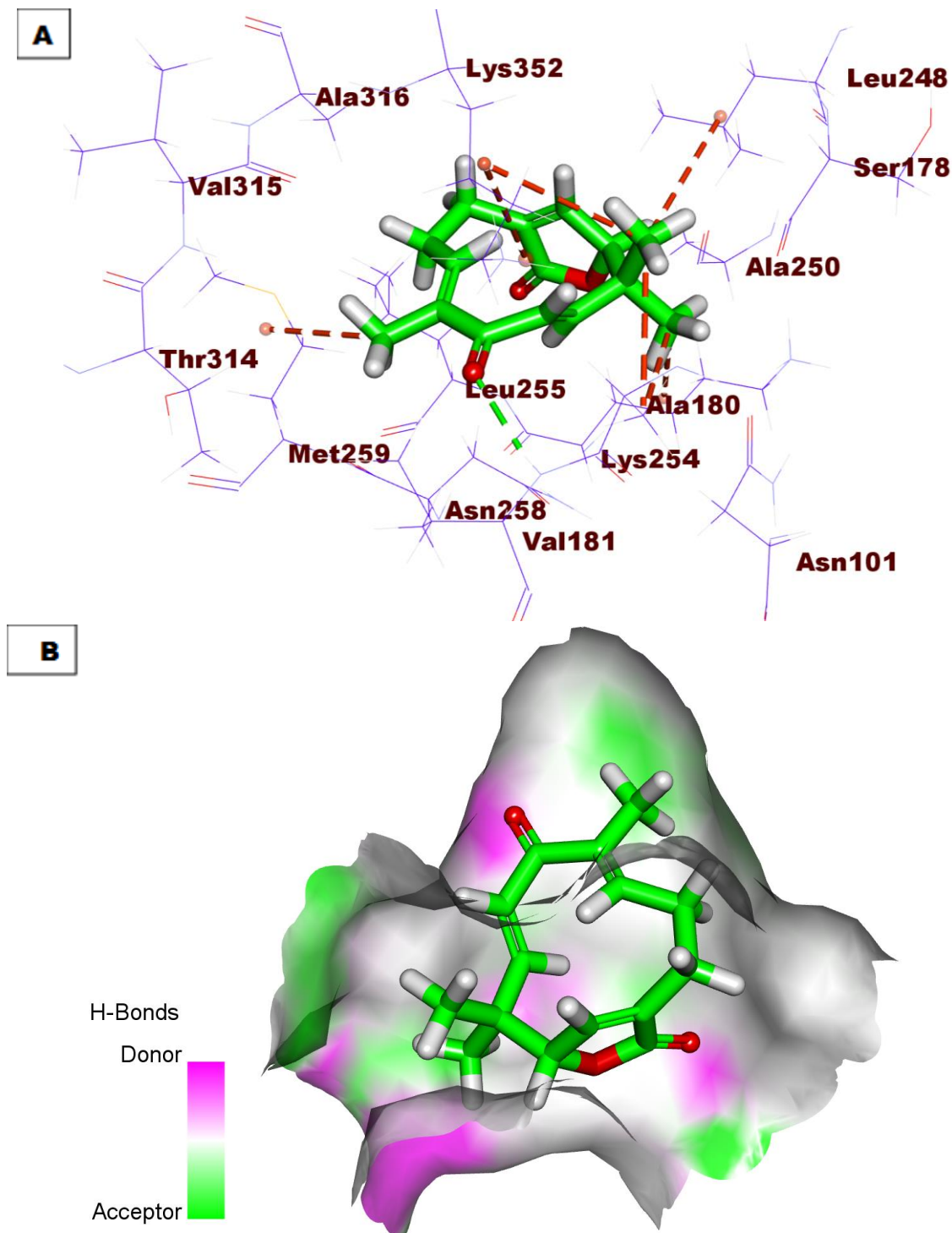
The results obtained from this experiment indicated that compounds **1** and **2** showed a similar position and orientation inside the putative binding site. The binding mode of the co-crystallized ligand, colchicine, exhibited an energy binding value of -8.18 kcal/mol. In detail, the trimethoxy phenyl moiety occupied the first pocket of the binding site forming a hydrogen bond with the amino acid Lys254. Also, it formed two hydrophobic interactions with both Ala180 and Leu248. The central ring structure and its acetamide side chain formed clear hydrophobic interactions with Ala180, Lys352, and Leu248 occupying the second pocket of the binding site. The tropolone ring occupied the third pocket of the binding site and formed two hydrogen bonds with both Gln247 and Thr353 (Figure 4).

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**Figure 4.** A) Co-crystallized ligand (colchicine) docked into the colchicine binding site of tubulin  
B) Mapping surface showing colchicine occupying the colchicine binding site of tubulin

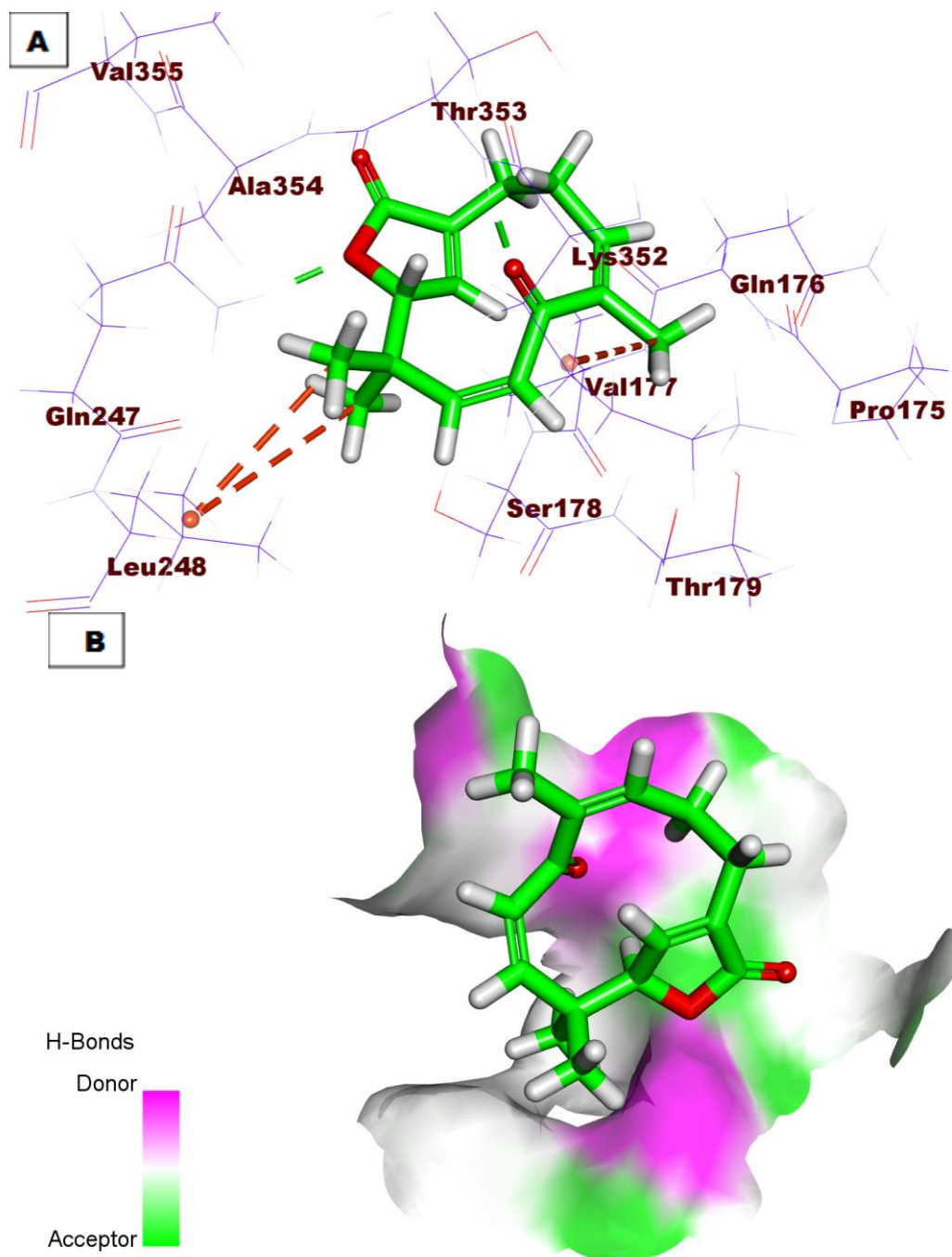
The proposed binding mode of compound **1** (affinity value of -5.62 kcal/mol with a hydrogen bond and seven hydrophobic interactions) was virtually the same as that of colchicine (Figure 5). The Furan-2(5*H*)-one moiety occupied the first pocket of the colchicine binding site. The methyl group and its adjacent carbonyl group occupied the second pocket of the colchicine binding site forming a hydrogen bond with Val181 and a hydrophobic interaction with Met259. The geminal dimethyl group occupied the third pocket of the colchicine binding site forming five hydrophobic interactions with Lys254, Ala180, Lue248, and Lys352.



**Figure 5.** A) Compound **1** docked into the colchicine binding site of tubulin  
B) Mapping surface showing compound **1** occupying the colchicine binding site of tubulin

Compound **2** (affinity value of -5.54 kcal/mol with two hydrogen bonds and three hydrophobic interactions) exhibited a binding mode like that of colchicine (Figure 6). The Furan-2(5H)-one moiety occupied the first pocket of the colchicine binding site forming a hydrogen bond with Gln247. The methyl group and its adjacent carbonyl group occupied the second pocket-forming a hydrogen bond with Thr353 and a hydrophobic interaction with Lys352. The geminal dimethyl group occupied the third pocket of the colchicine binding site forming two hydrophobic interactions with Lue248.

## Two antileishmanial asteriscunolides



**Figure 6.** A) Compound 2 docked into the colchicine binding site of tubulin  
B) Mapping surface showing compound 2 occupying the colchicine binding site of tubulin

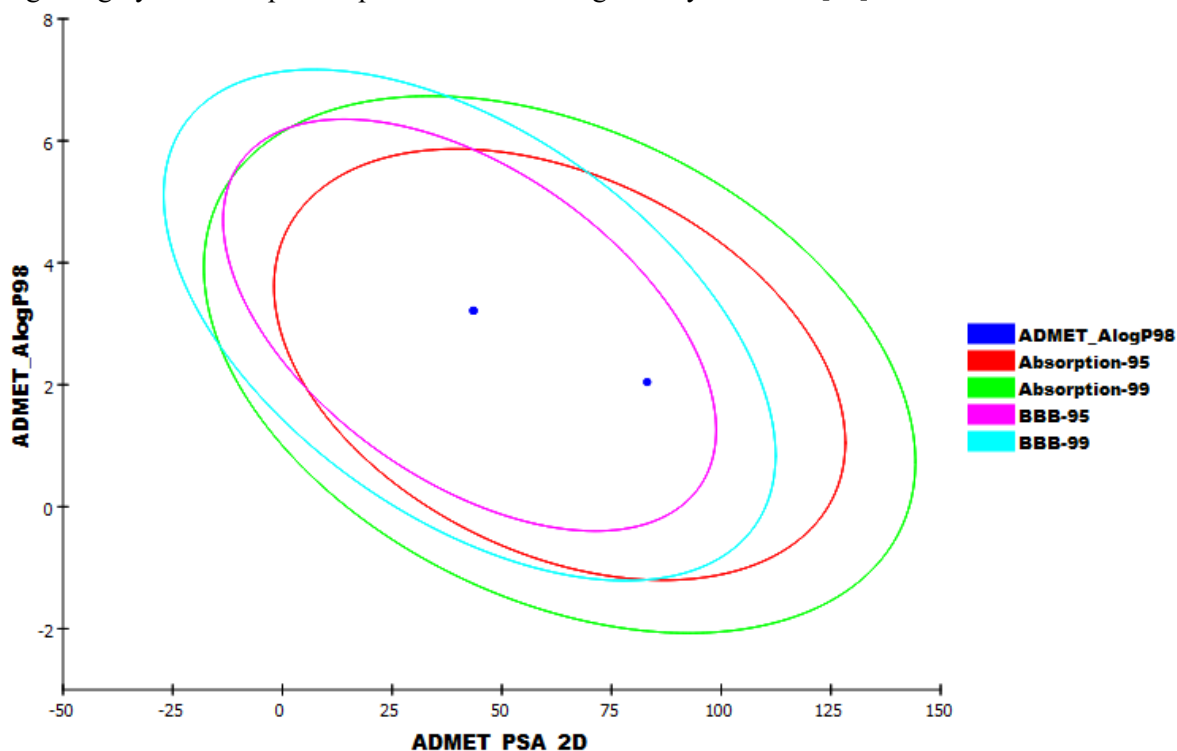
### 3.3.2. *In silico* ADMET Analysis

The excellent *in vitro* and *in silico* results encouraged us to run *in silico* ADMET studies at this early stage of compound design to reduce the risk of late stages attrition.

The conducted ADMET studies included i) Aqueous solubility which indicates the solubility of a compound in the water at 25°C. ii) Intestinal absorption which predicts the human intestinal absorption (HIA) for the tested compound after oral administration. iii) Blood-brain barrier penetration which predicts the ability of a molecule to penetrate the blood-brain barrier. iv) CYP2D6 binding predicts the potentiality of a drug to inhibit the cytochrome P450 2D6 enzyme. And v) Plasma protein binding predicts the fraction of drug bound to plasma proteins in the blood.

ADMET study revealed that compounds **1** and **2** exhibited low levels of aqueous solubility but predict that the compounds possessed good intestinal absorption levels. Furthermore, studies revealed that compounds **1** and **2** have high BBB penetration levels, compared to colchicine which showed low BBB penetration. Several reports have been published about the ability of the parasite *P. falciparum* to pass BBP causing cerebral malaria [68]. Also, some reports evidenced that *L. donovani* can infect and inflame the brain [69]. Thus, the potential of **1** and **2** to penetrate BBP gives them an excellent advantage to be an option in such cases.

CYP2D6 is an enzyme that has an essential role in the metabolism process of a wide range of molecules inside the liver. Therefore, its inhibition causes severe cases of drug-drug interaction [70]. Compounds **1** and **2** were predicted as non-inhibitors of CYP2D6, suggesting that the compounds possessed very low potentials for drug-drug interactions. Compounds **1** and **2** were predicted to bind plasma protein over and more than 90%. (Figure 7 and Table 4). Ghafourian and Amin indicated that a drug is highly bound to plasma protein if the binding affinity is  $\geq 90\%$  [71].



**Figure 7.** The expected ADMET study of compounds **1** and **2**

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**Table 4.** Predicted ADMET for compounds **1**, **2**, and colchicine

Compounds	BBB level <sup>a</sup>	Solubility level <sup>b</sup>	Absorption level <sup>c</sup>	CYP2D6 prediction <sup>d</sup>	PPB prediction <sup>e</sup>
<b>1</b>	1	2	0	false	true
<b>2</b>	1	2	0	false	true
<b>Colchicine</b>	3	3	0	false	false

<sup>a</sup> BBB level, blood brain barrier level, 0 = very high, 1 = high, 2 = medium, 3 = low, 4 = very low.

<sup>b</sup> Solubility level, 1 = very low, 2 = low, 3 = good, 4 = optimal.

<sup>c</sup> Absorption level, 0 = good, 1 = moderate, 2 = poor, 3 = very poor.

<sup>d</sup> CYP2D6, cytochrome P2D6, TRUE = inhibitor, FALSE = non inhibitor.

<sup>e</sup> PBB, plasma protein binding, FALSE means less than 90%, TRUE means more than 90%

### 3.3.3. Docking Against Cytochrome P450

Human cytochrome P450 3A4 (CYP3A4) is an essential metabolizing enzyme that is responsible for the oxidation of most xenobiotics. Consequently, the inhibition of CYP3A4 may lead to severe drug-drug interactions and toxicity [72]. Accordingly, further docking studies were carried out to investigate the binding ability of asteriscunolides A and C against Cytochrome P450 (CYP3A4).

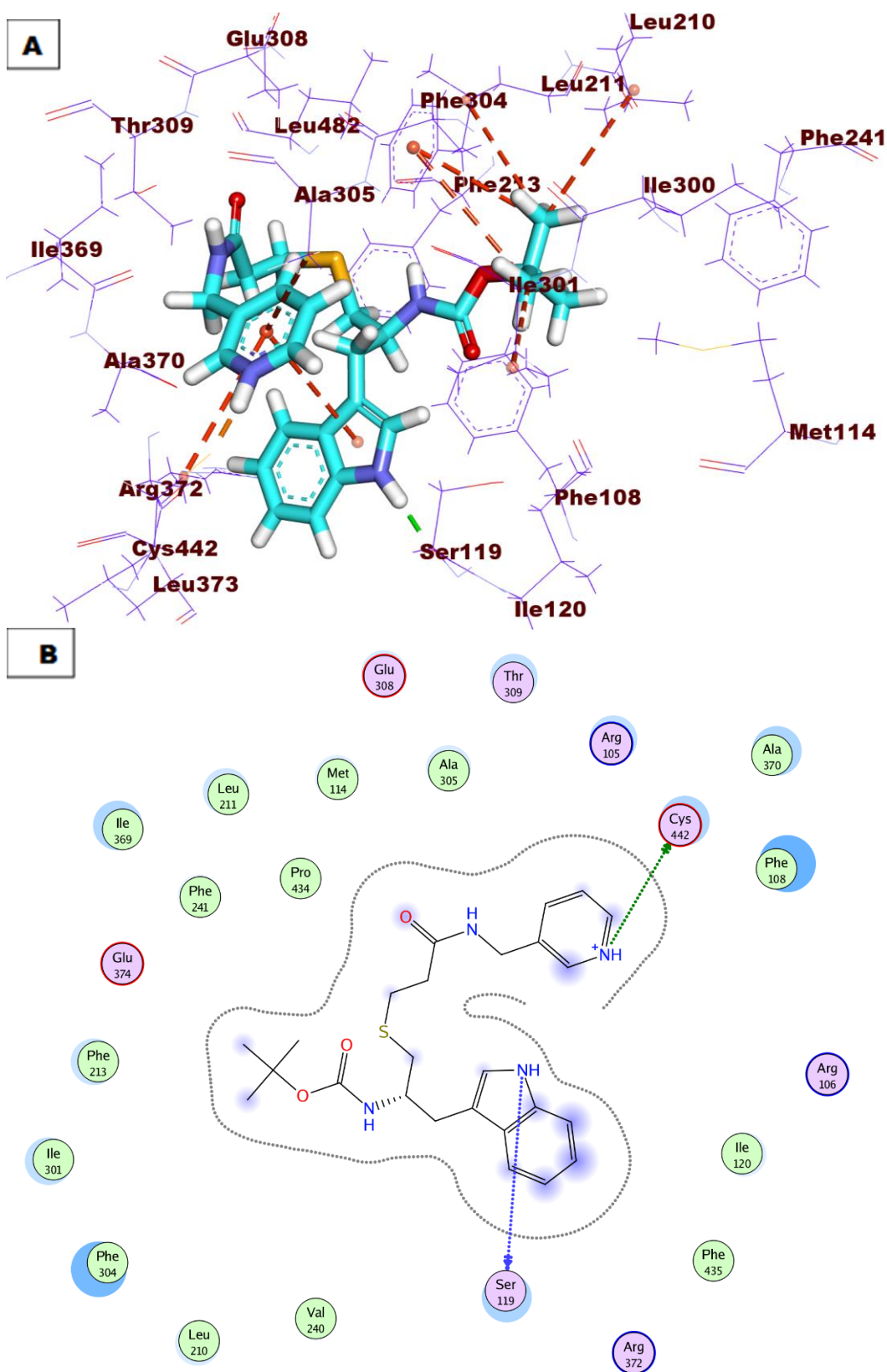
In this work, molecular docking investigational studies were performed against cytochrome P450 3A4 (CYP3A4) (PDB ID: 4D7D). The co-crystallized ligand (PKT) was used as a reference molecule and the binding free energies ( $\Delta G$ ) were reported in Table 5.

The proposed binding mode of PKT showed an affinity value equals to -29.53 kcal/ mol. It formed two hydrogen bonds with Ser119 and Cys442. In addition, it formed eight hydrophobic interactions with Leu210, Leu211, Ile301, Ala305, Cys442, and Phe304 (Figure 8).

The proposed binding modes of compounds **1** and **2** were illustrated in Figure 9 and Figure 10, with binding energy far less than that of the co-crystallized ligand. Compound **1** showed binding energy of -14.54 kcal/ mol and compound **2** exhibited binding energy of -15.05 kcal/ mol. In addition, the binding modes of these compounds are different from that of the co-crystallized ligand. These results revealed that the isolated compound cannot inhibit CYP3A4.

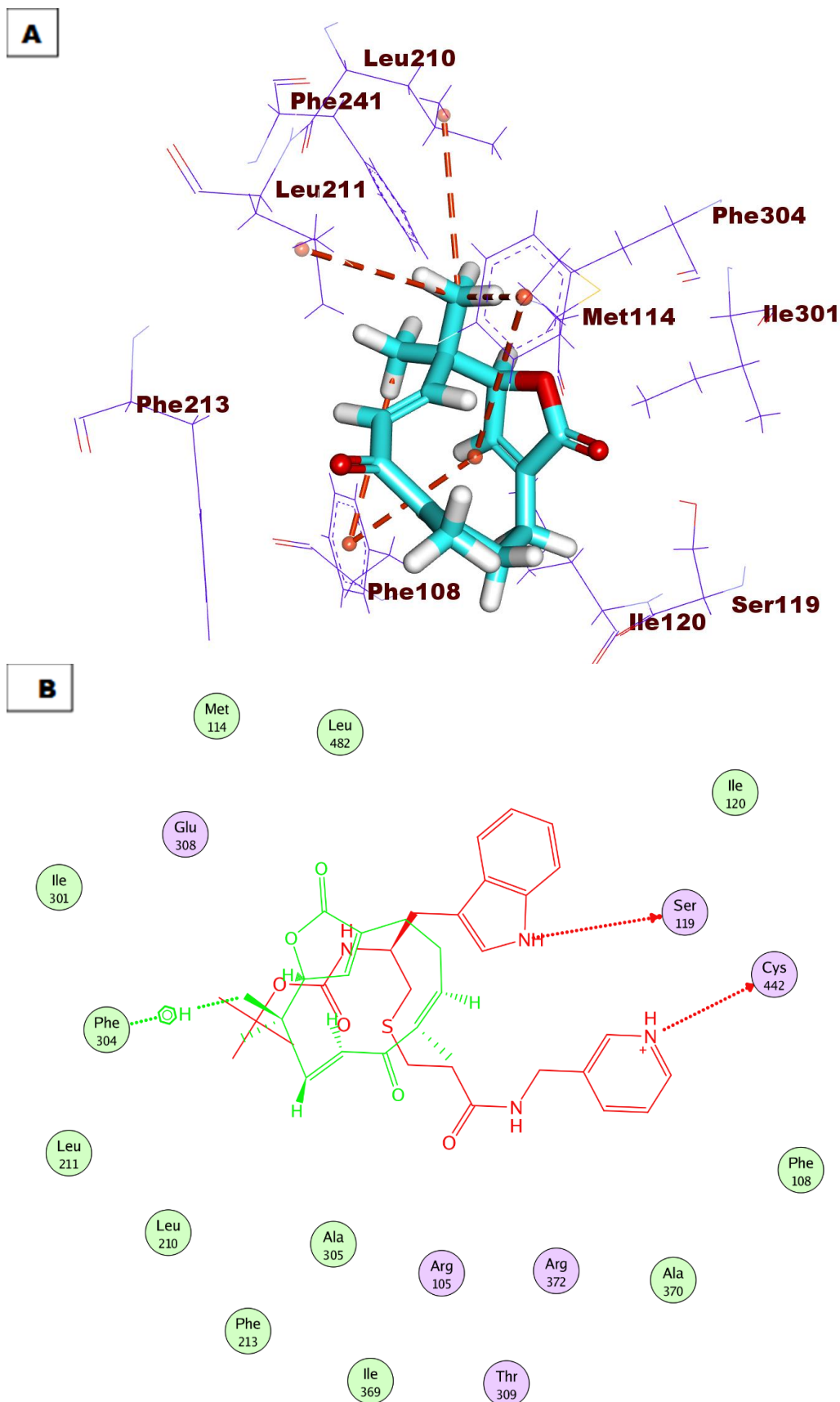
**Table 5.** The docking binding free energies of compounds **1** and **2** and co-crystallized ligand (PKT) against Cytochrome P450 (PDB ID: 4D7D)

Compounds	Binding free energy (kcal/mol)	No. of hydrogen bonds	No. of hydrophobic interaction
<b>1</b>	-14.54	0	8
<b>2</b>	-15.05	0	8
PKT	-29.53	2	8

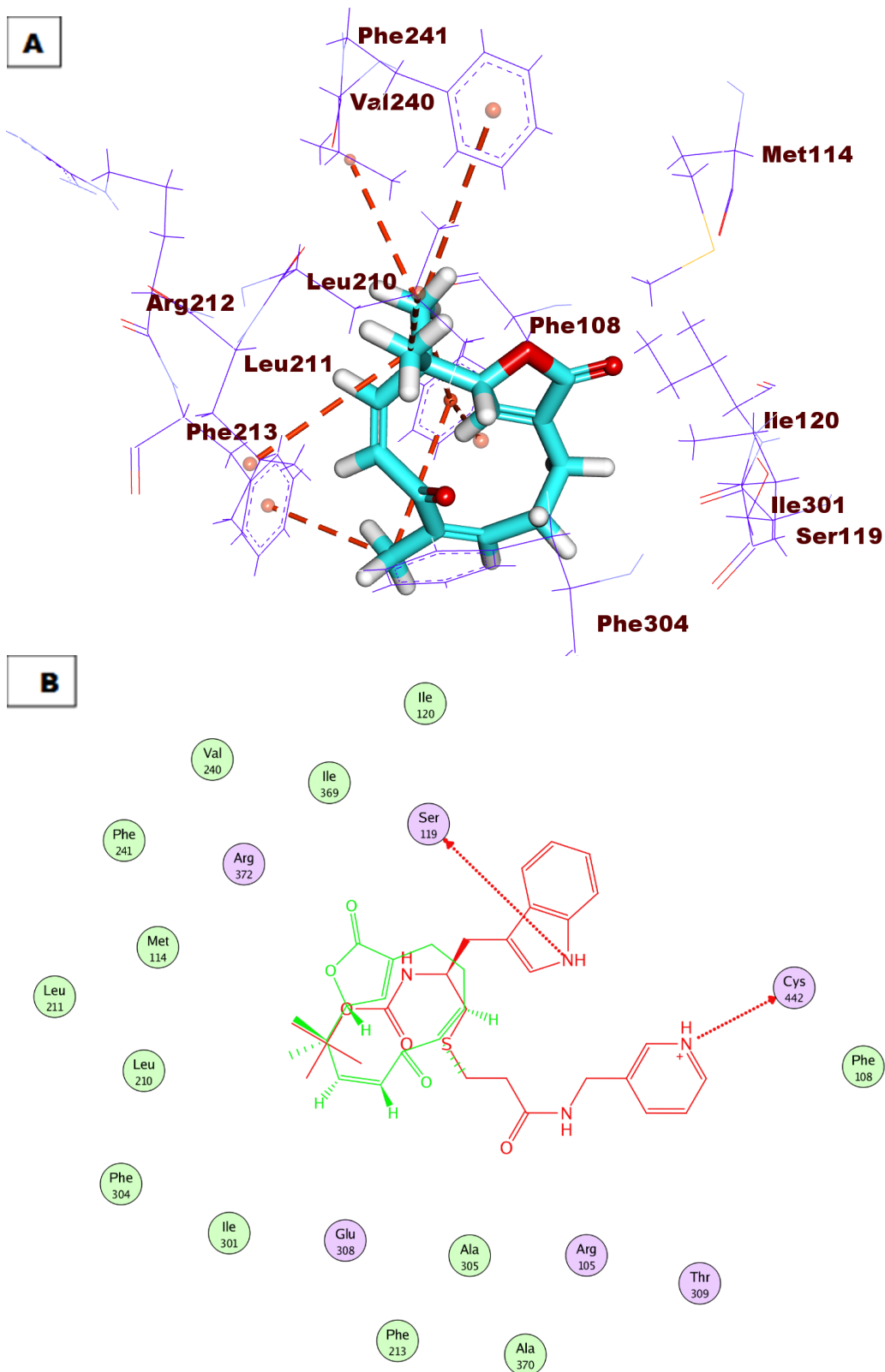


**Figure 8.** A) 3D Structure of co-crystallized ligand (PKT) docked into the active pocket of Cytochrome P450 B) 2D Structure of co-crystallized ligand (PKT) docked into the active pocket of Cytochrome P450

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**Figure 10.** A) 3D Structure of compound 2 docked into the active pocket of Cytochrome P450  
B) 2D Structure of compound 1 docked into the active pocket of Cytochrome P450.

## 4. Conclusions

This study reported the isolation of asteriscunolides A (**1**) and C (**2**) from *Asteriscus hierochunticus* for the first time to the best of our knowledge. The compounds exhibited excellent *in vitro* leishmanicidal against *L. donovani* promastigotes, axenic amastigotes, and intracellular amastigotes and also good antimalarial activities against both the sensitive and resistant strains of *P. falciparum*. Compounds **1** and **2** were observed to effectively bind to parasites tubulin from the docking studies, which has been reported to be the target proteins for various antileishmanial and antimalarial agents and could therefore serve as potential inhibitors of parasites tubulin and its polymerization. *In silico* ADMET and docking studies of the asteriscunolides A and C revealed low hepatotoxicity, low interactions with cytochrome p450, good intestinal absorption, and penetration of the blood-brain barrier. Asteriscunolides A and C could be good candidates for further investigations as potential leishmanicidal and antimalarial agents, especially in cases of cerebral malaria and leishmaniasis.

## Conflicts of interest

There are no conflicts to declare.

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## Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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