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In vitro antileishmanial activity and molecular docking studies of lupeol and monostearin, isolated from *Parkia biglobosa*

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ABSTRACT

Parkia biglobosa (Leguminosae) is used traditionally for managing leishmaniasis. However, there are no reports of the antileishmanial constituents from the plant. This study is aimed at isolating antileishmanial compounds, from the stem bark of *P. biglobosa,* and assessing their mechanism of action using molecular docking studies. Bioassay-guided fractionation led to the isolation of two known compounds, lupeol and monostearin. Their structures were elucidated using their mass spectra. Using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymathoxyphenyl)-2-(4-sulfophenyl)-2H-tretrazolium (MTS) assay, the evaluated antileishmanial effects of lupeol (1) and monostearin (2) showed IC_{50} values of 164.42 and 151.99 µg/mL respectively. The antileishmanial effects of the crude extract, non-polar, mid-polar and polar fractions were determined to be 64.43, 126.25, 725.65 and 167.52 µg/mL respectively. Molecular docking studies of the two isolates within the active sites of two validated targets in the *Leishmania* parasite: trypanothione reductase (TR) and pteridine reductase 1 (PTR1), showed that both compounds established important interactions with key amino acid residues in both proteins. Lupeol exhibited binding affinities of -7.1 kcal/mol and -10.2 kcal/mol for TR and PTR1, respectively, better than monostearin in both cases. Our findings back the claim that *P. biglobosa* stem bark possesses antileishmanial effects. Furthermore, the isolates lupeol and monostearin may be partly responsible for the observed activities.

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Introduction

Globally, an estimated 0.9 to 1.6 million cases of leishmaniasis occur annually and disproportionately affect the world's poorest people ([17].). Chemotherapy, the main form of control, is hampered by its unavailability, cost, unfavorable dosing regimen and parasite resistance [14]. Consequently, many of the sufferers turn to Traditional medicine particularly medicinal plants for the management of leishmaniasis.

Parkia biglobosa (Jacq) R. Br. ex G. Don (Leguminosae), commonly called the African locust bean tree, monkey cutlass tree or fern tree, is used traditionally by indigenous healers for various health disorders. Among other uses, *P. biglobosa* stem bark powder is applied to open wounds for their management [22], along with the leaves of the same plant for the treatment of malaria and fever [8].

Previous pharmacological studies of *P. biglobosa* have reported the antimalarial effects of phenolic compounds obtained from its methanolic extract [8]. In another study, the hydroethanolic and aqueous extract of the leaves and stem bark were active against enterobacteria [19]. The aqueous seed extract showed curative, but no protective effects, on gentamycin-induced kidney damage in Sprague Dawley rats [5]. Further, the seeds increase mating behavior and implantation rates, with no observable maternal and developmental toxicity in Sprague Dawley rats and ICR mice [6]. A recent antileishmanial screening assay revealed that *P. biglobosa* possessed cidal effects on the promastigote form of *Leishmania donovani* [20].

Previous phytochemical investigations of the plant have revealed the presence of 7–hydroxy-3,8,4′-trimethoxyflavone, 2′–hydroxy-3,7,8,4′,5′-penta-methoxyflavone and lupeol [18] as phytoconstituents. Additionally, 4-*O*-methyl–epi-gallocatechin, epi–gallocatechin, epi–catechin 3-*O*-gallate, and epi–gallocatechin 3-*O*-gallate [26] are phytoconstituents previously isolated from the plant.

However, the chemical compound(s) responsible for the antileishmanial effects and their possible mechanisms of action have not been investigated. Our work focused on these gaps in knowledge will address some of Africa Union's Agenda 2063 Goals and their linked Sustainable Development Goals, with many additional benefits for Ghana, Africa and the rest of the world. Phytochemical investigations that identify the antileishmanial constituents of *P. biglobosa* will further back its traditional use in the treatment of leishmaniasis. This is relevant for the practitioners (medical herbalists and traditional healers), and their clients, that employ the plant in antileishmanial herbal remedies with the added benefit of sustainable natural resource management and biodiversity conservation. In addition, the isolated antileishmanial compounds, with clearly elucidated mechanisms of action, could facilitate the development of new, effective and selective chemotherapeutic agents for leishmaniasis. The research and development required benefits education, training, innovation and has the potential for spin-outs generating sustainable and inclusive economic growth.

Consequently, the study investigated *P. biglobosa* extracts for their antileishmanial effects using the 3-(4,5-dimethylthiazol-2-yl)−5-(3-carboxymathoxyphenyl)−2-(4-sulfophenyl)−2H-tretrazolium (MTS) assay and isolated and identified the antileishmanial compounds from the active fractions using chromatographic and spectroscopic techniques. Additionally, molecular docking was also used to investigate the interactions of the identified compounds with 2 validated targets of the Leishmania parasite: pteridine reductase 1 (PTR1) and trypanothione reductase (TR).

Fractionation of the crude extract of the bark resulted in the isolation and characterization of the triterpene compound lupeol (**1**) and monoglyceride compound monostearin (**2**). These two compounds were further screened for their antileishmanial activities and docked against both PTR1 and TR. Our work highlights the first-time report of the in *silico* assessment of the two compounds, using molecular docking, against validated druggable protein targets.

Materials and methods

Plant material

The stem bark of *P. biglobosa* was collected from the road linking Kwahu Asakraka to Kwahu Adowso close to River Afram (N6°69′04.617″ and W0°61′83.967) in November 2019. The collected plant was authenticated, and the voucher specimen (KNUST/HM1/2019/SB024) was deposited in the Department of Herbal Medicine herbarium, KNUST.

General experimental procedures

EIMS data were recorded on a PerkinElmer GC Clarus 580 gas chromatograph interfaced to a PerkinElmer Clarus SQ 8 mass spectrometer equipped with ZB-5HTMS (5% diphenyl/95% dimethyl polysiloxane) fused capillary column (30 × 0.25 μm ID × 0.25 μm DF). IR spectra were carried out on a Bruker Alpha FT-IR spectrometer. Low-pressure column chromatography was carried out in glass columns using silica gel (70 – 230 mesh) and Sephadex – LH20 beads for normal phase and size exclusion chromatography respectively. Aluminum sheets precoated with silica gel 60 F_{254} with 0.25 mm thickness were used for analytical TLC.

Extraction, isolation and characterization of compounds

The dried and coarsely powdered stem bark of *P. biglobosa* (1 kg) was cold macerated using 4:1 methanol-chloroform mixture for 72 h, filtered, and evaporated to dryness. The crude extract (185 g) was suspended in water and further serially fractionated using petroleum ether, ethyl acetate and methanol.

Table 1
Protein targets and grid dimensions used in protein-ligand docking.

Protein Target	PDB ID	Bound Ligand	Grid Coordinates and Box Dimensions	
			Center (x, y, z) (Å)	Box Size (x, y, z) (Å)
PTR1	3H4V	DVP	−3.27175, −15.8258, 28.725	24.8, 24.8, 24. 8
TR	5EBK	RDS 777	24.9778, 49.1284, −9.79494	20.6, 20.6, 20.6

The pet ether fraction (12 g) was subjected to silica gel low - pressure column chromatography using a binary gradient consisting of pet ether and ethyl acetate, in increasing polarity. The fractions collected were monitored on analytical TLC and bulked into three sub-fractions, Fr. A, B and C. Fr A (0.05 g) yielded compound **1**.

The ethyl acetate fraction (23 g) was also subjected to silica gel low - pressure column chromatography using a binary gradient of pet ether and ethyl acetate, in increasing polarity. The gradient was started with 100% pet ether and gradually increasing to 100% ethyl acetate. Based on the TLC chromatograms, five bulked fractions (A – E) were obtained. Fraction C (0.72 g) was subjected to size exclusion separation on Sephadex LH-20 and eluted isocratically using chloroform : methanol (1:1) to obtain compound **2**.

Antileishmanial bioassay of extracts and isolates

Promastigote form of the *Leishmania donovani* culture was used in the assay as previously described [28]. The parasites were sub-cultured when they were 80 – 90% confluent. Aliquots of 50 μL of stationary-phase promastigotes were seeded in triplicate into 96-well microtiter plates at 1×10^7 parasites per well with 50 μL of extract (62.5 – 500 μg/ml) and isolates (12.5–200 μg/ml), in supplemented M199 medium and incubated at 28 ºC for 72 h. Subsequently, 10 μL of MTS solution was added to each well on the 96-well plate and the plates were re-incubated for 4 h. Amphotericin B served as positive control and was screened at concentrations of 0.78 –100 µg/mL and the culture medium was used as a negative control. The plate was read for absorbance at 490 nm using a microtiter plate reader (Infinite M200 Pro-Plate Reader, Tecan, Zurich, Switzerland). The percent growth inhibition was calculated from the absorbance readings compared to the negative control. The concentration of extract or compound that inhibited 50% of the parasites (IC_{50} values) of the extract was calculated using generated dose-response curves. Classification of the results was as follows: $IC_{50} \leq 100$ μg/ml, active, IC_{50} between 101 and 200 μg/ml, moderately active and $IC_{50} \geq 200$ μg/ml, inactive [10].

Molecular docking

Protein preparation

Pteridine reductase 1. The structure of PTR1 (PDB ID: 3H4V), with a resolution of 2.40 Å was obtained from the Protein Data Bank (PDB, https://www.rcsb.org/). The protein was co-crystallized with an inhibitor, methyl 1-(4-{[2,4-diaminopteridin-6-yl) methyl] amino} benzoyl) piperidine-4-carboxylate (referred to as DVP). The protein was prepared by removing all crystallographic water molecules and ligands, while polar hydrogens and charges were added [9].

Trypanothione reductase 1. The crystal structure of trypanothione reductase (TR) was obtained from the protein data bank (PDB ID: 5EBK) as a protein co-crystallized with a 6-(Sec-butoxy)−2-((3-chlorophenyl) thio) pyrimidine-4-amine (RDS 777). RDS 777 was bound to a pocket assumed to be the active site. The active site residues were obtained from the PDBSUM entry for 5EBK with catalytic residues namely: Glu466′, Cys57, and Cys52 for inhibitor RDS 777.

Ligand preparation

In preparation for the molecular docking simulation, the structure of each isolated compound was modeled in Spartan'14 (Wavefunction Inc., Irvine California, USA) ChemDraw interface, followed by SYBYL forcefield minimization as well as geometry optimization by equilibrium geometry estimation with Hartree-Fock 3–21G* basis set in a vacuum. Ligands were converted into protein data bank files (.pdb) and dock-prepared by including polar hydrogens and Gasteiger charges and saved as mol2 files [3].

Docking studies and estimation of the binding affinities of naturally occurring compounds

Molecular docking experiments were performed with AutoDock Vina [27] extended into UCSF Chimera [21]. Validation of docking protocols was made by re-docking ligands (inhibitors) from the protein-ligand complexes into their binding sites. In order to verify docked poses as representative of correctly bound ligand conformations, overlays were created with experimentally determined binding modes and root mean square deviations (RMSD) estimated in PyMol. The bound conformations of the re-docked ligands and an initial blind docking influenced the centers and sizes of grid box coordinates (Table 1) of the respective binding pockets chosen. The poses of the ligands for each target were ranked according to their binding energies by AutoDock Vina in UCSF Chimera. Ligands with very low binding energies and optimal interactions with the active site residues were selected as the best poses.

Table 2
Binding affinities and interactions of ligands docked against PTR1 and TR.

Protein Target	Binding affinity	Bound Ligand	Interacting residues	
			Hydrogen bond	Hydrophobic interactions
PTR1	−10.2 kcal/mol	Lupeol		Leu 14, Phe 107, Pro 209
	−5.7 kcal/mol	Monostearin	Leu 14, Gly 15, Asn 103, Ser 105, Ala 104	Tyr 179, Phe 107, Pro 209
TR	−7.1 kcal/mol	Lupeol		Leu 333, Pro 335, Ala 364
	−5.8 kcal/mol	Monostearin	Gly 13, Thr 51, Cys 52	Cys 57, Tyr 198, Leu 334, Pro 336, Phe 367

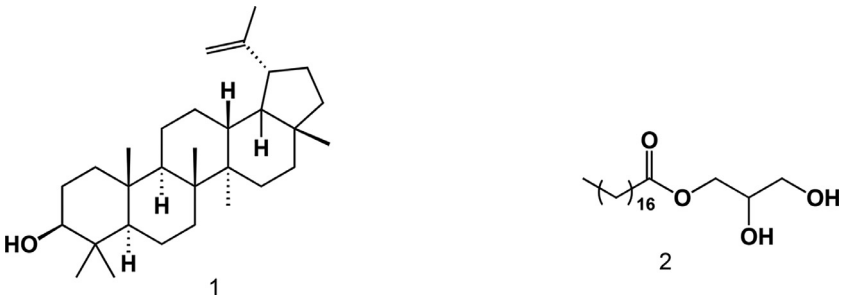

Fig. 1. Compounds isolated from the crude stem bark of P. biglobosa.

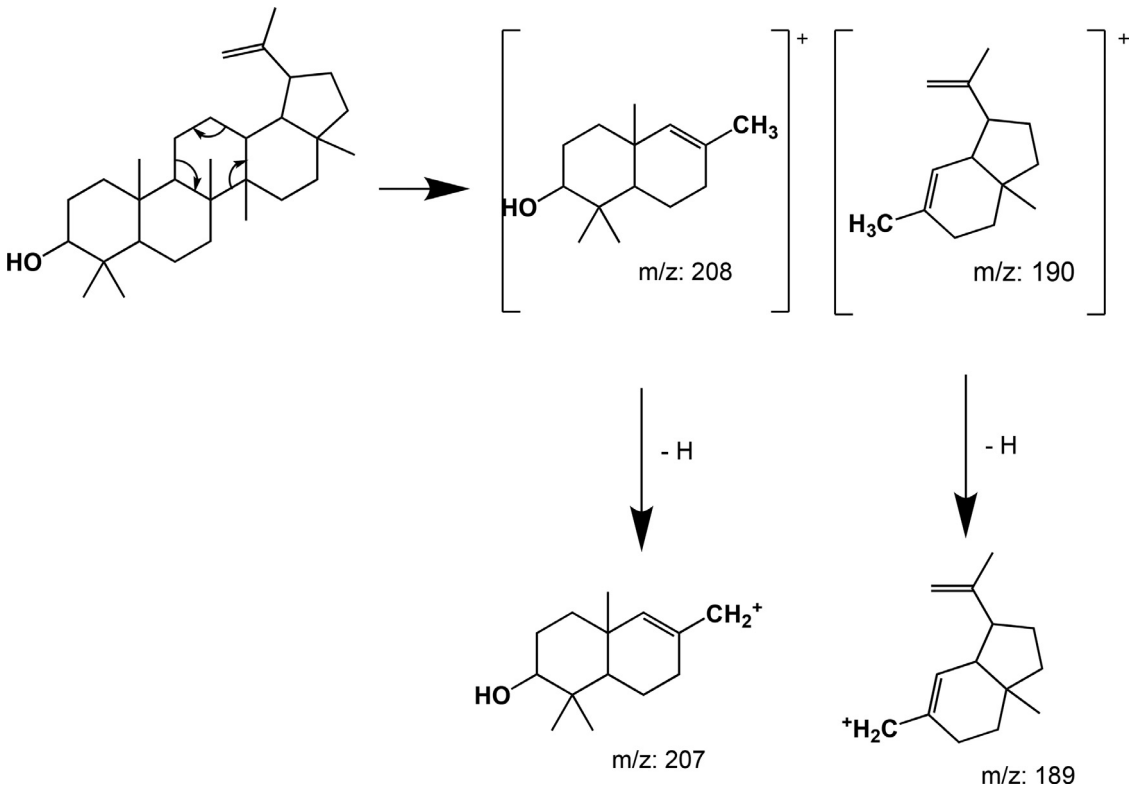

Fig. 2. Characteristic mass fragment pattern of saturated lupane molecule with unsaturated side chain.

Results and discussion

Characterization of the isolated compounds

The isolated compounds were identified as lupeol (**1**) and monostearin (**2**) by comparing their spectroscopic and mass spectra data to those previously published in the literature.

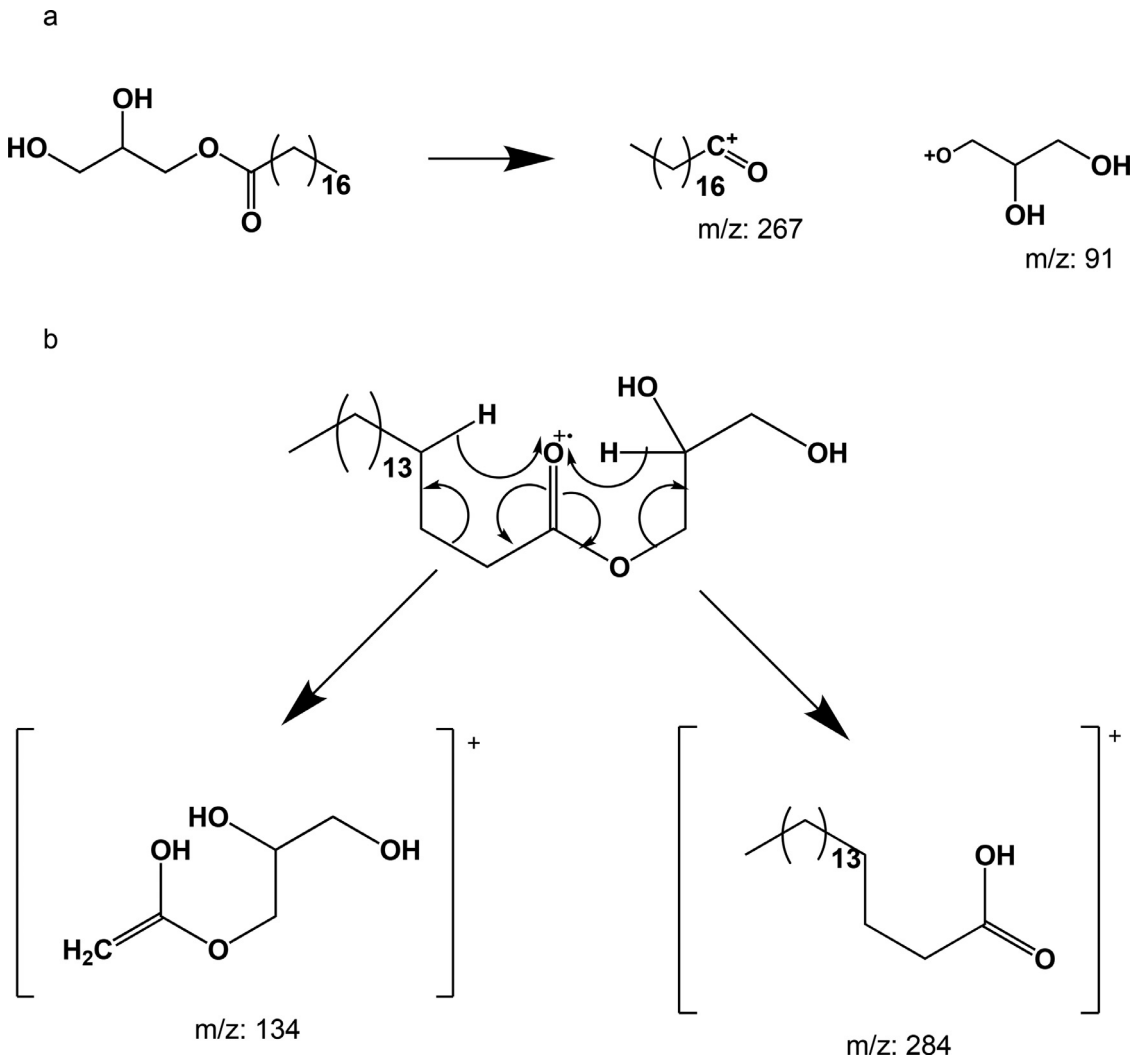

Fig. 3. Characteristic mass fragment pattern of monostearin showing a) formation of R-CO+ and b) McLafferty rearrangement in the alkyl and alkoxyl chains.

The FTIR spectra of compound **1** showed characteristic IR bands for the presence of double bond (1603 cm^{-1}) and hydroxyl (3277 cm^{-1}) functional groups. The GC–MS spectra revealed a characteristic fragmentation, yielding fragment ions at *m/z* 207 and *m/z* 189 corresponding to a saturated lupane with an unsaturated side chain [1]. The fragments are attributable to species following an initial a retro-Diels-Alder reaction in ring C, yielding *m/z* 208 and 190 amu respectively, accompanied by a loss of an additional H atom [7](Fig. 2).

The isopropenyl is further confirmed by the absence of *m/z* 43 (C_3H_7) in the mass spectra, which is characteristic of the lupane series in which isopropyl is attached to the ring E. Compound **I** was identified as lupeol.

The FTIR spectra of compound **2** showed characteristic IR bands for the presence of hydroxyl (3269.64 cm^{-1}) and ester 1602.54 cm^{-1}) groups.

Fatty acid esters were among the first natural products to be investigated by mass spectrometry [23]. The characteristic fragmentation reactions are formation of $R\text{-}CO^+$ and McLafferty rearrangement in the alkyl and in the alkoxyl chain, provided the chains are long enough [7].

The presence of ions at 267 amu, confirm an acyl ion, RCO^+ (Fig. 3). The MS spectra also revealed a more intense ion at 75 amu corresponding to the ion remaining after the loss of the acyloxy group from the molecular ion, $[M\text{- }RCOO]^+$. The neighboring signal at 74 amu corresponds to the loss of additional hydrogen, $[M\text{- }RCOOH]^+$. The additional presence of ions at 283 and 282 amu further suggest the acyloxy group as $CH_3(CH_2)_{15}COO^+$ (Fig. 3). Furthermore, McLafferty rearrangement in the alkyl chain yielded the fragment with signal at 134 amu.

An unusual feature of the mass spectra of triglyceride is an ion corresponding to the loss of water [15], the $[M\text{-}18]^+$ peak at 340 amu. This fragmentation is unique among the mass spectra of esters. The intensity of the $[M\text{-}18]^+$ ion is about equal

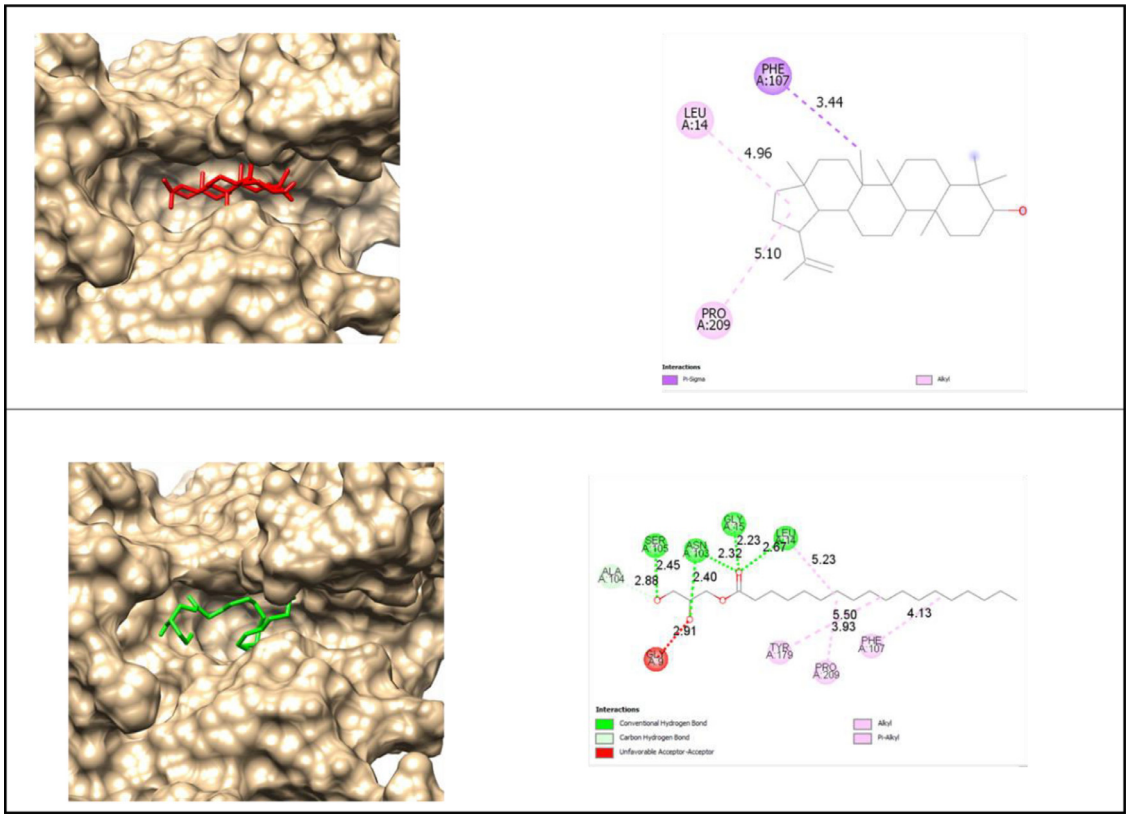

Fig. 4. 3D and 2D representation of protein-ligand interaction between lupeol (a) and monostearin (b) and PTR1 active sites.

to the intensity of the molecular ion for glycerides with saturated fatty acids, but its intensity decreases to almost zero as the degree of unsaturation increases. Compound **2** was identified as monostearin.

Antileishmanial activity

Antileishmanial activity of the crude extract, fractions, and isolates against promastigote forms of L. *donovani* revealed a dose-dependent response. The IC_{50} of the test samples are presented here: the crude extract, pet ether, ethyl acetate and methanol fractions showed IC_{50} values of 64.43, 126.25, 725.65, and 167.52 µg/mL respectively. The isolates, lupeol and monostearin, also showed IC_{50} values of 164.42 and 151.99 µg/mL respectively. Amphotericin B showed antileishmanial activity with IC_{50} of 42.69 µg/mL. Previous studies have showed *in vitro* antileishmanial activities on the aqueous-ethanol extract of the leaves and the stem bark of *P. biglobosa* [20]. Several studies have also highlighted the *in vitro* and *in vivo* antileishmanial effects of lupeol [11,12,16]. Additionally, monostearin has also been reported to have antileishmanial effects on the promastigote forms of L. *donovani* [4] Lupeol and monostearin may owe their antileishmanial effects to their lipophilicity. Lipophilic compounds have been previously reported to possess antiparasitic effects due to their potential to modulate the structure and function of ion channels, receptors and enzymes [13,25]

Molecular docking

To provide a molecular basis for the anti-leishmanial activity exhibited by the two compounds, an *in-silico* assessment of the phytoconstituents using a molecular docking approach was employed. There are a lot of protein targets used by the *Leishmania* parasite for various metabolic processes. Two of such proteins which have been validated as druggable targets are TR and PTR1. TR is fundamental for parasite survival in the host since it reduces trypanothione, a molecule used by the tryparedoxin/tryparedoxin peroxidase system of *Leishmania* to neutralize hydrogen peroxide produced by host macrophages during infection. Trypanothione (TS2) is reduced by NADPH in a TR-catalyzed reaction. The two NADPH electrons are transferred via FAD to the Cys52-Cys57 disulfide bridge. When TS2 binds to the protein, Cys52, deprotonated by the couple His461-Glu466, attacks the trypanothione disulfide bridge and this results in a mixed disulfide bridge. A final attack of the second protein cysteine (Cys57) on Cys52 produces the reduced substrate [2].

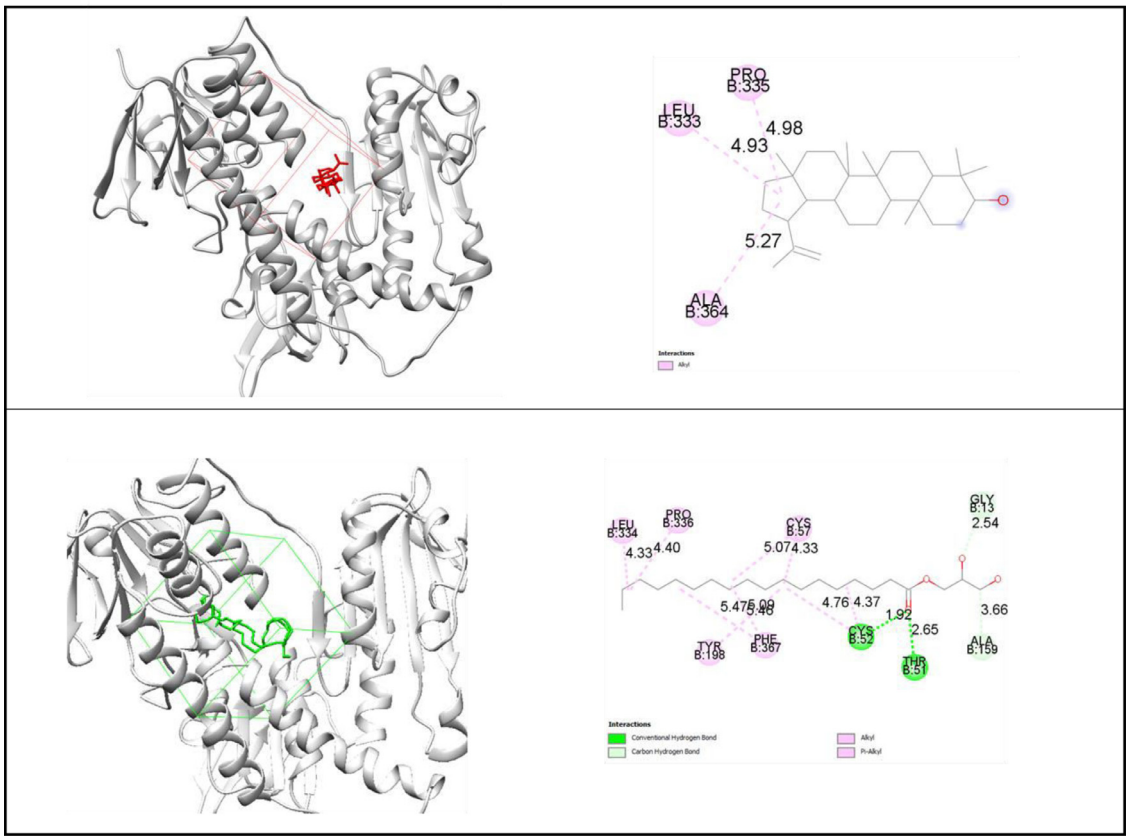

Fig. 5. 3D and 2D representation of protein-ligand interaction between lupeol (a) and monostearin (b) and TR active sites.

PTR1 is an NADPH-dependent short-chain reductase present in *Leishmania* that takes part in salvaging pterins needed for parasite growth [24]. In PTR1, Phe113 interacts with the nicotinamide cofactor to create the floor of the catalytic center needed for substrate binding. Nearby this center are three important residues: Tyr-194 is the active-site base which acts in concert with Asp-181 to acquire and pass on one reducing equivalent. Lys-198 positions the nicotinamide through hydrogen-bonding interactions with the cofactor ribose and may reduce the pKa of Tyr-194, thereby assisting catalysis.

The binding affinities of the two compounds when docked to PTR1 were −10.2 kcal/mol for lupeol and −5.7 kcal/mol for monostearin. For lupeol, hydrophobic interactions mediated its binding to PTR1, with pi-alkyl and alkyl interactions with Leu 14, Phe 107 and Pro 209 observed (Fig. 4). Hydrogen bond interactions between monostearin and Leu 14, Gly 15, Asn 103, Ala 104 and Ser 105, were observed in the PTR1-monostearin complex. Additional hydrophobic interactions between monostearin and Phe 107, Tyr 179 and Pro 209 were also observed (Fig. 4).

For the docking between TR and the two isolated phytoconstituents, the binding affinities recorded were −7.1 kcal/mol for lupeol and −5.8 kcal/mol for monostearin. Hydrophobic interactions between Leu 333, Pro 335 and Ala 364 of TR and lupeol were observed whereas both hydrogen bonding (with Gly 13, Thr 51 and Cys 52) and hydrophobic interactions (with Cys 57, Tyr 198, Leu 334, Pro 336 and Phe 367) were present in the monostearin-TR complex (Fig. 5). Against both proteins, lupeol exhibited very strong binding, even though the interactions were largely hydrophobic. Monostearin, against TR, interacted with the key amino acid residues, Cys 52 and Cys 57, which perform critical roles in the reduction of trypanothione. Together, the results of the docking study suggest that both compounds interact very well with the 2 druggable targets and may likely inhibit them via a competitive inhibition mechanism.

Conclusion

These two antileishmanial compounds in *P. biglobosa* may be responsible, at least in part, for the observed antileishmanial effects of the extracts and fractions of the plant. Additional antileishmanial compounds may exist in the plant, owing to the higher activities recorded for the extracts compared to the isolates. Therefore, future bioassay - guided isolation of antileishmanial compounds from *P. biglobosa* is warranted. Our findings further give credence to the ethnomedicinal use of *P. biglobosa* in the treatment of leishmaniasis. Monostearin and lupeol are potential hits against the promastigote form of the *L. donovani* parasites and could have a role in leishmaniasis chemotherapy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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