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RESEARCH ARTICLE

Steroids from *Diplazium esculentum*: Antiplasmodial activity and molecular docking studies to investigate their binding modes

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ABSTRACT

Diplazium esculentum is an edible fern commonly consumed by the local community in Malaysia either as food or medicine. Isolation work on the ethyl acetate extract of the stem of *D. esculentum* resulted in the purification of two steroids, subsequently identified as stigmasterol (compound **1**) and ergosterol-5,8-endoperoxide (compound **2**). Upon further testing, compound **2** displayed strong inhibitory activity against the *Plasmodium falciparum* 3D7 (chloroquine-sensitive) strain, with an IC_{50} of 4.27±1.15 µM, while compound **1** was inactive. *In silico* data revealed that compound **2** showed good binding affinity to *P. falciparum*-Sarco endoplasmic reticulum calcium-dependent ATPase (PfATP6); however, compound **1** did not show an antiplasmodial effect due to the lack of a peroxide moiety in the chemical structure. Our data suggested that the antiplasmodial activity of compound **2** from *D. esculentum* might be due to the inhibition of PfATP6, which resulted in both *in vitro* and *in silico* inhibitory properties.

Keywords: Antiplasmodial activity; *Diplazium esculentum*; ergosterol-5,8-endoperoxide; *in silico*; PfATP6.

INTRODUCTION

Malaria is an infectious disease that caused mortalities and morbidities worldwide. Increasing malaria-related deaths, especially in the tropics in recent years, are primarily associated with the most virulent *P. falciparum* infection (WHO, 2021). The alarming morbidity is attributed mainly to the development of parasite resistance towards antimalarial agents, including artemisinin, the current front-line drug against the disease (Ouji *et al*., 2018). The emergence of artemisinin resistance cases in the Greater Mekong regions urged a serious strategy to be taken to curb the transmission of the disease to other neighbouring countries (Imwong *et al*., 2017). The most effective means to prevent mortality and morbidity due to the resistant parasite infection is through chemotherapeutics since there is no fully licensed vaccine has been developed for malaria so far. Therapeutics for malaria derived from plants have high efficacy, and availability, low cost, and are generally safe for human consumption. Medicinal plants have been widely used to reduce malarial infections and symptoms for a long time, for example *Artemisia annua* (Septembre-Malaterre *et al*., 2020).

Diplazium esculentum (Retz.) Sw. (synonym *Diplazium malabaricum* Spreng., common name Paku Tanjung) belongs to the family Athyriaceae. It is a relatively large fern, commonly found in South East Asia farms and villages (Nur *et al*., 2018). Most people in South East Asia consume this fern either as food or medicine (Cicuzza, 2021). In Malaysia, several indigenous tribes in Borneo and Perak eat this fern as a traditional vegetable salad or "ulam" (Awang-Kanak & Abu Bakar, 2020). Local people in Malaysia traditionally used it to treat fever, diabetes, acne, asthma, scars, hemoptysis, and cough, and as a post-partum health tonic (Tag *et al*., 2012; Jasim *et al*., 2014). Phytochemical screening of *D. esculentum* revealed numerous bioactive components, such as steroids, phenolic compounds, alkaloids, flavonoids, tannins, glycosides, saponins, carbohydrates, and protein (Tongco *et al*., 2014; Junejo *et al*., 2015). Previous studies demonstrated that *D. esculentum* exhibited antiinflammatory (Kaushik *et al*., 2011), antioxidant (Roy *et al*., 2013), antidiabetic (Tag *et al*., 2012), and antibacterial activities (Amit *et al*., 2011). A recent antimalarial effect of *D. esculentum* aqueous extract in *Plasmodium berghei*-infected mice had been reported (Ramli *et al*., 2021). However, the antimalarial efficacy of this plant and its bioactive components against human malarial parasite has yet to be reported.

P. falciparum-Sarco endoplasmic reticulum calcium-dependent ATPase (PfATP6) is homologous to mammalian Sarco endoplasmic reticulum calcium ATPase (SERCA) with all essential motifs and sequences for the structure and function of a SERCA are conserved, for example, high-affinity Ca^{2+}-binding sites, a phosphorylation site and a nucleotide-binding site (Arnou *et al*., 2011). PfATP6 is the only SERCA-type Ca^{2+} ATPase enzyme that exists in the *Plasmodium* sp. One suggested mechanism of action of artemisinin that is commonly crucial for its activity is through the interaction of peroxide bonds with haem (Klayman, 1985; Meshnick *et al*., 1996). An oxy free radical generated upon hydrolysis of the peroxide linkage by the alkylation process in artemisinin is responsible for the lethal damage of the parasite (Jefford, 2001). Artemisinin also targets the specific plasmodial protein, PfATP6, which is known as another mechanism

of action for artemisinin (Eckstein-Ludwig *et al*., 2003; Naik *et al*., 2011). In the present study, PfATP6 was used as the target protein for the bioactive compounds from *D. esculentum* and the *in silico* computational data were compared with artemisinin.

Thus, the present study was performed to understand the potential of *D. esculentum* and the isolated compounds against *P. falciparum* 3D7 growth. Moreover, cytotoxicity activities and selectivity indexes of compounds obtained from the ethyl acetate extract of *D. esculentum* were reported. Molecular docking was also performed to determine the binding affinity of the identified compounds to an antimalarial drug target, the PfATP6 homology model. The results from *in vitro* and *in silico* activities of the isolated compounds were compared with the reference antimalarial drug, artemisinin. As of now, *D. esculentum* antimalarial effect on *P. falciparum* strain, identification of bioactive constituents from this plant, as well as *in silico* activities of the isolated constituents against PfATP6 have yet to be reported.

MATERIALS AND METHODS

Identification, extraction, and isolation of compounds

Diplazium esculentum was collected from a small rubber plantation at Sungai Buah, Sepang, Selangor in Peninsular Malaysia. The plant samples were identified by a certified botanist from Greenhouse Complex, Universiti Kebangsaan Malaysia, and a voucher specimen (NTF247) was deposited in the herbarium of the Faculty of Science and Technology, Universiti Kebangsaan Malaysia.

The stems, leaves, and roots of *D. esculentum* were air-dried at room temperature for 1 to 2 weeks and ground into powder using a grinder. The air-dried powder (300 g) was soaked with ethanol (ET) or ethyl acetate (EA). The extracts were dried by using a rotary evaporator and prepared for *in vitro* assays. Based on the moderate *in vitro* antimalarial activity, the stem EA extract was selected for further fractionation and subjected to vacuum liquid chromatography (VLC) over silica gel with increasing polarity of hexane: acetone (9:1–3:7) to yield eight fractions (fractions 1-8). Fraction 4 was re-crystallized to yield compound **1**. Fraction 3 was subjected to radial chromatography (RC) over silica gel, and eluted with mixtures of hexane: acetone (9:1 – 3:7) to yield eight fractions (fractions 1a–8a). Fraction 6a was subjected to column chromatography (CC) over silica gel with mixtures of hexane: chloroform (9:1–1:9) to yield ten fractions (fractions 1b–10b). Fraction 6b was purified using preparative thin-layer chromatography (PTLC) over silica gel with mixtures of hexane: acetone (8:2) to yield compound **2**.

NMR spectroscopic analysis

The structures of pure compounds were identified based on one-dimensional (1D) experiments consisted ^{1}H and ^{13}C NMR spectra captured by nuclear magnetic resonance (NMR) analyses using FT-NMR cryo-probe 600 MHz (Bruker). A pure sample was dissolved in 600 µL deuterated chloroform ($CDCl_3$) and transferred to a 5 mm Norell NMR tube (Sigma-Aldrich, USA) with TMS as an internal reference (Alhassan *et al*., 2019). NMR spectra of compound **1** (stigmasterol) and compound **2** (ergosterol-5,8-endoperoxide) were analysed with Topspin 2.1 software.

In vitro antiplasmodial assessment

Extracts from *D. esculentum* and isolated compounds were evaluated for antiplasmodial activities against *P. falciparum* 3D7 obtained from Malaria Research and Reference Reagent Resource Center (MR4), USA. *Plasmodium* sp. infected erythrocytes without treatment served as the positive control. The parasites were cultured in the synchronised ring phase at 2% haematocrit and 2% parasitaemia in extracts or pure compounds or an established antimalarial drug. As a negative control, unparasitised O+ erythrocytes without treatment were used in the assay. The concentrations of the test extracts used in the assay ranged from 0.0001 to 1000 µg/mL whereas compounds ranged from 0.0001 to 1000 µM. The reference compound, artemisinin used in the study ranged from 0.00035 to 35 µM. The assay was carried out according to lactate dehydrogenase enzymatic reaction (pLDH) (Makler & Hinrichs, 1993; Hashim *et al*., 2019). Colour changes were measured colorimetrically at 650 nm (Fluorostar OPTIMA, Germany) after one hour of incubation. Data were analysed for IC_{50} values (inhibition concentration at 50% parasite growth) through non-linear regression using Graphpad prism 5. All measurements were conducted in triplicates including those for the positive controls.

In vitro cytotoxicity assessment

Chang liver cells were collected from the American Type Culture Collection (ATCC), USA. Cytotoxicity of extracts and isolated compounds were measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Chang liver cells were seeded at 2×10^4 in a complete medium. The desired concentrations of the extracts and compounds in test wells ranged from 0.01 to 10 mg/mL and 0.01 to 10 000 µM, respectively. Mammalian cell cultures without test compounds served as a control. The cell culture was incubated with or without compounds for 48 hours (37°C, 5% CO_2). Then, the MTT-PBS reagent was mixed into each well. The plates were incubated for three hours at 37°C. The mixture was removed and replaced with dimethyl sulphoxide (DMSO) to dissolve the MTT formazan product. The mixture was vortexed for 15 min, and absorbance was measured at 540 nm (Fluorostar OPTIMA, Germany). IC_{50} values were measured through a non-linear regression curve using Graphpad prism 5. All measurements were conducted in triplicates including those for the positive controls. The selectivity index (SI) of the test compounds was calculated using the following formula; $Selectivity\ Index\ (SI) = \frac{IC50\ MTT}{IC50\ pLDH}$. SI values greater than 10 (SI>10) are considered promising and selective activities (Sarr *et al*., 2011; Shamsuddin *et al*., 2021).

Protein homology modeling and molecular docking studies

The amino acid sequence of *P. falciparum* ATP6 (PfATP6; 1228 amino acids) was obtained from the PlasmoDB database (http://plasmodb.org/plasmo/, accessed on 20 January 2022) (Aurrecoechea *et al*., 2008). The BLAST server (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 20 January 2022) was used to detect the sequence with high similarities between PfATP6 and SERCA (similarities index: 43.5%) (Altschul *et al*., 1997). The SERCA 3D structure (PDB: 2DQS, resolution 2.5 Å obtained from the RCSB database (http://www.rcsb.org/pdb/explore/explore.do?structureId=2DQS, accessed on 20 January 2022) was used as an ideal template to form a PfATP6 homology (Garah *et al*., 2009). The homology model constructed using SwissPDB Viewer 3.7 (Bordoli *et al*., 2009) includes loading the PfATP6 primary sequence, searching for templates in Protein Data Bank, performing structural alignment, and submitting it to the Swiss Model Server. The quality of the refined PfATP6 homology model was validated by PROCHECK analysis (Laskowski & Swindells, 2011). The Ramachandran plot analysis was used to determine the distribution of the Psi/Phi torsion angles of the homology model. The results for residues in most favoured regions (90.3%), additional allowed regions (8.8%), tolerated regions (0.6%), and disallowed regions (0.2%) were determined**.** PfATP6 homology structure for docking analysis was prepared by the default parameters, which resulted in the removal of all water molecules, adding hydrogen atoms, and adding partial charges using the Gasteiger United charges.

In this study, AutoDock Vina (Trott & Olson, 2010) was utilized for the docking study. The binding affinity scores of ligands to the homology of PfATP6 were determined, and interaction of the protein-ligand was performed using Discovery Studio and LigPlot Softwares (Laskowski & Swindells, 2011). Thapsigargin as a SERCA inhibitor, artemisinin as the antimalarial reference compound, and the isolated compounds was docked into the thapsigargin binding cleft of the PfATP6 homology model. Structures of the compounds

were placed inside the grid box of the binding site for the docking studies. The grid size was set to 30× 30× 30 (*x*-, *y*-, *z*-axes) points with 1 Å spacing and centered on the thapsigargin-binding cleft. The binding affinity (Kcal/mol) was calculated from the docking analysis, and the predicted inhibition constant (Ki_{pred}) was calculated based on the docking scores (ΔG values) using the following formula; $Ki_{pred} = e^{\frac{\Delta G}{RT}}$. R (gas constant) was equivalent to 1.98 cal*(mol*K)$^{-1}$, and T (room temperature) was equivalent to 298.15 Kelvin (Shityakov & Förster, 2014).

Statistical Analysis

The data were presented as the means ±SD of results from three independent experiments. Statistical analysis was performed using the one-way ANOVA of the Graphpad Prism 5 software. A $p<0.05$ value was considered statistically significant.

RESULTS AND DISCUSSION

The purified compounds were analysed by spectroscopic methods (NMR). The obtained data were compared to literature values. The two isolated steroids were identified as stigmasterol (compound **1**) (Forgo & Kövér, 2004) and ergosterol-5,8-endoperoxide (compound **2**) (Krzyczkowski *et al*., 2009). A previous study by Trung *et al*. identified that ergosterol peroxide is present in wild mushrooms such as *Fomitopsis dochmius*, *Daldinia concentrica*, *Ganoderma applanatum*, *G. lobatum*, *G. lucidum*, *Phellinus igniarius*, and *Trametes gibbose* (Trung *et al*., 2018; Dembitsky *et al*., 2021). As far as we know, this is the first report of the occurrence of an endoperoxide compound from the genus *Diplazium* sp. The structures of the compounds are shown in Figure 1, and one-dimensional NMR (^{1}H, ^{13}C) is depicted in the supplementary information.

The antiplasmodial activity was evaluated for compounds **1** and **2** using the plasmodium lactate dehydrogenase (pLDH) assay and cytotoxicity was evaluated against Chang liver cells (Table 1). The pLDH result showed that compound **2** was good at inhibiting replication of *P. falciparum* 3D7 (IC_{50}=4.27 ± 1.15 µM), whereas compound **1** showed no activity towards the *P. falciparum* 3D7 strain (IC_{50}=582.81 ± 1.64 µM). Both steroids (compounds **1** and **2**) exhibited low toxicity effects against Chang liver cells, with IC_{50} of 192.52 ± 3.13 and 684.23 ± 2.78 µM, respectively. Further calculation of selectivity indexes (SI) displayed that compounds **1** and **2** showed 0.33 and 160.17, respectively. Compound **2** showed high SI (>10) compared to compound **1**, suggesting that the antiplasmodial activity shown by compound **2** is specific and selective towards *P. falciparum*. The previous study by Kuria *et al*. revealed that ergosterol-5,8-endoperoxide possessed good antiplasmodial activity against CQ-sensitive *P. falciparum* (FCA 20/GHA strain) (IC_{50}=8.2 µM) (Kuria *et al*., 2002), and the IC_{50} is comparable with the IC_{50} value obtained in this study. For stigmasterol, a previous study revealed that this compound was inactive against *P. falciparum* (FcM29 strain) (IC_{50}>120 µM) (Banzouzi *et al*., 2015), which is also equivalent to the effect shown in this study. Ergosterol-5,8-endoperoxide previously showed no cytotoxicity effect against the human skin carcinoma A431 cell line (Kuria *et al*., 2002), whereas stigmasterol showed a

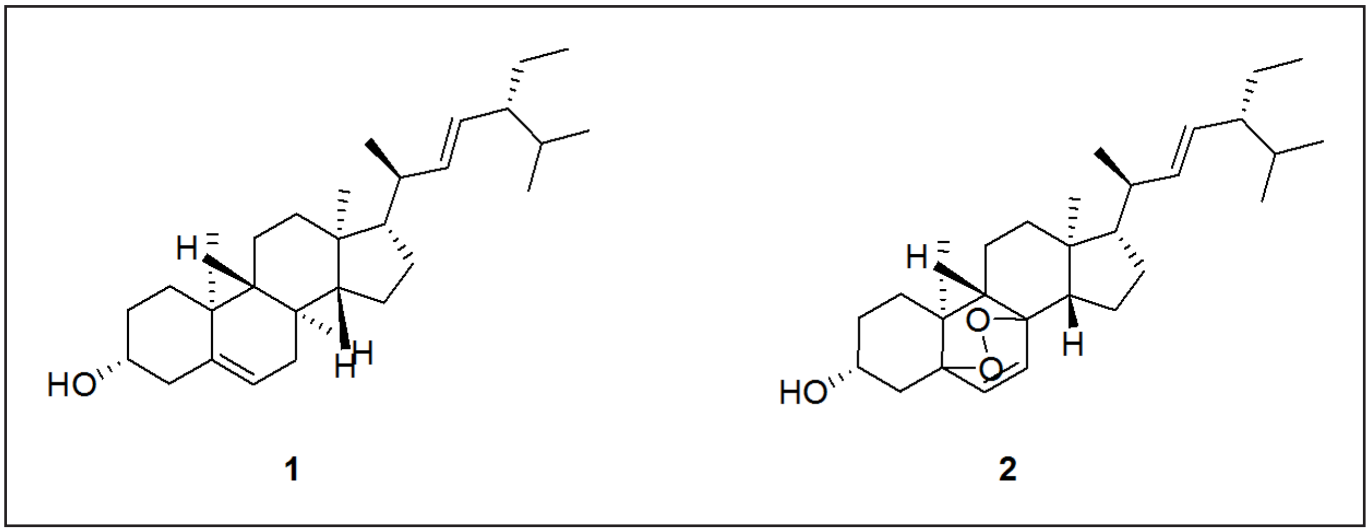

Figure 1. Chemical structures of compounds **1** and **2** from *D. esculentum*.

Table 1. *In vitro* antiplasmodial and cytotoxicity effects of extracts and isolated compounds from *D. esculentum*

Extracts/ Compounds	Antiplasmodial Activity against *P. falciparum* 3D7, IC_{50}±SD (µg/mL)	Cytotoxic Activity against Chang cells, IC_{50} ±SD (µg/mL)	Selectivity Index (SI), $\frac{IC50\ MTT}{IC50\ pLDH}$
Stems EA	39.98 ± 1.60 (moderately active)	420.51 ± 1.51	10.52
Leaves ET	>150 (inactive)	>200	ND
Leaves EA	>150 (inactive)	>200	ND
Roots ET	>150 (inactive)	>200	ND
Stigmasterol	582.81 ± 1.64 µM (inactive)	192.52 ± 3.13 µM	0.33
Ergosterol-5,8-endoperoxide	4.27 ± 1.15 µM (active)	684.23 ± 2.78 µM	160.17
Artemisinin (antimalarial reference drug)	0.55 ± 0.01 nM (potent)	250.10 ± 32.12 µM	>2000

All experiments were repeated thrice, each in triplicates. SD: standard deviation, ND: not determined. Antimalarial range; potent: IC_{50}<1 µM, active: 2–20 µM, moderately active: 21–100 µM, weak: 101–200 µM, inactive: IC_{50}> 201 µM (Dolabela *et al*., 2008; Katsuno *et al*., 2015).

low toxicity effect against both KB and Vero cells (Banzouzi *et al*., 2015). Artemisinin, the antimalarial reference drug, displayed potent antiplasmodial activity against *P. falciparum* 3D7. Artemisinin SI was ten times higher than compound **2** SI in parallel with the usage of artemisinin as frontline treatment in malaria.

It has been suggested that PfATP6 is a viable drug target for antimalarial chemotherapy (Dahlström *et al*., 2008). In addition, the homologous structure of PfATP6 was determined using the structure of the mammalian Sarco endoplasmic reticulum calcium ATPase (SERCA), identified from *Oryctolagus cuniculus*, the rabbit skeletal muscle (PDB: 2DQS, 2.5 Å) (Toyoshima & Nomura, 2002). Superposition of the PfATP6 homology model with the SERCA structure showed that both structures have highly similar arrangements of the transmembrane helices (RMSD=0.6 Å), where the thapsigargin (a reference ligand) binding site is located (Figure 2).

Computational docking analysis showed that steroids from *D. esculentum* were specifically bound at the binding sites of the PfATP6. The binding affinity and predicted inhibition constant of compound **2** in PfATP6 were -9.6 Kcal/mol and 0.09 µM, respectively (Table 2). This compound exhibited strong hydrogen bonding at the Lys260 amino acid and hydrophobic interactions at residues Asn706, Leu713, Ile271, Ile644, Leu707, Ile708, Phe264, Ile648 and Val651 of PfATP6 (Figure 3). Compound **1** also exhibited moderate binding affinity with PfATP6, with -7.6 Kcal/mol, and a predicted inhibition constant of 2.64 µM. Compound **1** showed hydrophobic interaction with amino acid residues Lys260, Leu263, Phe264, Ile271, Ala652, Val651, Asn647, Ile648, Ile708 and Leu713 (Figure 3). Compound **2** also showed promising binding affinity to mammalian SERCA protein, with a value of -9.4 Kcal/mol. Both compound **2** and artemisinin contain a common endoperoxide moiety in their chemical structures, which is not present in compound **1**. It was reported that the peroxide moiety in artemisinin may be responsible for plasmodial inhibition (Lell *et al*., 2011). In this study, compound **2** showed hydrophobic interactions with PfATP6 involving Phe264, Asn706, Ile708, Ile644, Leu707, and Lys260 amino acids, which were similar to artemisinin. For compound **1**, although the compound showed binding affinity on PfATP6 and SERCA, in *in vitro* cultures, the compound did not exhibit antiplasmodial activities on *P. falciparum* 3D7. Our data revealed that artemisinin significantly inhibited PfATP6 activity, with a predicted inhibition constant (Ki_{pred}) of 0.68 µM. The data obtained for artemisinin is in parallel with the potent antiplasmodial activity shown in the *in vitro* study. The previous study by Eckstein-Ludwig *et al*. revealed that artemisinin inhibited the PfATP6 of *P. falciparum* in *Xenopus* oocytes *in vitro* assay (Eckstein-Ludwig *et al*., 2003).

Endoperoxides such as artemisinin are mainly used in Southeast Asia and Africa areas, which are known to have multidrug-resistant *P. falciparum* cases (O'Neill & Posner, 2004; Imwong *et al*., 2017). Both compound **2** and artemisinin contain a common endoperoxide moiety in their chemical structures. The peroxide moiety in

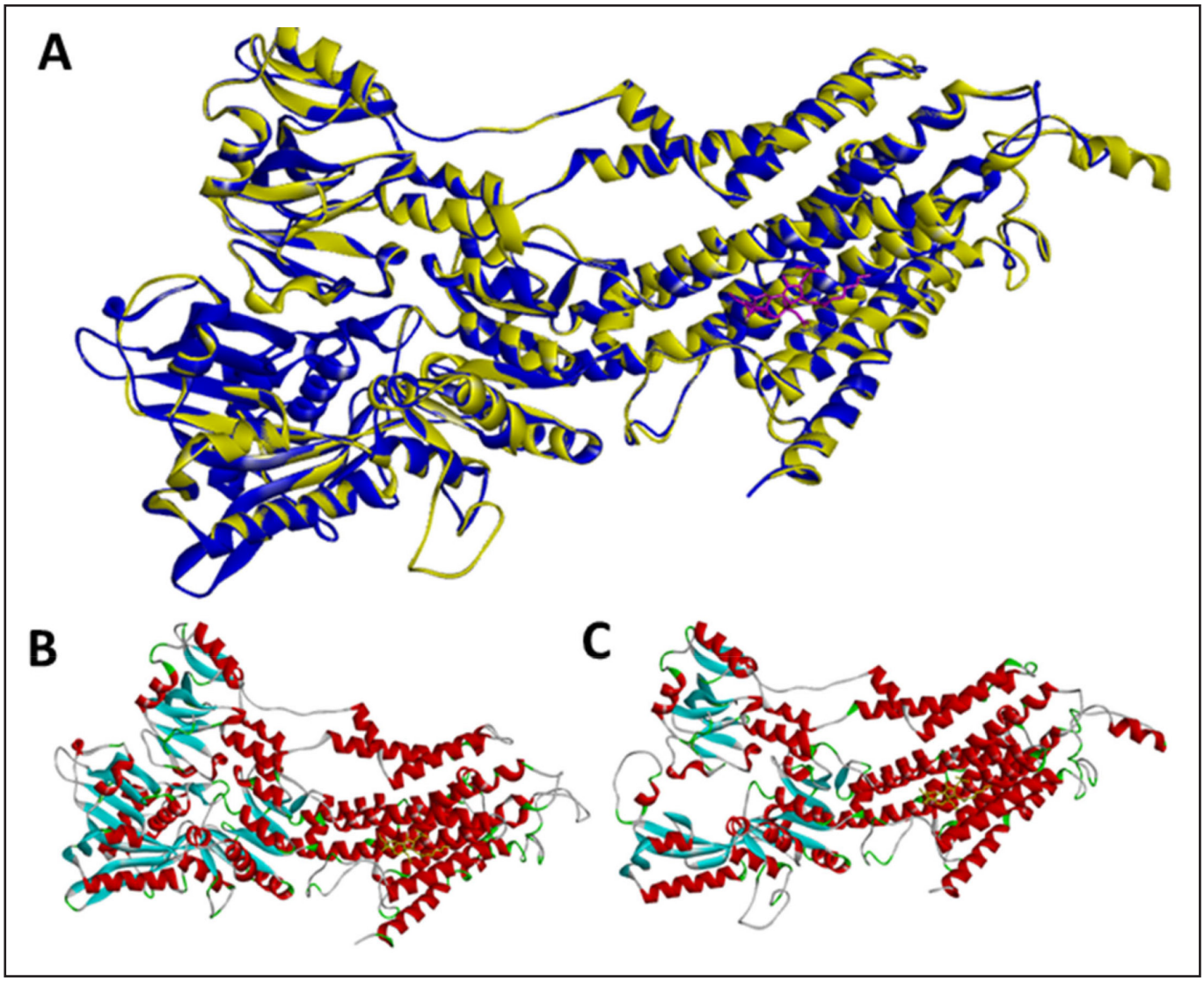

Figure 2. (a) Superimposition of the three-dimensional structure of the PfATP6 homology model (yellow), SERCA (blue) with thapsigargin (pink) in its binding site, (b) the mammalian SERCA, and (c) the parasite PfATP6 model were presented.

Table 2. Binding affinity (Kcal/mol) and predicted inhibition constant (Ki_{pred}) of compound **1** and **2** from *D. esculentum* against SERCA and PfATP6

Compounds	SERCA		PfATP6	
	Binding Affinity (Kcal/mol)	Predicted Inhibition Constant (Ki_{pred}, μM)	Binding Affinity (Kcal/mol)	Predicted Inhibition Constant (Ki_{pred}, μM)
Stigmasterol (1)	-8.1	1.13	-7.6	2.64
Ergosterol-5,8-endoperoxide (2)	-9.4	0.13	-9.6	0.09
Artemisinin	-8.1	1.13	-8.4	0.68
Thapsigargin	-9.5	0.11	-7.6	2.64

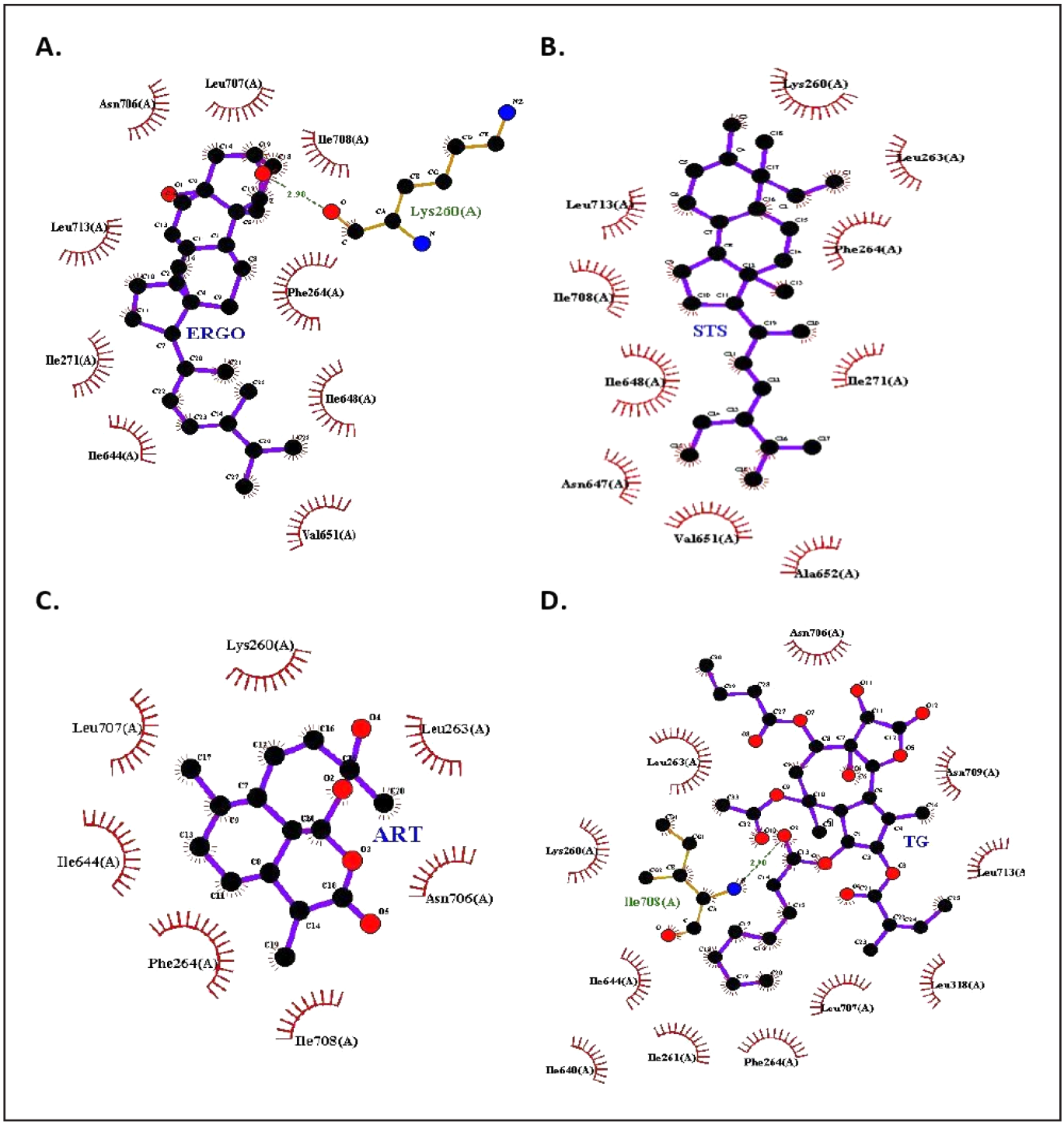

Figure 3. (a) Ergosterol-5,8-endoperoxide (**2**) (b) stigmasterol (**1**) (c) artemisinin and (d) thapsigargin interactions with amino acid residues on PfATP6 binding sites, analysed by the LigPlot docking software.

artemisinin is responsible for plasmodial inhibition (O'Neill *et al*., 2010). Attack by haem iron breaks the endoperoxide linkage of the drug to produce an oxy free radical, which is rearranged to yield a carbon-free radical. Carbon-free radical generated from the activation step further alkylates specific malarial proteins causing lethal damage to malarial parasites (O'Neill & Posner, 2004). Oxygen atoms from the endoperoxide bridge are vital for the formation of oxy free radicals that may cause lethal damage to the parasite. Artemisinin derivatives not only have one mode of action which is the alkylation of haem and proteins in the parasite (Yang *et al*., 1993), but they could also inhibit PfATP6, and *P. falciparum* translationally controlled tumor protein (TCTP), and impair the parasite mitochondrial functions (Gopalakrishnan & Kumar, 2015). The endoperoxide moiety is predicted to play an essential part in the antiplasmodial effect shown by compound **2**. We suggested that compound **2** acted similarly to artemisinin allowing the cleavage of the peroxide bridge by iron to generate carbon-centered radicals, leading to enzyme inactivation and parasite death. Artemisinin, a highly oxygenated sesquiterpene lactone peroxide contains one more oxygen atom compared to compound **2** (Wang *et al*., 2010). Thus, the lack of oxygen atoms in the endoperoxide bridge in compound **2** possibly reduces the antimalarial effect of the compound as compared to artemisinin.

Although the antimalarial activities of this plant had been reported against *P. berghei*, a rodent malarial parasite by Ramli *et al*. (2021), the present study would be the first report of antimalarial activities of *D. esculantum* against *P. falciparum* 3D7, human malarial parasite and cytotoxicity effect on Chang mammalian liver cells. The present findings showed the antimalarial activities of ergosterol-5,8-endoperoxide isolated from *D. esculentum* corroborated *in silico* activities of the compound on PfATP6. The findings from this study were in line with ergosterol peroxide's broad range of bioactivities, for example, antioncogenic, antiinflammatory (Kobori *et al*., 2007), antiviral, antimycobacterial, antiproliferative, and trypanocidal (Meza-Menchaca *et al*., 2019). Thus, our findings suggested ergosterol-5,8-endoperoxide may serve as a template in the search for new antimalarial drugs to combat *P. falciparum* multi-drug resistance cases. However, more investigations are required to determine the mechanism of action of ergosterol-5,8-endoperoxide against *P. falciparum* 3D7.

CONCLUSION

In conclusion, the present study represented the first report of the antimalarial activity of *D. esculentum* against *P. falciparum,* and the findings implicated ergosterol-5,8-endoperoxide as the bioactive component. Our investigation suggests that the antiplasmodial activity displayed by ergosterol-5,8-endoperoxide is associated with its peroxide moiety, which closely resembled the peroxide moiety in the antimalarial compound, artemisinin. We postulated that PfATP6 could be a protein target for ergosterol-5,8-endoperoxide, and the endoperoxide compound could serve as a template in search of new antimalarial drugs to combat parasite resistance in the future.

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Conflict of interest

The authors declare no conflicts of interest.

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SUPPLEMENTARY DATA

https://msptm.org/files/Vol39No4/tb-39-4-011-Safar-H-F-supplementary-data.pdf