*Article*

# Exploratory analysis into the *in vitro* and *in silico* activity of *E. fusca* Lour. (Fabaceae) elucidates substantial antiplasmodial activity of the plant

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**Abstract:** The exploration of alternative antimalarial therapeutics is a requisite for the emergence of resistance against Artemisinin. Considering the required cost and time length of classical small molecule drug discovery process, phytochemical screening of traditionally used medicinal plant which are repertoire of active compounds with antimalarial activity has become popular. To investigate the antimalarial property of traditionally used medicinal plants, a number of *Erythrina* spp have been reviewed systematically where less studied *E. fusca* has been selected for further analysis. Phytochemical investigation yielded five compounds namely; Phaseolin, Phytol, β-amyrin, Lupeol, and Stigmasterol. *In-vitro* antimalarial drug sensitivity HRP-II ELISA was carried out against chloroquine (CQ) sensitive 3D7 and CQ-resistant Dd2 strains. Extracts showed significant antimalarial activity against 3D7 and Dd2 strains ($IC_{50}$ 4.94 – 22 µg/mL) and these compounds have been reported here for the first time.. Molecular docking analysis showed high binding energy (−9.0 ± 0.32 kcal/mole) indicating high degree of interaction between Phaseolin and 14 clinically important *Plasmodium falciparum* proteins at the active site. Stable interaction was also observed between ligand and protein from molecular dynamics simulation analysis with high free energy (−75.156 ± 11.459) that substantiates the potential of Phaseolin as an antimalarial drug candidate.

**Keywords:** antimalarial; *Erythrina fusca*; phaseolin; molecular docking; phytochemical analysis

## 1. Introduction

Malaria is a well-known deadly tropical disease that causes approximately 409,000 deaths in 2019 [1]. This parasitic disease is caused by *Plasmodium* spp. whereby *Plasmodium falciparum* is responsible for 50% cases which might turn into fatal [2, 3]. Artemisinin (ART)-based combination therapy (ACT) is considered as the first line antimalarial treatment for *Plasmodium falciparum* (*Pf*) with optimum therapeutic outcome and reducing transmission [4]. Although ACT is currently effective in most of the countries of the world, treatment failure [5] and the spread of ART-resistant parasites have already been observed in South-East Asia that raises the concern for future resistance [6]. Based on some experimental findings, ART-resistance is attributed to naturally occurring point mutations in the *P. falciparum* K13-propeller-gene (pfkelch13) and in some cases the underlying drug resistance mechanism is yet to be revealed [7]. In addition to growing resistance, lack of

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sensitivity to ART agents is evolving and expanding globally due to the polymorphisms observed in the pfkelch13 gene [8, 9].

Since the magic efficacy against this deadly disease is likely to be dropped down due to soaring resistance, alternative potential therapeutic options are needed to be discovered or developed [10]. However, new drug development might require an investment of billions of dollar from the discovery of the lead to the final marketed drug formulation which is often tedious and time consuming process [11]. To overcome the challenges, reverse pharmacology has turned out to be a makeshift to facilitate the drug discovery process, as such many of the synthetic drugs including the derivatives of artemisinin are of plant origin [12]. Another plant derived prominent antimalarial drug, Quinine has many synthetic derivatives such as chloroquine and primaquine that have been found to be effective as well [13].

Discerning the pharmacological activity of plant or plant derivatives, traditional healers have been using many plants to treat malaria worldwide including Bangladesh [14]. Still now systematic investigation at medicinal compound level of the traditional plants is limited. In Bangladesh, the local healers in the endemic area use few plants to treat febrile diseases like malaria. We surveyed a few plants and also investigated them in our previous study [15]. However, one of the less studied species of *Erythrina* came into suggestion from this survey. Several previous studies suggest the potential of *Erythrina spp*. for their antimalarial activity. *E. abyssinica* showed antimalarial activity of some flavonoids *in vitro* with evidence of *in vivo* activity as well [16, 17]. Besides, literature suggests potential antiplasmodial activity of compounds from other species *E. variegata.* Thus, we aimed to investigate less studied traditionally used medicinal plant *E. fusca* Lour., locally known as "Harikakra" in Bangladesh. Ithas been used for its antibacterial, anxiolytic, antinociceptive, antioxidant etc. activities for decades **[18]**. Besides, previous study showed prenylated flavonoids derived from this plant to be effective against K1 strain of *Pf* **[19]**. The finding of these studies indicate the likelihood of other antimalarial compound/s and thus necessitates further phytochemical investigation of *E. fusca*.

In our current study we undertook a systematic investigation that entailed extraction, isolation, characterization using chromatographic and spectroscopic techniques **[20]**. Later on, we investigated the crude as well as different fractions of the extracts for antimalarial property. We originally planned for investigation of plant derived pure compounds for antiplasmodial activity, provided that the yield would have been very high. Due to very minimal amount of extraction of pure compound, we had to choose the *in silico* approach where we investigated the ligand-protein interaction with molecular dynamics simulation to further bolster the key findings.

## 2. Results:

The cascade of chromatographic separation yielded eight compounds of which five were spectroscopically analyzed and revealed as Phaseolin (1), Lupeol (2), Phytol (3), β-Amyrin (4), Stigmasterol (5) (Figure 1). The first four were derived from the chloroform fractions and last one from n-Hexane fractions. The compounds other than Phaseolin are very common among plants and easy to detect. However, Phaseolin has been reported for the first time from this plant and three grams of fraction yielded only 11.5049mg of pure Phaseolin (yield 0.0038% only) 9.6871mg (0.0032%) of mixture of lupeol and beta amyrin, 3.4603mg of phytol (yield 0.0012%), and 13.0622mg of stigmasterol (yield 0.0043%).

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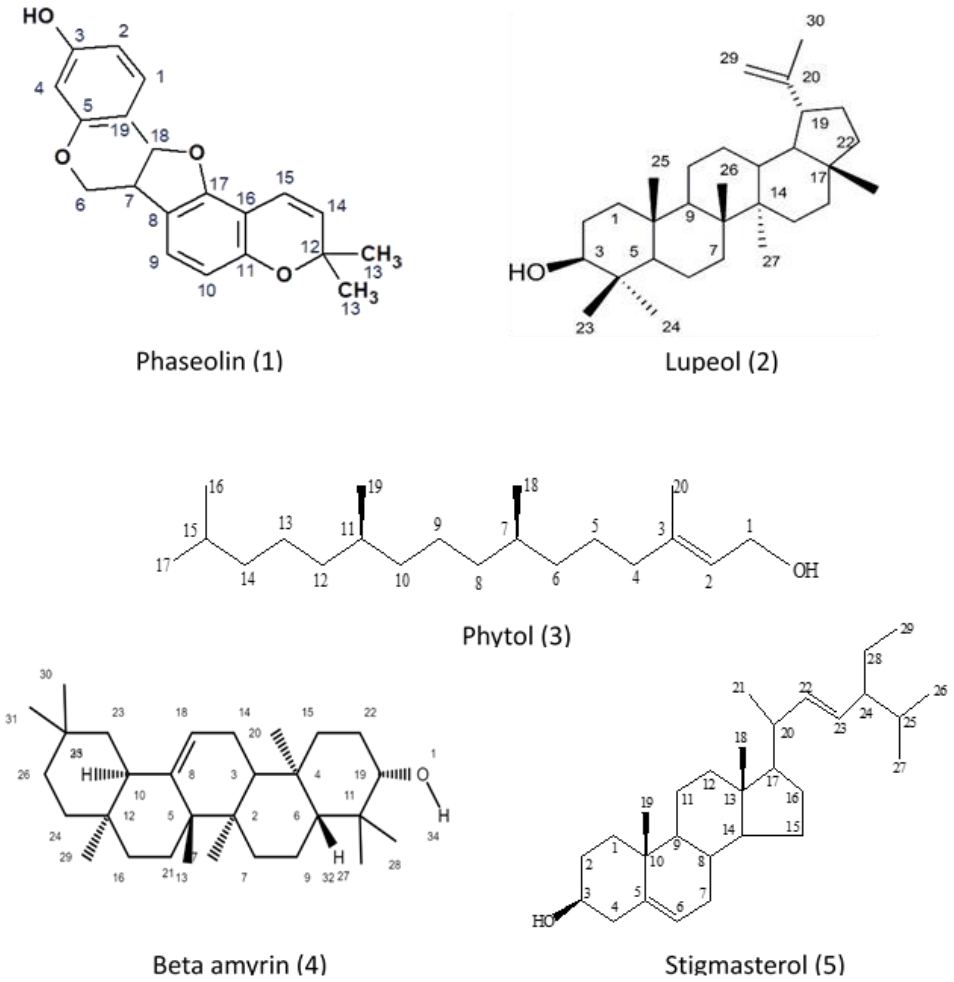


**Figure 1.** Chemical structure of isolated chemical compounds analyzed for docking analysis; Phaseolin, Lupeol, Phytol, Beta amyrin and stigmasterol.

*2.1. Antimalarial Activity of the Crude Extract and Fractions:*

we performed antimalarial assay of the crude extract and its different Kupchan fractions against CQ sensitive 3D7 and resistant Dd2 strain and observed significant antimalarial effect. Here, $IC_{50}$ 4.94 µg/mL in aqueous fraction against 3D7 and $IC_{50}$ 4.88 µg/mL in n-Hexane fraction against Dd2 strain were obtained. The extent of antimalarial activity among different fractions has been summarized in Table 1.

**Table 1.** Antimalarial activity of crude of *E. fusca* and its partitions against *Pf* strains.

| ***E. fusca* Extracts** | | **$IC_{50}$ values (µg/mL) ± SD** | |
|---|---|---|---|
| | | **3D7** | **Dd2** |
| Crude (methanol) | | 13.64 ± 0.67 | 8.22 ± 0.65 |
| Fractions | *n*-Hexane | 21.44 ± 5.74 | 4.88 ± 0.26 |
| | $CHCl_3$ | 22.55 ± 2.69 | 13.77 ± 0.71 |
| | Aqueous | 4.94 ± 0.74 | 18.77 ± 3.65 |

*2.2. Computational Investigations:*

The docking results of the compounds have been provided in the Table 2. As the compounds other than Phaseoline have been studied for antimalarial property, here we have emphasized on the remaining and first reported compound from this plant. High binding affinities were observed between the clinically important malaria proteins and the Phaseolin. Among the 14 proteins, **5JAZ5JAZ** (−9.3kacl/mole), **5K8S5K8S** (−8.8

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kcal/mole) and **6EE46EE4** (−8.7 kcal/mole) expressed excellent binding affinities which are presented in Table 2. The binding interactions has been depicted in Table 3 and Figure 2.

**Table 2.** Binding affinity of ligands and 14 target proteins of *Plasmodium falciparum*.

| Target Protein Molecules | Binding Affinity of Ligands | | | | |
|---|---|---|---|---|---|
| | **Phaseolin** | **Beta Amyrin** | **Lupeol** | **Phytol** | **Stigmasterol** |
| | **CID_4063834_uff_E=373.58** | **CID_73145_uff_E=694.14** | **CID_259846_uff_E=928.19** | **CID_5280435_uff_E=165.89** | **CID_5280794_uff_E=546.19** |
| 4PLZ | −7.9 | −8.4 | −8.4 | −5.7 | −8.8 |
| 4QT2 | −7.8 | −7.8 | −8.3 | −5.8 | −7.2 |
| 4R1E | −6.8 | −8.0 | −7.4 | −5.0 | −7.2 |
| 4R6W | −8.5 | −8.2 | −8.0 | −5.1 | −7.4 |
| 4WI1 | −8.1 | −9.6 | −9.1 | −5.6 | −9.0 |
| 4ZXG | −8.3 | −8.9 | −8.6 | −5.0 | −8.1 |
| 5E16 | −8.2 | −7.7 | −6.8 | −4.9 | −7.9 |
| 5JAZ | −9.3 | −8.4 | −7.4 | −4.7 | −8.2 |
| 5K8S | −8.8 | −8.1 | −7.7 | −6.3 | −7.5 |
| 5ZNC | −7.9 | −8.3 | −7.9 | −5.8 | −7.8 |
| 6AQS | −8.0 | −8.1 | −7.9 | −4.4 | −7.6 |
| 6EE4 | −8.7 | −10.6 | −9.5 | −6.2 | −9.2 |
| 6FBA | −7.5 | −8.2 | −7.7 | −4.5 | −8.2 |
| 6I4B | −7.5 | −8.0 | −7.9 | −7.5 | −7.5 |

**Table 3.** Nonbonding interaction of Phaseolin with 5JAZ, 5K8S and 6EE4 after flexible docking.

| Type of bond (5JAZ) | Distance | Type of bond (5K8S) | Distance | Type of bond (6EE4) | Distance |
|---|---|---|---|---|---|
| TRP296 O…H (Hydrogen bond) | 2.7965 | TYR374 O…H (Hydrogen bond) | 2.11073 | GLU526 O…H (Hydrogen bond) | 2.45449 |
| GLY272 (Amide-Pi Stacked) | 3.82631 | TYR374 (Pi-Orbitals) | 4.39305 | VAL459 (Pi-Sigma) | 3.61821 |
| PRO273 (Alkyl) | 4.02671 | LYS426 (Alkyl) | 3.85149 | HIS496 (Pi-Pi T-shaped) | 4.62618 |
| LYS295 (Alkyl) | 4.08652 | VAL427 (Alkyl) | 4.13821 | TYR580 (Pi-Pi T-shaped) | 5.0078 |
| LYS295 (Alkyl) | 4.52289 | VAL427 (Alkyl) | 3.99876 | VAL459 (Alkyl) | 3.9127 |
| LYS336 (Alkyl) | 5.30062 | | | VAL459 (Alkyl) | 4.62938 |
| PRO294 (Alkyl) | 4.98993 | | | HIS496 (Pi-Alkyl) | 4.46507 |
| MET298 (Alkyl) | 5.478 | | | TYR580 (Pi-Alkyl) | 5.14535 |
| PRO273 (Alkyl) | 3.55355 | | | VAL493 (Pi-Alkyl) | 5.44598 |
| TRP296 (Pi-Alkyl) | 5.45846 | | | | |
| TRP296 (Pi-Alkyl) | 4.88233 | | | | |
| PRO273 (Pi-Alkyl) | 5.398 | | | | |
| LYS295 (Pi-Alkyl) | 3.91567 | | | | |

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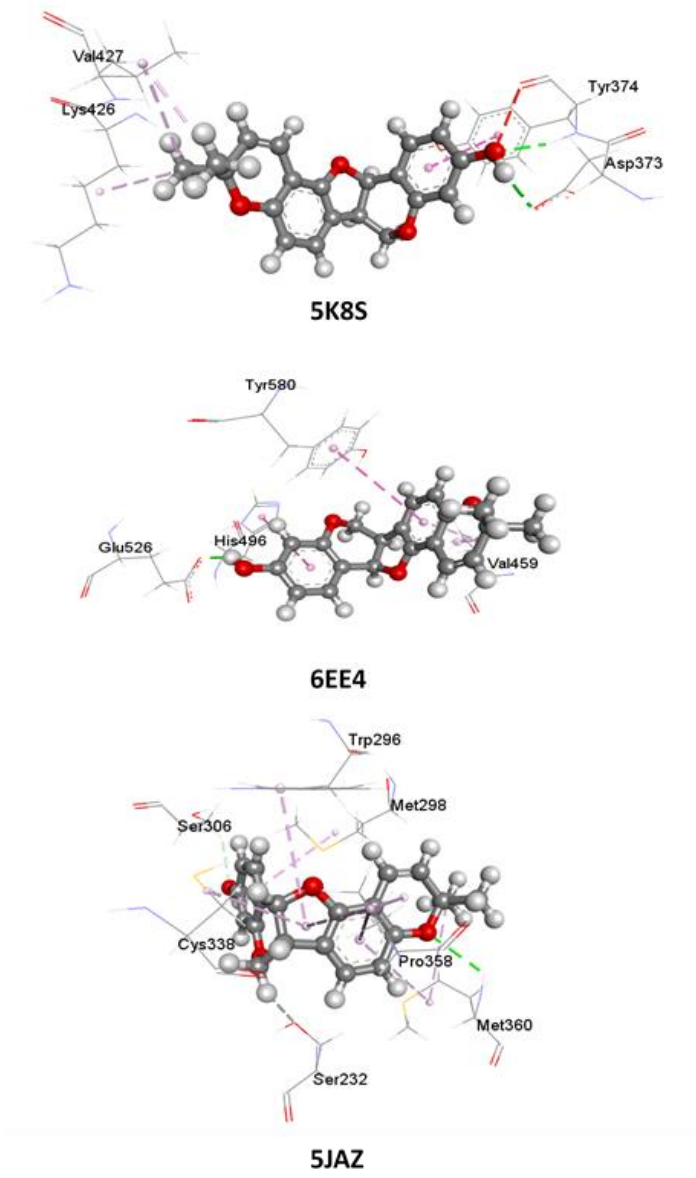


**Figure 2.** High affinity interactions between Phaseolin and clinically important Pf proteins (5JAZ, 5K8S and 6EE4).

*2.3. Drug-Likeness Property of Phaseolin*

The target of the drug likeliness evaluation was to predict if the bioactive molecule Phaseolin has a good ADME (absorption, distribution, metabolism, and excretion) properties. The compounds having good drug likeliness property should have a good aqueous solubility which is predicted by ESOL, (ALI) logS and (SILICOS-IT) logSw [21]. According to that, the ligand is found to be moderately soluble and drug-likeness parameters are good. It seems to follow Lipinski rule of 5 (molecular weight (MW) not more than 500 g/mol, hydrogen bond acceptors not more than 10, hydrogen bond donors not more than 5, LogP value less than 5, and number of rotatable bonds not less than 10) [22], Ghose, Veber, Egan, Muegge rule, with a bioavailability score 0.55 and also zero alert in the PAINS (Pan assay interference compounds). The SwissADME analysis also shows good GI (gastrointestinal) tract absorption and blood brain barrier permeability.

*2.4. Molecular Dynamics Simulation*

Molecular dynamics simulation can be used to aid the drug development process by giving insight into the dynamic interaction of drug molecules acting on the target protein. We performed MD simulation of the complexes that showed the best binding energy and

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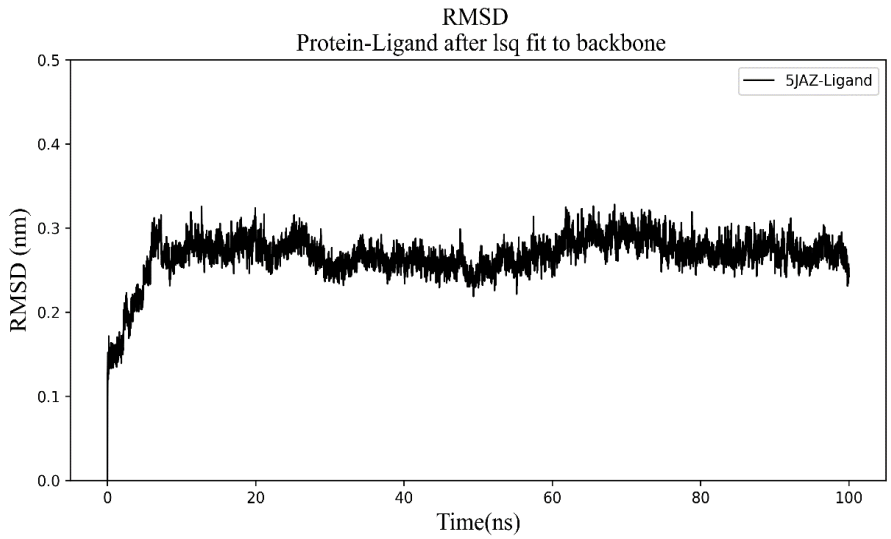
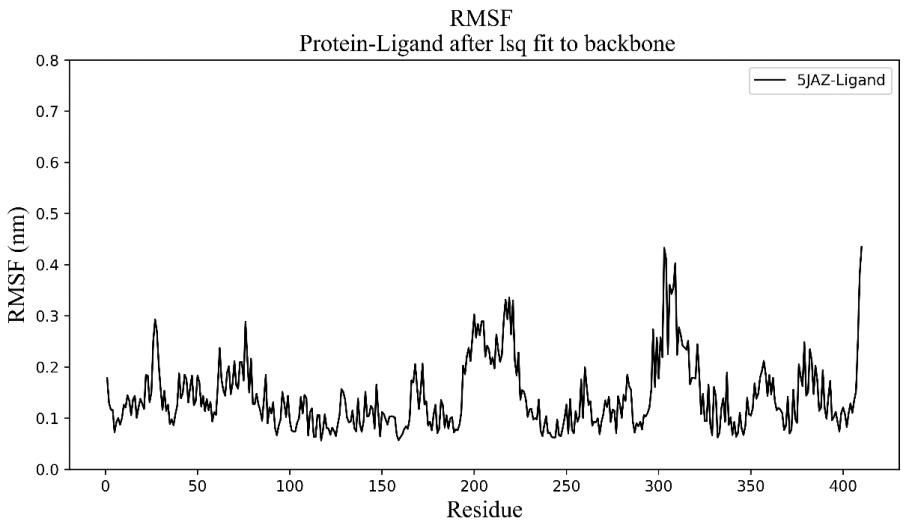
using the simulation trajectories, we plotted RMSD, RMSF, and Rg plots. The plots were drawn for each complex by using the trajectories created after the simulation processes.

Initially, the RMSD value (Figure 3(a)) for 5JAZ-Phaseolin increased to 0.3 nm. The simulation trajectory was stable for the entirety of the simulation after approximately 10. The RMSD values remained close to 0.3 nm for the most of the simulation period. The RMSF (Figure 3(b)) of 5JAZ-Phaseolin showed little deviation and major peaks were seen between the 295 to 350 number amino acid residues. The Rg (Figure 3 (c)) values had only 0.10 nm difference between the highest and lowest value. The position of the ligand Phaseolin in the 5JAZ protein in different timeframe of the simulation is shown in Figure 4.

(a)




(b)




(c)

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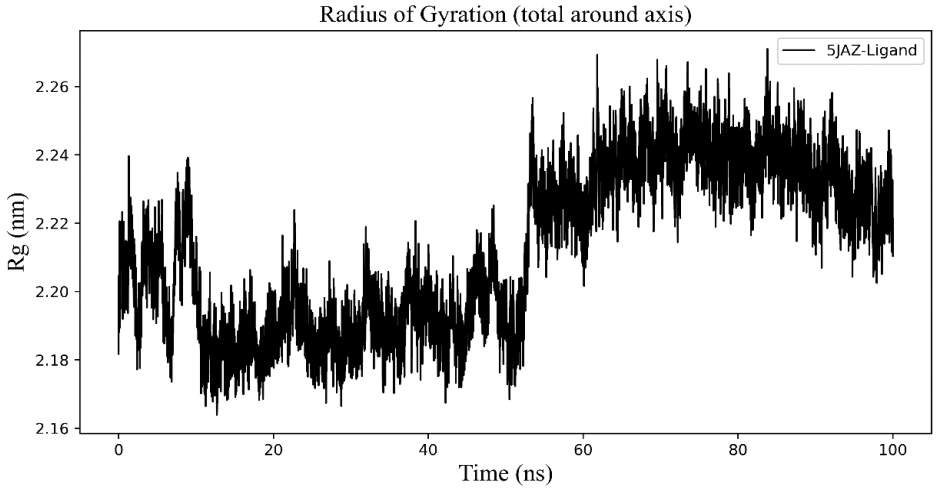


**Figure 3.** (a) Root Mean Square Deviation (RMSD), (b) Root Mean Square Fluctuation (RMSF), and (c) Radius of Gyration (Rg) of 5JAZ-Phaseolin complex after MD simulation using CHARMM36 force field.

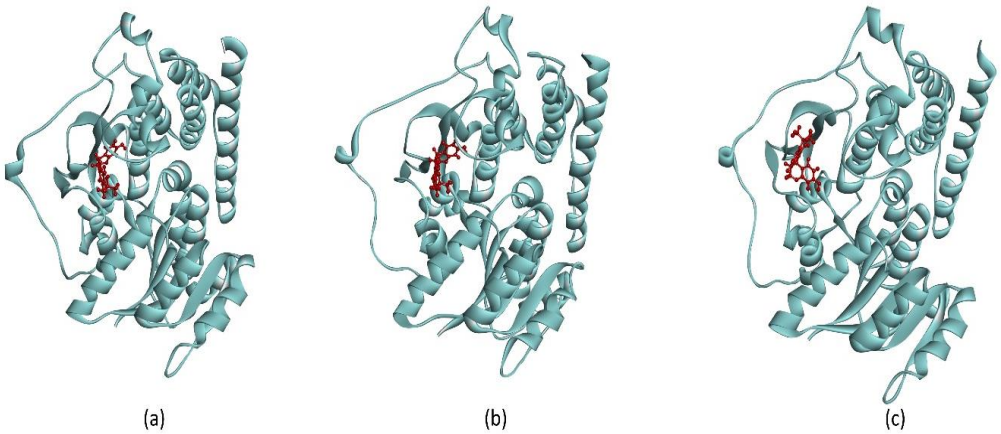


**Figure 4.** Position of Phaseolin onto 5JAZ in different timeframe in the simulation - (a) 0ns, (b) 50ns, (c) 100ns.

5K8SIn the first 20 ns of the simulation process the RMSD value ranged from 0.1 nm to 0.2 nm and again soared to 0.3 nm for 5K8S-Phaseolins complex (Figure 5(a)). The RMSD values normalized at around 20 ns and were stabilized for the rest of the simulation. The RMSF (Figure 5(b)) of 5K8S-Phaseolin showed little deviation. Peaks did not indicate major deviations and the peak RMSF values never reached more than 0.55 nm. The Rg (Figure 5(c)) values showed little deviation and stayed between 1.5 to 1.6 nm. The position of the ligand Phaseolin in the 5K8S protein in different timeframe of the simulation is shown in Figure 6.

(a)

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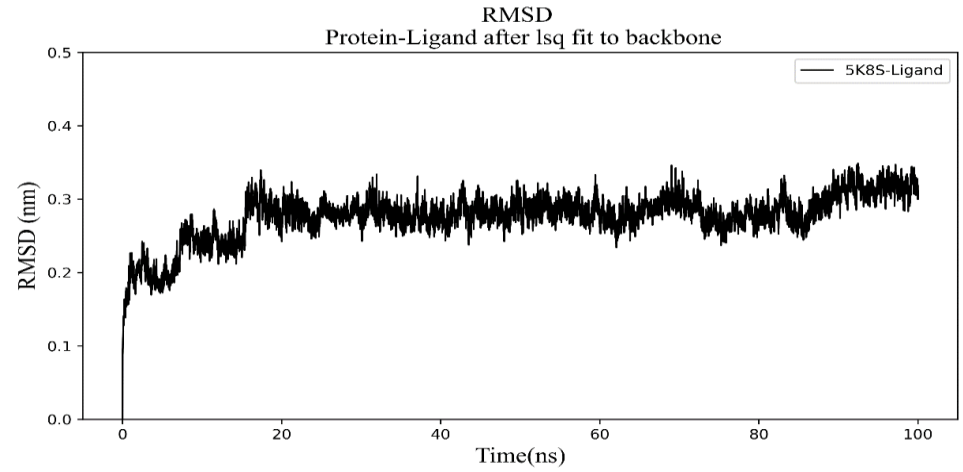
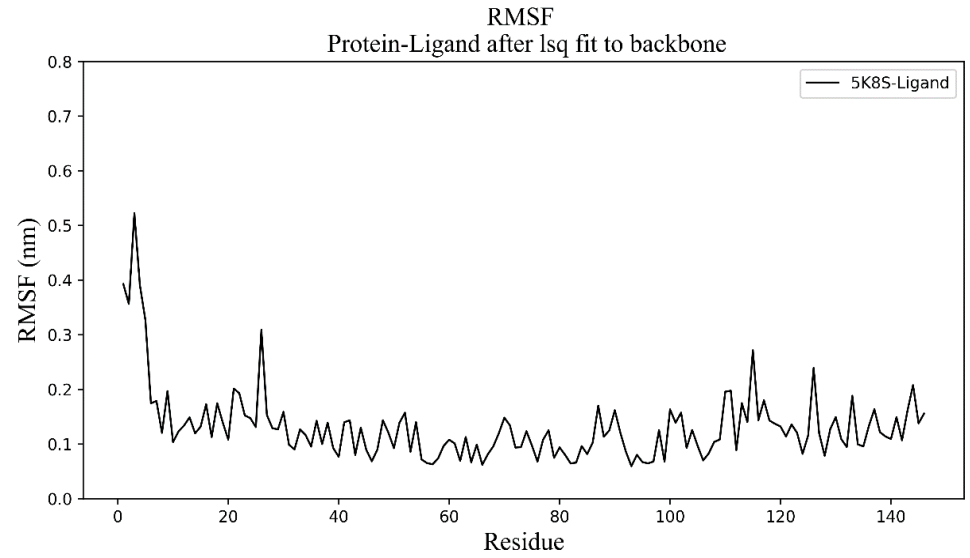



(b)

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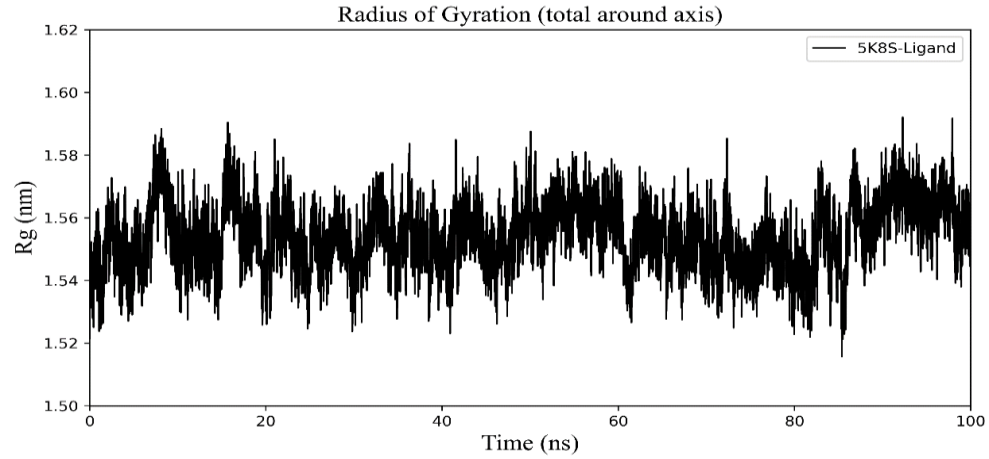


**Figure 5.** (a) RMSD, (b) RMSF, and (c) Rg of 5K8S-Phaseolin complex after MD simulation using CHARMM36 force field.

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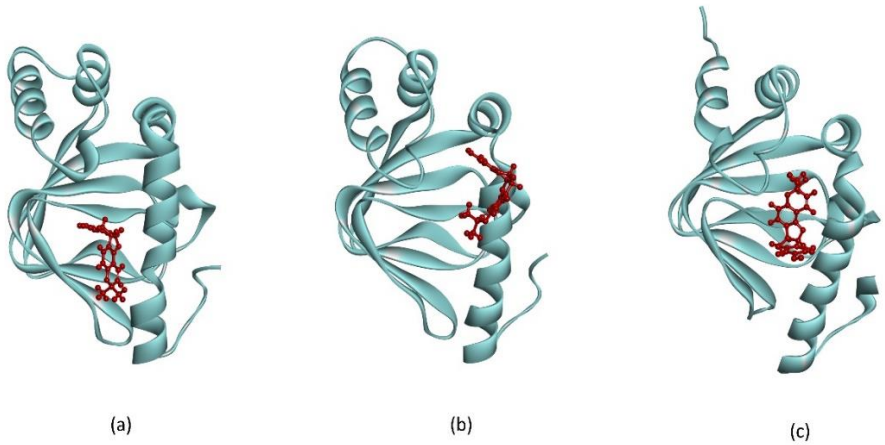
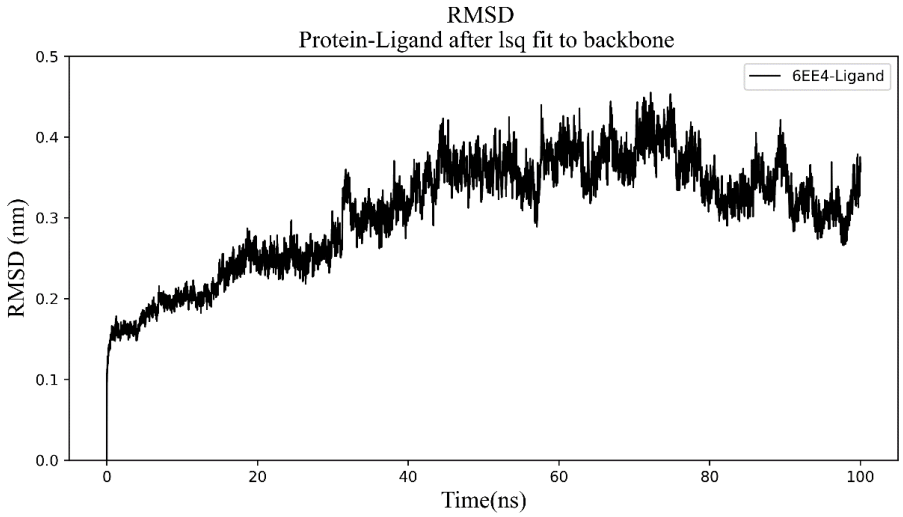



**Figure 6.** Position of Phaseolin onto 5K8S in different timeframe in the simulation - (a) 0ns, (b) 50ns, (c) 100ns.

RMSD values gradually increased to about 4.5 nm after 75 ns and later climbed down to 0.3nm and kept fluctuating Figure 7(a)) for 6EE4-Phaseolin complex. The RMSF of 6EE4-Phaseolin showed major peaks between the 450 to 500 number amino acid residues (Figure 7(b)). Rg values remained between 2.9 to around 3 nm which was the highest among the complexes investigated. Figure 8 represents the the positions of the ligand Phaseolin in the 6EE4 protein in different timeframe of the simulation.

(a)




(b)

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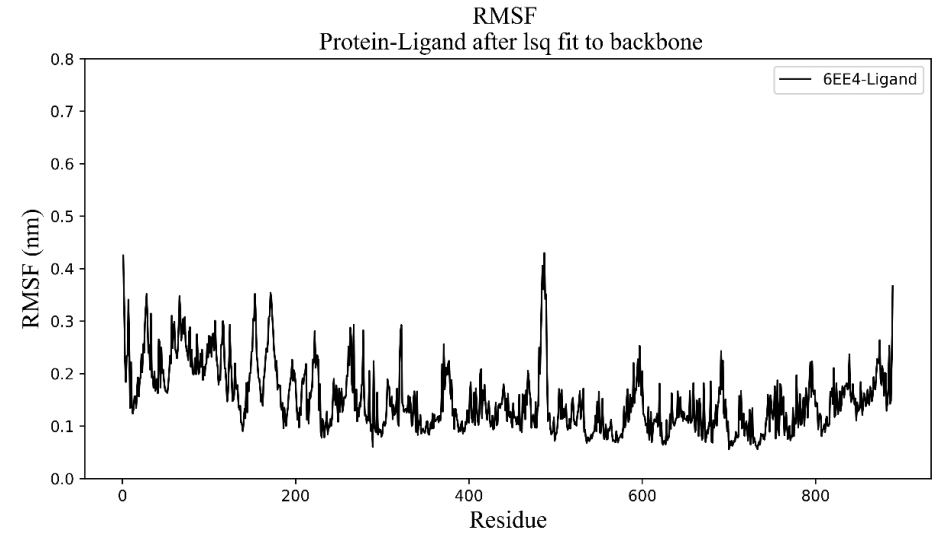


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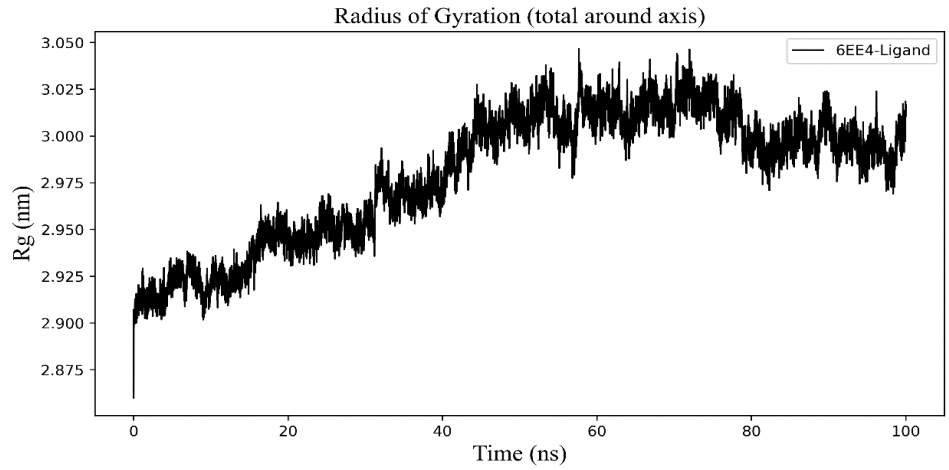


**Figure 7.** (a) RMSD, (b) RMSF, and (c) Rg of 6EE4-Phaseolin complex after MD simulation using CHARMM36 force field.

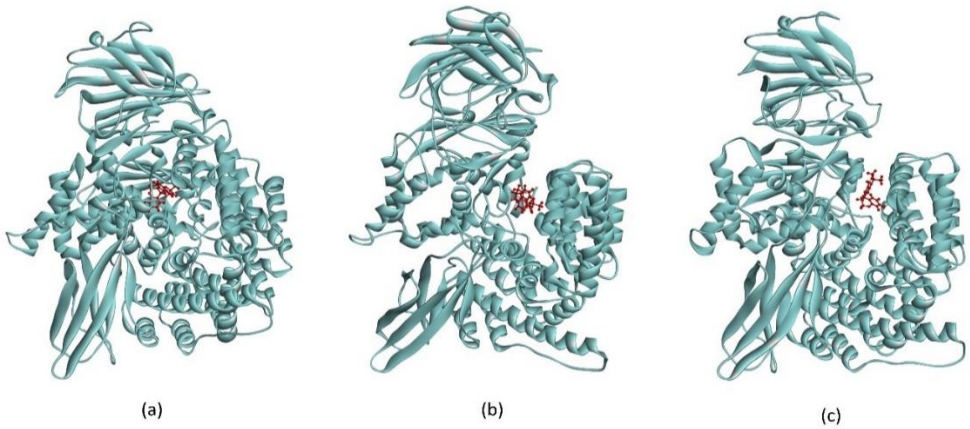


**Figure 8.** Position of Phaseolin onto 6EE4 in different timeframe in the simulation - (a) 0ns, (b) 50ns, (c) 100ns.

*2.5. Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) Calculations*

The MM-PBSA method was used to calculate free binding energy throughout the MD simulation. The free binding energy of Phaseolin with 5JAZ, 5K8S, and 6EE4 was −49.331, −75.156 and −63.762, respectively. The binding energy of the three complexes was summarized in Table 4.

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**Table 4.** Binding free energy components of Protein-Phaseolin systems (in units of kcal/mol).

| System | van der Waal energy | Electrostatic energy | Polar solvation energy | SASA energy | Binding energy |
|---|---|---|---|---|---|
| 5JAZ-Phaseolin | −103.870 ± 26.530 | −23.528 ± 15.998 | 92.147 ± 26.490 | −14.081 ± 2.351 | −49.331 ± 18.570 |
| 58KS-Phaseolin | −104.681 ± 11.813 | −4.266 ± 6.741 | 46.057 ± 14.881 | −12.267 ± 0.921 | −75.156 ± 11.459 |
| 6EE4-Phaseolin | −119.605 ± 10.669 | −41.002 ± 15.838 | 111.306 ± 21.174 | −14.461 ± 1.205 | −63.762 ± 19.082 |

## 3. Discussion

We undertook a series of experiments including phytochemical, *in vitro* and *in silico* studies to identify antimalarial compound/s from *E fusca*. The study explored the potential of the extracts as a source of future antimalarial therapeutics through *in vitro* investigations. The key finding is the identification and characterization of pterocarpan, Phaseolin, reported for the first time from *E fusca*. The other four compounds isolated from the plant extracts have already been studied for antimalarial activity. No previous studies have identified Phaseolin as an antimalarial drug, However, it has been reported in numerous stidues that the Phaseolin is a form of phytoalexin serves as a defensive molecule secreted by plants when attacked by pathogens and parasites. [23, 24]. β-Amyrin and Lupeol have been identified to show antimalarial activity [25, 26], whereas, Stigmasterol can show antiprotozoal activity [27]. Furthermore, both in vitro and in vivo studies have revealed that phytol and its derivatives have antiplasmodial activity [28].

We found significant to moderate activity of the extracts against CQ sensitive and resistant malaria strains from the *in vitro* antimalarial assay. Their antimalarial activity was categorized as: high ($IC_{50}$<5 μg/mL), promising (5< $IC_{50}$<15 μg/mL), moderate (15< $IC_{50}$<50 μg/mL) and inactive ($IC_{50}$>50 μg/mL) based on previous reports [29-31]. The aqueous fraction was most efficient against the CQ sensitive 3D7 strain, whereas the n-hexane fraction was most effective against the CQ resistant Dd2 strain. Between the two fractions, considerable activity shows the presence of the active compound or a combination of active compounds. However, the discrepancy showed by n-hexane fraction against the CQ sensitive and resistant strains can be attributed to the nonhomogeneous distribution of antimalarial compounds among the fractions. Moreover, the activity might differ due to the inherent mechanism of drug action against these strains. The earlier study conducted by Khaomek et al. [19] found antimalarial property in the ethyl acetate extract of the stem bark and prenylated flavonoid isolated from the *E. fusca* against multi-drug resistant *Pf* strain K1. However, only three of the isolated compounds showed activity at concentrations of 12.5g/mL in that study, whereas comparable activity was observed even at the fraction level in our study.

Chromatographic separation was performed with a quest to identify and characterize the active compounds from the fractions, and Phaseolin turned out to be most promising drug candidate. Further docking and molecular dynamics study substantiates the potential of the Phaseolin as an antimalarial agent. In terms of binding affinity from docking study, three of the proteins have been ranked as promising that further investigated through molecular dynamics simulation. Among them, the **5JAZ** protein is associated with parasite growth, **5K8S** is linked with parasite invasion in red blood cells (RBC), and **6EE4** plays a major role in *P. falciparum and P. vivax* by hemoglobin digestion [32-34].

The overreaching goal of our molecular dynamics (MD) simulation study was to investigate the stability and dynamic activity of the receptor protein in the ligand-protein complex system [35, 36]. 5JAZ5JAZ-Phaseolin complex indicates no substantial structure deviation in RMSD, suggesting a stable Phaseolin binding to the protein 5JAZ. The Rg values of the protein-ligand complex also showed promising results and had only 0.10 nm difference between the highest and lowest values which further implies the stable binding of phaseolin to 5JAZ. For most of the simulation time, the RMSD values of 5K8S-Phaseolin complex remained close to 0.3 nm, suggesting insignificant changes in the structure. RMSF of this complex was relatively stable compared to the other complexes, indicating very stable ligand bound complex. The corresponding Rg values of the 5K8S-Phaseolin complex found to be the lowest compared to two other complexes.

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The free binding energy was also evaluated for all the three complexes. All the complexes had negative free binding energy (Table 3) indicating a stable binding of the ligand onto the three proteins. 5K8S-Phaseolin complex showed the highest free binding energy that indicates greater binding affinity and stability among the three proteins-ligand complexes. Moreover, this complex shows the most stable results among all the evaluation methods suggesting that the Phaseolin would most likely to give the most effective inhibitory action against the 5K8S protein.

Eventually, the findings from molecular docking and dynamics study justify significant potential of the compound Phaseolin as a future antimalarial drug candidate against these targets. With no exception the findings of our study have been compromised due to some methodological limitations. However, further downstream studies are required to be taken under consideration to bolster the role of antimalarial drug property of the isolated compound, Phaseolin.

## 4. Materials and Methods

### *4.1. Plant Collection, Preparation of Extract and Fractions*

Bark was collected and taxonomically identified by the Bangladesh National Herbarium. with a voucher specimen accession number ID- DACB 48256. The bark was shade dried on the rooftop for a week and ground to a coarse powder. Subsequently, 500gm dried coarse powder was soaked in 2L of methanol with regular frequent stirring for extraction of compound(s) through cold maceration. The filtrate was then collected through multiple filtrations with cotton plug, Whatman no. 1 filter paper as well as decantation and evaporated to dryness using a rotary evaporator (**Büchi® rotary evaporator Model R-200**). The fractionation was done using a slightly modified version of the Kupchan method of solvent-solvent partitioning from the 28.5g of crude [37] and yielded three partitions; 7.8 g (yield 27.39%) of *n*-hexane, 6.6g (yield 23.16%) of chloroform, and leftover 5.3g (yield 18.59%) of mother methanolic aqueous fraction.

### *4.2. Size Exclusion Chromatography*

Three gm of n-hexane and chloroform extracts were subjected to size exclusion chromatography using lipophilic Silica 60G and later 600mg of similar fractions were subjected to Sephadex (LH-20). Gradient solvent systems of n-hexane, ethyl acetate, and methanol were used with changing polarity of the solvents from non-polar to fully polar combination resulting in 48 different fractions. For the Sephadex column, the solvent system was n-hexane: chloroform: methanol (2:5:1) initially and then 10% methanol in chloroform with a gradual increase by 10% up to 100% methanol likewise. The preparative thin-layer chromatography (PTLC) was performed on several fractions for purification purposes based on their TLC behavior. For that purpose, silica beads were coated on a glass slide, dried and spots were placed sequentially. Later, the bands were observed under UV after running them in gradient solvent as well. Purity was confirmed later using single aluminum based TLC plates. Later spectroscopic analysis was performed using 2d-NMR (supplementary S1). Eight of the purified compounds, four from n-hexane and four from chloroform partition, were spectroscopically analyzed.

### *4.3. Antimalarial Activity*

(i) Malaria Culture Maintenances

In this study, both chloroquine (CQ) sensitive *Plasmodium falciparum (Pf)* strain 3D7 (MRA-102) and CQ-resistant Dd2 (MRA-156) strains were used from BEI-resources (MR4/ATCC® Manassas, VA, USA). The continuous *in-vitro* culture of the asexual *Pf* blood stage was maintained as described by Trager and Jensen with slight modification [38]. In brief, malaria parasites were cultured *in vitro* using human O-positive erythrocytes and RPMI-1640 culture media (Gibco, Life Technologies, NY, USA). The media has been enriched with 10% heat-inactivated AB+ serum in addition to 0.5% Albumax II powder (Gibco, Life technologies, Grand Island, NY, USA), 11 mM glucose, 25 mM HEPES, 23.81

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mM NaHCO3, 200 µM hypoxanthine, and 41.876µM gentamicin solution incubated at 37°C in an anaerobic candle jar. Parasite growth was monitored every 24 hours incorporating fresh medium daily and parasitaemia was kept <5% with a haematocrit of 2% in 5mL.

(ii) Evaluation of In Vitro Antimalarial Activity

The antimalarial assay was performed using HRPII based enzyme-linked immunosorbent assay (ELISA) method described by Noedl *et al.* and WWARN [15, 39, 40]. The data is analyzed using GraphPad Prism 8.2 (La Jolla, CA 92037 USA) to construct a non-linear regression graph fit with a sigmoidal dose-response curve and determine the inhibitory concentration ($IC_{50}$) value for individual samples.

### *4.4. Molecular Docking Calculations*

**CID 4063834 (Phaseolin), CID 73145 (β-amyrin), CID 259846 (Lupeol), CID 5280435 (Phytol) and CID 5280794 (Stigmasterol)** were subjected to molecular docking study against various proteins of *Pf* due to its prospect as a drug candidate among five isolates. AutoDock Vina software was employed for flexible docking and docking calculations [41]. Search result for *Plasmodium falciparum* in rcsb pdb came with 787 proteins of which eliminating duplicate and common Uniprot ID, there were 96 proteins. Considering current important drug targets and published manuscript, we have shortlisted 14 proteins as final docking targets. Crystal structures were obtained from the Protein Data Bank (PDB ID-4PLZ, 4QT2,4R1E, 4R6W, 4WI1, 4ZXG, 5E16, 5JAZ, 5K8S, 5ZNC, 6AQS, 6EE4, 6FBA and 6I4B) and prior to docking ligand, water molecule removal and necessary energy minimization were performed. The center grid box was positioned to cover all the active sites in x,y and z directions and torsional rotation was allowed for optimal flexible docking.

### *4.5. Analysis of Drug-Likeness Using SwissADME Tool*

Chemical structure of compound, Phaseolin was downloaded from PubChem data bank in SDF (structure data format), having the conformer ID 4063834. After that, we run the SMILES in the SwissADME web page. The default parameters were chosen for determination of the drug likeliness and evaluation of the physicochemical property assessment [42].

### *4.6. Molecular Dynamics Simulation*

In our study, molecular dynamics simulation was carried out for the best three proteins and Phaseolin complexes as the other compounds have already antimalarial activity established in previous studies [23-28]. We used the GROMACS software [43] for running the simulations. The simulations were performed in Charmm36 [44] forcefield with TIP3 water molecules in a dodecahedron box for maintaining optimum conditions. In order to neutralize the system, $Na^+$ and $Cl^-$ ions were also introduced into the system. Energy minimization was a key step for minimizing the energy of the system and was carried out with a tolerance of 1000 kJ $mol^{-1}nm^{-1}$. The system was equalized with a constant number of particles, volume, and temperature (NVT) for each complex as well as a constant number of particles, pressure, and temperature (NPT).

For evaluation of the dynamic states of the complexes, root mean square deviations (RMSD), root mean square fluctuations (RMSF), and radius of gyration (Rg) plots were drawn using the MD simulation trajectories. The RMSD plots show the deviations of a simulated protein-ligand system from a reference structure in the simulation to evaluate stability. The fluctuations in the residues are determined by RMSF whereas the radius of gyration predicts the structure's overall compactness. For determining the stability of the bound structure, the RMSD, RMSF, and Rg plots of the three complexes were carefully analyzed.

### *4.7 MM-PBSA Calculations*

Free binding energy calculations can be used to evaluate the binding affinity of a ligand to its receptor in a given MD simulation system. The free binding energy calculations were calculated in order to determine the affinity of the binding of the ligands onto the respective three proteins in separate simulation trajectories. The calculations were done using the Molecular Mechanics-Poisson Boltzmann Surface Area (MM-PBSA) [45] method using the g_mmpbsa tool [46]. The calculations were performed using 50 snapshots from the last 20 nanosecond (ns) of the simulation trajectory.

## 5. Conclusions

The study is the first to isolate the five aforementioned compounds from *E.fusca*. . Substantial antimalarial activity of the fractions of the extracts of this plant have been found against both CQ-sensitive and resistant *Pf* strains. However, we were unable to evaluate the antimalarial activity of the pure compound, owing to limitations involving minimal amount of yield of the pure compound from the extracts. As a result, we resorted to the plethora of different *in silico* approaches, whereby we found high free energy and stable interactions of one of the isolated compounds, phaseolin, with three clinically important proteins *of P.falciparum.* Nevertheless, our findings warrant the need for further prospective investigations of the pure compounds for the evaluation of their potential as drug candidates to curb the emerging drug resistance among *P.falciparum* strains.

**Supplementary Materials:** Supplementary material S1 has been attached.

**Author Contributions:** SAS, OI, and MSA participated in the design of the study. SAS, OI, JS and AM carried out the laboratory experiments and data analysis. MAS, MAR, MSR performed phytochemical investigation. SAS and PG drafted the manuscript. SLB and MRHH reviewed the draft. PG revised the manuscript. SAS and MSA conceived the concept, designed, coordinated and supervised the study. All authors read and approved the final manuscript.

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