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ANTIMALARIAL AND ANTIOXIDANT EFFICACY OF MEDICINAL PLANT, *MORUS ALBA*: AN INTEGRATED STUDY USING HISTOPATHOLOGICAL, HEMATOLOGICAL, BIOCHEMICAL, AND MOLECULAR DOCKING APPROACHES

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Abstract

Malaria remains a major global health challenge due to increasing resistance. The present study evaluates the antiplasmodial potential of *Morus alba* methanolic extract (MAME) in a murine model. *Plasmodium berghei* NK65-infected BALB/c mice were treated with chloroquine (5 mg/kg) and *Morus alba* methanolic leaf extract (250, 500 and 750 mg/kg). The experimental grouping included Group A (Control), B (Negative control), C (Positive control), D1, D2, and D3 (All treated with 250, 500 & 750 mg/kg of *Morus alba* methanolic extract). Acute toxicity testing showed no mortality at 2000 mg/kg. Significant parasite inhibition in group D3 proved antiplasmodial potential of extract at a higher dose of 750mg/kg. Phytochemical screening revealed phenols, flavonoids, alkaloids, and terpenoids. The concentration of extract for 50% inhibition of biological activity was calculated as (IC_{50} was 14.01 ug/ml) which showed its potency as biological agent. *In silico* analysis of bioactive phytocompounds identified via GC-MS showed strong affinity for newly discovered antimalarial target, *Plasmodium falciparum glutathione S transferase PfGST*. These findings suggest dose-dependent antimalarial and antioxidant effects of *Morus alba*.

Key words: Plant extract; *Plasmodium berghei*; antiplasmodial activity; Antioxidant; Balb/c mice

Introduction

Malaria remains a major global health challenge and continues to cause significant morbidity and mortality globally. World Health Organization reported about approx. 282 million cases and 610000 deaths in 2024 globally which showed 12000 cases increase when compared with 2023 (Report, 2026). However increasing drug resistance limits the effectiveness of current antimalarial therapies (Gujjari *et al.*, 2022). Discovery and development of novel drugs have been in trend to fight against the malaria (Belete, 2020). Today's challenge of increasing drug resistance needs urgent findings for new treatment options to control this deadly disease (Shibeshi *et al.*, 2020). Medicinal plants are now being used as source of antimalarial drugs due to their bioavailability and least side effects.

Morus alba (MA) is continued to be a part of traditional medicine due to various therapeutic properties (Elachouri *et al.*, 2024). The pharmacotherapeutic, ethnopharmacological and toxicological profile of this plant has already been reported in previous studies (Dadhwal and Banerjee, 2023). Presence of Bioactive compounds emphasized its importance in disease management (Ramos *et al.*, 2021; Fatima *et al.*, 2024). It has been widely reported for its antioxidant, anti-inflammatory, and antimicrobial properties (Yu *et al.*, 2021). *Morus alba* leaves are rich in diverse antioxidants such as anthocyanins, phenolic compounds and flavonoids which may contribute to their biological activities (Chen *et al.*, 2021). Antimicrobial and bioactive potential of *Morus alba* have been demonstrated by many studies (Suriyaprom *et al.*, 2021; Zhu *et al.*, 2021) . An experimental study in culture media proved alkaloids isolated from *M. alba* (MA) have therapeutic benefits in managing malaria (Pradhan *et al.*, 2023). Number of studies reported the general antioxidant and pharmacological activities of *Morus alba* but integrated studies on evaluation of its antiplasmodial and antioxidant potential to malaria-induced oxidative stress and parasite suppression in murine models using both experimental and computational approaches are still lacking. Based on this research gap, we hypothesized that *Morus alba*, having strong antioxidant properties can alleviate malaria-induced oxidative stress and can also act as antimalarial drug in a *Plasmodium berghei*–infected murine model by targeting the key parasitic enzymes through its bioactive compounds. This integrated study aimed to evaluate the antimalarial and antioxidant effects of *Morus alba* leaf extract in *Plasmodium berghei*-infected mice through *In vivo* and *In silico* analysis.

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Materials and Methods

Plant sample collection and processing: The fresh leaves of *M. alba* were gathered from Quaid-i-Azam University area in June 2024. The plant was identified and authenticated by the Department of Botany, Quaid-i-Azam University, Islamabad for identify proofing. A voucher specimen with number 134057 was deposited at National Herbarium, Quaid -i-Azam University, Islamabad. The collected leaves underwent the process of rinsing and air drying at ambient temperature. Subsequently, the dried leaves were coarsely powdered with electrical grinder, and the final material was stored at normal temperature in airtight container till further processing. Extraction of 25g of powdered test sample was extracted with 250ml of 100% methanol, at a solvent-to-plant powder ratio of 1:10 (w/v) utilizing Soxhlet apparatus (SYD-260) adjusted at 6 cycles per hour maintaining the temperature at 65°C. Extract was filtered using Whatman No.1 filter paper and Rotary evaporator (R-205 V) was employed to concentrate the extract under pressure by evaporation. The concentrated extract was accurately weighed, and the percentage yield was determined. Standard formula (Abbas *et al.*, 2021) was used for calculation.

$$\text{Percentage yield (yield \%)} = \frac{\text{Weight of solvent free extract actual yield (g)}}{\text{Dried extract weight (g)}} \times 100$$

Experimental animal: Male Balb/c mice (6-8 weeks, 30-35g) were obtained from National Institute of Health Sciences (NIH) and maintained in animal house of Quaid-i-Azam university. Total thirty-six mice were used for this study. Mice were housed in steel cages under controlled environmental conditions (temperature 22 ± 2°C, a 12 h light/dark cycle and relative humidity 50–60%). Standard laboratory feed and water were provided ad libitum. Cleanliness of cages was maintained by replacing the wooden bedding and removing the residual feed and feces every third day to avoid any secondary infection. Animals were acclimatized for one week before starting the experiment.

Parasite: *Plasmodium berghei* NK 65 (chloroquine sensitive strain) was used for developing murine malaria model in this experimental study. This strain was obtained from Bei resources site which is managed by ATCC (American Type Culture Collection center).

Dose selection: Three doses (250mg/kg, 500mg/kg and 750mg/kg) of *Morus alba* extract were chosen for this study to evaluate the dose-dependent antiplasmodial effect of the *Morus alba* extract. These low to high doses were selected based on previously reported *In vivo* studies evaluating the antiplasmodial activity of crude plant extracts in murine malaria models (Chandel *et al.*, 2015; Johnson *et al.*, 2017; Nureye *et al.*, 2021; Shittu *et al.*, 2021) . Plant extracts are commonly administered within the range of 100–1000 mg/kg body weight to evaluate bioactivity and dose–response relationships (Aslam *et al.*, 2025). These doses were weighed accurately and dissolved in distilled water used as vehicle.

Parasite inoculation: Single dose of 50µl of *Plasmodium berghei* NK 65 was injected intraperitoneally to one Balb/c mouse to induce malaria and waited for the establishment of parasitemia as per protocol given in ATCC guidelines. Parasitemia was checked by counting the parasites in thin blood smear starting from 3rd post inoculation day. When 10-30% of parasitemia was established, blood containing 1×10^7 parasitized erythrocytes, was transferred to another group of mice for propagation of strain. Malarial parasites were usually in ring or trophozoite stage at this time. Cardiac puncture was performed to acquire blood from infected mice after euthanizing with isoflurane. Blood was now transferred to heparin coated syringes. After determining the cell concentration of collected blood, blood stock dilution was attained by adding phosphate buffer saline as such that 0.2ml of blood comprised 1×10^7 parasitized RBCs (Nureye *et al.*, 2021).

Rane's curative test: The curative potential of the methanolic crude extract was evaluated by adopting the standard Rane's curative test (Ryley & Peters, 1970). Animals were randomly assigned to control and experimental groups. Total of 72 mice were divided into six groups (n=12 per group): control, untreated infected, chloroquine-treated, and extract-treated groups. In Rane's test, 0.2 ml of infected blood with 1×10^7 parasitized erythrocytes were administered to all groups intraperitoneally except control group A of normal mice. At post infection day 03, experimental groups were given graded doses of plant extract while control positive group received standard chloroquine dissolved in vehicle (distilled water) through oral gavage for four days. Control negative group received same volume (10ml/kg) of vehicle (distilled water) (Table 1). Giemsa-stained thin blood smears of teil vein samples were prepared from all groups on day 07. Six mice from each group were kept under observation till 30th day (post infection) and their mean survival time was evaluated (Bantie *et al.*, 2014). Parasitemia and chemosuppression (parasitemia suppression) percentage were measured using the standard formula (Peters & Robinson, 1992).

$$\text{Percentage parasitemia} = \frac{\text{Total no. of parasitized RBCs}}{\text{Total no. of RBCs}} \times 100$$

Average parasitemia suppression was calculated:

$$\text{Average parasitemia suppression} = \frac{\text{\% Parasitemia day 3 - \% Parasitemia day 7}}{\text{Total no. of RBCs}} \times 100$$

Table 1. Dosage of Chloroquine (mg/kg) and *M. alba* (mg/kg) for each group.

Groups	Doses
A (normal)	NA
B (negative control)	PbNK65 infected + Distilled water (vehicle) given by oral gavage (10ml/kg)
C (positive control)	PbNK65 infected + Chloroquine (5mg/kg) given by oral gavage
D1	PbNK65 infected + Extract (250mg/kg) given by oral gavage
D2	PbNK65 infected + Extract (500mg/kg) given by oral gavage
D3	PbNK65 infected + Extract (750mg/kg) given by oral gavage

Hematological and biochemical analysis: At the end of the experiment on day 08 post-infection, six mice (n=6) from each group were euthanized by isoflurane inhalation followed by cervical decapitation. Blood samples (1-2ml) from each mouse were obtained via cardiac puncture and collected in EDTA-coated vacutainers for hematological and biochemical analyses. Hematological analysis was performed on blood sample using Auto VX-H360 Hematology Analyzer. Biochemical analysis was performed on blood serum, separated from blood by centrifugation at 300rpm for 15-20 min, using BIO-LA-TEST kit on chemistry analyzer.

Histopathological analysis: Additionally, liver, spleen, kidney tissues were harvested for histopathological examination. Organs' specimens were initially preserved in Neutral Buffered Formalin (NBF) overnight. These were then hydrated with ascending concentration of alcohol followed by clearing using xylene. Treated tissues were then embedded in paraffin wax and solidified to become blocks. 5µm thick sections were trimmed from blocks using microtome. Sections were then placed on glass slides in water bath. Xylene was used to deparaffinize the sections. Hematoxylin and eosin stain were used for final staining (Macsween, 1977). Prepared slides were then observed under light microscope at 10X and 40Xobjective magnification.

Acute toxicity test: The acute toxicity of MAME was tested in two main groups of normal Balb/c mice aged 6-8 weeks weighing about 30-35 g with 5 mice in each group. Mice were kept starved, fed with only water for 3 hours before giving extract dose. With the help of stomach gavage, extract in dose of 2000mg/kg mixed in distilled water (0.2ml) was administered orally to group I in single dose while same volume of only distilled water was given to group II. Mice were kept under close observation initially for 1 hour, 4 hours, then 24 hours and later for 14 days to check for change in physical behavior, motor activities, feeding habits, any signs of convulsions and death (Guideline, 2001)

Qualitative interpretation of phytochemicals: Phytochemical qualitative analysis of the methanolic extracts of *M. alba* was performed to check the presence of the phytochemicals like flavonoids, tannins, glycosides, alkaloids, saponins, sterols, quinones, terpenoids, anthraquinones using standard protocols (Sofowora, 1993).

Total flavonoid content: The aluminum chloride colorimetric method was used to quantify total flavonoid content (TFC) of the *M. alba* methanolic extract (MAME) (Zhishen *et al.*, 1999). 5% $NaNO_2$ (0.30ml), 10% $AlCl_3$ (0.3 ml) and 1M NaOH (2 ml) were added to the extract sequentially followed by incubating the extract for 20 minutes in the dark and then measure the absorbance at 510 nm using distilled water as a blank and quercetin as a standard. The Total flavonoid content was determined using Quercetin calibration curve (0.9, 1.8, 3.75, 7.5, 15 and 30 mg/50mL) which was then calculated to mg QE/g dry weight and measurements were in triplicate. Spectrophotometer was adjusted at 510nm, and each solution of quercetin was placed in it to determine the absorbance. The calibration curve was plotted accordingly.

Total phenolic content: Total phenolic content was quantified by using Folin–Ciocalteau method (Singleton and Rossi Jr, 1965). The Stock solution (50µl), with Folin-C reagent (5ml) and 7.5% Na_2CO_3 (4 ml) was heated at 45C for 30 min. The absorbance was noted after cooling at 765nm on UV spectrophotometer. The total phenolic content in *Morus alba* extract was calculated as gallic acid equivalents (GAE) from a calibration curve (50, 100, 150, 200, 250 and 300µg/mL) and expressed as µg GAE/mg of distilled water (µg GAE/mg of DW). The experiment was performed in triplicate.

Antioxidant activity

Total reducing power (TRP): The reducing power of the extract was determined based on the potassium ferricyanide colorimetric assay (Thaipong *et al.*, 2006) by incubating the solution of sample, 200µl phosphate buffer (0.2 M, pH 6.6) and 250µl potassium ferricyanide (1% w/v in distilled water) at water bath at 50 C and terminated with trichloroacetic acid and centrifugation (3000 rpm, 10 minutes). Ferric chloride was added to the supernatant and absorbance was recorded at 630nm with a spectrophotometer. A positive and negative control was taken as ascorbic acid and DMSO, respectively and the results were given as µg ascorbic acid equivalents per mg of the sample (Nmol AAE/nmol).

Total antioxidant capacity (TAC): TAC was calculated based on the phosphomolybdenum assay (Prieto *et al.*, 1999) by combining the extract (100µl) with TAC reagent (900µl), which included sulphuric acid, ammonium molybdate, and sodium phosphate and incubating it at 95ºC over a period of 90 minutes. Upon cooling, spectrophotometer was used to determine the absorbance of the solution at 630 nm, with the negative control being DMSO and ascorbic acid being the standard. The results were expressed in µg ascorbic acid equivalents per mg of sample (µg AAE/mg).

DPPH Free radical scavenging assay: The free radical scavenging ability of the extract was then determined (Arteaga *et al.*, 2012) by mixing the10µl extract with the 190µl DPPH solution then letting the mixture incubate at 37 o C over a period of 30 minutes. The absorbance at 517 nm was determined by a microplate reader and the scavenging activity was calculated as (1-*As*/*Ac*) x 100, where *As* and *Ac* *Ac* represent sample and control absorbance, respectively. The experiment was conducted thrice, and ascorbic acid was positive control.

Statistical analysis

Data was analyzed using one-way ANOVA followed by Tukey's post hoc test using graph pad prism 9.3.1. Results are expressed as mean ± SEM, and $p<0.05$ was considered statistically significant.

GC-MS analysis of Methanolic extract of *Morus alba* (MAME): Gas chromatography–mass spectrometry (GC–MS) analysis was carried out using an Agilent 6890N system equipped with an Agilent JW Scientific DB-5MS capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness) coated with (5% phenyl)-methylpolysiloxane as the stationary phase. Helium was employed as the carrier gas at a constant flow rate of 1.5 mL/min. Samples were injected manually into split mode with an injection volume of 5 µL.

The oven temperature was programmed from 120°C to 280°C at a rate of 10°C min^{-1}.

Identification of compounds: All the identified components were quantified using a percent relative peak area. The identification of compounds was performed by comparing their relative retention time and mass spectra with those of the NIST and Wiley library data of the GC-MS system(Olivia *et al.*, 2021).

Molecular docking studies: The target protein selected for this study was *Plasmodium falciparum Glutathione S transferase* (*PfGST*), an enzyme implicated in detoxification and oxidative stress regulation within the malarial parasite *Plasmodium falciparum* (Otun & Achilonu, 2025). The three-dimensional structure of *PfGST* was retrieved from the Protein Data Bank with PDB ID-10KT. To further validate, the biological significance of *PfGST*, a protein-protein interaction network, was constructed using the STRING database. String analysis was employed to identify interacting proteins and evaluate functional associations within biological pathways related to oxidative stress response and detoxification.

The protein structure was prepared by removing water molecules and co-crystallized ligands**.** Phytochemical compounds extracted from *Morus alba* through GC-MS were retrieved from PubChem in SDF format. Molecular docking was performed using AutoDock Vina integrated within PyRx (Zothantluanga *et al.*, 2021). The ligands were imported into PyRx where they were converted into three-dimensional confirmations and energy minimized using the Open Babel. A grid box was defined around the active site of *PfGST*, to allow ligand exploration within the binding pocket. Docking results were evaluated based on binding affinity energy values expressed in kilo Cal/mol. The ligand–protein complexes obtained through AutoDock Vina were subjected to further analysis using BIOVIA Discovery Studio. Two-dimensional (2D) and three-dimensional (3D) interaction diagrams were generated to visualize the binding interactions between the ligands and the amino acid residues within the active site of *Plasmodium falciparum Glutathione S-transferase* (*PfGST).* This analysis identified key interactions, including hydrogen bonds, hydrophobic interactions, π–alkyl interactions, and van der Waals contacts, which contribute to the stabilization of the ligand–protein complexes.

Results

Percentage yield: The percentage yield value of 25g of crude methanolic extract of *M. alba* was 14.8%.

In acute toxicity assessment: No **s**igns of toxicity or mortality were noticed within the 14 days of observation period.

Average Parasitemia, chemosuppression and mean survival time: Blood microscopy of thin smears of infected nontreated group B showed ring, trophozoite and schizont stages of *Plasmodium berghei* life cycle. Chloroquine treatment in group C caused significant average parasitic inhibition of 99.1± 1.1 (p<0.001). Maximum chemosuppression equal to 72.2 ± 3.0 among experimental groups was seen in group D3 which was being treated with high dose of 750mg/kg of extract reflecting the effectiveness of *Morus alba* leaves against the malarial parasite (Table 2, Fig. 1). Treatment with chloroquine and the extract increased the mean survival time significantly (p<0.001) reflecting the effectiveness of drug and extract against the parasite, but the effectiveness of extract was dose dependent.

Histological analysis of internal organ: Histopathological changes seen in internal organs (liver, kidneys, spleen) experimental groups in comparison with normal groups can be seen in Figs. 2, 3 and 4 respectively.

Hematological parameters: Malaria caused significant anemia, lymphocytopenia, and thrombocytopenia reflected by reductions in RBC count, Hb, PCV, MCV, MCH, MCHC, lymphocytes and platelets count (Table 3). Chloroquine treatment restored parameters near control values. MAME treatment resulted in dose-dependent improvement, with D3 showing the greatest recovery, although values remained slightly below normal.

Biochemical parameters: Liver enzymes (ALT, AST, ALP) were significantly elevated in infected untreated mice, indicating hepatic damage. Treatment with chloroquine and MAME reduced enzyme levels, with the highest improvement observed in D3. Similarly, renal markers (creatinine and uric acid) were elevated in infected mice and improved following treatment (Table 4).

Qualitative phytochemical screening: Phytochemical screening of *MAME* detected phenols, tannins, flavonoids, triterpenes, alkaloids, steroids, reducing sugars, saponins.

Total phenolic content: Total phenolic content of *Morus alba* methanolic extract appeared to be 3.45±0.08µg GAE/mg (Table 5).

Table 2. Parasitemia levels before and after dosage, percentage chemosuppression, and mean survival time across treatment groups (A, B, C, D1, D2, D3). Values are expressed as Mean ±SE.

Groups	Parasitemia before dosage % Mean ±SE	Parasitemia after dosage % Mean ± SE	Chemo suppression % Mean± SE	Survival time Mean ± SE
A	-	-	-	>27.0 ± 0.6$^{(a)[a]}$
B	29.9± 1.1	47.6 ±2.3	-	13.0 ± 0.9$^{\{a\}[a]}$
C	30.5 ± 1.4	2.5± 0.2	91.6± 1.1$^{(a)}$	>25.0 ± 0.6$^{\{a\}(a)}$
D1	31 ± 0.6	16.1±1.2$^{(a)[b]}$	47.6 ± 3.6$^{[a]}$	16.6 ±0.9$^{\{a\}(b)[d]}$
D2	29.5 ± 0.9	11.3 ± 1.0$^{(a)[c]}$	61.8 ± 4.1$^{[a]}$	20.0± 1.0$^{\{a\}(a)[e]}$
D3	31.0 ± 0.5	8.6 ± 0.7$^{(a)[e]}$	72.2 ± 3.0$^{[c]}$	22.0 ±0.7$^{\{a\}(a)[e]}$

The probability values given in brackets, (), and [] denote comparisons with normal group A, the infected group B treated with distilled water, and the Chloroquine-treated group C, respectively. Statistical results are indicated as follows: *a* = p<0.001, b = p<0.01, c = p<0.05, d = 0.1 and e = p>0.5

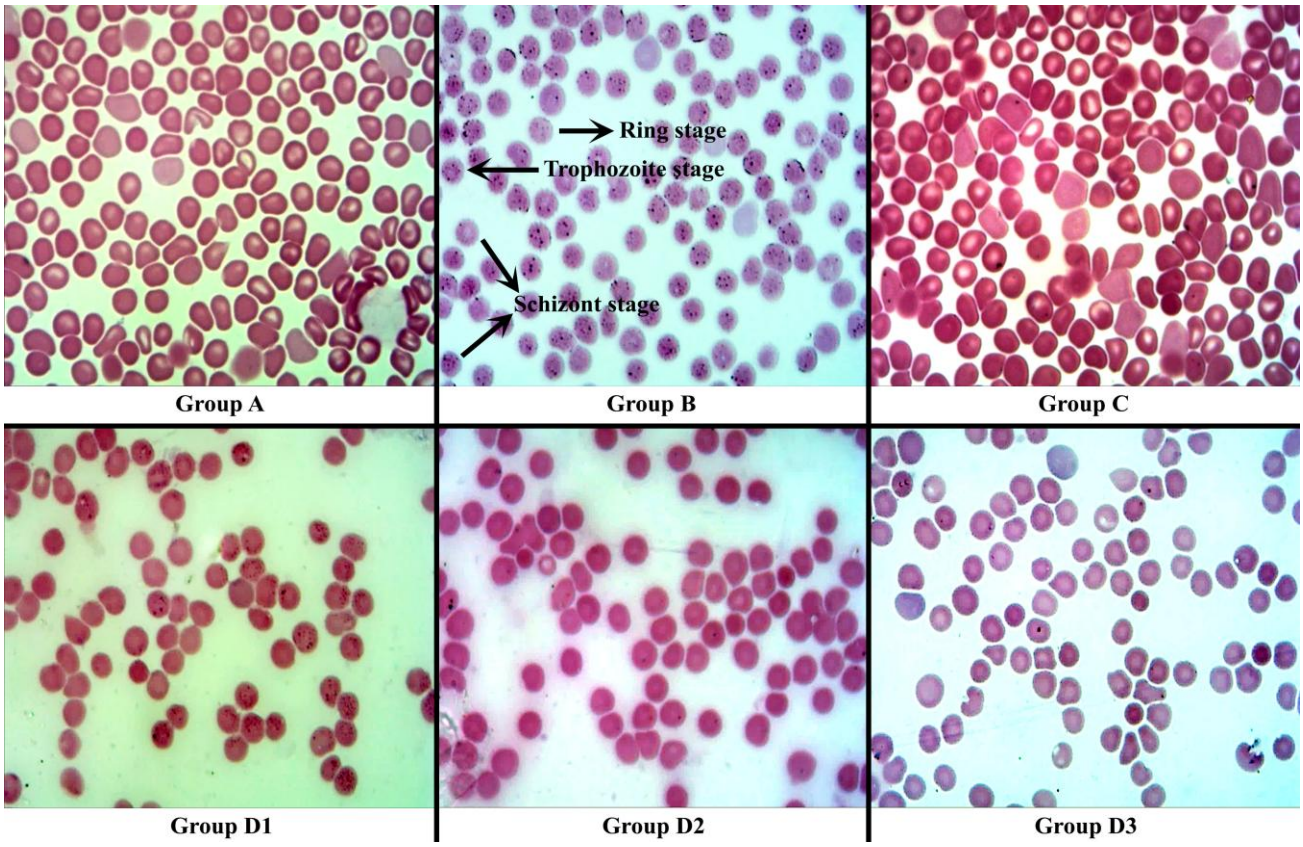

Fig. 1. Control group A, Negative control group B, Positive control group C, Groups D1, D2 and D3 treated with different doses of extract against *Plasmodium berghei.*

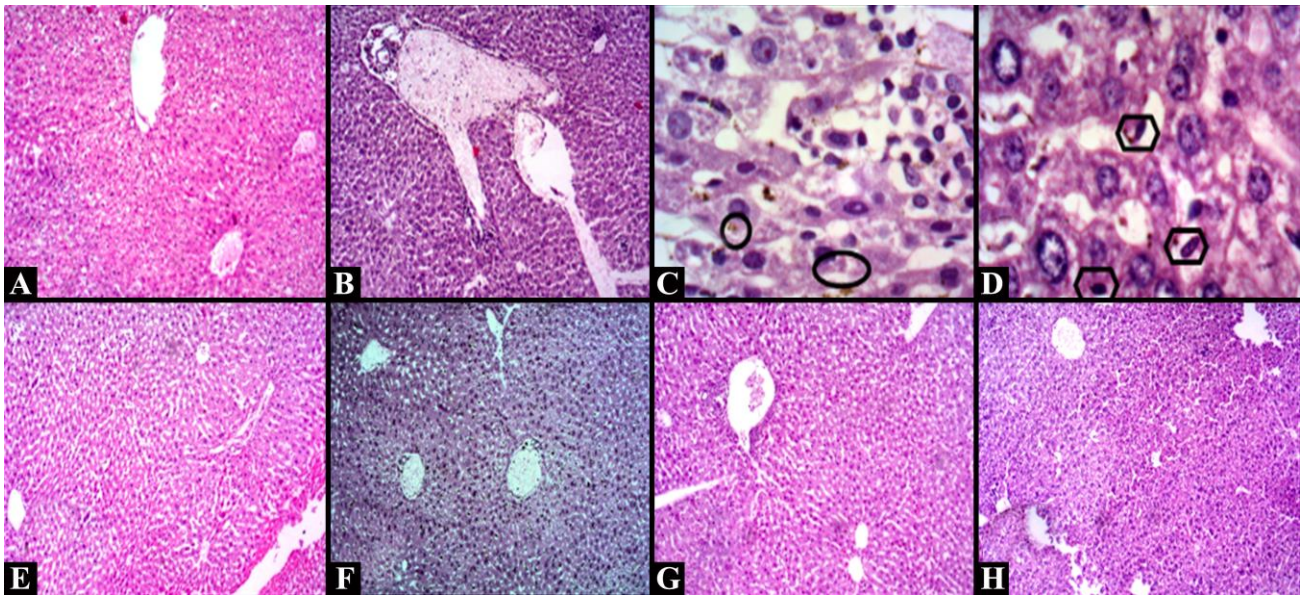

Fig. 2. Histopathological analysis of liver. (A) Control group (10X) showing normal liver architecture. (B) Negative control (10X) with periportal lymphocyte infiltration. (C–D) Negative control (40X) highlighting hemozoin deposition and Kupffer cell hypertrophy. (E) Positive control (10X) showing sparse periportal lymphocytes. (F) Group D1 (10X) with hemozoin pigment and moderate lymphocytic infiltration. (G) Group D2 (10X) showing absence of hemozoin with moderate infiltration. (H) Group D3 (10X) displaying mild diffuse lymphocytic infiltration.

Antioxidant activity: The antioxidant potential of *Morus alba* methanolic extract was quantified by different methods. Total reducing power of the extract was determined as 728.0 ± 0.0040µg AAE/mg of sample. Total antioxidant capacity measured was 910.2 ± 0.049µg AAE/mg of sample. The DPPH radical Scavenging activity of *Morus alba* was 74.8 ± 0.002 with IC50 value of 14.01(ug/ml), which confirmed the strong antioxidant potential of *Morus alba* methanolic extract (Table 7, Fig. 5).

Total flavonoid content: Total flavonoid content of *Morus alba* methanolic extract appeared to be 14.17±0.76 mg QE/g (Table 6).

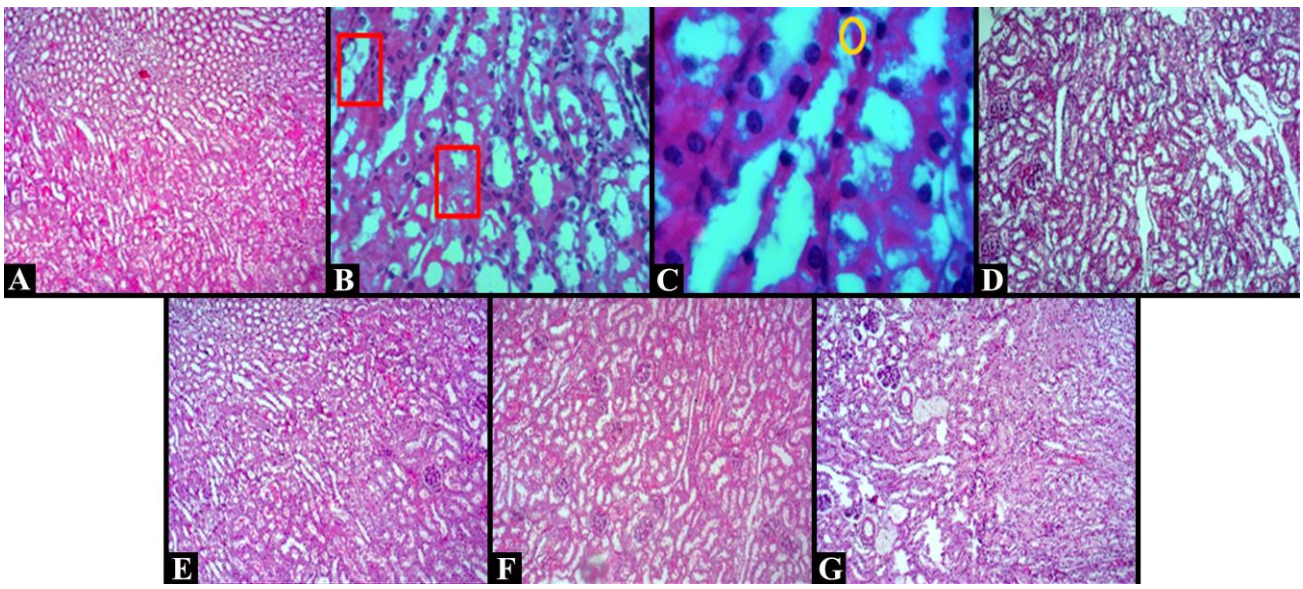

Fig. 3. Histopathological analysis of kidneys. (A) Control group (10X) showing normal kidney histology. (B–C) Negative control (40X and 100X) exhibiting acute tubular necrosis (marked). (D) Chloroquine-treated group (10X). (E–G) Extract-treated groups (10X) showing corresponding histological features.

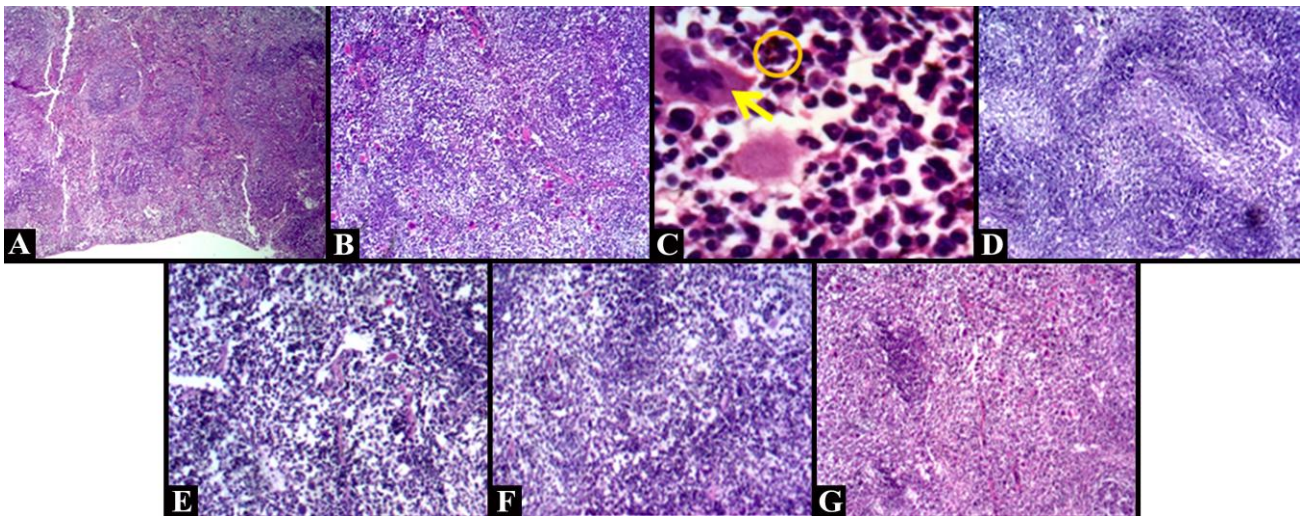

Fig. 4. Histological analysis of spleen. (Control at 4X, group A normal mice showed white and red pulp organization. Figure B and C (at 10X and 40X), showed hemozoin pigment and megakaryocytes in infected mice. Fig.4D, (at 10X) showing histological features of chloroquine treated group. Fig 4E, F and G (at 10X) showing histological features of extract treated groups.

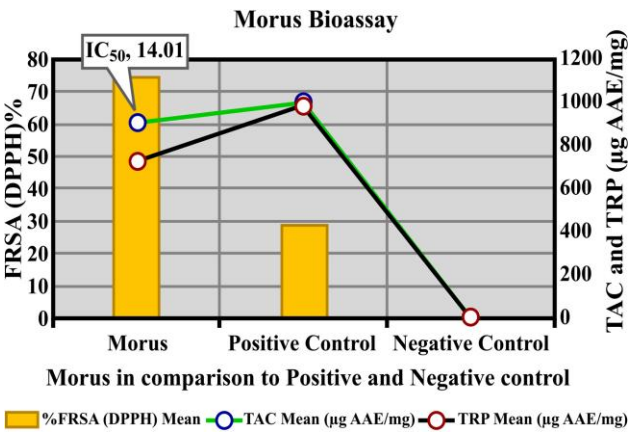

Fig. 5. FRSA(DPPH) values are shown as bars using the left Y-axis, while TAC and TRP means (µg AAE/mg) are plotted as blue and orange lines using the right Y-axis. IC_{50} values for *Morus alba* is added as data labels above respective bars.

GC-MS analysis of *Morus alba* methanolic extract (MAME): In GC-MS analysis, twenty-five compounds were identified (Fig. 6). Compounds with high peak percentage area were listed in Table 8.

Interaction Analysis of the top-ranked compounds: To study how the top compound interacts with the active site of *Plasmodium falciparum Glutathione S-transferase*, we picked the compound with the strongest binding from the docking analysis. From the *Morus alba* phytochemicals, 9,19-cyclolanost-24-en-3-ol acetate demonstrated highest binding affinity with a docking score of −9.0 kcal/mol. BIOVIA Discovery Studio was used to visualize the docked complex. Both 2D and 3D diagrams were generated to see ligand and protein interaction.

Table 3. Evaluation of differences of hematological parameters among control and experimental groups. The values in this table are expressed as Mean ± SEM (n=6). The probability values given in brackets{}, (), and [] denote comparisons with the normal mice of group A, the infected mice of group B treated with distilled water and the chloroquine-treated group C, respectively.
Statistical results are indicated as follows: *a* = p<0.001, b = p<0.01, c = p<0.05, d = 0.1 and e = p>0.5

Hematology Parameters	Groups					
	A	B	C	D1	D2	D3
RBC ($X10^6/\mu L$)	8.4± 0.1 (a) [e]	4.3 ± 0.2 {a} [a]	8.1 ± 0.2 {e} (a)	6.3 ± 0.2 (a){a}[a]	6.8 ± 0.3 (a){b}[b]	7.2±0.2 (a){b}[c]
HB (g/dL)	12 ± 0.5 (a)[b]	6.2 ± 0.2 {a}[a]	9.8 ± 0.3 {b}(a)	8.2± 0. (b){a}[d]	8.4 ± 0.3 (b){a}[e]	8.7 ± 0.4 (b){a}[e]
Hematocrit (%) (PCV)	38.8 ± 0.3 (a)[e]	27.1 ± 0.6 {a}[a]	36.5 ± 1.1 {e}(a)	34.8± 1.1 (a){b}[e]	35.0 ± 0.4 (a){c}[e]	35.3 ± 0.6 (a){c}[e]
MCV (fL)	51.8± 0.8 (a) [e]	42.3 ± 0.5 {a} [a]	49.0 ± 0.7 {e} (a)	46.3 ± 1.1 (c){b}[e]	47.7 ± 1.0 (b){c}[e]	47.0 ± 0.8 (a){d}[e]
MCH (Pg)	17.3 ± 0.4 (a) [e]	12.54 ± 0.5 {a} [a]	16.3 ± 0.4 {e} (a)	14.8 ± 0.3 (b){b}[e]	15.5 ± 0.3 (a){e}[e]	16.1 ± 0.4 (a){e}[e]
MCHC (g/dL)	32.7 ± 0.7 (a) [e]	27.3 ± 0.4 {a} [a]	31.8 ± 0.1 {e} (a)	28.7 ± 0.2 (d){a}[b]	29.7 ± 0.8 (b){b}[d]	30.5 ± 0.5 (b){d}[d]
Platelets ($x10^3/\mu L$)	351.3 ± 2.2 (a) [a]	242.6 ± 4.2 {a} [a]	312.3 ± 3.7 {a} (a)	261.3 ± 4.4 (b){a}[a]	269.6 ± 3.0 (a){a}[a]	282.5 ± 3.9 (a){a}[a]
Neutrophils (%)	26.3 ± 0.6 (a)[e]	32.6 ± 0.4 {a} [a]	27 ± 0.8 {e} (a)	30.5 ± 1.2 (e){b}[d]	29 ± 0.8 (d){e}[e]	28.5± 1.1 (c){e}[e]
Lymphocytes (%)	67.6 ± 1.0 (a) [e]	54.6 ± 2.1 {a} [a]	64 ± 1.4 {e} (a)	58.3 ± 0.8 (e) {a} [d]	58.6 ± 1.4 (e) {a} [d]	62.5 ± 1.4 (b) {d} [e]

RBC (X106/µL) HB (g/dL) Hematocrit (%) MCV (fL) MCH (Pg) MCHC (g/dL) Platelets (x103/µL) Neutrophils (%) Lymphocytes (%)

Table 4. Evaluation of differences of biochemical parameters among control and experimental groups. The values in this table are expressed as Mean ± SEM (n=6). The probability values given in brackets {}, (), and [] denote comparisons with the normal mice of group A, the infected mice of group B treated with distilled water, and the chloroquine-treated group C, respectively.
Statistical results are indicated as follows: *a* = p<0.001, b = p<0.01, c = p<0.05, d = 0.1 and e = p>0.5.

Groups names	Dosage	Liver function test (U/L) LFT			Renal function test (mg/dl) RFT	
		ALT	ALP	AST	Creatinine	Uric acid
A	None	32.7 ± 0.5 (a) [e]	50.8 ± 1.1 (a) [b]	80.1 ± 2.0 (a) [e]	0.7 ± 0.06 (a) [e]	2.5 ± 0.2 (e) [e]
B	Water	83.4 ± 0.6 {a}[a]	108.5 ± 1.7 {a}[a]	196.1 ± 5.3 {a}[a]	1.2 ± 0.1 {a}[c]	6.3 ± 0.3 {e}[a]
C	Chloroquine (5mg/kg)	36.2 ± 1.2 (a){e}	59.8 ± 0.4 (a){b}	89.1 ± 1.8 (a){e}	0.91 ± 0.1 (c){e}	3.3 ± 0.1 (a){e}
D1	250 mg/kg	44.7 ± 1.7 (a){a}[b]	76.0 ± 3.2 (a){a}[a]	136.4 ± 3.5 (a){a}[a]	1.1 ± 0.09 (e){c}[e]	4.2 ± 0.3 (a){a}[d]
D2	500 mg/kg	41.5 ± 2.0 (a){b}[d]	66.1 ± 1.9 (a) {a}[d]	106 ± 1.9 (a){a}[a]	1.0 ± 0.04 (e){e}[e]	3.8 ± 0.2 (a){c}[e]
D3	750 mg/kg	37.1 ± 1.5 (a){e}[e]	60.8 ± 1.1 (a){b}[e]	93.3 ± 2.5 (a){b}[e]	0.98 ± 0.1 (e){e}[e]	3.1 ± 0.2 (a){e}[e]

Table 5. Absorbance values, corresponding concentration (µg/ml), total phenolic content (TPC) expressed as µg GAE/mg of dry plant extract, and mean TPC with standard deviation.

Absorbance	Concentration (µg/ml)	TPC (µg GAE/mg of dry plant extract)	TPC (µg GAE/mg of dry plant extract) (mean ± SD)
0.707	70.7375	3.537	
0.681	67.4875	3.37	3.45 ± 0.08
0.694	69.1125	3.45	

Results were recorded as mean (n= 3) ± standard deviation

Table 5. Absorbance values, corresponding concentration (µg/ml), total phenolic content (TPC) expressed as µg GAE/mg of dry plant extract, and mean TPC with standard deviation.

Absorbance	Concentration (µg/ml)	TFC (mg QE/g of dry plant extract	TFC (mg QE/g of dry plant extract) (mean ± SD)
0.267	0.3	15	
0.240	0.29	14.5	14.17±0.76
0.219	0.26	13	

Results were stated as mean (n= 3) ± standard deviation

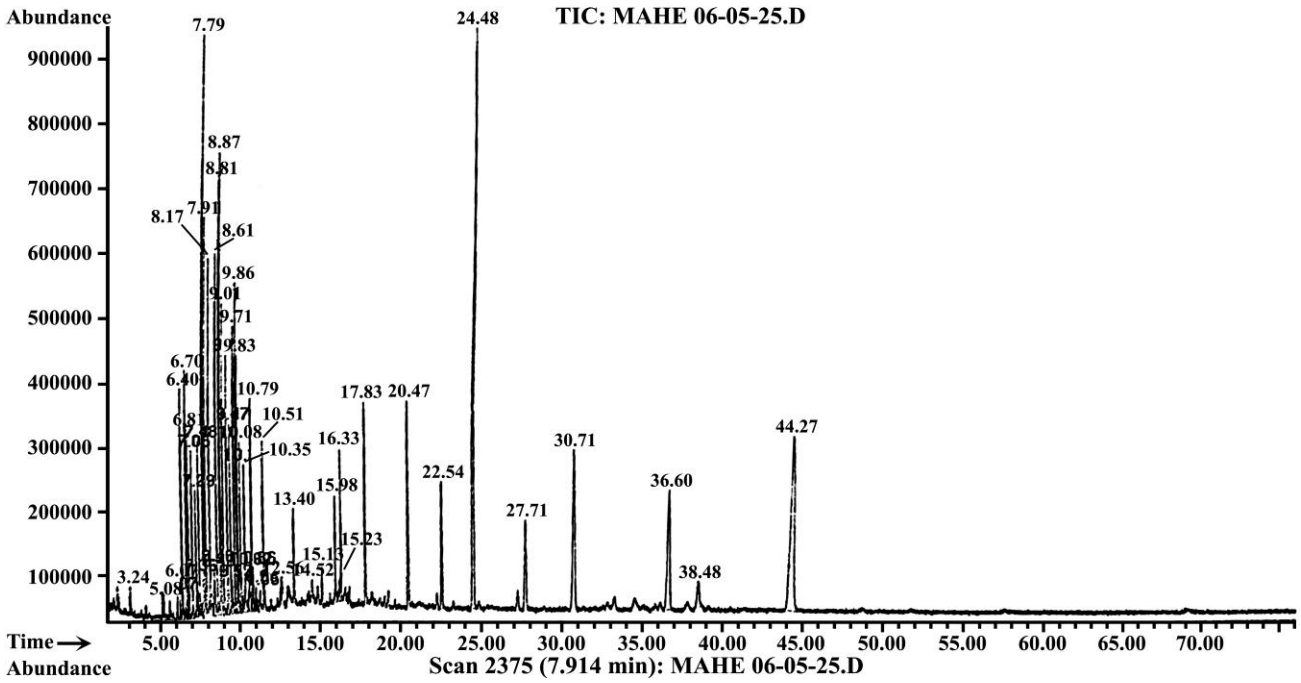

Fig. 6. GC-MS analysis of *Morus alba* leaf extract.

Table 7. Antioxidant activity parameters of the plant extract including total reducing power (µg AAE/mg sample), total antioxidant capacity (µg AAE/mg sample), percentage scavenging activity, and IC50 value (µg/ml).

Total reducing power (µg AAE/mg of sample) ± S.D.	728.0±0.0040
Total antioxidant capacity ± S.D. (µg AAE/mg of sample)	910.2±0.049
% Scavenging ± S.D.	74.8±0.002
IC50 (ug/ml)	14.01

Results were stated as mean (n= 3) ± standard deviation

String Network Analysis

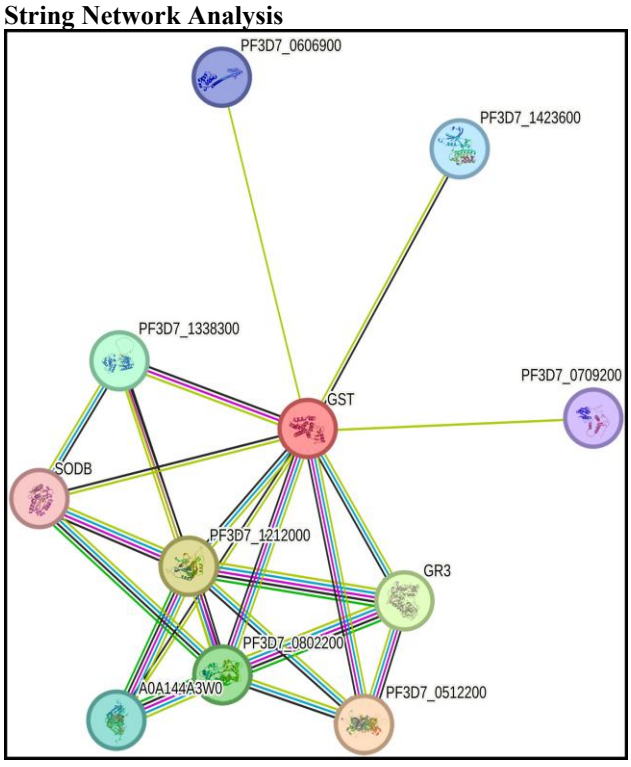

Fig. 7. Protein–protein interaction network of *PfGST* generated using STRING database. The network illustrates functional associations of *PfGST* with proteins involved in oxidative stress response, apoptosis regulation, and detoxification pathways.

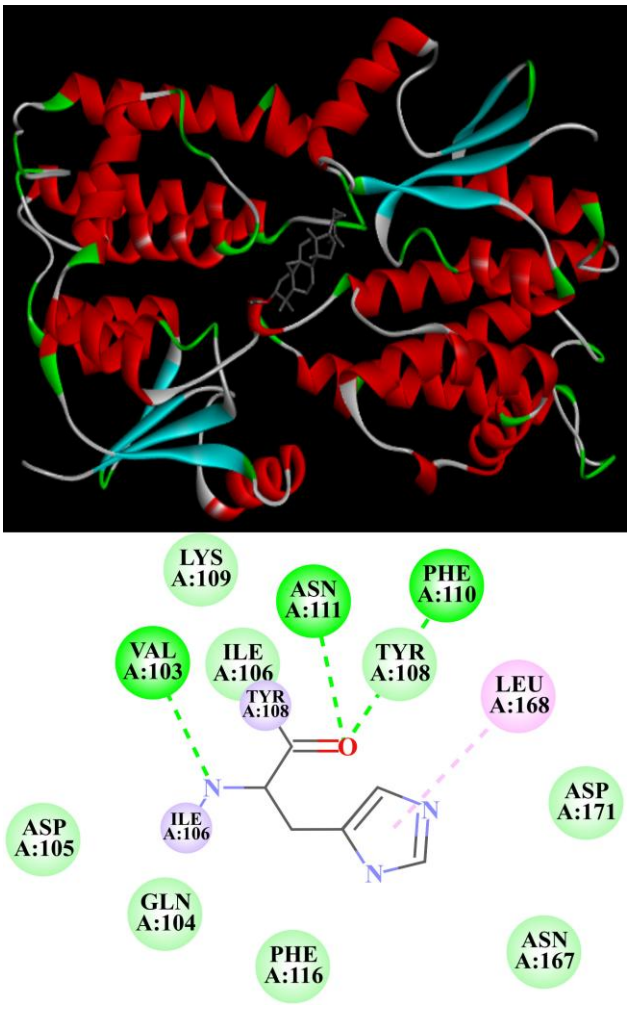

Fig. 8. 3D docking pose and 2D interaction diagram of 9,19-Cyclolanost-24-en-3-ol acetate (PubChem ID: 518616) docked with PfGST (PDB ID: 1OKT). The ligand formed hydrogen bonding interactions with **TYR A:108 and ASN A:111**, along with multiple hydrophobic interactions with residues **VAL, ILE, LEU, and PHE**.

Table 8. Compounds in *Morus alba* methanolic extract (MAME).

S. No.	Compound name	Molecular mass (g/mol)	Formula	Retention time (min)	% Area
1.	Benzene 1-Butylheptyl)-	232.40	$C_{17}H_{28}$	7.786	39.81%
2.	Benzene (1-propyloctyl)-	232.4	$C_{17}H_{28}$	7.914	19.62%
3.	benzene 1 butyloctyl)-	246.4	$C_{18}H_{30}$	8.866	22.84%
4.	benzene (1-pentyloctyl)-	260.46	C19H32	9.863	19.14%
5.	9-hexacosane	478.91	$C_{34}H_{70}$	22.543	18.09%
6.	Heneicosane	296.6	$C_{21}H_{44}$	24.479	100.00%
7.	Nonadecane	268.5	$C_{19}H_{40}$	30.709	43.48%
8.	9,19-cyclolanost-24-en-3-ol, acetate	468.8	$C_{32}H_{52}O_2$	36.607	40.74%

Molecular docking results

Table 9. Binding energies of selected compounds docked against PfGST (PDB ID: 10KT)
These results show that several *Morus alba* phytochemicals exhibit favorable binding affinity towards *PfGST.*

PubChem ID	Phytochemical compounds	Binding energy (kcal/mol)
518616	9,19-cyclolanost-24-en-3-ol, acetate	-9.0
17630	benzene 1 butyloctyl)-	-6.8
296567	9-hexacosane	-6.6
20654	Benzene (1-propyloctyl)-	-6.4
20661	Benzene 1-Butylheptyl)-	-6.4
20632	benzene (1-pentyloctyl)-	-6.1
12401	Nonadecane	-6.0
12403	Heneicosane	-5.7

Discussion

Malaria is vector borne spreading disease infecting significant numbers of people worldwide. There is a commonly available chloroquine drug used to control this disease, but this also has some negative impact on the patients. So, research is needed to replace this drug with natural herbs. Many plants were tested against the malaria induced by *P. berghei* in animals' models such as previous studies by (Ounjaijean *et al.*, 2021), (Ojueromi *et al.*, 2022) tested *Gymnema inodorum* and black seeds (*Nigella sativa*) leaf extracts modulating hematological parameters, managing anemia and other hematological complications associated with malaria. Similarly, current study used *Morus alba* as an anti-malarial plant in developing novel drug for malaria. A previous study by (Pradhan *et al.*, 2023) reported *Morus alba* as a potent anti-malarial plant after observing its biological efficacy. Studies conducted in lab setting can explain the pathogenesis and treatment of disease very well but for the purpose of assessing the biological activities of plant extract in physiological conditions of body, *In vivo* studies are essential. Various studies proved the similarity between the infections caused by *Plasmodium berghei* and *Plasmodium falciparum* in mice and humans respectively(Otun *et al.*, 2024). Furthermore, Balb/c mice genome is very similar to the human genome.

In our study, in acute toxicity evaluation, the median lethal dose (LD50) based on morphological and behavioral changes was estimated to be more than 2000mg/kg orally as no death was observed in 14 days of observational period with maximum oral dose of 2000mg/kg (Nomura *et al.*, 1983) calculated the LD 50 of *Morus alba* leaf equal to 2g/kg whereas in rats, dose up to 4000mg/kg/day didn't show any mortality. So, our results in similarity with previous studies declared the *Morus alba* as safe and non-toxic plant when administered orally.

Continuous reduction in mean survival time in parasitized mice are the clear markers of damage caused by increasing parasitemia. Continuous proliferation and replication of parasites in host cells may increase the metabolic demands of parasites from the host body which may contribute to weight loss of the host. As malarial infection causes severe damage to hematopoietic and immune systems of the host, it may contribute to reducing the host's mean survival time.(Quadros Gomes *et al.*, 2024) also correlated the parasitemia with mean survival time in parasite infected mice. In our research, *Morus alba* methanolic leaf extract significantly reduced the parasitic load and caused percentage inhibition in dose dependent manner. (Krettli *et al.*, 2009) declared a compound as antimalarial, having percentage inhibition of parasite equal 30% so our results proved the *Morus alba* as strong antimalarial agent with percentage inhibition of 72.2 ± 3.0 and this antimalarial activity may be due to the bioactive compounds present in it. This study showed presence of phenols, tannins, flavonoids, triterpenes, alkaloids, steroids, terpenoids, reducing sugars, saponins and our findings align with (Chan *et al.*, 2016) who revealed the presence of bioactive compounds including polyphenols, quercetin, kaempferol, flavonoids in *Morus alba* leaf extract and antimalarial potential of these compounds have been proved in various studies conducted on other medicinal plants (Chaniad *et al.*, 2021).

The extract-mediated improvement in hematological parameters suggests protection against malaria-induced anemia. Parasitic invasion and proliferation in RBCs cause direct and indirect destruction of RBCs harboring parasites and without parasite and their removal may happen due to cytokines produced in response to immune reaction of host cell to parasite(Ekvall, 2003). (Wang *et al.*, 2021) reported alterations in red blood count due to infection of *P. berghei* in mice. Furthermore, reported decrease in hemoglobin lead

to development of anemia during malaria affecting spleen, neutrophils and lymphocytes. (H Albohiri and A Alzanbagi, 2021) reported *P. berghei* as a pathogen which affects the liver and spleen. Our study reported a significant reduction in pack cell volume in response to infection but treatment with extract of *Morus alba* leaves showed restoration of pack cell volume near the control group. Previous study by(Ebenyi *et al.*, 2021) also reported decrease in packed cell volume in parasite infested mice. Mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) was significantly decreased in current research after infection and restored by the activity of *Morus alba*. In some relevant studies, it was reported by (Adebayo *et al.*, 2021), that *Plasmodium berghei* is a cause of alterations in hematological parameters in mice such as mean corpuscular volume, mean cell hemoglobin, mean cell hemoglobin concentration, highlighting the importance of recognizing the effect of this parasite on the host's blood parameters. There was a significant change in the neutrophils and lymphocytes of mice after parasite induction. This decrease in lymphocyte count with parallel increase in neutrophil count may result due to systemic inflammation induced by malarial infection (Berens-Riha *et al.*, 2014). Alterations in neutrophils in response to *Plasmodium berghei* infection were reported by (Su *et al.*, 2024). Infection with *Plasmodium berghei* K173 was reported to increase the percentage of neutrophils and decrease in the percentage of the lymphocytes in peripheral blood of mice(Wang *et al.*, 2021). The findings of (Ibraheem *et al.*, 2023) reported T cell-related immune responses in case of Plasmodium infection. Platelets were significantly reduced after infection and restored in treated groups. Defects in blood coagulation due to alteration in platelet count of mice infected with *Plasmodium berghei* was reported by (Boonyapranai *et al.*, 2022).

Plasmodium berghei infection has induced pathological changes in histological structure of liver, spleen and kidney which current research investigated. Previous studies demonstrated that *Plasmodium berghei* infection caused liver and spleen histopathological damage which disrupted normal organ structure, physiology as well as biomarker levels (Murshed *et al.*, 2024). *Plasmodium falciparum* and *berghei* induce liver injury in humans and mice respectively by causing activation of lymphocytes residing in liver through cytokines release. Plasmodium digests hemoglobin and releases free heme with production of hemozoin which is the fundamental reason of oxidative stress and immunosuppression (Olivier *et al.*, 2014). *Plasmodium berghei* infection in mice can lead to changes in liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in mice (Onyishi *et al.*, 2020)which is the indication of liver damage and dysfunction.(Trivedi *et al.*, 2021) showed restoration in ALP activity in infected mice with *Plasmodium berghei* by using the standard drug chloroquine and a natural product picroliv.

Urea and creatinine are biomarkers to assess the kidney function. Infection with *Plasmodium berghei* results in increase of these biomarkers. Previous investigations by (Ekasari *et al.*, 2022) showed that infection with *Plasmodium berghei* leads to malfunctioning of kidney, evidenced by changes in creatinine levels in mice. Plant extracts reported in previous studies played significant role in normalizing the kidney biomarkers (Boonyapranai *et al.*, 2021).

Plasmodium falciparum causes systemic complications in the host by inducing oxidative imbalance at cellular level (Percário *et al.*, 2012). This oxidative stress results due to the production of hydroxyl radicals in liver. Antioxidant compounds provide the underlying support in combating oxidative stress created by reactive oxygen radicals. *Morus alba* was reported with significant antioxidant activities due to rich sources of bioactive compounds (Chen *et al.*, 2022). A previous study by (Polumackanycz *et al.*, 2021) reported this plant with phenolic compounds. Our results of total phenolic content (TPC) showed that *Morus alba* contains a moderate quantity of phenolic components. The fruit and leaves of this plant exhibit high reducing power, indicating their potential as natural antioxidants. Antioxidant ability of plant extract is usually linked with quantity of phenolic compounds. Similarly, a study(Morante-Carriel *et al.*, 2024) featured its antioxidant power and importance of extraction methods in preserving antioxidant properties. In this study, *Morus alba* showed strong total reducing power, having good antioxidant capacity and significant number of total flavonoids and phenolic content. Furthermore, high scavenging capacity was observed to normalize the abnormalities induced by *Plasmodium berghei*. The total reducing capacity of this plant depends upon the phenolic and flavonoid content of fruits, leaves, and branches that contributes to scavenge free radicles and combat against oxidative stress (Hsu *et al.*, 2022) . It has been reported that polyphenols concentration in leaves fluctuates with the changing climate conditions such as precipitation amount, pollution, drought, heat and application of agricultural techniques (Abay & Eruygur, 2021). Our results may vary from other studies due to these factors. Another reason to report this plant as anti-malarial is due to its anti- inflammatory properties. The studies by (Umeyama *et al.*, 2021) showed that *Morus alba* bark extract was beneficial in reducing inflammation and cure ear edema in mice thus has anti-inflammatory and immunostimulatory effects. Compounds such as Kuwanon T and sanggenon A were reported to regulate NF-κB and HO-1/Nrf2 signaling pathways supporting anti-inflammatory properties (Ko *et al.*, 2021).

In addition to the *In vivo* findings, molecular docking showed that phytochemicals identified in *Morus alba* have favorable binding interactions with *Plasmodium falciparum Glutathione S-transferase* (*PfGST*), a protein involved in oxidative stress regulation in Plasmodium falciparum (Hiller *et al.*, 2006) .One compound, 9,19-cyclolanost-24-en-3-ol acetate had the strongest binding affinity (−9.0 kcal/mol) and interacted with key residues in the *PfGST* active site, suggesting potential inhibitory activity of *Morus alba* phytochemicals against parasite's detoxification enzyme.

Limitations: *In vitro* and *In vivo* models can emphasize foundational insights, but they cannot fully replicate human physiological conditions. Animal models of malaria do not completely create the complexity of human infection, thereby limiting the direct translational relevance of the findings. Furthermore, the bioavailability, metabolism, and pharmacokinetics of the plant bioactive compounds were not evaluated in this study, which may influence their actual therapeutic efficacy *In vivo*.

Conclusion

Morus alba leaf extract demonstrated dose-dependent antimalarial, antioxidant, and organ-protective effects in *Plasmodium berghei*-infected mice. Additionally, molecular docking of *Morus alba* phytochemicals with *PfGST* showed favorable binding interactions, which further justifies its anti-malarial activity.

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Conflict of Interest: Authors declared no conflict of interest.

Ethical approval: All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC) of Quaid-i-Azam university and approved under protocol number FBS-QAU2024-605. The study was conducted in accordance with ARRIVE guidelines and national regulations for laboratory animal care and use.

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