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Antiplasmodial effects of the aqueous ethanolic seed extract of *Ziziphus mauritiana* against *Plasmodium berghei* in Swiss albino mice

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Abstract
Ziziphus mauritiana is a fruit tree used traditionally since long back for wound healing, immunepotentiator, asthma, sedative, stomachic, styptic, as tonic etc. The present study determines the antiplasmodial effect of aqueous ethanolic seed extract against chloroquine sensitive *Plasmodium berghei berghei* nk65 infection in Swiss albino mice. Three different doses (100, 200, 400 mg/kg body weight) of the plant extract was chosen to study the blood schizonticidal activity in early infection and in established infection and was compared with chloroquine. The Prophylactic activity was also assessed and compared with pyrimethamine. In early infection, and in established infection the doses (100-400 mg/kg b.wt) was found to cause significant (P<0.001) suppression of infection in a dose dependent manner as compared to control. Although, the activity was lower than standard chloroquine. Similarly, the extract at all the doses caused the suppression in repository activity but was lower than pyrimethamine. The mean survival time was also increased in mice by 14 and 17 days at the doses of 200 and 400 mg/kg respectively, whereas the control group sustained only for 7 days. Thus, the seed extract showed the effectiveness against plasmodium infection.
Keywords: Antiplasmodial activity, *Ziziphus mauritiana*, Medicinal plant

1. Introduction
Malaria is a disease caused by *Plasmodium* infection and is still one of the important human parasitic diseases in the world. Each year, the disease infects about half a billion people and is responsible for about 1.5-2.7 million deaths[1]. According to WHO number of people worldwide infected with malaria is still increasing at the rate of about 5% annually[2]. The increasing multidrug resistant strains of *Plasmodium* and the undesirable side effects of existing anti-malarial drugs increases the encumber of disease and demand the search of new and effective anti-malarial agents.Although many drugs are available against malaria but they have side effects like nausea, vomiting, dizziness which are mild and transient. But the severs adverse reactions includes cardiovascular abnormalities, dyskinesia, ocular damage, neuromuscular disorders and loss of hearing [3]. Natural products are important sources of biologically active compounds, above all are generally safer to mammals. There are number of plants that are used traditionally as antimalarial agents[4].

Ziziphus mauritiana (Lamk.) is a tropical/ subtropical fruit tree of the *Rhamnaceae* family. It is widely distributed in Asia and Africa. The different plant parts has been used as folk remedy since long back for many diseases and ailments. Traditionally the plant is used for wound healing[5],fruit has been used as anodyne, anticancer, pectoral, refrigerant, sedative, stomachic, styptic, tonic, to cure vomiting, as mild laxative, applied on cuts and ulcers, to cure abdominal pains in pregnancy and has also been used against asthma, the bark paste is applied on sores[6-7]. Later the scientific findings emphasized the antioxidant potential of leaf extract[8-9] and seed extract[10]. The seed extract has also been reported for its cytotoxic efficiency[11-12] and immunmodulatory potential[13]. The *Ziziphus mauritiana* have been reported to possess betulinic acid[14] and there are reports showing the antiviral[15], antibacterial, anti-inflammatory and antimalarial activity[16] of betulinic acid.

The importance of the present study lies in the fact that the present work was deliberate with seeds as the test material which are usually thrown away as a waste.

Thus, keeping above in view, the present study was aimed to determine the possible antiplasmodial effects of aqueous ethanolic seed extract of *Ziziphus mauritiana* against Chloroquine sensitive *Plasmodium berghei berghei* nk65 induced infection.

2. Materials and Methods

2.1. Plant material

Fruits of *Ziziphus mauritiana* (Lamk.) variety Umran were collected from Botanical Gardens of Punjabi University, Patiala, Pb, India and authenticated by Dr. R.C. Gupta, Botany Department, Punjabi University, Patiala, Pb, India. Plant sample has been kept in Voucher specimen DOB (305) PUP at Punjabi University, Patiala.

2.2. Extract preparation

The pulp of *Ziziphus mauritiana* was peeled off, seeds were shade dried at room temperature and reduced to coarse powder. The dried and powdered seeds were percolated four times with ethanol: water (1:1) at room temperature. The combined extracts were filtered (whatmann paper), centrifuged (3200 × g, 4 °C, 30 min) and concentrated under reduced pressure in a thin film evaporator at 50±5 °C. The golden coloured paste so formed was dissolved in methanol and concentrated in thin film evaporator. Finally, the extract (ZMS) was completely dried under vaccum in the dessicator. The whole procedure yielded 9-10% (w/w) of the extract in terms of dried starting material.

2.3. Phytochemical Screening

Phytochemical screening of the crude seed extract was carried out employing standard procedures and tests[17] to investigate the chemical constituents such as alkaloids, tannins, terpenes, saponins, carbohydrates, sterols and Phytosterols.

2.4. Standardization of ZMS extract

Triterpenoid betulinic acid was used as a marker compound and was quantified in the extract[13]. as described earlier. Briefly, the ZMS extract was standardized on the basis of triterpenoid, betulinic acid using HPLC. The extract was dissolved in methanol:water (1:1) and filtered through 0.45 µm Millipore filter before injection into HPLC system. Marker compound was quantified at 30ºC employing Shimadzu HPLC system comprising of LC-10ATVP pump, diode array detector (SPD-M10 AVP), phenomenex C_{18} column (5 µm, 250 mm×4.0 mm, ID), column oven (CTO 10ASVP) and class VP software 6.10 by UV detection at 254 nm. The mobile phase consisted of acetonitrile:water.

2.5. Drugs

Tablets of chloroquine phosphate (Pfizer, India) and Pyremethamine sulphadoxin (Lupin Ltd., India) were dissolved in PBS to final doses of 5 mg/kg body weight and 1.2mg/kg body weight.

2.6. Animals

Swiss albino mice (25-30 gm) of either sex were obtained from CRI, Kasauli, India. The animals were housed in standard cages in the departmental animal house and acclimatized for a period of 10 days before the commencement of experiment. The mice were maintained on standard laboratory diet (Kisan Feeds Ltd., Mumbai, India) and water *adlibitum*. The experimental protocol was approved by Institutional Animal Ethics Committee and care of the animals was carried out as per the guidelines of Committee For the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No- 107/ 1999/ CPCSEA).

2.7. Parasite inoculation

The chloroquine sensitive *Plasmodium berghei berghei* nk65 strain was obtained from Zoology department Punjab University Chandigarh, India and was maintained in mice. The inoculum consisted of 5×10^7 *P.berghei berghei* parasitized red blood cells/ml. this was done by determining percentage parasitaemia and red blood cell count of the donar mouse. Each mouse was inoculated on day 0, intraperitoneally with 0.2 ml of infected blood containing about 1×10^7 *P.berghei berghei* parasitized red blood cells obtained from donar mouse having about 64% parasitaemia.

2.8. Parasitemia

Thin blood smear was prepared and stained with Giemsa stain. The percentage of parasitemia was determined by counting the number of parasitized RBC out of 1000 RBC in 10 random microscopic field[18].

2.9. Evaluation of suppressive activity on early infection (4-day test)

Suppressive activity of the extract was assessed as described by Knight and Peters[19]. The group of 30 animals were given standard intraperitoneal inoculum of 1×10^7 *P.berghei* infected erythrocytes. Then the animals were divided into five groups of 6 animals each. Group I, II and III received 100, 200and 400 mg/kg/day of the extract respectively. Group IV was given 5 mg/kg/day of chloroquine orally, and group V was given equal volume (0.2 ml) of distill water. All the extracts, drug and distill water were given for 3 days. On the 4^{th} day thin blood smears were made from blood samples obtained from the tail of animals. The smears were stained with Giemsa stain and examined under light microscope (100x) for the levels of parasitaemia. The average percentage suppression of parasitaemia was calculated in comparison to control as A= C-B/C ×100 where

A is average percentage suppression

C is average percentage parasitaemia in control group

B is average percentage parasitaemia in treated group

2.10. Prophylactic activity

The Prophylactic activity of the extract was determined as described by Peters[20]. All the animals were divided in five groups of six animals each. The animals were administered orally with 100, 200 and 400 mg/kg/day plant extract. The IV^{th} group received 1.2 mg/kg/day pyrimethamine and the V group served as control and fed with 0.2 ml distill water for three consecutive days. On day 4^{th} all the animals were inoculated with 1×10^7 *P.berghei* infected erythrocytes. 72 hours later thin blood smears were made and their percentage suppression of parasitaemia was calculated as described above.

2.11. Curative activity in during established infection: (Rane test)

The Schizonticidal activity of the extract during established infection was performed as described by Ryley and

Peters[21]. The animals were inoculated intraperitoneally with 1×10^7 *P.berghei* infected erythrocytes. On third day the animals were subsequently divided into five groups of six animals each. Group I, II and III received 100, 200 and 400 mg/kg/day of the extract respectively. Group IV received 5mg/kg/day chloroquine and group V received only 0.2 ml distill water daily. The drug and extract were administered for 5 days. Thin blood film stained with Giemsa stain were prepared from tail blood for each mouse daily for 5 days to monitor the parasitaemia level. The mean survival time for each group was determined in each group for a period of 28 days.

2.12. Statistical analysis

Data were analyzed using Graph Pad Prism software (Graph Pad Software, San Diego, CA, USA). All the results were expressed as mean ± S.E.M. Data of tests were statistically analyzed using one-way ANOVA followed by Tukey's multiple range test, applied for *post-hoc* analysis. A value of $P<0.001$ was considered to be statistically significant.

3. Results

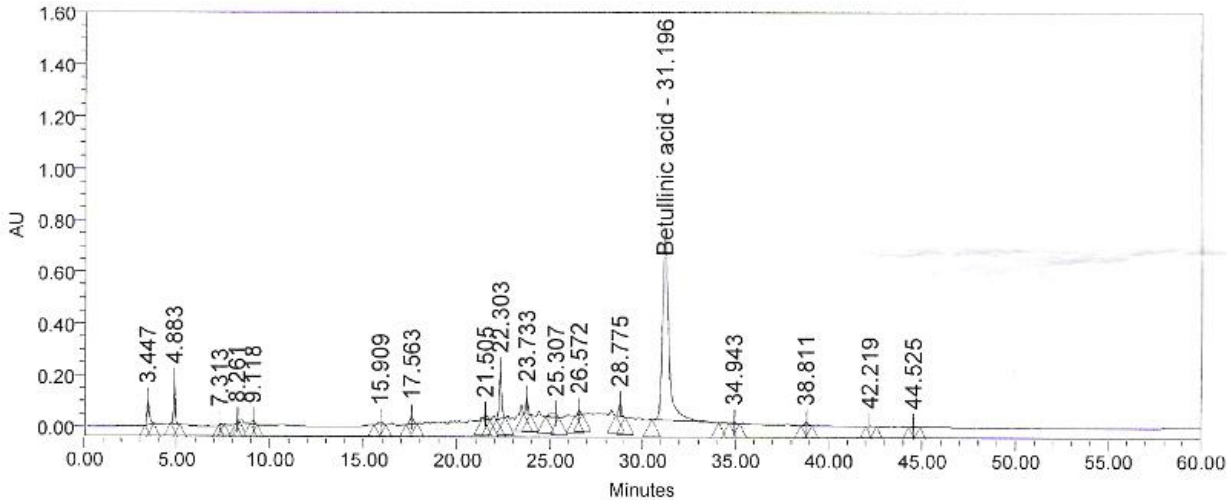
3.1. Phytochemical screening of ZMS extract

Phytochemical screening of aqueous ethanolic seed extract of *Ziziphus mauritiana* revealed the presence alkaloids, terpenes, tannins, flavonoids, saponins, sterols and phytosterols.

3.2. Standardization of ZMS extract

Chemoprofiling data of ZMS extract (Fig. 1) was generated based on naturally occurring triterpenoid betulinic acid. Seed extract of *Ziziphus mauritiana* was found to possess 2.62% betulinic acid.

Fig. 1. Chromatogram of ZMS employing HPLC profile of chemical marker.



3.3. Suppressive activity (4-day test)

The aqueous ethanolic seed extract of *Ziziphus mauritiana* produced a dose-dependent chemotherapeutic effect in a infected mice. The percentage chemosuppression observed were 38.90, 51.83, and 63. 6 for 100, 200, 400 mg/kg/day doses respectively. However the values obtained by treatment with plant extract at all the doses were lesser than standard drug (chloroquine 5mg/kg) which showed chemosuppression of 83.04 % but, caused a statistically significant ($P<0.001$) suppression of *P.berghei* activity in early infection as compared to control (Table 1).

Table 1. Suppressive activity of aqueous ethanolic seed extract of *Ziziphus mauritiana*

Drug/Extract	Dose (mg/kg/day)	% Parasitaemia	% Chemosuppression
Control(Dist. water)	0.2 ml	48.27 ± 0.87	
Ziziphus mauritiana seed extract (ZMS)	100	29.45 ±2.31[a]	38.90
	200	23.25 ±1.16[a]	51.83
	400	19.12 ±1.57[a]	63. 6
Chloroquine	5.0	8.27 ±2.43[a]	83.04

Values are expressed as mean ± S.E.M. Significance relative to control. [a] $P<0.001$, n=6.

3.4. Prophylactic activity

The aqueous ethanolic seed extract of *Ziziphus mauritiana* demonstrated a dose-dependent prophylactic activity at various doses employed, resulting in significant reduction of parasitaemia in extract treated groups. Chemotherapeutic effects of 34.86%, 45.91% and 50.29% were recorded respectively for the corresponding dose of extract (100, 200 and 400 mg/kg/day) (Table 2). Despite the fact that, the results shown by standard drug (Pyrimethamine) was higher than that of the extract treated group, the extract treated groups shown statistically significant ($P<0.001$) difference when compared with control group.

Table 2. Repository activity of aqueous ethanolic seed extract of *Ziziphus mauritiana*

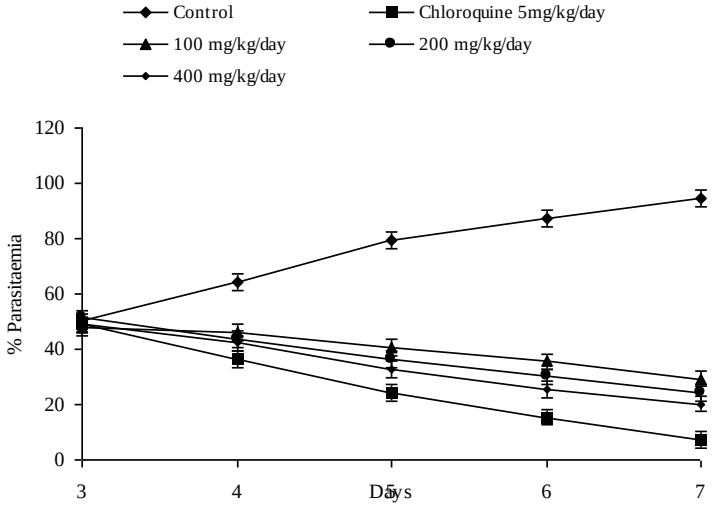
Drug/Extract	Dose (mg/kg/day)	% Parasitaemia	% Chemosuppression
Control (Dist. water)	0.2 ml	39.27 ± 2.01	
Ziziphus mauritiana seed extract (ZMS)	100	25.58 ± 1.35 [a]	34.86
	200	21.24 ± 3.65 [a]	45.91
	400	19.52 ± 1.76 [a]	50.29
Pyrimethamine	1.2	8.65 ± 0.76 [a]	77.97

Values are expressed as mean ± S.E.M. Significance relative to control. [a] P<0.001, n=6.

3.5. Curative activity

In the Rane test, the gradual decrease in percentage parasitaemia was observed from the day of treatment with extract and chloroquine. In the Rane test from the third day of treatment with plant extract and chloroquine gradual decline in parasitaemia was observed in all the groups (Fig. 2) and the reductions observed were statistically significant (P<0.001) when compared with control group . On the 7th day it was observed that control group showed 94.43% (average) parasitaemia while the treated groups treated with chloroquine, 100, 200 and 400 mg/kg/day showed 7.1%, 29.33%, 24.31% and 20.21% (average) parasitaemia respectively.

Fig 2. Antiplasmodial effect of various doses of aqueous ethanolic seed extract of *Ziziphus mauritiana* during established infection.



The results of mean survival time are shown in Table 3. The MST of the extract treated groups was significantly (P<0.001) longer than that of the control. Chloroquine treated group had MST value of 27.5 ± 0.63.

Table 3. Mean survival time of mice receiving various doses of aqueous ethanolic seed extract of *Ziziphus mauritiana* during established infection.

Drug/Extract	Dose (mg/kg/day)	Mean Survival time Days (MST)
Control (Dist. water)	0.2 ml	7.5 ± 0.26
Ziziphus mauritiana seed extract (ZMS)	100	11.5 ± 2.37 [a]
	200	14.5 ± 3.21 [a]
	400	17.5 ± 0.56 [a]
Chloroquine	5.0	27.5 ± 0.63 [a]

Data are expressed as mean ± S.E.M. Significance relative to control. [a] P<0.001, n=6

4. Discussion

The results of the present study suggest that the seed extract of *Ziziphus mauritiana* posses antiplasmodial activity against *P.berghei* parasite in early infection as well as in established infection. There are reports showing different phytochemicals present in *Ziziphus mauritiana*[10, 22]. The results of phytochemical analysis showed the presence of alkaloids, terpenes, tannins, flavonoids and saponins, sterols & Phytosterols and it is stated that the active principles present in plants like alkaloids, triterpenoids etc. may be responsible for the bioactivity[23] .

Earlier the alkaloid, Ziziphine has been reported to be present in *Ziziphus* and possess antiplasmodial activity[24], which supports the bioactivity shown by the plant extract. Another important principle present in *Ziziphus* is betulinic acid which has also been reported to possess antiplasmodial activity *in vitro*[25], although there are lesser instances of

antiplasmodial activity *in vivo* by *Ziziphus mauritiana*. But our study reveals that the crude extract of seeds of *Ziziphus mauritiana* has potency to reduce the percentage parasitaemia in 4 day test as well as in established infection, which is in corroboration of earlier studies[26] of Adzu *et al* 2007, who has shown the activity of *Zizyphus spina-christi* against the *P.berghei* infection. *Phyllanthus amarus* has also been reported to possess antiplasmodial activity showing its effect on 4 day test, established infection and in curative activity [27]. The Rane test relies on the ability of the standard inoculum of the *P.berghei* to kill the reciepient mouse within six days of inoculation. Thus, the extension of survival time beyond 12 days is regard as potential activity[28]. Our study indicated that the extract at the dose of 200 and 400 mg/kg helped the animals to sustain upto 14 and 17 days. Moreover it is documented earlier also that chloroquine which is a standard drug used against *plasmodium* infection is an antioxidant that helps in reducing the infection[29], which supports our findings as in our earlier findings we have reported the same extract as potent antioxidant[9].

Although at this preliminary stage exact mechanism of action of the extract has not been elucidated. Some plants are known to exert antiplasmodial action either by causing elevation of red blood cell oxidation[30]or by inhibiting protein synthesis[31] depending on their phytochemical constituents.

To conclude, the present study has shown that aqueous ethanolic seed extract of *Ziziphus mauritiana* possess antiplasmodial activity as observed from its ability to suppress *P. berghei berghei* infection in mice extract treated groups. The activity may be due to the presence of betulinic acid which has already been reported to possess antiplasmodial activity. Further investigations are in progress to identify the active constituents and the exact mechanism responsible for antiplasmodial activity of *Ziziphus mauritiana*.

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