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Antimalarial efficacy of *Albizia lebbeck* (Leguminosae) against *Plasmodium falciparum in vitro* & *P. berghei in vivo*

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***Background & objectives*: *Albizia lebbeck* Benth. (Leguminosae) has long been used in Indian traditional medicine. The current study was designed to test antimalarial activity of ethanolic bark extract of *A. lebbeck* (EBEAL).**

***Methods*: EBEAL was prepared by soxhlet extraction and subjected to phytochemical analysis. The extract was evaluated for its *in vitro* antimalarial activity against *Plasmodium falciparum* chloroquine (CQ) sensitive (MRC2) and CQ resistant (RKL9) strains. Cytotoxicity (CC_{50}) of extract against *HeLa* cells was evaluated. Median lethal dose (LD_{50}) was determined to assess safety of EBEAL in BALB/c mice. Schizonticidal (100-1000 mg/kg) and preventive (100-750 mg/kg) activities of EBEAL were evaluated against *P. berghei*. Curative activity (100-750 mg/kg) of extract was also evaluated.**

***Results*: Phytochemical screening revealed presence of alkaloids, flavonoids, phenols, saponins, terpenes and phytosterols. The extract exhibited IC_{50} of 8.2 µg/ml (MRC2) and 5.1 µg/ml (RKL9). CC_{50} of extract on *HeLa* cell line was calculated to be >1000 µg/ml. EBEAL showed selectivity indices (SI) of >121.9 and >196.07 against MRC2 and RKL9 strains of *P. falciparum*, respectively. LD_{50} of EBEAL was observed to be >5 g/kg. Dose-dependent chemosuppression was observed with significant (P<0.001) schizonticidal activity at 1000 mg/kg with ED_{50}>100 mg/kg. Significant (P<0.001) curative and repository activities were exhibited by 750 mg/kg concentration of extract on D7.**

***Interpretation & conclusions*: The present investigation reports antiplasmodial efficacy of EBEAL *in vitro* against *P. falciparum* as evident by high SI values. ED_{50} of <100 mg/kg against *P. berghei* categorizes EBEAL as active antimalarial. Further studies need to be done to exploit its antiplasmodial activity further.**

Key words *Albizia lebbeck* - chemosuppression - EBEAL - phytochemical - *Plasmodium berghei* - *P. falciparum*

Despite considerable progress in malaria control over the past decade, malaria remains one of the most important potentially fatal parasitic diseases in the world. It is one of the top three killers among communicable diseases, particularly in tropical Africa[1]. Appropriate selection of first- and second-line antimalarial medicines for country programmes is based entirely on the efficacy of various medicines against malaria. As the parasite evolves continuously to develop resistance to medicines, continuous global monitoring and reporting of drug efficacy and parasite resistance are needed.

Most of the medicines used today against malaria came from natural product lineages which can be traced back to herbal medicinal products: quinine, lapachol and artemisinin. There has been a resurgence in interest in indigenous phytomedicines, with a number of international and local initiatives actively exploring botanical resources, working with traditional healers to exploit known medicinal plants, as well as screening plants more generally for pharmacologically active compounds[2,3].

The present study was undertaken to evaluate the safety and efficacy of *Albizia lebbeck* (Benth) family: Leguminosae, as an antiplasmodial agent. It is native to tropical southern Asia, is a large, erect, unarmed, deciduous spreading tree found throughout India and has been used in Ayurveda, Sidha and Unani medicines[4]. *Albizia* species is reported to have many important medicinal properties mainly anti-inflammatory, antimicrobial (leaf extract) and analgesic[5]. It has been used traditionally due to its antiproliferative, nootropic, anxiolytic, haemolytic, anti-diarrhoeal, antioxidant, anti-arthritic and antifungal activities[6]. The leaf extracts of *A. lebbeck* have been reported to possess antihyperglycaemic and antidiabetic potential as well as nematicidal effects[7]. Ethanolic bark extract of *A. lebbeck* (EBEAL) was screened in the present study for its phytochemical composition, cytotoxic activity (CC_{50}) against *HeLa* cell lines, antimalarial activity against *Plasmodium falciparum in vitro* and acute toxicity (LD_{50}) against rodent host. Schizonticidal, preventive and curative activities of EBEAL against *P. berghei* were also evaluated.

Material & Methods

Plant materials: The permission for collection of stem bark of *A. lebbeck* was duly granted by Head of Forest Force (HOFF) for research purposes in accordance with provisions under Biological Diversity Act, 2002. The permission for collection of specified quantity of plant from Himachal Pradesh (HP) was also granted by Divisional Forest Officer, HP. The stem bark of plant was collected in the month of September, 2013, from Shimla, India. Voucher specimen for *A. lebbeck* (No.17865) was identified and authenticated by comparison with reference specimens in the herbarium of Department of Botany, Panjab University, Chandigarh, India.

Preparation of extract/Phytochemical screening: The bark of *A. lebbeck* was washed thoroughly with water, shade dried and powdered. Bark powder (110 g) was subjected to soxhlet extraction[8] using ethanol (500 ml) as solvent till extract in the siphon underwent complete discoloration. Ethanolic extract of *A. lebbeck* was evaporated to dryness *in vacuo* at 40° C in a rotary evaporator. The residue thus obtained was stored in screw capped vials at -4°C until used further.

Phytochemical examination of the extract was carried out for detection of alkaloids, phenols, flavonoids, tannins, saponins, phytosterols, terpenes, glycosides and steroids[9].

Animals and parasite strain: White Swiss albino mice (*Mus musculus)* of BALB/c strain (25-30 g), were obtained from Central Animal House, Panjab University, Chandigarh. Asexual blood stages of chloroquine (CQ)-sensitive strain (NK 65) of *P. berghei* were maintained by passaging intraperitoneal inoculation of 1×10^6 *P. berghei*-infected erythrocytes in citrate saline from infected to naive mice.

Cytotoxicity on HeLa cells: Cytotoxicity of EBEAL was evaluated on immortal cervical cancer cell line (*HeLa)* using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay[10]. *HeLa* cell line obtained from National Centre for Cell Science (NCCS), Pune, India, showed 70-72 per cent viability, and was suitable to perform cytotoxicity studies. Cell cytotoxicity was tested and the per cent cell viability was calculated using the following formula:

$$\text{Per cent cell viability} = \{(At-Ab)/(Ac-Ab)\} \times 100$$

where, At= Absorbance of test, Ac=Absorbance of control and Ab= Absorbance of blank. The per cent cell viability was calculated at various concentrations (10-1000 µg/ml) of the extract to determine CC_{50}. Cytotoxicity, CC_{50} for cell line, is the concentration of compound that causes a 50 per cent reduction in absorbance at 490 nm relative to untreated cells using MTT assay.

In vitro culture of P. falciparum and estimation of growth inhibition: MRC2 and RKL9, chloroquine sensitive and resistant strains of *P. falciparum*, respectively, obtained from National Institute for Malaria Research (NIMR), New Delhi, were kept in continuous *in vitro* culture according to the modified candle-jar method of Trager and Jensen[11]. Human red blood cells (blood type A+) in RPMI 1640 medium (Sigma Chemical Co., USA) supplemented with 25 mM HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Sigma), 0.2 per

cent sodium bicarbonate (Sigma), and 15 per cent complement inactivated human AB+ serum were used for parasite culture.

Stock solution of EBEAL was prepared with RPMI 1640 to achieve the required concentrations (5-100 µg/ml) before being tested in culture in duplicate in 96-well microtiter plates. The cultures, before testing, were synchronized by treatment with 5 per cent D-sorbitol (Sigma). After 48 h, thin smears were made from duplicate wells, fixed in methanol, stained with Giemsa stain, and observed through a microscope to calculate parasite inhibition at various concentrations of extract.

Fifty per cent inhibitory concentration (IC_{50}) values were determined graphically on dose-response curves with the help of probit analysis[12] by SigmaPlot 8.02 software (Systat Software Inc., USA). This activity was analysed in accordance with the norm of plants' antimalarial activity given by Lekana- Douki *et al*[13]. Based on WHO guidelines[13], antiplasmodial activity was classified as follows: high (IC_{50} <5 µg/ml), promising (5-15 µg/ml), moderate (15-50 µg/ml) and inactive (IC_{50} >50 µg/ml).

Selectivity index was calculated as the ratio of cytotoxicity of extract on *HeLa* cell line (cytotoxicity) to the IC_{50} of the extract against *P. falciparum* (antiplasmodial activity) strains.

Acute toxicity (LD_{50}): Limit test of Lorke[14] was employed to determine the acute toxicity of the extract. LD_{50} median tethal dose is defined as concentration of extract/drug corresponding to 50 per cent mortality as compared to control. The acute toxicity of the extracts was determined using four female BALB/c mice, by oral administration of 5 g/kg concentration. Dried extract residue was dissolved in standard suspending vehicle (SSV). Mice were fasted for 4 h. After administration of EBEAL, mice were examined for mortality and side effects. If the mice died, lower concentrations of extracts were administered to mice, till LD_{50} was determined.

Evaluation of in vivo schizonticidal, repository and curative activities of plant extract: Schizonticidal activity of the plant extract was assessed by the method described by Knight and Peters[15]. On day 0 (D0), all the mice were inoculated with 1×10^6 *P. berghei* infected erythrocytes and divided into seven groups containing six mice in each group. Different concentrations of the extract dissolved in formulation vehicle *i.e*., standard suspending vehicle (SSV)[16] were administered orally to mice. Treatment started one hour post-inoculation of parasite on same day (D0) and continued for four consecutive days (D0-D3) (Table). On D4, D7, D14, D21 and D28, respectively, thin blood smears were prepared

Table. Evaluation of schizonticidal, repository and curative activities (% average parasitaemia) of ethanolic bark extract of *A. lebbeck* (EBEAL)

Groups (n=6) Treatment dosage (0.2 ml/mouse/OD)	Schizonticidal activity D0: i.p. infection (1x10^6)*; D0-D3:dosage	Repository activity D0-D3: dosage;D4: i.p. infection (1x10^6)*	Curative activity D0: i.p. infection (1x10^6)* D3-D7: dosage
G1, Distilled water	35.3 ± 1.2	5.9 ± 1.4	15.5 ± 10.5
G2, EBEAL (100 mg/kg)	10.8 ± 3.9***	1.6 ± 0.9***	8.5 ± 3.2***
G3, EBEAL (250 mg/kg)	10.1 ± 2.4***	1.5 ± 0.2***	7.6 ± 5.2***
G4, EBEAL (500 mg/kg)	9.9 ± 1.2***	0.3***	7.5 ± 2.5***
G5, EBEAL (750 mg/kg)	7.1 ± 0.5***	1.4 ± 1.2***	4.03 ± 1.1***
G6, EBEAL (1000 mg/kg)	5.4 ± 0.4***	-	-
G7, CQ (5 mg/kg)	1.1 ± 0.2***	-	4.6 ± 2.3***
G8, Pyr (1.25 mg/kg)	-	1.7 ± 0.2***	-

*1x10^6 *P. berghei* infected erythrocytes
Data are expressed as mean ± SD (n=6 in each group)
P***<0.001 compared to control
Pyr, pyrimethamine

from the tail of each mouse, fixed in methanol and stained with Giemsa's stain. The percentage of chemosuppression was determined by the formula

$$100- \frac{\text{Number of infected erythrocytes}}{\text{Total number of erythrocytes}} \times 100$$

ED_{50} (median effective dose) of extract was determined as the concentration of the extract that caused 50 per cent growth inhibition of the parasite *in vivo*.

The repository or preventive activity of EBEAL was assessed using Peters' method[17]. The mice were divided into six groups of six mice each and were orally administered distilled water, SSV, pyrimethamine (1.25 mg/kg, positive control) and various concentrations of EBEAL for four consecutive days (D0-D3), respectively (Table). On the fifth day (D4), mice were inoculated with $1x10^6$ *P. berghei* infected erythrocytes. 72 h later, parasitaemia was assessed by studying Giemsa stained blood smears.

The suppressive activity of EBEAL in established infection of *P. berghei* was assessed using method described by Ryley and Peters[18]. On D0, mice were inoculated with $1x10^6$ *P. berghei* infected erythrocytes; 72 h later, mice were divided into six groups of six mice each and were orally administered distilled water, SSV, chloroquine (5 mg/kg, positive control) and various concentrations of EBEAL for four consecutive days (D4-D7), respectively (Table). On D7, parasitaemia was assessed by studying Giemsa stained blood smears.

Statistical analysis: Level of significance was determined by applying Student t test using Graphpad Software 3 (GraphPad Software, Inc, USA) and Probit analysis was done using SigmaPlot 8.02 software (Systat software Inc., USA). Kaplan-Meier (KM) estimator of survival was used to evaluate follow up among mice with censored (incomplete) data during schizonticidal activity.

Results

Plant extract/ Phytochemical screening: The collected 110 g dried stem bark of *A. lebbeck* (Voucher No. 17685) yielded 27.3 g of dried residue after ethanolic extraction and concentration in rota evaporator. Phytochemical screening of EBEAL showed the presence of alkaloids, phenols, flavonoids, saponins, phytosterols and terpenes.

Cytotoxicity on HeLa cells: The analysis revealed CC_{50} of >1000 µg/ml when per cent cell viability was plotted against various concentrations of the extract. Thus, according to criteria given by Osorio *et al*[19], the extract can be categorized as potentially non-toxic for further use.

In vitro culture of P. falciparum and estimation of growth inhibition : Continuous *in vitro* culture of *P. falciparum* was maintained to check the efficacy of different concentrations (5-100 µg/ml) of EBEAL. *In vitro* antimalarial screening of EBEAL exhibited IC_{50}= 8.2 µg/ml and 5.1 µg/ml against MRC2 and RKL9 strains (Fig. 1a, b) of the parasite, respectively. Therefore, the extract was classified as active[20] exhibiting selectivity indices of >121.9 and >196.07, respectively. Chloroquine, used as a reference antimalarial drug, tested in parallel had IC_{50} of 44 and 658 nM against sensitive and resistant parasite strains, respectively.

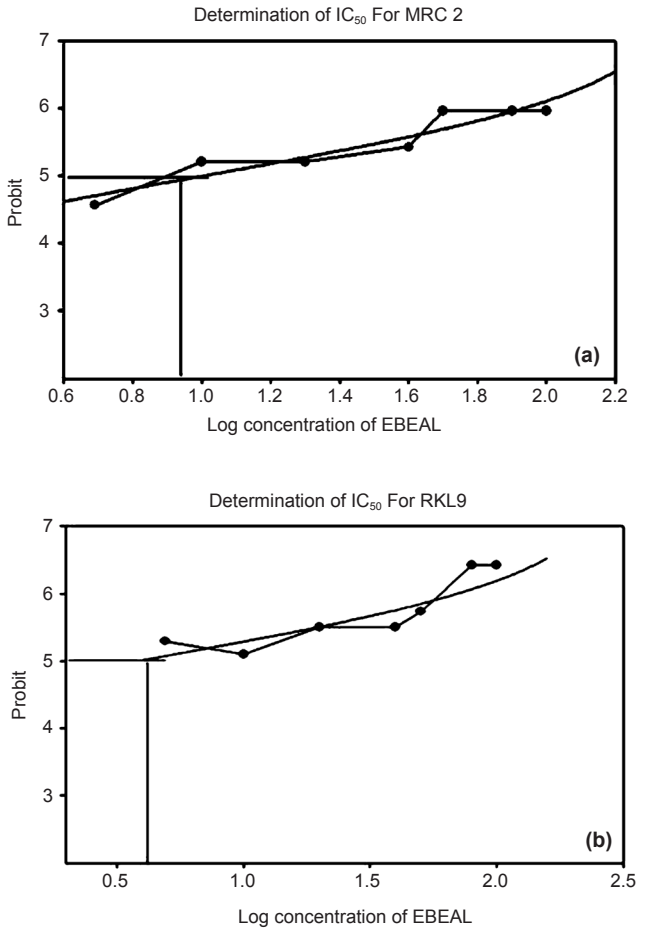

Fig. 1. Plots showing determination of **(a)** IC_{50} of EBEAL on MRC2 strain and **(b)** IC_{50} of EBEAL on RKL9 strain of *Plasmodium falciparum.*

Acute toxicity (LD_{50}): The median lethal dose for EBEAL was determined to be >5 g/kg. No mortality was observed with this concentration during the study period. These observations revealed the safety of *A. lebbeck* as a medicinal plant without severe side effects.

Evaluation of in vivo schizonticidal, repository and curative activities of plant extract: In the early infection, there was a dose-dependent decrease in the levels of parasitaemia on D7 as compared to control (Table) after oral administration of different concentrations of the extract. The standard drug chloroquine caused a chemosuppression of 96.8 per cent, whereas, concentrations of 100, 250, 500, 750 and 1000 mg/kg/day caused chemosuppression of 69.4, 71.4, 71.9, 79.8 and 84.7 per cent, respectively.

KM curve for EBEAL (Fig. 2) denotes survival as a function of time. The steep decline in the curve for G1 indicated that the cumulative probability of survival decreased from 0.6 to 0 by day 10 indicating poor prognosis from disease. Maximum censored observations (2) in G2, G3 and G6 during the experimental study showed the survival of mice beyond the follow up period. However, 50 per cent of mice of G5 survived till 28 days in contrast to mice of G6 which died between 20-21 days suggesting that concentrations of the extract higher than this might be toxic to decrease the survival of mice. This was comparable to mice of positive control (G7) where three mice survived beyond 28 days as supported by cumulative survival probability.

EBEAL produced significant (P<0.001) repository activity. Maximum chemosuppression was observed in G3 (94.9%) which was greater than that for standard drug pyrimethamine (71.2%). Low parasitaemia was recorded in all the extract-treated groups on D7. G4 (500 mg/kg) exhibited minimum parasitaemia of 0.3 per cent whereas maximum parasitaemia was observed to be 1.6±0.9 per cent in G2 (Table).

In established malaria infection, EBEAL exhibited dose-dependent reduction in parasitaemia levels on D7. Low parasitaemia was recorded in all the extract-treated groups on D28. The average chemosuppression values observed in extract-treated groups were 45.2, 50.9, 51.6 and 74 per cent on D7.

Discussion

In traditional medicine, whole plants or mixtures of plants are used rather than isolated compounds. There is evidence that crude plant extracts often have greater *in vitro* or/and *in vivo* antiplasmodial activity than isolated constituents at an equivalent dose[21].

Phytochemical analysis is required for all plant materials being evaluated, test materials and controls, to establish their nature, and hence the fundamental

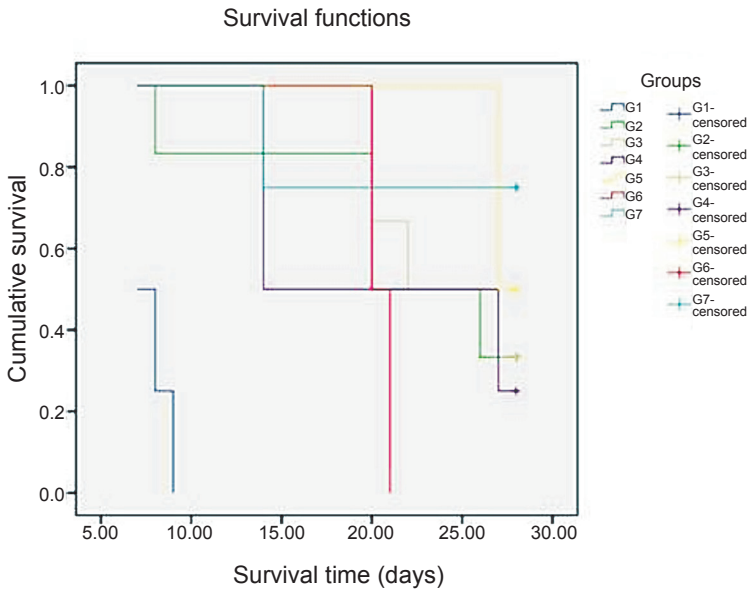

Fig. 2. Plot showing Kaplan-Meier curve for schizonticidal activity of mice of all the groups.

validity and reproducibility of the experiments[22]. Medicinal plants contain some organic bioactive substances[23] which have therapeutic value. In our earlier studies, *Xanthium strumarium* and *Ajuga bracteosa* plant extracts have been found to possess promising *in vitro* as well as *in vivo* antiplasmodial activities[24,25]. EBEAL contained alkaloids, phenols, flavonoids, saponins, phytosterols and terpenes as reported earlier[6]. The presence of alkaloids may be responsible for antimalarial activity of this plant. There are reports of flavonoids being promising antiplasmodial compounds within clinically tolerant and non-toxic concentrations[26] owing to their anti-inflammatory and antioxidant activities. Terpenoids, saponins and phenols exert antimicrobial properties and together with alkaloids in synergistic manner inhibit growth of the pathogens[27].

EBEAL showed CC_{50} of >1000 µg/ml depicting safety of the extract[19] *in vitro* against human *HeLa* cell line. Fulfilling WHO criteria, EBEAL can be categorized as a promising antimalarial as *in vitro* antimalarial screening showed IC_{50} of 8.2 and 5.1 µg/ml against MRC2 and RKL9 strains of the parasite, respectively. To estimate the potential of extracts or molecules to inhibit parasite growth without toxicity, selectivity index was introduced. Therefore, the extract was classified as active exhibiting selectivity indices of >121.9 and >196.07, respectively, according to classification given by Valdes *et al*[20]. Selectivity indices indicate that antiplasmodial activity is probably due to activity against parasite rather than due to cytotoxicity.

The median lethal dose for EBEAL was determined to be >5g/kg which pointed towards good safety profile of *A. lebbeck* in the rodents. According to Munoz *et al*[28], *in vivo* antiplasmodial activity can be categorized as moderate, good and very good if the extract displays a per cent growth inhibition equal to or greater than 50 per cent (ED_{50}) at a dose of 500, 250 and 100 mg/kg, respectively. Based on this classification, EBEAL displayed good antimalarial activity which was evident by maximum chemosuppression of 84.7 per cent at 1000 mg/kg/day concentration of the extract in a dose-dependent manner. However, the standard drug chloroquine caused a chemosuppression of 96.8 per cent on D7. This antimalarial activity might be attributed to the presence of alkaloids or flavonoids in this plant; or even a combined action of more than one secondary metabolites.

For the evaluation of repository activity, the standard drug pyrimethamine (1.2 mg/kg) was used as reference drug. It is an antifolate and prevents DNA replication of parasite by binding to dihydrofolate reductase (DHFR), which interferes with the folic acid mechanism necessary for DNA and RNA synthesis in parasite leading to its death[29]. EBEAL produced considerable repository activity. The extract was found to exhibit 94.9 and 76.2 per cent chemosuppression at concentrations of 500 and 750 mg/kg, respectively, which was greater than that for standard drug pyrimethamine (71.2%). G4 exhibited minimum parasitaemia (0.3%) in contrast to pyrimethamine (1.7±0.2%) on D7. In curative study also, dose-dependent reductions in parasitaemia on D7 were observed. Even after the follow up period, low parasitaemia of 7.2 ±2.1 per cent was observed in G2 supporting the antimalarial potential of the extract in established infection of *P. berghei*.

In conclusion, the findings of the present study exhibited significant antiplasmodial activity of *A. lebbeck* which should be exploited as a potential source of useful antimalarial drug in the future.

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***Conflicts of Interest*:** None.

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