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ANTIMALARIAL ACTIVITY OF *ALBIZIA LEBBECK* BENTH AGAINST CHLOROQUINE SENSITIVE *PLASMODIUM FALCIPARUM* 3D7 STRAIN

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ABSTRACT

The present study aimed to evaluate the antiplasmodial activity of medicinal plant *Albizia lebbeck* against chloroquine (CQ)-sensitive *Plasmodium falciparum* 3D7 strain and cytotoxicity against THP-1 cell line. The plant *Albizia lebbeck* was collected from Acharya Nagarjuna University Campus, Nagarjunanagar, Guntur district, Andhra Pradesh, India. Crude extracts from dried leaves, leaf petiole and stem bark of *Albizia lebbeck* was prepared through soxhlet extraction using methanol, ethyl acetate and aqueous sequentially. These extracts were tested *in vitro* against laboratory adopted *P. falciparum* 3D7 strain. The crude extracts were also tested for their cytotoxicity against THP-1 cell line. The phytochemical screenings were also conducted with standard methods. The methanol extract of leaf and leaf petiole; ethyl acetate extract of leaf and stem bark showed IC_{50} values from 5-50 µg/mL with good antimalarial activity and were significant at $P < 0.05$ and < 0.001. The ethyl acetate extract of leaf petiole and the aqueous extract of stem bark showed IC_{50} values ranging from 50 to 100 µg/mL which shown mild activity. Out of all the extracts, the ethyl acetate extract of leaf (IC_{50} = 19.22 µg/mL) has shown excellent antimalarial activity. The aqueous extract of leaf and leaf petiole; methanol extract of stem bark were inactive. All the extracts were non toxic to THP-1 cells. The phytochemical screening has revealed the presence of alkaloids, triterpenes, flavonoids, tannins, coumarins, carbohydrates, phenols, saponins, phlobatannins and steroids. It is concluded that the Ethyl acetate extract of leaf is potent for the development of antimalarial drugs.

KEYWORDS: *Albizia lebbeck*, antiplasmodial activity, IC_{50}, cytotoxic activity, selectivity index.

INTRODUCTION

Malaria is a curable, preventable and oldest recorded disease can be found even in ancient Indian medical literature like Charaka Samhita. The name malaria was originated from Italian words “mal” and “aria” which means bad air.[1] Malaria is very risky parasitic disease caused by protozoan parasites *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale* and the parasite has transmitted from human to human by the bite of infective *Anopheles* mosquito.[2] Each year 300 to 500 million new cases are being diagnosed and nearly 1.5 million people died; mainstream of deaths reported from Sub Saharan African countries, the majority of them were children Less than 5 years and pregnant women. [3] Malaria has an enormous impact on child health in malaria endemic countries and contributes to illness, respiratory infection, diarrhoeal disease and malnutrition.[4]

The prevalence of malaria increased in 1980s and 1990s as the parasites developed resistance to the most frequently used antimalarials and the vectors became resistance to insecticides.[5] The first effective drug is chloroquine and its resistance was reported in 1957, consequently distributed all over the world and reported from India in 1976.[6,7] Now artemisinin and its derivates are used as first line treatment according to World Health Organization Proceedings of Malaria Treatment. Unfortunately artemisinin-resistant strains has been reported from Thai- Cambodia in 2009 and hasten the need for new Antimalarials.[8]

Historically and traditionally plant parts have always been used as an important source in the medicine against malaria. About 30% of the world drug sales are based on natural products. It is estimated that there are about 2, 50,000 species of higher plants throughout the world, and most of them have not been examined in detail for their pharmacological activities.[9] Most effective antimalarial drugs such as chloroquine, quinine and artemisinin are derived from plants. The first effective malarial drug quinine was extracted from Cinchona tree; based on this structure chloroquine and primaquine were synthesized. The other effective drug artemisinin was extracted from Chinese herbal tree *Artemisia annua* in 1972[10] Artemisinin and its derivatives are now

recommended by World Health Organization (WHO) worldwide, in combination with other drugs such as lumefantrine, amodiaquine, mefloquine, sulphadoxine-pyrimethamine (SP) as the first line treatment of malaria.[11] This fact has encouraged the continuing search for new natural product-derived antimalarial drugs. Several plants are used in traditional medicine for the treatment of malaria and fever in malaria endemic areas.[12]

The present plant *Albizia lebeck* Benth is a species of flowering tree in the pea family belonging to the family Mimosoideae and is a large tree that grows in large size. It is widely distributed in India and is also found in South Africa and Australia. Barks are used in toothache, piles, diarrhoea and diseases of gum. Decoction of the leaves and barks are protective against bronchial asthma and other allergic disorders.[9] The present study evaluated the antiplasmodial activity of methanol (MeOH), ethyl acetate (EtOAc) and aqueous (Aqus) extracts from leaves, leaf petiole and stem bark of *Albizia lebbeck* Benth.

MATERIALS AND METHODS

Plant Collection

Fresh samples of leaves, leaf petiole and stem bark from *Albizia lebbeck* were collected from ANU campus, Nagarjunanagar of Guntur district, Andhra Pradesh, India. The plant *A. lebbeck* were deposited in the Department of Botany, Acharya Nagarjuna University and voucher specimen was deposited in the department. All the collected plant parts were washed thrice with tap water and twice with distilled water to remove the adhering salts and other associated animals. The authentication of the plant species were done by Prof. K. Khasim, Department of Botany, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.

Extract preparation

Shade-dried plant samples were subjected for in 90% different organic solvents methanol, ethyl acetate and water at 50-60°C in a Soxhlet apparatus. After complete extraction, the filtrates were concentrated separately by rotary vacuum evaporation (>45°C) and then freeze dried (−80°C) to obtain solid residue. The extraction percentage was calculated by using the following formula:

$$\text{Percentage of extraction} = \frac{\text{weight of the extract (g)}}{\text{weight of the plant material (g)}} \times 100$$

The extracts of plant were screened for the presence of phytochemical constituents by following the method of Sofowora (1982) and Kepam (1986).[13,14] The plant extracts were dissolved in dimethyl sulphoxide and filtered through millipore sterile filters (mesh 0.20 µm, Sartorious Stedim Biotech GmbH, Germany). The filtrate was used for testing at different concentrations of 200, 100, 50, 25 and 12.5 µg/mL. [15]

Parasite cultivation

The antiplasmodial activity of plant extracts was screened against CQ-sensitive *P. falciparum* 3D7 strain obtained from ongoing cultures in the laboratory. They were cultured according to the method of Trager and Jenson (1976)[16] in candle jar desiccator. *P. falciparum* were cultivated in human O^{Rh+} red blood cells using RPMI 1640 medium (Sigma Laboratories Private Limited, Mumbai, India) supplemented with O^{Rh+} serum (10%), 5% sodium bicarbonate and 50 µg/mL of gentamycin sulfate. Hematocrits were adjusted at 2% and cultures of parasite were used when they exhibited 2% parasitemia.[17]

In vitro antimalarial screening

The *P. falciparum* malaria parasite culture suspension of 3D7 (synchronized with 5% sorbitol to ring stage) was seeded (200 µL/well) in 96 well tissue culture plates. Plant extracts were added in µL/well to get different concentrations of extract (200, 100, 50, 25, 12.5 µg/mL). Chloroquine treated parasites were kept as positive controls and DMSO treated parasites were kept as negative control. The parasites were cultured for 30 h in candle jar desiccator. The cultures were incubated at 37°C for 48 hours in an atmosphere of 2% O_2, 5% CO_2 and 93% N_2. 18 h before termination of the assay [^{3}H] Hypoxanthine (0.5 µCi/well) was added to each well of 96 well plate. The effects of extracts in the cultures were evaluated by the measurement of [^{3}H] Hypoxanthine incorporation into the parasite nucleic acids.[18] Each treatment has four replicates; at end of experiment one set of the pRBC cells collected from wells and smears were prepared. These smears were fixed in methanol and air dried. The smears were stained with Acradine Orange (AO) stain. Stained smears were observed under UV illumination microscope (Carl Zeiss - Germany) for confirmation of [^{3}H] data of Hypoxanthine assay, remaining other three replicates were used for [^{3}H] Hypoxanthine assay. The experiment was terminated and the cultures were frozen and stored in -20^{0}C. The parasites were harvested on glass filter papers using NUNC Cell Harvester and CPM counts were recorded in gamma scintillation counter. Control readings were considered as 100% parasite growth and calculated the parasite inhibition in plant extract treated wells. The parasite inhibition was calculated as follows.

$$\% \text{ Inhibition} = \frac{\text{Average CPM of Control} - \text{Average CPM of plant extracts}}{\text{Average CPM of Control}}$$

The IC_{50} values were determinate by plotting concentration of extract on X-axis and percentage of inhibition on Y-axis with dose-response curves using Minitab 11. 12. 32. Bit software.

Cytotoxicity of extracts on THP-1 monocyte cells

The assays were carried out using 96-well flat-bottom tissue-culture plates. Cytotoxic properties of active plant extracts were assessed by functional assay [19] using THP-1 cells. The cells were cultured in RPMI-1640

medium which contained 10% fetal bovine serum, 0.21% sodium bicarbonate (Sigma), and 100 µg/mL penicillin and 50 µg/mL gentamicin (complete medium). Briefly, cells (0.2×10^6 cells/200 µL/well) were seeded into 96-well flat-bottom tissue-culture plates in complete medium. Drug solutions (200, 100, 50, 25 and 12.5 µg/mL) were added after 24 h of seeding and incubated for 48 hours in a humidified atmosphere at 37°C and 5% CO_2. DMSO as negative inhibitor ellipticine as a positive inhibitor was added to each well. At end of experiment ten micro liters of a stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 µg/ml in 1x phosphate-buffered saline) was added to each well, gently mixed, and incubated for another 4 h. After spinning the plate was centrifuged at 1,500 RPM for 5 min, the supernatant was discarded, subsequently added 100 µL of DMSO (stopping agent). After formation of formazan was read on a micro titer plate reader (Versa max tunable multiwell plate reader) at 570 nm and the percentage of cell viability calculated using the following formula.[20] The selectivity index of *in vitro* toxicity was calculated for each extract as the IC_{50} for THP-1 cells / IC_{50} for *P. falciparum.*

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

The IC_{50} values were determinated by plotting concentration of extract on X- axis and percentage of cell viability on Y- axis with dose response curves using Minitab 11. 12. 32. Bit software.

RESULTS

The phytochemical studies revealed that the methanol ethyl acetate and aqueous extracts of leaf, leaf petiole and stem bark of *A. lebbeck* have variety of phytochemical constituents namely alkaloids, triterpenes, flavonoids, tannins, coumarins, carbohydrates, phenols, saponins, phlobatannins and steroids represented in Table 1.

Table 1: Preliminary phytochemical screening of *Albizia lebbeck* in different extracts from leaves, leaf petiole and stem bark.

Tested compounds	Leaves			Leaf Petiole			Stem Bark		
	Me	EtAc	Aqs	Me	EtAc	Aqs	Me	EtAc	Aqs
Alkaloids	+	-	+	+	+	-	-	+	+
Coumarins	+	-	+	-	+	-	-	-	-
Carbohydrates	-	-	+	-	-	+	-	-	-
Phenols	+	-	-	-	+	-	-	+	-
Saponins	-	-	+	-	-	-	+	+	-
Tannins	-	+	-	-	-	-	+	-	-
Flavanoids	+	-	+	+	-	+	-	-	+
Terpenoids	-	+	-	+	-	-	+	+	-
Phlobatannins	-	-	-	+	+	+	+	-	-
Steroids	-	+	-	+	-	-	-	+	-

Me= Methanol; EtAc= Ethyl Acetate; Aqs= Aqueous
+ Present, - Absent

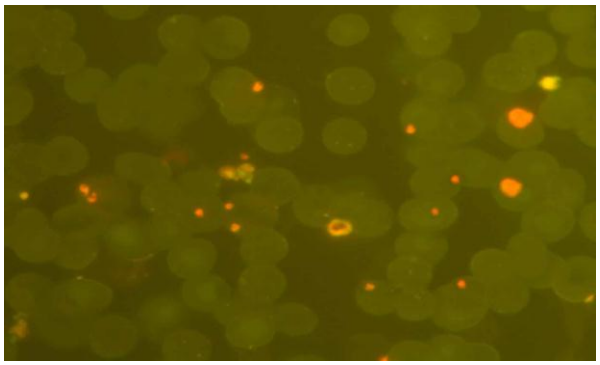
In the present study, crude extracts of methanol, ethyl acetate and aqueous from leaves, leaf petiole and stem bark of *A. lebbeck* were evaluated for their antimalarial potencies. The IC_{50} values of the tested plant extracts against *P. falciparum* are listed in Table 2. The *in vitro* antiplasmodial activity of biological active substances is categorized into four groups basing on IC_{50} value (<5 µg/mL-very active, <50 µg/mL-active, 50-100 µg/mL-weakly active, >100 µg/mL inactive).[21] Based on this categorization, the IC_{50} values of all the crude extracts of leaves, leaf petiole and stem bark (excepted aqueous extract of leaf and methanol extract of stem bark) of *A. lebbeck* showed a range of inhibitory concentrations against CQ-sensitive *P. falciparum* 3D7 strain. The methanol extract of leaf (33.45 µg/mL) and leaf petiole (41.00 µg/mL); the ethyl acetate extract of leaves (19.22 µg/mL) and stem bark (33.30 µg/mL) showed good antimalarial activity and are significant at $P<0.05$ and $P<0.001$. The ethyl acetate extract of leaf petiole (58.10µg/mL) and aqueous extract of stem bark (65.31 µg/mL) shown moderate antimalarial activity. Among these extracts, the ethyl acetate extract of leaves showed excellent antimalarial activity (IC_{50} = 19.22 µg/mL). The aqueous extracts of leaves and leaf petiole; methanol extracts stem bark shown inactive antimalarial activity with IC_{50} values >100 µg/mL.

The *in vitro* cytotoxicity studies against THP-1 cell line were conducted for all the extracts. All extracts showed IC_{50} value >200 µg/mL. An extract is classified as non toxic when the IC_{50} value is >20 µg/mL. Based on this, all the plant extracts are not harmful to *in vivo* studies. The selectivity indices indicate the low toxicity of tested extracts and safer for therapies (Table 2).

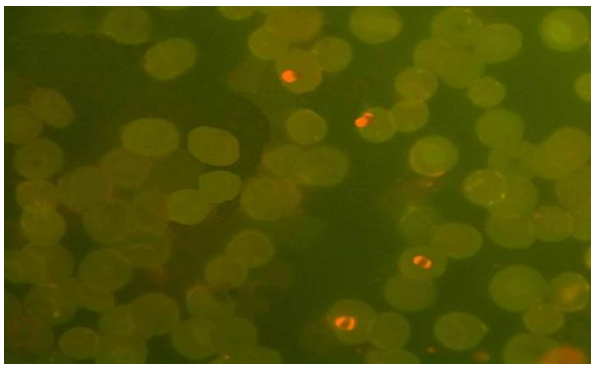
Table 2: Antiplasmodial activity against CQ-sensitive *P. falciparum* 3D7 strain and cytotoxicity against THP-1 cell line of different crude extracts from *Albizia lebbeck*.

Plant parts	Crude Extracts	% of Yield	IC_{50} 3D7 strain (µg/mL)	IC_{50} THP-1 cells (µg/mL)	SI
Leaves	Methanol	12.11	33.45±1.44	>200	>6.06
	Ethyl Acetate	3.23	19.22±3.44	>200	>10.52
	Aqueous	2.20	>100	>200	>2.00
Leaf petiole	Methanol	8.14	41.00±5.42	>200	>4.87
	Ethyl Acetate	2.16	58.10±1.19	>200	>3.44
	Aqueous	6.81	47.23±3.51	>200	>4.25
Stem Bark	Methanol	5.47	>100	>200	>2.00
	Ethyl Acetate	3.78	33.3±4.43	>200	>6.06
	Aqueous	4.15	65.31±7.18	>200	>3.07

Values are represented as mean±standard deviation, SI- selectivity index, $SI_{Plasmodium} = IC_{50\ THP\text{-}1}/ IC_{50\ P.\ falciparum\ 3D7}$



Control (Positive)



Leaf extract treated

Fig 1: Micrograhs of synchronized ring stage *P.f*3D7 culture treated with ethyl acetate leaf extract (200 mg/µL) of *Albizia lebbeck* for 48 h showing inhibition of ring stages

DISCUSSION

Malaria is still the most dangerous parasitic infectious disease which causes two million deaths every year. It is a great burden to developing nations, a number that could rise due to the increasing multi-drug resistance to all antimalarial drugs currently available.[22] There are several genetic polymorphisms identified in *P. falciparum* and *P. vivax* that can be providing reliable data about the prevalence of drug resistance. Amongst all, the pfcrt, pfmdr1, pfdhfr and pfdhps associated with drug sensitivity, have great role in drug resistance mechanisms in parasites and is directly connected to treatment failure.[23]

From the past 20 years, many strains of *P. falciparum* have become resistant to chloroquine and other anti-malarial drugs. The development and spread of drug resistant strains of *P. falciparum* has limited effectiveness to the currently used malarial drugs. In view of this fact, the emergence and spread of parasites resisting to antimalarial drugs has caused an urgent need for novel effective alternative antimalarial drug compounds to be discovered and developed with minimal side effects.[24]

Plants have over the years, proved to be a good source of chemotherapeutic agents. Today, many of the drugs have been derived from plants resources such as quinine, chloroquine and artemisinin. Historically, medicinal plants have provided a source of inspiration for novel therapeutic drugs, as plant-derived medicines have made large contributions. According to WHO, now a days, 80% of the world's population rely on plants for their primary health care.[25] Plants are producing secondary metabolites for their defense, which play an important role of physiological activities in human body.[26] The medicinal value of plants is due to the substances that it contains, which produce a physiological action on the human body. Some examples of these plants are alkaloids, essential oils, tannins, resins and many others.[27] India had remarkable biodiversity and rich cultural traditions of plant use. Interestingly, today many of the pharmaceutical companies are utilizing such plant-based formulations in treatment of various diseases and disorders worldwide.[28]

The present investigation has evaluated the *in vitro* antiplasmodial activity of *A. lebbeck* with different extracts such as methanol, ethyl acetate and aqueous form of leaves, leaf petiole and stem bark. According to Rasoanaivo et al. (1992),[21] the *in vitro* antiplasmodial IC_{50} results classifies the biological active substances into four groups (<5 µg/mL-very active, <50 µg/mL-active, 50-100 µg/mL-weakly active, >100 µg/mL-inactive). Based on the hypothesis, out of the 9 extracts tested, five showed good (IC_{50} = 19.22-47.23 µg/mL), two exhibited moderate (IC_{50} = 58.10-65.31.98 µg/mL), while two displayed inactive (IC_{50} = >100 µg/mL) antiplasmodial activity as shown in Table 1. Previously

Al-Musayeib et al. reported antimalarial activity of same plant against CQ-sensisitive strain of *P. falciparum* (3D7)[29].

The *A. lebbeck* has been used in traditional medicine for treatment of leprosy, dysentery, bronchitis, asthma, leucoderma, muscular tremors and rheumatism. It has been proved to possess anthelmintic, antibacterial, antifungal, antiviral and anticancer activities.[9] Albizia spices have also been used in indigenous system of medicine as a folk medicine for various ailments. The bark is dry, acrid, bitter with sharp taste; antihelmintic tonic; cures leprosy, dysentery, asthma, leucoderma, piles, tremors of the muscle and wandering of the mind. The flowers are pounded mixed with sugar and used during pregnancy as safeguard against miscarriage. The ashes of the bark are rubbed over the skin to remove hair. The smoke of the leaves is good for eye troubles. Fresh leaf juice mixed with lemon juice is used for dyspepsia; extract of crushed pods is used for ear ache, tooth ache, pain relief from fractured bones. Aqueous extract of bark and leaves applied externally to treat skin disease disinfects wounds and promotes healing.[29]

The *in vitro* antiplasmodial activity of the *A. lebbeck* ethyl acetate leaf extract may be due to the presence of major chemical classes such as phenols and alkaloids. Hence alkaloids are strong antiplasmodial compounds. Except the alkaloids, the major chemical classes such as coumarins, phenols, polysaccharides and flavanoids also exerted strong antiplasmodial activities.[30] Bandaranayake (2002)[31] reported about the bioactive compounds and chemical constituents of mangrove plants. Some of the traditional medicine involves the use of crude plant extracts which may contain an extensive diversity of molecules, often with indefinite biological effects. However, most of the available information regarding the medicinal potency of these plants is not provided with credible scientific data. For this reason, several researches have been reported the toxicity of medicinal plants.[32] In the present study, the *in vitro* cytotoxic effect against THP-1 cell lines showed IC_{50} >200 µg/mL. According to Falade et al., the ctyotoxicity >20 considered as non toxic to animals and safer for further studies. Thus, all the plant extracts are not harmful and safer for therapies.[33] Similarly, Bhagavan et al. reported the antimalarial activity of methanol, hexane, chloroform, acetone, and ethyl acetate extracts of *Citrus sinensis* (peel), *Leucas aspera*, *Phyllanthus acidus* (leaf), *Ocimum sanctum*, *Terminalia chebula* (seed) against CQ-sensitive (3D7) strain of *P. falciparum* and tested for their cytotoxicity on human laryngeal cancer cell line (HEp-2) and normal cell line (Vero).[34] Out of the 25 test extracts, they have concluded the leaf methanol and ethyl acetate extracts of *L. aspera*; ethyl acetate, methanol, and acetone extracts of *P. acidus*; and seed acetone extract of *T. chebula* had good antiplasmodial activity (IC_{50} ranged from 4.76 - 22.76 µg/mL) with selectivity indices ranged from 2.04- 9.97 for HEp-2 and >2 to <12 for Vero cells, respectively. The mechanism of action might be due the inhibition of hemozoin biocrystallization by the alkaloids and inhibition of protein synthesis by triterpenoids (Samuel et al., 2012).[35] Additional *in vitro* and *in vivo* work aimed at understanding the mechanisms of action of the active plant extracts, isolating and characterizing the bioactive constituents is underway in our laboratories and will be reported in due course of time.

CONCLUSION

In conclusion our shows that the ethyl acetate extract of leaves of *A. lebbeck* exhibits good *in vitro* antiplasmodial activity against CQ-resistant strain of *P. falciparum*. Further evaluation of the extract may provide potential molecule for therapy of malaria.

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