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EVALUATION OF IN VITRO ANTIMALARIAL ACTIVITY OF *CALOTROPIS GIGANTIA AND CITRUS AURINTOFOLIA* FLOWER EXTRACT AGAINST *PLASMODIUM FALCIPARUM 3D7*

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ABSTRACT

The alarming rate at which *Plasmodium falciparum* has developed resistance to chloroquine and other synthetic antimalarial drugs makes it necessary to search for more effective antimalarial compounds. In the present investigation the *in vitro* antiplasmodial activity of flower extract from *Calotropis gigantia* (Asclepiadaceae) and *Citrus aurintofolia* (Christm.) Swingle (family Rutaceae) was evaluated against chloroquine sensitive *Plasmodium falciparum* 3D7 strain at 24 h of incubation by light microscopy. The methanolic extract of both the plants *C. aurintifolia* and *C. gigantia* exhibited higher antimalarial activity (IC_{50} ranged between 47.64 and 117.52 µg/mL) as compared to aqueous extract (IC_{50} ranged between 71.31 to 162.63 µg/mL) respectively. The toxicity potentiality exhibited by flower extract was very negligible or did not show any apparent toxicity as compared to control. Future work should aim to discover the anti-malarial substances present to improve traditional use of these plants for malaria treatment.

KEYWORDS: Malaria, *Calotropis gigantia, Citrus aurintofolia, Plasmodium falciparum, antimalarial.*

INTRODUCTION

Malaria remains a major public health problem, especially in tropical and subtropical regions because of widespread antimalarial drug resistance.[1] Malaria is responsible for 500 million new cases and 2 to 3 million deaths every year, mostly among children under five years and pregnant women.[2] *Plasmodium falciparum* the most widespread etiological agent for human malaria has become increasingly resistant to standard anti-malarials. Consequently, new drugs or drug combinations are urgently needed today for the treatment of malaria.[3]

Medicinal plants are cheap and renewable sources of pharmacologically-active substances Plant extracts are still widely used in the treatment of malaria and other ailments and up to 80% of the world population use traditional medicines for their primarily health care.[4] Since little scientific data exist to validate antimalarial properties of these medicinal plants, it is important that their claimed antimalarial properties are investigated, in order to establish their efficacy and determine their potential as sources of new antimalarial drugs.[5]

In the present investigation flowers of *Citrus aurantifolia* (Christm.) and *Calotropis gigantia* were tested against *Plasmodium falciparum* 3D7 strain by in vitro method. Both these plants are widely distributed in India and known for its therapeutic effectiveness in a variety of diseases in traditional medicine. Furthermore, this plant is among the frequently used medicinal plant with antimalarial properties in Nigeria.[6] Lime extract of Citrus has an antimicrobial activity against upper respiratory tract bacterial pathogens.[7] The roots of *C. aurantifolia* used in traditional medicine for the treatment of fever. A wide range of bioactive ingredient from the citrus species has been found to exert anti-infection and anti-inflammatory activities.[8] The plant has shown anti-allergic, anti-inflammatory, anti-cancer, anti-bacterial and anti-protozoal properties.[9] Several anti-oxidant products have also been identified in the seeds of Citrus plant.[10]

Calotropis gigantia (Asclepiadaceae) is a plant widely distributed in tropical and subtropical regions of Africa and Asia with a long history of use in traditional medicine. A wide range of chemical compounds including cardiac glycosides, flavonoids, phenolic compounds, terpenoides have been isolated from this species.[11] The plant from this species are commonly known as milkweeds because of the latex they produce. *C. gigantea* is reported to exhibit mosquito controlling properties against *Culex gelidus* and *Culex tritaeniorhynchus* mosquitoes.[12] However, scientific information on the antimalarial activity from this plant has not been documented.

In this study we explored the in vitro antimalarial potential of flower extract of plant *Calotropis gigantia* and *Citrus aurintifolia* against *Plasmodium falciparum* 3D7 chloroquine sensitive strain.

Plant collection and Identification

Plant *Calotropis gigantia* (Asclepiadaceae) was collected from district Rajsamand located between latitudes 24˚46′ to 26˚01′ N and Longitudes 73˚28′ to 74˚18′ N of the state

Rajasthan, India. And *Citrus aurantifolia* (Christm.) Swingle (family Rutaceae) was collected from Mumbai (18º 55´ N, 72º 54´ E) India.

Identification of plant: The plants were identified by Dr. U. C. Bapat, Head, Department of Botany and Director, Blatter Herbarium, St.Xavier's College, Mumbai. The sample voucher specimen of *Calotropis gigantia* (Accession No. NI 1718) and of *Citrus aurantifolia* (Accession No. R2399) was deposited in the Blatter herbarium.

Extraction of plant material

Dry flowers were ground to coarse powder. Methanol and aqueous extracts were prepared and solvents were removed under reduced pressure by rotary vacuum evaporator.[13] The percentage yield for each sample was determined and the crude extracts were stored at 4^0C until their use in antimalarial assay.

***Plasmodium falciparum* culture and maintenance**

The *Plasmodium falciparum* 3D7 strain was procured as a generous gift from Indian Institute of Technology (IIT), Mumbai and was maintained in continuous culture by the modified method of Jensen and Trager.[14] With backup stored in liquid nitrogen. Culture consist of 5% hematocrite suspension of O+ human erythrocytes in RPMI 1640 medium (Gibco-BRL) supplemented with L-glutamine (4.2mM), HEPES (25 mM), $NaHCO_3$ (25 mM) hypoxanthine (6.8 M), 0.5% AlbumaxII (Invitrogen) and 50µg/ml Gentamicin. Cultures were incubated at 37^0 C in an atmosphere of 5% CO_2, 91% N_2, and 3% O_2.

***In vitro* anti-plasmodial assay**

The in vitro activity of flower extracts of both the plants were evaluated against *P. falciparum* by means of the Mark III test, as developed by the WHO.[15] Briefly, the parasite cultures, prior to experimentation, were synchronized to the ring stage by treatment with 5% D-sorbitol.[16] The two fold serial dilutions of the extracts were prepared in duplicate in 96 well flat bottomed micro plates. Solvent control of 0.5% DMSO and 0.1% ethanol to check the effect of solvents on parasite maturation. Filter sterilized extracts (100, 50, 25, 12.5, 6.25 and 3.125 µg/mL) were incorporated in 96 well tissue culture plate containing 200 µL of P. falciparum culture with 2% haematocrit and 1% ring stage infected erythrocytes (parasitemia). The plates were incubated at 37^0 C in CO_2 incubator for 24 h. Parasitemia was evaluated after24 h by light microscopy using Giemsa-stained smears as described by Le Bras and Deloron.[17] The antiplasmodial activities of extracts were expressed by IC_{50}, the

inhibitory concentrations of the drug that induced 50% reduction in parasitemia compared to control (100% parasitemia).

Brine shrimp toxicity bioassay

Inhibition of the growth of brine shrimps (*Artemia salina*) was used as a measure of the toxicity of the extract using brine shrimp lethality bioassay.[18] The eggs of the brine shrimp were collected from an aquarium shop (Mumbai, India) and hatched in artificial sea water (38g/lit saline) under constant aeration for 48 h. After hatching active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the bioassay. Extracts were dissolved in DMSO and then serially diluted in artificial sea water to the desired concentrations. The final DMSO concentration did not exceed 1%, which was shown not to have any harmful effect on the larvae. About 0.5 ml of diluted test solution was added to the pre marked test tubes containing 4.5ml of artificial sea water. Finally 10 active shrimps were added into each test tube. A vial containing 50µl DMSO diluted to 5 ml was used as control. Potassium permanganate (LC_{50} =5 µg /mL) was used as positive control, and vials containing shrimps, but without any extracts were included in each test. After 24 h the numbers of the survivors were counted using a magnifying glass and % mortality was calculated for each dilution as well as for control. The toxicity of each extract was determined from LC_{50} values (Lethal concentration of sample required to kill 50% of brine shrimp) in a set of three test tubes per dose.

Selectivity index (SI) = Ratio LC_{50}/IC_{50}

Chemical injury to erythrocytes

To assess morphological changes in erythrocytes due to extract concentration- RBCs were incubated with the highest concentration of the extract used in the anti-plasmodial assay along with, control uninfected erythrocytes without extract. After 48 hours of incubation, thin blood smears were stained with Giemsa and morphological findings were compared with control.[19]

RESULTS

The present study was investigated with methanol and aqueous extracts derived from flowers of *Calotropis gigantia* and *Citrus aurintifolia*. The basic measurement of antimalarial activity used in this study was the reduction in number of parasitized erythrocytes in extract treated test cultures as compared to control without drug at 24 h of incubation period. The methanolic extract of both the plants *C. gigantia* and *Citrus aurintifolia* exhibited higher

antimalarial actitvity (IC50 ranged between 47.64 and 117.54 µg/mL) as compared to aqueous extract (IC50 ranged between 71.31 to 162.73 µg/mL) respectively. The IC_{50} value of chloroquine was less than 0.36 µg/mL.

The microscopic observation of the uninfected erythrocytes incubated with highest concentration of extracts used in assay as compared to normal erythrocytes without extract showed no morphological difference at 24 h of incubation in both methanol and aqueous extracts. The antiplasmodial activity of both the plants demonstrated decreased in parasitemia with increasing concentrations of the extracts, reflecting an inhibitory activity on the parasite replication.

Table 1: In vitro antiplasmodial activity, cytotoxicity, and selectivity index of two plants against Plasmodium falciparum 3D7.

Plant name	Solvent extract of flowers	Concentration of Extracts (µg/mL)						Antiplasmodial activity IC_{50} µg/mL	Artemia salina LC_{50} (µg/mL)	Selectivity index (SI) (LC_{50}/IC_{50})
		6.25	12.5	25	50	100	200			
		Mean % inhibition of parasitemia								
Citrus aurintifolia	**Methanol**	**12.7**	**27.6**	**45.9**	**69.4**	**88.9**	**100**	**47.64**	**250.28**	**5.25**
	Aqueous	**7.4**	**21.8**	**40.8**	**57.2**	**73.7**	**86.7**	**71.31**	**287.90**	**4.03**
Calotropis gigentia	**Methanol**	**2.1**	**18.9**	**27.5**	**38.9**	**52.1**	**68.5**	**117.52**	**371.62**	**3.16**
	Aqueous	**0.2**	**13.3**	**20.2**	**29.1**	**43.9**	**52.9**	**162.73**	**349.34**	**2.14**

IC_{50}: the inhibitory concentration of extract that induced 50% reduction in parasitemia.

LC_{50}: the lethal concentration that reduced the number of viable shrimp larvae by 50%

Selectivity index (SI) = Ratio LC_{50}/IC_{50}.

Results of cytotoxicity

A brine shrimp cytotoxicity assay was used to predict the potential toxicity of the extracts. The selectivity index (SI) is defined as the ratio of the brine shrimp toxicity to the anti-plasmodial activity and is determined by dividing the LC_{50} values for the brine shrimp nauplii by the IC_{50} value for *Plasmodium falciparum*. The extract with higher selectivity (high SI value) indicate potentially safer therapy. In brine shrimp lethality bioassay, % mortality increased gradually with increase in concentration of the test samples in comparison to positive control KMnO4. The toxicity potentiality exhibited by flower extract was very negligible or did not show any apparent toxicity as compared to control. The results suggest that flower extract of both *Citrus aurantifolia* and *Calotropis gigantia* possesses considerable antiplasmodial activity and negligible cytotoxicity with selectivity index (SI value) ranged from 5.25 to 2.14 (table 1).

DISCUSSION

Crude extracts are the simplest of available medications and are still promoted by WHO policies as emerging alternative systems of medicine to reach the large population not covered by formal medical care in remote areas. Developing countries, where malaria is endemic, depend strongly on traditional medicine as a source for inexpensive treatment of this disease.[20,21] The present study aids important impetus to the area of new antimalarial agents from plants source.[22] Earlier report claims that both these plants are used in traditional medicine and cultivated throughout India.[23] The anti-plasmodial activity from the same genus but different species was reported from *Calotropis procera* and *citrus limon* at higher concentrations.[24,25]

Flower extract of both the plants exhibited promising antimalarial activity. The methanolic extract induced a significant decrease of parasite proliferation. However, aqueous extracts, traditionally used for medication did not show high antimalarial activity. This might have resulted from the lack of solubility of the active constituents in aqueous solution. All the tested extracts were found to be non-cytotoxic or with negligible toxicity on Brine shrimp larvae. This observation may be an indicator of their safety as drugs for mammalian organism. In the best of our knowledge this is the first study of anti-plasmodial activity of flower extract of both the plants. The results are encouraging and further work is needed for complete isolation, identification, characterization to elucidate the active compound.

CONCLUSION

The present study adds important impetus to the area of new antimalarial agents from plants source. The plant extracts however possess moderate antimalarial activity when compared with standard drug chloroquine. The results are encouraging and further work is needed for complete isolation, identification and characterization to elucidate the active compound and their in vivo anti-malarial efficacy in murine malaria model with comprehensive animal toxicity study. To the best of our knowledge no positive reports are available on antiplasmodial activity of both these plant.

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