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ANTIMALARIAL ACTIVITY OF PARTHENIUM HYSTEROPHORUS L AGAINST CHLOROQUINE SENSITIVE PLASMODIUM FALCIPARUM 3D7 STRAIN

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ABSTRACT

The present study aimed to evaluate the antiplasmodial activity of medicinal plant Parthenium hysterophorus against chloroquine (CQ)-sensitive Plasmodium falciparum 3D7 strain and cytotoxicity against THP-1 cell line. The plant P. hysterophorus was collected from Acharya Nagarjuna University Campus, Nagarjunanagar, Guntur district, Andhra Pradesh, India. Crude extracts from dried leaves, stem bark and root of P. hysterophorus was prepared through soxhlet extraction using methanol, ethyl acetate and chloroform 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, stem bark and root; ethyl acetate extract of leaf and root showed IC_{50} values from 5-50 µg/mL with good antimalarial activity and were significant at $P < 0.05$ and < 0.01. The ethyl acetate extract of stem bark and the chloroform 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 methanol extract of leaf (IC_{50} = 12.15 µg/mL) has shown excellent antimalarial activity. The chloroform extracts of leaf and root were inactive. All the extracts were non toxic to THP-1 cells but methanol extract of leaf (IC_{50} = 11.50 µg/mL) and methanol extract of root (IC_{50} = 10.23 µg/mL) shows cytotoxicty against THP-1 cell lines. The phytochemical screening has revealed the presence of alkaloids, triterpenes, flavonoids, tannins, coumarins, carbohydrates, phenols, saponins, phlobatannins and steroids. It is concluded that the further work will be essential to methanol extract of leaves for the development of antimalarial drugs due to slight cytotoxicity.

KEYWORDS: Parthenium hysterophorus, 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 under 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 antimalarial.[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.[12,13,14]

The present plant P.hysterophorus L is a species of flowering plant belonging to the family Asteraceae. It is a fast maturing annual herb with a deep tap root and may eventually reach a height of 2 m. The seed of parthenium mainly disperses through flooding, water currents, movement of vehicles, machinery, livestock, grain, fodder, and to a lesser extent by wind .P. hysterophorus L. is originated as a result of natural hybridization between Parthenium confertum and Parthenium bipinnatifidum. It adapts various agro-climatic conditions and almost distributed itself to variety of growing environmental conditions [15]. Some of the countries where this plant is distributed includes: India, China, Taiwan, Pakistan, Nepal, Sri Lanka, Bangladesh, Vietnam, Pacific Islands, Ethiopia, Kenya, Madagascar, South Africa, Somalia, Mozambique, Zimbabwe and several countries of South and Central America . This noxious weed is known for its adverse effect on environment, biodiversity, agriculture, and health of animals and human beings. This is because of the toxic chemicals produced by parthenium plant however some medicinal uses reported previously.[16] The present study evaluated the antiplasmodial activity of methanol (MeOH), ethyl acetate (EtOAc) and chloroform (CH) extracts from leaves, stem bark and roots of P. hysterophorus L

MATERIALS AND METHODS

Plant Collection

Fresh samples of leaves stem bark and root from P. hysterophorus were collected from ANU campus, Nagarjunanagar of Guntur district, Andhra Pradesh, India. The plant P.hysterophorus was 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 chloroform 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).[17,18] 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 100, 50, 25, 12.5, 6.25 µg/mL.[19]

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)[20] in candle jar dissector. 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.[21]

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 (100, 50, 25, 12.5, 6.25 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.[22] 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] Hypoxanthine assay data, 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 carrying out using 96-well flat-bottom tissue-culture plates. Cytotoxic properties of active plant extracts were assessed by functional assay [23] 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 (100, 50, 25, 12.5 and 6.25 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 formazon was read on a micro titer plate reader (Versa max tunable multiwall plate reader) at 570 nm and the percentage of cell viability calculated using the following formula.[24] 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 determinates 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, stem bark and root of P.hysterophorus 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 constituents of Parthenium hysterophorus in different extracts from leaves, leaf petiole and stem bark.

Tested compounds	Leaves			Stem Bark			Root		
	Me	EtAc	CH	Me	EtAc	CH	Me	EtAc	CH
Alkaloids	+	+	+	+	+	+	+	+	+
Coumarins	+	-	+	-	+	-	-	-	-
Carbohydrates	-	+	+	+	-	-	-	+	-
Phenols	+	+	-	+	+	-	+	+	-
Saponins	-	+	-	-	-	-	-	+	-
Tannins	+	+	-	-	-	-	+	-	-
Flavanoids	+	-	+	+	-	+	-	+	+
Terpenoids	+	+	-	+	-	+	+	-	-
Phlobatannins	-	-	-	+	+	+	+	-	-
Steroids	-	+	+	+	-	-	-	+	+

Me= Methanol; EtAc= Ethyl Acetate; CH= Chloroform + Present, - Absent

In the present study, crude extracts of methanol, ethyl acetate and chloroform from leaves, stem bark and root of P. hysterophorus 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).[25] Based on this categorization, the IC_{50} values of all the crude extracts of leaves, stem bark and root (excepted chloroform extracts of leaf and root) of P. hysterophorus showed a range of inhibitory concentrations against CQ-sensitive P. falciparum 3D7 strain. The methanol extract of leaf (12.15 µg/mL), stem bark (45.33 µg/mL) and root (22.40 µg/mL); the ethyl acetate extract of leaves (41.00 µg/mL) and roots (26.68 µg/mL) showed good antimalarial activity and are significant at $P<0.05$ and $P<0.01$. The ethyl acetate extract of stem bark (69.67µg/mL) and chloroform extract of stem bark (87.53 µg/mL) shown moderate antimalarial activity. Among these extracts, the methanol extract of leaves showed excellent antimalarial activity (IC_{50} = 12.15 µg/mL). The chloroform extracts of

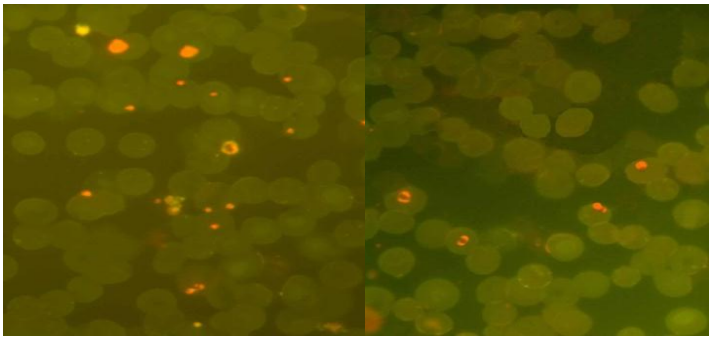
leaves and root shown inactive antimalarial activity with IC_{50} values >100 µg/mL.

The in vitro cytotoxicity studies against THP-1 cell line were conducted for all the extracts. All extracts showed IC_{50} value >20 µg/mL (except two extracts methanol leaves and roots). An extract is classified as non toxic when the IC_{50} value is >20 µg/mL. Based on this, the methanol extracts of leaves and roots toxic to cells, left behind 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 Parthenium hysterophorus.

Plant parts	Crude Extracts	% of Yield	IC_{50} 3D7 strain (µg/mL)(95%cl)	IC_{50} THP-1 cells (µg/mL) (95%cl)	SI
Leaves	Methanol	11.20	12.15±0.75 (10.29-14.00)	11.50±2.18 (6.09-16.91)	0.94
	Ethyl Acetate	3.35	41.00±4.58 (29.62-52.38)	57.60±5.24 (44.58-70.62)	1.33
	chloroform	2.80	>100	>100	00
Stem Bark	Methanol	7.50	45.33±4.73 (33.59-57.07)	88.43±2.23 (82.90-93.97)	1.95
	Ethyl Acetate	1.79	69.67±3.79 (60.26-79.07)	>100	>1.43
	chloroform	1.32	87.53±1.50 (83.80-91.66)	>100	>1.14
Roots	Methanol	2.94	22.40±3.68 (13.26-31.53)	10.23±0.68 (8.54-11.92)	0.45
	Ethyl Acetate	1.10	26.68±2.93 (19.41-33.94)	ND	ND
	chloroform	2.15	ND	>100	ND

Values are represented as mean±standard deviation, SI- selectivity index, $SI_{Plasmodium} = IC_{50\ THP\text{-}1} / IC_{50\ P.\ falciparum\ 3D7}$, Upper-Lower 95% Confidence interval level.



Control (Positive) Leaf extract treated

Fig 1: Micrograhs of synchronized ring stage P.f3D7 culture treated with methanol leaf extract (100 mg/µL) of Parthenium hysterophorus 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.[26] 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.[27]

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. [28]

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.[29] Plants are producing secondary metabolites for their defense, which play an important role of physiological activities in human body.[30] 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.[31] 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.[32]

The present investigation has evaluated the in vitro antiplasmodial activity of P. hysterophorus with different extracts such as methanol, ethyl acetate and chloroform form of leaves, stem bark and root. According to Rasoanaivo et al. (1992),[24] 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, six showed good (IC_{50} = 12.15-45.33 µg/mL), two exhibited moderate (IC_{50} = 69.67-87.53 µg/mL), while two displayed inactive (IC_{50} = >100 µg/mL) antiplasmodial activity as shown in Table 1. Previously Niharika et al reported antimalarial activity of same plant against CQ-sensisitive and resistant strain of P. falciparum (3D7).[33]

P. hysterophorus L. (Asteraceae) is an invasive weed throughout the world commonly known as altamisa, carrot grass, bitter weed, star weed, white top, wild feverfew, and the congress grass. Plant has been used as folk remedy for the treatment of infectious and degenerative diseases. All parts of the plant are reported to be used as bitter tonic, febrifuge, emmenagogue, antidysenteric, and so forth. Some researchers have reported its use in traditional medicine for treatment of wounds, ulcerated sores, anemia, fever, and heart troubles. In India and many other countries extracts of P. hysterophorus are used as ethno medicine against inflammatory, skin, neural, and female reproductive problems. In Maharashtra and Gujarat (India) the plant is used in the treatment of diabetes mellitus. P. hysterophorus has been found to be pharmacologically active as analgesic in muscular rheumatism and as vermifuge and therapeutic for neuralgia.[34].

The in vitro antiplasmodial activity of the P. hysterophorus methanol leaf extract may be due to the presence of major chemical classes such as phenols and alkaloids. Hence alkoloids are strong antiplasmodial compounds. Except the alkaloids, the major chemical classes such as coumarins, phenols, polysaccharides and flavanoids also exerted strong antiplasmodial activities.[35] Bandaranayake (2002)[36] 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.[29] In the present study, the in vitro cytotoxic effect against THP-1 cell lines showed IC_{50} >20 µg/mL. According to Falade et al., the ctyotoxicity >20 considered as non toxic to animals and safer for further studies. Thus, maximum the plant extracts are not harmful and safer for therapies.[37]

Similarly, Sree Rekah et al. reported the antimalarial activity of methanol, ethyl acetate and aqueous extracts of Albezia lebbeck against CQ-sensitive (3D7) strain of P. falciparum and tested for their cytotoxicity on human THP-1 cell line (HEp-2). Out of the 9 test extracts, the ethyl acetate extract of leaf (IC_{50} = 19.22 µg/mL) has shown excellent antimalarial activity. The aqueous extract of leaf and methanol extract of stem bark were inactive. All the extracts were non toxic to THP-1 cells. They have concluded the leaf ethyl acetate had good antiplasmodial activity (IC_{50} 19.22 µg/mL) with selectivity index ranged >10.52 for THP-1 cells, respectively.[38]

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).[39] 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 methanol extract of leaves of Parthenium hysterophorus 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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