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In vitro ANTIMALARIAL ACTIVITY OF *Pergularia daemia* (L.) AGAINST CHLOROQUINE-SENSITIVE (3D7) AND CHLOROQUINE-RESISTANT (K1) STRAINS OF *Plasmodium falciparum*

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author PB designed the study, searched the literature, collected the plant, prepared the crude extracts for phytochemical screening, analyzed the results and wrote the first draft of the manuscript. Author KS wrote the protocol and corrected the manuscript for final publication. All authors read and approved the final manuscript.

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ABSTRACT

Objective: To find out the *in vitro* antiplasmodial activity of *Pergularia daemia* leaf extract against *Plasmodium falciparum* chloroquine sensitive 3D7 strain and *Plasmodium falciparum* chloroquine resistant K1 strain and cytotoxicity against Vero cell line.
Methods: The *P. daemia* plant was collected from Kadaparajupalle at Dornala mandal, Prakasam District, Andhra Pradesh, India. Leaf crude extracts prepared in Soxhlet apparatus with hexane, chloroform and methanol solvents. These extracts were tested for *in vitro* antiplasmodial activity against 3D7 and K1 strains by standard laboratory protocol. *In vitro* Cytotoxicity of the leaf extract was also tested by following standard laboratory method.
Results: Hexane, chloroform and methanolic extracts of leaf shown good antiplasmodial activity against 3D7 strain i.e., hexane leaf extract shown IC_{50} of 11.04 µg/ml, chloroform leaf extract of 7.98 µg/ml and methanolic leaf extract of 6.33 µg/ml. Hexane, chloroform and methanolic extracts showed very active antiplasmodial activity against K1 strain with IC_{50} values of 4.79 µg/ml, 4.01 µg/ml and 2.91 µg/ml respectively. And all the leaf extracts were non-toxic against Vero cell line with CC_{50} >20 µg/ml.
Conclusion: The methanolic extract of leaf is effective and shown excellent antimalarial activity for the development of new antimalarial drug policies.

Keywords: *Pergularia daemia*; antiplasmodial activity; *Plasmodium falciparum* and cytotoxicity.

1. INTRODUCTION

According to WHO 2017 report in 2016, 91 countries reported a total of 216 million cases of malaria, an increase of 5 million cases over the previous year. The global tally of malaria deaths reached 445,000 deaths, about the same number reported in 2015 [1].

In the early 2000s *Plasmodium falciparum* parasites resistant to mefloquine, chloroquine, quinine,

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proguanil, atovaquone and sulphadoxine-pyramethamine, but not artemisinins were reported [2].

The introduction of artemisinin-based combination therapy has resulted in a substantial decline in malaria during the last decade. Artemisinin originally derived from plant *Artemisia annua*, which is the most potent antimalarial class of drugs available today and they exhibit a substantially faster elimination of parasites compared to other compounds [3]. However, the recent gains in antimalarial therapy are threatened by the emerging artemisinin resistant *P. falciparum* malaria in Southeast Asia. Consequently, new drugs and drug combinations are urgently needed for the treatment of malaria. Ideally, these drugs should have novel modes of action and be chemically different from drugs in current use [4].

Indeed, people from developing countries often do not have access to modern therapeutics such as Artemisin-based Combination Therapy (ACT) to treat malaria because of financial, geographical and/or cultural obstacles. The WHO estimates that up to 80% of the world's population depends on traditional medicinal products for some aspects of primary health care. Better knowledge of plants from traditional pharmacopoeias and validated traditional remedies in ITM (Improved Traditional Medicine) could lead to access to effective, standardized, available and affordable therapeutics for management of malaria by local populations [5].

Pergularia daemia is a hispid perennial herb that grows along the road sides of India and other tropical and subtropical regions. In Andhra Pradesh, it is commonly available and used as an ethnomedicinal plant in Nallamalais forest in Prakasam District, Andhra Pradesh, India [6].

Earlier reports indicate various beneficial uses of the herb, validating it as a medicinal herb. Traditionally the whole plant is used as anthelmintic, antipyretic, laxative and expectorant and to treat infantile diarrhoea and malarial fever. Dried leaf of this plant is used as an emetic agent and is effective in treating bronchitis, asthma [7], rheumatic amenorrhea, dysmenorrheal [8] wounds and to facilitate parturition. Fresh roots and shoots are found to be useful in treating whooping cough [9]. The shoots of the plants are considered to be an effective agent for abortion [10]. Stem bark is used in treating malaria [11] and twig is affective as an antipyretic agent and serves as a good appetizer [12].

The present study aims to find the *in vitro* antiplasmodial activity of *Pergularia daemia* leaf extract against *Plasmodium falciparum* chloroquine sensitive 3D7 strain and *Plasmodium falciparum* chloroquine resistant K1 strain and cytotoxicity against Vero cell line.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material and Preparation of Crude Extract

Plant *P. daemia* was collected from a place called Kadaparajupalle at Dornala mandal, Prakasam district, Andhra Pradesh, India (Fig. 1). The collected plant leaves were washed thrice with tap water and twice with distilled water to remove the dirt and adhering materials. Then the leaves were shade dried at room temperature for two weeks. The dried leaves were chopped into smaller pieces and then ground into powder. The samples were then stored in jars at room temperature until extraction. Shade-dried leaf sample was subjected for in 90% different organic solvents i.e., chloroform (60ºC-62ºC), hexane (65ºC-70ºC) and methanol (65ºC) in a soxhlet apparatus (Borosil). For *P. daemia* plant part extraction, 100 g of leaf powder was weighted accurately and used for the extraction in each of the above solvents. After complete extraction, the filtrates were concentrated separately by rotary vacuum evaporation at >45ºC and then freeze dried at -20ºC to obtain solid residue.

Fig. 1. The plant *Pergularia daemia* (L.) Forsk

2.2 *In vitro* Cultivation of *P. falciparum*

The *in vitro* cultures of both CQ - sensitive (3D7) and CQ - resistant (K1) strains of *P. falciparum* are routinely maintained in medium RPMI supplemented with 25mM HEPES,0.2% D-glucose,0.21% Sodium Bicarbonate and 0.5% ALBUMAX-II [13]. The stock (5 mg/mL) solution of compound was prepared in

DMSO and required dilutions were prepared in culture medium. For evaluation of 50% Inhibitory Concentration (IC_{50}) of the compound, Malaria SYBR Green-1 based fluorescence (MSF) assay [14] was carried out.

2.3 *In vitro* Antimalarial Assay

The highest concentration of extract used was 50.0 µg/mL. Two-fold serial dilutions of test sample were made in 96-well plate and incubated with 1.0% parasitized cell suspension containing 0.8% parasitaemia (Asynchronous culture with more than 80% ring stages). The plates were incubated at 37ºC in CO_2 incubator in an atmosphere of 5% CO_2 and air mixture. 72 hours later 100 µL of lysis buffer containing 2x concentration of SYBR Green-1(Invitrogen) was added to each well and incubated for one hour at 37ºC. The plate was examined at 485±20 nm of excitation and 530±20 nm of emission for relative fluorescence units (RFUs) per well using the Fluorescence Plate Reader (FLX800, BIOTEK).The inhibitory concentration (IC_{50}) values were obtained by logit regression analysis of dose-response curves. Chloroquine diphosphate (SIGMA) was used as the reference drug.

The *in vitro* antimalarial activity of biological active substances is categorized into four types based on IC_{50} value as follows: <5 µg/mL is very active, >5 to 50 µg/mL is active, >50 to 100 µg/mL is weakly active, >100 µg/mL is inactive [15].

Criteria for selection of promising lead: IC_{50} ≤10.0 µg/mL.

2.4 Evaluation of *in vitro* Cytotoxicity Assay

Cytotoxicity of the test samples was carried out using Vero cell line (C1008; Monkey kidney fibroblast cells) following the method as mentioned is Sharma et al. [16]. The cells were incubated with test sample dilutions for 72 h and MTT was used as reagent for detection of the cytotoxic activity. The highest concentration of test samples used was 100 µg/mL. 50% cytotoxic concentration (CC_{50}) was determined using dose–response curves. Podophyllotoxin (SIGMA) was used as the reference drug.

Selectivity Index (SI) can be calculated as: SI = CC_{50}/IC_{50}

Criteria for selection: SI >50.0

3. RESULTS

The percentage yield of leaf hexane, chloroform and methanol extracts of *P. daemia* was 15%, 20% and 25% respectively out of 100 g of leaf powder. More yield was obtained with leaf methanolic extract of *P. daemia* (Table 1).

Phytochemical screening of the leaf hexane, chloroform and methanolic extracts of *P. daemia* showed the presence of various medicinally bioactive constituents. A total of 11 phytochemicals were analyzed. The leaf hexane extract showed the presence of one compound, leaf chloroform and leaf methanol extracts of *P. daemia* showed the presence of five compounds each (Table 2).

Table 3 represents the IC_{50} values of the standard drug 'Chloroquine' (Control Positive) against CQ–sensitive (3D7) and CQ–resistant (K1) strains of *P. falciparum* with reference to DMSO (Control Negative).

The DMSO treated samples showed 100% survival of the parasite hence there is no inhibition or suppression of the parasite observed. Thus the final RFU of DMSO was 1791.75 which correspond to 100% of survival of the parasite without any inhibition against 3D7 strain of *P. falciparum*. And the Chloroquine treated samples has shown $IC_{50}$of 1.74 ng/mL against 3D7 strain of *P. falciparum* with 50% inhibition of the parasites (Table 3).

Similarly, the final RFU of DMSO was 2683.03 which correspond to 100% of survival of the parasite without any inhibition against K1 strain of *P. falciparum*. And the Chloroquine treated samples have shown IC_{50} of 0.83 ng/mL against K1 strain of *P. falciparum* with 50% of inhibition of the parasite (Table 3).

Table 4 reveals the *in vitro* antimalarial activity of leaf crude extract of *P. daemia* against CQ-sensitive *P. falciparum* 3D7strain. The leaf hexane extract has shown $IC_{50}$of 11.04 µg/mL, leaf chloroform extract has shown IC_{50} of 7.98 µg/mL and leaf methanol extract has shown IC_{50} of 6.33 µg/mL against 3D7 strain. All these extracts have exhibited good antimalarial activity with IC_{50} ranging from >5 to 50 µg/mL. Hence all the three extracts of *P. daemia* can be used for the treatment of CQ-sensitive 3D7 *P. falciparum* malarial infection.

Table 5 reveals the *in vitro* antimalarial activity of leaf crude extract of *P. daemia* against CQ-resistant *P. falciparum* K1 strain. The leaf hexane extract has shown IC_{50} of 4.79 µg/mL, leaf chloroform extract has shown IC_{50} of 4.01 µg/mL and leaf methanol extract has shown IC_{50} of 2.91 µg/mL against K1 strain. All these extracts have exhibited excellent antimalarial activity with IC_{50} value of <5 µg/mL. Thus, all the three extracts found to be promising for the use as

drug for the treatment of CQ-resistant (K1) *P. falciparum* malarial infection.

The microscopic observation of inhibition of *P. falciparum* in infected RBCs treated with methanolic leaf extract of *P. daemia* at higher concentration i.e., 50 µg/mL at 1000x magnification was shown in Figs. 2 and 3.

The *in vitro* cyototoxicity studies against Vero cell line were conducted for all the extracts. All the extracts showed CC_{50} value >300 µg/mL (Table 6). An extract is classified as non-toxic when the CC_{50} value is >20 µg/mL. Based on this, all leaf extracts were not harmful for *in vivo* studies.

Also the Selectivity Index (SI) values of all the extracts were >50 except the leaf hexane extract for 3D7 strain (Table 6). Hence these extracts can be selected for further use as drugs for treatment against CQ-sensitive (3D7) and CQ-resistant (K1) strains of *P. falciparum* except leaf hexane extract of *P. daemia*.

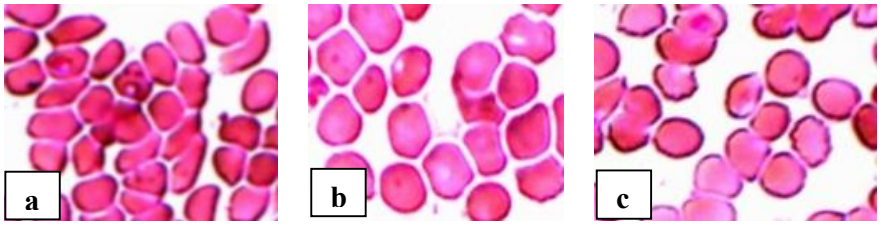

Fig. 2. Micrographs of *P. falciparum* CQ-sensitive 3D7 infected RBCs treated with methanolic leaf extract of *Pergularia daemia* at highest concentration (50 µg/mL)

a = Control Negative, b = Control Positive, c = Extract treated

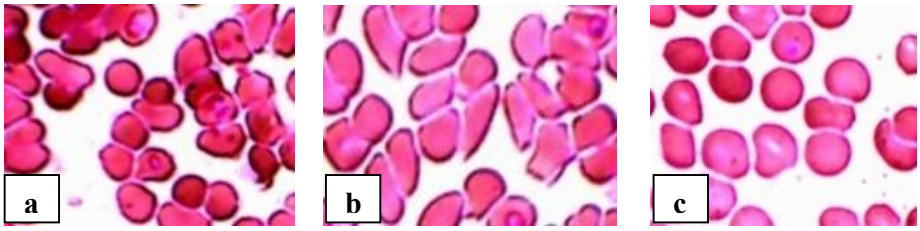

Fig. 3. Micrographs of *P. falciparum* CQ-resistant K1 infected RBCs treated with methanolic leaf extract of *Pergularia daemia* at highest concentration (50 µg/mL)

a = Control Negative, b = Control Positive, c = Extract Treated

Table 1. Percentage yield of leaf crude extracts of *Pergularia daemia*

Plant part	Solvent	Initial weight (g)	Yield of the extract (g)	Percentage of yield (%)
Leaf	Hexane	100	15	15
	Chloroform	100	20	20
	Methanol	100	25	25

Table 2. Phytochemical screening of leaf extract of *Pergularia daemia*

Phytochemical constituent	Leaf hexane extract	Leaf chloroform extract	Leaf methanol extract
Alkaloids	-	+	+
Saponins	-	-	+
Terpenoids and Steroids	-	+	+
Tannins	-	+	+
Anthocyanidins	-	-	-
Phenolic compounds	-	-	-
Flavonoids	+	+	+
Coumarins	-	-	-
Quinones	-	-	-
Resins	-	-	-
Glycosides	-	+	-

Table 3. *In vitro* antimalarial activity of the standard drug Chloroquine against CQ–sensitive (3D7) and CQ–resistant (K1) strains of *Plasmodium falciparum*

Compound	Conc. of the compound (ng/mL)	Total RFU (RBC with *P. f.* infection)	Blank RFU (RBC without *P. f.* infection)	Final RFU (Total RFU –Blank RFU = Only *P. f.* infected RBC)	% Survival	% Inhibition	IC_{50} (ng/ mL)
***Plasmodium falciparum* CQ-sensitive 3D7 strain**							
DMSO	0.07	2096.0	304.25	1791.75	100.00	0	-
(Control Negative)	0.15	(Mean)	(Mean)				
	0.31						
	0.625						
	1.25						
	2.50						
	5.0						
Chloroquine	0.07	2085	304.25	1780.75	99.38	0.62	1.74
(Control Positive)	0.15	2082		1777.75	99.22	0.63	
	0.31	2049		1744.75	97.37	2.63	
	0.625	1939		1634.75	91.24	8.76	
	1.25	1785.5		1481.25	82.67	17.33	
	2.50	550		245.75	13.71	86.83	
	5.0	377.5		73.25	4.09	95.91	
***Plasmodium falciparum* CQ-resistant K1 strain**							
DMSO	0.07	3109.2	426.17	2683.03	100.00	0	-
(Control Negative)	0.15	(Mean)	(Mean)				
	0.31						
	0.625						
	1.25						
	2.50						
	5.0						
Chloroquine	0.07	3078.0	426.17	2651.83	98.84	1.16	0.83
(Control Positive)	0.15	3055.0		2628.83	97.98	2.02	
	0.31	2957.0		2530.83	94.33	5.67	
	0.625	2261.0		1834.83	68.39	31.61	
	1.25	1073.0		646.83	24.11	75.89	
	2.50	1018.5		592.33	22.08	77.92	
	5.0	943.0		516.83	19.26	80.74	

RFU = Relative Fluorescence Units

Table 4. *In vitro* antimalarial activity of leaf crude extract of *Pergularia daemia* against Chloroquine-sensitive *Plasmodium falciparum* 3D7 strain

Compound	Concentration of the extract (µg/ mL)	Total RFU (RBC with *P. f.* infection)	Blank RFU (RBC without *P. f.* infection)	Final RFU (Total RFU –Blank RFU = Only *P. f.* infected RBC)	% Survival	% Inhibition	IC_{50} (µg/ mL)
DMSO	-	2096.0	304.25	1791.75	100.00	0	-
Leaf Hexane extract	0.78	2002.0	304.25	1697.75	94.75	5.25	11.04
	1.56	1896.0		1591.75	88.79	11.21	
	3.12	1808.0		1503.75	83.93	16.07	
	6.25	1719.5		1415.25	78.99	21.01	
	12.5	1087.0		782.75	43.69	56.31	
	25.0	413.0		108.75	6.32	93.68	
	50.0	359.0		54.75	3.05	96.95	
Leaf Chloroform extract	0.78	1972.5		1668.25	93.11	6.89	7.98
	1.56	1860.5	304.25	1556.25	86.85	13.15	
	3.12	1808.0		1503.75	83.93	16.07	
	6.25	1413.0		1108.75	61.88	38.12	
	12.5	810.0		505.75	28.23	71.77	
	25.0	346.0		41.75	2.33	97.67	
	50.0	310.0		5.75	0.32	99.68	
Leaf Methanol extract	0.78	1943.5		1639.25	91.49	8.51	6.33
	1.56	1936.5	304.25	1632.25	91.09	8.91	
	3.12	1766.5		1462.25	81.61	18.39	
	6.25	1213.0		908.75	50.71	49.29	
	12.5	531.5		227.25	12.68	87.32	
	25.0	348.5		44.25	2.46	97.54	
	50.0	341.0		36.75	2.05	97.95	

RFU = Relative Fluorescence Units

Table 5. *In vitro* antimalarial activity of leaf crude extracts of *Pergularia daemia* against Chloroquine-resistant *Plasmodium falciparum* K1 strain

Compound	Conc. of the compound (µg/ mL)	Total RFU (RBC with *P. f.* infection)	Blank RFU (RBC without *P. f.* infection)	Final RFU (Total RFU –Blank RFU = Only *P. f.* infected RBC)	% Survival	% Inhibition	IC_{50} (µg/ mL)
DMSO	-	3109.2	426.17	2683.03	100.00	0	-
Leaf	0.78	3041.0	426.17	2614.83	97.46	2.54	4.79
Hexane	1.56	2929.0		2502.83	93.28	6.72	
extract	3.12	2490.0		2063.83	76.92	23.08	
	6.25	1316.0		889.83	33.16	66.84	
	12.5	1108.5		682.33	25.43	74.57	
	25.0	888.0		461.83	17.21	82.79	
	50.0	748.5		322.33	12.01	8799	
Leaf Chloroform	0.78	2898.5	426.17	2472.33	92.15	7.85	4.01
extract	1.56	2707.0		2280.83	85.01	14.99	
	3.12	2081.0		1654.83	61.67	38.33	
	6.25	1210.5		784.33	29.23	70.77	
	12.5	999.0		572.83	21.35	78.65	
	25.0	811.5		385.33	14.36	85.64	
	50.0	659.0		232.83	8.67	91.33	
Leaf Methanol	0.78	3068.0	426.17	2641.83	98.46	1.54	2.91
extract	1.56	2588.0		2161.83	80.57	19.43	
	3.12	1674.5		1248.33	46.53	53.47	
	6.25	1096.5		670.33	24.98	75.02	
	12.5	899.5		473.33	17.64	82.36	
	25.0	742.0		315.83	11.77	88.23	
	50.0	531.0		104.83	3.91	96.09	

RFU = Relative Fluorescence Units

Table 6. Cytotoxicity of leaf crude extracts of *Pergularia daemia* against Vero cell line and Selectivity Index (SI) corresponding to CQ-sensitive (3D7) and CQ-resistant (K1) strains of *Plasmodium falciparum*

Compound	CC_{50} against Vero cell line	SI (3D7 strain)	SI (K1 strain)
Hexane leaf extract	>500	45.28	104.38
Chloroform leaf extract	440.55	55.20	109.86
Methanol leaf extract	>500	78.98	171.82
Chloroquine (Standard drug)	365.6	211.33	440.48

4. DISCUSSION

India had remarkable biodiversity and rich culture traditions of plant use. Interestingly, today many of the pharmaceutical companies are utilizing such plant based formulations for treatment of various diseases and disorders worldwide [17]. Plants are producing secondary metabolites for their defence, which play an important role in physiological activities of human body [18]. The medicinal value of plants is due to the substances that it contains, which produce a physiological action in human body. Some examples of these plants are alkaloids, phenols, tannins, saponins, essential oils, resins and many others [19].

In Africa and other countries where malaria is endemic, traditional medicinal plants are frequently used to treat or cure malaria [20]. It is a fact that conventional antimalarials such as quinine and artemisinin derivatives originated from plants. It is therefore important to investigate the antimalarial activity of medicinal plants in order to determine their potential as source of new antimalarial agents [21].

Selection of the plant species to be studied is obviously a crucial step for the ultimate success of the investigation. Three strategies are currently pursued: random collection of plant material, targeted collection based on consideration of chemotaxonomic relationships and the exploitation of ethnomedical information [22]. Identification of new plant derived antimalarial using an ethnopharmacological approach appears to be more predictive compared with random [23].

Kantamreddi and Wright studied the leaf methanol extract of *P. daemia* against 3D7 and K1 strains of *P. falciparum* and indicated it to be poorly antiplasmodial (IC_{50} = 203.8 and 244.1 µg/mL respectively) [24]. Naveen et al. screened the leaf extractions of *P. daemia* with ethyl acetate resulted in about 10-fold potentiation of antiplasmodial activity against *P. falciparum* 3D7 with IC_{50} of 21 µg/mL [25].

The present investigation has evaluated the *in vitro* antimalarial activity of *P. daemia* leaf hexane, chloroform and methanol extracts. According to Rasoanaivo et al., the excellent *in vitro* antimalarial against *P. falciparum* CQ – resistant K1 strain was observed in all the three extracts and good antimalarial activity was observed against P. falciparum CQ – sensitive 3D7 strain.

Alkaloids are strong antiplasmodial compounds and apart from alkaloids, the major chemical classes such as coumarins, phenols, polysaccharides and flavonoids also exerted strong antiplasmodial activities [26]. This correlates with the present study that the phytochemical analysis of the extracts revealed the presence of alkaloids, saponins, terpenoids & steroids, tannins, flavonoids and glycosides which demonstrated effective antimalarial activity [27].

And also the *P. daemia* plant is used as anthelmintic, laxtative, antipyretic, expectorant, to treat infantile diarrhoea and malarial intermittent fevers [28,29,30]. Madhuri et al. investigation indicates that the *P. daemia* aqueous and ethyl acetate extracts offered significant potential for the development of novel antibacterial therapies and the treatment of several diseases caused by microorganisms [31].

Similarly, Pravin and Akkewar reported the phytochemical screening of methanol, ethyl acetate, chloroform, hexane and aqueous extracts of *P. daemia* which showed the presence of alkaloids, terpenoids, tannins, phenolic compounds, carbohydrates, saponins, anthraquinones, glycosides and flavonoids. Various extracts of this plant were examined using agar disk diffusion method against Gram positive, Gram negative and fungus microorganism [32].

Packirisamy and Moorthy reported the antimicrobial activities of 3 different concentration (250, 500, 750 µg/mL) of hydro alcohol (1:1) extracts of *P. daemia* which showed wide spectrum of activity against test organisms namely *Staphylococcus aureus, Bacillus substilis*, *Staphylococcus pyogenes* (gram positive), *E. coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae*, *Aeromonas hydrophila* and *Vibrio harveyi* (gram negative) [33]. Whereas in our study, the leaf extracts of *P. daemia* has shown antimalarial

activity. Hence in future, this plant can be subjected to the isolation of major antimicrobials and antimalarial constituents for further pharmacological evaluation.

Several medicinal plants have been reported to have antiplasmodial activity. Kaushik et al. [34] evaluated the antiplasmodial activity of medicinal plants from North Indian Buchpora and South Indian Eastern Ghats. Chinsembu reported the antimalarial activity of the plants from Sub-Saharan Africa [35].

5. CONCLUSION

Thus increasing global spread of multi-drug resistant malaria parasite showed that there is a need for new chemotherapeutic agents to combat malaria. So the present study aimed to screen the *in vitro* antimalarial activity of *P. daemia* for the scope to develop new antimalarial drugs. The study revealed that all the leaf extracts of *P. daemia* exhibited excellent antimalarial activity against *P. falciparum*. Further evaluation of the extract may provide potential molecule for therapy of malaria.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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