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EVALUATION OF *IN-VITRO* ANTIPLASMODIAL ACTIVITY OF SELECTED ETHNOBOTANICALLY IMPORTANT MEDICINAL PLANT EXTRACTS

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Keywords:

Antiplasmodial, Medicinal Plants, *Albizia lebbeck*, SYBR-Green I, *Tecomella undulata*, Cytotoxicity, Chloroquine resistance

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ABSTRACT: Purpose: A serious cause of morbidity and mortality, malaria remains a major public health concern. Failure of available treatments due to the rapid emergence of drug-resistant parasite strains emphasizes the need for a continuous search for new, safe, accessible, and affordable drug treatments. **Methods:** In this context, an ethnobotanical survey was conducted, different parts of seventeen medicinal plants were collected, and their methanolic extracts were prepared. These extracts were evaluated for anti-plasmodial activity against two strains of *Plasmodium falciparum*: chloroquine-sensitive 3D7 and resistant INDO using SYBR-Green I assay. The extracts were also assessed for cytotoxic effects against HEK293 mammalian cell lines using MTT assay. **Results:** Two plant extracts: *Albizia lebbeck* and *Tecomella undulata* exhibited good activity with IC_{50} ranging from 10-20µg/ml. Among others, eight showed moderate activity (IC_{50}= 20.1- 50µg/ml), while eight extracts either showed poor activity or were not active upto IC_{50}=100µg/ml against *Pf*3 D7. The extracts with good to moderate effects showed equipotent effects against *Pf* INDO and were non-toxic to mammalian cell lines with selectivity indices ranging from 2.3 to >13.3. This study validated the traditional usage of the selected plants especially leaves of *A. lebbeck* and *T. undulata*. **Conclusion:** The results obtained have presented a starting material for identification and purification of active compounds that might provide an alternative drug therapy to fight against malaria.

INTRODUCTION: Malaria continues to be the most prevalent parasitic disease. Currently, 87 countries are malaria- affected with children below the age of five being most vulnerable as they accounted for 67% of deaths [1].

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Although recent advancements in the treatment and control strategies malaria remains a major concern due to the constant emergence of resistant *Plasmodium* strains against available antimalarials [2-4].

The challenges of developing new therapeutics to reduce disease severity and eradicate malaria persist. Out of five *Plasmodium* species that cause human malaria, *P. falciparum* has developed resistance to almost all the available drugs, including current frontline artemisinin derivatives [5]. This constant emergence of resistant strains and

difficulty in developing an effective vaccine highlights the need for novel antimalarial compounds. Human life depends upon four basic things *i.e.,* food, clothes, shelter and good health. The fourth one is provided by plant kingdom and that is why nature and natural products are foremost solution in healthcare system for centuries [6]. Traditional knowledge and application of medicinal plants to treat diseases has increased success rate of drug development. Previous accomplishments of isolating quinine from bark of *Cinchona* tree and artemisinin from *Artemisia annua* are good examples of natural products as a source of lead candidates for drug development [7, 8]. Therefore, a plant product having specific clinical activity is considered as an initial point for a discovery and development.

India is among the richest countries in flora and since time immemorial, numerous medicinal plants have been used to treat a wide variety of diseases [9-11]. Therefore, the present study aimed to collect and screen medicinal plants from different regions of India against Plasmodium falciparum to identify a potent source for developing lead antimalarial candidates. A total of seventeen medicinal plants were tested against CQ- sensitive strain and CQ resistant strain of *P. falciparum*. The plants were also found to be non-cytotoxic to mammalian cells. The results obtained in this study reveal medicinal plants in India that have antimalarial potential. On further evaluation, they would lead to the identification and isolation of novel, a cost-effective antimalarial drug.

MATERIALS AND METHODS:

Collection of Medicinal Plants: An ethnobotanical survey using standardized questionnaire [11] was conducted at Kasauli, Himachal Pradesh; Noida, Uttar Pradesh; Sanjay Van, New Delhi. Locals were interviewed to identify plants that are traditionally used for treating malaria-associated symptoms such as fever, chills, anemia. Following standard sustainable collection protocol, plant materials were collected and identified by Dr. Sunny Dhir, Maharishi Markandeshwar (Deemed to be University), Mullana, India using reference samples that were preserved and deposited at Amity Institute of Virology and Immunology, Amity University Uttar Pradesh, Noida, Uttar Pradesh, India.

Preparation of Medicinal Plant Extracts: Each plant part was air dried in shade and powdered in a grinder. Dried and powdered plant material were extracted using methanol as solvent of extraction [11, 12]. Briefly, powdered leaf samples (12- 16g) were suspended in 200 ml of methanol and kept for overnight shaking in orbital shaker. The filtrate was then collected using Whatman filter paper No. 1 and the residue was again suspended in 200 ml methanol the process was repeated 5 times to extract each sample in total 1L(200 ml × 5) of methanol. The filtrate collected from each sample was pooled and, solvent from the filtrate was then evaporated using rotary-evaporator at 42°C. The obtained dry residue was weighed and stored at 4°C.

***In-vitro P. falciparum* Culture Maintenance:** Two strains of *Plasmodium falciparum* namely3D7 (CQ sensitive) and INDO (CQ resistant) were bought from Malaria Research and Reference Reagent Resource Center (MR4) and cultivated in laboratory set up using a modified Trager and Jenson method [13]. The parasites were suspended in fresh erythrocytes (obtained from Rotary Blood Bank, Tughlakabad Institutional Area, New Delhi, India) at 4% hematocrit using complete media. Complete media was prepared by combining 16.2 g/L Roswell Park Memorial Institute (RPMI) 1640 powder (Gibco), 0.5% albumax I (Gibco), 0.2% sodium bicarbonate, 0.005% hypoxanthine (MERCK), 10mg/l gentamicin. The culture was then incubated at 37°C and a gaseous mixture of 5% O_2, 5% CO_2 and 95% N_2. Media was refreshed daily and parasitemia was monitored microscopically using Giemsa stain.

Preparation of Test Samples: Stock solutions of test samples (25 mg/ml) were prepared in dimethyl sulfoxide (DMSO). Ten-fold dilution from the stocks were prepared to obtain 2.5 mg/ml in 10% DMSO. Following this, two-fold dilutions were prepared in a culture medium and 4 µl from each of these were added to the assay plated to obtain working concentrations of 100 -12.5 µg/ml. The final concentration of DMSO in each well was 0.4% which is found to be non-toxic to the parasite.

For controls: stocks of artemisinin were prepared in DMSO and that of CQ in sterilized distilled water.

Growth Inhibition Assay against *P. falciparum*: Antimalarial efficiency of methanolic extracts was tested against *P. falciparum* using SYBR green I fluorescence-based assay [14]. Using 5% sorbitol, parasites were synchronized at ring stages and incubated at 2% hematocrit, 1% parasitemiain a 96-well plate. Dilutions of each of the test samples were then added to this in triplicates in increasing order of concentration. DMSO (0.4%) was used as a negative control. For positive controls: 50nM artemisinin and 100nM CQ were used. Following this, the test plates were incubated at 37°C with standard culture conditions for 48 h. After incubation, 100 µl SYBR Green I solution was added to each well following which the plates were incubated at 37°C in dark for 1 h. Subsequently, fluorescence was measured on Perkin Elmer fluorescence plate reader at 485 nm excitation and at 530 nm emission. Dose-response curves were plotted using fluorescence counts and drug concentration to determine the inhibitory concentration (IC_{50}).

***In-vitro* Cytotoxicity of the Plant Extracts:** Cytotoxic effects of plant extracts were evaluated on HEK293 mammalian cell lines using MTT assay [15]. The cell lines were maintained in complete media containing DMEM (Gibco), 10% fetal bovine serum (Gibco), 100 Units/ml penicillin, 100 µg/ml streptomycin (Gibco) and incubated at 37 C, 5% CO_2. For the assay, cells were trypsinized using 0.25% trypsin (Gibco), and 10^4 cells/100µl/well were seeded in a 96-well tissue culture-treated flat bottom plate. The plates were incubated at standard culture conditions. Following 24 h incubation, test samples in the concentration of 200 µg/ml to 25 µg/l, in triplicates, were added to each well, and plates were incubated for 24 h. Subsequently, 0.5 mg/ml of MTT-PBS solution was added to the wells and the plates were then incubated for 4 h. After this, 100 µl of DMSO was added to every single well and mixed thoroughly to solubilize formazan crystals. The absorbance was read at 570nm using a multi-well plate reader, and dose-response curves were plotted to determine TC_{50}.

RESULTS:

Survey and Collection of Samples: After conducting an ethnobotanical survey at three different locations in India and interviewing 50 locals, details of plants used to relieve headache, fever, nausea, *etc*. were recorded. In addition, details of methods used to prepare the extracts, mode of administration, doses administered were also collected. Based on that, eighteen parts from seventeen plantswere collected to test their antimalarial potential **Fig. 1, Table 1.**

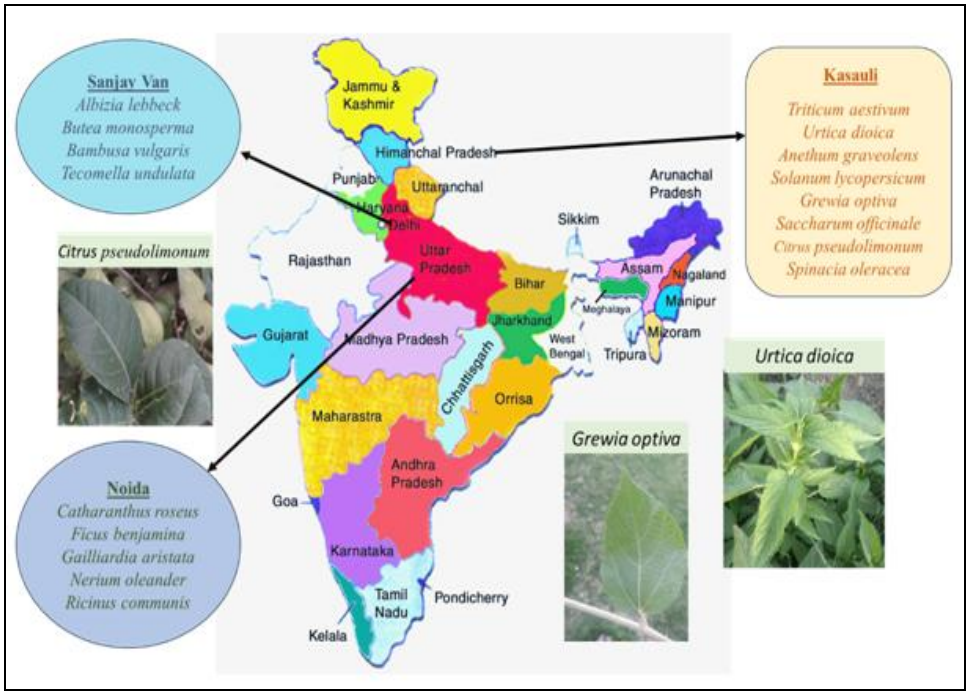

FIG. 1: SITES OF PLANT SAMPLE COLLECTION

TABLE 1: TRADITIONAL USES OF SELECTED MEDICINAL PLANTS

S. no.	Plant Name	Parts Used	Local/Common name	Traditional Usage
1	*Albizialebbeck* (L.) Benth./Leguminosae	Leaves	Saras	Bark is used to treat respiratory disorders. Fruit juice is used in case of snake bites and asthma.
2	*Anethumgraveolens* L./Apiaceae	Leaves	Dill	Decoction of dill plant is used to treat vomiting and indigestion. Mixture of powdered seeds of dill and fenugreek are used to regulate blood pressure.
3	*Bambusa vulgaris*Schrad./Poaceae	Leaves	Bamboo	Leaf decoction is used to treat fever, malaria. Decoction of shoot is used to treat respiratory disorders
4	*Butea monosperma* (Lam.) Taub./Leguminosae	Leaves	Palas	Leaf decoction is used for sore throat, leaf ointment is used for swelling. Juice of leaves, bark and roots are used to intestinal worm infections.
5	*Catharanthus roseus* (L.) G.Don/Apocynaceae	Leaves	SadaBahar	Leaf decoction is used in diarrhea, fever. Flower decoction is used in case of asthma and tuberculosis.
6	*Citrus pseudolimonum* Wester/Rutaceae	Leaves	Hill Lemon	Juice of fruit is used to treat fever. Leaves are infused to treat headaches and cold.
7	*Ficusbenjamina* L./Moraceae	Leaves	Weeping fig/Pukar	Fruits are used to treat malaria, vomiting. Paste of leaves and bark is used to relieve rheumatic headaches. Juice made from bark is used in case of liver diseases.
8	*Gaillardia aristataPursh*/ Compositae	Leaves, Flower	Blanket flower	Root and leaf infusions are used to treat stomach disorders.
9	*GrewiaoptivaJ.R.Drumm. ex Burret*/ Malvaceae	Leaves	Biul	Powdered stem and bark are used to treat fungal infections. Fruits are used to relieve fever.
10	*Nerium oleander* L./Apocynaceae	Leaves	Kaner	Bark is used as febrifuge. Leaves are used to treat bacterial infections.
11	*Ricinus communis*L./ Euphorbiaceae	Leaves	Castor Bean	Leaf decoction is used to relieve headaches. Paste made from leaves are used to treat rheumatic swellings, oral sores.
12	*Saccharumofficinarum* L./ Poaceae	Leaves	Sugar cane	Macerated leaves are drunk to treat anemia. Leaf decoction is used to treat urinary disorders. Stem juice is used to relieve sore throat.
13	*Solanum lycopersicum*L./Solanaceae	Leaves	Tomato	Fruit paste is used to treat anemia. Leaf Paste is used to treat worm infections. Mixture of leaves and castor oil is used to treat leprosy spots.
14	*Spinaciaoleraceae*L./Amaranthaceae	Leaves	Paalak	Leaves are used to treat febrile conditions, lung inflammation, worm infections. Seeds are used to relieve fever.
15	*Tecomellaundulata* (Sm.) Seem./*Bignoniaceae*	Leaves	Roheda	Vapors from rushed leaves are used to treat cough.
16	*Triticum aestivum*L./ Poaceae	Leaves	Wheat	Juice made from seedlings is used to treat anemia.
17	*Urticadioica*L./Urticaceae	Leaves	Bichchhu Buti	Leaf and fruit ash are used to treat fever. Leaves and seeds are used to treat allergy, liver disorders, arthritis.

***In-vitro* Antiplasmodial and Cytotoxic Effects of the Extracts:** Out of eighteen plant extracts assessed against *Pf*3D7, leaves of two plants *Tecomella undulata* and *Albizia lebbeck* showed good activities with IC_{50} ranging from10.1 to 20µg/ml, eight extracts namely, leaves of *Nerium oleander, Ricinus communis, Saccharum officinarum, Solanum lycopersicum, Citrus pseudolimonum, Bambusa vulgaris, Urtica dioica, Grewia optiva* were moderately active with IC_{50}= 20.1 to 50 µg/ml, four extracts *viz.,* leaves of *Ficus benjamina, Catharanthus roseus, Spinacia oleracea, Butea monosperma* showed poor antimalarial effects with IC_{50} ranging from 50-100µg/ml and four plant extracts namely, leaves of *Triticum aestivum, Anethum graveolens* and leaf and flower extracts of *Gaillardia aristata* were not active up to 100µg/ml **Table 2, Fig. 2.**

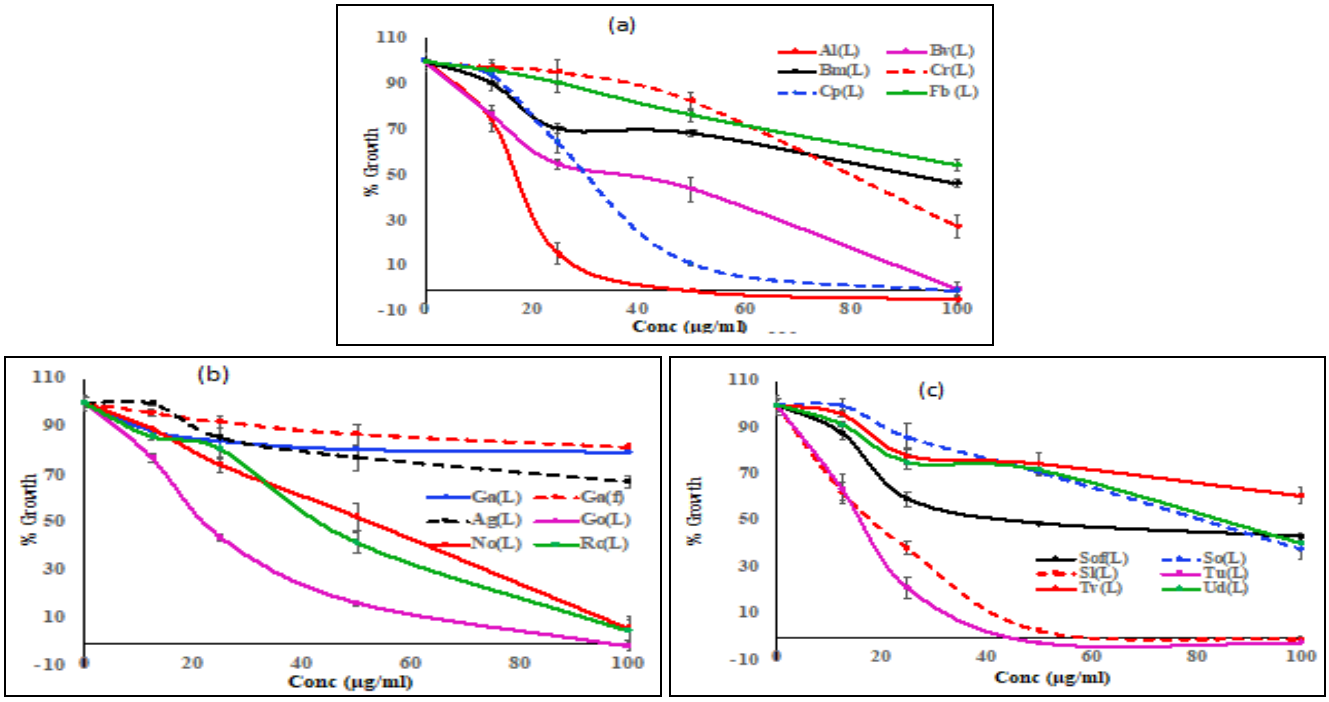

FIG. 2: DOSE-DEPENDENT GROWTH INHIBITION CURVES OF *PLASMODIUM FALCIPARUM* TREATED WITH PLANT EXTRACTS. CURVES OF *PF*3D7 TREATED WITH METHANOLIC EXTRACTS OF (A) LEAVES OF *A. LEBBECK* [AL(L)], *B. VULGARIS* [BV(L)], *B. MONOSPERMA*[BM(L)], *C. ROSEUS* [CR(L)], *C. PSEUDOLIMONUM* [CP(L)], *F. BENJAMINA* FB(L)]; (B) LEAVES AND FLOWER OF *G. ARISTATA* [GA(L) AND GA(F)], LEAVES OF *A. GRAVEOLENS G. OPTIVA*[GO(L)], *N. OLEANDER* [NO(L)], *R. COMMUNIS* [RC(L)]; (C) LEAVES OF *S. OFFICINARUM* [SOF(L)], *S. OLERACEAE* [SOL(L)], *S. LYCOPERSICUM* [SL(L)], *T. UNDULATE* [TU(L)], *T. AESTIVUM* [TV(L)], *U. DIOICA* [UD(L)]

Ten extracts that exhibited good to moderate effects against *Pf*3D7 were assessed against CQ resistant *Pf*INDO and six of them *i.e., T. undulata*, *A. lebbeck*, *N. oleander*, *C. pseudolimonum*, *G. optiva* and *B. vulgaris* showed were found to be equipotent with IC_{50}< 50 µg/ml. These extracts were also evaluated against HEK293 mammalian cell lines and were found to be non-toxic with selectivity index ranging from 2.3 to >13.3 **Table 2.**

TABLE 2: *IN-VITRO* ANTIPLASMODIAL AND CYTOTOXIC ACTIVITY OF SELECTED MEDICINAL PLANTS

S. no.	Plant name	Parts used	*Plasmodium falciparum* IC_{50} (µg/ml) 3D7	INDO	Cytotoxicity to HEK293 (TC_{50} in µg/ml)
1	*Albizialebbeck*	Leaves	17	18.17 (1.06) *	>200 (11.7)#
2	*Anethumgraveolens*	Leaves	>120	-	-
3	*Bambusa vulgaris*	Leaves	30	35.9 (1.1)	150 .0)
4	*Butea monosperma*	Leaves	90	-	-
5	*Catharanthus roseus*	Leaf	85	44.1 (0.5)	>200 (2.3)
6	*Citrus pseudolimonum*	Leaves	30	30.5 (1.0)	>200 (6.6)
7	*Ficusbenjamina*	Leaf	100	-	-
8	*Gaillardia aristata*	Leaf	>100	-	-
9	*Gaillardia aristata*	flower	>100	-	-
10	*Grewiaoptiva*	Leaves	22	32 (1.4)	>200 (9.0)
11	*Nerium oleander*	Leaf	50	24.9 (0.4)	200 4)
12	*Ricinus communis*	Leaf	40	55.8 (1.3)	>200 (5.0)
13	*Saccharumofficinale*	Leaves	50	56 (1.1)	>200 (4.0)
14	*Solanum lycopersicum*	Leaves	35	>100	-
15	*Spinaciaoleraceae*	Leaves	60	64.8 (1.0)	-
16	*Tecomellaundulata*	Leaves	15	15.3 (1.0)	>200 (>13.3)
17	*Triticum aestivum*	Leaves	130	-	-
18	*Urticadioca*	Leaves	43	58 (1.3)	>200 (4.6)

* Numbers in parenthesis denote resistance index (IC_{50}*Pf*INDO/IC_{50}*Pf*3D7), # Numbers in parenthesis denote SI (TC_{50} HEK293/IC_{50}*Pf*3D7).

DISCUSSION: Natural products have always played a important part in disease management as plant-derived metabolites are used for treating wide range of diseases [9, 16]. Hence, drug discovery begins with collection of plants on the basis on their traditional knowledge. In this context, seventeen medicinal plants were screened for antimalarial activity against CQ-sensitive 3D7 and CQ resistant INDO strain of *Plasmodium falciparum*. *Albizia lebbeck* (L.) Benth. (Mimosoideae) is a traditional medicine for arthritis, asthma, cold and cough, cancer [17, 18]. Here, *A. lebbeck* showed good activity with IC_{50} = 17 and 18.1 µg/ml against both *Pf*3D7 and *Pf* INDO respectively. Active antimalarial nature of *A.lebbeck*has previously been recognized by Kalia *et. al* who noted significant *in-vitro* activity with IC_{50}= 8.2 and 5.1 µg/ml against *Pf*MRC2 (CQ sensitive) and *Pf*RKL9 (CQ resistant) strain of *P. falciparum* by ethanolic extract of this plant [19].

Tecomella undulata (Sm.) Seem (Bignoniaceae) is a well-recognized medicinal plant traditionally used to treat liver disorders, cancer, hemorrhoids, diabetes, as a blood purifier [20]. In our study, *T. undulata* showed good antiplasmodial activity against both *Pf*3D7 and *Pf*INDO with IC_{50}= 15 µg/ml and 15.3 µg/ml respectively.

Saccharum officinarum is a constituent of polyherbal formulation known as SAABMAL used as an antimalarial in Nigeria. Recently, a study showed 95% suppression of parasitemia on treating *P. berghei*-infected mice with 400mg/kg of SAABMAL [21]. Our study, however, showed moderate activity of leaves of *S. officinarum* with IC_{50}= 50 and 56 µg/ml against *Pf*3D7 and *Pf*INDO respectively.

Leaf water extract of *F. benjamina* collected from Cameroon showed significant antimalarial effects with IC_{50}= 12 µg/ml and 26 µg/ml against *Pf*3D7 and *Pf*INDO; petroleum ether extract of leaves collected from Punjab, India showed IC_{50}=14 µg/ml which improved substantially on bio-guided fractionation with IC_{50}=4 µg/ml of hexane fraction and 7 µg/ml of chloroform fraction against *Pf*3D7 [23]. On the contrary, our study, where both location and solvent used for extraction were different, showed poor activity of leaf methanolic extract with IC_{50}=100 µg/ml against Pf3D7.

Nerium oleander L. is a drought-tolerant plant and is known to have numerous bioactivities such as anti-inflammatory, antibacterial, immuno-modulatory, neuroprotective, *etc*. Here, we have shown good to moderate antiplasmodial effects of leaves of *N. oleander*. Previous studies have reported the active nature of this plant against malaria vectors, *i.e.,* larval stages of *Anopheles stephensi* with LC_{50}- 0.58 g/l of chloroform leaf extract, 0.55 g/l of benzene flower extract [24], 94.6 mg/l of acetone flower extract [25] and larval stages of *A. gambiae* with LC_{50}= 127.8 ppm of aqueous leaf extract and 281.5 ppm of aqueous flower extract [26].

This plant is extremely poisonous due to the presence of toxic compounds such as oleandrin [27, 28]; however, leaf methanolic extract in our study was found to be non-toxic to mammalian cell lines and moderately toxic against malaria parasite. Likewise, *Ricinus communis*, which is widely distributed throughout tropics and temperate regions of the world has shown to be effective against malaria vector. Sogan *et al,* observed 35 and 45% mortality of *A. culicifacies* on treatment with leaf (LC_{50}= 65.62 ppm) and seed (LC_{50}= 9.37 ppm) dichloromethane extract of *R. communis* respectively. Further, treatment with aqueous seed extract on Day 1 showed 65- 100% mortality of *A. gambiae* for 5 days with LD_{50}= 74.1 ppm [26]. However, antiplasmodial of *R. communis* has not been evaluated yet, and in the present study, we showed moderate effects of leaf extract against *Pf*3D7and good effects against *Pf*INDO.

Grewia species are widely distributed in tropical and subtropical regions and are among the most important ingredients in traditional medicines. Many species of *Grewia* have been reported to exhibit antimalarial activity for instance chloroform fraction of *Grewiabila mellata collected by chromatography* inhibited growth of D6 and W2 strains of *P. falciparum* with IC_{50}=2.3 and 1.7 µM respectively [29]. However, *G. optiva* has not been studied yet despite its common use as a traditional herb to treat malaria. In our study, methanolic crude leaf extract showed moderate activity which might be attributed to its constituents. *Solanum lycopersicum* originated in Andes and now is common all around the world. This economically important crop has a high nutritive value and has

been found to exhibit bioactivities such as antibacterial, antioxidant [30]. Larvicidal activity of *S. lycopersicum* has been reported against *Aedes*and *Culex* [31]; however, antimalarial or larvicidal against malaria vector has not been assessed yet. Here, leaves of this plant has shown moderate effects against *Plasmodium* parasite.

Urtica dioica is a widespread perennial plant that grows on light soil and humus. Extracts of *U. dioica* have been used for treating anemia, pain, rheumatism [32]. It has also exhibited antiviral properties against dengue virus [33]. Despite its traditional usage, antimalarial potential of this has not been evaluated yet. Here, leaf extract showed moderate effects against *P. falciparum* validating its traditional usage.

Bambusa vulgaris is the widely distributed throughout the tropical and subtropical regions and is the most common among all *Bambusa species*. The leaves of this plant are traditionally used to treat malaria [34], typhoid [35], venereal diseases [36], haematuria as a febrifuge agent. Previous studies have shown that aqueous leaf extracts from Ghana and ethanolic leaf extract from Cuba inhibited growth of *Pf*3D7 with IC_{50}= 7.5 and 4.7 µg/ml, respectively [37, 38]. Additionally, Anigboro *et a*l; reported a decrease in parasite load by 87% compared to positive control on treatment with 300 mg/kg of ethanolic leaf extract of *B. vulgaris* [39]. However, in our study, methanolic leaf extracts exhibited moderate antimalarial effects with IC_{50}= 30 µg/ml. The variation in activity might be attributed to: 1) choice of solvents that extracted out different components and their interaction influenced their activity, 2) different locations of sample collection.

Butea monosperma is a deciduous tree widely distributed in India, Ceylon, Burma. This plant is used in Unani, Ayurveda, Homeopathy and modern medicine [40]. The plant is used to treat helminthiasis, filariasis, diarrhea, and dysentery [41]. Previous studies have reported in vivo activity of this plant where a methanolic extract of leaves of *B. monosperma* showed only 6% suppression of *P. berghei* parasitemia on day 4 at a dosage of 500 mg/kg [42]. In line with that, in our study, the leaf extract of *B. monosperma* showed poor *in-vitro* activity against *Pf*3D7.

CONCLUSION: Rampant multi-drug resistant *Plasmodium* parasites calls for the continuous search of novel antimalarial drugs. This study revealed remarkable antimalarial efficacy of selected plants that were used by traditional healers and studied. *In-vitro* assessment of two medicinal plants: *A. lebbeck* and *T. undulata* showed antimalarial potential that justify their usage and healing properties against malaria-like symptoms. The antimalarial activity of these plants might be due to the molecules present in the plant extracts. Therefore, further studies on isolation and characterization of the principal components contributing to their active nature are likely to yield novel compounds that could be developed as alternative chemotherapy for treating malaria.

Statements & Declarations:

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CONFLICTS OF INTERESTS: Authors Declares No conflicts of interest.

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