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The Antimalarial Potential and Phytochemical Composition of Mangroves from Southeast India: An *In vitro* Study

Hridya, V.K.[1*], Prince Godson S.[1], Chandrasekar, N.[1] and Kumaresan, S.[2]

[1]*Centre for GeoTechnology, Manonmaniam Sundaranar University, Tirunelveli, India*
[2]*Department of Chemistry, Manonmaniam Sundaranar University, Tirunelveli, India*
**Email: vkhridya@gmail.com*

Abstract

The proven activity of mangrove plants which are being used in folklore medicines for the treatment of diseases like epilepsy, smallpox, asthma, diabetes, rheumatism, stomach pains, fevers, malaria, cholera, hepatitis, etc, will be a lead factor for the mangrove ecosystem conservation in future. Four mangrove plants, which are therapeutically important, were collected from Pichavaram, Tamil Nadu, India, at monsoon times according to its lesser impact of anthropogenic signatures by analyzing its elemental composition of leaves by ICP-AES. *In vitro* antimalarial activities of isolated fractions were screened against chloroquine-sensitive (MRC-2) and chloroquine-resistant (RKL-9) isolates of *Plasmodium falciparum* by the growth assessment of erythrocytic stage. The IC_{50} values of the active extracts against *P. falciparum* are 1.05, 2.18, 2.21, and 2.51µg/ml. The phytochemical screening using HPTLC technique reveals the presence of secondary metabolites such as alkaloids, terpenoids (especially betulin and lupeol) and flavonoids and this supports the biological activity of the selected mangroves. The cell cyto-toxicity data clearly suggest that the exhibited activity might not be due to the general toxicity of the extracts. These findings support the need for exploring and preserving mangroves could be the potential of lead moieties of this species.

Keywords: *Acanthus ilicifolius; Lumnitzera racemosa; Rhizophora mucronata*; *Plasmodium falciparum*; Betulin; Lupeol

1. Introduction

The mangrove plants are being used in folklore medicines [Kirtikar & Basu 1935; Chopra *et al*,1956], and recently extracts from mangroves and mangrove-dependent species have proven activity against human, animal, and plant pathogens (Nabeelah Bibi *et al*, 2019). The Arabs developed a rich pharmacopoeia from many different species of mangroves [Bandaranayake, 1999] to explore its scientific value to be developed as new natural resources. The collected ethnobotanical information of true Indian mangrove species of Pichavaram, Tamil Nadu have been reported by Prabhakaran *et al* [2012]. The reported traditional medicinal uses and the lack of phytochemical and pharmacological studies towards malaria research on mangrove species prompted this investigation into the phytochemistry and the antimalarial activities of various mangrove species from South India. The analytical data regarding the toxicity of the plant should be relevant in the case of plants that are used in drug discovery. It is necessary to ensure their mineral elemental composition of mangroves which have been widely used as folklore and traditional medicines, in connection with the impact of anthropogenic effects due to growing urbanization near estuaries.

Malaria is the most infectious disease in the developing world, affecting more than 229 million people and causing about 0.4 million deaths annually over recent years [WHO, 2020]. Almost all malaria cases in the country are caused by *Plasmodium falciparum*, considered to be the leading cause of death worldwide (WHO, 2008b). The continuous spread of *Plasmodium falciparum* resistance to antimalarial drugs poses a serious threat to malaria control programs. Traditional herbal medicines have been used to treat malaria for thousands of years in various parts of the world. The author has previously reported two antimalarial active compounds such as betulin and lupeol from *Rhizophora mucronata* [Hridya *et al*, 2012] by HPTLC method. This current study gives an analytical data for proving the non-toxical characteristics of mangrove plants and leading to enhancing the medicinal values as well as the mangrove ecosystem conservation.

2. Materials and Methods

2.1 *Collection of Plant Material:* The leaves of selected mangrove species such as *Acanthus ilicifolius* (A), *Excoecaria agallocha* (E), *Lumnitzera racemosa* (L), and *Rhizophora mucronata* (R), collected from Pichavaram (Lat. $11^0$27'N, Long. $79^0$47' E) of Tamil Nadu, India, based on their ethnomedical history.

2.2 *Elemental Analysis:* The plant materials were air-dried for 7–10 days in the shade at the environmental temperatures, dried, and were powdered mechanically using a commercial electrical stainless-steel blender. Elemental analysis of these powdered leaves was performed with Thermo Electron IRIS INTREPID II XSP DUO model Flexible axial and radial view ICP-AES instrument, with high concentration capabilities of spectral range 165 to >1000nm. Blank sample and laboratory standard sample (MES-23) were also subjected to the same procedure. The precision assessed by replicate analyses was within 3%.

2.3 *Plant Extraction:* Each plant powder (~500g) was extracted with petroleum ether, chloroform, and methanol in a simultaneous Soxhlet apparatus (boiling point range

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60– 80°C) for 18h separately until exhaustion. The extract was concentrated under reduced pressure in a Heidolph Rotary Evaporator at 45°C, and the residue obtained was stored at 4°C.

2.4 *Phytochemical Analysis:* The preliminary qualitative phytochemical studies of four mangrove crude extracts were done [Harborne, 1973] for alkaloids, triterpenes, flavonoids, phenols, sugars, saponins. Chromatograms of the spotted TLC plates were developed with the corresponding mobile phases [Wagner & Bladt, 1995; Bernard Fried, Joseph Sherma, 2003] in a twin-trough chamber up to 60mm under laboratory conditions, and then subjected to scanning using a CAMAG TLC scanner 3(CAMAG, Switzerland), in the absorbance-reflectance scan mode.

2.5 *In vitro antiplasmodial assay:* Chloroquine-sensitive (MRC-2) and chloroquine-resistant (RKL-9) isolates of *P. falciparum* provided from the National Institute of Malarial Research (NIMR), Delhi, India were used for *in vitro* culture. Parasites were grown in uninfected O^+ RBCs as host cells and maintained in RPMI-1640 medium containing 25mM HEPES supplied with 2mg/mL gentamicin, 0.5% $NaHCO_3$ (HiMedia), and human serum. The culture was maintained at 37°C in a CO_2 incubator. Parasitaemia was determined qualitatively and quantitatively using light microscopy (Giemsa stain). The activity is based on the evaluation of the effect of samples on the growth of the synchronized culture of *P. falciparum* [Usha Devi *et al*, 1996]. A log dose-response curve was generated and used to determine its IC_{50}.

2.6 *In vitro cytotoxicity:* The anti-proliferative effects of active fractions were determined by MTT cell viability assay [Arung *et al*, 2012]. MTT assays of the extracts were done using L929 fibroblast cells purchased from NCCS, Pune, was maintained in Dulbecco's modified eagles' media and grown to confluency at 37°C and 5 % CO_2 in a humidified atmosphere in a CO_2 incubator, then it trypsinized (500µl of 0.025% Trypsin in PBS/ EDTA solution). Samples were added to grown cells at a concentration of 10µg, 50 µg, 100µg and 200µg from a stock of 5mg/ml and incubated for 24 hours. OD was read at 540 nm.

3. Results and Discussion

Mangroves are degrading due to urbanization, pollution, and industrial development; public awareness needs to be raised regarding the value of mangroves. The elemental compositions of the leaf of selected mangrove plants used for anti-malarial studies were analyzed (Table 1). Leaves of the selected mangrove species were collected according to their non-toxicity on monsoon seasons for the present work. The concentration of toxic elements such as As, Sn, and Hg were below the detection limit in plants. The concentration of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), and iron (Fe) are rich in the selected plants and are essential to human health. Sulfur is found in important metabolic molecules [Abdallah *et al.*, 2010], and these molecules play important roles in the plant lifecycle and the protection of plants from different environmental stresses and pathogens [Lee *et al.*, 2011]. The Ni concentration in *Excoecaria agallocha* is a little higher. The higher concentration of Ni in plants may be due to anthropogenic activities. EPA has recommended daily intake of Ni should be less than 1mg beyond which is toxic [McGrath & Smith, 1990]. The elemental analysis of mangrove species shows the non-toxic characteristics of these selected species.

The phytochemicals profiling for all fractions of four selected plants were done using High-Performance Thin Layer Chromatography (HPTLC) (Fig.1a, 1b & 1c) and this study [Harborne, 1973] reveals the availability of various phytochemical constituents, namely, alkaloids, terpenoids, and flavonoids in mangroves. The rich abundance of these secondary metabolites may enhance the pharmacological activity towards *P. falciparum.*

The *in vitro* anti-plasmodial activities of different fractions

Table 1. Elemental composition of selected mangrove plants

Elements	*Acanthus ilicifoius*	*Excoecaria agallocha*	*Lumnitzera racemosa*	*Rhizopora mucronata*	Unit	Detection Limit in ppm
As	*BDL	*BDL	*BDL	*BDL	ppm	0.03
Ca	0.874	1.04	0.837	0.979	%	0.01
Cd	0.04	0.08	0.05	0.04	ppm	0.01
Co	1.04	1.87	0.08	0.04	ppm	0.01
Cr	0.74	0.88	0.03	0.04	ppm	0.01
Cu	124.03	104.38	19.44	173.83	ppm	0.01
Fe	226.25	332.23	77.87	186.65	ppm	0.01
Hg	*BDL	*BDL	*BDL	*BDL	ppm	0.01
K	5932.19	5220.52	853.66	7697.82	ppm	0.01
Li	0.03	*BDL	*BDL	1.63	ppm	0.01
Mg	4147.6	3663.34	1716.22	6415.67	ppm	0.01
Mn	354.28	246.72	221.44	415.38	ppm	0.01
Na	1.02	0.48	0.39	1.52	%	0.01
Ni	1.85	3.29	0.71	0.59	ppm	0.01
P	827.39	626.18	1415.63	1426.9	ppm	0.01
Pb	1.05	0.48	0.391	1.519	ppm	0.01
S	1682.84	791.55	1091.72	2496.05	ppm	0.01
Sn	*BDL	*BDL	*BDL	*BDL	ppm	0.01

*BDL-Below Detection Limit

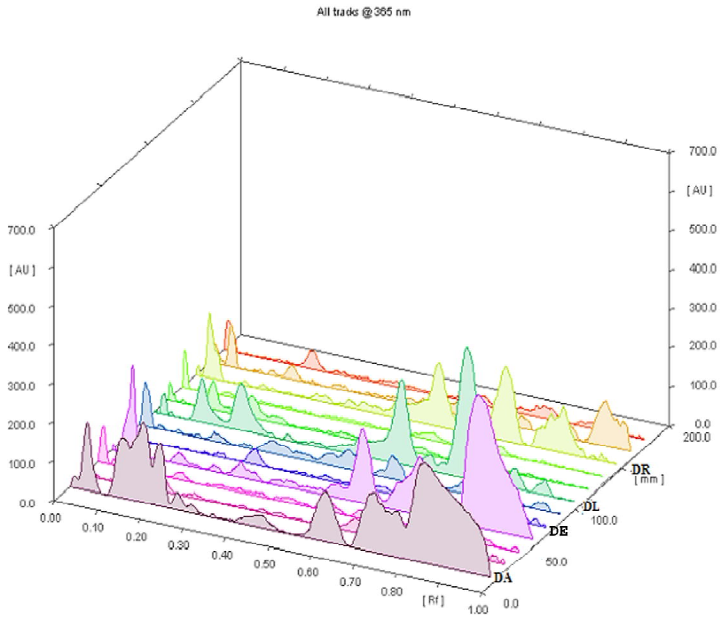

Fig. 1a. HPTLC planar chromatogram for alkaloids

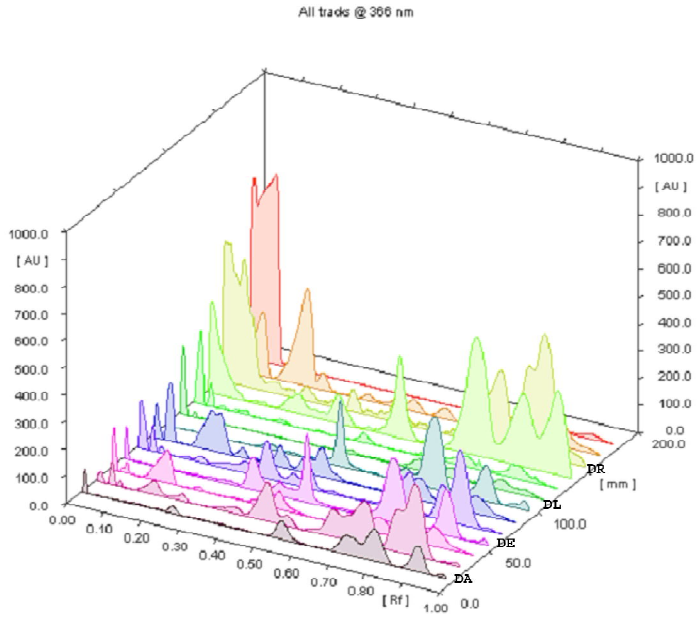

Fig. 1b. HPTLC planar chromatogram for flavonoids

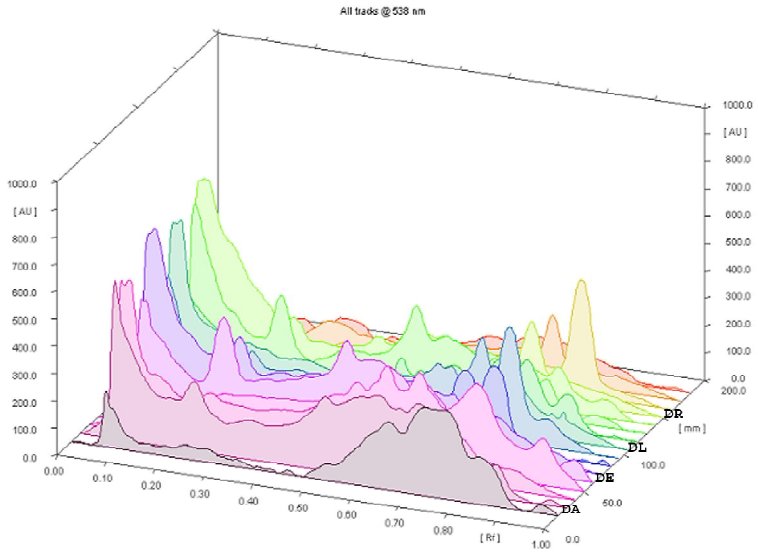

Fig. 1c. HPTLC planar chromatogram for terpenoids

Table 2. *In vitro* antimalarial activity of selected mangrove species

Plant Species	Extracts used	Concentration used	Average % inhibition MRC-2	RKL-9
Acanthus ilicifolius	Petroleum ether	2-100µg/ml	23.78	12.25
	Chloroform		41.64	54.09
	Methanol		39.76	31.52
Excoecaria agallocha	Petroleum ether	2-100µg/ml	-	-
	Chloroform		18.14	9.88
	Methanol		15.69	24.26
Lumnitzera racemosa	Petroleum ether	2-100µg/ml	-	-
	Chloroform		34.38	63.23
	Methanol		51.35	40.12
Rhizophora mucronata	Petroleum ether	2-100µg/ml	26.08	11.29
	Chloroform		49.22	47.34
	Methanol		58.41	70.09

of the selected four plants were done against chloroquine-sensitive (MRC-2) and chloroquine-resistant (RKL-9) isolates. Table 2 shows the average inhibition percentage of fractions in serial dilution method of various concentrations from 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625ìg/ml [Ouattara *et al*, 2006]. The highest schizont inhibition concentration exhibited by methanol fraction of *R. mucronata*; 70.09% towards RKL-9 and 58.41% against MRC-2, and that for chloroform fraction was 47.34% and 49.22 towards respective isolates. The chloroform fraction of *L. racemosa* and *A. ilicifolius* showed an inhibition percentage of 63.23% and 54.09% respectively towards RKL-9 isolates. All these fractions were shown a dose-dependent action towards both chloroquine-sensitive and chloroquine-resistant isolates. The fractions, which showed the highest inhibition percentage on these preliminary tests, were repeatedly tested against chloroquine resistant *P. falciparum*. Their schizonticidal activities towards RKL-9 isolates were plotted in Fig. 2.

The schizonticidal activity of extracts was expressed by the inhibitory concentrations at 50 (IC_{50}), representing the concentration of drug that induced a 50% parasitaemia decrease compared to the positive control culture referred to as 100% parasitaemia. The IC_{50} values were determined graphically on dose-response curves (concentration versus percent inhibition curves) with non-linear analysis. This activity was analyzed in accordance with the norm of plants antimalarial activity of Rasaonaivo *et al*. [1992]. According to this norm, a sample is very active if IC_{50}<5µg/ml, active 5µg/ml < IC_{50}< 50µg/ml, weakly active 50µg/ml < IC_{50}<100 µg and inactive IC_{50}>100 µg/ml.

Here, chloroform extract of *A.ilicifolius* shows the highest inhibition concentration 94% with an IC_{50} of 2.06µg/ml. This activity arises due to the synergic effect of secondary metabolites present in those mangrove plants individually or collectively. The second highest active chloroform fractions of *L.racemosa* were also again tested for IC_{50} concentration and get a value of 4.51µg/ml with 92% as the highest inhibition concentration. The chloroform fraction of *R.mucronata* shows 89% as the highest concentration while that of methanol extract shows 88% as the highest with IC_{50} values 4.62µg/ml and 5.69µg/ml respectively. The author has already been reported two antimalarial compounds (Fig. 4) from *R.mucronata* [Hridya *et al*, 2012], which boosts the possibility of more active components. Ravikumar *et al.* (2010d) reported that the ethanolic bark extract of *R. mucronata* exhibited an antiplasmodial activity. The major chemical classes, such as alkaloids, phenols, polysaccharides, and flavonoids also exerted strong antiplasmodial activities (Basak *et al*. 1996; Scalbert 1991; Cowan 1999). The reported antiplasmodial activities of some selected mangrove species against *P. berghei* (Muhaimin *et al*, 2019) indicate it as a potent source for natural antimalarial therapy. Some other

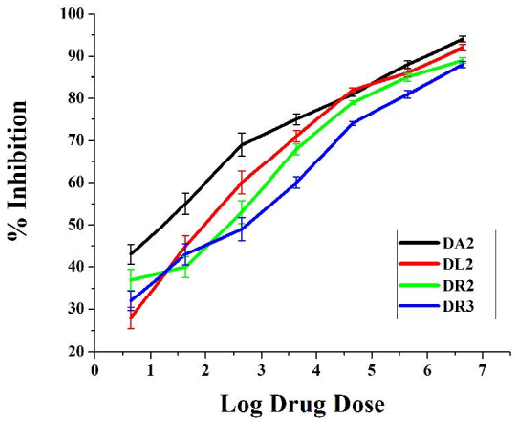

Fig. 2. *Schizont* inhibition percentage Vs concentration (log base2)

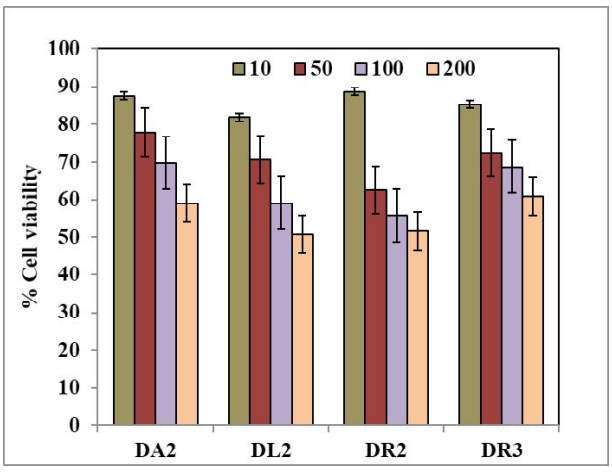

Fig. 3. Cytotoxicity (MTT) assay for active extracts

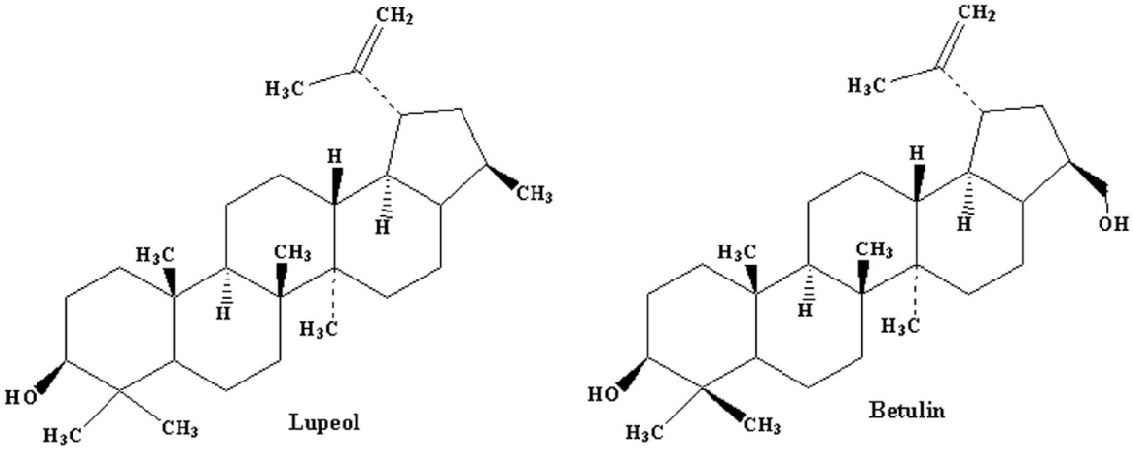

Fig. 4. a) Lupeol, b) Betulin

reported activities of mangroves are immune-stimulatory, anti-complementary, anti-inflammatory, hypoglycemic, and antiviral activities [Bandaranayake, 2002], larvicidal activity [Nazar *et al.*, 2009], antimalarial activity [Ravikumar *et al.*, 2011], antimicrobial activity [Ravikumar *et al.*, 2009], anticoagulant activity [Kathiresan *et al.*, 2006], and potential antiplasmodial activity [Otoguro *et al.*, 2004].

The cytotoxicity data (Fig. 3) clearly suggests that the exhibited activity might not be due to the general toxicity of the extracts. It is concluded that the present study has made a challenge to report a higher activity on mangrove leaves of three mangrove plants against both chloroquine-sensitive and chloroquine-resistant *P. falciparum* on Pichavaram mangrove forests. *E. agallocha* exhibited less activity among the selected mangroves may be due to the presence of any toxicants, which made a higher influence than its active principle.

4. Conclusion

In the present study, the chloroform and methanol extracts of mangrove leaves have exhibited the anti-plasmodial activity in the *in vitro* culture and it will lead to the development of new drugs. The leaf extract of mangrove plants showing promising activity against both chloroquine-sensitive and chloroquine-resistant *P. falciparum* is suitable for the Indian system of the formulation i.e., Siddha/Ayurveda in the treatment of malaria/fever and needs to be carried out a toxicological evaluation. The antimalarial compounds such as betulin and lupeol were identified using the HPTLC method in our laboratory enhancing the antimalarial activity of the selected mangrove species. The medicinal value of mangroves will be a lead factor for mangrove ecosystem conservation in the future. Thus, mangroves are indeed very special not only because of its vital roles in biodiversity enrichment, coastal protection and fisheries resource development but also for its potential phyto-pharmacological relevance's as well.

Acknowledgements

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