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Original Article

Bioassay Guided Fractionation and *in vitro Anti-plasmodial* Activity of *Ficus deltoidea* and *Ficus benjamina*

Hayat Mohammad Mukhtar[1], Amandeep Singh[2*], Hardeep Kaur[3]

ABSTRACT

Background: Malaria is infectious vector born disease affecting 212 million people belonging to 97 countries globally in the year 2016. Although this number has reduced progressively from last one decade but recent failure of currently available antimalarial drug therapy has accentuated the urgent need to explore different novel approaches in *Anti-plasmodial* drug discovery. **Objective:** The aim of the present study was to evaluate the *Anti-plasmodial* activity of traditional medicinal plants *Ficus deltoidea* and *Ficus benjamina*. **Materials and Methods:** Crude petroleum ether and hydro alcoholic extract of both the plant species were evaluated for *Anti-plasmodial* activity by schizont maturation inhibition assay using 3D7 *plasmodium* strains**. Results:** It was observed that petroleum ether extract of *F. benjamina* leaves showed most promising inhibitory effect on the growth of schizonts with IC_{50} 14.5 µg/ml. Bio-assay guided fractionation of petroleum ether extract of *F. benjamina* led to the hexane and chloroform fraction with high *Anti-plasmodial* activity (IC_{50} 4.0 µg/ml and IC_{50} 7.8 µg/ml respectively). Further, phytochemical investigation of *F. benjamina* indicated the presence of various valuable phytochemicals belonging to class of steroids, terpenoids and phytosterols. **Conclusion:** This study has revealed the *Anti-plasmodial* activity of *F. deltoidea* and *F. benjamina* for the first time. Significant *Anti-plasmodial* activity and preliminary phytochemical studies of *F. benjamina* indicates its rich chemical diversity which make this plant a good candidate for isolating new molecule that could serve as new lead in *Anti-plasmodial* drug discovery.

Key words: Malaria, Antiplasmodial, Schizont maturation inhibition assay, *Ficus deltoidea Ficus benjamina*, Bioassay guided fractionation.

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INTRODUCTION

Malaria is still considering a grievous parasitic disease caused by *Plasmodium falciparum* and other species of plasmodium, killing 429000 of the total 212 million infected people globally in the year 2016.[1] Although this morbidity and mortality rate is gradually reducing every year but still *P. falciparum* is regularly gaining attention due to its high adaptive character, fast sexual reproduction and rapid development of resistance against most of the currently available antimalarial drugs. However, World Health Organization (WHO), succeed somewhere in controlling the resistance by prescribing combination of various synthetic and semi synthetic drug molecules in the form of Artemisinin combination therapy (ACT).[2] But recent failure reports of ACTs, rapid re-emergence of malaria in certain ACT treated malarial patient and decreasing efficacy of standard antimalarial drug chloroquine (CQ) convinced the global chemists to focus on exploring some other new strategies in *Anti-plasmodial* drug discovery.[3,4] Various approaches such as development of new scaffolds, structural modification of existing molecules, better understanding of parasite life cycle and its mechanism of drug action, synthesizing hybridized drug molecules, designing of new ACT and plant based antimalarial drug discovery can be adopted to fulfill the need of timely requirement. Among the several strategies followed for the development of new drugs for the malaria, isolation and identification of novel biomolecules from plant sources is gaining much more importance than other approaches.[5,6] Traditional medicinal plants gives us most effective antimalarial drugs in the form of quinine and artemesinin isolated from *Cinchona calisya* and *Artemisia annua* respectively.[7,8] Similarly, large number of such plants like *Azadirachta indica*, *Tinospora cordifolia*, *Carica papaya* with acclaimed antimalarial activity used in the traditional medicine were extensively evaluated scientifically and have now become a part of the modern world health care system.[9,10,11] However, still there are large numbers of plants which are used traditionally to cure malarial fever and not yet explored and reported scientifically. Hence, nature is considered as an ever-evolving source of medicinally important plant secondary metabolites.

Ficus is one of the largest genus in the *Moraceae* family which comprises of approximate 800 species globally and approximate 115 species are distributed in India.[12] Several members of this genus were employed as one of the economical source of medicine because of its rich chemical diver-

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sity and therapeutic potential. Some species of genus *Ficus* demonstrated in the treatment of malarial fever by showing significant inhibition in the growth of *Plasmodium* strains Table 1.[13,14,15,16,17] *F. deltoidea* (FD) and *F. benjamina* (FB) are two morphologically similar perennial trees, grown up to the height of 4-8 m; widely distributed in the plain area of India, Southeast Asia, Malaysia and Northern Australia. FD and FB are commonly known as *Ficus* mixed and Benjamin's fig/ Java fig/ Pimpri/weeping fig respectively in India. Both of the plants were utilized traditionally in India for the treatment of malaria and other parasitic disease.[18,19] Ethnomedical studies revealed the significance of *F. benjamina* leaves (FBL) and *F. deltoidea* leaves (FDL) in the management of respiratory disorders,[20] burn injury,[21] diabetes,[22] cancer,[23] and inflammation.[24] The presence of wide range of chemical compounds such as flavnoids, coumarins phytosterols and phenolics are also considered as an important factor to accept the ethnopharmacological claims and modern pharmacological studies.[25,26,27,28,29] However, there is no scientific data yet reported in literature which specifies the *Anti-plasmodial* activity of FD and FB. With the above prospective of searching traditional medicines for the better *Anti-plasmodial* molecules, the various extracts of FDL, FBL and fractions of most active crude extract are evaluated for *In vitro Anti-plasmodial* activity by schizont maturation inhibition assay.

MATERIALS AND METHODS

Collection of Plant material

Plant materials were selected based on their ethnobotanical properties, medicinal uses and biological activities. Fresh leaves of plant species were collected from Botanical garden, Khalsa College Amritsar, Punjab, India in April 2016. The plant samples were taxonomically identified by Prof. Parveen Kumar Ahuja, senior taxonomist, Faculty of life sciences, Khalsa University Amritsar, Punjab, India Figure 1, Table 2. Specimens of the same were preserved in the herbarium section of the Khalsa College of Pharmacy, Amritsar, and Punjab for further reference.

Preparation of plant extracts and fractions

Procured leaves were cleaned properly and shade dried in open air for 6-7 days and then, pulverized in mixture grinder to get a coarse powder in dry form. 500 g of dried powder was successively extracted with petroleum ether and 80% ethanol for 24 h by hot percolation method using soxhlet apparatus. Both the crude extracts were concentrated under reduced pressure at 45°C and freeze dried to produce powder form of extract. The residues obtained were weighed accurately and stored at 4°C. The crude plant extracts were fractionated into different fractions based on their *Anti-plasmodial* activity. The petroleum ether extract of FBL was suspended in water and this aqueous solution was extracted successively with hexane, chloroform and butanol by partition extraction method Figure 2.

Phytochemical screening

All the crude extracts and fractions were filtered and examined for the presence of major phytoconstituents as per standard protocol of preliminary phytochemical analysis.[30]

In vitro cultivation of *Plasmodium* parasites

In vitro blood stage culture of CQ-sensitive strain (3D7) of *P. falciparum* was used to test the *Anti-plasmodial* activity of different plant extracts and its fractions. The culture of erythrocytic stage of malaria parasite was maintained at Malaria Parasite Bank, National Institute of Malaria Research, New Delhi, India using modified method of Trager and Jensen.[31] Isolated 3D7 strain of *P. falciparum* was cultivated in human AB +ve red blood cells using RPMI 1640 medium supplemented with AB Rh+ serum (10 %), 5 % sodium bicarbonate and 40 µg/ml of gentamycin sulphate. The culture was incubated at 37°C under a gas mixture of 2% O_2, 5% CO_2, and 93% N_2 in CO_2 incubator. Initial parasitemia was maintained between 0.5% - 1.0%. The growth of the parasite was daily observed by microscopic examination of thin blood smear treated with Geimsa stain and when the parasitaemia is above 3% in the initial culture it should be subcultured by replacing the old media with fresh RBCs and complete media. Percentage parasitaemia is calculated by counting the infected erythrocytes in a total of 10,000 erythrocytes.

Percentage parasitemia = [No. of infected erythrocytes/ total no. of erythrocytes] × 100

Table 1: Antiplasmodial activities of some species of genus ficus reported in literature.

Plant (Plant part)	Solvent	IC_{50} of AMP (µg/ml): CQ^S strain of Plasmodium	IC_{50} of AMP (µg/ml): CQ^R resistant strain of Plasmodium	Ref
F. religiosa (Bark)	Ethyl acetate	12.5: 3D7	16: INDO	13
F. benghalensis (Bark)	Ethyl acetate	19: 3D7	17: INDO	13
F. racemosa (Leaves)	Ethyl acetate	48: 3D7	–	13
F. thonningii (Leaves)	Methanol	5.3: NF54	21.1: K1	14
F. thonningii (Leaves)	Hexane	2.7: NF54	10.4: K1	14
F. thonningii (Leaves)	Ethyl acetate	5.3: NF54	15.3: K1	14
F.fistulosa (Leaves+Bark)	Chloroform	7.330: D6	3.760: W2	15
F. pyrifolia (Leaves)	Hydroalcoholic	-	18: FCM 29 clone 1 16: FCM 29 clone 3 20: FCM 22	16
F. polita (Leaves)	Hydroalcoholic	17.8: 165* 19.7: 111* 20: 62* 22.1: 83* 24.2: 151*	-	17

*P. falciparum strain isolate number

Figure 1: Morphology of FDL and FBL a) entire plant of FD and FDL b) entire plant of FB and FBL.

Table 2: Taxonomy of selected species of genus *Ficus*.

Taxonomy	*F. deltoidea*	*F. benzamina*
Kingdom	Plantae	Plantae
Sub kingdom	Viridiplantae	Viridiplantae
Division	Tracheophyta	Tracheophyta
Class	Magnoliopsida	Magnoliopsida
Order	Rosales	Rosales
Family	Moraceae	Moraceae
Genus	Ficus	Ficus
Species	Deltoidea	*benjamina*
Variety	Diversifolia	-
Binomial name	*Ficus deltoidea* L. var. diversifolia.	*Ficus benjamina* L.

Schizont maturation inhibition assay

Stock solution (1000 µg/ml) of test extracts and fractions were prepared by dissolving 1 mg of lyophilized crude extracts and its fractions in 100 µl of DMSO and 900 µl of incomplete media (without *Plasmodium* strains). The 100 µl of this stock solution was placed into the 96 well plate previously inoculated with 100 µl of *Plasmodium* cultured blood mixture media in two-fold serial dilution to obtain drug concentrations of 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 2.0 and 0.9 µg/ml. Two initial concentrations of 500 µg/ml and 250 µg/ml were not considered for the *Anti-plasmodial* drug evaluation because of its possibility of high toxicity to RBCs as well as to follow the WHO protocol of *Anti-plasmodial* drug screening.[32] Prepared well plates were incubated in the controlled gas mixture at 37 °C for 24-30 h for schizont maturation. After 24 h of incubation, a thick smear was prepared from the control well to observe maturity of schizonts and if more than 10% schizonts (with more

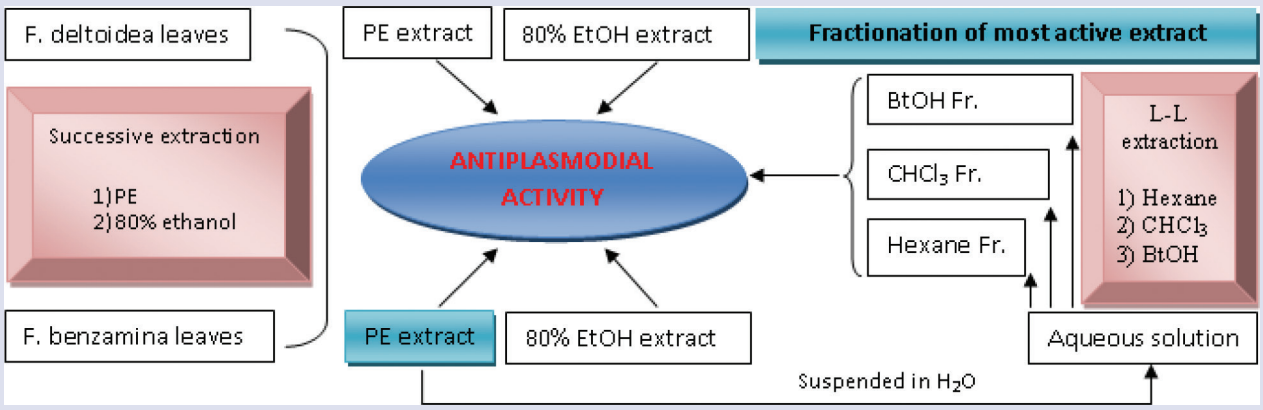

Figure 2: Extraction protocol of FDL, FBL and its fractions.

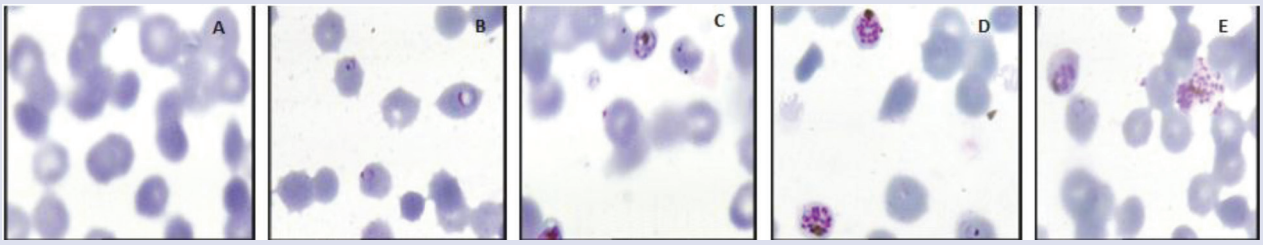

Figure 3: Microscopic view of different stages of parasite development A) Fresh RBC B) Ring stage C) Trophozoite stage D) Schizont stage E) Merozoite stage.

than three nuclei) were seen in the control wells, the experiment was considered valid for study Figure 3. The thin smear of each well was then prepared and after Giemsa staining the schizonts was counted out of a total 200 asexual parasites. IC_{50} values, indicates the concentration of the test sample where 50% of schizont maturation is inhibited as compared with parasite development in positive control. Negative control is maintained with fresh red blood cells, positive control is maintained with parasitized blood cell culture without any treatment.

RESULT AND DISCUSSION

In the present study, the crude extracts of FDL and FBL were evaluated for *Anti-plasmodial* activity by schizont maturation inhibition assay Table 3. According to WHO guidelines, *Anti-plasmodial* activity was classified as follows: highly active/good at IC_{50} < 5 µg/ml, promising at 5–15 µg/ml, moderate at 15–50 µg/ml and inactive at >50 µg/ml.[33] Based on this classification, the crude hydro-alcoholic extract (FDM) of FDL have IC_{50} greater than 50 µg/ml whereas its petroleum ether extract (FDPE) showed moderate *Anti-plasmodial* activity with IC_{50} 26 µg/ml. This result indicates that FDPE is more active than FDM. Furthermore, hydro-alcoholic extract (FBM) of FBL exhibited moderate inhibitory effect with IC_{50} 31.8 µg/ml and its petroleum ether extract (FBPE) showed most promising *Anti-plasmodial* effect with IC_{50} 14.5 µg/ml. The comparison of activity of FDL and FBL suggests that FBL extracts (FBM/FBPE) are more active against *P. falciparum* than FDL. Of the most active plant extract, different fractions of FBL petroleum ether extract (FBPE) was prepared and examined for *Anti-plasmodial* activity. Hexane (FBF_h), chloroform (FBF_c) and butanol (FBF_b) fractions of FBPE were prepared by successive liquid liquid extraction method and it was observed that FBF_h and FBF_c fractions of FBPE showed good to promising inhibitory effect on the maturity of schizonts with IC_{50} of 4.0 µg/ml and 7.8 µg/ml respectively whereas its FBF_b fraction was found to exhibit moderate *Anti-plasmodial* activity (IC_{50} 41.4 µg/ml).

Table 3: Antiplasmodial activity of FDL, FBL and its various fractions against *P. falciparum*.

Plant extracts	IC_{50} [a, b] (µg/ml)
FDM	53.4
FDPE	26
FBM	31.8
FBPE	14.6
FBFh	4.0
FBFc	7.9
FBFb	41.4
Chloroquine	0.03

[a]mean of three observations, [b]R^2 > 0.97

Further, phytochemical investigations of most active petroleum ether extract of FBL and its bio fractions revealed the presence of various group of chemical compounds Table 4. The preliminary phytochemical analysis confirms the presence of steroids, terpenoids, alkaloids and phytosterols in FBPE and its bio-fractions except butanol fraction (FBF_b).

The results obtained from this study revealed the rich chemical diversity of FBL which could be responsible for the *Anti-plasmodial* activity of FBPE and its bio-fractions. Literature also implicated several members of steroids, terpenoids and phytosterols have showed high significant inhibitory effect on the growth of *Plasmodium* parasite.[34,35] It could be considered that the *Anti-plasmodial* activity of FBL and its bio-fractions can be ascribed to the phytochemical constituents present in them, which further justified the use of FBL for the treatment of malaria in the tradi-

Table 4: Phytochemicals screening of FBPE and its bio-fractions.

Chemical class	Tests	FBL PE	FBF_h	FBF_c	FBF_b
Glycosides	Modified Borntrager's test	-	-	+	+
	Legal test	-	-	+	++
Phytosterols	Salkowski's test	+++	++	++	-
Steroids	Killer Killiani Test Liebermann Burchard test	+++	+++	+++	+
Phenols	Ferric chloride test	++	-		
Tannins	Gelatin test	++	++		
Flavonoids	Alkaline reagent test	-	-	+	+
	Lead acetate	-	-	+	++

tional medicine. Since, crude petroleum ether extract of both the plants and various bio-fractions of FB showed significant inhibitory effect on *Plasmodium* growth but both traditional drugs were found to be less effective in comparison to standard antimalarial drug. Thus, further efforts are needed for the isolation and identifications of chemical constituents of most active bio-fraction of FBPE such as FBF_h and FBF_c. Hence, to get an actual finding, more refining of plant constituents could be required to develop novel plant based drug molecules with high *Anti-plasmodial* activity against sensitive as well as resistant strains of *Plasmodium* parasite.

CONCLUSION

The present study demonstrates the successful application of traditional plant based drugs in the treatment of malaria. *In vitro Anti-plasmodial* drug screening of *F. deltoidea* and *F. benjamina* leaves justifies its traditional usefulness in modern medicine. The crude petroleum ether extract of *F. benjamina* was found to be more active of two allied species of genus *Ficus*. Moreover, its hexane and chloroform fractions can be considered as an important lead in the discovery of new antiplasmoidal drug molecule which could only be possible by isolating pure active constituents. Thus, a further study on the isolation of *Anti-plasmodial* molecules from the active fractions of *F. benjamina* is in process and will be reported in near future.

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CONFLICTING INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS USED

FD-*Ficus deltoidea*, **FDL**- *Ficus deltoidea* leaves**, FB**- *Ficus benjamina*, **FBL**- *Ficus benjamina* leaves**, FBPE**- *Ficus benjamina* Petroleum ether extract**, FBFh**- *Ficus benjamina* hexane fraction**, FBFc**- *Ficus benjamina* chloroform fraction**, FBFb**- *Ficus benjamina* butanol fraction.

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GRAPHICAL ABSTRACT

SUMMARY

Antiplasmodial activity of both *F. benjamina* and *F. deltoidea* leaves were evaluated and it was determined that *F. benjamina* petroleum ether extract exhibited most promising inhibitory effect on the growth of schizonts with IC_{50} 14.5 µg/ml. Bio-assay guided fractionation of petroleum ether extract of *F. benjamina* was performed and highest activity was showed by its hexane and chloroform fraction with very good antiplasmodial activity having IC_{50} 4.0 µg/ml and IC_{50} 7.8 µg/ml respectively.

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