Advance Access Publication 2 May 2008

eCAM 2010;7(4)441–445
doi:10.1093/ecam/nen030

Original Article

In Vitro Anti-plasmodial Activity of *Trigonella foenum–graecum* L.

M. Palaniswamy[1], B. V. Pradeep[1], R. Sathya[1] and J. Angayarkanni[2]

[1]Department of Microbiology, Karpagam Arts and Science College (Autonomous), Coimbatore 641 021 and
[2]Department of Biotechnology, Bharathiar University, Coimbatore 641 046, Tamilnadu, India

Developing countries, where malaria is one of the most prevalent diseases, still rely on traditional medicine as a source for the treatment of this disease. For the present study, *Trigonella foenum-graecum* L. (fenugreek) were collected from Coimbatore, Tamilnadu, India. The test plant has been used in India by traditional healers for the treatment of fever as well as other diseases. The active principle was extracted out in different solvent systems to assess the anti-plasmodial potential, with an aim that they can further be utilized to formulate drugs. *In vitro* anti-plasmodial assay of the extracted fractions of fenugreek leaves was carried out using laboratory adapted chloroquine sensitive and resistant *Plasmodium falciparum* isolates. Schizont maturation inhibition assay was adopted to analyze the potential of the extracts. Ethanol extract (50%) seemed to possess profound anti-plasmodial activity with IC_{50} value of $8.75 \pm 0.35\,\mu g\,ml^{-1}$ and $10.25 \pm 0.35\,\mu g\,ml^{-1}$ against chloroquine sensitive and resistant *P. falciparum* isolates, respectively. Among the investigated six fractions of the plant extracts, two were found to have significant anti-plasmodial activity with IC_{50} values $<10\,\mu g\,ml^{-1}$, namely ethanol and butanol extracts. Two extracts chloroform and ethyl acetate showed moderate activity with IC_{50} values ranging from 10 to $20\,\mu g\,ml^{-1}$, and the other two extracts, hexane and water appeared to be inactive with IC_{50} values $>85\,\mu g\,ml^{-1}$. In addition, preliminary phytochemical screening of the various extracts indicated the presence of alkaloids, saponin, tannin like phenolic compounds, flavonoids and steroids.

Keywords: anti-plasmodial activity – malaria – medicinal plants – phytochemical screening – *Plasmodium falciparum*

Introduction

Malaria is one of the most prevalent, devastating parasitic infectious diseases in the world. Each year, 300–500 million clinical cases and 1.5–2.7 million deaths associated with malaria are reported globally (1). According to the World Health Organization (WHO; 2), malaria is endemic in 91 countries, predominantly in Africa, Asia and Latin America, with about 40% of the world's population at risk (3). The problems of controlling malaria in these countries are aggravated by inadequate health structures and poor socioeconomic conditions. It is distributed widely, mainly due to the multi-drug resistance developed by *Plasmodium falciparum*. Of the four species of protozoan parasite *Plasmodium* that cause malaria in humans, *P. falciparum* is so far the most virulent and probably the best studied pathogen after human immunodeficiency virus and *Mycobacterium tuberculosis* (4). Despite over 22 years of efforts, a human malaria vaccine has not yet gone into routine use; nevertheless a considerable progress has been made (5). The situation has become even more complex over the last few years with prevalence of multi-drug resistant strains and the cases of adverse reaction of available anti-malarial drugs (6,7).

Mortality and morbidity due to malaria are a matter of great concern throughout the world, especially in tropical and subtropical regions. Even though casualty in children below the age of 5 years is very high, the disease affects

For reprints and all correspondence: M. Palaniswamy, Department of Microbiology, Karpagam Arts and Science College (Autonomous), Coimbatore 641 021, Tamilnadu, India. Tel: +91-422-2611146; Fax: +91-0422-2611043; E-mail: m.palaniswamy@gmail.com

all age groups. The pathogenesis occurs during erythrocytic stages. A peculiarity of *P. falciparum* is its ability to adhere to vascular endothelium (cytoadherence) of erythrocytes infected with maturing parasites. Now the severe and complicated cerebral malaria due to *P. falciparum* is compounded by the chloroquine-resistant parasites. Chloroquine, though effective as a blood schizontocidal, is ineffective or partially effective in resistant cases. Spread of multi-drug-resistant strains of *Plasmodium* and the adverse side effects of the existing anti-malarial drugs have necessitated the search for novel, well tolerated and more efficient anti-malarial drugs (6,8). Development of new therapeutic approaches to malaria is very much needed, since resistance of parasites to different anti-malarials is fast developing. This initiated intensive efforts for developing new anti-malarials from indigenous plants (i.e. medicinal plants for tackling the ever-burning problem and thus needless to say) has become one of the prime focuses of research in malaria.

Herbal medicine remains one of the common forms of therapy available for much of world's population. According to the WHO, about three-quarter of the world's population currently uses herbs and other forms of traditional medicine to treat diseases (9). The therapeutic properties of higher plants offer a virtually untapped reservoir of potentially useful sources of drugs that will continue to serve humankind into the 21st century as they have done since dawn of history. Plants medicinal potions have been exploited in treating maladies from eczema and malaria to respiratory disorders. The present study has been focused on evaluating the potential of various extracts of *Trigonella foenum-graecum* L. for anti-parasitic effects.

Materials and Methods

Plant Materials

The young leaves of *T. foenum–graecum* L. were harvested in April 2004 from Coimbatore, Tamilnadu, India, based on ethnomedical data and interview with local communities. This was authenticated by Botanical Survey of India, Coimbatore division. The young leaves were collected and washed thoroughly with water and air-dried under shade and ground using a kitchen blender.

Plants Crude Extracts

The dried and ground plant materials (50 g) were extracted with 50% ethanol (600 ml) for 48 h using soxhlet apparatus till the solvent became colorless in the siphon. The ethanol extract was filtered through Whatmann No. 1 (Whatmann International Ltd, Maidstone, UK) paper and filtrates were freeze-dried using lyophilizer to yield 36.86% w/w referred as crude extracts. The residue was dried over night and then extracted with 500 ml water by shaking in a water bath shaker at 70°C for 2 h. Ten grams of crude extracts was dissolved in 200 ml of 50% ethanol and put in a separating funnel. Hundred milliliter of hexane was added to the above solution and shaken thoroughly for 5 min and kept for 1 h at room temperature. The upper layer was collected as hexane fraction. To the remaining lower fraction 100 ml of chloroform was added and the whole process was repeated. In this extraction process, the upper layer was collected as chloroform fraction. Another extraction was done with the addition of 100 ml ethyl acetate and upper layer was collected as ethyl acetate fraction. To the left over fraction 100 ml of butanol was added to proceed in a similar manner and upper layer was collected as butanol fraction. The extracts were preserved at -20°C until use.

Phytochemical Screening of Plant Extracts

A preliminary phytochemical analysis of the plant extracts was carried out using thin-layer chromatography (TLC). Standard screening tests using conventional protocol (10) were utilized for detecting the presence of alkaloids, saponins, tannins/phenolic compounds, flavonoids and steroids.

Cultivation of *P. falciparum* and *In Vitro* Anti-Plasmodial Tests

Laboratory adapted chloroquine sensitive and resistant *P. falciparum* isolates were used for this study. The parasites were maintained in continuous culture in human red blood cells (O^+) diluted to 5% haematocrit in RPMI 1640 medium supplemented with 25 mM HEPES, 30 mM $NaHCO_3$ and 10% human AB^+ serum (11). The anti-plasmodial activity was performed in triplicate in a 96-well microtiter plate, according to WHO method that is based on assessing the inhibition of schizont maturation (2). The culture was synchronized using 5% aqueous solution of sorbitol (one portion of pellet and nine portions of sorbitol) and kept for 5 to 7 min at room temperature. This ensures killing of all other stages except rings. It was centrifuged for 5 min at 1500 r.p.m. The supernatant was discarded and the pellet was washed with incomplete media twice. Parasitaemia was adjusted to about 1% for the assay by diluting with freshly washed RBCs. Extracts were dissolved in DMSO (20 mg ml^{-1}) and diluted in medium to final concentration between 150 and 1 μg ml^{-1}. For the positive control wells, parasitized red blood cells were devoid of plant extracts and compounds whereas only non-parasitized red blood cells were prepared for the negative control wells. Fifty microliters from blood mixture media was added to each well in plate and incubated in CO_2 condition at 37.5°C for 24–36 h. After incubation,

contents of the wells were harvested and stained for 30 min in a 2% Giemsa solution pH 7.2. The developed schizonts were counted against the total asexual parasite count of 200. The percent inhibition at each concentration was determined and the mean of the least three IC_{50} values of parasite viability was calculated using mathematical log-concentration–response probit analysis (12).

Results

Phytochemical Screening

The phytochemical screening of medicinal plant showed the presence of alkaloids, saponin, tannin like phenolic compounds, flavonoids and steroids. The presence of alkaloids, tannin like phenolic compounds, steroids, in ethanol extracts and alkaloids and tannin like phenolic compounds in butanol extracts may contribute the anti-plasmodial activity of this traditional herb (Table 1).

In vitro Anti-Plasmodial Studies

The results of *in vitro* anti-plasmodial activity of the extracts are shown in Fig. 1. The most interesting anti-plasmodial activity was obtained with 50% ethanol extract against chloroquine sensitive and resistant *P. falciparum* with the least IC_{50} value of $8.75 \pm 0.35\,\mu g\,ml^{-1}$ and $10.25 \pm 0.35\,\mu g\,ml^{-1}$, respectively. Similarly, butanol fraction showed good IC_{50} value of $9.25 \pm 0.35\,\mu g\,ml^{-1}$ activity against sensitive *P. falciparum;* and moderate activity against resistant *P. falciparum* IC_{50} value of $26.25 \pm 1.77\,\mu g\,ml^{-1}$. The chloroform and ethyl acetate extract showed the IC_{50} against chloroquine sensitive *P. falciparum* was 16 ± 0.00 and $16.50 \pm 0.07\,\mu g\,ml^{-1}$ where as IC_{50} value against chlroroquine resistant *P. falciparum* isolate was $23.75 \pm 1.76\,\mu g\,ml^{-1}$. The other two extracts, hexane and water appeared to be inactive with IC_{50} values $>85\,\mu g\,ml^{-1}$.

Table 1. Phytochemical screening of the various extracts of *T. foenum–graecum*

Extracts	Alkaloids	Saponin	Tannin/ Phenolic compound	Flavonoids	Steroids
Ethanol	+	–	+	–	+
Water	–	+	–	–	–
Hexane	–	–	–	–	+
Chloroform	–	–	–	+	–
Ethyl acetate	–	–	–	+	–
Butanol	+	–	+	–	–

'+' Present; '–' absent

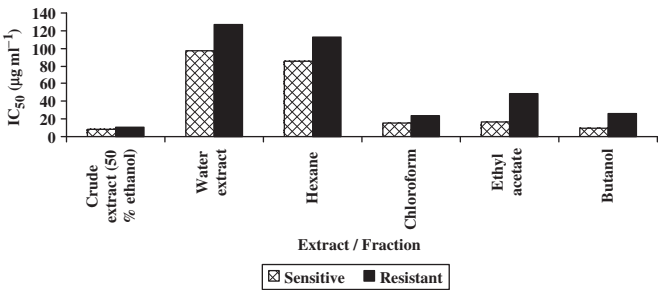

Figure 1. *In vitro* anti-plasmodial effect of the crude extract/fraction of *T. foenum–graecum* against chloroquine sensitive and resistant *P. falciparum isolates* (Choroquine IC_{50} value = $0.23\,\mu g\,/ml^{-1}$ positive control).

Discussion

WHO experts say that the number of people worldwide infected with malaria is still increasing at the rate of about 5% annually despite the extensive programs conducted by them (13). Preventing transmission by mosquitoes, vaccination and drug treatment are the three rapidly developing areas of malaria research. Of the various means available for the control of malaria, the use of effective drugs against the parasite remains the most important and is likely to remain so for a considerable time to come. A research for newer and more efficient drugs has become an important aspect of anti-malarial research (14). Plants have been used for decades as traditional medicine for the treatment of malaria. Malarial drugs such as quinine and artemisinin have also been derived from plants and continue to be effective in treating malaria. In the present study, an *in vitro* assay of the anti-plasmodial activity of *T. foenum–graecum* has been carried out.

The phytochemical screening of medicinal plants showed presence of various components in the different extracts, which may be responsible for the anti-plasmodial activity. Alshawsh (3) reported that presence of tannins, polysaccharides and proteins isolated from Yemeni medicinal plants were responsible for the anti-plasmodial activity. MacKinnon (15) demonstrated that the presence of limnoids, and triterpenoids from *Azadirachta indica* plant showed anti-malarial effect *in vitro*. Some alkaloids (krukovin and limacrin) isolated from *Abuta grandifolia* have been reported to be active against *P. falciparum* (16). Previous phytochemical investigations on *Stephania abyssinica* revealed the presence of hasubanan alkaloids (17–19) in the ethanol extracts of the roots. In the present study, the alkaloid fraction of ethanol and butanol extracts have been found to exhibit the highest activity against *P. falciparum*. From *Piper* species, isolation of pseudo-dipllapiol, benzoic-acid derivatives, flavanones and chalcones has been reported with anti-plasmodial effect (20,21). Muregi *et al.* (22) have reported that the methanolic extracts of leaves and root bark of *Clerodendrum myricoides* showed good

activity against all the test isolates. *C. myricoides* has been also reported to be useful in the management of other parasitic diseases such as theileriosis.

In most reviews of plants used as anti-malarial, ethanobotanical listings have been made with experiments conducted against *P. falciparum* in individual studies (23).

The anti-plasmodial activity of *S. abyssinica* and *Ajuga remota* was previously reported by Muregi *et al.* (22). They found that the aqueous extract of plant leaves showed the IC_{50} values $>20\,\mu g\,ml^{-1}$ which is in agreement with our results. Previous bioassay-guided phytochemical investigations showed anti-plasmodial (24) activity.

Siems *et al.* (25) have reported that the highest anti-parasitic activity *in vitro* was detected in extracts of *Siparuna andina* with an IC_{50} of $3.0\,mg\,ml^{-1}$ and $3.9\,mg\,ml^{-1}$ for *P. falciparum* strain poW and Dd2, respectively, and also they have reported that prolonging the incubation time of the anti-plasmodial assay to 72 h slightly increased the selectivity indices.

Ekebergia capensis hexane extract was found to exhibit no anti-plasmodial activity *in vitro* against *P. falciparum*. However, the chloroform, ethyl acetate, methanol and water extracts gave good IC_{50} values ($<5\,\mu g\,ml^{-1}$) suggesting that the plant extracts have a high *in vitro* anti-plasmodial activity. The methanolic extract of *C. myricoides* leaves showed good anti-plasmodial activity (19). Similar findings were observed in the present study, with high anti-plasmodial activity in ethanol, butanol, chloroform and ethyl acetate extract.

The *n*-butanol extract of *Eurycoma longifolia* roots displayed higher anti-plasmodial activity of $0.34\,\mu g\,ml^{-1}$ than its diethyl ether extract of $1.50\,\mu g\,ml^{-1}$. Both these extracts were more potent than chloroquine diphosphate ($2.50\,\mu g\,ml^{-1}$) against the Gombak A isolate of *P. falciparum* (12). Alshawsh (3) demonstrated that the anti-plasmodial activity of aqueous extracts of *Acalypha fruticosa* ($IC_{50} = 1.6\,\mu g\,ml^{-1}$), *Azardirachata indica* ($IC_{50} = 2.0\,\mu g\,ml^{-1}$) and of *Dendrosicyos socotrana* ($IC_{50} = 2.3\,\mu g\,ml^{-1}$).

The specific changes in morphology produced by particular extract hints at different modes of action of the putative active principles in the extracts. The parasitaemia also decreased with increasing concentration of the extract reflected an inhibitory activity on parasite replication. This may be indicative of a significant potential for isolating purer compound. There are cases where the individual isolated components may not exhibit activity unlike their combinations in the crude extracts. It is therefore necessary to carry out detailed phytochemical studies to identify the active constituent of the test plant.

Crude extracts are the simplest of available medications and are still promoted by WHO policies as emerging alternative systems of medicine to reach the large population not covered by formal medical care in remote areas. Crude plant extract that showed lower activity upon fractionation have yielded purer compounds with potent anti-malarial activity.

In conclusion, some of the alkaloids and tannins like phenolic compounds from *T. foenum–graecum* L. showed potential anti-plasmodial properties against *in vitro* culture of chloroquine sensitive and resistant *P. falciparum*. Further studies on these extracts are important since they can probably serve as biochemical tools for the understanding of the chloroquine resistance and the mechanism of reversal in *P. falciparum*.

In this regard the result of this preliminary study is very much encouraging. This is the first report on *in vitro* anti-plasmodial effect of *T. foenum–graecum*. Further studies can be carried out for the isolation of active principle and elucidation of chemical structure with an objective of exploring the possibility of using the component as oral/parenteral drug for treating malarial infection.

Acknowledgements

M.P. is sincerely grateful to the Management, Karpagam Arts and Science College (Autonomous), Coimbatore, Tamilnadu, India for providing support and encouragement. This article is dedicated to Late K.G. Purnima, who has contributed to this work to a greater extent.

References

1. World Health Organization. *Fact sheet No. 94*. Geneva: WHO, 1998.
2. World Health Organization. *In Vitro Micro Test (MarkIII) for the Assessment of the Response of Plasmodium falciparum to Chloroquine, Mefloquine, Quinine, Amodiaquine, Sulfadoxine/Pyrimethamine and Artemisinin*. Geneva: WHO, CTD/MAL/97, 20, 2001.
3. Alshawsh MA, Mothana RA, Al-shamahy HA, Alsllami SF, Lindequist U. Assessment of antimalarial activity against *Plasmodium falciparum* and phytochemical screening of some Yemeni medicinal plants, 2007, 1–4. Advance Access published online (October 22, 2007), doi:10.1093/ecam/nem148.
4. Russell FD. The parasite genome: the grant assault. *Nature* 2002;419:493–4.
5. Greenwood B, Mutuabingwa T. Malaria in 2002. *Nature* 2002; 415:670–2.
6. Bickii J, Njifutie N, Foyere JA, Basco LK, Ringwald P. In vitro antimalarial activity of limonoids from Khaya grandifoliola C.D.C. (Meliaceae). *J Ethnopharmacol* 2000;69:27–33.
7. McGregor IA. Epidemiology, malaria and pregnancy. *J Trop Med Hyg* 1984;33:517–25.
8. Olliaro PL, Trigg PI. Status of antimalarial drugs under development. *Bull. World Health Organ* 1995;73:565–71.
9. Rao CGG, Motiwale AV, Satyanarayana D, Subrahmanyam EVS. Formulation of taste masked oral suspension of quinine sulphate by complexation. *Indian J Pharm Sci* 2004;66:329–31.
10. Wagner H, Bladt S. *Plants Drug Analysis: A Thin Layer Chromatography Atlas*, 2nd edn. Berlin: Springer, 1996, 306–64.
11. Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976;193:673–5.
12. Chan KL, Choo CY, Abdullah NR, Ismail Z. Antiplasmodial studies of *Eurycoma longifolia* Jack using the lactate dehydrogenase assay of *Plasmodium falciparum*. *J Ethnopharm*. 2004;92:223–7.
13. World Health Organization. *Malaria distribution. A weekly epidemiological record*, Vol. 71. Geneva: WHO, 1996,17–24.

14. Biagini GA, Neill PM, Nzila A, Ward SA, Bray PG. Antimalarial hemotheraphy: Young Guns are back to the future? *Trends parasitol* 2003;19:479–87.
15. MacKinnon S, Durst T, Arnason JT, Angerhofer C, Pezzuto J, Sanchez-Vindas PE, et al. Antimalarial activity of tropical Meliaceae extracts and gedunin derivatives. *J Nat Prod* 1997;60:336–41.
16. Steele JC, Simmonds MS, Veich NC, Warhurst DC. Evaluation of the anti-plasmodial activity of bisbenzylisoquinoline alkaloids from *Abuta grandifolia*. *Planta Medica* 1999;5:413.
17. Kupchan SM, Leipa AJ, Fujita J. New phenolic husubanan alkaloids from Stephania abyssinica. *J Org Chem* 1973;38:151–3.
18. Southon IW, Buckingham J. *Dictionary of Alkaloids*. London: Chapman and Hall, 1989,1001.
19. Dagne E, Ganatilaka AAL, Kingston DGI, Alemu M. 4′-Omethylstiphavanine from Stephania abyssinica. *J Nat Prod* 1993;56:2022–5.
20. Vieira PC, De Alvarenga MA, Gottlieb OR, Gottlieb HE. 4-Hexadecenylphenol and flavonoids from *Piper hispidum*. *Planta Medica* 1980;39:153–6.
21. Burke B, Nair M. Phenylpropene, benzoic acid and flavonoid derivatives from fruits of Jamaican *Piper* species. *Phytochemistry* 1986;25:1427–30.
22. Muregi FW, Chhabra SC, Njagi ENM, Lang'at-Thoruwa CC, Njue WM, Orago ASS, et al. Anti-plasmodial Activity of Some Kenyan Medicinal Plant Extracts Singly and in Combination with Chloroquine. *Phytother Res* 2004;18:379–84.
23. Bradeley D, Warhurst D. Guideline for the prevention of malaria in travelers from United Kingdom. *CDR Rev* 1997;7:138–51.
24. Kuria KAM, De Coster S, Muriuki G, Masengo W, Kibwage I, Hoogmartens J, et al. Anti-malarial activity of Ajuga remota Benth. (Labiatae) and Caesalpinia volkensii (Caesalpiniaceae) in vitro confirmation of ethnopharmacological use. *J Ethnopharm* 2001; 74:141–8.
25. Siems KJ, Mockenhaupt FP, Bienzle U, Gupta MP, Eich E. *In vitro* antiplasmodial activity of Central American medicinal plants. *Trop Med Int Health* 1999;4:611–15.

Received December 11, 2007; accepted March 11, 2008