Current Trends in Biotechnology and Pharmacy
Vol. 17(4) 1404-1408,October 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2023.4.75

Anti-malarial Activity of Indian Medicinal Plant Extracts by Haemazoin Inhibition Assay

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Abstract

Many traditional plants are promising source of antimalarial drugs. Present study is proposed to evaluate *in vitro* antimalarial effect of selected plant extracts. Methanol extract of nine plants were evaluated for antimalarial activity against Chloroquine-sensitive (MRC-2) and chloroquine-resistant (RKL-9) strains of *P. falciparum*. Evaluation was carried out by colorimetric quantification of haemazoin inhibition. Results indicated that all the plant extracts showed antimalarial activity in dose dependent manner. Methanol extract of *E. hirta*, *E. thymifolia* and *B. variegate* showed moderate antimalarial activity against MRC-2 and RKL-9 strains. Methanol extract of leaves of *B. variegata* demonstrated highest activity with lowest IC_{50} 21.85 µg/mL and 26.51 µg/mL against MRC-2 and RKL-9, respectively amongst all extracts. This result potentially justifies the antimalarial use of this plant.

Keywords: Antimalarial, Haemazoin, Plant extracts, *P. falciparum*

Introduction

Malaria is a critical health trouble in tropical and developing countries. Every year more than 400 000 people die due to malaria, in spite of preventable and treatable disease (1). Malaria is caused by parasites of the genus *Plasmodium*. There are five *Plasmodium* species that cause malaria in humans; out of these *P. falciparum* is most pathogenic and responsible for the greater number of cases and mortality in tropical and subtropical regions (2). Malaria control is currently depending on mosquito vector prevention and chemotherapy but resistance toward insecticide and antimalarial drugs is a bigger challenge (3-5). To overcome this situation every year thousands of compounds are screened for antimalarial drug discovery but out of them very few are effective and successfully rich to the final stage. Developing completely new moieties from a ground is so complicated process and need 12–15 years as well as costs more than $1 billion till finished product in the market (6). There is necessity to discover new molecule with promising mechanism of action at starting phase. There are many methods for screening an antimalarial potential of drugs like isotopic assays, *p*LDH enzymatic assay, *p*LDH DELI assay, HRP2 assay, DNA dye assay, histidine-rich protein II antibody assay and many more (7). However, these methods involve complex protocol and not affordable for developing countries, which makes them incompatible for screening potential of antimalarial drugs. One simple and inexpensive method for the development of new treatments against malaria is to study the *Plasmodium* hemozoin (8).

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During erythrocytic stage of malaria parasites, parasites use hemoglobin as a source of amino acid; degrade the hemoglobin and converted Fe^{+2} into Fe^{+3}. Fe^{+3} (ferric form) is oxidatively very active and harmful for host and parasite both (9). It may cause death of parasite also. Parasites remove this harmful and toxic haem via detoxification mechanism like neutralization with histidine rich protein-2 (10), reduced glutathione (11), peroxidative degradation or crystallization into haemozoin. Haemozoin (water insoluble pigment) formation is most common pathway for detoxification of heam (12). Quantification of haemozoin formation is a very simple, cheap and affordable colorimetric method for *in vitro* screening of antimalarial drugs or plant extracts.

Materials and Methods

Chemicals

RPMI-1640 media with phenol red, glutamine and HEPES buffer, albumax II, hypoxanthine, Sodium bicarbonate ($NaHCO_3$), sorbitol, Dimethyl sulphoxide (DMSO) and Giemsa stain were of cell culture grade and purchased from Himedia Laboratories. Quinine diphosphate and artemisinin were purchased from Sigma-Aldrich. All other chemicals and solvents were of analytical grade.

Plant materials and extraction

Leaves of *Ailanthus excelsa*Roxb (SU/DPS/Herb/58), *Bauhinia variegate*(Herb/61), *Alstoniascholaris*(L.) BR. (Herb/32),and *Caesalpinia crista* L. (Herb/65), Aerial parts of *Euphorbia hirta* (Herb/56), *Euphorbia thymifolia*(Herb/56), *Fagoniaarabica*(Herb/63/B) and *Leucasaspera*(Herb/18)and root of *Demiaextensa* (Herb/63/A)were collected from Saurashtra region, Gujarat, India. Voucher specimens were deposited in the Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, India. The collected plants were washed with water and dried under shade. The dried plant material of each plant species was grounded into fine powder (60 meshes). Each plant were extracted at 60°C with methanol by hot maceration method.The filtrates were dried at 40 °C by using rotatory vacuum evaporator.

In vitro cultivation of Plasmodium falciparum

Antimalarial activity of plant extracts was performed against chloroquine sensitive (MRC-2) and chloroquine-resistant (RKL-9) strain of *P. falciparum*, procured from the National Institute of Malaria Research (NIMR), New Delhi, India. Parasites were maintained using a standard protocol of Trager and Jenson (13) in complete parasite medium with 5% hematocrit (human red blood cells)(RPMI-1640 with phenol red, 40 mg/L gentamycin, 20 mM HEPES buffer, 25 mg/L hypoxanthine, 0.4% albumax II and 2.1 g/L) and incubated at 37°C in 1% O_2, 5% CO_2 and 94% N_2 gas mixture. Cultures were examined using microscopy (Giemsa stain) at every 6, 12, 24 and 48 h for regular development of parasite stages. Synchronization was maintained with 5% sorbitol in cultures throughout the course of the study.

Correlation between parasitemia and hemazoin level

P. falciparum cultures (MRC-2 & RKL-9) were serially diluted with uninfected RBC with complete media (5% haematocrit and 0% to 10% parasitemia). To the 200 µL of above diluted culture add 800 µL of 2.5% SLS (sodium lauryl sulphate) in 0.1M $NaHCO_3$, pH 8.8. Incubate at room temperature for 15 min and then centrifuge at 13,000 rpm for 10 min., after centrifugation remove supernatant and repeat this procedure. Add 200 µL of 5% SLS in 50 mM NaOH, incubate at room temperature for 30 min. after 30 min transfer 200 µL of each solution to 96 well plate and scanned at 405/750 nm. The amount of haemozoin in the infected erythrocytes was presented as the absorbance at 405/750 nm and then plotted against parasitaemia.

Evaluation of antimalarial potential of plant extracts by colorimetric quantification of P. falciparum hemazoin level

For screening of extracts, stock solution of extracts was prepared in dimethyl sulphoxide and was then serially diluted with complete culture medium (10 to 500 µg/mL). 10 µL of serially diluted drug/extract solution was added into 200 µL of asynchronous parasite culture. Dimethyl sulphoxide was also tested by adding a similar amount tocontrol wells. Chloroquine and artemisinin were used as the positive control. 96 well plates were incubated at 37°C for 48 h in CO_2 incubator. The haemozoin of infected erythrocytes was quantified as per above mentioned protocol. IC_{50} values were calculated by the log dose response curve using the Graph Pad Prism 5 software. Each analysis was performed in triplicate (n =3) (8).

Results and Discussion

During the erythrocytic cycle of malaria parasite, hemoglobin degradation is occur due to metabolic activity of parasite, this haemoglobin degradation release free heme, α-hematin (ferriprotoporphyrin IX) which is toxic for parasite (14). As self-defence, parasites detoxify heme using several pathways, polymerization of heme converting it to haemozoin (β-haematin). β-haematin is unreactive and water insoluble crystalline material called malaria pigment (15,16). Inhibition of β-haematin formation is very suitable target for malaria treatment. Many antimalarial drugs, chloroquines, 8-aminoquinolines, bisquinolines, pyrimethamine, and the combination therapy sulfanilamide–pyrimethamine inhibit the hemazoin formation (17,18). Hemazoin content is directly correlate with parasitemia. Many *in vitro* assay based on quantification of hemazoin have been mentioned in earlier research.

Relationship between hemazoin level and amount of parasites in infected culture was measured as an absorbance in both the strain. Both the strain showed linear relationship. Figure 1 represents a very good linear relationship of heam and parasitemia level in MRC-2 strain, it indicate that this assay method is best suited for in vitro assay. Figure 2 represents a linear relationship of heam and parasitemia level in RKL-9 strain, it indicate less linearity than the MRC-2 strain. This assay detected the absorbance of 1% parasitemia, it represents its sensitivity of this method.

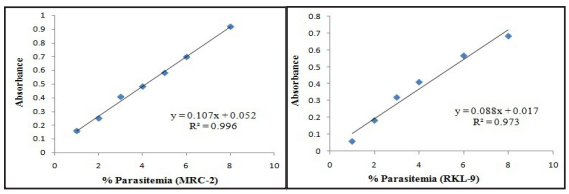

Figure 1: Linear relationship between hemazoin level and growth of parasites (% parasitemia) in (A) MRC-2 strain (B) RKL-9 strain

In current research nine plants were selected on the bases of traditional claim and ethnomedicinal uses. Methanol extracts of nine plants were evaluated for its antimalarial activity against chloroquine sensitive (MRC-2) and chloroquine resistant (RKL-9) strains by an *in vitro*colorimetric quantification of *P. falciparum*-hemazoin.Results of both the strain indicated linear relation between hemazoin content and parasitemia level. Based on this results we conclude that hemazoin inhibition assay of parasites is suitable for monitoring parasite growth as well as for screening new antimalarial compounds. All selected plants have been screened first time for hemazoin inhibition. Results of Table 1 indicated that methanol extract of *E. hirta*, *E. thymifolia* and *B. variegate* were showed most potent activity with IC_{50}24.03 µg/mL, 30.57 µg/mL and 21.85 µg/mL against MRC-2 strain, respectively and 37.01 µg/mL, 35.83 µg/mL and 26.51 µg/mL against RKL-9 strain respectively. As reported by previous studies, antimalarial activities of plant extracts were classified as promising activity (IC_{50} ≤ 15 µg/ml), moderate activity (IC_{50} = 15-50 µg/ml), weak activity (IC_{50} > 50 µg/ml), and inactivity (IC_{50} > 100 µg/ml) (19). Present study indicated that moderate antimalarial activity of *E. hirta*, *E. thymifolia* and *B. variegate* against MRC-2 and RKL-9 strains. *C. crista* and *A. scholaris* possess weak activity against MRC-2 and RKL-9 strains while *A. excels* showed weak activity against MRC-2.

Table 1: *In vitro* antimalarial activity of Plant extracts against *P falciparum* by haemozoin-based colorimetric method

Methanol extract	***P falciparum* MRC-2** IC_{50} (µg/mL)	***P falciparum RKL 9*** IC_{50} (µg/mL)
A. excelsa	84.72	>100
B. variegata	21.85	26.51
C. crista	98.13	87.23
A. scholaris	72.09	74.19
L. aspera	ND	ND
D. extensa	>100	>100
F. arabica	>100	>100
E. hirta	24.03	37.01
E. thymifolia	30.57	35.83
Chloroquine	0.051	0.082
Artemisinin**A**	0.0093	0.0098

B

B. variegate leaves, most active of nine plants, belongs to Fabaceae family. In the previous study, two phytoconstituents dihydrodibenzoxepins and flavanones were isolated from Bauhinia species and exhibited antimalarial activity (IC_{50} 5.8-11.2 *µ*M) (20). Leaves extract of *B. variegate* exhibited *in vivo* schizonticidal action against *P. berghei* in mice (21).

Conclusion

There is ongoing research focusing on the identification of effective plant extracts to treat malaria. Present method is rapid and required less expensive reagents and minimum sophisticated instrumentation. For the first time, present studyreports the *in vitro* antimalarial activity of leaves of *B. variegate* on haemozoin-based colorimetric method. The methanol extract possesses moderate antimalarial activity againstboth CQ- sensitive (MRC-2) and resistant (RKL-9) strains of *P. falciparum*. Further research required to isolate the bioactive compounds and throw light on their mechanisms of action.

References

1. WHO team (2019) Global Malaria Programme, World Malaria Report. Geneva. World Health Organization.
2. Snow, R.W. (2015) Global malaria eradication and the importance of *Plasmodium falciparum* epidemiology in Africa. BMC Med 13(23). https://doi.org/10.1186/s12916-014-0254-7
3. Tun, K.M., Jeeyapant, A., Imwong, M., Thein, M., Aung, S.S.M. et al.(2016) Parasite clearance rates in Upper Myanmar indicate a distinctive artemisinin resistance phenotype: a therapeutic efficacy study. Malar J 15(1):185.
4. Recht. J., Siqueira, A.M., Monteiro, W.M., Herrera, S.M., Herrera, S. et al. (2017) Malaria in Brazil, Colombia, Peru and Venezuela: current challenges in malaria control and elimination. Malaria Journal 16(1):273.
5. He, Y., Campino, S., Diez Benavente, E., Warhurst, D.C., Beshir, K.B, et al. (2019) Artemisinin resistance-associated markers in *Plasmodium falciparum* parasites from the China-Myanmar border: predicted structural stability of K13 propeller variants detected in a low-prevalence area. PLoS One 14(3). e0213686.
6. Di Masi, J.A., Grabowski, H.G. and Hansen, R.W. (2016) Innovation in the pharmaceutical industry: new estimates of R & D costs. J Health Econ 47:20–33.
7. Shweta, S., Phulen, S. and Rakesh, S. (2017) Development in Assay Methods for in Vitro Antimalarial Drug Efficacy Testing: A Systematic Review. Front Pharmacol 8:754.
8. Tran Thanh Men, Nguyen Tien Huy, Dai ThiXuan Trang, Mohammed Nasir Shuaibu, Kenji Hirayama, *et al*. (2012) A simple and inexpensive haemozoin-based colorimetric method to evaluate anti-malarial

drug activity.Malar J 11:272.

9. Tripathi, A.K., Khan, S.I., Walker, L.A. and Tekwania, B.L. (2004) Spectrophotometric determination of de novo hemozoin/b-hematin formation in an in vitro assay. Anal. Biochem 325(1):85–91.

10. Huy, N.T., Serada, S., Trang, D.T., Takano, R., Kondo, Y. et al. (2003) Neutralization of toxic heme by Plasmodium falciparum histidine-rich protein 2. J Biochem 133(5):693–698.

11. Atamna, H. and Ginsburg, H. (1995) Heme degradation in the presence of glutathione. A proposed mechanism to account for the high levels of non-heme iron found in the membranes of hemoglobin opathic red blood cells. J BiolChem 270 (42):24876–24883.

12. Egan, T.J. (2002) Physico-chemical aspects of hemozoin (malaria pigment) structure and formation. J Inorg Biochem 91:19–26.

13. Trager, W. and Jensen, J.B. (1976) Human malaria parasites in continuous culture. *Science*, 193(4254):673-675.

14. Orjih, A.U. (2001) On the mechanism of hemozoin production in malaria parasites: activated erythrocyte membranes promote beta-hematin synthesis. Exp Biol Med 226(8):746–752.

15. Sonnet, P. and Mullié, C. (2011) *In vitro* antimalarial activity of ICL670: a further proof of the correlation between inhibition of *β*-hematin formation and of peroxidative degradation of hemin. Experimental Parasitology. (2011)128 (1):26–31.

16. Egan, T.J. (2008) Haemozoin formation. Mol Biochem Parasitol 157(2):127–136.

17. de Villiers, K.A. and Egan, T.J. (2009) Recent advances in the discovery of haem-targeting drugs for malaria and schistosomiasis. Molecules 14(8):2868–2887.

18. Kim, Y.F. and David, W.W. (2013) Hemozoin and antimalarial drug discovery Future. Med Chem 5(12):1437–1450.

19. Jonville, M.C., Kodja, H., Humeau, L., De Mol, P., Cao, M. et al. (2008) Screening of medicinal plants from Reunion Island for antimalarial and cytotoxic activity. J Ethnopharmacol 120(3):382–386.

20. Boonphong, S., Puangsombat, P., Baramee, A., Mahidol, C. andRuchirawat, S, (2007) Bioactive Compounds from *Bauhinia purpurea* Possessing Antimalarial, Antimycobacterial, Antifungal, Anti-inflammatory, and Cytotoxic Activities. J Nat Prod 70(5):795-801

21. Banyal, H.S., Sharma, R. and Nisha Devi (2015) Antimalarial effect of *Ocimum sanctum* Linn. And *Bauhinia variegata* Linn. On *Plasmodium berghei*. IOSR J Pharm 10(2):70-72.