PHARMATUTOR
PHARMACY INFOPEDIA
ISSN: 2347 - 7881

Research Article

In-vitro screening of Dhatura Inoxia for its anti-Malarial activity against P. falciparum

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ABSTRACT

The natural products are important source of biologically active compounds having potential for developing a novel malaria drug. Malaria is one of the most important infectious vector born parasitic disease caused by *Coccidian protozoan* parasite of genus *Plasmodium* carried out by mosquito, invade red blood cells. Malaria is thousand times more complex than any disease. In the current study, a natural ingredient of herb; *Dhatura Inoxia* is analyzed, for its anti-malarial activity by preparing crude extract of water and ethanol, analyzed on schizont stage of plasmodial life cycle against two Chloroquine strains: sensitive and resistant of *Plasmodium falciparum* isolates. The results revealed that IC_{50} values of crude water extract was recorded as; 71± 1.41µg/ml and 105±7.07 while as the ethanol crude extract produced IC_{50} concentrates: 52±3.53 µg/ml and 59.5± 70 µg/ml against sensitive and resistant *P. f.* isolates, respectively. The results showed that this herb plant possess significant antiplasmodial activity in terms of IC_{50} as recorded in this study.

Key words: Anti-malarial activity, schizont, IC_{50}, herbs, crude extracts.

INTRODUCTION

Malaria still remains one of the greatest causes of illness and death in this world. It is estimated that malaria kills 1.5 to 2.7 million people globally; mostly children below five years and 300 to 500 million people suffer annually (WHO, 1996). Despite the considerable progress in malaria control over the past decade, malaria remains a disease of priority, particularly in Africa where about 90% of clinical case occurs. One of the greatest challenges facing malaria control worldwide is the spread and intensification of parasite resistance to antimalarial drugs. The limited number of such drugs has led to increasing difficulties in the development of antimalarial drug policy and adequate disease management (WHO, 2000). Developed countries are relatively free of malaria, but it remains well entrenched across the tropical world and greatest burden fall on Africa. Malaria parasite is one of the few infective agents of human that invade human red blood cells and completes their life cycle. All four species of malarial parasites, which infect human have a similar life cycle that alternates between human and mosquito. The clinical symptoms and sign are produced by the asexual forms of parasite which invades and destroy red blood cells, localized in critical organs and tissues across the body. The parasite invasion induces the release of many pro-inflammatory cytokines, produce fever and pain (Wright, C.W. and Phillipson D, 1990).

How to cite this article: SO Pratap, R Gaur, R Stephan, JL bhatt, CR Pillai, U Devi, In-vitro screening of Dhatura Inoxia for its anti-Malarial activity against P. falciparum, PharmaTutor, 2014, 2(4), 93-98

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The *P. Falciparum* is a predominant species which causes 120,000,000 deaths per year globally and in 1989, the World Health Assembly urged priority states and WHO to increase control efforts. Each year this disease infects upto half a billion people and resulting in two million death (Anon.,1979). The eradication of the disease has been hampered by the emergence and spread of multidrug resistant malarial parasites, especially *P. falciparum* strains resistant to many antimalarial drugs (Olliaro,1996).

The epidemiology of malaria depends upon a complex interplay between the host (Human), vector (Mosquito) and the complexity in the life cycle of malaria parasite. Sporozoites infected mosquitoes, bites to man, exo-erythrocytic parasites which subsequently develop in liver and sexual forms of parasite (macro and micro-gametocytes), which arise from the asexual forms do not cause clinical disease. Jenson, (1976) states that malaria is thousand times more complex ailment than anything else. The Artemisinin groups of a new drug target the parasite when it is in its ring-like form. early in the asexually dividing part in the blood stream (Pillai, 2001). The parasite is thus destroyed before it reaches its immature gametocyte stage in which, it is ingested by anopheles mosquitoes as blood meal. Artemisinin drug developed by the scientists of CDRI and CIMAP, Lucknow have short half-life in the human body and they are eliminated from the body rather quickly (1.6-2.6 hrs).

The main problem for malaria control, at present, is the antimalarial drug resistance, especially of Plasmodium falciparum, the most deadly malaria parasite (Krettli, 2001). Another important reason for the persistence of malaria in Africa is the presence of the vector, Anopheles gambiae, although social and economic factors are also worth mentioning. The female A. gambiae feeds preferentially on humans and is long-lived, making it particularly effective at transmitting malaria from one person to another. The spread of multi drug-resistant P. falciparum has highlighted the urgent need to develop new antimalarial drugs, preferably inexpensive drugs that are affordable for developing countries, where malaria is prevalent (Miller, 1992)

In view of the problems associated with antimalarial drug resistance, new drugs or drug combinations are urgently required today for the treatment of malaria. Preferably, new drugs should have novel modes of action or be chemically different from the drugs using currently (Wright and Phillipson, 1990). Development of new therapeutic approaches to malaria is very much needed; since the resistance of parasites has increased a lot against mostly used different antimalarial compounds like; primaquine, proquanil, pyrimethamine, metloquine etc. so there is an urgent need to produce alternative drugs with minimal side effects for humans (Usha Devi, 1996).

Due to limited availability or affordability of pharmaceutical medicines in many tropical countries, majority of the populations depend on traditional medical remedies (WHO, 2002; Zirihi *et al*., 2005), mainly from plants extracts since beginning. There are a series of new synthetic antimalarials that have been developed either or undergoing for trails against on different stages of *Pasmodial falciparum.* Most of the antimalarial drugs in use at the moment, such as quinine and artemisinin were either obtained directly from plants or developed by using plant-derived chemical compounds as template (Basco *et al*., 1994) which are widely acceptable as a potent treatment against malaria.

Plants have always been considered to be a possible alternative which found rich source of new valuable drugs. First time a natural product gained wide acceptance as a treatment for the Malaria was in the 16th century when the therapeutic action of the bark of Cinchona tree,

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ISSN: 2347 - 7881

disclosed by the natives of Peru to J. missionaries (Connelly, 1996).

The sincere intensive efforts are much needed for the development of new antimalarials from indigenous plants hence having much potential for the development of novel anti-malarial drugs (Singh & Panday, 1998). The natural products are important source of biological active compounds and have potential to be novel antimalarial drugs using by local people from many years to overcome malaria (Kalyman, 1985).

In this regard, preliminary study has been carried out for crude extracts/fractions of *D. innoxia* to analyse its antiplasmodial activity against chloroquine sensitive and resistant strains of *P. falciparum* isolates and encouraging results have been recorded.

MATERIALS AND METHODS

Plant Extracts:

Plants of D*. Innoxia* were collected from the lower altitude forests of Uttaranchal which is identified for its anti-malarial activity by local people. Plant material like; leaves and barks were dried in the shelter for preserving their chemical nature. The crude ethanol extracts (by cold extraction methods) and water extract (by boiling) were prepared respectively. From the crude ethanol extracts different fractions like hexane, chloroform, ethanol and butanol were also prepared by using separating funnels.

Preparation of Blood cells for culture:

The AB^+ blood is collected from patient's having *P. falciparum* infection in an anticoagulant and dispensed into 4-5 aliquots followed by spinning at 1500 rpm for 10 minutes and plasma & buffy clots removed with sterile pasture pipette. The washing media is added and supernatant was removed. Equal amount of complete media was again added to obtain 50% cell suspension and the cells are ready for the culture.

The infected blood from the malaria patient is collected and processed exactly like the normal erythrocytes and finally a suspension of 50% was prepared. An appropriate volume of the infected cells are added to the uninfected erythrocytes to get an initial parasitaemia of 0.1%. This suspension is diluted with complete media to make an 8% cell suspension and dispensed in the culture vials. The vials are placed into the desiccators with a lit candle and cover put on, after applying silicon grease, with the stopcock open. The incubation is carried out at 37^0C. The medium of culture was exchanged after every 24 hrs and parasitaemia is estimated by making the slides which reached on 2-5% after 96 hrs. (Connelly, 1996; Meshnick, 1997; Greenwood, 1992).

***In-vitro* screening of Antimalarial activity:**

The culture was synchronized by using 5% aqueous solution of Sorbitol for the degeneration of all other stages except ring. In this way, the similar stages were achieved to the culture and all degenerated stages are removed through centrifugation at 500 rpm for 5 minutes and the culture was dispersed in all the 96 wells. The material to be tested is dissolved in the 100µl of DMSO. The different concentration of plant extracts was dispersed in 96 well plates and first well remained as a control. The synchronized parasite was inoculated to all the wells including control well to get a final concentration of 5% hematocrit. The plates were incubated at 37^0C for 24-30 hrs depending on the maturation pathway of schizont and after the confirmation of their presence the smear was stained with Jeimsa stain.

Staining of Blood smear:

Blood smear, isolated from the patient stained with JSB-1 and JSB-2 stain for observing parasite morphology to determine parasitaemia. The smear was fixed with methanol and allowed to dry. Then smear was dipped into JSB-2 for few seconds and immediately washed with DW repeatedly. Then blood smear was dipped in

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ISSN: 2347 - 7881

JSB-1 strain for 40 to 50 seconds followed by proper washing with DW. Examination was carried out of the dry smear under the oil immersion for the morphological study of the parasites. The schizonts were counted per 200 asexual stages of parasites. The values were compared between control and test wells. The inhibition percentage of schizonts per each concentration of extract /fraction was calculated as,

Inhibition% = 100- A

Where A is the % of growth of schizonts in the test well which is determined by the following formula (Annon, 1979).

$$A = \frac{\textit{Number of Schizonts present in the test well}}{\textit{Number of schizonts present in the control}} \times 100$$

The IC_{50} values, the concentration at which the inhibition of parasite growth represents 50% were calculated from the plot of the probit of Chloroquine activity and logarithm of drug concentration by linear regression analysis (Usha Devi, 1996; Pillai, 2001; Trager, 1976).

Fluorescent microscopy:

200cells/field for each well were selected for the analysis of Schizonts by using high-power fluorescent microscopy and oil immersion was used the better observation. The percentage of growth inhibition with respect to the control was determined by simple arithmetic calculations.

Results:

Study of parasite morphology revealed out that different fractions of selected plant have recorded for the differential antimalarial activities (Table 2) carried out as number of parasites in the form of mature schizonts out of total of 200 parasites. The crude extracts of *D. Innoxia in* water and ethanol was recorded in the current study to analyze antimalarial activity in terms of IC_{50} values. The crude extract of water was obtained such as; 71±1.41µg/ml for Chloroquine sensitive while remain (105±7.07) against Chloroquine resistant isolates of *P. falciparum.* The ethanol crude extract produced IC_{50} concentrates: 52±3.53 µg/ml and 59.5±70 µg/ml against sensitive and resistant *P. f.* isolates, respectively.

Current study also revealed the analysis of others different fractions of *D. Innoxia* in chloroform, butanol, hexane, ethanol and ethyl acetate solvents tested further which were recorded as; 7.25± 0.35 µg/ml, 69±7.07µg/ml, 51± 1.41 µg/ml, 86± 8.48 µg/ml, 32.5 ±3.35 µg/ml against chloroquine sensitive while 9.75± 0.35 µg/ml, 19±0.70 µg/ml, 90± 0.00 µg/ml, 140± 14.12µg/ml, 69±4.24 µg/ml respectively for the chloroquine resistant strains of *P. falciparum* isolates. IC_{50} values of 16 µg/ml or less is considered to be effective and thus the results of this preliminary study are very much encouraging. Not more work has been published on *D. Innoxia* for its antimalarial effect against *P. falciparum* isolates.

Table-1. *In-vitro* anti-plasmodial effect of the water and ethanol extract/fractions of *Datura innoxia* against chloroquine resistant and susceptible isolate of *P. falciparum*

Material Tested	I C_{50}(µg/ml)	
	Susceptible	Resistant
Crude extract 100% Ethanol	71±1.41	105±7.07
Crude water extract 100%	52±3.53	59.5±70
Chloroform fraction	7.25±0.35	9.7±0.35
Butanol fraction	69±7.07	119.5±0.70
Hexane	51±1.41	90±0.0
Ethanol fraction	86±8.48	140±14.41
Ethyl acetate	32.5±3.53	69±4.24

DISCUSSION

The result of above studies indicates that *Datura innoxia* possess significant antiplasmodial activity as recorded in term of IC_{50}. In most reviews of plants used as antimalarials, ethno-botanical listings have

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ISSN: 2347 - 7881

been conducted against *P. berghei* and cannot be applied to *P. falciaprum* in individual studies (web). The utilization of crude extracts were the simplest method of available medications by the tribals people for treatment and are still also promoted by WHO policies as emerging alternate systems of medicine to reach the large population not covered by formal medical care in remote areas. The Indian system of Ayurveda, Siddha, Unani and Homeopathic have listed also such plants because they have many active biological active components requires much scientific validation. Several new methods of in-vitro assessment have been introduced with the advent tools of Biological sciences to make reliable identification and confirmation of drug resistant parasite and assessment of a new drug. The newest generation of drug-sensitivity assay might provide one step in this direction to discover potent malarial drugs as the drug resistance ability of *P. falciparum* has become an issue of utmost concern.

Awe and Makinde, (1997) reported the dose-dependent and seasonal variation in the activity of *M. lucida* which includes anthraquinones which showed activity against *P. falciparum* and also possess antifungal properties *invitro* assessment. The relatively lower inhibition was observed for the organic extract of *Azadirachta indica* as compare to the current study may be due to its more activity as an antipyretic rather than as a schizonticidal agent for malaria therapy (Okpaniyi and Ezeuku, 1981). However, Makinde and Obih, (1985) reported that boiled water extract of *A. indica* showed schizonticidal activity against chloroquine-sensitive *P. berghei*. It is therefore possible that the strain of the parasites or the species accounted for the differences observed.

In addition it is not uncommon that some plants which are popularly used to treat fever or malaria in some areas may be found to be inactive or toxic in mice (Krettli, 2001). One probable explanation is the unsuitability of the *invivo* rodent malaria models to demonstrate the expected activity. Truly, no in vitro drug sensitivity test can entirely mimic the *in-vivo* situation, but these *in-vitro* methods should ideally utilize both uniform drug exposure and a test medium that approximates the *in-vivo* milieu. Additional *in-vivo* models may be needed to adequately evaluate these antimalarial plants (Dow, 1999).

The isolation and elucidation of the chemical structure of different active components found in the medicinal plants is of most important values to identify as a new lead compound which can be developed further in to candidate antimalarial drugs. This requires a team work drawn from various disciplines ranging from Ethanobatany, Phytochemistry, Biochemistry, Biological Sciences and their use in alternate system of medicine and eventually scientific and clinical evaluation (Pillai *et al.,* 2001). Malaria vaccine research has traditionally been in the forefront in the development of new technologies applicable to the human population (Sharma, 1997). It is no surprise therefore that first Recombinant vaccine produced from bacterial polypeptide will be the first synthetic vaccine that has been tested in humans (Web.)

CONCLUSIONS

This study adds important information to the area of malaria research, which always is in need of alternative anti-malarial drugs and drug combinations to combat with the drug resistant parasites. In this study, crude ethanol extract were recorded better as against the crude water extract against *P. falciaprum* isolates. Although it is premature to conclude at this stage that these herbal combinations can be used as effective anti-malarials, this finding provides a foundation for further exploration of a new effective herbal drug or drug combination with curcumin for protection from the development of resistance among malarial parasites.

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ISSN: 2347 - 7881

Acknowledgement: This work was performed at Malaria Research Centre, New Delhi to fulfillment of M.Sc. degree in Biotechnology. Authors are grateful to Director of Malaria Research Centre, New Delhi to giving me permission of current research work. We are also thankful to Dr.Y. K. Jaiswal and Dr. K.C. Nand, Department of Biotechnology, Jiwaji University Gwalior and Sophia College Gwalior for their encouragement during the research work. The help and assistance received from my colleagues Prabhakar Mittal and Ruchi Gaur is gratefully accredited.

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