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IN VITRO ANTIMALARIAL ACTIVITY OF ANNONA RETICULATA FRUITS EXTRACTS BY SYBER GREEN I BASED FLUORESCENCE METHOD

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ABSTRACT

The most serious form of malaria caused by *Plasmodium falciparum* continuous to be a major public health problem. As a result, this study aimed to assess the antimalarial potential of ethanol leaf extracts. The cytotoxicity study of crude drug extracts accessed against Vero cell line and their antimalarial activity investigated against *Plasmodium falciparum* 3D7 and KI strain. Syber Green I Based fluorescence *In vitro* antimalarial study was used to access the efficacy of *Annona reticulata* extract. The plant extracts had no cytotoxicity effect on *the Vero* cell line (CC50= 200µg/ml). This study showed moderate antimalarial activity of ethanolic extract with IC50 values of 3D7 and KI are 38.5 and 52 respectively. The present study indicates that *Annona reticulate* could be a good source of antimalarial agent.

KEYWORDS: *Annona reticulate,* anti-malarial, Chloroquinediphosphate, Syber Green I, MTT assay, *Vero* cell line.

INTRODUCTION

Malaria is a major public health concern affecting a large paste of the population. Ethnomedicine is a potential source of antimalarial compounds and provides templates for the synthesis of novel antimalarial molecules. The first step in the antimalarial drug discovery process is to evaluate the antimalarial activity of the test compounds or plant extracts. Malarial drug resistance has emerged as one of the greatest challenges facing malarial control.[1] Natural products play an important role in the field of new drug research and development. Plants also provide raw materials for our buildings and in the manufacture of biofuels, dyes, perfumes, pesticides, drugs. The use of the plant in traditional medicinal practice has a long drawn history and remains the main stage of primary health care most of the third world.[2]

Traditional medicines are used by about 60% of the world population in both developing and developed countries. Parasitic infections are still one of the major causes of mortality in underdeveloped countries. Parasitic protozoan belongs to the genus plasmodium such as *Plasmodium falciparum, plasmodium vivax, plasmodium malariae, plasmodium ovale, plasmodium knowleri, plasmodium* cynonolgi,[3] cause malaria, one of the most severe tropical diseases. The malaria parasites are transmitted by the female anopheles mosquito. *Annona reticulata* is commonly known as Bullock's heart. It is a small evergreen tree cultivated throughout India for its fruits. It is traditionally used for the treatment of epilepsy, dysentery, cardiac problem, parasitic and worm infestation, constipation, bacterial infection, dysuria, fever, ulcer, and insecticides.[4]

MATERIALS AND METHODS

The present study was carried out to evaluate the antimalarial activity of *Annona reticulata*. Qualitative analysis was done by using total ethanolic extract and maceration. The details of the material used and the method followed is described below

Collection of plant material

Leaves of *Annona reticulata* were collected in September 2019 from Mannancherry, Alappuzha (Kerala). The fresh leaves of *Annona reticulate* were dried for 17 days at room temperature.

Extraction of plant materials

The maceration process involves the separation of medicinally active portions of crude drugs, Based on the immersion of crude drug in the bulk of solvent or menstruum. Solid drug material was taken in a stoppered container with about 750ml of menstruum and allowed to stand for at least 3-7 days in a warm place with frequent shaking. The mixture of crude drug-containing solvent is filtered until most of the liquid drains off. The filtrate and washing are combined to produce 1000ml of the solution.

Coarse Solid drug material was taken in a stoppered container with about 750ml of Menstruum and allowed to stand for at least 3-7 days in a warm place with frequent shaking. The mixture of crude drug-containing

solvent is filtered until most of the liquid drains off. The filtrate and washing are combined to produce 1000ml of the solution. The crude drug material is charged in the extractor, which is connected with a circulatory pump and spray distributor, along with several connected tanks to receive the extraction solution. This is known as multiple stage extraction because the solvent added and circulated in the extractor containing drug is removed as extracted solution and is stored in the receiver tanks. This operation is repeated thrice. When the crude drug material is charged in the extractions, the stored solution is once again circulated through the fresh drug and then removed as an extract. Likewise, after three extractions, the drug and removed from the extractor, again recharged with fresh drug and the whole cycle is repeated.[5]

Preliminary phytochemical screening

Qualitative analysis for determining the presence of alkaloids, tannins, Flavanoids, terpenoids, steroids, glycosides, saponins, resin, and oil in the plant extracts, were carried out using standard methods 0.5 g of the dried extracts were dissolved in 20 ml distilled water, filtered and used for various qualitative tests.[6]

BIOLOGICAL SCREENING

Cytotoxicity study by MTT Assay[7]

The antimalarial activity of samples on VERO cells was determined by the MTT (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) assay was used to assess the cytotoxicity Horiuchi et al.,(1988). Cells (1 × 105 /well) were plated in 0.2 ml of medium/well in 96-well plates. For the MTT assay, the medium from the wells was removed carefully after incubation. Each well was washed with MEM (w/o) FCS 2-3 times and 200µl of MTT (5mg/ml) was added. The plates were incubated for 6-7 hrs in a 5% CO2 incubator for cytotoxicity. After incubation, 1ml of DMSO (solubilizing reagent) was added to each well and mixed well by micropipette, and left for 45sec. The presence of viable cells was visualized by the development of purple color due to the formation of formazan crystals. The suspension was transferred to the cuvette of a spectrophotometer and the OD (optical density) values were read at 595nm by using DMSO as a blank. Measurements were performed and the concentration required for a 50% inhibition of viability (IC50) was determined graphically Standard Graph was plotted by taking a concentration of the drug in the X-axis and relative cell viability on the Y-axis.

***In Vitro* Antimalarial Activity[8]**

Aseptic procedures all procedures (except centrifugation) have been performed in a level II biosafety cabinet with the biosafety cabinet surface which has been wiped down with aseptic solution at the beginning and the end of every day. The valves on gas cylinders at the end of each day have been closed. The incubator and the storage surfaces were cleaned at least every 3 months.

Preparation of Lysis buffer (1 L)

15.76 gm of Tris-HCl has been dissolved completely in about 700 mL cell culture water using a magnetic stirrer. pH has been adjusted to 7.5 using concentrated hydrochloric acid. 20 mL 0.5 M EDTA was added to give a final concentration of 10mM (2% w/v). 160 mg saponin (0.016 % w/v final) has been added. 16.0 mL Triton X-100 (1.6 % v/v final) has been mixed. Cell culture has been added in water to bring the final volume to 1 Litre. The solution was mixed thoroughly, avoiding the creation of bubbles. Vacuum filtration has been done with the solution using 0.2µ pore to remove any particulate matter and store it indefinitely at RT.

Lysis buffer containing SYBR Green I (15 ml)[9]

This solution should be made fresh in a darkened room. Thawed one 30 µlaliquot of SYBR Green I. Added 30 µl SYBR Green I to 15 ml lysis buffer (20x final SYBR Green concentrations). 15 ml lysis buffer is adequate for one 96 plates. Pipetted to mix, avoiding the creation of bubbles

Preparation of malaria cultures and sensitivity assay

Determined % parasitemia of malarial culture. For fresh field isolates ≤ 0.3%, run the assay at 2% hematocrit in complete medium without reducing the parasitemia. If parasitemia of culture-adapted samples or fresh field isolates is>0.3, dilute to 0.3% or 0.15% parasitemia using complete culture medium for 72 or 96hr incubations respectively at 2% hematocrit in complete medium. A 72hr assay is adequate for most drugs; 96hr incubation can be used for slow-acting drugs like antibiotics. Fresh field isolates are not washed before the assay. Using an automated liquid handler or manually, add 100 µl malaria-infected erythrocytes to each well on a pre-dosed drug plate. Incubate cultures for 72hr or 96hr at 37ºC in a humidified chamber, under a gas mixture of 90% N2, 5% O2, and 5% CO2 or in a candle jar. After the 72hr or 96hr incubation, added 100 µlLysis buffer containing 20x SYBR Green I to each well, in a dark room. Incubate the plates at RT in the dark for 24 hrs. Fluorescence on a fluorescence plate reader has been recorded with excitation and emission wavelength bands centered at 485 and 530 nm, respectively. Data was transferred into a graphic program (EXCEL) and IC50 values were obtained by Log regression analysis. Chloroquine diphosphate (SIGMA) was used as the standard reference drugs.

Selective Index

Selectivity Index (SI) was calculated as: SI = CC50 / IC50

RESULTS

Preliminary Phytochemical Evaluation

Qualitative chemical tests were carried out in total ethanolic extract. The result of the chemical test for extract was performed and reported the presence of Alkaloids, glycosides, phenolic, flavonoids, Terpenoids

Cytotoxicity study

To evaluate the cytotoxicity activity of ethanolic extracts of Annona reticulata against the Vero cell line respectively were incubated with different concentrations of the extract. Cell viability was determined by the MTT assay. The CC50 value was determined as the concentration of *Annona reticulata* required to inhibit the formation of MTT formazan by 50% was found to be 200µg/ml

Table 1: CC50 Values (µg/ml) of ethanolic extract of *Annona reticulata* leaves on Vero cell line by MTT assay.

Sl no	Sample	MTT Method (CC50)
1	A.reticulata	200µg/ml
2	Chloroquine diphosphate	110µg/ml

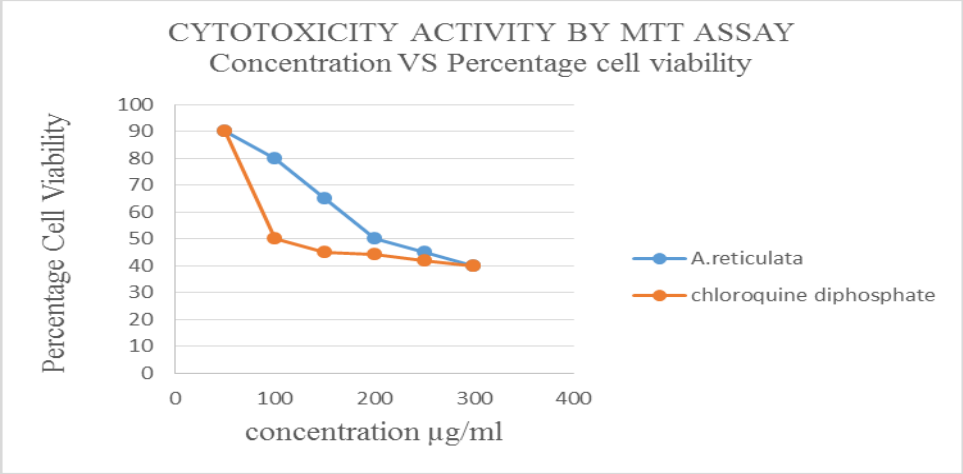

Fig 1: Cytotoxicity activity of ethanolic extract of *A.reticulata* Vero cell line by MTT Assay method.

***In Vitro* Antimalarial Activity**

Syber Green I Based Fluorescence (MSF) assay

The extract of *A.reticulata* was analyzed for its antimalarial activity and its compared with that of the standard drug chloroquine diphosphate. The fluorescence reading of the plate will be noted and percentage growth inhibition calculated various ranges of concentration of *A.reticulata.*

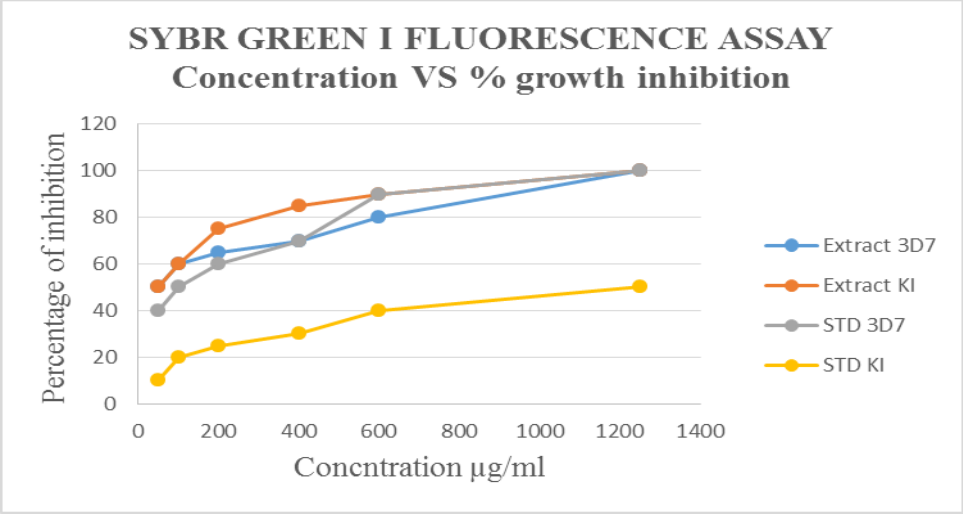

Fig 2: Percentage growth inhibition of ethanolic extract of *Annona reticulata* leaves and standard drug in various range of concentration using SYBER Green I Fluorescence Assay (MSF).

Table 2: IC50 values (µg/ml) of ethanolic extract of *Annona reticulata* leaves using Malaria SYBR Green I-based Fluorescence (MSF) methods.

Sl no	Sample	MSF Method	
		3D7	KI
1	Ethanolic extract of *A.reticulata*	38.5	52
2	Chloroquinediphosphate	0.21	0.019

Selective index

Selective index of plant extract and standard drug by using different stains. Calculated by using formulae and compared values of plant and standard drug.

Table 3: Selective index of antimalarial MSF method by using a different strain of standard drug and ethanolic extract of leaves of A.reticulata.

Sl no	Sample	Selective index	
		3D7	KI
1	Ethanolic extract of A.reticulata	5.71	4.23
2	Chloroquinediphosphate	486.34	4600

DISCUSSION

Literature survey of *Annona reticulate* leaves shows more phenolic and flavonoids. So the extracts were used for carrying the biological activity studies. So phytochemical screening is very useful for the evaluation of some bioactive compounds of some medicinal plants.[10] The preliminary phytochemical screening of extract showed the presence of various chemical constituents such as alkaloids, glycosides, phenolics, flavonoids, steroids. This shows a high amount of its possible medicinal value. Phytochemical screening shows the presence of anti-parasitic compounds. Alkaloids might be responsible for the antimicrobial activity.[11]

In vitro antimalarial study using Syber Green I fluorescence MSF method, the result in IC50 values of the total ethanolic extract showed 38.5µg/ml in strain 3D7 and 52µg/ml in strain KI of *Plasmodium falciparum.* Several criteria have been proposed for considering a compound as active.[12] The compound is considered to be active when it shows an IC50 value >200 where those with an IC50 of >25µg/ml have low activity. IC50 values ranges 10-25µg/ml moderate activity IC50 of <10µg/ml good activity. The results of the study indicated that the extract of the *Annona reticulate* possesses moderate antimalarial activity.[13] The total ethanolic extracts of the 3D7 strain showed more activity than that of strain KI. The selective index is defined as the ratio of CC50 to IC50. The higher the SI the more promising is the extract due to its selective action as malarial parasites. The plant extracts were evaluated for cytotoxicity displayed 5.71 against 3D7 and 4.23 against KI strain. Total ethanolic extracts *Annona reticulate* leaves have antimalarial activity but none of them are potential as standard drug chloroquinediphosphate.[14]

CONCLUSIONS

The present study has found that the leaves of *Annona reticulate possess moderate antimalarial activity* with good selective index against both strains of *plasmodium* by using Syber Green I Fluorescence (MSF) Assay. Cytotoxicity study on Vero cell line using MTT assay showed total ethanolic extracts has activity. The preliminary phytochemical study showed alkaloids (responsible for anti-parasitic components), phenolics, flavonoids (responsible for antiprotozoal activity) terpenoids (antiplasmodial activity), and saponins.

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REFERENCES

1. Kunle Okaiyeto and Anthony I. Okoh. In Vitro Assessment of Antiplasmodial and Antitrypanosomal Activities of Chloroform, Ethyl Acetate and Ethanol Leaf Extracts of Oedera centifolia. Applied Sciences, 2020; 10: 1-13.
2. Ronan Batista, Ademir de Jesus Silva Júnior and Alaíde Braga de Oliveira. Plant-Derived Antimalarial Agents: New Leads and Efficient Phytomedicines. Part II. Non-Alkaloidal Natural Products. Molecules, 2009; 14: 3037-3072.
3. Bethel Kwansa-Bentum, Kojo Agyeman, Jeffrey Larbi-Akor, Claudia Anyigba, and Regina Appiah-Opong. In Vitro Assessment of Antiplasmodial Activity and Cytotoxicity of *Polyalthia longifolia* Leaf Extracts on *Plasmodium falciparum* Strain NF54. Malaria Research and Treatment, 2019; 1: 1-9.
4. Prasad G. Jamkhande, Amruta S. Wattamwar. Annona reticulata Linn. (Bullock's heart): Plant profile, phytochemistry, and pharmacological properties. Journal of Traditional and Complementary Medicine, 2015: 1-9.
5. Solanki R and Nagori BP: New Method for extracting phytoconstituents from plants. International Journal of Biomedical and Advance Research, 2010; 03(10): 770-774.
6. Prashanti, P. Anitha, S. Sashidhara, S. Effect of Rotulaaguatica Lour on experimental kidney

stone.International Journal of Pharmacognosy and phytochemical research, 2015; 7(6): 1142-1146.
7. P. Senthilraja, K. Kathiresan. In vitro cytotoxicity MTT assay in Vero, HepG2, and MCF -7 cell lines study of Marine Yeast. Journal of Applied Pharmaceutical Science, 2015; 5 (03): 080-084.
8. Matthias, G. Vossen. Sandra P Ferschy. Peter Chiba and Harald Noed. The SYBR Green I Malaria Drug Sensitivity Assay: Performance in Low Parasitemia Samples. The American journal of tropical medicine and hygiene, 2010; 82(3): 398–401.
9. Owuor, B.O. Ochanda, J. O. Kokwaro, J .O. Cheruiyot, A.C. Yeda, R..A. Okudo, C A. Akala, H M. In vitro antiplasmodial activity of selected Luo and Kuria medicinal plants. Journal of Ethnopharmacology, 2012; 144: 779–781.
10. Aja, P .M. Okaka, A.N.C. Onu, P.N. Ibiam, U. Urako, A.J. Phytochemical Comp Talinumtriangulare (Water Leaf) Leaves. Pakistan Journal of Nutrition., 2010; 9(6): 527-530.
11. Jindal A.P Kumar. Antimicrobial activity of alkaloids of Tridaxprocumbens. International journal of pharmaceutical sciences and research, 2012; 3(9): 3481-3485.
12. Syamsudin Abdillah. Risma Marisi Tambunan. Yunahara Farida. Ros Sumarny and Rita Marleta Dewi. In Vitro and In Vivo Antiplasmodial Activities of Garcinia parvifolia (MIQ) MIQ Leaves From Indonesia. Journal of Pharmacy and Biological Sciences, 2014; 9(6): 28-32.
13. Toghueo Kouipou Rufin Marie, Heroine Mbetyoumoun Mfouapon, Eugenie Aimée Madiesse Kemgne, Cedric Derick Jiatsa Mbouna , Patrick Valere Tsouh Fokou, Dinkar Sahal , and Fabrice Fekam Boyom. Anti-Plasmodium falciparum Activity of Extracts from 10 Cameroonian Medicinal Plants. Medicines, 2018; 115(5): 1-14.
14. Valdes, A.F.C. Martínez, J.M, Lizama, R.S, Gaiten, Y.G, Rodríguez, D.A. & Payrol, J.A. - In vitro antimalarial activity and cytotoxicity of some selected Cuban medicinal plants. Rev. Inst. Med. Trop. Sao Paulo, 2010; 52(4): 197-201.