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Research Article

IN-VITRO ANTIMALARIAL EVALUATION AND PHYTOCHEMICAL ANALYSIS OF RHIZOMES OF *CURCUMA AERUGINOSA ROXB*

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A B S T R A C T

The present work showed antimalarial activity and cytotoxicity study of total ethanolic extract from rhizomes of *Curcuma aeruginosa*. Roxb Antimalarial study was evaluated by SYBER GREEN I-Based Fluorescence method and *In vitro* cytotoxicity study of total ethanolic extract by MTT Assay. Physio-chemical evaluation of the drugs for detecting adulteration, which includes, Total ash value, extractive value and fluorescence analysis. To analyse qualitative and quantitative, Phytochemical analysis such as Total Phenolic content, preliminary phytochemical analysis of total ethanolic extract of *C. aeruginosa* revealed a presence of Alkaloid, glycoside, phenolic, flavonoids, Terpenoids, steroids etc. Total ethanolic extract of *C.aeruginosa* inhibit the formation of MTTformazan by 50% was found to be 220µg/ml compared to 110µg/ml standard drug. Antimalarial study by SYBER Green I- Based Fluorescence (MSF) method shows IC_{50} value of ethanolic extract and chloroquinediphosphate against strain 3D7 38.5, 0.23 respectively and strain K1 shows 52.0 and 0.025. selective index of *Curcuma aeruginosa* was found to be 5.71 and 4.23 compared to 478.26 and 4400 chloroquinediphosphate.

INTRODUCTION

Malaria is a mosquito borne infectious diseases of human and other animals. The term malaria originates from medieval *mala aria*means bad air**.** According to World Health Organization, there were an estimated 243 million cases Worldwide by 2015. Malaria caused by unicellular protozoan parasites belonging to the genus Plasmodium. The diseases are one of the leading causes of morbidity and mortality in the world. More human death and diseases is caused by malaria parasites than by all over eukaryotic pathogen combined. About 3.4 billion peoples are at risk of malaria infection in about 109 countries and territories **(**Diarra N*et.al., 2015***)**. By 2009, malaria infected about 300-500 million people Worldwide, killing about 1 million people annually.

Parasitic infections are still one of the major causes of mortality in the poor or underdeveloped countries. Parasitic protozoan belonging to the genus *Plasmodium* causes malaria, one of the most severe tropical diseases. *Plasmodiums* are organized in the phylum of Apicomplexa, sub order of Heamosporidae and family of Plasmodiiae. The four identified species of the parasite responsible for inflicting human malaria are *plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malaria.*

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The malaria parasites are transmitted by the female Anopheles mosquito. They possess very complex life cycle. The natural ecology of the malaria parasites involves the Plasmodium infecting successfully two different hosts, human and the female *Anopheles* mosquito.

Artemisinin and its derivatives have been used extensively in China and Southeast Asia, where there are high levels of resistance to the majority of the quinoline containing drugs and to all the antifolate drugs. The artemisinin-type compounds currently in use are either the natural extract artemisinin itself or the semi-synthetic derivatives (dihydroartemisinin, artesunate, artemether) **(**Saifi *et al*., 2013**).** *Curcuma aeruginosa*is commonly known as "pink and blue ginger". It is a perennial herb distributed in south Indian region of 50-60 meter altitude. It is widely used in Indian system of medicine. It is traditionally used for the treatment of leucoderma, asthma, tumour, piles, and bronchitis. Paste of rhizome is applied on rheumatic pain. The plant is reported to antimicrobial, anti-inflammatory

MATERIALS AND METHODS

Plant Collection

Rhizome part of *Curcuma aeruginosa* was collected from Thiruvambady in Calicut district, Kerala on 20/12/2015 at 6am to 8am and rhizome was authenticated by A K Pradeep, Assistant Professor, Department of Botany, University of

Calicut and the voucher specimen number 88454, has been submitted to the Department of Pharmacognosy, Jamia Salafiya Pharmacy College for future references.

Physico-Chemical Evaluation of the Crude Drug

To evaluate physico-chemical evaluation of crude drugs by evaluation of foreign matters, moisture content, determination of extractive values, determination of Ash values and fluorescence analysis (Dr.CK Kokate)

Total ethanolic extraction

Extraction of rhizomes of *Curcuma aeruginosa* was carried out by continuous extraction method using Soxhlet apparatus. 250 g rhizome size reduced and shade dried for seven days. Extracted with 1 litter of ethanol in the round bottom flask and extraction was continued for 8 hours. The extract obtained was collected and concentrated by gentle heating. The concentrated extract was weighed and stored in refrigerator. Thus total extract is obtained (Asokan Bhagavan *et al*., 2011)

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 outusing standard methods 0.5 g of the dried extracts were dissolved in 20 ml distilled water, filteredand used for various qualitative tests.(V Anu *et al*., 2019), (Prashanthi P *et al*., 2015)

Estimation of Total Phenolic Content

10 mg of the extracts were weighed, dissolved in methanol and made up to 10 ml with methanol. 1ml was pipette out from each extract solution and 5 ml of Folin Ciocalteau reagent was added. After 5 minutes, 4 ml of sodium carbonate solution was added and incubated at room temperature for 2 hours. Then, absorbance was measured at 750 nm and the values obtained were interpreted in the standard graph of Gallic acid to get the milligram equivalents of gallic acid.

Estimation of total Flavonoid Content

10 mg of the sample extracts were weighed dissolved in methanol and made up to 10 ml with methanol. 1ml was pipette out from each dissolved samples and 4 ml of water followed by 0.3 ml of sodium nitrate was added. After 5 minutes, 0.3 ml of 10% aluminium chloride solution and at the 6th minute 2 ml of 1M sodium hydroxide was added, mixed well and the absorbance was measured at 510 nm and the values were interpreted in the standard graph of Rutin to get the milligram equivalents of Rutin.(M. Atanassova *et al*., 2011)

Cytotoxicity Sudies (Mtt Assay)

The monolayer cell culture was trypsinized and the cell count was adjusted to 3-lakhcells/ml using medium containing 10% newborn calf serum. To each well of 96 well microtitre plates, 0.1ml of diluted cell suspension was added. After 24 hours, when the monolayer formed the supernatant was flicked off and 100 µl of different test compounds were added to the cells in microtitre plates and kept for incubation at 37ºC in 5 % CO2 incubator for 72 hour and cells were periodically checked for granularity, shrinkage, swelling. After 72 hour, the sample solution in wells was flicked off and 50µl of MTT dye was added to each well. The plates were gently shaken and incubated for 4 hours at 37oC in 5% CO2 incubator. The supernatant was removed, 50 µl of Propanol was added, and the plates were gently shaken to solubilise the formed formazan. The absorbance was measuredusing a microplate reader at a wavelength of 490 nm. The percentage of viability was calculated using the formula below **(**Sanjay patel*et al*., 2007)

Percentage of cell viability = OD of test / OD of control x 100%

In Vitro Anti Malarial Activity By Sybr Green I Fluorescence (MSF) Assay

Procedure

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 indefinitely at RT. (Matthias G. Vossen *et al*., 2010)

SYBR Green I stock solution

10000x SYBR Green I concentrate has been thawed at room temperature in laminar flow hood in a darkened room. Aliquot 30 µl into amber-coloredEppendorf tubes, label with the day's date and store at -20 ºC for up to 6 months.

Lysis buffer containing SYBR Green I (15 ml)

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 mlLysis buffer (20x final SYBR Green concentrations). 15 mlLysis buffer is adequate for one 96 plate. 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 are >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 isolate are not washed prior to the assay. Using 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 37 ºC 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 cantered at 485 and 530 nm, respectively. Data was transferred into a graphic programme (EXCEL) and IC50 values were obtained by Log regression analysis. Chloroquinediphosphate (SIGMA) was used as the standard reference drugs (B O Owuor *et al*., 2012)

Selective Index (SI)

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

RESULTS

Physioco- Chemical Evaluation Of The Drug

Table 1 physico-chemical parameters of drugs

Sl No	Physico Chemical Parameters	Percentage
1	Evaluation of foreign matter	9.82±0.020.
2	Moisture content (Loss on drying)	53.1±0.07
3	Water soluble extract	7.83±1.35
4	Alcohol soluble extract	6.37±0.23
5	Total ash	5.8±0.01
6	Acid insoluble ash	0.61±0.02
7	Water soluble ash	3.71±0.04

Fluorescence Analysis

Fluorescence analysis of different types of reagent mixed with plant rhizome powder. And observed the mixture on day light, long and short UV and observed the colour change of each mixture on day, long and short UV light.(Gayathri and D Kiruba 2015)

Preliminary phytochemical evaluation

Qualitative chemical test 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, steroids.

Estimation of Total Phenolic Content

The total Phenolics content in the extracts were determined by Folin Ciocalteau method. The absorbance values obtained for different concentrations of standard gallic acid are tabulated. The absorbance values obtained for *C.aeruginosa* rhizomes extract was 0.646±0.003 and the total Phenolics content in gallic acid equivalents are 70.52.

Estimation of Total Flavonoid Content

Estimation of total Flavanoids in the extracts was carried out by Aluminium chloride colorimetric method. The absorbance values obtained for different concentration of the standard Rutin are tabulated. The absorbance obtained for the *C.aeruginosa* rhizome was 1.244±0.003 and the total Flavanoids content in Rutin equivalent are 57.12.

Cytotoxicity studies

MTT assay Cytotoxicity of *Curcuma aeruginosa* was estimated by MTT assay on vero cell line (C1008; monkey kidney fibroblast) cells. Percentage cell viability was determined by a colorimetric MTT assay at 540 nm.CC50 was determined as concentration of *C.aeruginosa* required to inhibit the formation of MTT formazan by 50% was found to be 220 µg/ml

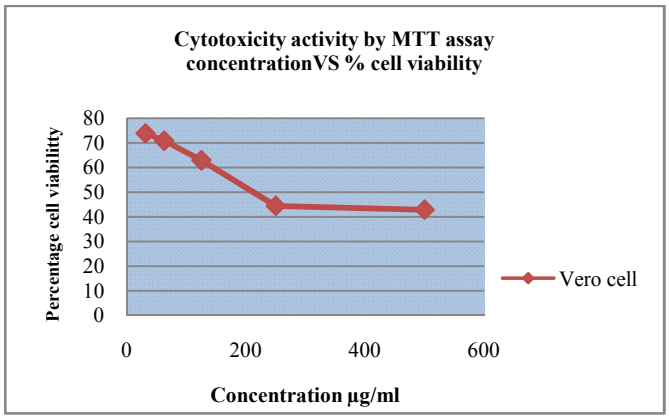

Fig 1 Cytotoxicity activity of ethanolic extract of *C.aeruginosa*on Vero cell line by MTT assay

Table 2 CC_{50} values (µg/ml) of ethanolic extract of *C.aeruginosa* rhizome on Vero cell line by MTT assay

Sl.no	Sample	CC_{50}
1	Extract of *C.aeruginosa*	220µg/ml
2	Chloroquinediphosphate	110µg/ml

In Vitro Antimalarial Activity

Sybr Green I Based Fluorescence (Msf) Assay

The extracts of *Curcuma aeruginosa*were analyzed for its antimalarial activity and it is compared with that of the standard Chloroquinediphosphate. The fluorescence reading of plate will be noted and percentage growth inhibition calculated. From the results of present study it can be stated that the extracts of*C.aeruginosa*.

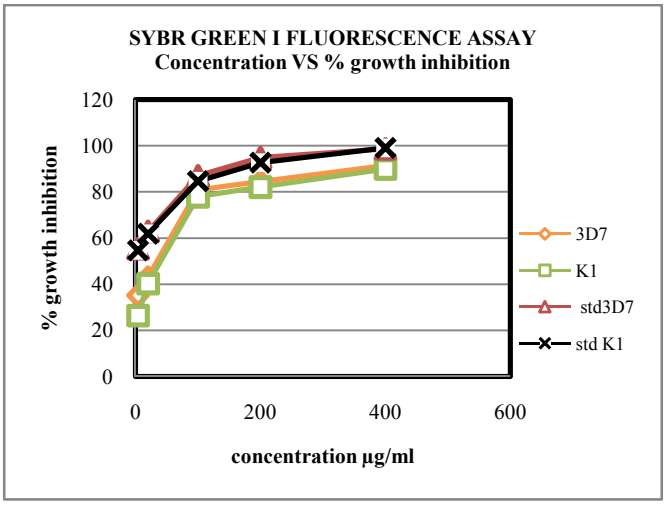

Fig 2 Percentage growth inhibition of ethanolic extract of *C.aeruginosa* rhizomes and standard drug in various range of concentration using SYBR GREEN –I FLUORESCENCE ASSAY

Table 3 IC50 values (µg/ml) of ethanolic extract of *C.aeruginosa* rhizome using Malaria SYBR Green I-based Fluorescence (MSF) methods Sample

Sl.no	Sample	MSF method	
		Strain 3D7	Strain K1
1	Ethanolic extract of *C.aeruginosa*	*38.5*	52.0
2	Chloroquinediphosphate	0.23	0.025

Selective Index (SI)

Table 4 Selective index of antimalarial MSF method by using different strain of standard drug and ethanolic extract of *C.aeruginosa* rhizome

Sl.no	Sample	Selective Index	
		3D7	K1
1	*C.aeruginosa*	5.71	4.23
2	Chloroquinediphosphate	478.26	4400

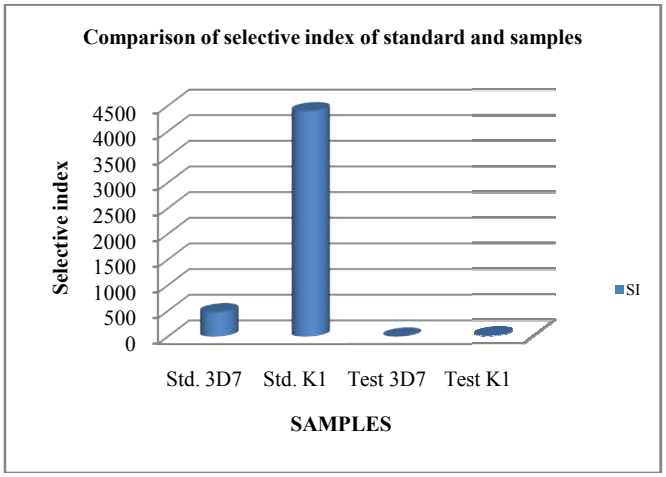

Fig 3 Comparison of selective index of antimalarial MSF method by using different strain standard drug and *C.aeruginosa* rhizomes

DISCUSSION

From the quantitative evaluation of total phenolics and flavonoids it was clear that the ethanolic extracts showed more of phenolics and flavonoids and so the extracts were used for carrying the bioactivity studies. So phytochemical screening is very useful in the evaluation of some active biological components of some medicinal plants **(**P M Aja *et al*., 2010**).** The qualitative and quantitative analysis of various extracts of *C.aeruginosa* was carried out and extracts showed the presenceof various chemical constituents such as alkaloids, glycosides, phenolics, flavonoids, carbohydrates and steroids. This shows high level of its possible medicinal value. Phytochemical screening showed the presence of anti-parasitic components, alkaloid might be responsible for the antimicrobial activity (Jindal A *et al*., 2012) In the presence of Flavanoids present in the plant extract might be responsible for antiprotozoal activity**.** The presence of terpenoids in the plant extract might be responsible for antiplasmodial activity (S Ganapaty *et al*., 2009)

Antimalarial activity assay using Malaria SYBR Green I-based Fluorescence (MSF) methods. The result IC50 values of ethanol extract were 38.5µg/ml in strain 3D7 and 52.0µg/ml in K1 strain of *P. falciparum.* As seen in Table 13. . Several criteria have been proposed for considering a compound as active. Generally, a compound is considered to be inactive when it shows an IC50 > 200 µM, whereas those with an IC50 of 100-200 µM have low activity; IC50 of 20-100 µM, moderate activity; IC50 of 1-20 µM good activity; and IC50 < 1 µM excellent/potent antimalarial activity. The ethanolic extract from 3D7 strain showed better antimalarial activity than that of K1strain**. (**Syamsudin Abdillah *et al*., 2014) Chloroquinediphosphate was selected as a standard drug and IC50 of it was found 0.23 and 0.025, CC50 was found110µg/ml and Selectivity Index was found 478.26 and 4400. The same values of ethanolic extracts are lower than chloroquinediphosphate. So it can be said that plant extracts have antimalarial activity but none of them are as potential as chloroquinediphosphate.

CONCLUSION

Many of the curcuma species have been reported for antimalarial activity. However, they have no report on the *Curcuma aeruginosa.* Roxb. Various Physico-chemical parameters were evaluated such as foreign matter, moisture content, extractive values, ash values and fluorescence analysis of the drug. Phytochemical studies showed alkaloids, glycosides, Phenolics, Flavanoids, carbohydrate, terpenoids, steroids. Cytotoxicity activity done on *Vero* cell line (African monkey kidney normal cell line) using MTT assay showed ethanolic extract have considerable activity. It shows 220µg/ml was 50% cytotoxicity activity. After the cytotoxicity evaluation fixes the biological activity performed concentration. And done antimalarial studies using SYBR green I Fluorescence (MSF) assay show total ethanolic extract has good antimalarial activity. Selective index of the plant extract and standard were done.

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