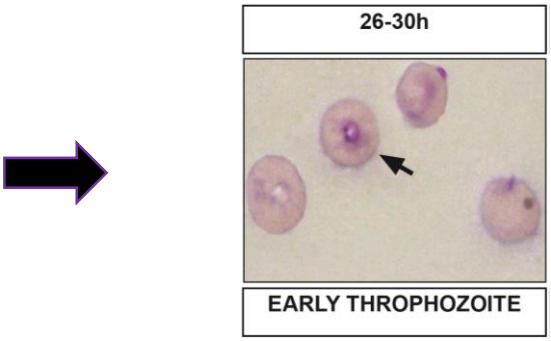
Chapter 2

Screening of medicinal plants against *Plasmodium falciparum*



2.1 Introduction

Plants are a store-house of myriads of molecules with diverse pharmacological properties. The use of plants as primary health care property is quite common everywhere, especially in Asia, Latin America and Africa. Due to accessibility and affordability, traditional medicines play a significant role in developing countries especially in rural areas for the treatment of diseases including malaria. The usage of plants described traditionally or the ethnic practices implicate certain properties of plants which can give us direction for the selection of plants to reach new compounds with the desired property. To validate this proposition, are the two remarkable discoveries of quinine and artemisinin, the active antimalarial principles isolated from *Cinchona* bark and *Artemisia annua* respectively. The long history of dependence on plants to search drugs along with the enormous flora that remains unexplored, indicates a strong possibility for the presence of potent and powerful compounds in plants. India, with a gamut of environmental conditions, is a region known for its high plant diversity and a high degree of endemism. India holds the status of being the origin of two ancient systems of medicine: Ayurveda and Siddha. Apart from them, Unani is another medicinal system originally from Greece, which is widely practiced in India. Consultation of texts and literature on medicinal plants and consideration of ethnopharmacological practices assisted us to select plants for investigation of antiplasmodial activity. In addition to the plants traditionally characterized as antimalarials, the plants with antiparasitic, anthelmintic, antipyretic and hepatoprotective activity were also explored in the current study. Many such plants, possessing activity related to antimalarial activity were found to exhibit antiplasmodial activity.

2.2 Materials and methods

2.2.1 Selection of plants

The plants described as antimalarials or possessing properties that indicated the possibility for the presence of antimalarial principles namely antiparasitic, anthelmintic, antipyretic and hepatoprotective property were selected for the study (Table 2.1).

Table 2.1: Plants selected for the study that are used to treat malaria and malaria like symptoms in Indian traditional system of medicine.

Plant species	Family	Common name	Traditional use
Aristolochia bracteolata Lam.	*Aristolochiaceae*	Kitamar	Anthelmintic
Andrographis paniculata (Burm.f.) Nees	*Acanthaceae*	Kalmegh, Kirayat	Antimalarial
Cassia angustifolia M.Vahl	*Leguminosae*	Senna, Senai	Antimalarial, Vermifuge, Antipyretic
Cassia tora L.	*Leguminosae*	*Chakunda*	Antiparasitic, Antipyretic
Centella asiatica (L.) Urb.	*Apiaceae*	*Brahmi*	Antipyretic, Blood purifier
Commiphora wightii (Arn.) Bhandari	*Burseraceae*	*Guggul*	Antiparasitic, Increases white blood cells
Curcuma zedoaria (Christm.) Roscoe	*Zingiberaceae*	Jangli haldi	Anthelmintic, Antipyretic, Hepatoprotective, Blood purifier
Cyperus rotundus L.	*Cyperaceae*	Mutha, Motha	Antiparasitic, Anthelmintic, Harmonizes liver, spleen and pancreas
Glycyrrhiza glabra L.	*Leguminosae*	Mulaithi	Antimalarial, Blood purifier
Ficus religiosa L.	*Moraceae*	Peepal	Antihelmintic
Ocimum sanctum L.	*Lamiaceae*	Tulsi	Antimalarial, Antipyretic, Anthelmintic, Blood tonic
Phyllanthus amarus Schumach. & Thonn.	*Phyllanthaceae*	Bhumi amla	Antimalarial
Terminalia chebula Retz.	*Combretaceae*	Harad	Antiparasitic, Anthelmintic, Antipyretic, Used in liver disorders, Blood purifier
Terminalia bellirica (Gaertn.) Roxb.	*Combretaceae*	Bahera	Antiparasitic, Anthelmintic, Used in liver disorders
Tinospora cordifolia (Willd.) Miers	*Menispermaceae*	Giloy	Antimalarial
Vitex negundo L.	*Lamiaceae*	Nirgundi	Antiparasitic, Antimalarial, Antipyretic, Anthelmintic, Liver disorders

Basis-Ayurvedic encyclopedia (Tirtha, 1998).

2.2.2 Collection of plant material

Different parts of the selected plants (Table 2.2, Table 2.3) were collected in the post-monsoon season (October-December) from Vadodara, Gujarat, India. Prior to the biomass collection, the plants were authenticated at the herbarium of The Maharaja Sayajirao University of Baroda (Biodiversity Collection Index Code: BARO), Vadodara, Gujarat, India.

2.2.3 Preparation of extracts

The plant materials collected were washed thoroughly under tap water to remove adhered dirt and finally washed under distilled water. The plant parts were air-dried at room temperature. Extracts were prepared by subjecting 20 g of dried and finely ground plant material to sequential solvent extraction in the order of increasing polarity, using 200 ml each of n-hexane (HEX), dichloromethane (DCM), ethyl acetate (EtOAc), ethanol (EtOH) and water (AQ) for 24 h at 40°C. After filtration, the extracts were concentrated under reduced pressure at 40-45°C using rotary evaporator (BUCHI). The extracts were further dried to constant weight. Extract yields were determined and the extracts were stored in airtight vials at 4°C until further used.

2.2.4 *In vitro* antiplasmodial activity screening

The antiplasmodial activity of the extracts was checked by their effect on *P. falciparum*. For this the *P. falciparum* CQ-sensitive 3D7 strain was procured from ICMR-National Institute of Malaria Research (NIMR), New Delhi, India. The parasite culture and maintenance were done using methods from Moll et al. (2013) with some modifications. The protocols followed are mentioned in detail as follows.

2.2.4.1 In vitro cultivation of parasite

(a) Culture media

Incomplete medium was prepared by adding 10.4 g Roswell Park Memorial Institute (RPMI)-1640 medium, 5.9 g HEPES buffer, 4.0 g glucose and 44 mg hypoxanthine to 1 l of autoclaved Milli-Q water. Gentamicin powder at a final concentration of 50 µg/ml was added to the medium. After getting stirred for 1 h the medium was sterile filtered using an autoclaved filter unit and a peristaltic pump. The incomplete medium thus prepared was stored at 4°C until used. To prepare complete medium 2.5 ml of sterile-filtered 5% sodium bicarbonate ($NaHCO_3$) was added to 87.5 ml of incomplete medium and the pH was adjusted to 7.2 using HCl. This was followed by the addition of 10 ml of heat-inactivated plasma. The resultant complete medium was stored at 4°C and kept for no longer than five days.

(b) Heat inactivation of plasma

Plasma (AB) was procured from Suraktam blood bank, Vadodara, Gujarat. It was heat-inactivated by placing it at 56°C in a preheated water bath for 2 h followed by sterile filtering. Then 10 ml aliquots of the filtrate were aseptically transferred to 15 ml cryogenic tubes and stored at –80°C until used.

(c) Treatment of erythrocytes

Fresh blood from healthy volunteers with O+ group was procured from Suraktam Blood Bank, Vadodara, Gujarat, in acid citrate dextrose (ACD) tubes and was centrifuged at 500 g for 10 min at 4°C to separate the blood into its 3 component layers: plasma, buffy coat and erythrocytes. The plasma and buffy coat were aspirated using a Pasteur pipette. The erythrocytes were washed with two volumes of sterile-filtered phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4), pH 7.2 to remove remaining white blood cells. The wash cycle was repeated three times. The erythrocytes were then resuspended in one volume of incomplete medium and were then stored at 4°C for no longer than two weeks.

(d) Preparation of culture from frozen stocks

Frozen stock cultures of the *P. falciparum* 3D7 strain in 1.5 ml cryogenic tubes were thawed in a water bath preheated at 37°C. The thawed culture was transferred to a 15 ml tube. For each ml of culture, 100 µl of sterile-filtered 12% NaCl was added dropwise while swirling gently and then the suspension was left at room temperature for 5 min. To this, nine volumes of sterile-filtered 1.6% NaCl was added, gently mixed and the solution was centrifuged at 500 g for 10 min at 20°C. The supernatant was removed and the stabilate was resuspended in nine volumes of a filter-sterilized 0.2% glucose solution prepared in 0.9% NaCl and centrifuged as before. After aspiration of supernatant, the stabilate was resuspended in 750 µl freshly washed uninfected erythrocyte suspension prewarmed to 37°C. The suspension was then transferred to a tissue culture flask ($35cm^2$) and was made up to 5 ml with a prewarmed complete medium at 37°C containing 10% AB plasma. Further for culturing, Trager-Jensen's candle jar method was used, wherein the culture flask and a lit candle were placed in a glass desiccator. The desiccator was then sealed tightly. The burning candle consumes some of the O_2 inside the desiccator and produces CO_2, which extinguishes the fire. Once the CO_2 inside the desiccator reaches around 5% from its fresh air content of

0.036-0.039%, the candle stops burning. This creates perfect conditions for the culture of the malaria parasite. The culture was incubated for 48 h at 37°C.

(e) Culture maintenance

The old spent medium of the culture was replaced daily with fresh complete RPMI-1640. The culture flask was transferred to a sterile hood. The flask was gently tipped and without stirring the cells into suspension the medium was aspirated through a sterile pipette. To monitor the parasite stage and calculate parasitemia, a smear of culture was prepared on a glass slide, which was examined later. Fresh complete medium, prewarmed to 37°C in a water bath was added to the flask. The settled cells were resuspended in the medium by gentle aspiration and flushing down through the pipette and by gentle swirling. The flask was returned to the candle jar and incubated again at 37°C. It was always ensured that the media was preheated to 37°C and the culture handling time outside the candle jar at 37°C was minimized.

The parasites were subcultured when the parasitemia reached about 5%. For this, the culture from the flask was collected into a centrifuge tube and spun at 500 g for 10 min at room temperature. The packed infected erythrocytes were suspended in complete medium to make 50% suspension. The suspension of infected erythrocytes was then diluted with washed uninfected erythrocytes prewarmed to 37°C to obtain parasitemia from 0.5-1%. Further, complete medium was added to prepare a suspension of 4% hematocrit. Approximately 5 ml of this suspension was transferred to the culture flask ($35cm^2$) and the culture was maintained as mentioned before.

(f) Synchronization of the parasite culture

P. falciparum is usually asynchronous during *in vitro* culture and a synchronized parasite culture is necessary to evaluate results of experiments. So, culture was synchronized by sorbitol treatment (Lambros and Vandenberg, 1979) which lyses the late-stage parasite-infected erythrocytes, preferentially selecting for erythrocytes with early ring-stages. The selection effect is due to permeability of the infected erythrocytes and sensitivity of the parasite to sorbitol. Briefly, the culture with majority ring-stage population and parasitemia around 10% was transferred to a centrifuge tube and centrifuged at 500 g for 10 min at room temperature. After aspiration of supernatant, five volumes of 5% D-sorbitol was added to the pellet and gently mixed. The mixture was incubated at room temperature for 5 min at

37°C and centrifuged as before. The cell pellet was washed twice with complete medium. A thin smear was made to determine the percent parasitemia by quick staining. The culture was diluted approximately with the addition of 50-100 µl fresh-packed erythrocytes. Complete medium was to adjust the hematocrit to 4%. The culture was incubated in a candle jar as mentioned earlier.

(g) Calculation of parasitemia

To monitor the parasite cultures thin smears from the cultures were prepared daily and stained with Giemsa stain. A small amount of oil was put at the end of the thin smear which contained single-layered cells. The slide was observed under a 100X oil immersion objective in a microscope. Infected erythrocytes and total erythrocytes (Infected and uninfected erythrocytes) were counted in five random fields containing more than 100 erythrocytes per field. The percentage parasitemia for a field was calculated as the number of infected erythrocytes per 100 erythrocytes. The percentages of parasitemia from the five fields were used to calculate an average percentage parasitemia.

(h) Staining of the parasite

Two methods of Giemsa staining, the standard staining method and the rapid staining method were followed. The standard staining method was used for monitoring the culture on a routine basis and evaluation of slides from screening assays. The rapid staining method was used whenever a quick assessment of the culture was needed such as during the procedure of culture synchronization.

(i) Giemsa standard staining

Commercially supplied Qualigens Giemsa stain was used for staining. The working solution was always freshly prepared. Thin-film prepared was air-dried and fixed in methanol for 30 s. After methanol fixing, the smear was completely dried before staining to avoid membrane artifacts in erythrocytes. Giemsa stain was prepared fresh by diluting the stain 1:10 with sodium phosphate buffer, pH 7.2. The slide was stained with this diluted Giemsa stain for 30 min. The slide was removed from the stain and rinsed thoroughly under running tap water while letting the stream of water run at the backside of the slide and taking care of not washing the smear off the slide. The stained slide was dried completely in air and observed under a microscope using a 100X oil immersion lens. The parasitemia was calculated as the average from five random sectors on the stained slides.

(ii) Rapid Giemsa staining

For rapid Giemsa staining, the air-dried blood film was stained with undiluted Qualigens Giemsa stain for 1-2 min. The slide was placed in deionized water for 2-4 min, until the stain diluted out to give the preferred color. The slide was rinsed under tap water as mentioned for the standard staining method. The slides were air-dried completely and evaluated under a microscope using a 100X oil immersion lens. The parasitemia was calculated as the average from five random sectors on the stained slides.

2.2.4.2 Growth inhibition assay

The antiplasmodial activity of the extracts was evaluated by *in vitro* parasite growth inhibition assay. For this, the growth of the *P. falciparum* over one growth cycle was studied in presence of extracts. Assays were performed in triplicate in flat-bottom 96-well microtiter plates. Briefly, parasite culture synchronized at ring-stage with 2% parasitemia and 4% hematocrit was used for the assay. Extract stocks of 250 µg/ml were prepared in incomplete RPMI-1640 medium and the dissolution was assisted with DMSO (final concentration < 1%). Parasite culture that received CQ diphosphate at the final concentration of 1.5 ng/ml was used in positive controls and that which received no treatment was used in negative controls. Uninfected erythrocytes suspension was kept as a blank. At first, 20 µl of the extract stocks were pipetted in the test wells to achieve the final test concentration of 25 µg/ml. The positive control wells received 20 µl of CQ diphosphate suspension. To the negative control and background wells, 20 µl of incomplete RPMI-1640 medium was added. The vehicle control wells received 1% DMSO. Then 180 µl of the parasite suspension was added to all except the background wells. Following this 180 µl of uninfected erythrocytes were added to the background wells. Screening for all the extracts was performed at once in multiple plates. The plates were swirled gently to mix the contents. The plates were incubated in a candle-jar following the method mentioned previously, for 72 h at 37°C. After incubation, the plates were removed from the candle-jar. To estimate the growth of the parasite, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-phenazine methosulfate (PMS) assay was performed.

The protocol for the MTT-PMS assay proposed by Lazaro and Gay (1998) was used with some modifications to monitor the growth of the parasite. Initial screening of the plant extracts for antiplasmodial activity was done by mitochondrial dehydrogenase based assay where the parasitic mitochondrial dehydrogenase enzyme metabolically reduced the yellow-

colored tetrazolium dye MTT to form water-insoluble blue formazan crystals. The formazan crystals formed were dissolved and read, which estimated the levels of mitochondrial dehydrogenase activity in the culture. The levels of parasitemia and mitochondrial dehydrogenases activity expressed in terms of formazan absorbance, follow a positive linear correlation (Lazaro and Gay, 1998), hence the estimation of activity of these enzymes can be used to assess the parasitemia in the culture. The assay was performed in microtiter plate format. In brief, after 72 h of incubation, the culture was mixed properly and 50 µl of the medium was aspirated in other microtiter plates. To prevent the reduction of MTT caused by blood hemoglobin sodium nitrite was added. So, 20 µl of 100 mg/ml sodium nitrite solution prepared in PBS buffer, pH 7.4 was added to each well and mixed. The plates were transferred to candle jar for 30 min at 37°C. Following incubation, 20 µl of freshly prepared MTT-PMS solution (5 mg/ml MTT and 0.25 mg/ml PMS) was added and mixed. The plates were again incubated in candle jar for 3 h at 37°C. To dissolve the blue formazan crystals formed after incubation, 100 µl of 10% SDS solution prepared in 0.01 N HCl was added to each well and mixed gently. The resulting blue colored solution was read at 570 nm. The background absorbance read at 690 nm was subtracted from the absorbance read at 570 nm. The final absorbance values were obtained by subtracting the average absorbance of blank wells from average absorbance of the control and test wells. The percentage inhibition of the parasite growth was calculated according to the following equation: % Parasite inhibition = $[1 - (A_S/A_{NC})] \times 100$, where A_S is the final absorbance of the test sample or positive control, A_{NC} is the final absorbance of the negative control.

2.2.5 Antiplasmodial IC_{50} studies

The extract(s) found to possess antiplasmodial property after performing the growth inhibition assay were studied for their ability to inhibit the recombinantly expressed PMs from *P. falciparum*, mPM I and mPM II (expression discussed in Chapter 3). The extract(s) demonstrating PM inhibition property were assessed for their antiplasmodial IC_{50} values using dose-response analysis. The analysis was performed using ring-stage synchronized parasite culture with 2% parasitemia and 4% hematocrit. To prepare stock solutions, 1 mg of extracts were dissolved in 1 ml of incomplete RPMI-1640 medium. For some extracts, the dissolution was assisted with DMSO (final concentration < 1%). Then, for each extract, a series of six concentrations of 20 µl each were made in the microtiter plate wells using the stocks and their 2-fold dilutions, to result in the final test concentrations spanning from 100-

3.125 µg/ml. The dilutions were prepared in incomplete RPMI-1640 or in a solution of incomplete RPMI-1640 and DMSO (final concentration < 1%), wherever needed. Similarly, 20 µl of aliquots with various concentrations of the CQ diphosphate which served as a positive control drug, were added to the positive control wells to get the final test concentration from 1.51-0.048 ng/ml. Negative controls were the wells where parasite culture did not receive any treatment and wells with uninfected erythrocytes were kept for background. The negative control wells and background wells received 20 µl of incomplete RPMI-1640 medium. Subsequently, 180 µl of parasite suspension was added to all but background wells. Then 180 µl of uninfected parasite suspension was added to the background wells. The plates were swirled gently and incubated in candle-jar for 72 h at 37°C. After incubation, thin films of the contents of each well were prepared and were Giemsa stained. The slides were examined under 100X oil immersion lens and parasitemia for each slide was calculated as described earlier. Mean % parasitemia was determined from an experiment performed in triplicates. The percentage inhibition of the parasite growth was calculated according to the following equation: % Parasite inhibition = $[1 - (P_S/P_{NC})] \times 100$, where P_S is the parasitemia of test sample or positive control, P_{NC} is the parasitemia of negative control.

IC_{50} values were calculated using dose-response analysis in Graph pad Prism 6.0. This was done by plotting mean % parasitemia against log concentration of the extracts or control drug. The dose-response curves were normalized by defining the lowest parasitemia value in the data set which was approximately 0% parasitemia as 0% and the highest parasitemia value in the data set which also had no significant difference from the parasitemia obtained from untreated negative control, as 100%. For each data set curve fitting was done either by using linear regression analysis or non-linear regression analysis where log(inhibitor) vs. normalized response (variable slope) model was selected to derive the regression equation. Diagnostics were performed to ensure that the model well-fitted the data. This included inspecting the precision of best-fit values of the parameters, quantifying goodness of fit by R^2, checking the Gaussian distribution of residuals and assessing the random distribution of residuals by plotting the residual plots. The results of curve fitting and diagnostics were calculated by the software. A well-fitted model was used to estimate IC_{50} values of the extracts or the control drug. The percentage potency of an extract was calculated in reference to the standard drug CQ diphosphate using the following formula: % Potency = (IC_{50} of CQ diphosphate/IC_{50} of the extract) × 100.

2.2.6 *In vitro* cytotoxic activity

Assessment of cytotoxic activity of plant extract(s) with promising PM inhibition property was done against Human embryonic kidney-293 cell lines (HEK-293) by an assay performed in triplicate. Briefly, 200 µl (10,000 cells/well) cells were seeded in Minimum Esstential Medium, in 96-well microtiter plates. After 24 h of incubation, the spent medium was aspirated. Then 200 µl of various concentrations of test extracts prepared in medium, ranging from 10-50 µg/ml final concentration were added to the respective wells and the plates were incubated. Cells treated with cisplatin at the final concentration of 15 µg/ml were used as standards, cells treated with 1% DMSO were kept as vehicle controls and untreated cells were kept as negative controls. Wells with the medium without cells were kept as blanks. After 24 h, 10% MTT reagent was added to the wells to a final concentration of 5 mg/ml for 3 h, thereafter formazan crystals formed were collected and solubilized in 100 µl of DMSO. The final absorbance was determined after subtraction of background absorbance taken at 690 nm from the absorbance taken at 570 nm. After subtracting the blank absorbance, the percentage cell viability was calculated according to the following equation: % Cell viability = $(A_S/A_{NC}) \times 100$, where A_S refers to the absorbance of the test sample or the standard and A_{NC} is the absorbance of the negative control. The IC_{50} value of an extract on cell growth was calculated from the dose-response curve generated by plotting the % cell viability against the log concentration of the extract. After curve fitting, estimation of IC_{50} value was performed by the procedure described previously. The selectivity of an extract for parasite versus HEK-293 cells expressed as selectivity index (SI) was determined by the ratio of the IC_{50} value for HEK-293 cells to the IC_{50} value for *P. falciparum* 3D7.

2.2.7 *In vitro* hemolytic activity

The hemolytic effect of plant extract(s) with PM inhibition activity was evaluated using human erythrocytes in a triplicate assay. In brief, erythrocytes collected from Suraktam blood bank, Vadodara, Gujarat, were suspended in PBS, pH 7.2. The erythrocytes suspension (2% hematocrit) was then treated with different concentrations of each plant extract, ranging from 50-500 µg/ml at 37°C for 1 h. After incubation, erythrocytes were pelleted at 3000 g for 10 min at 4°C. Erythrocytes suspended in PBS were considered as negative controls, having 0% hemolysis and erythrocytes treated with Triton X-100 (0.1%) were kept as positive controls having 100% hemolysis of the cells. Erythrocytes treated with

1% DMSO were kept as vehicle controls. To quantify the amount of hemoglobin released due to erythrocyte lysis if any, the supernatant was collected and absorbance at 450 nm was read spectrophotometrically. The percentage hemolysis was determined as the percentage relative to the hemolysis caused by Triton X-100 using the following equation: % Hemolysis = $[(A_S-A_{NC})/(A_{PC}-A_{NC})] \times 100$, where A_S is the absorbance of a test sample, A_{NC} is the absorbance of the negative control and A_{PC} is the absorbance of the positive control. The concentration of extract which exhibited 50% hemolysis, (HC_{50}) was calculated from a dose-response curve, by plotting the % hemolysis against the log of the extract concentration. After curve fitting, the HC_{50} analysis procedure was the same as described before. Extract with HC_{50} value lower than 200 µg/ml was considered as hemolytic.

2.2.8 Statistical analysis

Data were expressed in terms of mean ± standard deviation. Comparison of data and statistical significance was determined using one-way analysis of variance (ANOVA) and two-tailed t-test (GraphPad Prism 6.0, GraphPad Software Inc., USA). IC_{50} and HC_{50} values were calculated by using dose-response analysis (GraphPad Prism 6.0, GraphPad Software Inc., USA).

2.3 Results and discussion

2.3.1 Preparation of extracts

Different parts of the plants selected for the study were sequentially extracted with solvents of different polarities (Table 2.2). A yield resulting after extraction with a solvent implicated the amount of the compounds with polarity similar to that of the solvent. The residue plant material indicated the portion in the plant that did not solubilize in the entire spectrum of solvents used and it mainly represented the insoluble fiber. Overall % yields were found to be 4.22 ± 5.62%, 3.48 ± 2.28%, 3.92 ± 4.65%, 5.88 ± 6.17% and 21.85 ± 10.98% from HEX, DCM, ETOAc, ETOH and AQ respectively. The values suggested the abundance of polar components in plants in general as compared to mid-polar and non-polar components.

2.3.2 *In vitro* antiplasmodial activity screening

The parasite, *P. falciparum* CQ-sensitive 3D7 strain was cultured *in vitro* and synchronized at the ring-stage (Figure 2.1). After 6 h of parasite reintroduction into the medium, the parasite was found to reestablish itself and thrive again into the culture medium. The

screening of plant extracts for antiplasmodial activity was performed using this culture. Among all the 129 extracts tested, 22 extracts were found to exhibit > 70% parasite inhibition (Table 2.3). No significant effect of the vehicle was observed on the parasite growth.

Plants contain myriads of phytochemicals broadly categorized into different classes, which can be selectively extracted using different organic solvents, hence the five fractions obtained after sequential extraction varied in components. The *in vitro* screening results indicate the potential as well as the class of an antiplasmodial agent in a plant. They also provide validation to the traditional usage of some of these plants for the treatment of malaria.

Table 2.2: Percentage yield of fractions obtained after extraction of plant material using different solvents.

Plant Species	Plant part used	Yield of plant extract residues (%)					Residue (%)
		HEX	DCM	EtOAc	EtOH	AQ	
Aristolochia bracteolata	Leaves	4.30	0.50	5.60	2.50	11.40	60.00
	Roots	15.80	0.60	0.40	0.40	12.80	64.60
Andrographis paniculata	Aerial parts	2.00	3.50	8.80	2.40	15.30	67.40
Cassia angustifolia	Leaves	3.00	2.70	2.40	7.10	21.00	62.00
	Flowers	1.80	2.40	0.20	8.00	27.10	60.00
	Seeds	3.80	4.70	0.40	1.20	50.30	39.00
Cassia tora	Leaves	4.10	3.40	4.40	7.70	8.90	65.70
	Seeds	1.00	1.70	1.00	10.66	24.00	56.70
Centella asiatica	Leaves	3.00	2.90	8.00	6.40	22.00	57.40
	Roots	4.60	5.00	3.00	5.60	22.40	58.20
Commiphora wightii	Stem	1.70	11.10	0.80	1.70	24.09	59.70
Curcuma zedoaria	Leaves	1.90	2.50	2.50	14.40	15.00	60.00
	Rhizome	2.60	6.00	9.50	2.20	14.20	65.10
Cyperus rotundus	Leaves	3.40	2.10	14.90	1.90	19.10	48.90
	Roots	3.00	5.60	1.80	1.40	6.20	80.60
Glycyrrhiza glabra	Roots	1.40	2.70	1.00	3.00	11.00	70.20
Ficus religiosa	Leaves	27.80	2.50	1.50	2.10	29.00	29.80
	Roots	4.60	2.80	3.40	2.40	10.20	75.20
Ocimum sanctum	Leaves	3.60	7.20	0.60	3.60	36.00	44.30
Phyllanthus amarus	Aerial parts	1.10	2.30	1.70	3.50	14.00	74.20
Terminalia chebula	Leaves	2.50	1.90	1.90	4.40	35.60	50.70
	Fruits	0.20	1.50	19.00	30.00	14.80	31.00
Terminalia bellirica	Leaves	5.00	4.10	3.60	5.30	39.30	41.00
	Fruits	0.20	1.30	0.40	7.40	39.00	44.20
Tinospora cordifolia	Aerial parts	2.40	4.70	2.10	3.70	23.00	51.20
Vitex negundo	Leaves	4.90	4.80	3.10	14.00	22.30	50.10

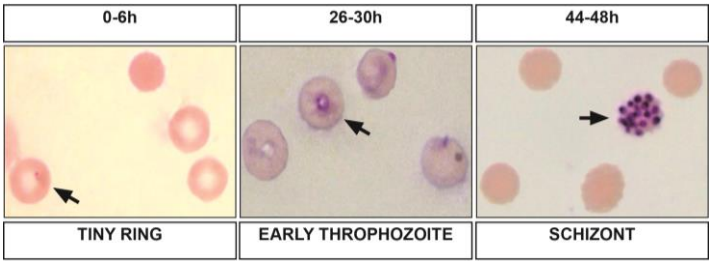


Figure 2.1: Various stages of *Plasmodium falciparum* 3D7 observed under microscope with 100X magnification during culture.

Table 2.3: Inhibition of *P. falciparum* 3D7 following 72 h exposure to plant extracts *in vitro* at the final concentration of 25 µg/ml.

Plant Species	Plant part used	Parasite inhibition (%)				
		HEX	DCM	EtOAc	EtOH	AQ
Aristolochia bracteolata	Leaves	43.45 ± 1.08	40.22 ± 3.98	41.68 ± 1.89	68.94 ± 0.84	81.52 ± 0.34
	Roots	51.77 ± 2.33	54.43 ± 3.08	74.03 ± 2.90	55.38 ± 1.12	53.89 ± 1.26
Andrographis paniculata	Aerial parts	52.85 ± 0.74	66.16 ± 1.13	63.62 ± 0.67	88.04 ± 0.28	73.80 ± 0.51
Cassia angustifolia	Leaves	40.05 ± 0.85	41.21 ± 0.97	42.22 ± 1.04	45.72 ± 1.66	47.89 ± 0.39
	Flowers	44.32 ± 0.91	43.57 ± 1.25	45.05 ± 0.97	44.49 ± 1.35	43.22 ± 1.26
	Seeds	44.32 ± 1.46	44.65 ± 0.98	42.02 ± 0.87	42.09 ± 1.48	-
Cassia tora	Leaves	40.58 ± 4.04	41.23 ± 4.52	42.78 ± 4.86	52.97 ± 3.73	80.70 ± 1.49
	Seeds	42.81 ± 3.93	44.44 ± 2.33	72.43 ± 2.24	61.46 ± 4.66	58.16 ± 4.07
Centella asiatica	Leaves	30.35 ± 1.78	29.93 ± 4.27	31.20 ± 2.08	30.19 ± 4.16	32.13 ± 0.85
	Roots	32.26 ± 1.41	41.78 ± 3.18	45.81 ± 1.63	44.40 ± 1.41	45.21 ± 2.47
Commiphora wightii	Stem	59.20 ± 1.28	64.68 ± 0.34	66.11 ± 0.53	78.52 ± 0.49	85.32 ± 0.97
Curcuma zedoaria	Leaves	46.05 ± 1.17	46.27 ± 1.04	43.44 ± 3.60	43.84 ± 0.49	55.16 ± 1.35
	Rhizome	57.38 ± 0.97	86.97 ± 0.67	74.75 ± 1.29	65.73 ± 1.71	74.38 ± 1.17
Cyperus rotundus	Leaves	52.42 ± 1.83	52.02 ± 3.64	53.79 ± 1.77	54.98 ± 0.31	56.86 ± 0.49
	Roots	56.83 ± 0.99	54.45 ± 0.39	55.06 ± 0.65	62.94 ± 0.75	66.39 ± 0.97
Glycyrrhiza glabra	Roots	56.48 ± 1.22	53.61 ± 0.81	44.94 ± 0.81	51.60 ± 4.30	78.84 ± 1.66
Ficus religiosa	Leaves	78.75 ± 0.67	55.41 ± 0.97	64.49 ± 1.35	64.05 ± 1.17	53.62 ± 0.49
	Roots	52.97 ± 0.67	61.70 ± 4.95	75.87 ± 2.79	75.91 ± 0.96	78.98 ± 0.36
Ocimum sanctum	Leaves	43.82 ± 0.06	76.90 ± 1.41	50.76 ± 1.48	60.17 ± 3.45	50.36 ± 0.19
Phyllanthus amarus	Aerial parts	51.41 ± 0.65	87.45 ± 0.53	72.90 ± 0.70	60.66 ± 0.90	64.46 ± 1.07
Terminalia chebula	Leaves	42.95 ± 0.49	45.11 ± 1.59	49.38 ± 4.53	45.22 ± 2.08	45.47 ± 4.27
	Fruits	46.35 ± 4.53	51.22 ± 2.08	50.25 ± 4.40	52.56 ± 1.31	74.08 ± 1.71
Terminalia bellirica	Leaves	42.83 ± 1.35	42.20 ± 0.67	42.75 ± 1.29	43.86 ± 1.46	35.92 ± 1.04
	Fruits	45.72 ± 0.97	44.95 ± 1.84	44.54 ± 0.73	65.75 ± 1.82	68.69 ± 0.40
Tinospora cordifolia	Aerial parts	43.73 ± 0.43	54.41 ± 1.93	78.05 ± 0.32	61.60 ± 0.19	52.37 ± 0.26
Vitex negundo	Leaves	55.86 ± 1.13	60.76 ± 1.17	62.51 ± 1.35	63.01 ± 1.13	76.94 ± 1.04

Data are represented as mean ± standard deviation from an experiment performed in triplicate.

2.3.3 Antiplasmodial IC_{50} studies

The 22 extracts which demonstrated > 70% parasite inhibition during the growth inhibition assay were tested for their ability to inhibit the recombinantly expressed enzymes mPM I and mPM II *in vitro*. The results suggested four extracts viz. *A. paniculata* EtOH (aerial parts), *C. wightii* AQ (stem), *C. zedoaria* DCM (rhizome) and *P. amarus* DCM (aerial parts) extracts that inhibited both the enzymes efficiently, and thus the extracts were further assessed for their antiplasmodial IC_{50} values.

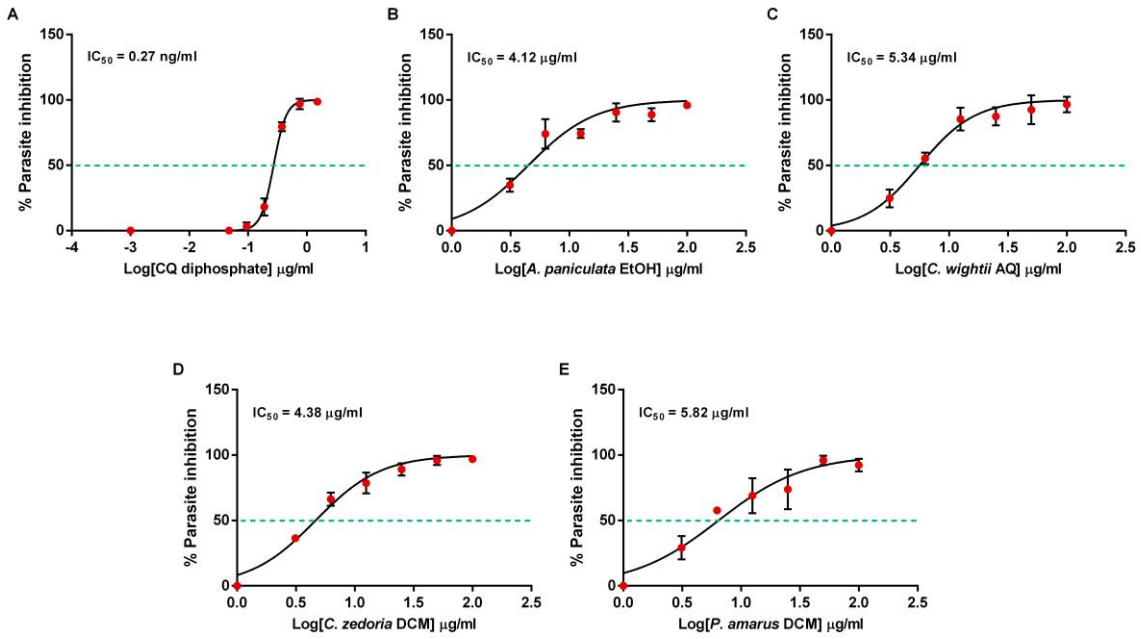


Figure 2.2: IC50 determination of CQ diphosphate and plant extracts against *P. falciparum* 3D7 *in vitro*.

The four plant extracts which were found to have antiplasmodial property and exhibited PM inhibitory activity were evaluated for their antiplasmodial IC_{50} values against *P. falciparum* 3D7 *in vitro*. CQ diphosphate was used as a positive control. Values are represented as mean ± standard deviation from an experiment performed in triplicate.

The IC_{50} values for all the four extracts were found to be in the range of 4-6 µg/ml (Figure 2.2). The standard antimalarial drug CQ diphosphate inhibited *P. falciparum* 3D7 with an IC_{50} value of 0.27 ng/ml. Based on the classification given by (Rasoanaivo et al., 2004) for antimalarial activity, the extracts were classified as class III antimalarial (IC_{50} > 1-10 µg/ml), with good to moderate antimalarial activity. The EtOH extract from *A. paniculata* showed the highest antiplasmodial activity with a potency of 0.0066% of that of CQ diphosphate. The *C. zedoaria* DCM extract, *C. wightii* AQ extract and *P. amarus* DCM extract exhibited

0.0062%, 0.0051% and 0.0046% antiplasmodial potency of that of CQ diphosphate, respectively.

The IC_{50} value for *A. paniculata* EtOH extract was found to be of 4.12 µg/ml. The finding demonstrates the folk practice in Tamil Nadu, India where AQ infusion of the plant is given internally to treat malaria (Okhuarobo et al. 2014). Water boiled extracts from *C. schimperi* (O. Bergman) Engl. has been used by the Maasai community of Kenya to treat malaria and the methanol derived chloroform extract from this plant was reported to have antiplasmodial activity (Koch et al. 2005). In addition to the current study where antiplasmodial activity was found in the *C. wightii* AQ extract with an IC_{50} of 5.34 µg/ml, these reports suggest the presence of antiplasmodial agents in the *Commiphora* genus which are polar in nature. The DCM extract from *C. zedoaria* was found to have an IC_{50} of 4.38 µg/ml. DCM extract of rhizome of the same plant, expected to have similar composition was found to inhibit the parasite *in vitro* with an EC_{50} of 2.38 µg/ml (Mohd Abd Razak et al., 2014). The IC_{50} of *P. amarus* DCM extract was found to be 5.82 µg/ml. A previous study from our laboratory has reported antiplasmodial activity from polar; AQ extract of *P. amarus* aerial parts (Keluskar and Ingle, 2012). In another study, the non-polar DCM extract of *P. niruri* L. (possibly *P. amarus*) whole plant was found to exhibit more antiplasmodial activity than the polar; EtOH extracts (Tona et al., 1999). These findings indicate the presence of two or more antiplasmodial principles which are also of diverse nature, in the plants of the genus *Phyllanthus*. Altogether the reports support the selection of extracts that are antiplasmodial and the extracts can be explored further to find out the antiplasmodial agents that act by inhibiting PM I and PM II.

2.3.4 *In vitro* cytotoxic activity

The cytotoxic activity of the selected four plant extracts with class III antimalarial activity was assessed using the HEK-293 cell line. The *A. paniculata* EtOH and *C. wightii* AQ extracts did not demonstrate any significant effect on the cell viability, hence they exhibited no cytotoxic activity (Figure 2.3A, B). However, the *C. zedoaria* and *P. amarus* DCM extracts were found to exhibit a cytotoxic effect on the HEK-293 cells (Figure 2.3C, D). No significant effect of the vehicle was observed on the cell viability. The microscopic examination also suggested similar results, as was concluded by the loss of cell confluency observed in the case of *C. zedoaria* and *P. amarus* DCM extracts along with the cell lysis which was more pronounced in the former (Figure 2.3E). Compounds isolated from the

EtOH extract of *A. paniculata* did not exhibit any significant cytotoxicity at viricidal concentrations ranging from an IC_{50} of 7.97-11.1 µg/ml (Wiart et al., 2005), which are also the concentrations well above then that required for antiplasmodial effect as determined from the current study. Toxicities from *C. wightii* have been reported only from the resin derived extracts which include, mild gastrointestinal discomfort, thyroid problems and generalized skin rash (Nohr et al., 2009). Since the presence of resin is practically impossible in the AQ extracts, it supports the lack of toxicity observed in the case of *C. wightii* AQ extract in our study.

In the absence of any effect on cell viability in the tested range of concentrations, the SI for the *A. paniculata* EtOH and *C. wightii* AQ extracts was concluded to be > 10. With the IC_{50} values 53.36 µg/ml and 53.41 µg/ml for the *C. zedoaria* DCM and *P. amarus* DCM extracts respectively, the SI was found to be 12.18 and 9.18 respectively. A SI value above 10 suggests better safety of the product (Monzote et al., 2014) while, a SI below 2.0 indicates that an extract may possess strong antimalarial activity but it may be a general toxin (Koch et al., 2005). Based on these criteria the *A. paniculata* EtOH, *C. wightii* AQ and *C. zedoaria* DCM extracts were found to be highly selective for the parasite and the *P. amarus* DCM extract was found to be comparatively less selective but it could not be categorized as a general toxin. However, considering the microscopic observations performed during the analysis along with the results from the MTT assay both the *C. zedoaria* DCM and *P. amarus* DCM extracts were concluded to be cytotoxic against the HEK-293 cell line.

2.3.5 *In vitro* hemolytic activity

The selected four plant extracts with PM inhibition activity were tested for their effect on erythrocytes using *in vitro* hemolytic activity assay (Figure 2.4). None of the four extracts displayed significant hemolytic activity, when the extract concentrations were varied from 50-250 µg/ml. Hemolytic activity was detected in the case of all the extracts at 500 µg/ml, as suggested by the highest level of hemolysis of 13.27 ± 1.46% with the *A. paniculta* EtOH extract followed by significantly equal levels ($p < 0.5$) of 4.5 ± 1.48%, 5.37 ± 2.42% and 4.28 ± 2.50% with the *C. wightii* AQ, *C. zedoaria* DCM and *P. amarus* DCM extracts respectively. The vehicle showed no significant hemolytic activity. The absence of any significant hemolytic activity over the range of concentration spanned suggested the HC_{50} values of all the extracts analyzed to be above 500 µg/ml, which as a result indicated SI values of more than 10.

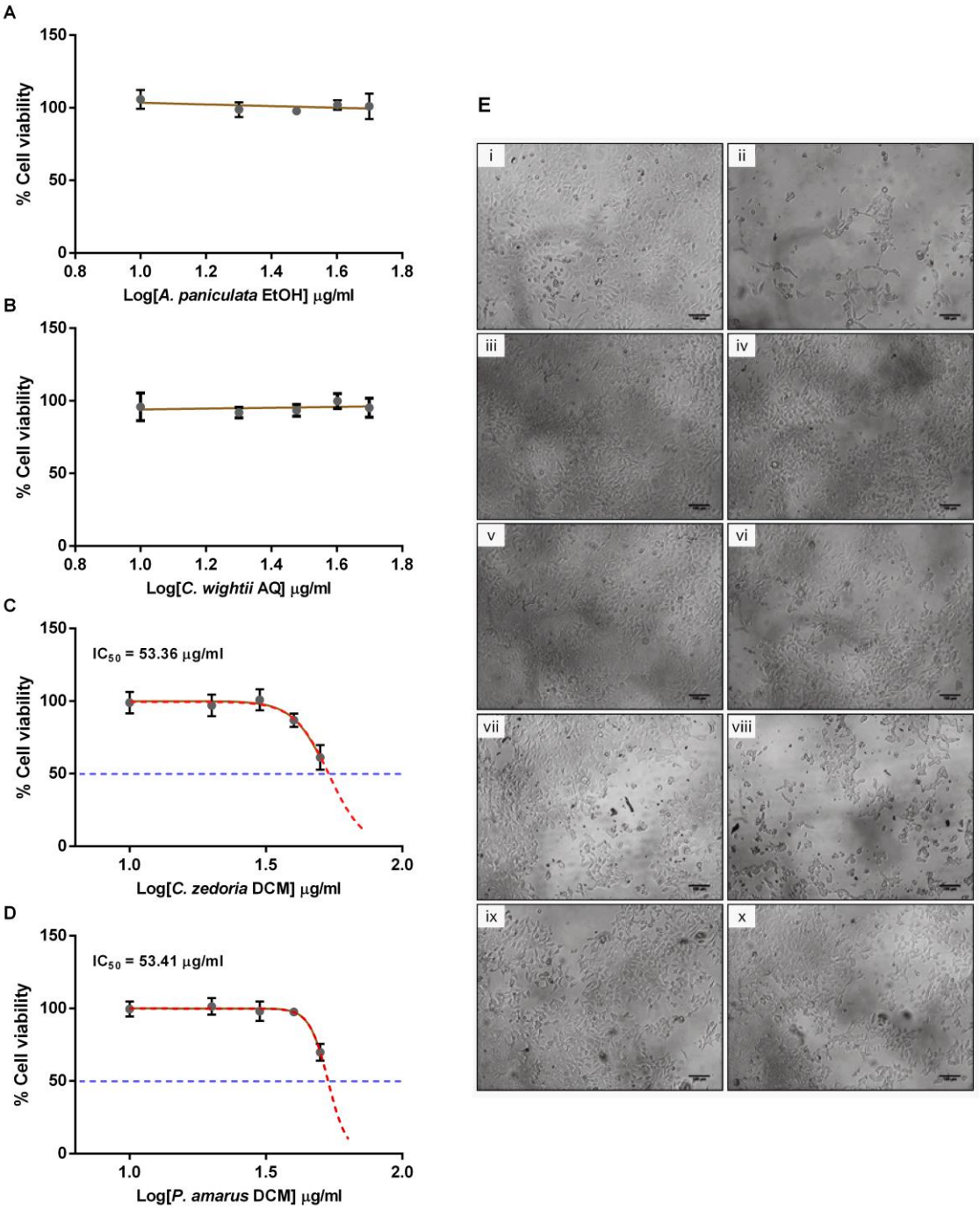


Figure 2.3: Effect of plant extracts with PM inhibition activity on HEK-293 cell line studied *in vitro*.

(A-D) Dose-response curves of the plant extracts obtained with MTT-assay. Values are represented as mean ± standard deviation from an experiment performed in triplicate.
(E) Representative microscopic images of the HEK-293 cells treated with the plant extracts. Untreated cells (i); Positive control cisplatin (15 µg/ml) (ii); *A. paniculata* EtOH extract [(iii) 40 µg/ml and (iv) 50 µg/ml]; *C. wightii* AQ extract [(v) 40 µg/ml and (vi) 50 µg/ml]; *C. zedoaria* DCM extract [(vii) 40 µg/ml and (viii) 50 µg/ml]; *P. amarus* DCM extract [(ix) 40 µg/ml and (x) 50 µg/ml].

Assessment of hemolytic activity for plant extracts is necessary, especially where the targets are intraerythrocytic in position. The extent of hemolytic activity of an antiplasmodial agent can be translated to its ability to target the parasite located inside an erythrocyte without causing any side effects to normal erythrocytes. The four extracts studied for their hemolytic effect did not display any significant hemolytic activity against human erythrocytes till 250 µg/ml, which also indicates a SI value of more than 10. Altogether, the results from the cytotoxicity analysis and the hemolytic activity analysis suggest that the *A. paniculata* EtOH and *C. wightii* AQ extracts were highly selective for the parasite and recommended their further analysis to identify the phytocompounds conferring them the PM inhibition property.

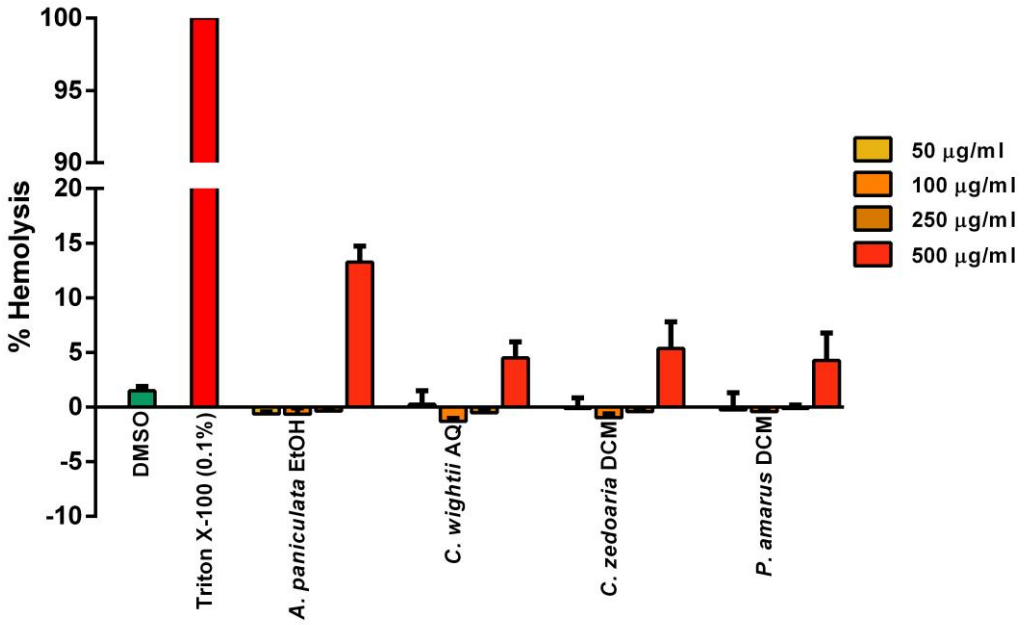


Figure 2.4: Effect of plant extracts with PM inhibition activity on human erythrocytes studied *in vitro*.

The percentage hemolysis of human erythrocytes upon treatment with the plant extracts at various concentrations was determined. Triton X-100 (0.1%) was used as a positive control. Values are represented as mean ± standard deviation from an experiment performed in triplicate.

In conclusion, plants that are prevalent in the traditional medicinal system for antimalarial and other related properties were studied for their antiplasmodial potential. Out of all the 129 extracts derived from the plants, 22 were found to significantly inhibit the growth of *P. falciparum* 3D7 *in vitro*. The findings provide validation to the traditional usage of some of these plants for the treatment of malaria and also support the method of extraction to

obtain the bioactive components from them in a traditional setup. The antiplasmodial extracts with PM I and II inhibition property, *A. paniculata* EtOH and *C. wightii* AQ were found to be non-toxic to HEK-293 cells and human erythrocytes. Exploration of these two extracts may lead to novel antimalarials that act by inhibiting PMs.