CHAPTER II

Materials and Methods

Plant Material

The *Tylophora indica* shoot, *Plumaria rubra* leaves, stem and flowers and *Xanthium strumarium* bur were collected from Gujarat University campus. The plant material to be used was selected based on literature survey and traditional medicinal uses of these plants. Care was taken to avoid damaged, infected or denatured plant material and suitable precautions were taken during processing, extraction and storage in the laboratory.

Authentication of Plant Material:

Samples of plant material were submitted to the Botany Department, Gujarat University, Ahmedabad, India for identification and taxonomic authentication.

Certification of the verified, authenticated plants has been attached as Appendix I.
Table 1: Details of collection of Plant Material:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Plant Parts</th>
<th>Collection Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Leaves</td>
<td>Leaves were collected from above the ground, free from mud or heavy dust infection. Tender or pale yellow leaves were avoided. Leaves were collected with petiole by giving them force in opposite direction.</td>
</tr>
<tr>
<td>2.</td>
<td>Flowers</td>
<td>Only fresh and open flowers were collected with pedicel.</td>
</tr>
<tr>
<td>3.</td>
<td>Tender stem</td>
<td>Approximately 1 foot long tender stem was cut.</td>
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<tr>
<td>4.</td>
<td>Aerial shoot</td>
<td>The entire 1 foot to 3 feet tender aerial shoot was collected with leaves, flowers and tender stem.</td>
</tr>
<tr>
<td>5.</td>
<td>Bur</td>
<td>Completely dry bur without any infection was collected.</td>
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</tbody>
</table>

The collected plant parts were washed under running tap water and dried in hot air oven at 42°C. The internal circulation of hot air oven dried the samples uniformly and fast. The materials were reshuffled twice in a day to prevent the fungus from growing in wet regions. The dried plant materials were powdered and kept in airtight bottles in 4°C until use with proper labeling on container about name of plant, part, collection place, collection date and season of collection.
Preparation of the extracts:

Plant extracts were prepared using different extracting solvents (Harbonne, 1973).

A) Petroleum ether extracts.

The powdered dry plant material (20 gram) was extracted with 250 ml of petroleum ether for 72 hours, reflux at $50^\circ$C using soxhlet extractor. The crude solvent extract collected in the flask was concentrated at reduced pressure in rotary evaporator for recovery of concentrated extract. The yield collected after drying was kept at $-4^\circ$C until further use.

B) Diethyl ether extracts.

The powdered dry plant material (20 gram) was extracted with 250 ml of diethyl ether for 72 hours, reflux at $40^\circ$C using soxhlet extractor. The crude solvent collected in the flask was concentrated at reduced pressure in rotary evaporator for recovery of concentrated extract. The yield collected after drying was kept at $-4^\circ$C until further use.

C) Chloroform extract.

The powdered dry plant material (20 gram) was extracted with 250 ml of chloroform for 72 hours, reflux at $30^\circ$C using soxhlet extractor. The crude solvent collected in the flask was concentrated at reduced pressure in rotary evaporator for recovery of concentrated extract. The yield collected after drying was kept at $-4^\circ$C until further use.
D) Acetone extract.

The powdered dry plant material (20 gram) was extracted with 250 ml of acetone for 72 hours, reflux at 45°C using soxhlet extractor. The crude solvent collected in the flask was concentrated at reduced pressure in rotary evaporator for recovery of concentrated extract. The yield collected after drying was kept at -4°C until further use.

E) Hydro-alcohol extract.

The powdered dry plant material (20 gram) was extracted with 250 ml of methanol:water (70:30) for 8 hours, reflux at 80°C using soxhlet extractor. The crude solvent collected in the flask was dried at reduced pressure in rotary evaporator or lyophilized for recovery of concentrated extract. The yield collected after drying was kept at -4°C until further use.

F) Alcohol extract.

The powdered dry plant material (20 gram) was extracted with 250 ml of methanol/ethanol for 72 hours, reflux at 50°C using soxhlet extractor. The crude solvent collected in the flask was concentrated at reduced pressure in rotary evaporator for recovery of concentrated extract. The yield collected after drying was kept at -4°C until further use.

G) Aqueous extract.

The powdered dry plant material (20 gram) was extracted with 250 ml of de-ionised water for 18 hours, reflux at 80°C using soxhlet extractor. The crude solvent collected in the
flask was lyophilized for recovery of dried extract. The yield collected after drying was kept at \(-4^\circ\text{C}\) until further use.

**Phytochemical studies:**

Qualitative analysis for determining the presence of alkaloids, tannins, flavonoids, terpenoids, steroids, glycosides, saponins, resin, and oil in the plant extracts, were carried out using standard methods as described by Harborne (1973), Trease and Evans (1978) and Sofowora (1993). 0.5 gm of the dried extracts were dissolved in 20 ml distilled water, filtered and used for various qualitative tests.

**Test for Alkaloids:**

The test was performed with Mayer’s, Wagner’s and Dragendorff’s reagents. Observation of white, brown, orange coloration indicated the presence of alkaloids.

**Test for Tannins:**

To 2 ml of filtrate few drops of 0.1% ferric chloride was added and observed for brownish green, blue or blue-black color. Development of color indicates presence of tannins.

**Test for Flavonoids:**

5 ml of dilute ammonia solution was added to 2 ml aqueous filtrate followed by the addition of concentrated sulphuric acid. A yellow coloration observed indicated the presence of flavonoids.
Test for Glycosides:

The extract was hydrolyzed with HCl solution and neutralized with NaOH solution. A few drops of Fehling’s solution A and B were added and solution was heated. Red precipitate indicates the presence of glycosides.

Test for Steroids:

2 ml of acetic anhydride was added to 2 ml of plant extract followed by 2 ml of H_2SO_4. The color changed from violet to blue or green indicates the presence of steroids.

Test for Saponins:

Plant extract was boiled with 5 ml water for few minutes, the mixture was cooled and mixed vigorously. The formation of frothing indicates the presence of saponins.

Test for Resins:

The extract was mixed with the same amount of copper acetate solution and the mixture was shaken vigorously and allowed to separate. A green color indicates the presence of resin.
Test for terpenoids:

Five milliliter of extract was taken in a test tube and 2 ml of chloroform was added to it followed by the addition of 3 ml of concentrated Sulfuric acid. Formation of reddish brown layer at the junction of two solutions confirms the presence of terpenoids.

In-vitro Malaria Parasite Culture.

Equipments:

1) Biological safety cabinet Class II Type (Patel Scientific Instruments).
2) Low temperature Incubator (Electroequip).
3) Deep Freeze (-20°C) (Voltas).
4) Cryocans (INOX).
5) Autoclave.
6) Hot air oven.
7) Room Air Purifier (HiMedia).
8) Biological Stereo Microscope (Lawrence and Mayo).
9) Biological Inverted Microscope (Lawrence and Mayo).
10) UV-Vis Spectrophotometer (Systronics 167).
11) Centrifuge (REMI C24).
12) Laboratory centrifuge (Remi R8).
13) Millipore Filter Assembly.
14) Water bath.
15) Desiccators.
16) Microbalance Citizen CY 64

17) Vortex mixer-Remi Instruments

18) Temperature controlled magnetic stirrer.

Chemicals:

1. Culture media  RPMI-1640 powder with L-glutamine, without Sodium bicarbonate - (Gibco BRL Life Technologies)

2. HEPES (N-2 hydroxyethyl peprazine N-2 ethane sulphonic acid) (Gibco)

3. Sodium Bicarbonate - (HiMedia).

4. Gentamycin sulfate - Genticyn 80 mg/2 ml.

5. Glycerol AR (Astron).

6. Sorbitol (HiMedia).

7. Hypoxanthine (HiMedia).

8. Geimsa powder (Sigma Fine Chemicals)

9. Glycerol(Glycerine)

10. Eosin (Water soluble)(Himedia)

11. Methylene blue (Medicinal)

12. Potassium dichromate (K,Cr,O$_7$)

13. Disodium Hydrogen Phosphate dihydrate (Na$_2$HPO$_4$.2H$_2$O)

14. Dimethyl Sulphoxide (DMSO) (Merck)
15. Immersion oil.
17. Hydrochloric acid (HCl).
18. Sulphuric acid (H₂SO₄).
19. Sodium Chloride (NaCl).
20. Heparin (heparin Sodium Injection BEPARINE 5000 IU/5ml).
21. Xylene.
22. Methanol
23. Ammonia solution
24. Formaldehyde solution
25. Sodium hypochlorite
26. Potassium permanganate
27. Calcium chloride
28. Copper sulphate.
29. Microscopic Slides.

All reagents used for the culture purpose were of the tissue culture grade.
Washing and sterilization of glassware:

All the glassware used for the experiments were soaked in chromic acid overnight and washed in running tap water for one hour. These glassware were then transferred to boiling soap water for 2 hours followed by cleaning them in running tap water. To remove the soap if remaining, these glassware were dipped in 5% HCl overnight followed by cleaning with running tap water, single distilled water, double and triple distilled water. They were dried in the oven at 180°C for 24 hours and autoclaved at 15 lb pressure for 30 minutes and again transferred them to high temperature incubator for 24 hours at 180°C.

Preparation of medium (RPMI-1640):

Stock media:

10.4 gm. of RPMI-1640 powder was dissolved in 900 ml of autoclaved triple distilled water. 2.5 ml of HEPES (1M) buffer was added along with 1.0 gm. of glucose to the RPMI-1640 solution. The volume was maintained up to 960 ml. To avoid contamination 40 μg/ml of Gentamycin sulfate was also added to the media ensuring proper stirring of media to dissolve all the constituents properly. The final volume was made up to 1 litre. The media was filtered using 0.22 μm filter and stored at 4°C till further use.

Incomplete media (washing media):

96 ml of stock RPMI 1640 media in a glass media bottle was mixed with 4.2 ml of 5% sodium bicarbonate. The Sodium bicarbonate was added to the RPMI media only at the
Complete Media preparation:

4.2 ml of 5% sodium bicarbonate and 10 ml of plasma were added to 96 ml of the RPMI stock media in a glass media bottle. Complete media was always freshly prepared and filtered prior to use.

Preparation of RBCs and Plasma:

O\textsuperscript{+ve} blood was obtained from the blood bank. Plasma was separated by centrifuging at 3000 rpm for 10 minutes. The clotting elements were removed from fresh plasma by adding, one drop at a time, 1 ml of 10% \( \text{CaCl}_2 \) per 100 ml plasma. After stirring for 30 minutes the clot was removed and the plasma was filtered and kept frozen at \(-20^\circ\text{C}\) for further use (Jensen, 1979; Hui et al., 1984). The buffy coat along with some RBCs was removed from the centrifuged blood. The remaining RBCs were washed 3-4 times with the washing media (incomplete media). The washed RBCs were stored for further use.

Plasmodium Culture initiation:

The \textit{P. falciparum} strains MRC-2 and 3D7 were obtained from Malaria Parasite Bank of National Institute of Malaria Research Center (NIMR) New Delhi. The infected RBCs were washed with incomplete media every time before use. The culture was initiated using
fresh RBCs with an initial parasitemia of 1%. The culture was maintained in glass vials in a
dessicator at 37°C (Trager and Jensen, 1976).

**Continuous culture process:**

Normally the media replacement was done once in a day but if the growth of parasites
was not seen properly, then the media was changed twice a day. Routine checking for
increase in parasitemia was performed by counting the numbers of schizonts and ring stages
using Jaswant Singh Battacharji (JSB) stain on thick/thin smear.

**P.falciparum subculture:**

When the parasitemia was above 3%, the parasites were subcultured by dispensing
into fresh vials with a parasitemia of 1%. For sub-culturing 0.2 ml washed RBC was taken
with fresh 2ml complete medium and 100μl infected blood from old vial was added.

**Cryopreservation of adapted parasites:**

The culture with more than 50% ring stages was selected for cryopreservation. The
infected blood was centrifuged at 1500 rpm for 10 min, supernatant removed and cells were
resuspended in an equal volume of cryoprotectant. The cells were distributed in small
aliquots in screw cap vials and frozen quickly by immersion in liquid nitrogen.
**Revival of cryopreserved parasites.**

The cryovials were thawed quickly and initiated with 15% plasma in the complete media followed by reducing the amount of plasma 10 % in the complete media.

**In-vitro schizonticidal assay of plant extracts:**

**Synchronization of parasites:**

Synchronization of *P. falciparum* to obtain only the ring stages was done by treating 1 ml of infected RBCs with 5% sorbitol for 10 minutes (Lambros and Vanderberg, 1979). The sorbitol treated samples were centrifuged at 1500 rpm for 5 minutes, the supernatant was decanted, the pellet was washed thrice in washing media and the culture was initiated using fresh RBCs as mentioned earlier.

**In vitro screening of antiplasmodial property of plant extracts:**

The culture was synchronized using 5% aqueous solution of sorbitol (Lambros and Vanderberg, 1979). All other stages except ring stages were found to degenerate. Degenerated stages were removed by centrifuging for 5 minutes at 1500 rpm. The supernatant was discarded, and the pellet was washed thrice with incomplete media. Parasitemia was adjusted to about 1% for assay by diluting with fresh washed RBCs. The plant crude extracts were dissolved with 10% DMSO in media having a final concentration of 1mg/ml. The tests were performed using a 96-well micro plates (flat-bottomed) (Desjardins *et
(al., 1979) with 2 fold serial dilutions. All tests were run in triplicate. Synchronized parasite culture was incubated in test well plates with each extract for 24 hours.

**In-vitro Experimental test series:**

The following plant extracts were evaluated *in-vitro*:

1. TPH : *T. indica* shoot hexane
3. TPDE : *T. indica* shoot Diethyl ether.
4. TPC : *T. indica* shoot Chloroform
5. TPA : *T. indica* shoot Acetone
6. TPM : *T. indica* shoot Methanol
7. TMPW : *T. indica* shoot Hydromethanol
8. TPW : *T. indica* shoot aqueous
9. FRPLC : Fresh *P. rubra* leaves and stem chloroform
10. FRPLE : Fresh *P. rubra* leaves and stem ethanol
11. FRPLM : Fresh *P. rubra* leaves and stem methanol
12. FRPLW : Fresh *P. rubra* leaves and stem aqueous
13. DRPLPE: Dry *P. rubra* leaves and stem Petroleum ether
14. DRPLM : Dry *P. rubra* leaves and stem methanol
15. DRPLFM: Dry *P. rubra* flowers methanol
16. XPE : *X. strumarium* bur Petroleum ether
17. XC : *X. strumarium* bur Chloroform
18. XM : *X. strumarium* bur methanol
19. XMW : X. strumarium bur hydromethanol
20. XW : X. strumarium bur aqueous

The in-vitro experimental tests carried out included:

RBC : Normal cultured RBC
IRBC : RBC infected with plasmodium
SnRBC : Synchronised infected RBC

IRBC + TPM : Infected RBC treated with T.indica methanolic extract
IRBC + DRPLM : Infected RBC treated with dry P.rubra methanolic extract
IRBC + TPW : Infected RBC treated with T.indica aqueous extract
IRBC + XW : Infected RBC treated with X.strumarium aqueous extract
IRBC + XM : Infected RBC treated with X.strumarium methanolic extract
IRBC + FRPLW: Infected RBC treated with fresh P.rubra aqueous extract
IRBC + FRPLC: Infected RBC treated with fresh P.rubra chloroform extract

**In-vitro Efficacy Evaluation** :

After 24 hours of each culture, Normal RBC alone, Plasmodia Infected RBC, Synchronised infected RBC and Cultures of infected RBC with test extract, the slides were prepared, stained and scored according to the WHO guidelines (WHO, 1997). The values were compared between control and test wells. The inhibition percentage of schizont per each
concentration of extract/fraction was calculated as: Inhibition=100-A, where A is % of schizont in the test wells, which were determine by the following formula

\[ A = \frac{Z}{m} \times 100 \]

Where Z is the number of schizonts per 200 asexual parasites in the test wells, m is the number of schizont in control wells (WHO, 1997). From dose – response curve, IC\(_{50}\) and IC\(_{90}\) values (concentration at which the inhibition of parasite growth represent 50% and 90% respectively) were derived for each extract/fraction by plotting % inhibition against concentration of the test material (Sharma and Sharma, 1999).

**Preparation of Jaswant Singh Battacharji (JSB) stains:**

The stain solutions were prepared to stain the blood smears and study the parasite morphology and determine percent parasitemia from stained smears.

**Composition for JSB- I:**

Methylene Blue (medicinal): 0.5 gm

Potassium dichromate (K\(_2\)Cr\(_2\)O\(_7\)): 0.5 gm

Disodium hydrogen phosphate dihydrate (Na\(_2\)HPO\(_4\).2H\(_2\)O): 3.5 gm

Sulphuric acid (H\(_2\)SO\(_4\)) 1%: 3 ml

Distilled water (Double distilled water): 500 ml
Preparation:

500 ml of water was measured in a flask with measuring cylinder. 0.5 gm. methylene blue was added to 500 ml of distilled water and dissolved properly. 3 ml. of sulphuric acid (1%) was added in three parts (1 ml at a time) and solution was stirred thoroughly. 0.5 gm potassium dichromate was added and mixed properly by shaking till blue color turn violet until precipitation observed, then 3.5 gm. disodium hydrogen phosphate dehydrate was added, and solution was shaken till precipitate dissolves. Once the solution becomes clear (no precipitate) it was condensed for at least 1 hour using a reflex condenser, till the color of the solution deepens. This solution was filtered and stored in the brown bottle in room temperature for at least 2 days for proper stain maturation.

Composition for JSB- II:

Eosin yellow (water-soluble): 1.0 gm.

Distilled water: 500 ml.

Preparation:

1 gm of eosin was dissolved in 500 ml of distilled water filtered with Whatman filter paper no. 1 and stored in a bottle (brown color), allowed for maturing for 48 hours before use in dark at ambient temperature, as freshly prepared eosin solution did not yield as satisfactory results.
Staining technique using JSB stains:

The stain solutions JSB-II, JSB-I and buffered water were taken in three staining jars and labeled accordingly. Thin smear was fixed with methyl alcohol (Methanol). Fixed blood smear was dipped in JSB-II (Red) stain for few seconds (two or three dips) and immediately washed in buffer water (approximately ten times). Then smeared slide was dipped in JSB-I (Blue) stain for 40-45 seconds and washed well in buffer water. The blood smear was allowed to dry. Examination of the dried slides was carried under oil immersion (objective 100X) in biological microscope. The duration of staining required at JSB-I and JSB-II was varied from batch to batch, and it was also dependent on the maturation of stain. Hence the staining time was predetermined by employing varied time sequences, to every batch of stain. Filtration of the stains was done daily before use through Whatman filter paper No.-1.

Preparation of Giemsa stain:

Composition:

Giemsa powder (Azure B type): 3.8 g

Glycerol, Pure: 250 ml

Methyl alcohol: 250 ml

Preparation:

The stain was prepared by mixing methyl alcohol and glycerol, with gradually adding small quantities of Giemsa powder in a porcelain mortar. Grinding was done till the powder dissolves. This was then filtered and stored in a brown bottle for maturation in room temperature for 15 days. Stock solution was diluted with the distilled water before use.
Staining technique using Giemsa stain:

1:10 dilution of Giemsa stain was prepared from the stock solution. The methanol fixed slides were subjected with the stain for 45 to 60 minutes. The stain was washed off thoroughly with distilled water and kept for drying. After drying slide was observed under the microscope in oil emersion (objective 100 X).

Diagnostic methods in malaria:

The definite diagnosis of plasmodia infection was established on the finding of parasite in the blood, since malaria can be confused with any other fever. Thus, the diagnosis was always a matter of clinical judgment. The simplest and surest test was the time-honoured peripheral blood smear study for malarial parasites. There are other newer tests though, but none have surpassed the gold standard peripheral smear study.

Peripheral smear study for malarial parasites:

The Malaria parasite test was the gold standard in diagnosing malarial infection. It involves collection of a blood, making of smear, its staining with Romanowsky or Giemsa stains and examination of the red blood cells for intracellular malarial parasites (Chwatt, 1985).

Thick blood films:

The drops of the blood was touched with glass slide held above the blood drop and then after reversing the slide the blood was spread evenly with a corner of another slide to
make square or a circle patch of moderate thickness that will just allow one to read through it. The slide was kept horizontal while drying and was protected from dust and high temperature (Chwatt, 1985).

**Thin blood films:**

The drop of blood was kept smaller than the thick film. Smooth end of another clean glass slide was applied to the drop of the blood at an angle of 45°, slide was touched to the drop of the blood until its spread along the edge. The spreader was pushed forward keeping it at the same angle. Thin film was dried by waving it in the air (Chwatt, 1985).

**Examination of blood film from the infected patient:**

The parasites are easily detected in the thick film but they may be more difficult to identify than in thin film. This is due to the fact that the red blood cells are not visible, as a result of hemolysis subsequent to staining an unfixed film. The only elements that are seen in the film are leucocytes and the parasites. However, the appearance of the latter is somewhat altered because of dehaemoglobinisation and slow drying in the course of the preparation of the film. Thus the young trophozoites appear as incomplete ring or spots of blue cytoplasm with detached red chromatin dot. In late trophozoites of *P. vivax* the cytoplasm may be fragmented and Schuffner’s stippling may be less obvious; the band form of *P. malariae* are less characteristic. However, the schizonts and gametocytes of these species retain their usual appearance and the same goes for the crescents of *P.falciparum*. Although the thick film is recommended as a routine method, it may be supplemented by taking a thin film, which could be of value when the correct identification of some parasite species is of importance.
Quantitative aspect of thick and thin blood films for examination of malaria parasites:

Based on the examination of 100 microscopic fields under oil immersion, with a magnification of 1000 times, the numerical threshold, at which malaria parasites can be detected by an experienced technician in well stained blood films is about 100 parasite per 1 μl of blood; for thick film the threshold is lower about 10-20 parasite per 1 μl of blood.

Changes in red blood cells infected with human malaria parasites as seen in the thin film (Chwatt, 1985).

**P. vivax:** Large than normal, paler, often slightly distorted. Schuffner’s dots present in nearly all infected cells except for young rings. Multiple infections by several parasites uncommon. Pigment brownish in short scattered rods.

**P. malaria:** About normal size or slightly smaller. Stippling not seen by normal staining. No multiple infections on erythrocytes, as a rule. Pigment seen in early stages, dark granules rather than rods, often seen at the periphery of the cell.

**P. falciparum:** Normal in size. Multiple infections of erythrocyte very frequent. Some cells yellowish, seem to have a thicker rim (brassy cells). No Schuffner’s stippling but irregular clefts (Maurer’s dots) may be seen in overstrained films. Pigment
granular with tendency to coalesce. In gametocytes (crescents) the outline of erythrocyte barely seen.

**P. ovale:** Many infected erythrocytes enlarged and definitely oval in shape while the parasite is round or elongated. The outline of infected cells often ragged (fimbriated). Schuffner’s dots prominent at all stages of the parasite. Pigment brownish similar to that of *P. vivax*.

**Oxidative stress parameters:**

**Lipid Peroxidation (LPO) - Thiobarbutiric acid reactive species assay (TBARS):**

The Thiobarbutiric acid reactive species (TBARS) levels in normal RBCs, infected RBC (IRBCs) and treated RBCs were detected by the method of Okhawa *et al.* (1979).

**Principle:**

The method is based on the formation of a red chromophore that absorbs at 532 nm following the reaction of thiobarbituric acid (TBA) with malonyl dialdehyde (MDA) and other breakdown products of peroxidised lipids collectively called as thiobarbituric acid reactive substances (TBARS).
Method:

Preparation of hemolysate:

Normal, IRBCs and treated RBCs were washed with incomplete media. RBC lysate was prepared by lysing a known amount of RBC’s with cold hypotonic phosphate buffer, pH 7.4. The hemolysate was separated by centrifuging at 3000× g for 10 min at 4°C. (Devasena et al 2006).

0.01 ml of hemolysate was added to the tubes containing 0.01 ml of 8.1% sodium dodecyl sulphate (SDS). 0.075 ml of 20% acetic acid solution (adjusted to pH 3.5 with 1N NaOH) and 0.075 ml of 1% thiobarbituric acid (TBA) solution. The blank was prepared for each sample by substituting TBA solution with distilled water. The final volume was made to 0.2 ml with distilled water. The solution was mixed and heated in a water bath at 95°C for 60 minutes. The tubes were then immediately cooled and 0.2 ml of the aliquot was transferred to a centrifuge tube to which an equal volume of 10% TCA was added. The solution was mixed and centrifuged at 1000 g for 15 minutes. The aliquot of the resulting supernatant fraction was read against blank on Systronics Digital Spectrophotometer 167 at 532 nm.

Calculation:

\[
Concentration\ of\ MDA = \frac{O.D.\ of\ sample \times \text{dilution factor} \times 10^9 \times 100}{E \times \text{tissue wt. (}mg\text{)} \times \text{Aliquot vol}}
\]

Where, dilution factor =1

\[E=\text{extinction\ coefficient\ for\ MDA}\ (1.56 \times 10^5)\]

The results were expressed as nanomoles of MDA/mg tissue weight /60 minutes.
Superoxide Dismutase (SOD; E.C. 1.15.1.1):

The activity of superoxide dismutase (SOD) in normal RBCs, infected controlled RBCs (IRBCs) and treated RBCs were assayed by the method of Kakkar et al.(1984).

Principle:

In this method, the formazan formed at the end of the reaction indicates presence of the enzyme. One unit of the enzyme activity is defined as the enzyme concentration required to inhibit 50% of the optical density of chromogen formed in one minute at 560 nm under the assay condition.

Method:

Hemolysate was prepared as mentioned earlier. In the assay system, the control consisted of 1300µl phosphate buffer (0.052M sodium pyrophosphate buffer at a pH 8.4 adjusted with 0.052M NaH₂PO₄ H₂O), 50µ ml of freshly prepared phenazine methosulphate (PMS: 186 µM in double distilled water), 150 µl of nitroblue tetrazolium (NBT: 300 µM in double distilled water) and 100µl of fresh NADH (780µM in double distilled water). To the sample system 100 µl of hemolysate was added. Then the reaction was stopped by addition of 1ml of glacial acetic exactly 90 seconds after the addition of the hemolysate. 2ml of n-butanol was added to the tubes and shaken vigorously to extract the formazan. Then the tubes were
centrifuged for 10 minutes at 2000 rpm and supernatant was used for the measurement of optical density at 560 nm against butanol on a UV-vis spectrophotometer systronics 167.

Calculations

\[ X = \frac{O.D \text{ of std}}{O.D \text{ of sample}} \times \text{Accuracy factor} \times \frac{\text{dilution}}{\text{standard enzyme units}} \]

Where, Accuracy factor=1.0; dilution=2.5; Standard Enzyme Unit =3.0

\[ \text{Enzyme activity} = \frac{X}{mg \text{ protein}}. \]

The activity was expressed as units SOD/mg protein

Total Protein Estimation:

Total Protein content was estimated in RBCs, infected control and treated samples by the method of Lowry et al. (1951) using bovine serum albumin as standard. The blue colour that developed is quantitatively proportional to the total protein present and was measured at 540 nm. The protein contents was expressed as mg/100 mg tissue weight.

Hemoglobin (Hb) Determination:

Hemoglobin determination, or hemoglobinometry, is the measurement of the concentration of hemoglobin in the blood. The synthesis of hemoglobin takes place in the developing red cells located in bone marrow. Hemoglobin (Hb) is converted to acid haematin
by addition of 0.1 N hydrochloric acid and resulting brown colour is compared with standard brown glass reference blocks of a Sahli's hemoglobinmeter. The level of the fluid was noted at its lower meniscus and reading corresponding to this level on the scale was recorded as g/dl (Mulchay, 1970).

**Thin-layer chromatography (TLC):**

Thin-layer chromatography consists of a stationary phase immobilized on a glass or plastic plate, and a solvent system. The choice of suitable solvent system was made by running elutropic series of different solvents on the TLC plates. 5µl of the extract in a solvent was deposited as a spot on the stationary phase the chromatographic precoated with silica on an aluminium plate (60F254,10cm×10cm). The bottom edge of the plate was placed in a solvent reservoir, and the solvent moved up the plate by capillary action. When the solvent front reached the other edge of the stationary phase, the plate was removed from the solvent reservoir. The separated spots were visualized with ultraviolet light (UV-254nm and UV-366nm). The different components in the extract moved up the plate at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase.
High Performance Thin Layer Chromatography (HPTLC):

HPTLC provides a resourceful, quick and reliable technique for the quantitative and qualitative analysis of new and unknown phyto-chemicals in plant extracts. Thus the technique is now widely used in phyto-chemistry, toxicology and forensic laboratory (Pawar et al., 2008). The plant metabolites with several uniqueness (alkaloids, terpenoids, flavanoids, steroids, saponins, tannins etc) can be used as a medicine. This study helps to identify active metabolites involved and as an analytical tool in quality control aspects of the developed product (Pattanaya et al., 2010). Each constituent in the sample is separated on TLC (Thin Layer Chromatography) plate through selected solvent system considering the polar, non-polar or the intermediate nature of the metabolites. This is then followed by scanning, retention factor ($R_f$) value and $\lambda$ max profile metabolites (Sharma and Patel, 2009). The HPTLC technique is supportive to evaluate profiles of the crude plant extracts made by using various solvents for the maximum extraction of the desired metabolites in selected solvents. The comparative analysis with different solvent extracts would also serve for value addition of the product (Harborne, 1984).

Function of solvent system:

Depending on the polarity of the compounds in the extract, a compound travels at diverse distances up the plate. More polar compounds will attach to the polar silica gel and thus travel up to short distances on the plate. While non-polar substances will spend more time in the mobile solvent phase and travel larger distances on the plate (Sabnis and Daniel,
The measure of the distance a compound travels on the plate is called the $R_f$ value. The recognition of separated compounds in HPTLC is mainly done on the basis of retention factor parameter ($R_f$), colors of spots and the computerized fingerprints of samples. These HPTLC generated fingerprints can be saved as electronic images (Harborne, 1984; Cimpoiu, 2006). $R_f$ value of each spot is considered by calculating and dividing the distance traveled by the solute (compound) from the baseline upon distance traveled by the solvent from the baseline (solvent front). Qualitative analysis was performed with the assistance of HPTLC instrument. The HPTLC system (Camag, Switzerland) comprises of (1) TLC Scanner connected to a PC running WinCATS software under MS Windows NT; (2) Linomat V Sample applicator. The HPTLC analysis requires sample and solvent preparation.

**Sample preparation:**

All reagents used in this preparation were of analytical grade. Each extract was redissolved at the concentration of 50-100 $\mu$g/ml in individual solvent in which they were extracted in narrow glass vial and used for plate application.

**Plate activation:**

Aluminum sheet back coated with silica gel 60$\ F_{254}$ plates were taken for the study. The plates were activated in an oven at 80 °C (10 min) before use. This process assists to remove the moisture and activates the active sites of silica gel for better separation.
Sample application:

Camag Linomat V was used for nitrogen gas-assisted and controlled application of samples on to TLC plate. The sample extracts were streaked in form of narrow bands on the precoated silica gel 60F254 aluminum TLC plate, at a stable application rate of 50-250 μl/s. Samples were applied at the height of 10 mm from the base, having specific band width and space flanked by two bands.

Plate development and chromatographic conditions:

Following sample application, the plates were subjected to linear ascending development, in a selected solvent system (ethyl acetate: methanol: ammonia), up to a distance of about 80 mm. Twin trough glass chamber (with 10 min prior saturation with the solvent system) was utilized at room temperature.

Scanning of plate:

Succeeding to the development, the TLC plates were dried in a current of air. Densitometric scanning was done using Camag TLC Scanner III (Camag, Switzerland) in the absorbance mode at 254 nm wavelength with a scanning speed of 20 mm/s, data resolution 100 μm/step and a specific slit dimension. The source of radiation used was deuterium and tungsten lamp. All other measurement parameters were at default settings. The
chromatograms statistical data were generated using WinCATS evaluation software (Version 1.4.6.8121).

**Photo documentation of plate:**

Following scanning, images were taken at wavelength 254 and 366 nm by UV/Vis. Lamp, in Ultra violet cabinet attached with smart Digital Photographic unit (Camera).

**Post chromatographic derivatization of TLC plate:**

Post-chromatographic derivatization of developed TLC plates was also done wherever necessary. Anisaldehyde: sulfuric acid spray reagent was utilized to mark visible spots visible on the plate.

**Molecular docking and bioavailability studies of selected phytochemicals against Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1)**

**PREPARATION OF PROTEIN TARGET STRUCTURE AND LIGANDS:**

The X-ray crystal structure of Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) was recovered from the Protein Data Bank (PDB ID- 2YK0)( Bernstein et
were subjected to energy minimization using GROMOS96 utility (without reaction field) executed in Swiss-Pdb Viewer 4.0.1.(Guex and Peitsch, 1996).

The series of these plant bioactives was based upon the availability of literatures quoted experimentally authenticated anti malarial action and/or the conventionally used medicinal plants in which the active constituent(s) accounted for treating malaria. Ligand structures were regained in Structure Data Format (SDF) from NCBI PubChem (Bolton et al., 2008) and wherever appropriate, drawn using ChemSketch v. 10 (ACD/ChemSketch, version 10 2006) with reference to 2D structures obtainable in the literature. The dataset comprised of 10 ligands (Septicine, Tylophoridinedine, Tylophorin,Tylophorinine, Xanthumin, Plumericin, Isoplumeric, fulvoplumericin, Quercetin, Xanthydrol) were energy minimized using molecular mechanics geometry optimization module executed in HyperChem (2011.10. Hyperchem 8.0.7-Licensed version at The Gujarat Cancer Research Institute, Asarwa, Ahmedabad). AMBER force field with distant reliant dielectric constant, scale factor for electrostatic and Van der Waals forces set to 0.5 and without any cutoffs to bond types and its lengths were selected to finish global minimum energy conformation. For that cause, all the structures were minimized and exported to hard disk.

Active site prediction

This calculation was not approved of for protein structures co-crystallized with ligand as the ligand binding site was occupied as active site for docking with the ligand dataset. Structures with unbound ligands were computationally analyzed for active site using
Q-SiteFinder (Alasdair et al., 2005). It differentiates pockets on the protein surface through calculation of van der Waals interaction energies using a methyl probe and probes with favorable interaction energies were gathered and ranked.

**Virtual screening**

Ligand dataset selected for studying were virtually screened (docked) into the binding site of the target protein *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) using Argus Lab 4.0.1 from Planaria Software LLC. To allow fast sampling, the binding site was constructed which consist of all remnants that have at least one atom within 3.5 Å from any atom in the co-crystallized ligand. This method of constructing binding site was helpful for protein structures with co-crystallized ligand. A varied approach was performed for proteins with unbound ligand whose active site was predicted using Q-Site Finder. Best 3 scored pockets was computationally examined for each protein target using Jmol Java plugin executed in Q-SiteFinder and the amino acids entrenched in the predicted cavity volume was utilized as active site residues. These two methods generally gave a good representation of the important residues in the binding pocket for a protein target. A grid box of size 22 X 15 X 16 with atom scaling of 0.40 Å was produced and high precision Argus Dock engine with A Score as scoring function were chosen. After grid generation, the ligands were flexibly docked with the protein and 1000 poses were created, among which best 10 poses of low-energy which were collected in rank 1, were examined. Argus Dock engine makes use of ligand torsionality as a hierarchical tree in which the root’s node (group of bonded atoms that do not have rotatable bonds) is placed in a search point inside a grid containing residues of
the active site. A set of varied and energetically favorable translations are generated and poses that survives in torsional search through an estimated thorough search are retained and finally clustered.