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Chemicals:

The chemicals used in the present study were procured from Gibco BRL Life Technologies, Hi-Media Laboratories Pvt. Ltd. (Mumbai, India) and Sisco Research Laboratories Pvt. Ltd. (Mumbai, India).

Instrumentation:

Biological safety cabinet (Patel Scientific Instruments), Low temperature Incubator (Electroequip), Deep Freeze (-20°C) (Voltas), Cryocans (INOX), Room Air Purifier (HiMedia), Hi Performance Thin Layer Chromatography System (CAMAG), Biological Stereo Microscope (Lawrence and Mayo), Biological Inverted Microscope (Lawrence and Mayo), UV-Vis Spectrophotometer 117 (Systronics), Centrifuge (REMI C24), and Laboratory centrifuge (Remi R8).

Parasite Culture and Maintenance:

Reagents:

The important reagents required for the malaria parasite cultures are listed below. It is vital to observe strict quality controls while choosing chemicals as well as preparing and processing the reagents.

1. RPMI-1640 powder with L-glutamine, without Sodium bicarbonate - Gibco BRL Life Technologies
2. HEPES (N-2 hydroxyethyl peprazine N-2 ethane sulphonylic acid) (Gibco)
3. Sodium Bicarbonate - (HiMedia)
4. Gentamycin sulfate - Genticyn 80 mg/2 ml.
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5. Glycerol AR (Astron)
6. Sorbitol (HiMedia)
7. Hypoxanthine (HiMedia)
8. Eosin (Water soluble)
9. Methylene blue (Medicinal)
10. Potassium dichromate (K$_2$Cr$_2$O$_7$)
11. Disodium Hydrogen Phosphate dihydrate (Na$_2$HPO$_4$·2H$_2$O)
12. Chromic acid
13. Hydrochloric Acid (HCl)
14. Sulphuric Acid (H$_2$SO$_4$)
15. Sodium Chloride (NaCl)
16. Xylene
17. Methanol
18. Ammonia solution
19. Formaldehyde solution
20. Sodium Hypochlorite
21. Potassium permanganate
22. Calcium chloride

All reagents used for the culture purpose were of the tissue culture grade.

Washing and sterilization of glassware:

All the glass ware 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
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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) solution:

Procedure:

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. Filtered the media using 0.22 μm filter and stored at 4°C till further use.

Preparation of Washing Media (Incomplete 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 time of requirement and was prepared fresh. The media was once again filtered and maintained the pH of 7.4.

Preparation of Complete Media:

4.2 ml of 5% sodium bicarbonate was added to 96 ml of the RPMI stock media in a glass media bottle, 10 ml of plasma was added and stored at 4°C till use. Complete was always freshly prepared and filtered prior to use.
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**Preparation of RBCs and Plasma:**
O\(^+\)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, 1ml of 10 % 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°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.

**Initiation of Plasmodium Culture:**

The *P. falciparum* strains MRC-2 and RKL-9 were obtained from Malaria Parasite Bank of NIMR (National Institute of Malaria Research Center) 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). Routine checking for increase in parasitemia was performed by counting the numbers of schizonts and ring stages using Jaswant Singh Battacharya (JSB) stains.

**Subculture of *P. falciparum***:

When the parasitemia was above 3% the parasites were subcultured by dispensing into fresh vials with a parasitemia of 1%.

**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 min.
remove the supernatant, washed the pellet thrice in washing media and the culture was initiated using fresh RBCs as mentioned earlier.

Cryopreservation and revival of parasites:

The culture with more than 5 % ring stages were cryopreserved for further use and revived as and when required by standard protocol. During the revival and initiation of the culture 15 % plasma was used in the complete media followed by reducing the amount of plasma upto 10 % in the complete media.

In vitro screening of antiplasmodial property of plants:

Plant material:

The plants were identified morphologically and were examined properly and the collected plant materials were authenticated in the Botany Department, School of Science, Gujarat University and in the Biosciences Department of Vallabh Vidyanagar, Anand. The herbarium was prepared and deposited in the Herbarium sections of the respective departments. The crude extraction was carried out with ethanol and water in soxhlet apparatus. The standard method for crude extraction was followed according to Harborne (1973) and Raman (2006). Further extraction was carried out by the liquid-liquid separation technique in separating funnel, these solvents were decided according to the polarity and non polarity index of the solvents or the literature survey.

Preparation of ethanol and aqueous extract from whole plants of Ephedra, Vitex sps plants:

The plants were collected, washed and shade dried under ambient temperature. After complete drying the plants were sliced and ground with a steal blender. The ground powders were sieved to get fine powder material. 100 gm. powdered plant material was
capsulated in filter paper and kept in the thimble, 800 ml solvent (ethanol and water) was added into the flask, and the continuous extraction was carried out in the soxhlet apparatus for 72 hrs. (till the color of the solvent in the siphon becomes colorless), then 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 weighed and kept at -20°C until further use.

**Preparation of ethanol and aqueous extract of latex of Calotropis plant:**

Latex was collected in solvents (ethanol/water) at 1:1 v/v (latex:solvent). The twig of the plant and stem barks were small incisioned and the latex was allowed to flow, which was collected in the collecting tube with the solvents upto mark. After the collection, the tubes were allowed to settle and centrifuged at 1500 rpm for 10 min till the rubber like material settles down the clear supernatant was collected, and the pellet was resuspended in 10 times the volume with solvent and soxhleted for 24 hrs to get the maximum extraction out of it and the filtered and individual supernatant was collected. The collected solvent was concentrated using rotary evaporator or lyophilizer, and the dried extract was weighed and kept at -20°C until further use.

**Preparation of ethanol and aqueous extract from fruits of Colocynthis and seeds of Lantana plants:**

**Colocynthis:**

The fruits were washed properly after slicing them in small pieces allowed to dry in ambient temperature in sterile room. The dried fruits were steel blended and the powdered material was sieved and stored in the air tight glass bottles till further use. The dried powder material was extracted as described above.
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**Lantana:**

The seeds of the lantana were collected washed properly with distilled water to remove the dust and soil particle if any, and processed further as described above.

**Preparation of ethanol and aqueous extracts from roots of the Sida sps. plant:**

The roots from the plants were separated out and were washed properly with distilled water to remove the soil particles and were sliced to small pieces and shade dried in ambient temperature. The dried roots were grounded properly and were extracted with ethanol and water respectively as described earlier.

**Extraction of crude extract:**

5 gm. of concentrated crude extract was dissolved in 100 ml of 50% ethanol and was shaken well in a separating funnel. To it was added 50 ml of hexane and after shaking for 5 min. it was kept for 1 hr. The upper layer was collected as hexane fraction and was repeated thrice. Same procedure was followed for chloroform, ethyl acetate and butanol respectively.

**In vitro screening of extracts against P. falciparum:**

The culture was synchronized using 5% aqueous solution of sorbitol (Lambros and Vanderberg, 1979). All other stages except ring stages are being degenerated. Degenerated stages have been removed by centrifuging for 5 min. 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 and the polar and non polar extracts were dissolved with 10% DMSO in media having a final concentration of 1mg/ml. The tests was performed using a 96-well micro plates (flat-bottomed) (Desjardins et al., 1979) with 2 fold dilutions
serial dilutions. Synchronized parasite culture was incubated in test well plates with each extract for 24 hours and after that the slides were 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:

\[ \text{Inhibition} = 100 - A \]

where \( A \) is % of schizont in the test wells, which were determined 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, and \( m \) 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 extraction/fraction by plotting % inhibition against concentration of the test material (Sharma and Sharma, 1999).

**Preparation of 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 \( (K_2Cr_2O_7) \): 0.5 gm
- Disodium hydrogen phosphate dehydrate \( (Na_2HPO_4.2H_2O) \): 3.5 gm
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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 atleast 1 hrs. in reflex condenser till the color of the solution deepens. This solution was filtered and stored in the brown bottle in room temperature for atleast 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's 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).
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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 an employing varied time sequences, to every batch of stain. Filtration of the stains was done daily before use through Whatman's filter paper No.-1.

Preparation of Giemsa's 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, that 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 with dust and flies (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* (Chwatt 1985). 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 µl if a thin film technique is used; for thick film the threshold is lower about 10-20
parasite per µl of blood, but here the experience of the microscopist is an important factor (Chwatt 1985).

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

- **P. vivax**: Larger 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. malariae**: 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*.

**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 aqueous and alcoholic 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 Dragendroff’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 Phenols:**

To filtrate, few drops of neutral 5% ferric chloride solution was added. Dark green color indicates the presence of phenolic compounds.

**Test for Steroids:**

2 ml of acetic anhydride was added to 2 ml of plant extract and 2 ml of H2SO4 was added. The color changed from violet to blue or green indicates the presence of steroids.

**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. Red precipitate indicates the presence of glycosides.
Test for Saponins:
Plant crude extract was boiled with 5 ml water for few min., the mixture was cooled and mixed vigorously. The formation of frothing indicates the presence of saponins.

Test for Resins:
The extract was obtained in a test-tube, the same amount of copper acetate solution was added and the mixture was shaken vigorously and allowed to separate, a green color indicates the presence of resin.

Test for Oils:
Press the small quantity of extract between two filter papers, the oil on filter paper. Indicates the presence of fixed oils.

Estimation of Total Protein:
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 developed is quantitatively proportional to the total protein present and was measured at 540 nm. The protein contents were expressed as mg/100 mg tissue weight.

Determination of Hemoglobin (Hb):
A routine test performed on practically every patient is the hemoglobin determination. Hemoglobin's main function in the body is to carry oxygen from the lungs to the tissues and to assist in transporting carbon dioxide from the tissues to the lungs. Hemoglobin determination, or hemoglobinometry, is the measurement of the concentration of hemoglobin in the blood. The formation 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).

**High Performance Thin Layer Chromatography (HPTLC):**

HPTLC provides an efficient, fast and consistent 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 various characteristics (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 component 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 helpful to compare profiles of the crude plant extracts prepared 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).

**Role of solvent system:**

Depending on the polarity of the compounds in the extract, a compound travels at different distances up the plate. More polar compounds will stick to the polar silica gel...
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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, 1990; Sharma and Patel, 2009). The measure of the distance a compound travels on the plate is called the \( R_f \) value. The identification 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 calculated 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 help of HPTLC instrument. The HPTLC system (Camag, Switzerland) consists of (1) TLC Scanner connected to a PC running WinCATS software under MS Windows NT; (2) Linomat V Sample applicator. The HPTLC analysis needs sample and solvent preparation.

**Sample preparation:**

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

**Plate activation:**

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

Camag Linomat V was utilized 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 constant 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 between two bands.

Plate development and chromatographic conditions:

After 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 used at room temperature.

Scanning of plate:

Subsequent to the development, the TLC plates were dried in a current of air. Densitometric scanning was carried out 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 utilized was deuterium and tungsten lamp. All remaining measurement parameters were at default settings. The chromatograms were integrated statistical data were generated using WinCATS evaluation software (Version 1.4.6.8121).

Photo documentation of plate:

After 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 performed wherever necessary. Anisaldehyde: sulfuric acid spray reagent was used to mark visible spots visible on the plate.

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity:

To defend cells and organs from the oxidative stress induced by reactive oxygen species (ROS), living individuals have advanced with an extremely efficient and highly sophisticated system, the so-called “antioxidant defensive system” composed of a group of compounds and enzymes strong enough to remove free radicals. These components function interactively and synergistically to counterbalance free radicals before they cause damage (Percival, 1998). Antioxidants are substances that neutralize free radicals or their action (Sies, 1996). A broader definition of an antioxidant is “any substance which, when present at low concentration compared to those of oxidizable substrates”. The term oxidizable substrate includes DNA, lipids, proteins and carbohydrates, which are essential building blocks of a biological system (Halliwell et al., 1995). To avoid oxidative stress, antioxidants can play an important role conferring beneficial healthy effects as there are of naturally occurring and synthetic antioxidants known. These antioxidants belong to different classes of compounds, such as carotenoids, polyphenolics, gallic acid derivatives, tannins and catechins. Examples include phytic acid, lipoic acid, melatonin, quercetin, rutin and butylated hydroxytoluene (Vaya and Aviram, 2001).

DPPH assay with TLC was used to assess the antioxidant activity of extracts shown effective as antiplasmodial. Extracts were redissolved at the concentration of 50-
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100 μg/ml in respective solvent in which they were extracted. 15 μl of this dilution were applied on the precoated silica gel 60F_{254} aluminum TLC plate with the fine capillary. The plates were subjected to linear ascending development, in a selected solvent system (methanol: ethyl acetate: ammonia - 77:15:8 v/v/v). The plates were dried at room temperature and sprayed with 0.2% DPPH in methanol. Purple color of DPPH reagent bleaching by yellow spots is the indication of positive antioxidant activity (Bektaş et al., 2005).