Materials & Methods
1. In vitro culture of malaria parasite- *Plasmodium falciparum*

1.1 Culture initiation from cryopreserved infected blood cells

- The culture of malaria parasite was obtained from Malarial Research Centre (MRC), Delhi in cryopreserved condition.
- The cryopreservation would preserve malaria parasite in a viable condition for a long time without continuous culture during transportation.
- The cryopreserved vial was taken out from dry ice and the frozen liquid was thawed by rolling the vial between two palms.
- Transfer the culture in centrifuge tube and centrifuge at 1500 rpm for 10 min, discard the supernatant.
- Give wash in the same way by 3.5% NaCl and then two washes with complete culture media.
- The washed RBC cells were inoculated to the complete medium and transferred to the culture vial.

1.2 Maintenance of *P. falciparum* continuous culture

- Once the culture is established the fresh media has to be provided twice in a day. Remove the old media carefully from the culture vial without disturbing the settled RBC layer and add 1-2ml fresh media (Figure 1;d). Shake gently to mix the culture properly. Normally the media replacement once a day was enough but if the growth of parasite was not seen properly, then the media has to be changed twice a day.
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- To examine from a culture take a drop on slide and make a smear. Fix the smear by giving a dip in methanol and process the same for staining. Observe the stained slide for counting parasitemia percentage in oil immersion (100X) lense. The proper time of sub-culturing was when the parasitemia percentage becomes more than 1% (more than three parasites in one oil immersion field).
- For sub-culturing take 0.2 ml washed RBC with fresh 2 ml complete medium and 100μl infected blood from the old vial. Discard the old vial with the remaining blood.
- Shift the culture in CO$_2$ incubator with 5.0 % CO$_2$ and 98 % humidity level. In the absence of CO$_2$ bottle the candle-jar method (Trager and Jensen, 1976) was applied (Figure 1;e). The culture vials were arranged in desiccators with burning candle and closed the lead and allowed the candle to extinguish. Close the cork of the lead. Grease the lead margin twice in month to maintain the vacuum. The candle consumes the oxygen of the internal environment resulting in the rise of CO$_2$ level up to 5.0%.
- The basic requirements needed in routine culture are shown in Figure 1;a, from left to right- filtering flask, filtering unit, culture vials, filters (0.22μm), pipette holder, sterile pipette (10ml), sterile pasture pipette (3ml), 96-well plate with covering steakor, RPMI-1640 culture media, blood, serum, sodium bi carbonate (5%), gentamycin and hand vacuum pump.
1.3 Media preparation and sterilization procedure

- Dehydrated powder of media RPMI-1640 (Hi-Media/GIBCO) used for parasite culture (16.2g/pkt) contains RPMI-1640 (10.4 g/l) with L-glutamine and 25mM HEPES buffer (5.8g/l), without sodium bicarbonate.
- The entire packet was dissolved into 960ml sterile distilled water in laminar flow; rinse the pack with water to remove the traces of media.
- Add 2g glucose and 40µg/ml gentamycin sulfate (1.2ml from 80mg/2ml injection vial available at local chemist). Gentamycin is one of the potent antibacterial which has no effect on parasite growth and thus prevents the risk of contamination.
- Use sterilized measuring cylinder (1 liter capacity) and flask (2 liter capacity) to measure distilled water and pour in flask for sterilization. Cool the sterilized water by tap water and transfer it in laminar flow.
- The media is sterilized through nylon membrane filter of 0.22µm porosity (Millipore) by media filtration assembly (Tarson). The assembly was fitted on filtering flask and through vacuum pump the media is pressurized to pass through membrane and collect in filtering flask (Figure 1,b). The filtration assembly with nylon membrane and filtering flask was sterilized and cooled in laminar flow before use.
- Distribute the sterile media in two screw caped, tissue culture disposable bottles (Tarson) (500ml) and store at 4-8 °C in refrigerator. Seal the mouth of bottles with parafilm and cover it with aluminum foil.
1.4 Preparation of complete media for culture maintenance

- 40ml of media is taken separately in 50ml capacity sterile bottle from main stalk in sterile conditions for routine use. The pH of media is adjusted between 7.2 to 7.4 with the help of 5% sterile tissue culture grade sodium bicarbonate solution (Hi-Media).
- The proper pH is judged by conversion of yellow media to light pinkish orange colour and confirmed by keeping drop on pH strip having the desirable pH range.
- The solution of 5% sodium bicarbonate is sterilized at 121°C for 15min in autoclave and stored in refrigerator.
- The media with adjusted pH is known as incomplete media. Such incomplete media is used for RBC washing. When the media is supplemented with proper serum then it is called complete media used for parasite culturing (Figure 1;c).
- Complete media having 15% serum (1.5ml in 8.5ml media), is adjusted for pH after addition of serum.
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a. Basic requirements for *P. falciparum*, in vitro culturing

b. RPMI-1640 media sterilization in vitro culturing through 0.22μm filtration

c. Complete media preparation with addition of serum and pH adjustment

d. Routine media changing in continuous culture of *P. falciparum*

e. Anaerobic atmosphere created by candle-Jar method

f. Schizonticidal testing of plant extracts in 96-well plate

Figure 1: *In vitro* culture technique of *P. falciparum*
1.5 Collection and procedure for human erythrocytes

- Any blood cell type can be used for parasite culture, but as the original culture obtained from MRC, Delhi was maintained in A⁻ve RBC cells, I have continued with A⁺ve cells.
- 10ml A⁺ve whole blood is collected in sterile centrifuge tube and centrifuged at 1500rpm for 10 min to separate plasma and the yellowish buffy layer of WBC (on the top of RBC) was carefully removed and thrown with plasma. Another two wash was given to RBC cells with incomplete media (adjusted pH).
- The washed RBC cells were stored with even volume of complete media in the same centrifuge tube and kept in refrigerator.

1.6 Collection and procedure for human serum

- The parasites were cultured in A⁺ve blood cells therefore the serum type used was either A⁺ve, AB⁺ve or O⁺ve which were compatible to A⁺ve cells.
- One unit (350ml) of blood was collected without anticoagulant and kept in refrigerator in the lower rack (8°C) for 24hrs to get separate serum from blood.
- The bag was opened in laminar flow and serum was transferred in 50ml sterile centrifuge tubes. Maximum four tubes are required for this, so the rest of the coagulated blood remaining in the bag was discarded.
- The tubes were centrifuged at 3000 rpm for 30min at 8-10 °C in refrigerated centrifuge. The tubes were carefully taken out and opened in laminar flow.
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- The serum was carefully sucked by sterile pipette (10ml capacity, Tarson) without disturbing settled blood cells and transferred in sterile tissue culture bottles (60ml capacity, Tarson).
- The separated serum was inactivated at 56 °C for 30min in water bath. This treatment destroys the immune related proteins which may hamper in culture.
- The inactivated serum was stored in frozen condition in deep freeze.

1.7 Stain preparation for malaria parasites

The stains used for routine parasite observation were Field A&B (Field et al., 1963) and JSB A&B (Singh & Battacharji, 1944).

1.7.1 Preparation of Field stain

Dissolve the listed ingredients in water to prepare stains and stored in tight bottles.

Remove the necessary amount in staining jars for daily use. There are two solutions.

**Solution A:**
- Methylene blue: 0.8g
- Azure I: 0.5g
- Di-sodium hydrogen phosphate (anhydrous) $\text{Na}_2\text{HPO}_4$: 5.0g
- Potassium di-hydrogen phosphate $\text{KH}_2\text{PO}_4$: 6.25g
- Distilled water: 500ml

**Solution B:**
- Eosin: 1.0g
- Di-sodium hydrogen phosphate (anhydrous): 5.0g
- Potassium di-hydrogen phosphate: 6.25g
- Distilled water: 500ml
Staining procedure:

- Fill both the solution individually in separate staining jars
- Dip the slide with fixed smear in solution A for 45 sec.
- Take it out and remove the excess stain by dipping it in tap water.
- Dip it in solution B for 10 sec and again rinse it in tap water.
- Counter stain by solution A again for 15 sec. Rinse in tap water and slide is ready to observe under microscope.

1.7.2 Preparation of JSB stain

<table>
<thead>
<tr>
<th>Solution A:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene Blue</td>
<td>0.5g</td>
</tr>
<tr>
<td>Potassium dichromate ((K_2Cr_2O_7))</td>
<td>0.5g</td>
</tr>
<tr>
<td>Sulphuric acid ((1% \text{ by volume}))</td>
<td>3.0ml</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate ((\text{anhydrous}) \ Na_2HPO_4)</td>
<td>3.5g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500ml</td>
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</tbody>
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Dissolve methylene blue in water and gradually add drop by drop sulphuric acid with proper stirring to ensure thorough mixing. Next potassium dichromate is added which gives purple precipitate, then disodium hydrogen phosphate is added. Stir the solution and in some time the precipitate gets dissolved. Put the solution on reflux for boiling exactly for one hour, count the time from when the boiling starts. Store in amber bottle and use it at least after 2 weeks when it gets mature. Always filter JSB-A before use.

Solution B: Just dissolve the powder in water and stored in amber bottle.

| Eosin | 1g |
| Distilled water | 500ml |

Staining procedure:

- Stain in JSB-B for 5 sec. Rinse in water
- Stain in JSB-A for 30sec. Rinse in water. Dry and observe in microscope.

2. Extractions derived from the selected plant parts

2.1 Selection and processing of plant material

The plant parts used were apical bud, flowers, apical twig and dry latex. The apical buds consisted terminal leaf pairs up to 4th internode with maximum leaves length of 8.5cm. The apical twigs about 12” long of Calotropis procera were collected, washed under running tap water, and dried in hot-air oven at 42 °C (one feet long apical twig includes apical bud, leaves, flowers, tender stem and tender bark). The latex of plant was collected in clean glass or plastic bottles and dried in drying dishes at 42 °C in oven. After 10-12 days, the dried plant materials were powdered and kept in airtight bottles until used.

2.2 Extraction derived by aqueous and organic solvents

2.2.1 Aqueous extraction

The plant material(s) was mixed with water in 1:16 (g/ml) ratio to prepare a “Kwath” (water decoction). The mixture was heated on electric coil until one fourth of the volume remained in the container. Cooled the container for 10-15 min and filtered it with four folds of Nylon cloth. The filtrate was dried in oven at 42 °C in drying plates over anhydrous sodium sulphate (Na$_2$SO$_4$).
2.2.2 Partition of aqueous decoction with organic solvents

The dried filtrate obtained from the water decoction of most active plant sample (latex) evaluated by schizonticidal screening, was again extracted with acetone. The acetone extraction was carried out for 3 hrs on soxhlet apparatus at 50°C with solvent cycle rate of 5cycles/hr. Then the dried water extract taken out from soxhlet apparatus and crushed with acetone in mortar with pestle and again extract that powder in soxhlet for another 3hrs with acetone in same conditions. This acetone fraction was combined and dried in oven at 38°C. The dried acetone fraction was again re-dissolved in hot water. The aqueous solution of acetone fraction was transferred into a separating funnel for the further partition with n-hexane, chloroform, ethyl acetate and n-butanol successively. Three washes were given by each solvent and than combined and dried at 38 °C in oven over anhydrous sodium sulphate and stored in refrigerator until used. All fractions were tested for schizonticidal activity.

Testing of antibacterial efficacy the additional extractions were carried out from the dried filtrate obtained from water decoction of apical twig and latex. It was again extracted with acetone followed by methanol. In additional set of experiment, instead of methanol, ethanol was used for both the plant samples to check the comparative antibacterial effect of methanol and ethanol fractions against test bacterial strains.

2.2.3 Solvent extraction

The dry powder of plant samples were extracted through soxhlet apparatus. The solvents used were acetone and methanol in serial order. All samples were extracted
for 12 hrs with keeping coil temperature 50°C for acetone (b.p.56°C) and for 4 hrs with keeping coil temperature 70°C for methanol (b.p.78°C) that keeps the rate of solvent cycles from thimble at 5cycles/hr. The powder was completely dried before adding the next solvent. All solvents used in the research were obtained from MERCK.

3. **In vitro schizonticidal testing of plant extracts**

Schizont is one of the parasite stages that is inhibited by the extract, and activity of the extracts are compared on the basis of reduction in schizont percentage (Desjardins *et al.*, 1979).

3.1 **Synchronization of culture**

- Continuous culture has three erythrocyte stages continuously developing from one to another stage named as ring, trophozoid and schizont which has to synchronize for only ring stage before starting an assay.
- Observe the culture and predict appropriate time when the rings would develop maximum in culture.
- Prepare 5% sorbitol in distilled water.
- Mix culture of two vials in one centrifuge tube and centrifuge at 1500rpm for 10min. Discard the old media and add sorbitol in 1:9 proportions to the centrifuge tube.
- Keep for 7min to lyse RBC containing parasite stages of 18hr (i.e. trophozoids & schizonts) and the entire culture will be synchronized for rings only.
- Centrifuge to remove sorbitol from the culture and wash twice with media.
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- Add serum in the equal quantity of RBC and the culture is ready for schizonticidal testing.

3.2 Schizonticidal testing of plant extracts

- The extract is dissolved in dimethyl sulphoxide (DMSO) at 100mg/ml concentration. The repetitive dilutions were prepared in incomplete RPMI-1640 media (pH 7.2) up to 2mg/ml concentration.
- Add initial 100μl incomplete media in all eight wells of the vertical line of 96-tissue culture well plate (Figure 1f).
- From 2mg/ml concentration 100μl was loaded in the third well of 96-tissue culture well plate. The first well was of control containing only infected blood with medium. Second well was of experimental control having DMSO dilution without drug to know the level of inhibition through DMSO.
- From third to eight well proceed with double dilution method. The range of extract concentration after double dilution from 3rd to 8th well was 0.1 to 0.003 mg/well (ie. 1 to 0.03mg/ml).
- Finally add 10μl synchronized culture in each well.
- Close the plate and put in incubation for 36 hrs for the development of schizonts from rings.
- In between 36 to 40 hrs opened the plate and removed the media and prepared slides from all wells.
3.3 Derivation of IC$_{50}$ value

- IC$_{50}$ value is the concentration of compound that required for inhibiting 50% schizont maturation in the parasite population.
- Count up to 200 infected RBC from each slide of eight wells.
- Calculate schizont inhibition percentage from the formula:
  
  \[
  100 - \left[ \frac{(\text{No. of schizonts in test} \times 100)}{\text{No. of schizonts in control}} \right]
  \]

- Plot schizont inhibition percentage on Y-axis and log$_2$ (logarithm of the base 2) of the double diluted extract concentrations on X-axis. The shape of graph achieved was of sigmoid shape. The linear portion of the graph was used to obtain IC$_{50}$ through linear regression analysis with the help of Microsoft Excel.
- The IC$_{50}$ value obtained from plotted graph was actually the log of concentration based on two. Therefore it was converted first in the natural log and than, after application of anti-log it was converted to real value of extract in mg/ml.
- Data of schizont inhibition are in the form of serially increasing data therefore it was directly plotted on axis with equal interval. While the data of double dilution can't be plotted on the axis with the equal division therefore its log on the base of two was taken. The concentration of extract tested was in double dilution therefore logarithm based on two of each extract was plotted on graph. The formula used to find log$_2$X was log$_2$X = log$_e$X/loge 2 (loge2 = 0.69).
4. In vivo extract testing against *P. berghei* infected mice

The active extracts were tested for curing acute as well as cerebral malaria in mice model.

4.1 Initiation of infection in mice model

The rodent parasite *Plasmodium berghei* ANKA clone obtained from Tata Institute of Fundamental Research, TIFR, Mumbai was used to induce blood staged malaria (acute malaria) in Swiss White mice (SW). The same clone failed to induce cerebral malaria in SW mice and therefore *Plasmodium berghei* ANKA clone was obtained from Dr. Pied, Pasteur Institute which was reported for cerebral malaria induction in C57Bl/6 mice. Subsequent experiments in C57Bl/6 mice obtained from three places viz., ACTREC (Mumbai), NIV (Pune) and NCLAS (Hyderabad) indicated that there were some differences in the C57Bl/6 mice from each source and all mice do not develop cerebral features. It was seen that majority of the mice from NCLAS developed the most visible and reproducible clinical symptoms of cerebral malaria. For future experiments for cerebral malaria, breeding pairs of C57Bl/6 mice have been obtained from NCLAS to build up the mouse colony. Therefore SW mice were used for blood stage malaria and C57Bl/6 mice were used for cerebral malaria.

Strain of *P. berghei* was maintained in liquid N\textsubscript{2} and at the time of experiment it was thawed and injected in healthy mice. The mice were daily examined for percentage of parasite load in blood. When the parasite load reach up to the
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desirable level than the appropriate volume of blood is withdrawn and immediately transfused to an experimental mouse. Some amount of blood was stored again in liquid N\textsubscript{2} for use in future experiment.

4.2 Base line values for body weight, rectal temperature and hemoglobin of mice

Before \textit{in vivo} extract(s) evaluation against acute malaria (blood staged malaria) in mice model, baseline values were obtained for body weight, rectal temperature and hemoglobin of healthy mice of different ages and from both sexes. To obtain a standard graph for hemoglobin, Drabkin's cyanohemoglobin method was used.

Simultaneously, dissection and histology of normal C57B1/6 mouse brain was done for comparison with cerebral malaria stage.

4.3 Standardization of blood stage and cerebral malaria

Infected blood used for infection was collected from infected mice showing trophozoite stage with 40-60% parasitemia and this was injected in experimental mice in volume of $\geq 100$ $\mu$l which was the most suitable for experiment. Experiments were started initially using two routes of infection viz. intra peritoneal (ip) and intra venous (iv). Finally infection with $10^5$ parasites/mouse was standardized for SW mice to induce blood staged malaria and $10^6$ parasites/mouse was standardized for C57 mice to induce cerebral malaria through i.p root of infection. Finally i.v. was not used in experiment.
4.4 Standardization of Chloroquine regimens for blood stage malaria in SW mouse

Intra-peritoneal injection with $10^5$ parasites/mouse was given to SW mice. Since the herbal preparation to be tested will be given orally, chloroquine was also given orally to infected mice after day 5 post infection when the parasite load in the blood was 1-2%. Three different doses of chloroquine tried were viz.,

1) 30 mg/kg body weight on day 5 and 10 mg/kg body weight on day 6 and 7 post infection.
2) 30 mg/kg body weight from day 5 to 7 post infection.
3) 10 mg/kg body weight from day 5 to 9 post infection.
4) 30 mg/kg body weight from day 5 to 9 post infection.

The survival rate of mice was maximum at 30mg/70 kg/5 days; therefore this regimen was finally selected for comparison with plant extracts.

4.5 Extracts evaluated for in vivo anti-malaria testing

The extracts was stored in refrigerator at 6-8° C. At the time of experiment it was dissolved in distilled water with 1mg/ml concentration for orally administration to the experimental mice. In every experiment the extracts given from 5th day of infection up to subsequent 5 days. Each experiment contains total 25 animals; 7-Control (Without any treatment), 7-Chloroquine treated, 7-plant extract testing, 4-plant extract (no parasite). The extracts selected for in vivo activity against P.burghei in mice were as follows:
1. Water decoction of dry latex powder (oven dried, 42°C)

Latex decoction was evaluated on cerebral staged malaria in C57 mice model with 500mg/70kg/day dose orally fed for 5 days from day 5, post infection.

2. Cold water extract of fresh apical bud (spray dried, 60°C)

Fresh apical buds were crushed in cold water and left over night at 8°C in refrigerator in air tight container. Next day it was filtered through muslin cloth and dried in spray drier to get fine power. This powder of apical bud tested for acute blood stage malaria in Swiss White mouse strain with dose 385mg/70kg/day for 5 days after 5 days of post infection.

5. Chemical profile of active extracts

The molecules existing in the active extract derived from the proposed plant drug were identified through HPLC, GC-MS, IR and NMR analysis. The extracts have shown maximum activity in \textit{in vitro} anti-malarial testing was selected for the detection of chemical compounds present in it. The selected extracts were:

(i) Water decoction and its further fractions of latex and apical bud: Subjected for IR to identified range of organic groups present in organic fractions derived from water decoction. The most active ethyl acetate fraction of latex decoction was further subjected for HPLC analysis and compared with six standard compounds.
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**Specification of HPLC analysis:**

1. Solvent system: Methanol : Water (95:5)
2. Flow rate: 1ml/min
3. Detection: UV 205 nm
4. Column: C8 (250mm Length)
5. Standards compared: β-sitosterol, cetile alcohol, tri-palmitin, methyl stearate, methyl palmitate, di-palmitin.

(ii) Acetone extract of dry latex and apical bud subjected to GC-MS for separation and detection of compounds.

The extract contains many compounds and therefore to get proper resolution the dried acetone extract was partitioned between hexane and 90% ethanol combined in 1:5 ratios. n-Hexane layer was separated and solvent evaporated to get liquid oily mixture of hydrocarbons. The dried n-Hexane layer was again re-dissolved in Acetone and remaining part dissolved in n-Hexane with Methanol combined in 9:1 ratios in warm condition (60°C). Both the parts of n-hexane fraction of acetone extract were injected for GC-MS analysis. The entire hexane fraction was subjected to NMR and IR for the confirmation of organic groups present that provides supporting evidence to GC-MS analysis.

**Specification of GC-MS analysis:**

1. GC-MS model: Perkin Elmer Autosystem XL with Turbomass
2. Column type: PE-5MS
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Column Material: 5% Phenyl polysiloxane

Column Length: 30 meters

Column inner diameter: 0.250 mm

3. Flow rate (N2): 1ml/min

4. Temperature of injector: 230°C

Temperature of detector: 280°C

Temperature of source: 280°C

Temperature of transfer: 280°C

5. Programming rate:

Starting from 78°C for 5min

Increasing temperature with rate 10°C/min up to 280°C and hold for 20min

Retention time: 45min

The standard compounds applied for peak identification of GC are as follows:

A : α-amyrine (RT: 20.42 min)

B : B-sitosterol (RT: 22.21 min)

C : Methyl Tridecanoate (RT: 17.21 min)

D : Methyl ricinoleate (RT: 24.41 min)

E : Histamine (RT: 16.38 min)

F : Methyl palmitate (RT: 20.56 min)

G : Methyl Palmitoleate (RT: 20.33 min)
6. Pharmacognosy of the plant parts used in herbal formulation

The fingerprinting of the range of chemical compounds present in the used plant material was generated with HPTLC, IR, NMR and GC instruments. Detection of elements with present heavy metals investigated with XRF and AAS. The additional pharmacognosy parameters include extractable matters, ash values and growth rate of apical bud were carried out. The majority of pharmacognosy methods were followed according to WHO (Anonymous, 2002).

6.1 Sample preparation and solvent systems for HPTLC fingerprinting

HPTLC fingerprinting was obtained from the apical buds C. procera. The sample from powder of dried apical buds was prepared according to the method described by Harborne (1998) with little modifications to separate terpenoids and phenols. Powder of dry apical buds was homogenized for 5 min in MeOH-H2O (4:1) (10 X wt.). Extract was filtered with Whatman No.1 filter paper and evaporated to 1/10 vol (<40°C). Condensed extract was acidified with 2-3 drops of H2SO4. Extract it with CHCl3 (X 3) in separating funnel (125ml capacity). Collect the layer of chloroform and discarded aqueous acidic layer. The chloroform layer was moderately polar extract contains terpenoids and phenolics. This fraction of phytochemicals was used for HPTLC fingerprinting of apical buds. Latex sample was characterized by n-hexane fraction of acetone extract for HPTLC fingerprinting.

The solvent system used for apical bud extract was petroleum ether: ethyl acetate: acetone: n-propanol: ethanol: water (40:4:5:0.5:0.2) and for latex extract was n-hexane: di-ethyl ether: acetic acid (80:20:1v/v). The latex extract was compared with standard
compounds oleic acid, triolein and β-sitosterol. The separation was achieved on precoated Si-Gel plate (E-Merck, F253) 0.5mm thick, on which sample was loaded with the help on Linomat IV. The plate was developed in two twinge Cammage TLC chamber. The developed spots were observed under UV366 and UV254. The chromatographic plate of apical bud extract was developed with Anisaldehyde to obtain various colour spots according to the characteristic of the compounds and latex extract developed with concentrated sulphuric acid (Wagner and Bladt, 2002).

6.2 IR and NMR specification for fingerprinting of powdered dry latex of C. procera

IR spectra of latex samples were obtained on Perkins Elmer FTIR (spectrum GX). The solid powder of dried latex was mixed with Sodium bromide (NaBr) in 1:100 ratio and made palate under heavy pressure. The pallet was subjected under the beam of infrared with wavelength 400-4000nm in FTIR instrument. Spectra obtained on NMR at 60MHz with TMS as internal standard and CDCl₃ and DMSO as solvent.

6.3 Extraction of essential oil from latex and characterization through GC

Essential oil was obtained from the dried latex of C. procera into the Clavenger apparatus. Clavenger apparatus with 5ml capacity collection tube and 1L capacity round bottom flask was used. The dried latex powder 100g was boiled in 500ml distilled water for 2hrs. The vapor of boiling water was collected with 4ml/min in collection tube. The brown droplet collected into the collection tube on the top of water was characterized through GC with the same method describe elsewhere in the text.
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6.4 Method of extraction for extractable value determination

Powder of dry plant material 10g was extracted with soxhlat apparatus for 3hrs in solvents series. Extraction was achieved from apical bud in descending order with ethanol, water, petroleum ether, chloroform and acetone and from latex in the order of acetone, water, ethanol, chloroform and petroleum ether. The powder was taken out from soxhlat and completely dried before adding another solvent for extraction. The extracts were dried in oven (42°C) to determine the extract value in percentage (g/g). The extractive value was calculated to find out yield of particular solvent extraction.

6.5 Ash value determination

Total ash:

5g dried powered apical bud and mature leaves taken in two different sets in separate crucibles. Ignite it at 500-600°C until the formation of white ash. Weigh the ash with crucible and deduct the weight of crucible to obtain exact ash value in mg/g of dried material.

Acid-insoluble ash:

In the same crucible with ash, add 25ml HCl (70g/l), cover with a watch-glass and boil gently for 5 min. Rinse the watch-glass with 5ml of hot water and add this liquid to the crucible. Collect the insoluble matter on an ashless filter-paper and wash the crucible with hot water to recover remaining residues on filter paper. Ignite the ash less filter paper with acid insoluble content in the same crucible until none of the residue of ash-less filter paper seen in crucible. Allow to cool in desiccator for 30 min and weigh immediately.
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Weigh crucible with acid insoluble ash and deduct the weight of crucible to obtain amount of acid insoluble ash in mg/g of dried plant powder.

Water-soluble ash:
In another set of crucible with total ash, add 25ml of water and boil for 5 minute. Collect the insoluble matter on ashless filter-paper. Ignite it in crucible for 15 min at 450°C. Subtract the weight of this residue in mg from the weight of total ash. Calculate the content of water-soluble ash in mg/g of dried material.

6.6 Growth rate of apical bud of Calotropis procera

The growth pattern of plant height with leaves expansion was studied continuously for 37 days on infant plant. Height of plant measured was from base (soil) to the first node and not up to the leaf apex. Leaf growth measured was noted from nodal attachment to the apex from abaxial (outer) side. The study was performed in the month of November and December 2003 in the winter season.

7. Clinical trial of herbal regimen

The present protocol for clinical assessment of herbal antimalarial drug is based on the recommendation by The WHO unit on Anti-infective Drug Resistance Surveillance and Containment (DRS) for assessing the in vivo response. The criteria take into account the traditional WHO 28 day in vivo test (Anonymous, 1973), the WHO criteria (Anonymous, 1996), and the views of malariologists. The criteria involve (i) clinical criteria for study entry and withdrawal, and (ii) parasite counts during follow-up.
Clinical trial establishes the drug efficiency on human volunteers. It has three phases:

Phase-I: It covers 80-100 patients to judge drug efficacy on proposed disease.

Phase-II: It covers 100-300 patients with totally controlled clinical trial conducted by doctors to scientifically prove drug efficacy and recognize side effects.

Phase-III: It covers 300-1000 patients in mass scale to find out the maximum range of drug efficacy and toxicity in population.

The phase-I open clinical trial was conducted for proposed herbal regimen with the involvement of doctors to prove its anti-malarial efficacy. The study initiated by the pilot scale evaluation with enrolment of few patients and after the success of herbal treatment the large number of victims enrolled for clinical trial. The large scale distribution of herbal regimen was conducted through NGO named “People’s Health and Development Trust” and “Kutch Nav Nirman Abhiyan (KNNA)” where the drug was dispensed through health workers in the interior regions of Kutch, where the modern medication was far away.
7.1 Criteria for examination and medication of patients

According to the FDA Rules & Regulations the herbal medicine to be used for human consumption has no need to take any permission before going for clinical assessment through doctors. The present protocol for clinical assessment of herbal antimalarial drug is based on the recommendation by The WHO unit on Anti-infective Drug Resistance Surveillance and Containment (DRS).
Following criteria have been followed for this:

1. The study population consists of male and female with simple *Plasmodium vivex* infection or with acute uncomplicated *falciparum* malaria aged between 8 to 70 years who consented to participate in the studies.

2. No conventional anti-malarial medicine has to be continued with this treatment.

3. Drug was administered after meal.

4. Terminated the treatment if severe malarial symptoms persisted on 2\textsuperscript{nd} day and patient was considered in danger.

5. With complains like diarrhoea, cough-cold, headache, stomachache, etc. the treatment with the same dose was continued.

6. In the case of other disease symptoms along with malaria viz. dengue, typhoid, jaundice, tuberculosis, treatment of other disease was continued with the running medication for malaria.

7. Medication for diabetes, cardiac problem, renal problem, HIV infection etc. can be continued along with this medication.

8. Pregnant women and infants were not enrolled for treatment. The cases of cerebral malaria were also not enrolled in the conducted study.

9. For anaemia, epilepsy and neurological problems, or when patient is hospitalized, malarial treatment was continued with other treatment.

10. If the patient vomited within the first 30 minutes post administration, the treatment was repeated with the same dose. If the patient vomited between 30 minutes and 1 hour after dosing, half dose was administered. The patient with persistent vomiting should be excluded from the study. These data were recorded in the study.
11. If the drug was not found suitable with patient’s physiology or the progress of the patient was unsatisfactory, then the treatment was discontinued and the appropriate suitable medication was continued with the help of the doctor.

12. Medication was completed either on 3rd day or on 7th days in the case of tablets or capsules respectively.

13. Patient’s peripheral blood parasitaemia (asexual and sexual form) should be assessed once daily on days 0, 1, 3, 5, 7 and during follow-up on the days 14, 21 and 28 for confirmation of relapse. Parasite count should be recorded.

14. The body temperature was noted from the three sides of body; from neck, fold of elbow and from folded palm.

15. In malaria prone areas one capsule on alternate days up to four capsules for seven days were given as preventive to the healthy persons of patient family. The same prophylactic effect of the capsules was proved on students of college during monsoon. Follow up was taken after monsoon to find the occurrence of malaria in the treated group in comparison of the untreated group of the same institute.

16. The supportive Ayurvedic medicines given were:

1. Vomiting: Chhardi ripu 2 tablests 3 times a day. In case of severe vomiting dose could be repeated 2 tabs every hour.

2. Anorexia, colic: Agnitundi vati: 2 tabs twice a day after meals.

3. Hyperacidity: Sutsekhar ras (Suvarna Rahit): 2 tabs twice a day after meals. (Headache is many a times associated with hyperacidity)

4. Diarrhoea: Kutaj Ghanvati: 2 tabs 2-3 times a day. In case of acute diarrhoea karpur ras vati (2 tabs) can be coupled with Kutaj Ghanvati.
17. In case of patient’s absence for follow-up, the patient was excluded from the case study.

18. The privacy of patients is maintained during the representation of results. The data of patients are strictly limited for research purpose.

7.2 Blood smear collection, parasite identification and counting

7.2.1 Collection, fixing and staining of blood smears

Middle finger of left hand is normally selected for pricking. Clean the finger tip with spirit swab and prick it with sterile disposable needle. Remove the first spot of blood with cotton swab and twist the finger little to allow the blood come out on the tip. Hold the finger properly and touch clean glass slide on the top of fresh blood drop. Give three touch for thick smear preparation and single touch for thin smear preparation on the same slide. Use the corner of another slide to spread the blood film in square shape to prepare a thick film. For preparation of thin film, put another slide edge touching to blood spot at right angle and spread it to make thin film.

Fix thin film by deeping in methanol, care should be taken that it won’t touch to thick film. Allow thick smear for 12hrs before de-haemoglobinization for proper drying. Then wash with distilled water drop by drop passing from thick smear to remove haemoglobin and fix with methanol by a single dip in coupling jar. The purpose of thin film is to confirm the species of *Plasmodium* either *falciparum* or *vivex*. The stain gives colour to haemoglobin of RBC, WBC and malarial parasite.
While in the thick film the haemoglobin was washed out therefore the staining is imparted to parasite and WBC. This condition is used for counting percentage of parasite from thick film. Stain the slide as shown in the section 1.7.1 and 1.7.2.

7.2.2 Identification of parasite from thin film

There are certain characteristics for right identification of malaria parasite. Before declaring the smear nil for malaria parasite minimum 100 fields from thin film has to be viewed carefully.

<table>
<thead>
<tr>
<th>Characteristic of \textit{P. falciparum}</th>
<th>Characteristic of \textit{P. vivex}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two Chromatin</td>
<td>Single Chromatin</td>
</tr>
<tr>
<td>Multiple Infection</td>
<td>Single parasite in RBC</td>
</tr>
<tr>
<td>One sixth of RBC</td>
<td>One third of RBC</td>
</tr>
<tr>
<td>Thin Cytoplasm</td>
<td>Thick Cytoplasm</td>
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<tr>
<td>RBC Not Enlarged</td>
<td>RBC Enlarged</td>
</tr>
<tr>
<td>Banana shaped Gametocytes</td>
<td>Spherical Gametocytes</td>
</tr>
<tr>
<td>High Parasitaemia</td>
<td>Low Parasitaemia (Max 1-2%)</td>
</tr>
<tr>
<td>Rarely found Schizont in peripheral blood</td>
<td>All stages found in peripheral blood (ring, trophozoid, schizont)</td>
</tr>
</tbody>
</table>

7.2.3 Malaria parasite count from thick film

Stained thick film is examined to quantify the parasitaemia. Parasitaemia is measured by counting the number of asexual parasites (rings, trophozoids and schizonts) against a number of WBC in the thick blood film, based on a putative count of 8,000 WBC per $\mu$l as an adequate mean WBC in the population under investigation. The number of asexual parasites is counted against at least 200 WBC (enumeration on the field being counted when reaching 200 WBC must be completed). The parasitaemia (in $\mu$l) is calculated using the formula:
Materials and Methods

Parasitaemia (μl) = \( \frac{\text{Number of parasites} \times \text{WBC count (8,000)}}{\text{Number of WBC counted (200)}} \)

If > 500 parasites have been counted without having reached 200 WBC, the count is stopped after completion of count in the last field. The parasitaemia is calculated according to above formula. If \( P. falciparum \) gametocytes are seen, a gametocyte count is performed against 1,000 WBC.

7.3 Involvement of doctors

Consent of doctors was obtained with their official seal and signature on the proposed protocol as shown below:

18. One tablet per day can be given, dose according to the age, as prophylactic in the malaria prone areas or during malarial epidemic outbreak.

19. Supportive herbal medicine are suggested:
   1. Vomiting: Chhardi ripu 2 tablets 3 times a day. In case of severe vomiting dose could be repeated 2 tabs every hour.
   2. Anorexia, colic: Agaisanthi vali: 2 tabs twice a day after meals.
   3. Hyperacidity: Sanechar ras (Suvra Rishi): 2 tabs twice a day after meals. (Headache is many times associated with hyperacidity)
   4. Diarrhoea: Kairi Ghamari: 2 tabs 2-3 times a day. In case of acute diarrhoea karpas vali (2 tabs) can be coupled with Kutaj Ghamari.

20. Researcher agrees to maintain the privacy of the patient. He also agrees to use this data strictly for the research purpose.

I have carefully read the above submissions and willing to give my help for this research programme.

Name:
Designation:
Clinic Address:
Residential Address:
Permanent Address:
Phone - Clinic:
   Residence:
   Mobile:
E-mail:

Signature and Seal
List of the doctors participated in the research programme of clinical trial of herbal anti-
malarial treatment are as under.

**Doctors of Government Ayurvedic OPDs**

**Anand District**

<table>
<thead>
<tr>
<th>Name of the Doctors</th>
<th>Centers of OPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Dr. Pravinbhai J. Desai</td>
<td>Sojitra</td>
</tr>
<tr>
<td>2. Dr. D.B.Vyas</td>
<td>Umaretha</td>
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<tr>
<td>3. Dr. G.L.Vyas</td>
<td>Mogri</td>
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<tr>
<td>4. Dr. Ramesh M. Patel</td>
<td>Petlad</td>
</tr>
<tr>
<td>5. Dr. J.D.Panara</td>
<td>Khanhat</td>
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<tr>
<td>6. Dr. Nirupama N. Joshi</td>
<td>Borsad</td>
</tr>
<tr>
<td>7. Dr. Mulajibhai V. Rabari</td>
<td>Petlad</td>
</tr>
<tr>
<td>8. Dr. V.D.Upadhyaya</td>
<td>Kathal</td>
</tr>
</tbody>
</table>

**Kheda District**

<table>
<thead>
<tr>
<th>Name of the Doctors</th>
<th>Places of Clinic</th>
</tr>
</thead>
<tbody>
<tr>
<td>9. Dr. Prakash B. Parekha</td>
<td>Apadwanj</td>
</tr>
<tr>
<td>10. Dr. K.R.Bavrya</td>
<td>Hariyala</td>
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<tr>
<td>11. Dr. Barot Kavita M.</td>
<td>Virpur</td>
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<tr>
<td>12. Dr. S.B.Bhatt</td>
<td>Thasru</td>
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<tr>
<td>13. Dr. N.N.Nayak</td>
<td>Makra</td>
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<tr>
<td>14. Dr. M.G.Shaikhr</td>
<td>Kaira</td>
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<tr>
<td>15. Dr. N.R.Nimavat</td>
<td>Matar</td>
</tr>
</tbody>
</table>

**Private Practitioners**

<table>
<thead>
<tr>
<th>Name of the Doctors</th>
<th>Places of Clinic</th>
</tr>
</thead>
<tbody>
<tr>
<td>16. Dr.Sailesh Panchal</td>
<td>Nadiad</td>
</tr>
<tr>
<td>17. Dr.Dattatray Trivedi</td>
<td>Nadiad</td>
</tr>
<tr>
<td>18. Dr.Hamandra Patel</td>
<td>Tarapor</td>
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<tr>
<td>19. Dr.Piyush Patel</td>
<td>Nal</td>
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<tr>
<td>20. Dr.Milin Soni</td>
<td>Nadiad</td>
</tr>
<tr>
<td>21. Dr.Ravindra Patel (M.B.B.S.)</td>
<td>Mogari</td>
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<tr>
<td>22. Dr.Vakharia</td>
<td>Bordoli</td>
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<tr>
<td>23. Dr.Janmajay Patel</td>
<td>Nadiad</td>
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<tr>
<td>24. Dr.Rajesh Maheta</td>
<td>Ahmdabad</td>
</tr>
</tbody>
</table>
Materials and Methods

7.4 Design of forms for data collection during treatment

1. Registration form

It includes patient name, enrollment number, date of enrollment and incoming
dates of follow up during treatment as well as after treatment. This form serves the
purpose of attendant sheets for complete follow up as well as index to search
patient enrollment number from their names.

2. Patient's consent

The signature of patient or parents/guardian is taken on informed consent form
before administering medication

3. Observation form

It contains complete information of patient, present symptoms, running treatment
for any other disease, past malaria history and any other problem present.

4. Follow-up form (during treatment)

This form is filled in during follow-up on alternate days. It gives the complete
scenario of the patient condition (progressive/regressive) during the treatment.
5. Treatment completion form

At the completion of study this form is filled. It gives the information about drug efficacy to see whether patient was cured or not, and gives details of the consumed doses by patient (regularly/irregularly). This helps in to the validation of drug efficacy and concludes the final result.

6. Follow up form (after treatment)

Information of malaria relapse after the completion of treatment up to 28 days is covered in this form.

7. Laboratory assessment form

It includes laboratory assessment of malaria parasite (diagnosis P.f./P.v.) from thin blood film and percentage of parasite from thick blood film.

8. Record of dispensed drug

It provides information about the quantity of medicine supplied to the doctors and number of patients enrolled by them.
Registration Form

Name of Doctor:

Place:

Contact No.:

<table>
<thead>
<tr>
<th>No</th>
<th>Date 1st Day</th>
<th>Patient name</th>
<th>3rd Day</th>
<th>5th Day</th>
<th>7th Day</th>
<th>14th Day</th>
<th>21st Day</th>
<th>28th Day</th>
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</tbody>
</table>
Patient's Consent

I hereby give my consent for me / my relative to take herbal anti-malarial medication to cure/prevent malaria infection.

Signature

Date:

Enrolment No.:

Name of Patient:

Name of Doctor:

Signature and Seal
Observation Form

Date:
Time:

No:
Name:
Address:
Phone:
E-mail:
Date of Birth:
Age: Sex: M / F
Weight: kg Height: Blood group: A / B /
AB / O / RH

Clinical Evaluation:
Fever: Shivering: Periodicity of fever: ___ hr Bodyache: Headache:
Giddiness: Anorexia: Dyspnoea: Nausea: Vomiting: ___/day Weakness:
Hepatomegaly: Splenomegaly: Seizure: Diarrhoea: ___ motions/day
Cough:

Any Other Symptoms/Complications:
Days of symptoms started: Days
Body Temperature (axillary): °C / °F
Respiratory rate: /min
Pulse rate: /min
Malarial Parasite: P.V. / P.F. /
Dose: mg (Tab/Cap) t.d.s. / b.d.s./O.D.
Duration: Days

Drug taken for malarial fever/ other fever during last 7 Days: N / Y
Any other treatment (running / administered):
Malarial fever within the preceding 6 months: Y / N
Antimalarial therapy in the preceding 4 weeks: Y / N
Frequency of earlier occurrences of malaria:
At what age?:
Type of Plasmodia infection diagnosed?:
Drugs taken for malaria in Past:

Physical Examination:
Malnutrition: Mucous membranes: Gastrointestinal diseases:
Allergic: Skin: Blood pressure: Diabetic: Cardiac:
Renal: Hepatic: Spondylosis: Joint pain: Oedema:
Asthmic: Pneumonic: Tuberculosis: Surgery: Other:

Additional Remarks:

Name of Doctor:
Signature and Seal

77
Follow-up Form
(during treatment)

No.:
Name of Doctor :
Name of Patient :
Parasite Diagnosed : P.V. / P.F. / Type of Medication : Tab/Cap
Date of Enrolment :
Date of Examination:
Day of Examination : 3/5/7

1. Is he regularly taking the doses : Y / N Gap: dt: Time:
2. Treatment is further continued : Y / N Reason:
   Specification:
3. Malarial symptoms if reappeared:
   Fever: Shevering: Other: dt: Time:
4. Body temperature during fever:
5. Any Complain after drug administration:
   Nausea: Vomiting: Headache: Stomachache: Diarrhoea: Constipation:
6. Any other Complain:
7. Parasite stage present in peripheral blood: Asexual / Sexual
   Stage Name:
   Dt:
8. Any other observation:
9. Additional Remarks:

Signature and Seal
## Treatment Completion Form

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Name of Doctor</th>
<th>Name of Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Parasite Diagnosed</th>
<th>Type of Medication</th>
<th>Date of First visit</th>
<th>Day of Examination</th>
</tr>
</thead>
</table>

10. Has he completed the dose? : Y / N  
11. Is he completely cured with the initial 3 / 7 days dose of the given mg? : Y / N  
12. Was the treatment terminated?:  
   - No:   - Yes:   - Date of Termination:   - No of Dose  
     - Taken:   - Reason:  
     - Parasitimia:  
13. Any Complain after drug administration:  
   - Nausea:   - Vomiting:   - Headache:   - Stomachache:   - Diarrhoea:   - Constipation:  
14. Any other Complain:  
15. Any other observation:  
16. Any changes in dose was necessary during treatment: N / Y  
   - Reason:  
17. Any other medicine for malaria was administered:  
   - No:   - Yes:   - Generic/trade name:   - Dose:   - mg  
     - t.d.s. / b.d.s.  
     - Date of Medication:   - Time:   - Duration:   - Termination:  
     - Reason:  
     - No of herbal doses administered:   - Herbal Drug is continue: Y / N  
18. Any other drug for other diseases were administered by doctor or patient it self:  
   - No:   - Yes:   - Disease(s):   - Drug(s):  
19. Any other emergency: N / Y  
   - Blood transfusion:   - Saline:   - Hospitalization:  
   - Reason:   - Dt:   - to-  
20. Additional Remarks:  

Signature and Seal
Follow-up Form
(after treatment)

No.:
Name of Doctor:
Patient's Name :
Date of Enrolment :
Date of Examination:
Day of Examination: 14/21/28
Parasite Diagnosed: NONE / P.V. / P.F. /

1. Parasitemia: Stage:
2. Date of parasite diagnosed (Blood Smear):
3. Date of the completion of the last course:
4. Days from the completion of the last course:
5. Is the parasitemia was nil on the 7th day blood smear: Y / N
6. Dose of the last course (Tab/Cap): mg t.d.s. / b.d.s./O.D.
7. Dose of the New Course: Same / Change: (Tab/Cap) mg t.d.s. / b.d.s.
8. Duration: Initial was days + days medicine given

Additional Remarks:

Signature and Seal
Laboratory Assessment

<table>
<thead>
<tr>
<th>Field</th>
<th>WBC Up to 200</th>
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Name of Doctor:
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Signature and Seal
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7.5 Assessment of efficacy

Treatment failure criteria

Treatment failure is defined by the occurrence of any of the following:

1. Development of danger signs or severe malaria on continuous 3rd day after the starting of treatment, in the presence of parasitaemia.

2. Axillary temperature > 37.5°C on Day 3 in the presence of parasitaemia.

3. Parasitaemia on Day 3 > 25% comparison to day 0 count.

Treatment success Criteria

Cumulative cure rate with the completion of treatment and no recurrence by Day 28.

Efficacy end-points

1. Parasite clearance time (PCT: days): defined as time between instituting therapy and clearance of (asexual *P. falciparum*) from peripheral blood film detectable by microscopy and clearance of asexual parasites (negative) throughout the follow up period.

2. Proportion of patients with parasitaemia (asexual *P. falciparum*) by 24 hours (Day 1), and 72 hours (Day 3).

3. Fever clearance time (FCT: days): defined as time required for axillary temperature to fall below 37.5°C and remain so for at least 72 hours.
7.6 Computer software design for management and processing of data

The huge data of different parameters recorded during clinical trials were managed and analyzed with the help of computer software, specially designed by software engineer for the specific purpose of this study. The main entry pages of the software are shown here.
8. Commercial production of medicine with quality control standards

Commercial production includes the complete data on economics of labor charges, collection time for desirable quantity, yield after drying and processing of the raw material, etc. On the basis of these data drug costing was calculated. Quality control standards maintained during drug production includes standard physical conditions of raw material (texture, order, test of dry powder and viscosity, specific gravity and pH of fresh latex). In addition, physical parameters of prepared tablets and capsules were also measured, that includes diameter/length, thickness, hardness and total dissolution time (T.D.T.). The produced medicines were tested for total microbial load before medication.

8.1 Physical parameters of raw material and prepared medicines

Texture, order and test was observed personally and documented. Viscosity of fresh latex was measured on viscometer, specific gravity was ascertained manually by dividing weight of 1ml latex with the weight of 1ml distilled water. The pH of fresh latex was measured with pH strip range 3.5 to 6.0. The spot of latex was put on the surface of pH strip and colour change was observed from the reverse side of the strip. The pH was measured in morning in between 8.00 to 10.00 am. Physical parameters of prepared medicines measured on ‘hardness tester’ for hardness measurement and TDT on ‘disintegration test machine’.
8.2 Total Microbial burden

Aerobic bacteria and enterobacteria (gas forming) were calculated by MPN (Most Probable Number) test and moulds were calculated by plate count method. 1g powder of apical bud/dry latex mix with 10ml sterile distilled water in 10ml capacity sterile centrifuge tube in laminar flow. Centrifuge it at 1500 rpm for 5min to settle the powder and take 1ml from it and add in 9ml sterile distilled water. Now the tube contains microbial burden of 100mg powder. In the same way prepare another two dilution for 10mg and 1mg powder’s microbial burden. Add 1ml from the three dilutions in appropriate test tubes of MPN test.

Most probable number test:

Twelve tubes were prepared, each containing 9ml Nutrient broth and a Delham’s tube. All 12 tubes were divided in to 4 groups each group had 3 tubes. First group was control and was added with sterile distilled water (diluent of sample), remained as it was. In second group, 1ml from first sample tube was added (equal to 100mg powder). Same way in third and fourth group, 1ml solution was added equal to 10mg and 1mg sample from dilution tubes. Incubate the entire set at 37°C in incubator for 5 days. Note the number of tubes from each set for bacterial growth (turbid) and gas bubble trapped in Delham’s tube. The table was used to determine the bacteria count (not shown here, it is available in many books) (Anonymous, 2002).
Plate count method for moulds:

In the same way 1ml from different dilutions of powder solution which give 100mg and 10mg powder concentration add in to warm potato dextrose agar (45°C) 15ml and spread in petri plates (10cm diameter) for the calculation of fungal spores. Streptomycin (100μg/disk) was added to prevent bacterial growth, as it may interfere the fungal colony counting. Two replicates were prepared for each dilution. Incubate at 25°C in BOD incubator. Each new colony developed from a single spore and so the statistical calculation gives the appropriate number of fungal spores present in pre gram powder. The plates were observed for 5dyas for the development of new fungal colonies.

9. Antibacterial testing of plant extracts

In the present work antibacterial effect of extracts was evaluated in addition to schizonticidal effect on *P. falciparum*.

9.1 Test organisms

The selected bacterial strains were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The selected bacterial species are listed as under:

A. Opportunistic purulent wound bacterial strains:

1. *Staphylococcus aureus* (Isolate) Gram Positive-Cocci
2. *Staphylococcus epidermidis* (ATCC 155) Gram Positive-Cocci
3. *Bacillus cereus* (ATCC 11778) Gram Positive-Bacilli
4. *Pseudomonas aeruginosa* (ATCC 25668) Gram Negative-Bacilli
5. *Klebsiella pneumoniae* subsp. (ATCC 15380) Gram Netgative-Bacilli
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6. Serratia marcescens (Isolate) Gram Negative-Bacilli
7. Enterobacter aerogenes (ATCC 13048) Gram Negative-Bacilli
8. Escherichia coli (ATCC 25922) Gram Negative-Bacilli

B. Pathogenic bacterial strains not involved in wound infection:

9. Salmonella paratyphi A (MTCC 735) Gram Negative-Bacilli
10. Salmonella typhi (NCTC 8394) Gram Negative-Bacilli

C. Non-pathogenic bacterial strains:

11. Bacillus subtilis (ATCC 6051) Gram Positive-Bacilli
12. Micrococcus luteus (ATCC 4698) Gram Positive-Bacilli

The bacterial strains were maintained in glass test tubes on prepared slants of Nutrient agar with wax sealed cotton plug. The bacterial slants were stored in refrigerator until used.

9.2 Antibacterial test

Antibacterial tests were carried out using the disk diffusion method (Bauer et al., 1966). Mueller–Hinton broths with bacterial inoculums were incubated for 16hrs at 37°C. Bacterial broth for each bacterial strain was diluted in 1ml normal saline (0.85%NaCl) up to the density adjusted at 0.5 McFarland turbidity standards (10^8 colony-forming units (CFU)/mL). The 0.5 McFarland standard is prepared by mixing 0.05 ml of 1.175% barium chloride dihydrate (BaCl_2·2H_2O), with 9.95 mL of 1% sulfuric acid (H_2SO_4). Bacterial inoculum 0.1ml inoculated on solid Mueller–Hinton No.2 agar (Hi Media) in petriplates. The bacterial inoculum was well spread by glass spreader until totally absorbed in agar layer for the development of uniform bacterial growth. Discs of 5mm diameters were made from Whatman paper no.3 with the help of punching machine. Each plate had maximum four discs with appropriate distance. The dry fractions were dissolved
in DMSO (Dimethyl sulfoxide) as it shows no inhibitory effect on tested microbes. The fix volume of 40μl (4mg from 100mg/ml DMSO) of each fraction was loaded on each disc immediately after putting the disc on agar surface (Alzoreky and Nakahara, 2002). The eight antibiotic compounds (Octodiscs Hi-Media OD 053) were used as positive control. The 40μl/disk DMSO was used as negative control. Petriplates were incubated at 37°C for 16hrs for development of visible bacterial growth. The bacterial inhibition zones measured in diameter included with disk from the average of two replicates.

9.3 Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) defined as the lowest concentration of drug which inhibits the visible growth of bacterium in liquid medium. The MIC values were determined according to the NCCL guidelines (Anonymous, 2000(c)) by double dilution method in the range of 2-0.0625mg/ml extract.

10. Micropropagation of Calotropis procera from node explants

The nodes of C. procera were collected from natural habitat, surrounding of Vallabh Vidyanagar. Isolated nodes, used as explant, were thoroughly washed with Tween-40 in running tap water for 15min, and then treated with the mixture of three antimicrobial agents named 0.5% (v/v) Savlon, 0.1% (w/v) Bavistin and 0.1% (v/v) Roger solution for 10min. These were washed thoroughly under running tap water and finally treated with 0.5% (v/v) Sodium hypochloride for 15min and then rinsed with sterile distilled water. This entire process makes the pre-sterilization of the
explants. Surface sterilization of the explants was done with 0.1% (w/v) Mercuric chloride treatment for 5 min and repeatedly washed with sterile distilled water. The explants were inoculated on MS basal medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 0.8% (w/v) agar-agar with different hormone combinations. The pH of the medium was adjusted to 5.8 and autoclaved at 1.1 kg/cm² (121°C) for 15 min. The inoculated tubes were incubated at 25°C±2°C under 16 hr photoperiod provided by fluorescent light of about 3000 lux intensity. Sub-culturing was done into fresh solid medium at every four weeks (George, 1996). The auxins used were 2,4-dichlorophenoxyacetic acid (2,4-D) and indole 3- butyric acid (IBA) with cytokinins named benzylaminopurine (BAP) and kinetin (Kn) in different combinations. Each combination was replicated three times with twelve explants per replicate. The growth parameters were measured after 30 days and showed length from mean of replicates (n=36) in cm with standard error (S.E.). The results were evaluated with Dunnett Test at P<0.05 level of significance for the judgment of appropriate composition of growth regulators.

11. Statistics applied in the study

11.1 Significance of IC₅₀ and IC₉₀ values

IC₅₀ values help to compare the in vitro anti-malarial activity of two different extracts. IC₉₀ values were used to judge the appropriate dose for in vivo testing of clearance of parasite with comparison of IC₉₀ value of well known anti-malarial drug like Chloroquine. The calculation was performed with the help of Microsoft Excel.
11.2 Mean with Standard Deviation (S.D.) or Standard Error (S.E.)

The data narrated in the present research are of mean from the studied replicates with S.D. for mice experiments and with S.E. for tissue culture experiment.

i) Mean is defined as the sum of observations divided by their number. Formula for calculation of mean is, $\text{Mean} = \frac{X_1+X_2+...+X_n}{n}$.

ii) The step wise method for calculating S.D. is,

a) Find mean of the replicates

b) Find deviation from the mean for each value

c) Square the each value of deviation (from the mean)

d) Sum the total square values of each deviation (from the mean) and divided with (n-1) observation. The value obtained is called as variance.

Formula of Variance = \[ \frac{\text{sum of squares of deviations from the mean}}{(n-1)} \]

e) S.D. is square root of variance that was shown with the sign of $\pm$ with value of mean for each result.

iii) S.E. is used when more than one samples were drawn from a population and the S.D. for each mean differ slightly and not the same. The formula is, $\text{S.E.} = \frac{\text{S.D.}}{\sqrt{n}}$.

11.3 Tukey Test

The Tukey Test (for one-way layout design) was applied for pair wise comparisons of each dilution of extracts to judge significant variation between means of schizonticidal activity ($P<0.001$). Probability for the occurrence of the results differs is significance with
Materials and Methods

$P \leq 0.001$ that indicates the occurrence of such results by chance is 1 or less than 1 out of 1000 chances. The same test was applied for evaluation of anti-malarial activity in mice model with $P \leq 0.05$ level of significance.

11.4 Dunnett Test

This test is applied to compare the experimental results with control group in tissue culture experiment for judgment of proper combination. Tukey and Dunnett Test were applied by using the statistical software “KyPlot”.
12. Abbreviations used in the text

%T: Percentage Transmission
ASS: Atomic Absorbance Spectrophotometer
B.D.: twice a day
CAS: Chemical Abstract Service
CQ: Chloroquine
EI-MS: Electron impact mass spectra
GC: Gas Chromatography
GC-MS: Gas Chromatography with Mass Spectrometer
HCl: Hydrochloric acid
HEPES buffer: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC: High Performance Liquid Chromatography
HPTLC: High Performance Thin Layer Chromatography
ip: Intra peritoneal
IR: Infrared Spectroscopy
iv: Intra venous
Na₂SO₄: Sodium sulphate
NaCl: Sodium chloride
NMR: Nuclear Magnetic Resonance
O.D.: Once daily
P.f.: Plasmodium falciparum
P.v.: Plasmodium vivax
RPMI-1640: RPMI-1640 was developed at Roswell Park Memorial Institute in 1966 by Moore and his co-workers, hence the acronym RPMI.
S.D.: Standard deviation
S.E.: Standard error
SW: Swiss White Mice strain
t.d.s.: three times a day
XRF: X-Ray Fluorescence Spectroscopy