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ANTIPLASMODIAL ACTIVITY OF *AGANOSMA CYMOSA*

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Keywords:

Aganosma cymosa, Antiplasmodial activity, 4 day suppressive test and Rane's test

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ABSTRACT: Ethylacetate leaf extract of *Aganosma cymosa* was screened for antimalarial activities *in-vivo* in rats inoculated with red blood cells parasitized with *Plasmodium falcipuram* using 4-day Suppressive test and Rane's test. The result of mean parasitemia by 4-day Suppressive test for the Group AC600, AC400 & AC200 was 4.62 ± 3.63, 13.34 ± 2.42, 19.29 ± 3.23 and percentage suppression were 80.1%, 42.54% and 16.9% respectively. However, standard drug chloroquine (15 mg/kg) was found to exert a considerable lower percentage parasitemia of 2.73 ± 1.6 and higher percentage suppression of 88.24%. From the results of the Ranes test, the parasitemia levels on day 7 for AC200, AC400, AC600 were 16 ± 0.4, 12 ± 0.4 and 2 ± 0.6 and the percentage inhibition was found to be 23.80, 45.45 and 65 respectively. While the chloroquine treated group had a mean parasitemia 6 ± 0.2 and percentage inhibition of 70%.The plant extracts showed significant dose-dependent antiplasmodial activity with the decrease in parasitemia, rectal temperature, body weight and increase in suppression, packed cell volume in all treated groups *viz.* AC200, AC400 & AC600 when compared to standard chloroquine and control groups by both the models.

INTRODUCTION: Malaria continues to pose a serious threat to the human population of the tropical and subtropical regions. The disease is caused by *Plasmodium* species and is transmitted by female *Anopheles* mosquitoes. The alarming rate at which *Plasmodium falciparum* has developed resistance to chloroquine and other synthetic antimalarial drugs makes it necessary to search for more effective antimalarial compounds. The World Health Organization recommended Artemisinin Combination Therapies (ACTs) as the first line of treatment for uncomplicated malaria caused by human *Plasmodium falciparum*.

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Like quinine, artemisinin is derived from a plant but is a structurally distinct compound. The pharmacophore consists of peroxide within a 1, 2, 4-trioxane configuration, which leads to several suggestions of how these antimalarials might work. Antimalarials such as chloroquine, artesunate, antifolates and tetracyclines, were identified either by their chemical relationship to natural products or from their activity against other infectious pathogens. It is therefore imperative to continue the search of new chemical entities that can qualify as candidate anti-malarial drugs as remedies with little or no side effects [1].

Aganosma cymosa also known as *Echites cymosa (Apocynaceae*) is a climbing shrub found commonly growing in hill slopes. The distribution is restricted to the peninsular region in India and Bengal. The traditional plant is used as an emetic and anthelmintic. It is also used in the treatment of bronchitis, leprosy and skin diseases.

The flower is said to be effective in diseases of the eye. The plant has been reported to contain the highest oil content of about 10.3% [2, 3, 4]. *Aganosma cymosa* is used as antiseptic, emetic, anthelmintic, in bronchitis, leprosy, skin diseases, ulcer, inflammations, arthritis, purulent discharges from the ear and diseases of the mouth, eye diseases, biliousness, fever, Anodyne, sedative properties, also used for paraplegia, sciatica and Neuralgia.

The study presents an evaluation of the *in-vivo* antiplasmodial activity of ethylacetate leaves extract of varying doses of *Aganosma cymosa* in rats using 4 day Suppressive test and Rane's test

MATERIALS AND METHODS:

Plant Materials: Fresh leaves of *Aganosma cymosa* were collected from local areas of Warangal and it was identified and authenticated (KU/No.DB1/VPC/cog/2018/46), at the Herbarium of the Department of Botany, Kakatiya University. The leaves were air-dried over 14 days under the shade at room temperature and then pulverized using a milling machine. About 350 grams powder *of A. cymosa* was subjected to Soxhlet extraction to prepare Ethylacetate extract. The solvent of the extract was evaporated to dryness in the Rota evaporator. The dried residue of about 42 grams was stored in screw-capped vials at –4 °C.

Animals: The animals (Wistar rats) of either sex were used for these experiments. Four-week-old rats (16-20 g) were used for a malaria study. The animals were housed in standard cages and were maintained on a standard pelleted feed (Guinea Feed) and water *ad libitum*. Permission and approval for animal studies were obtained from the institutional animal ethical committee (1663/PO/ Re/S/12/CPSCEA: 21-11-2015).

Phytochemical Screening: The crude ethyl acetate extract was analyzed for the presence of important phytochemicals following the protocol. Briefly, tannins and flavonoids were determined by boiling 0.5 g of an extract with 20 ml of distilled water for 5 min in a water bath. Several drops of 0.1% ferric chloride and hydroxide solutions were added to 2 ml of the filtrate, respectively. Brownish green or blue-black coloration indicated the presence of tannins, while yellow coloration showed the presence of flavonoids. The presence of saponins was determined by frothing upon boiling of 2 g of a crude extract with 20 ml of distilled water in a water bath for 10 min. The sample was filtered and allowed to cool. The filtrate (10 ml) was diluted with distilled water (1:1 v/v) and shaken vigorously until the formation of froth, which was stable for a few minutes. The frothing was mixed with 3 drops of olive oil and shaken vigorously for the formation of an emulsion. Alkaloids were determined by boiling 1 g of an extract with distilled water and acidified with 5 ml of 1% HCl in a water bath. Several drops of Meyer's reagent were added to 2 ml of the filtrate. The formation of creamy white/turbid precipitate indicated the presence of alkaloids.

Terpenoids were determined by mixing 5 ml of extract solution with 2 ml of chloroform. Concentrated H_2SO_4 (3 ml) was then carefully added to form layers. A reddish-brown precipitate at the interface indicated the presence of terpenoids. The presence of cardiac glycosides was determined by dissolving 5 mg of extract in 2 ml of glacial acetic acid containing 1 drop of ferric chloride solution. The mixture was then layered with 1 ml of concentrated H_2SO_4. A brown ring at the interface indicated the presence of cardiac glycosides. The presence of phlorotannins was determined by the deposition of a red precipitate when several drops of dilute HCl were added to 2 ml of extract [5, 6].

Determination of Acute Toxicity in Rats: This was done by determining the median lethal dose (LD_{50}) of the extract using the method of Lorke's (1983). This involved intra-peritoneal (i.p.) administration of different doses of the extract (100-1000 mg/kg) to groups of five rats each. The animals were observed for the manifestation of physical signs of toxicity such as writhing, decreased motor activity, decreased body/limb tone, decreased respiration and death. The number of deaths in each group within 24 h was recorded [7, 8].

Evaluation of *in-vivo* Antimalarial Activity of Methanol Crude Leaf extract of *Aganosma cymosa:* Parasite Inoculation: Wistar rats earlier infected with *Plasmodium berghei*, ANKA strain, (parasitemia level of 20-30%) were used as a donor. The donor rats were then sacrificed with

ethyl ether anesthesia and blood was collected by cardiac puncture into heparinized vacutainer tube. The blood was then diluted with physiological saline (0.9%) based on parasitemia level of the donor rats and the red blood cell (RBC) count of normal rats, in such a way that 1 ml blood contains 5×10^7 infected RBC [13]. Each mouse was then given 0.2 ml of this diluted blood intraperitoneally, which contained 1×10^7 *P. falcipuram* infected RBCs [9].

Grouping and Dosing of Animals:

Four-Day Suppressive Test: This test was used to evaluate the schizontocidal activity of the crude extract against *P. falcipuram* infected rats. Antiplasmodial activity of the test extract was performed in a 4-days suppressive standard test. Male Swiss Albino rats weighing 22-29 g were inoculated on the first day (Day 0), intraperitoneally, with 0.2 ml of infected blood. The rats were then divided randomly into five groups of six rats per group. Three groups (II, III and IV) were assigned as test groups whereas the other two groups (I & V) were used as control (negative and positive) groups. Three hours after infection 200, 400 and 600 mg/kg/day of ethyl acetate leaf extract of *A. cymosa* were administered to the test groups. Chloroquine at the dose of 15 mg/kg/day and an equivalent volume of vehicle (0.2 ml 7% tween 80 solutions) were administered to the positive and negative control groups, respectively, for four consecutive days (Day 0-3). On the fifth day (on day 4, 24 h after the last dose *i.e.* 96 h post-infection), thin blood smears were made from the tail of each mouse, fixed with methanol and stained with 10% Giemsa. The parasitemia level was determined by counting the number of parasitized erythrocytes out of 100 erythrocytes in random 8 fields of the microscope.

Parasitaemia was determined by light microscopy using a 100 × objective lens and the following equation:

% Parasitemia = Number of Parasitized RBC/Total Number of RBC Counted × 100

Average percentage chemo-suppression = 100 [(A−B) / A]

Where A is the average percentage parasitemia in the negative control group and B is the average percentage parasitemia in the test group. The capability of the various doses of the crude extract in preventing body weights and packed cell volume (PCV) reduction of the rats as a result of rise in parasitemia was followed. The weight, PCV and rectal temperature were taken at D_0 and D_4 [10-13].

Test for Curative Activity (Rane's Test): The chemotherapeutic activity of ethyl acetate crude extract was carried out in established infection. On the first day (Day 0), a 0.2 ml standard inocula of 1×10^7 *P. falcipuram* infected erythrocytes was inoculated in rats intraperitoneally. Seventy-two hours later (Day 3), rats were randomly divided into three test groups (II, III and IV) of six rats each. Group I and V were assigned as negative and positive controls, respectively.

Then, the test groups were dosed with AC200, AC400, AC600 mg/kg/day of the extract. Negative and positive controls received vehicle (0.2 ml 7% tween 80) and chloroquine 15 mg/kg/day orally, respectively. Giemsa-stained thin blood film was prepared from the tail of each mouse on days 3 and 7 to monitor parasitemia level [14].

Determination of Packed Cell Volume*:* Packed cell volume (PCV) was measured to predict the effectiveness of the test extract in preventing hemolysis resulting from increased parasitemia. Heparinized capillary tubes were used for the collection of blood from the tail of each mouse. The capillary tubes were filled with blood up to $3/4^{th}$ of their volume and sealed at the dry end with sealing clay. The tubes were then placed in a micro-hematocrit centrifuge with the sealed end outwards and centrifuged for 5 min at 12,000 rpm. The tubes were then taken out of the centrifuge and PCV was determined using a standard micro-hematocrit reader. PCV is a measure of the proportion of RBCs to plasma and measured before inoculating the parasite and after treatment using the following relationship:

PCV = Volume of erythrocytes in a given volume of blood/Total blood volume

Monitoring Body Weight: In the 4-day suppressive test, bodyweight of each infected rats was measured before infection on day 0 and after treatment on day 4. However, for Rane's test the body weight was measured after infection on day 3 and after treatment on day 7. The body weight of

each mouse was measured using a sensitive digital weighing balance [15].

Determination of Rectal Temperature: In a 4-day suppressive test, the body temperature of each mouse was determined before infection on day 0 and after treatment on day 4 using digital thermometer [16].

Monitoring Mean Survival Time: In all of the *in-vivo* antiplasmodial models, mortality was monitored and the number of days from the time of inoculation of parasite up to death was recorded for each mouse in the treatment and control groups for 30 days. The mean survival time (MST) for each group was calculated as follows:

MST = Sum of Survival Time of All Rats in a Group (days) / Total Number of Rats in the Group [17]

Data Analysis: Data were analyzed using Graph Pad Prism v 8.1.2. Comparisons were made among negative and positive controls as well as treatment groups using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Mean PCV, rectal temperature and body weight before and after infection and treatment were compared by two-tailed paired *t*-test. The result was considered statistically significant at a 95% confidence level and $P < 0.0001$.

RESULTS AND DISCUSSION:

Phytochemical Screening: The dark green ethyl acetate extract of about 42 grams was obtained by Soxhalation. The percentage yield was 12%. Phytochemical screening of the crude extract revealed the presence of chemical constituents such as alkaloids, flavonoids, tannins, terpenes, saponins and cardiac glycosides. The acute toxicity studies with the extract of *A. cymosa* showed no altered manifestations with the doses up to 1000 mg/kg.

Antiplasmodial Effect by 4 day Suppressive Test with the Ethylacetate Leaf extract of *Aganosma cymosa*: In the suppressive test the extract showed a dose-dependent antiplasmodial effect. The extract and chloroquine-treated groups showed a reduction in the percentage parasitemia when compared to that of the control, where a prominent increase was recorded as shown in **Table 1**. This reduction in percentage suppression was statistically significant relative to the control ($p<0.0–0.001$). The crude ethyl acetate extracts of varying doses significantly decreased parasitic load in *P. falciparum* infected rats, compared to the standard chloroquine and negative control group (23.22 ± 5.21). The result of mean parasitemia for the Group AC600 was 4.62 ± 3.63 and percentage suppression is 80.1%. The result of mean parasitemia for AC400 & AC200 was 13.34 ± 2.42, 19.29 ± 3.23 and the percentage suppression was 42.54% and 16.9% respectively. However, standard drug chloroquine (15 mg/kg) was found to exert a considerable lower percentage parasitemia of 2.73 ± 1.6 and higher percentage suppression of 88.24%. The mean survival time of rats treated with varying doses of extracts was found to be 10 to 18 days. Survival time for the chloroquine treated group was 23 days. The extract treated rats survived significantly longer than rats in the negative control. The temperature and weight changes were observed to be prominently decreasing whereas packed cell volume was increasing with all doses.

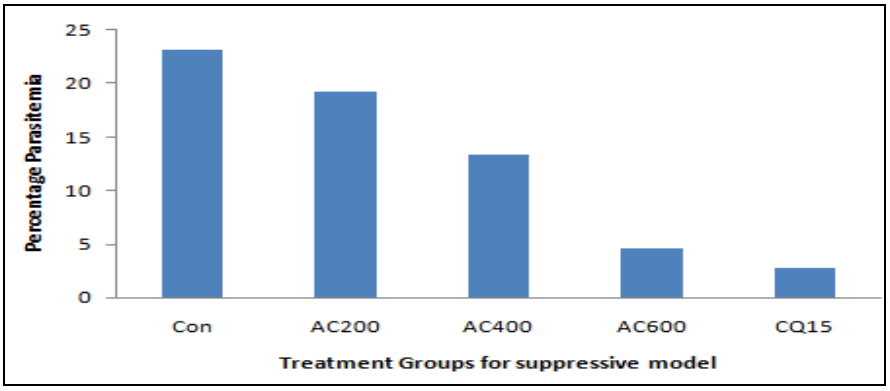

FIG. 1: FOUR DAY PARASITAEMIA LEVELS OF DIFFERENT DOSES OF CRUDE EXTRACT AT DIFFERENT DOSES OF *AGANOSMA CYMOSA IN P. FALCIPURAM* INFECTED RATS AS COMPARED TO THE CONTROL (CON) AND THE STANDARD TREATMENT, CHLOROQUINE (CQ). Each bar represents the Mean ± SD for each group of rats, n =6**.** Significant difference: * $p < 0.001$.

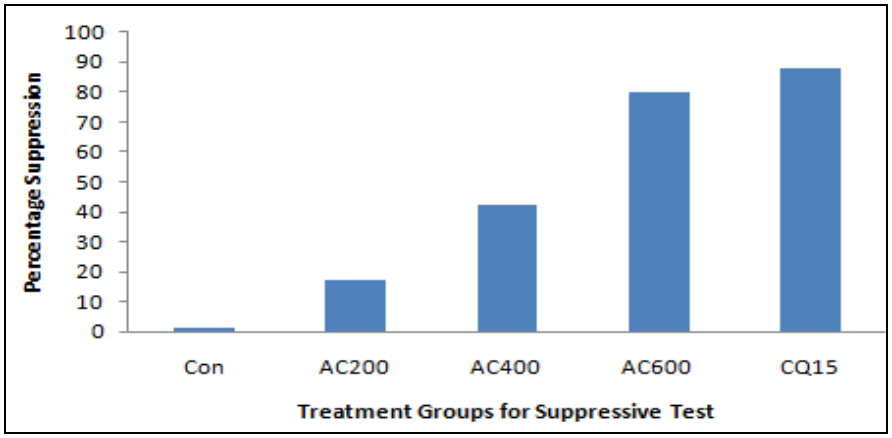

FIG. 2: PERCENTAGE PARASITEMIA SUPPRESSION OF *A. CYMOSA* VARIOUS EXTRACTS (200, 400, 600 mg/kg/day) *ON P. FALCIPURAM* INFECTED RATS AS COMPARED TO THE (CON) AND THE STANDARD TREATMENT CHLOROQUINE (CQ). Each bar represents the Mean ± SD for each group of rats, n = 6. Significant difference: * and #p < 0.001. Data were analyzed by one way ANOVA followed by Dunnett's test (*n* = 6); $^{\#}P < 0.001$ as compared to control.

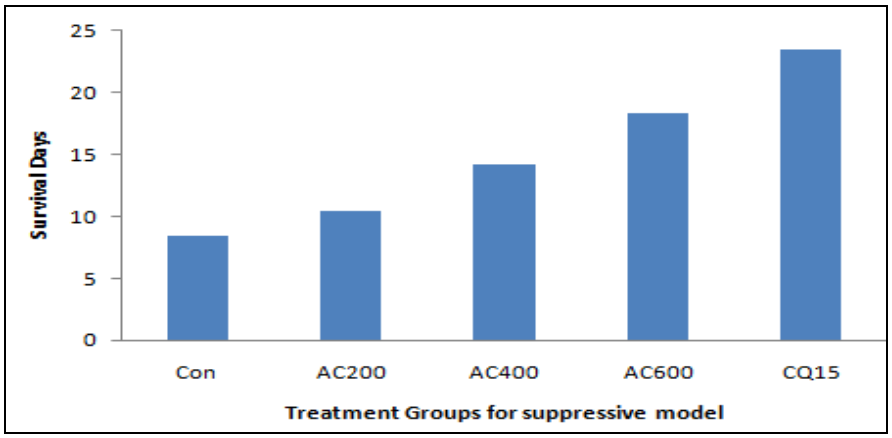

FIG. 3: MEAN SURVIVAL TIME OF *AGANOSMA* AS COMPARED TO THE CONTROL (CON) AND THE STANDARD TREATMENT CHLOROQUINE (CQ)* Values are significantly different at P < 0.001.

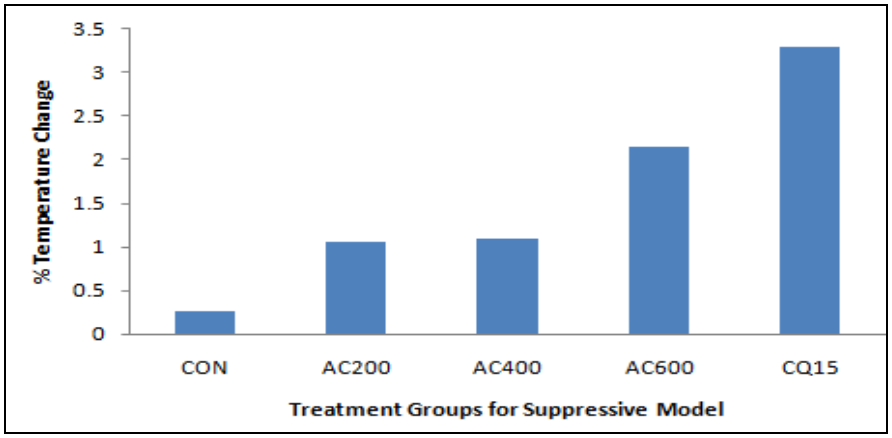

FIG. 4: THE EFFECT OF CRUDE EXTRACT OF *AGANOSMA* ON RECTAL TEMPERATURE OF *P. FALCIPURAM* INFECTED RATS ON DAY 5 IN SUPPRESSIVE TEST AS COMPARED TO THE CONTROL (CON) AND THE STANDARD TREATMENT, CHLOROQUINE (CQ). Each bar represents the Mean ± SD for each group of rats, n =6. Significant difference: * p < 0.001.

TABLE 1: MEAN PARASITEMIA ± SEM AND PERCENTAGE SUPPRESSION OF *AGANOSMA CYMOSA* TREATED GROUPS BY 4 DAY SUPPRESSIVE TEST

	Control	CQ 15 mg/Kg	AC200 mg/kg	AC400 mg/kg	AC600 mg/kg
Mean parasitemia count	23.22 ± 5.2	2.73 ± 1.6	19.29 ± 3.23	13.34 ± 2.42	4.62 ± 3.63
Percentage suppression	000	88.24%	16.9%	42.54	80.1%

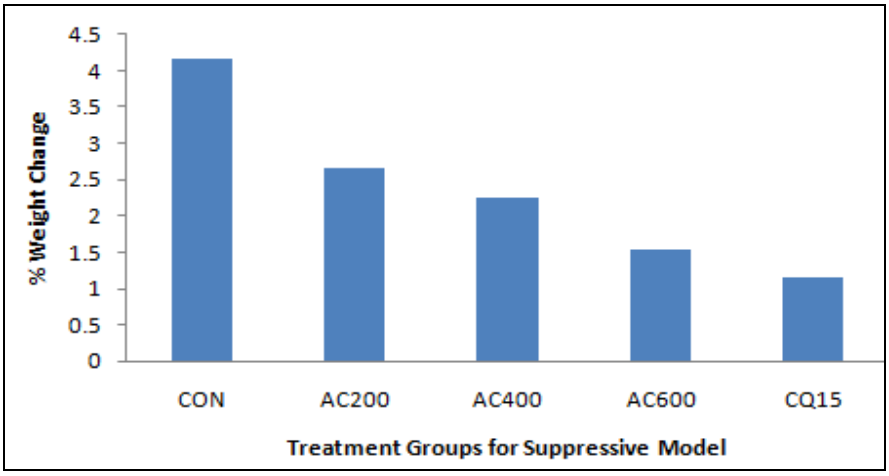

FIG. 5: EFFECT OF CRUDE EXTRACT OF *AGANOSMA* ON BODY WEIGHT OF *PLASMODIUM FALCIPURAM* INFECTED RATS IN 4 DAY SUPPRESSIVE TEST. Results presented as mean ± SD; n =6. Significant difference between Day 1 and Day 5 ($P < 0.001$)**.** As compared to CON; as compared to CQ.

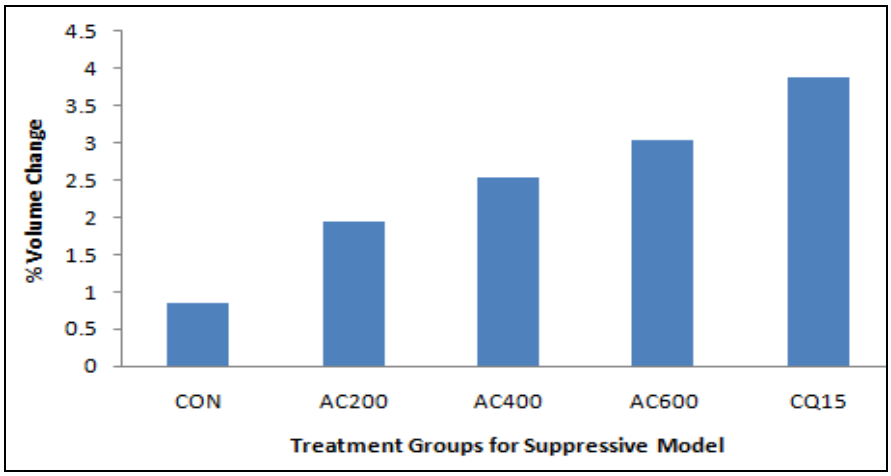

FIG. 6: EFFECT OF CRUDE EXTRACT OF *AGANOSMA* ON PCV OF *PLASMODIUM FALCIPURAM* INFECTED RATS IN 4 DAY SUPPRESSIVE TEST. Results presented as mean ± SD; n =6. Significant difference between Day 1 and Day 5 ($P < 0.001$). As compared to CON; as compared to CQ.

Antiplasmodial Effect by Rane's Test with the Ethylacetate Leaf Extract of *Aganosma cymosa*: From the results of Ranes test, the parasitemia levels on day 7 for AC200, AC400, AC600 were 16 ± 0.4, 12 ± 0.4 and 7 ± 0.6 and the percentage inhibition was found to be 23.80, 45.45 and 65 respectively. While the chloroquine treated group had a mean parasitemia 6 ± 0.2 and percentage inhibition of 70%. The highest mean parasitemia observed for the control group which was 36 ± 0.2.

The mean survival time for Chloroquine 15mg/kg was found to be 27 ±0.2 days when compared to 12 ± 0.2, 16 ± 0.6 and 22 ± 0.6 for AC200, AC400, & AC600 respectively. Inhibition is inversely related to parasitemia. So, the leaf extract has shown reduced parasitemia corresponding to high inhibition. The rats in the control group had a mean survival time of 8 ± 0.4. There was a prominent decrease in the temperature and weight pattern and PCV results were increased from Day 0 to Day5 for AC600, AC400 and AC200.

Similar to the results of the suppressive test, the percentage inhibition was increased with an increase in the dose levels of the extract, indicates that the extracts are capable of a reduced level of parasitemia by both the models.

TABLE 2: MEAN PARASITEMIA AND PERCENTAGE PARASITEMIA SUPPRESSION BY CURATIVE TEST

	Control	CQ 15mg/kg	AC200 mg/kg	AC400mg/kg	AC600 mg/kg
Mean parasite count	36 ± 0.2	6 ± 0.2	16 ± 0.4	12 ± 0.4	7 ± 0.6
Percentage parasitemia	000	82.78	35.55	65	80.5

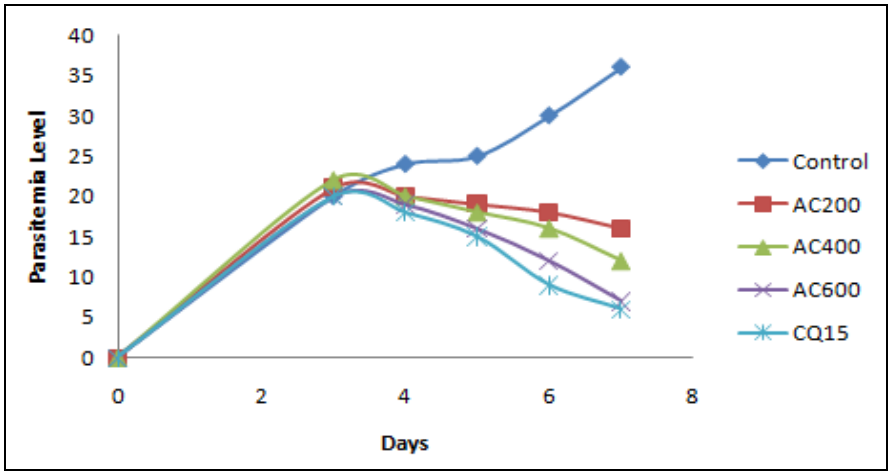

FIG. 7: PARASITAEMIA LEVELS OF DIFFERENT DOSES OF CRUDE EXTRACT OF *AGANOSMA CYMOSA IN P. FALCIPURAM* INFECTED RATS AS COMPARED TO THE CONTROL (CON) AND THE STANDARD TREATMENT, CHLOROQUINE (CQ) BY RANE'S TEST. Each bar represents the Mean ± SD for each group of rats, n =6. Significant difference: * p < 0.001.

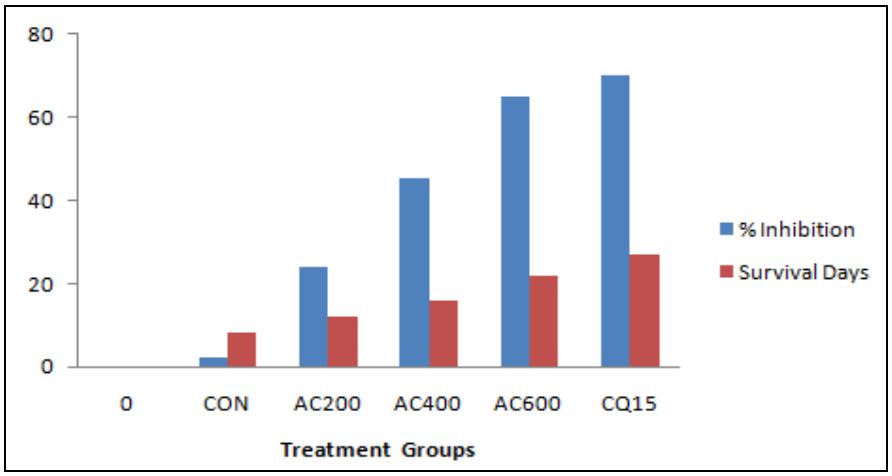

FIG. 8: PERCENTAGE PARASITEMIA SUPPRESSION OF *AGAMOSMA CYMOSA* VARIOUS EXTRACTS (200,400,600 mg/kg/day) *ON P. FALCIPURAM* INFECTED RATS AS COMPARED TO THE (CON) AND THE STANDARD TREATMENT CHLOROQUINE (CQ) BY RANE'S TEST. Each bar represents the Mean ± SD for each group of rats, n =6. Significant difference: * p < 0.001. Data were analyzed by one way ANOVA followed by Dunnett's test (*n* = 6); $^{*}P < 0.001$ as compared to control.

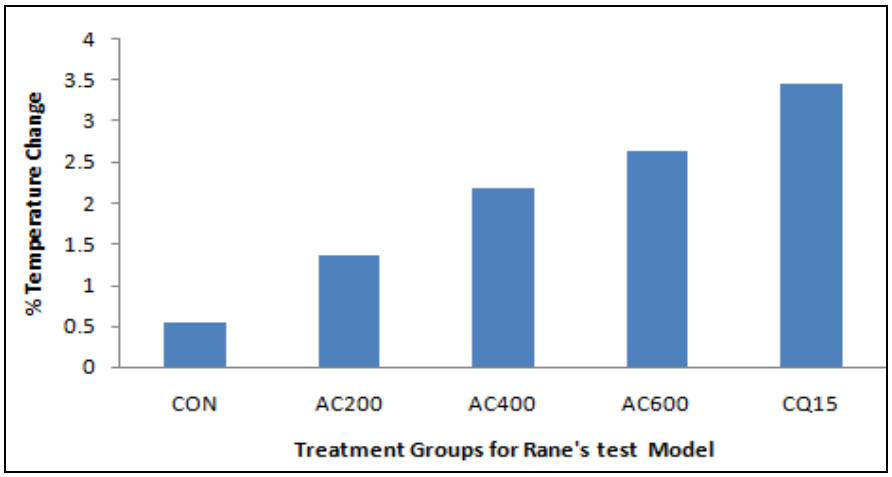

FIG. 9: EFFECT OF CRUDE EXTRACT OF *AGANOSMA* ON BODY WEIGHT OF *PLASMODIUM FALCIPURAM* INFECTED RATS IN RANE'S TEST. Results presented as mean ± SD; n =6. Significant difference between Day 1 and Day 5 (P < 0.001). As compared to CON; as compared to CQ.

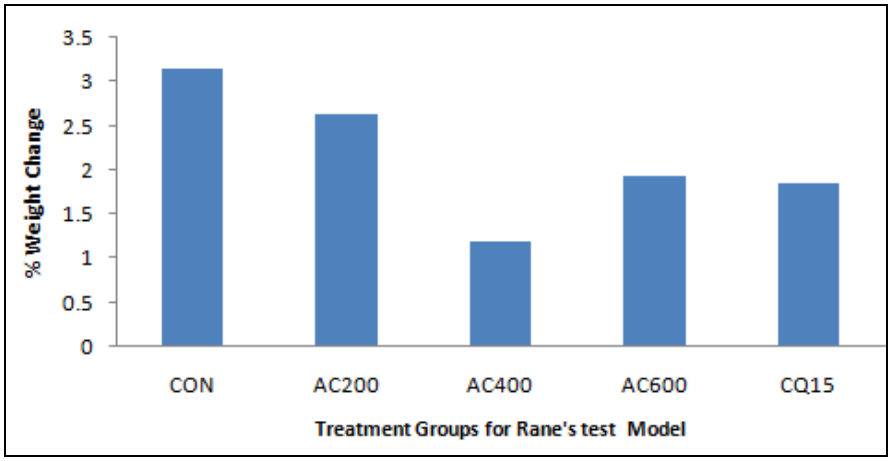

FIG. 10: EFFECT OF CRUDE EXTRACT OF *AGANOSMA* ON BODY WEIGHT OF *PLASMODIUM FALCIPURAM* INFECTED RATS IN RANE'S TEST. Results presented as mean ± SD; n =6. Significant difference between Day 1 and Day 5 ($P < 0.001$). As compared to CON; as compared to CQ.

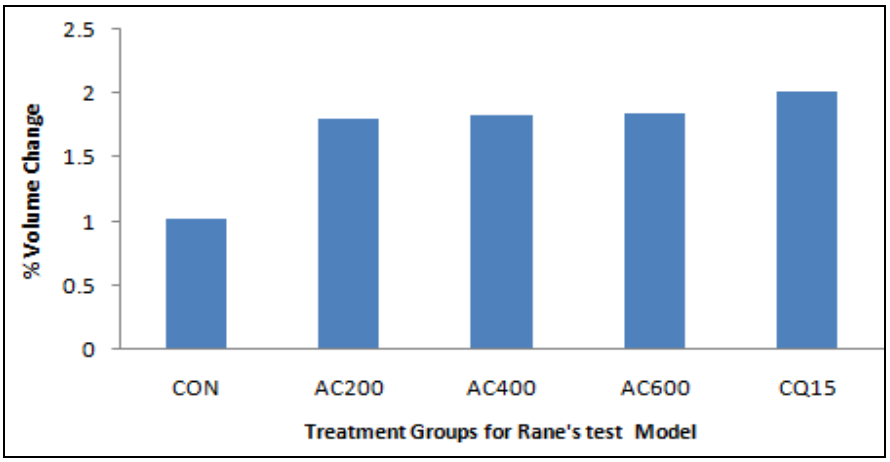

FIG. 11: EFFECT OF CRUDE EXTRACT OF *AGANOSMA* ON PCV OF *PLASMODIUM FALCIPURAM* INFECTED RATS IN RANE'S TEST. Results presented as mean ± SD; n =6. Significant difference between Day 1 and Day 5 ($P < 0.001$)**.** As compared to CON; as compared to CQ.

CONCLUSION: The results obtained in this study, indicate that the leaves of *Aganosma cymosa* possess significant antiplasmodial activities against chloroquine-resistant strains of *P. falciparum*. In conclusion, our results showed that the extract was not only found to inhibit parasitemia in a dose-dependent manner but also enhanced the mean survival time period of treated rats. These findings justify and confirm the usage of this plant in the treatment of malaria. Further studies are required on the extract to isolate, characterize and identify the lead compound which could serve as a useful agent against resistant malaria infections.

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CONFLICTS OF INTEREST: Nil

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