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Antimalarial efficacy of *Nyctanthes arbor-tristis* and its effect on combination with Artesunate in *Plasmodium berghei* K173 induced mice model.

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ABSTRACT

Background

Malaria is caused by parasite plasmodium species which is transmitted via the bite of infected mosquitoes, the key approach to treat malaria include prompt and effective treatment with Artemisinin based combination therapy. The search for additional antimalarial from plant extracts and their combination with Artemisinin derivatives must continue to help in effective treatment of the disease.

Aim

To evaluate the antimalarial efficacy of *Nyctanthes arbor-tristis* (NAT) and its effect on combination with Artesunate.

Materials and Methods

The study was done in the Department of Molecular Bioprospection, CSIR-CIMAP, Lucknow, Uttar Pradesh, India. Leaves of *Nyctanthes arbor tristis* (NAT) were chosen, dried, grounded and extracted with solvent of variable polarity. The bioactivity of NAT leaf extracts was carried out through *Plasmodium berghei* induced malaria in albino swiss mice animal model. The two independent experiments gave us the result to plan the third experiment on combination therapy. From the first experiment IC₅₀ of Artesunate was derived and from the second experiment the Methanol 80% extract of NAT was found to be indicative of the plant bioactive. The experiment involving the combination therapy was taken up by combining 20mg/kg of Artesunate with 250mg/Kg and 500mg/Kg of Methanol 80% extract of NAT.

Results

Our study showed that by combining Methanol 80% extract of NAT with sub effective dose of Artesunate results in improvement of mean survival time, parasitaemia and haemoglobin levels.

Conclusion

Our experiment helped us to conclude that NAT leaves can be used as a lead for combination therapy. However, further detailed experiments related to the safety and pharmacokinetics of combination need to be considered.

Keywords: Artesunate, Malaria, *Nyctanthes arbor-tristis*, Plasmodium

INTRODUCTION

Malaria is a hematologic infectious disease where *Plasmodium falciparum* infection is one of the most frequent acquired red blood cell (RBC) disorders worldwide. [1] About 3.2 billion people – almost half of the world's population – are at risk of malaria. [2] It is endemic in most part of India and other tropical countries. Malaria is caused by 4 species of protozoan parasite Plasmodium i.e. *P. vivax*, *P. malariae*, *P. falciparum* and *P. ovale*. *P. knowlesi*, [3] is now considered the fifth species of Plasmodium causing malaria in humans.

In many countries, progress in malaria control is threatened by the rapid development and spread of antimalarial drug resistance. To date, parasite resistance to artemisinin – the core compound of the best available antimalarial medicines – has been detected in 5 countries of the Greater Mekong subregion [4]

Artemisinin based combination chemotherapy is preferred due to the rapidity of action, but non-artemisinin-based chemotherapy may be considered as alternative regimen. In India, the National Malaria Control Programme recommends artemisinin based combination therapy as Artesunate Plus Sulfadoxine–Pyrimethamine combination except in North-eastern states where Artemether Plus Lumefantrine combination. [5]

Bioprospection of medicinal plants may disclose active compound that may serve as lead to develop a new drug that can be used in ACT. One such promising plant is *Nyctanthes arbor-tristis* Linn. (Oleaceae) also known as night jasmine is a wild hardy large shrub or a small tree distributed in sub-himalayan regions and southwards to Godavari in India. [6] The scientific literature on the plant *Nyctanthes arbor tristis* (NAT) has already proven the ethnopharmacological potential which includes anthelmintics [7] antimicrobial [8], antileishmanial, [9] antiviral and antimalarial [10] activity.

Because our understanding of the scientific principles of these herbal drugs is still unsatisfactory, resulting in their wider spared use in patients. The present study has been taken up to prospect antimalarial effect of leaf extract from NAT and the effectiveness of the combination of leaf extract from NAT along with ED₅₀ of Artesunate.

AIM AND OBJECTIVES

General Objective

- Bioevolution (Efficacy) of antimalarial activity of plant extracts from *Nyctanthes arbor-tristis* against *P.berghei* K173 in mice model.
- Specific Objective
- To find out the effective dose of Artesunate in the above-mentioned model system.
- Evaluate the effectiveness of the combination of extracts along with ED₅₀ of Artesunate.

MATERIALS AND METHODS

The study protocol was approved from Institutional Animal Ethical Committee and experiments on animals were carried out as per standard ethical IAEC and CPCSEA [12] guidelines. haemoglobin concentration [**were measured on Day 12.**] The study was carried out in the Department of Molecular Bioprospection, CSIR-CIMAP, Lucknow, Uttar Pradesh, India for a period of six months from January 2012 to June 2012.

Plant material

The plant *Nyctanthes arbor-tristis* (NAT) material was collected and authenticated by taxonomist Dr. SC Singh, Botany and Pharmacognosy, Department of CSIR-CIMAP Lucknow (U.P) India. The NAT leaves were dried in shed, ground to powder and stored in an airtight container until further use.

Animal model

Swiss albino mice were obtained from “JEEVANIKA”, CSIR-CIMAP Lucknow. The animals were kept at room temperature of $22 \pm 3^{\circ}\text{C}$ with 50 – 70% relative humidity and 12:12 hrs. of light and dark cycles. The animals were fed with rodent pellet diet procured from M/S Dayal industries, Lucknow, India and water ad libitum. Male mice weighing 19-24 gm. were used for the study.

Extraction of NAT leaves

The NAT leaves was macerated successively with six solvents viz. Hexane, Chloroform, Ethyl acetate, Acetone and 100% Methanol and 80% Methanol selected based on polarity.

STUDY DESIGN

Early malaria infection (4-day suppressive test) model

This is the most widely used preliminary test, in which the efficacy of a compound is assessed by comparison of blood parasitaemia and mouse survival time in treated and untreated mice.^[13]

Experiment 1

To estimate the ED₅₀ of Artesunate in *P.berghei* mice model. Six groups of six mice each were formed. Group I as control treated with 0.7% Carboxy methyl cellulose and Group II-VI were treated with Artesunate 10mg/kg, 20mg/kg, 40mg/kg, 80mg/kg and 100mg/kg respectively.

Experiment 2

To evaluate the efficacy of the NAT leaf extracts in *P.berghei* mice model. Eight groups of six mice each were formed. Group I as control treated with 0.7% Carboxy methyl cellulose, Group II-VII were treated with 500mg/kg of NAT leaf extract of Hexane (IVT 4), Chloroform (IVT 5), Ethyl acetate (IVT 6), Acetone (IVT 7), 100% Methanol (IVT 8) and 80% Methanol (IVT 9) respectively and Group VIII as standard treated with Chloroquine 5mg/kg.

Experiment 3

To evaluate the efficacy of the combination of the active extract of NAT leaf with ED₅₀ of Artesunate in *P.berghei* mice model. Five groups of six mice each were formed. Group I as control treated with 0.7% Carboxy methyl cellulose, Group II treated with 500mg/kg of active extract of NAT leaf, Group III treated combination of 250mg/kg of active extract of NAT leaf and Artesunate 20mg/kg, Group IV treated

with combination of 500mg/kg of active extract of NAT leaf and Artesunate 20mg/kg and Group V treated with standard Chloroquine 5mg/kg.

Each mouse of each group was inoculated with the parasite *Plasmodium berghei* K173 at the commencement of the experiment (day 0). After 2-4 hr post-infection, the experimental groups were treated with a single dose of test compound by the intraperitoneal (i.p) route. On day 1 to 3, the experimental groups were treated again (with the same dose and same route) as on day 0. [14] Blood samples from the animals' caudal vein were taken on Day 4, 6, 8, 10 and 12 for estimation of parasitaemia and haemoglobin concentration was measured on Day 12. Blood drops were transferred on slides, thus, making thin film from each mouse and staining with Geimsa stain. Parasitaemia is determined microscopically by counting 4 fields of approximately 100 erythrocytes per field. For low parasitaemia's (<1%), up to 4000 erythrocytes must be counted. From day four, the animals were fed ad libitum and observed for 28 days. Any death that occurred during this period was noted and used to determine the mean survival time.

Three parameters namely parasitaemia, haemoglobin and mortality were considered for assessing the antimalarial activity of Artesunate or various NAT leaf extracts and their combinations.

STATISTICAL ANALYSIS

Values were expressed as Mean \pm SEM. The one-way ANOVA test was used to analyse and compared the results at a 95% confidence level. Values of $p < 0.05$ were considered significant.

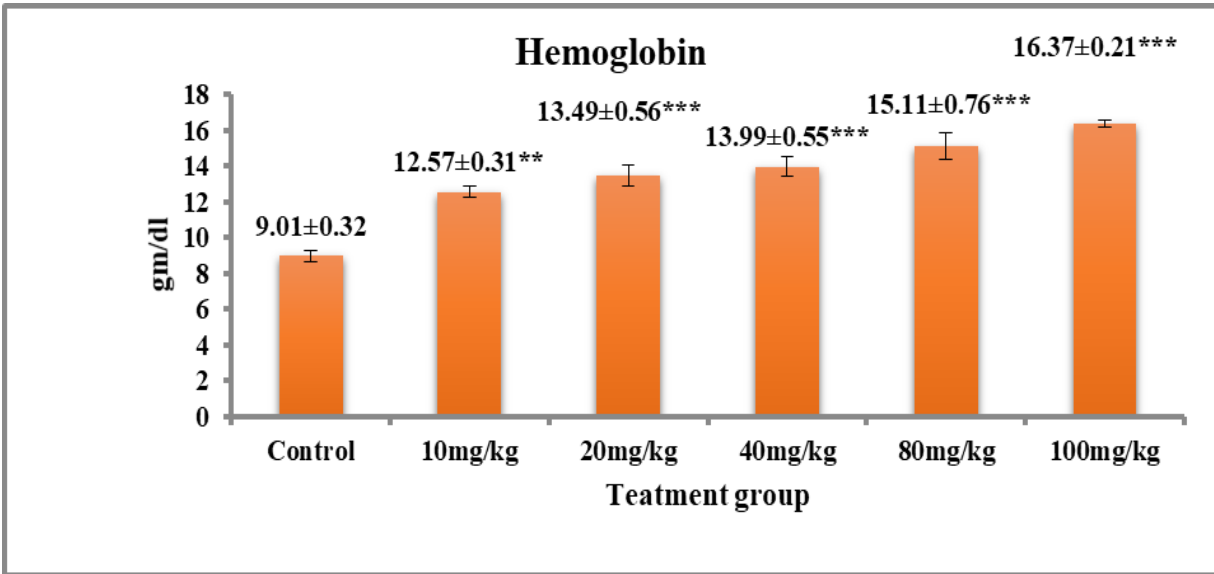
OSERVATIONS AND RESULTS

Table 1: The effect on Parasitemia (%) in different doses of Artesunate against *P.berghei* in swiss albino mice

Groups	Day 4	Day 6	Day 8	Day 10	Day12
Control†	4.78 \pm 0.68	14.65 \pm 1.82	33.40 \pm 1.93	41.21 \pm 1.93	42.06 \pm 1.29
10mg/kg	0.40 \pm 0.07**	0.45 \pm 0.06**	0.60 \pm 0.21**	11.32 \pm 0.48**	27.24 \pm 1.55*
20mg/kg	0.00**	0.00**	0.00**	0.00**	9.31 \pm 2.65**
40mg/kg	0.00**	0.00**	0.00**	0.00**	0.00**

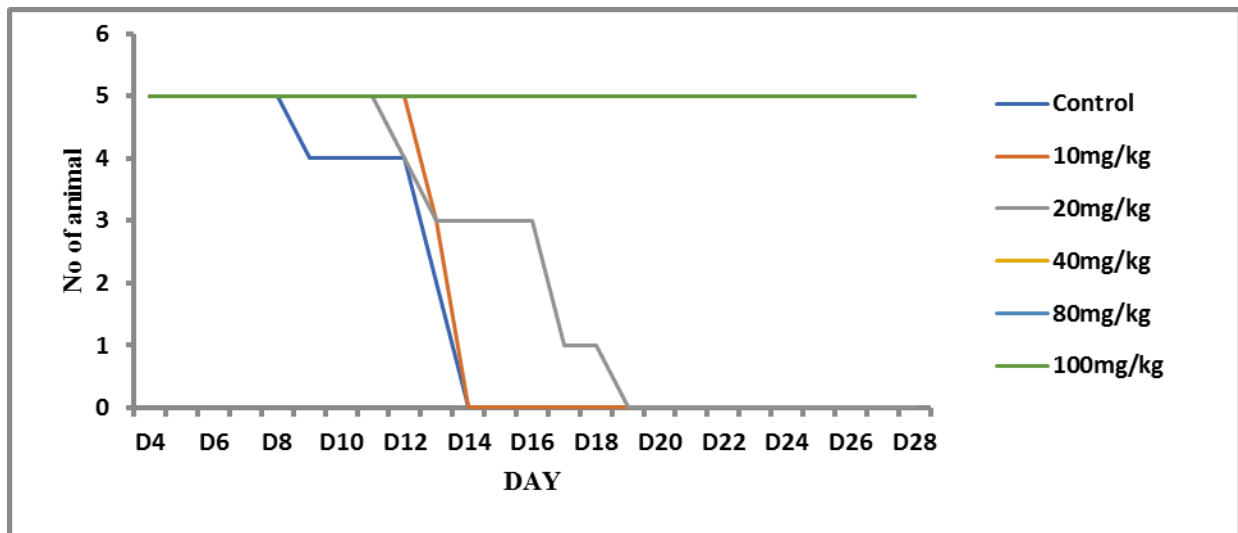
80mg/kg	0.00**	0.00**	0.00**	0.00**	0.00**
100mg/kg	0.00**	0.00**	0.00**	0.00**	0.00**

†Control (0.7% Carboxy methyl cellulose); *p<0.01 and **p<0.001 (Control Vs Treatment)



** p<0.01 Control Vs. Treatment, ***p <0.001 Control Vs. Treatment

Graph 1: Haemoglobin concentration in different groups treated with Artesunate

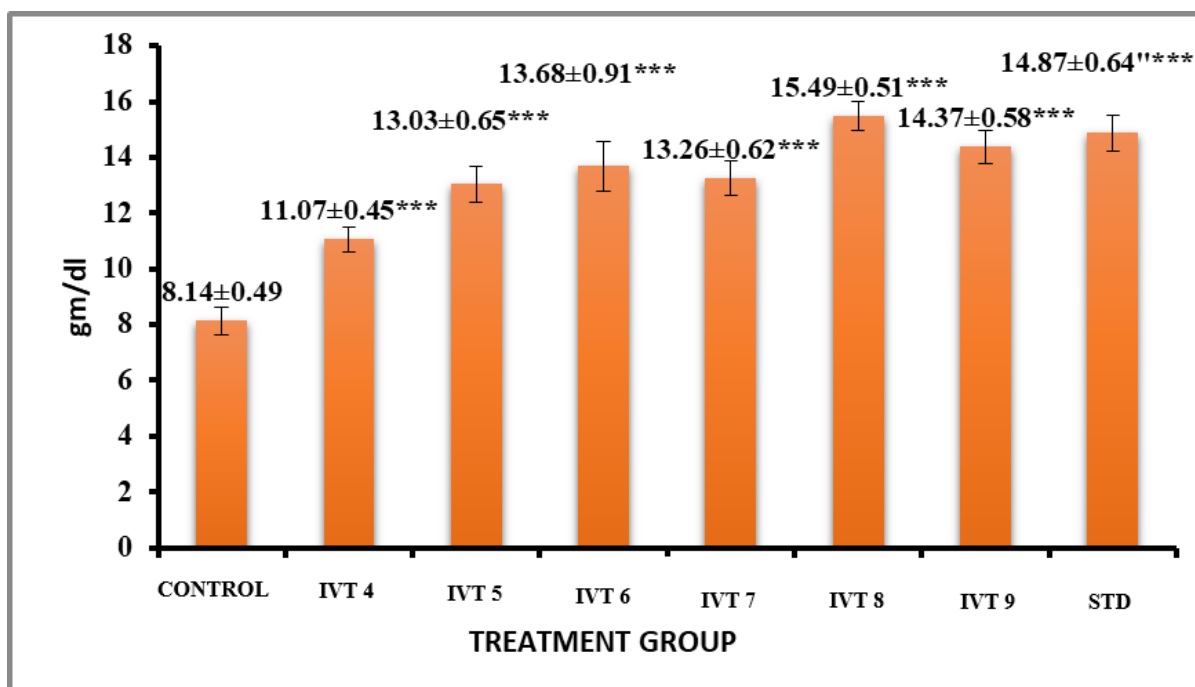


Graph 2: Survival kinetics of Artesunate

Table 2: The effect on Parasitemia (%) in various extracts of Nyctanthes arbor-tristis plant against P.bhergai in swiss albino mice.

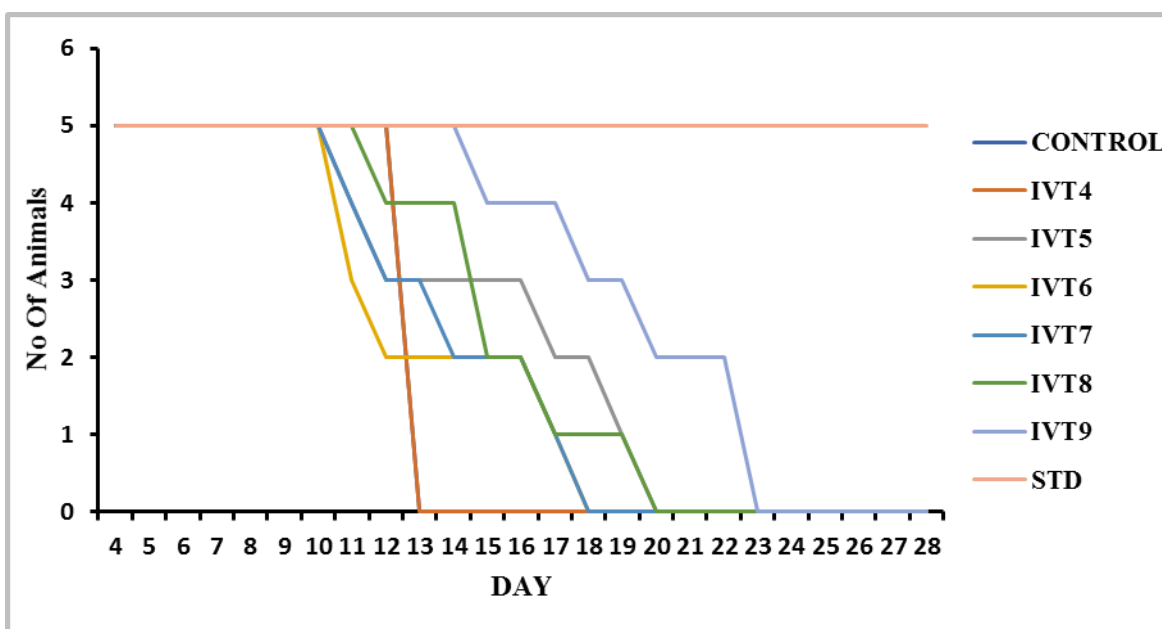
Groups	Day 4	Day 6	Day 8	Day 10	Day12
Control	0.99±0.07	4.73±1.49	25.36±1.13	35.3±1.23	42.4±1.26
Hexane (IVT 4)	0.05±0.03**	1.99±1.05	27.99±0.73	35.57±1.62	45.7±1.11
Chloroform (IVT 5)	0.06±0.04**	0.27±.17**	13.83±0.79**	18.24±1.98**	29.9±1.20**
Ethyl acetate (IVT 6)	0.05±0.03**	0.30±0.16**	16.84±0.84**	27.84±1.75**	37.3±2.04**
Acetone (IVT 7)	0.00**	0.41±0.20**	16.81±0.79**	28.71±1.75**	37.6±2.17**
Methanol (100%) (IVT 8)	0.00**	0.22±0.11**	5.76±0.30**	5.92±0.91**	18.23±1.39**
Methanol (80%) (IVT 9)	0.00**	0.16±0.14**	1.68±0.22**	4.5±1.15**	9.2±1.33**
Standard‡	0.00**	0.00**	0.00**	0.00**	0.00**

†Control (0.7% Carboxy methyl cellulose); *p<0.01 and **P<0.001 (Control Vs Treatment); ‡Standard (Chloroquine 5mg/kg)



*** P<0.001 Control Vs. Treatment

Graph 3: Haemoglobin concentration in various extracts of Nyctanthes arbor-tristis plant.

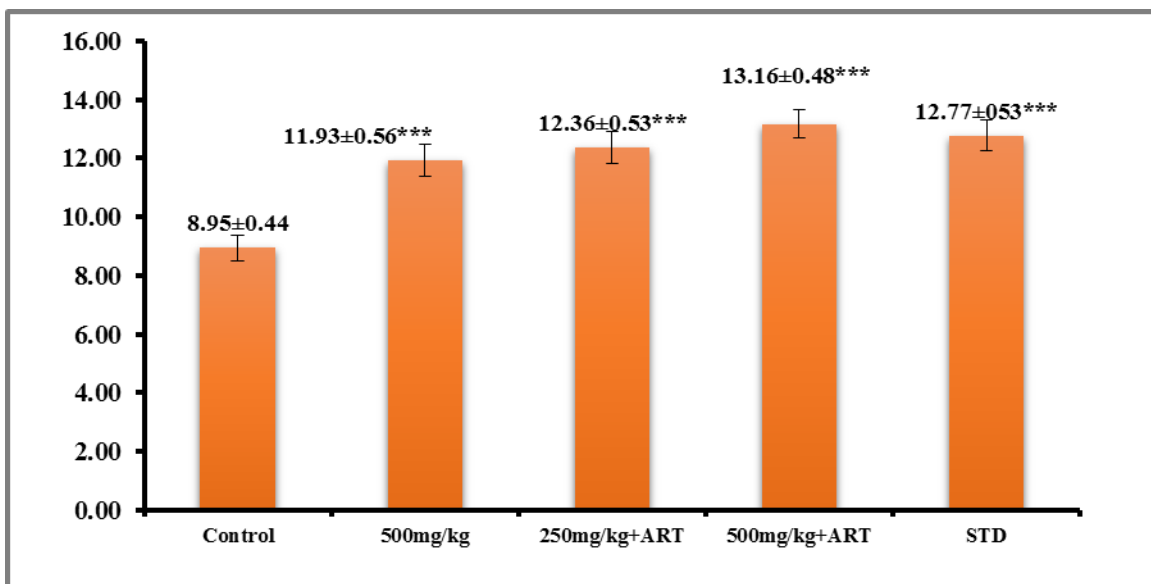


Graph 4: Survival kinetics of various extracts of Nyctanthes arbor-tristis plant.

Table 3: Parasitemia (%) in 80% methanol extract of Nyctanthes arbor-tristis plants with Artesunate against P.bergai in swiss albino mice.

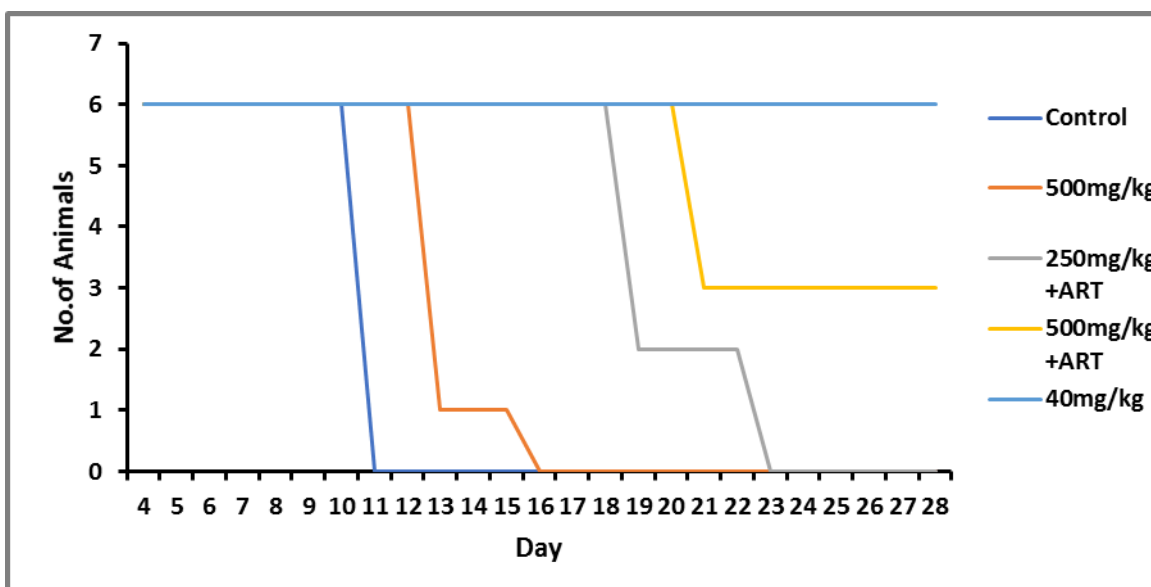
Groups	Day 4	Day 6	Day 8	Day 10	Day12
Control†	1.27±0.45	19.24±2.71	45.96±1.18	37.64±0.99	46.56±0.99
500mg/kg (IVT 9)	0.49±0.13**	1.33±0.36**	3.02±0.73**	11.47±1.28**	21.43±2.91**
250mg/kg (IVT 9) + 20mg/kg (Artesunate)	0.48±0.09**	0.75±0.16**	1.83±0.41**	4.69±0.70**	8.52±1.32**
500mg/kg (IVT 9) + 20mg/kg (Artesunate)	0.00**	0.63±0.32**	0.33±0.15**	2.01±0.91**	2.96±1.4**
Chloroquine 5mg/kg	0.00**	0.00**	0.00**	0.00**	0.00**

†Control (0.7% Carboxy methyl cellulose); **P<0.001 (Control Vs Treatment)



P <0.01 Control Vs. Treatment, * P<0.001 Control Vs. Treatment

Graph 5: Haemoglobin concentration in different groups NAT and NAT + Artesunate combination



Graph 6: Survivability in different groups NAT and NAT + Artesunate combination

The dose of Artesunate 40 mg/kg body weight (BW) was found to be statistically significant (p<0.001) to produce the best effect and were able to cure animal by reducing parasitaemia % (Table 1), improving haemoglobin levels (Graph 1) and showing good survival kinetics with no death observed (Graph 2). Thus, the sub effective dose or ED₅₀ of Artesunate came out to be 20mg/kg. Even in the higher group of ≥40 mg/kg BW showed a deviated result, and this might be due to the toxicity at higher dose (Graph 2).

Amongst the six solvent, hydroalcoholic 80% methanolic extract in 500mg/kg dose was found to be optimally effective (p<0.01) in reducing the parasitaemia (Table 2) with improved both haemoglobin levels (Graph 3) survival kinetics with less death observed (Graph 4) when compared to all three parameters viz. parasitaemia % (Table 1), haemoglobin levels (Graph 1) and survival kinetics i.e. number of death occurred (Graph 2) of sub effective dose of Artesunate 20mg/kg.

The effect of combination therapy of 500mg/kg 80% Methanolic NAT extract with sub effective dose of Artesunate 20mg/kg was found to be statistically significant ($p < 0.001$) in reducing parasitaemia % (Table 3), improving haemoglobin levels (Graph 5) and better survival kinetics (Graph 6) was far greater than the effect as monotherapy.

DISCUSSION

In our study we took both the nonpolar as well as a hydroalcoholic extract of NAT leaves both of which showed statistically significant ($p < 0.01$) antimalarial activity in animal models. Mostly in previous studies only nonpolar extracts of NAT leaves were taken. One study of crude ethanolic extract (CEE) of NAT leaves showed only modest potency ($IC_{50} 77 \pm 7 \mu\text{g/mL}$) against *P.falciparum* 3D7 which was thought to be related to the fact that crude plant extracts contain several complex molecules, only a few of which may be active [15] or the resultant activity of crude extract may increase/decrease/abolish depending on synergistic/antagonistic interactions among/between various types of complex molecules present in extract. [16] Even as the crude extract ($IC_{50} 77 \pm 7 \mu\text{g/mL}$) was found to be largely inactive, it was subjected to activity guided RPHPLC fractionation in the hope of finding some molecules which may be present in trace amounts and yet be quite potent. [15]

However, some studies support the antimalarial activity of NAT leaves extract as in a study by Badam et al [16] alreported the minimal efficacy dose (MED_{50}) of ethanol extract of NAT leaves against CQ sensitive strain of *P.falciparum* in the range of 1000–1200 $\mu\text{g/mL}$ which is further supported by another study by Mishra et al [18] reported the inhibition of *P.falciparum* by the 50% ethanolic extract of NAT leaves both *in vivo* and *in vitro*.

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Our study showed that 80% methanolic NAT leaf extract prolongs the survival time (Graph 4) in malaria infected mice which was supported by a similar study where the ethanolic extract of NAT leaves was used [19] Early *in vitro* study conducted concomitantly during the exploratory phase showed antiparasitic activity against drug sensitive (3D7) and drug resistant (Dd2) *Plasmodium falciparum* strains with nonpolar extracts of NAT leaves at minimum inhibitory concentration ($MIC_{50} 25\text{--}35 \mu\text{g/mL}$). [20]

Moreover, some human clinical studies also showed the antimalarial activity. In one study the leaf juice of NAT was used to treat malarial fever, [21] while in another study a paste of five fresh leaves thrice a day for seven days has shown the parasitic clearance of 76.7% within seven days. [11]

In our study we found that the hydroalcoholic Methanol 80% extract of NAT leaves has statistically significant ($p < 0.01$) antimalarial activity in the dose of 500mg/kg. The combination of Methanol 80% extract of NAT leaves 500mg/kg with Artesunate 20mg/kg showed highly statistically significant ($p < 0.001$) decrease in parasitaemia %, nearly equal Haemoglobin concentration and increased survivability then Artesunate 20mg/kg monotherapy which can be hypothesized that the extract would increase the bioavailability of the artemisinin derivatives.

CONCLUSION

In the present study we can conclude that hydroalcoholic 80% methanolic NAT extract in 500mg/kg dose was found to be optimally effective as an antimalarial agent and showed even better results in combination therapy with Artesunate 20mg/kg. Furthermore, detailed studies are needed to isolate and characterize the molecule(s) present in hydroalcoholic 80% methanolic NAT extract responsible for the antimalarial activity is suggested.

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