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EVALUATION OF NEEM- ARTEMETHER COMBINATION FOR ANTIMALARIAL ACTIVITY IN *PLASMODIUM BERGHEI* INFECTED MICE

Sanjay Balasaheb Bhawar*[1] and Bhanudas Shankar Kuchekar[2]

[1]Research Scholar, Vinayaka Missions University, Ariyanoor, Salem, Tamilnadu, India.
[2]Department of Pharmaceutical Chemistry, MAEER's Maharashtra Institute of Pharmacy, Kothrud, Pune, Maharashtra, India.

***Correspondence for Author: Sanjay Balasaheb Bhawar**
Research Scholar, Vinayaka Missions University, Ariyanoor, Salem, Tamilnadu, India.

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ABSTRACT

Studies have shown that neem possess antimalarial activity and a new combination therapy with of artemisinin derivatives and neem is unique. Neem leaf extract was prepared by Continuous hot extraction method and evaluated for antimalarial activity. The mice survival and % parasitemia were studied in *Plasmodium berghei (P. berghei)* infected albino mice treated with neem leaf extract and combination of neem extract with artemether.The mean survival time in mice infected with *P. berghei* was compared after treatment with neem extract and combination of neem-artemether. Oral administration of neem leaf extract prolonged the survival of *P. berghei* infected mice. The survival rate in mice treated with neem leaf extract (5mg/day) and artemether (1000 µg) was found 80% after 40 days post infection and they recovered with no detectable parasitemia. Administration of neem artemether combination reduced the parasitemia in mice more effectively compared to that in mice treated with a single drug. A single dose of 1000 µg of artemether in combination with neem gives complete protection in *P. berghei* infected mice. Suppressive action exerted by combination was superior to that of administration of single drug at the same dose.

KEYWORDS: artemether, mice survival, neem leaf extract, % parasitemia.

INTRODUCTION

The increase in prevalence of multidrug resistance malaria dramatically illustrates the continuous need for new antimalarial agents. One possible approach is the identification of new antimalarial drug candidates in plants, empirically used to treat malaria. Artemisinin (qinghaosu) derivatives have all been used in combination with other antimalarial drugs for the treatment of malaria. Artemisinin derivatives are eliminated rapidly and has a short half life. When given in combination with a longer half-life "partner" antimalarial drug allows a reduction in the duration of treatment, while at the same time enhancing efficacy and reducing the likelihood of resistance development[1,2].

Azadirachta indica (A. indica), is one of the most promising medicinal plants, having a wide spectrum of biological activity. Every part of the neem tree has been known to possess a wide range of pharmacological properties. Neem has been extensively used in Ayurveda, Unani and Homeopathic medicine. The Sanskrit name of the neem tree is 'Arishtha' meaning 'reliever of sickness'. The importance of the Neem tree has been recognized by the US National Academy of Sciences, which published a report in 1992 entitled 'Neem-a tree for solving global problems'[3]. It was showed that nimbidin, azadirachtin and gedunin present in neem possess antimalarial activity[4]. An active ingredient irodin A from Neem leaves was isolated, which was found toxic to causative strains of malaria. Components of the alcoholic extracts of leaves and seeds are effective against both chloroquine-resistant and sensitive strains of malarial parasite[5]. The antimalarial potential of neem in combination with artimisinin derivative has been explored with major conclusion that combination is more effective[6].

MATERIALS AND METHODS

Animals

Inbred albino rats were obtained from the animal house of Pravara Medical College, Pravaranagar. The research was conducted in accordance with standard institutional guidance given by the Institutional Animal Ethics Committee (IAEC). The Labs used for the purpose was approved by Committee for the purpose of control and supervision of experiments on animals, Ministry of social justice and empowerment, Govt. Of India (Registration No. -448/01/c/CPCSEA).

Collection of plants, processing and their extraction

The leaves of azadirachta indica was collected from ahmednagar district. The leavces of plant were dried

under shade away from direct sunlight. The dried parts were cleaned and coarsly powdered in grinder and powder material was passed through 120mesh to remove fine powders and coarse powder was used for extraction.

Extraction of crude drugs

The leaves A. indica were collected and shade dried and then pulvirised in grinder. About 100gm powdered leaves utilised for extraction was passed through 120 mesh sieve and used for extration.

Solvent used: petrolium ether and ethanol

Method : Continuous hot extraction method

The powdered neem leave were extracted with petroleum ether for removal of coloring matter by defattation process using continous soxhleat extraction method. The compltion of extraction was indicated by taking sample out of siphon tube on TLC plate and placing it in iodine chamber. Absence of colored spot on plate indicates complete exctraction. The defatted leaves were refluxed with water and alcohol (1:1) for 3hr to get hydroalcoholic extract. The extraction temperature was maintained at 50 degree C with constant shaking. The extract was filtered and concentrated to get thick paste and after it freeze dried to get powder. The extract was stored in air tight container.

Characterization of neem extract

FTIR analysis

FTIR spectroscopy of neem leaf extract was performed on FTIR (Jasco FT/IR-4100) spectrophotometer. About 5 mg of sample was mixed with 100 mg of Potassium bromide (KBr) and compressed to form pellets. The spectra of sample were scanned from a wave number range of 650 to 4000 cm^{-1}.

Antimalarial activity

Mice survival study[7,8, 9]

Male albino mice were used for the study. Tap water and mouse feed were provided ad libitum. *P. berghei* (ANKA strain) erythrocytic stages were maintained by serial passing of infected blood in male albino mice. Animals were divided into nine groups based on the treatment. Mice were injected intraperitonally (i. p.) with 10^7 *P. berghei* infected mouse erythrocytes. Control group (group no 8) was only given 5% tween 60. Mice in group 1 received an oral suspension of neem extract in corn oil at a dose equivalent to 5 mg of neem extract on day 1, 2 and 3. Animals in group 2, 3 and 4 received by oral route a combination of neem extract (equivalent to 5mg neem extract on day 1, 2 and 3) and i. p. injection of artemether at single dose of 0.5, 1 or 1.5 mg, respectively (on day 1). While artemether was injected intraperitonally as a suspension in 5% tween 60 to animals in group 5, 6 and 7 with a dose of 0.5, 1 or 1.5 mg (on day 1) respectively. The survival time (over 40 days post treatment) of mice infected with the erythrocytic stages of *P. berghei* was compared in different groups.

Determination of mean survival time

Mortality was monitored daily and the number of days from the time of incoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow up period. The mean survival time (MST) for each group was calculated as:

$$MST = \frac{\text{Sum of survival time in all mice in group (days)}}{\text{Total number of mice in that group}}$$

Percent mean parasitemia in mice[10]

Parasitemia was monitored by light microscopy (oil immersion, 1000× magnification) by examining thin smears of blood from the tail veins of the mice. Blood films are made by applying 4.5 microlitres of blood to microscope slides as soon as the specimen is received. Thin blood films were fixed in methanol and stained with Giemsa stain immediately after slide production. The parasitemia level was determined by counting, in random fields of the microscope, the number of parasitized RBCs. The percent of infected RBCs were determined by enumerating the number of infected RBCs in relation to the number of uninfected RBCs. A minimum of 500 RBCs was counted per sample.

$$\% \text{ Parasitemia} = \frac{\text{Number of infected RBC}}{\text{Total number of RBC counted}} \times 100$$

Inoculation of *P. berghei* to mice was done on day 0, while percent mean parasitemia was measured on day 1,2,3 and 4. The blood samples were collected after 4 hours of receiving treatment as per specified above and at the same time on next days.

Statistical analysis

All data were expressed as mean ± SD. The statistical analysis of all the observations was carried out using one-way ANOVA followed by a multiple comparison test of Tukey, where necessary. $P<0.05$ was considered as significant compared with the control group and all data were analysed at a 95% confidence interval.

RESULTS AND DISCUSSION

FTIR analysis

Neem extract into powdered form was scanned between 4000cm^{-1} to 450 cm^{-1}. The resultant spectrum obtained shown in figure 2. Presence of peaks for aldehydic C-H stretching (around 2940 cm^{-1}), C=C group (around 1625 cm^{-1}) and Geminal methyl group (around 1350 cm^{-1}) were indicative of terpenoid group of compounds present in the aqueous neem extract.

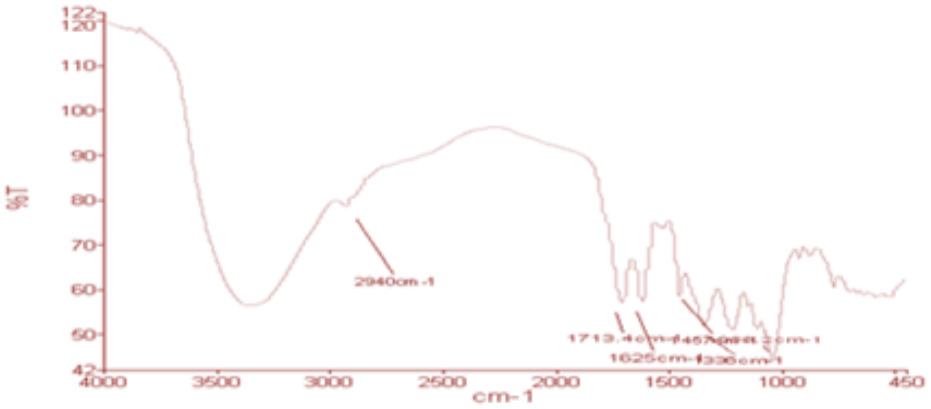

Fig 1: FTIR spectra of A. hydroalcoholic extract of *A. indica*

Antimalrial activity

Mice survival study

The survival time (over 40 days post treatment) of mice infected with the erythrocytic stages of *P. berghei* was compared in different groups as showed in table 1. All mice in control group died in 8 days post exposure to infection. Animals treated with only neem extract failed to survive beyond 10 days. While animals treated with 0.5 mg of artemether have survived little longer than treated with neem extract with 40% survival on day no 16. Animals in the group 7, showed 40% survival on day 20 and 100% mortality by day 25. Survival rate for animals in group 6 was 40% on day 25 but 100 % mortality by day 40. Combination of neem–artemether prolonged the survival time as compared to monotherapy at same dose while survival rate in group 3 and 4 were found 60% and 80% respectively by the day 40.

Table 1. Mean survival time in *P. berghei* infected mice

Group No	Treatment	Route	Dose mg	MST
1	Neem extract	oral	3 Days 5mg/ d	8.6
2	neem extract + artemether combinaion	Oral i.p.	3 Days 5mg/ d +1.5mg	36.4**
3		Oral i.p.	3 Days 5mg/ d +1 mg	32.8**
4		Oral i.p	3 Days 5mg/ d +0.5mg	16.2**
5	Artemther	i.p.	1.5 mg	31.8*
6		i.p.	1.0 mg	18.2*
7		i.p.	0.5 mg	10.6*
8	Control			6.8

**P<0.05, **P<0.001 compared to control*

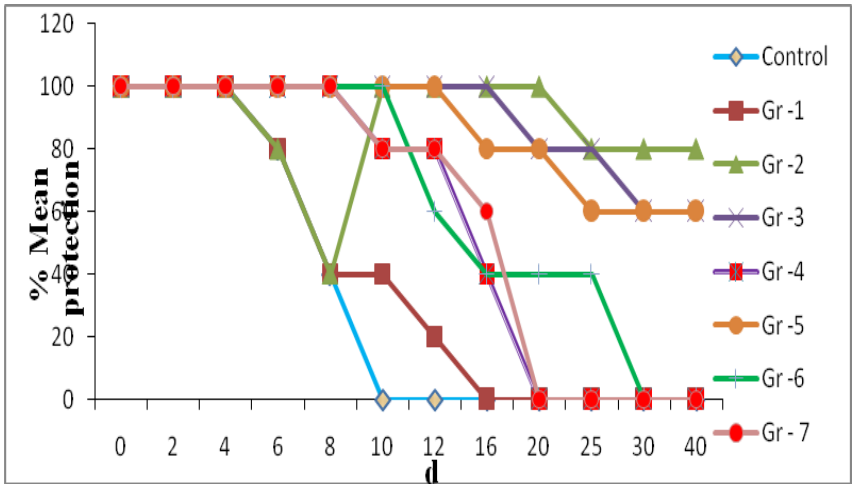

Fig. 2: Percent mean protection in mice receiving different treatments over time (40 days post treatment), * (n=5)

Percent mean parasitemia in mice

There was marked variation in percent parasitemia in mice receiving control, artemether, neem extract and combination of neem- arthemether for the first 3 days as showed in table no 2. The mean percent parasitemia is measured after 2 hrs of i.p. injection of p. *berghei* on day 0 while after 4 hours after treatment on day 1,2 and 3. There was drastic increase in percent mean parasitemia in control mice within 3 days. Administration of neem reduced the percent mean parasitemia in mice as compared to that in control mice. Administration of neem-artemether combination reduced the percent mean parasitemia in mice more effectively as compared to mice treated with mono drug therapy. Suppressive action was superior to that of artemether alone at the same dose.

Table 2: Percent mean parasitemia in mice receiving different treatments on 4th day

Treatment	Route	Dose	No. Of mice tested	% mean Parasitemia on 4th day
Control			5	56.51 ± 5.2
Neem extract	oral	3 Days 5mg/ d	5	46.12 ±4.9*
neem extract + artemether combinaion	Oral i.p.	3 Days 5mg/ d +1.5 mg	5	7.16 ± 0.9
	Oral i.p.	3 Days 5mg/ d +1.0 mg	5	10.58 ± 1.8
	Oral i.p	3 Days 5mg/ d + 0.5 mg	5	19.91 ± 1.9
Artemther	i.p.	1.5 mg	5	9.05 ± 1.5**
	i.p.	1.0 mg	5	13.12 ± 2.2**
	i.p.	0.5	5	25.26 ±2.2

*Data are expressed as mean ±SD (n = 5); *P<0.05, **P<0.001 compared to control*

CONCLUSIONS

In both mice survival and % parasitemia inhibition study, it was found that neem extract has weak antimalarial activity. Administration of combination of neem extract and artemether have increased survival rates and reduced the parasitemia more effectively in mice. Such suppressive action was superior to that of administration of single drug at the same dose.

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