PHARMACOLOGICAL STUDIES ON MELIA AZADIRACHTA, LINN*

N. O. MELIACEAE

By

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Synonyms : *Azadirachta indica* A Juss; Nimba (Sans), Neem, Margosa (Eng:); Nimb (Hindi); Vembu (Tam.); Vepa (Tel.); Bevinamara (Can.)

PART I

Antibacterial, antifungal and antitubercular activity of Neem oil and its fractions

Melia azadirachta is an evergreeen tree indigenous to and cultivated nearly all over India. Every part of the tree, the bark, the leaves, young branches, flowers, unripe and ripe fruits, oil and the gum is reported to have medicinal properties.[1]

Investigations have been carried out by several workers[2-18] with a view to isolate the bitter principles which are generally believed to be the therapeutically active ingredients of neem. The work upto about 1940 was controversial and has been reviewed by Siddiqui and Mitra[10]. As a result of extensive investigations, Siddiqui and his co-workers isolated four bitter principles, nimbin, nimbinin, nimbidin and nimbidol[10] from the neem oil. Some of these bitter principles were shown by them to be present in root bark and trunk bark also [11,12]. By mild alkaline hydrolysis of nimbidin (which was considered by them to be the main active principle of neem oil from preliminary bacteriological tests)[13] they obtained neonimbidin, nimbidic acid and nimbidinic acid. From nimbidinic acid which is the main product of hydrolysis of nimbidin, they prepared sodium, potassium, quinine and other nimbidinates[10].

From neem blossoms also different bitter principles have been isolated by different workers [14,15]. The flavonol aglycons present in neem blossoms have been identified by circular paper chromatography by Pankajamani and Seshadri[17]. Recently the structure of neem gum has also been investigated[18].

The literature on the clinical, pharmacological, insecticidal and other similar studies of neem oil and its bitter principles is scanty and controversial. Clinical trials under controlled conditions, proved the juice of the leaves and the neem oil to be ineffective as vermifuges[19]. According to Chatterji[2,3,4] ethyl ester of margosic acid and some margosates were beneficial in leprosy,

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syphilis, skin diseases and cancer. They exhibited no *in vitro* bactericidal activity, but were effective against some protozoa, viz. *Paramoecium* and *Prowzekia*[2]. Siddiqui and Mitra[10] stated that preliminary pharmacological investigations carried out with nimbidin and the water soluble sodium nimbidinate showed that they were non-toxic and stimulated uterine contractions. Neem leaves were shown to possess considerable repellent and insecticidal action on *Calandra orysae* which attack grains during storage even though they cannot be entirely depended upon as grain preservatives[20]. Bhat and Broker[21],[22] showed that aqueous extract of neem leaves was ineffective against the coagulase property of *staphylococci* but full saturation of the extract with ammonium sulphate yielded a fraction whieh delayed the clotting time of plasma by coagulase from either staphylococci or Russel's viper venom. Recently Choudhury *et al*[23] reported that water extract of neem leaves had a stimulatory effect and the alcohol extract decreased the force of concentration and rate of isolated toad heart while both the extracts inhibited the tone of the intestinal musculature.

This paper records our observations on some pharmacological properties of the neem oil and its bitter principles isolated by the method of Siddiqui and his co-workers[10].

MATERIALS AND METHODS

The bitter principles were isolated from neem oil according to the method of Siddiqui and Mitra[10] and Siddiqui[24]. These authors decribed the procedure for the isolation of four bitter principles, nimbin, nimbinin, nimbidin and nimbidol. However, with the sample of oil used by us, we could isolate only nimbidin and nimbidol.

Antibacterial activity in vitro. The antibacterial action of the neem oil and its fractionates was evaluated by the conventional filter paper disc method using nutrient agar medium and some pathogenic gram-positive and gram-negative organisms. In some instances the cup plate and serial dilution techniques were also used to evaluate the antibacterial activity.

Antifungal activity. The two pathogenic fungi *Candida albicans* and *Tinea rubrum* were used for these studies. Of the two, *C. albicans* grew quickly on Sabouraud's glucose agar[25] of the following composition: dextrose 40 gm., agar 35 gm., peptone 10 gm. and distilled water 1000 ml.

However, *T. rubrum* took about 2—3 weeks to show enough growth. Hence with this fungus readings were taken after 2 weeks. For inoculation with *T. rubrum*, mycelium from a 17 day old culture was made into a suspension with sterile water by means of a sterile mortar and pestle and 0.1 cc. of this suspension was used. The well known fungicidal salicylic acid, in alcoholic solution was included in the experiment as a positive control.

(a) *Antitubercular activity in vitro* : This was done in Youmans' medium[26]. Except nimbidin all other fractions were made as emulsions in water (1 in 100) and then the required dilutions were prepared with the medium itself. Nimbidin in a concentration of 1 in 100 was dissolved in alcohol and further dilutions were made with the medium. Corresponding amounts of alcohol incorporated in the medium were kept as controls. Some tubes without any drug and some tubes containing different concentrations of sodium para-aminosalicylate were also included. All the tubes were then autoclaved at 15 lbs. pressure for ½ hour. By means of a sterile platinum loop the tubes were inoculated by gently floating on the top, approximately equal amounts of inocula from 12–16 days culture of *Mycobacterium tuberculosis* var. hominis $H_{37}R_v$ grown in Youmans' medium. All the tubes were then sealed with paraffin and incubated for 3 to 4 weeks. Nimbidin fraction which exhibited inhibitory action on *M. tuberculosis* was investigated in detail. This includes the nature of its action whether bacteriostatic or bactericidal, the influence on the acid fastness of the mycobacteria and its effect in combination with streptomycin.

(b) *In vivo action on the experimental tuberculosis in mice* . 25 mice were divided into 5 groups of 5 animals each, such that the initial weight in each group was approximately equal. Mice, 5–7 weeks old and all weighing about 18-22 gm. were used for the experiment. They were infected with a 14 day old culture of the $H_{37}R_v$ strain of *M. tuberculosis* by intravenous injection into the tail vein of 0.1 ml. of a uniform suspension containing 1 mg. wet weight of the Mycobacteria for each animal. Group I was not given drug and served as an untreated control. Group 2 was treated with neem oil 250 mg./kg. body weight/day, Group 3 with nimbidin 60 mg./kg. and Group 4 with nimbidol 250 mg./kg. Group 5 received 250 mg./kg. per day sodium para-aminosalicylate and served as treated control. All the drugs were given by the drug diet method.

The survival time, the weight curves, the macroscopic and microscopic examination of the tissues were considered for the evaluation of the activity of the compounds as previously described[28].

(c) *Effect of neem oil on the sensitivity to tuberculin in $H_{37}R_v$ infected guinea pigs.* It is possible that drugs might favourably influence the course of tuberculosis infection by reducing the hypersensitivity though they may not possess direct antimycobacterial action and thus act as suitable adjuncts for other chemotherapeutic measures.

With this idea in view, the effect of the neem oil on tuberculin hypersensitivity has been investigated.

The method followed is essentially that of Long and Martin[39] with slight modifications. Four guineapigs infected with *M. tuberculosis* $H_{37}R_v$ strain by intramuscular route about one month earlier and found to be tuberculin positive were used for these tests. 1.0 ml of neem oil was given subcutaneously to two guinea pigs. The other two guinea pigs served as untreated controls. Five hours after giving the neem oil injections, all the animals in both the groups were given intracutaneously four doses of old tuberculin 50, 100, 200 and 500 international units in a volume of 0.1 ml. and the diameter of the lesions produced was measured after 24 hours.

RESULTS

Antibacterial activity. Neither the bitter principles, nimbidin and nimbidol nor the neem oil and other fractionates, viz. residual oil and fatty ballast showed any activity against the following 9 bacterial species :

Escherichia coli, Micrococcus pyogenes var *aureus, Streptococcus pyogenes* (hemolyticus), *Salmomella tytphosa, Salmonella paratyphi* A, *Shigella dysenteriae* (sonnei), *Klebsiella pneumoniae, Alcaligenes fecalis, and Proteus vulgaris.*

Antifungal activity. While *Candida albicans* was not inhibited by any of the fractions tested, nimbidin in a concentration of 1/1000 prevented the growth of *Tinea rubrum.*

TABLE I

Effect of neem oil and its fractions on the *in vitro* antitubercular activity at the end of third week.

Drug	Concentration			
	1/1,000	1/10,000	1/100,000	1/1,00,000
Neem oil	3+	4+	4+	4+
Residual	4+	4+	4+	4+
Nimbidin	–	1+	2+	4+
Nimbidol	3+	4+	4+	4+
Fatty Ballast	3+	3+	3+	4+
PAS	–	+	2+	2+
Alcohol 1/100	4+			
Control	4+			

– Indicates total inhibition
± Indicates very slight growth
1+ Indicates growth over half the surface area
2+ Indicates growth over the entire surface area
3+ Indicates growth all over the surface and also extending on the sides of the tube
4+ Same as 3 but extending on the sides to a greater extent.

The results at the end of the first week indicate that nimbidin completely inhibited the growth in dilutions upto 1/100,000, partial inhibition was shown by neem oil, nimbidol and the fatty ballast upto 1/100,000 while residual oil had entirely no effect. By the end of second week, nimbidin 1/1000 still showed complete inhibition. In higher dilutions upto 1/100,000 the effect was partial. Neem oil and nimbidol in 1/1000 and the fatty ballast upto 1/1,000,000 concentration manifested a partial activity at this period. The same trend, partial inhibition by neem oil and nimbidol in 1/1000, by the fatty ballast upto 1/100,000 and complete inhibition by nimbidin at a concentration of 1/1000 was observed even at the end of three weeks (Table I).

Studies on the nature of this inhibition caused by nimbidin revealed that nimbidin at 1/1000 dilution was bactericidal, as the subcultures were found to be nonviable.

Not much variation in the staining character of the inhibited organisms was noticed.

The results of the *in vitro* antitubercular action of the various concentrations of streptomycin and nimbidin revealed that there was no synergistic activity between nimbidin and streptomycin.

TABLE II

The influence of Nimbidin and streptomycin in various concentrations on the growth of M. tuberceulosis (Readings at the end of 3 weeks).

Nimbidin concentration.	Streptomycin concentration				
	10^{-5}	10^{-6}	10^{-7}	10^{-8}	0
10^{-3}	–	–	–	–	–
10^{-4}	–	–	+	+	+
10^{-5}	–	–	+	2+	2+
10^{-6}	–	–	+	4+	4+
0	–	–	+	4+	4+

In vivo action on the experimental tuberculosis in mice. The growth rate of the individual groups during treatment is shown in fig 1. The initial increase in weight during the first 3 days was a feature common to all animals both treated and untreated. During the next three

days the untreated and neem oil treated groups were stationary while the groups treated with other fractions and PAS continued to show the gain in

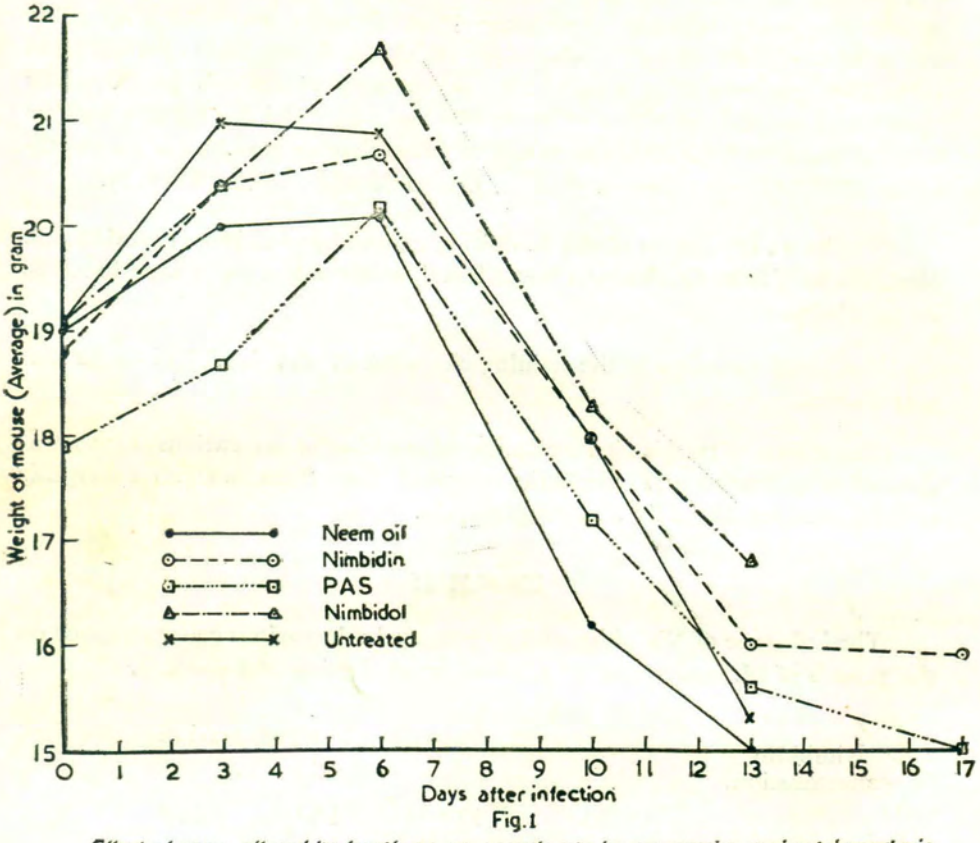


Fig.1

Effect of neem oil and its fractions on growth rate in progressive murine tuberculosis

weight indicating probably a slight degree of inhibitory action on the progress of the disease. After 6 days there was loss of weight in all the groups.

In Table III are recorded the average survival time, average weight loss or gain and the average macroscopic lesions in lungs. At the time when all the controls had died, all the five animals in the nimbidin and PAS treated groups were still alive as also one in the neem oil group and 3 in the nimbidol group. Average survival period evaluation indicates that nimbidin had a definite suppressive effect, while oil by itself had no action. No appreciable difference either in weight loss or in the extent of tuberculosis between the PAS treated and nimbidin treated mice was observed.

TABLE III

Evaluation of the activity of neem oil and its fractions in murine tuberculosis.

Group	Drug administered	No. of mice in each group	Average survival time (days)	Average weight loss or gain (gm)	Average amount of macroscopic tuberculous lesions in lungs*
1	Untreated ..	5	13.0	–3.8	3.6 +
2	Neem oil ..	5	18.6	–4.0	3.5 +
8	Nimbidin ..	5	17.0	–2.9	3.5 +
4	Nimbidol ..	5	15.8	–2.3	3.4 +
5	PAS ..	5	17.2	–2.9	3.3 +

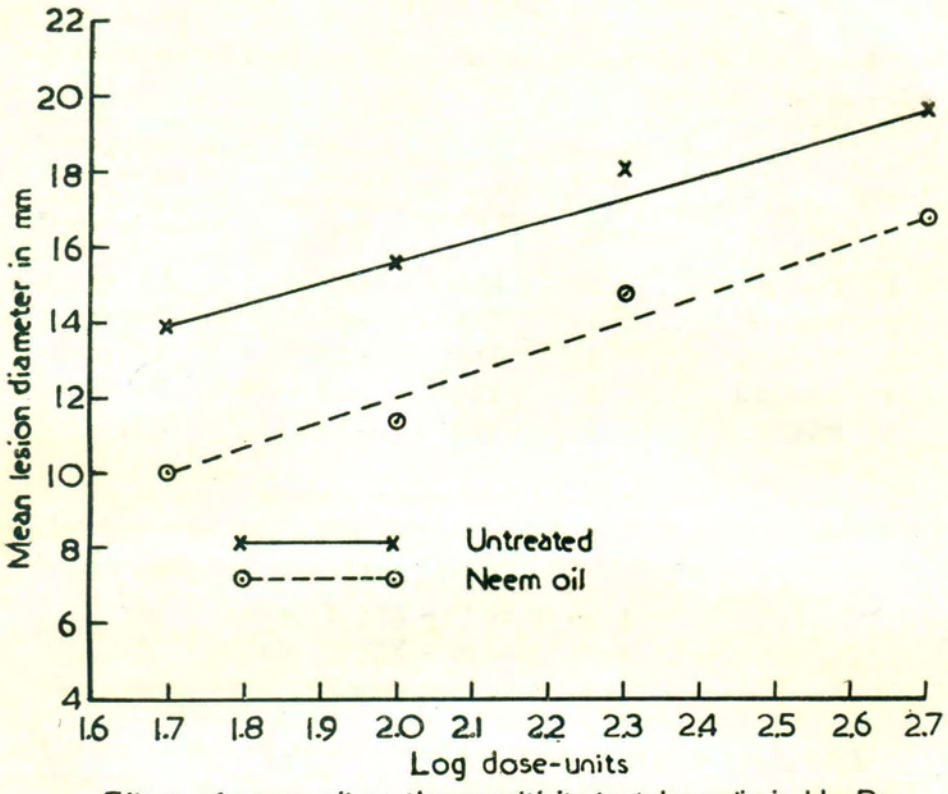
* 0 = Apparently normal
1+ = less than 10% of the organ replaced by grossly pathologic substance
1 to 2 = 10 – 25% of the organ do
2 to 3 = 25 – 50% do do
2 to 4 = more than 50% do

Effect of neem oil on the sensitivity to tuberculin in $H_{37}R_v$ *infected guinea pigs.* The mean lesion diameters produced by 50, 100, 200 and 500 international units of old tuberculin in guineapigs infected with $H_{37}R_v$ show that neem oil depressed to some extent the sensitivity to tuberculin after 24 and 48 hours. The values after 24 hours are recorded in Table IV. The results after 24 hours are also presented in a graphical way in Fig. 2 with log dose units as the abscissae and mean lesion diameters in mm. as the ordinates. The readings fall more or less in a straight line. The graph also indicates in a more clear way that in $H_{37}R_v$ infected guinea pigs neem oil 1 ml. when given subcutaneously reduces the sensitivity to tuberculin.

TABLE IV

Results of the experiment on the effect of neem oil on the sensitivity to tuberculin in $H_{37}R_v$ infected guinea pigs at the end of 24 hours.

Treatment	No. of guinea pigs	Mean lesion diameter (mm) produced by International units of old tuberculin			
		50	100	200	200
Untreated	2	14.0	15.8	18.3	20.0
Neem oil 1 cc.	2	10.0	11.5	16.0	17.0



Effect of neem oil on the sensitivity to tuberculin in $H_{37}Rv$ infected guinea pigs after 24 hours

Fig.2

DISCUSSION

The absence of any *in vitro* antibacterial action of the neem oil and its fractionates on the common pathogenic bacteria, at first would seem to throw doubts on the claimed clinical efficacy of the neem as an antiseptic dressing. Probably the explanation lies in the observations of Bhat and Broker [21,22] that a fractionate of the neem oil alters the coagulase activity of the staphylococci and reduces the virulence of the organism.

Of the various fractionates tested nimbidin appears to be the most active principle. This bitter substance inhibits the growth of *Tinea rubrum* and offers an explanation for the use of neem oil in some fungal affections of the skin.

The same principle, nimbidin, exhibits an antituberculous activity of a bactericidal nature at higher concentrations and bacteriostatic at lower levels; the other principles as also neem oil are only bacteriostatic even in high concentrations. This *in vitro* antitubercular activity of nimbidin is also reflected in its ability to retard the progress of experimental tuberculosis in mice as exhibited by the survival time and the loss in weight of the experimental animals. Neem oil, which showed no inhibitory action in acute experiments with mice, was found to depress the tuberculin sensitivity in guinea pigs. Since the hypersensitive state, by its allergic menifestations due to the interaction with products of tubercle bacilli, is held to be responsible for the severity of the symptoms in tuberculosis, a dimunition of this hypersensitive state would naturally lessen the severity and it is possible that the neem oil may indirectly be beneficial in the disease.

SUMMARY

1. Neither neem oil nor its fractions showed any *in vitro* antibacterial activity against 9 species of gram-positive and gram-negative organisms tested.

2. Nimbidin inhibits the growth of the fungus *Tinea rubrum*, grown in a liquid medium-sabouraud's glucose broth. Neem oil and its fractions do not appear to possess appreciable activity against *Candida albicans*.

3. In the *in vitro* experiments with *Mycobacterium tuberculosis* $H_{37}R_v$ nimbidin in a concentration of 1/1000 completely inhibited the growth for 3 weeks, while higher dilutions showed only partial inhibition. Neem oil, nimbidol and fatty ballast even in very high concentrations of 1/1000 exhibited partial inhibitory influence only. Residual oil was inactive.

4. Nimbidin 1/1000 was bactericidal against *Mycobacterium tuberculosis* but did not alter the staining character of the inhibited organisms.

5. No synergism between nimbidin and streptomycin was observed.

6. *In vivo* experiments on the course of the progressive experimental tuberculosis in mice revealed that nimbidin prolonged the survival period, while the neem oil by itself had no effect. No appreciable difference either in weight loss or in the extent of tuberculosis was noticed between the PAS treated and nimbidin treated mice, at the dosage levels tried.

7. Neem oil depressed the hypersensitivity to tuberculin in sensitized guinea pigs.

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