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Anti-microbial activity of a new vaginal contraceptive NIM-76 from neem oil (*Azadirachta indica*)

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Abstract

Efficacy of NIM-76, a spermicidal fraction from neem oil, was investigated for its antimicrobial action against certain bacteria, fungi and Polio virus as compared to whole neem oil. The NIM-76 preparation showed stronger anti-microbial activity than the whole neem oil. It inhibited growth of various pathogens tested including *Escherichia coli* and *Klebsiella pneumoniae* which were not affected by the whole neem oil. NIM-76 also exhibited antifungal activity against *Candida albicans* and antiviral activity against Polio virus replication in vero cell lines. It also protected mice from systemic candidiasis as revealed by enhanced % survival and reduced colony forming units of *C. albicans* in various tissues. This shows that NIM-76 has a potent broad spectrum anti-microbial activity. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: NIM-76; Contraceptive; Anti-microbial activity

1. Introduction

There is currently a worldwide upsurge in the use of herbal preparations and active ingredients of medicinal plants in health care. This is particularly true in the rural areas of Asian countries where herbal medicine are in most cases, the only choice for treating human ailments. Among these, neem (*Azadirachta indica*) is one of the most widely used plant and aqueous extracts of its stem

bark are used as tonic, stimulant and as a remedy against various skin ailments (Dhawan and Ratnaik, 1993). Studies made with neem showed that it possesses anti-inflammatory, antipyretic, hypoglycaemic and diuretic activities (Binde et al., 1958; Murthy et al., 1978; OKpanyi and Ezeukwa, 1981). The stem bark possesses anti-tumour and interferon inducing activities (Fujiwara et al., 1982) and other plant parts have been reported to have antibacterial, antifungal, anti-malarial and anticancer effects (Siddiqui et al., 1992; Udeinya, 1993; Kusamran et al., 1998). Regarding neem oil, it has been reported to have

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anti-fertility activity and to stimulate cell mediated immune response (Upadhyay et al., 1992).

During our investigations on the development of contraceptives from neem, a fraction from neem oil, coded as NIM-76, was isolated and found to possess only spermicidal activity (Riar et al., 1991) while neem oil has spermicidal, anti-implantation as well as abortifacient effects (Riar et al., 1984; Sinha et al., 1984a, b). As a consequence, NIM-76 has been developed as a pre-coital antifertility formulation for human use which is about to undergo Phase I clinical trials. As a complement to this previous study, we report here the antimicrobial (antibacterial, antifungal and antiviral) effects of NIM-76 which in addition to its spermicidal action could be useful to maintain vaginal health and hygiene thus contributing to the well-being of women.

2. Materials and methods

2.1. Determination of antibacterial and anti-fungal activity

2.1.1. Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of NIM-76 was determined by serial broth dilution methods as described by Irobi et al. (1996). The NIM-76/neem oil diluted in DMSO was added to 5 ml sterile nutrient broth to give various concentrations 0.25–15 mg/ml. Later, 0.5 ml of the exponentially growing microbial broth culture (1×10^6 cells/ml in case of bacterial strains and 1×10^4 cells/ml in case of *C. albicans*) was introduced into respective test tubes. Another set of tubes containing only the growth medium without DMSO (control) and with DMSO (solvent control) and each of the test microbe was set up separately. Ciprofloxacin (10 µg/ml) and ketoconazole (50 µg/ml) were used as positive control for bacterial strains and *C. albicans*, respectively. The tubes were incubated at 37°C for 24 h and the growth was measured by measuring optical density at 520 nm using GBC spectrophotometer. The MIC was regarded as the lowest concentration of the extract which inhibit the growth of bacteria or fungi.

2.1.2. Minimum microbicidal concentration

The minimum microbicidal concentration (MCC) of NIM-76 was determined by a modification of the methods described by Irobi et al. (1996). Subcultures made from diluted (1:10) samples, obtained from those test tubes which did not yield any visible turbidity (growth) in the MIC assays, were streaked on freshly prepared nutrient agar plates and incubated at 37°C for 48 h. The MCC was regarded as the lowest concentration of the extract that did not permit any colony growth on the surface of the nutrient agar.

2.2. Determination of antiviral activity

It was carried out against polio virus (vaccine strain) in vero cell line. The virus was incubated with various concentrations of NIM-76/neem oil (50–250 µg/ml) overnight. After establishing a confluent layer of vero cells in 96 well tissue culture plate, the virus of various dilutions (10^{-2} – 10^{-4}) was added to the cells in triplicate and incubated in CO₂ incubator at 37°C overnight. Alternatively, NIM-76/neem oil was added to the vero cells after virus has been adsorbed (after 1 h of virus addition) and incubations were carried out over night. The cells were then washed three times with sterile Hanks Balance Salt Solution (HBSS). Later 200 µl of DMEM with 10% foetal bovine serum containing 0.04% neutral red was added to the wells and the cells were incubated at 37°C for 1 h. After incubation, the cells were washed with HBSS three times. The cells were then lysed using ethanol:acetic acid (50:1) solution to release the neutral red taken up by the live cells and the colour was measured at 570 nm by Dynatech ELISA reader. The amount of neutral red taken is directly proportional to the number of viable cells and inversely to the virus titre.

2.3. In-vivo antifungal activity

Systemic candidiasis was induced in Swiss albino mice ($n = 10$) by administering 10^5 cells per ml of the culture suspensions of *C. albicans* (0.2

ml) intravenously through the tail vein. The test compound (NIM-76) was administered orally as solution in polyethylene glycol-200 (PEG-200) at the dose level of 60 mg/kg, twice daily for 7 days with the help of a gastric canula. The positive control ketoconazole (60 mg/kg) and solvent control (PEG-200) were maintained throughout the experiment. The animals were observed for morbidity and mortality for 16 consecutive days and the total leukocyte counts (TLC) and differential leukocyte counts (DLC) were performed using a haemocytometer.

The mice were killed at the end of day 16 by cervical dislocation and dissected immediately. The kidneys, lungs and liver were removed and homogenised in 5 ml of sterile saline. The number of viable units of *C. albicans* (c.f.u) in each homogenate was determined by plate counts on Sabouraud's dextrose agar (SDA) containing 0.05 mg/ml of chloramphenicol and the results were recorded after 24 h incubation at 37°C.

2.4. Experimental vaginal candidosis

The mice were brought to pseudoestrous by injecting 0.2 ml of oestrodioldipropionate (2.5 mg/ml), sub-cutaneously for 4 days. On the fifth day, all the animals were inoculated vaginally with 10^5 – 10^6 cells of *C. albicans* in 0.1 ml of sterile saline. NIM-76 was applied intra-vaginally as 2% and 4% mixture in polyethylene glycol 200 (PEG 200), twice daily, for 9 days, 24 h after the inoculation of vagina with *C. albicans*. Samples of vaginal scrapings were taken with a wire loop on days 3, 6 and 9 and were suspended aseptically into 10 ml of sterile normal saline. Tenfold dilution of this suspension is then plated on SDA containing 0.05mg/ml Chloramphenicol and incubated for 2 days at 37°C. The number of colony forming units were determined and scored to assess the intensity of infection. Clotrimazole 2% solution was used as positive control and a solvent control (PEG 200) was also maintained throughout the experiment.

All the experiments were carried out in duplicate on two different occasions and Student's *t*-test was used for statistical analysis.

3. Results

3.1. Antibacterial and antifungal activity of NIM-76

The result indicate that NIM-76 inhibited all the microbes tested more effectively than neem oil especially *E. coli* and *Pseudomonas aeruginosa* which are not inhibited by neem oil even at 15 mg/ml. In general, gram positive bacteria were more sensitive than gram negative bacteria. Although the MIC of NIM-76 for various microbes was 0.25–2.0 mg/ml, the MCC was achieved at much higher concentration i.e. 0.6–5.0 mg/ml (Table 1).

3.2. Antiviral activity

The antiviral activity of NIM-76 and neem oil was tested against polio virus (vaccine strain) in vero cell line. Crude neem oil did not inhibit the viral multiplication at all concentrations tested. In-contrast, NIM-76 inhibited the viral multiplication appreciably as revealed by enhanced neutral red uptake by vero cells (Fig. 1). However, NIM-76 did not inhibit the virus once the absorption is initiated, i.e. after 1 h of virus addition to the cells.

3.3. In-vivo antifungal activity

The studies with experimental systemic candidiasis in mice revealed that NIM-76 at a dose of 60 mg/kg was able to effectively control systemic candidiasis in mice and was comparable to that of the ketoconazole (anti-fungal drug) which served as positive control. This is revealed by the low percentage of mortality in NIM-76 treated animals (30% in NIM-76, 40% in ketoconazole) compared with 60% mortality in control group. There was an insignificant increase in total leukocyte counts in NIM-76 treated group compared to the control group (Table 2). Further, the data of colony forming units obtained from various tissue homogenates revealed that NIM-76 reduced the fungal growth significantly when compared with the untreated group indicating its strong antimicrobial action (Fig. 2).

Table 1
In-vitro anti-microbial activity of NIM-76 vis-a-vis neem oil^a

Organism	NIM-76 (mg/ml)		Neem oil (mg/ml)	
	MIC	MCC	MIC	MCC
Gram negative bacteria				
<i>E. coli</i>	2.0	4.0	NE ^b	NE
<i>S. typhi</i>	1.5	2.5	8.0	12.0
<i>S. dysenteroides</i>	1.5	2.5	10.0	12.0
<i>P. vulgaris</i>	1.0	2.0	8.0	10.0
<i>P. aerogenosa</i>	2.0	5.0	NE	NE
Gram postive bacteria				
<i>S. faecalis</i>	1.0	2.5	6.0	10.0
<i>S. aureus</i>	1.0	1.5	4.5	7.0
Fungus				
<i>C. albicans</i>	0.25	0.60	1.2	2.5

^a Keteconazole was used as a positive control for *C. albicans* (MIC value 50 µg/ml) and ciprofloxacin (10 µg/ml) as positive control for bacterial strains.

^b NE; not effective even at 15 mg/ml.

3.4. Vaginal candidosis

In control and solvent treated groups, there is a progressive increase in the number of colony forming units of *C. albicans* with time. NIM-76, at both 2 and 4% concentrations effectively inhibited the growth of *C. albicans* from the 3rd day itself and its activity is comparable to that of clotrimazole (Fig. 3).

4. Discussion

Neem has been reported to have antibacterial and antifungal effect. It has been shown to be active against pathogenic bacteria such as *Staphylococcus aureus* and *Salmonella typhi* (Chopra et al., 1956; Patel and Trivedi, 1962) and against various pathogenic fungi belonging to the genera *Trichophyton*, *Epidermophyton*, *Microsporum*, *Geotrichium* and *Candida* (Khan and Wassilew, 1987). In-addition, neem leaf extract was found to be active against a number of viruses such as small pox, chicken pox, fowl pox, poliomyelitis, herpes viruses etc (Rao et al., 1969; Kaii-a-Kamb et al., 1992).

The present study aimed at comparing anti-microbial activity of NIM-76 and neem oil. In general, NIM-76 was more active than neem oil against all microbes tested. Further, NIM-76 inhibited the growth of certain pathogens such as *E. coli* and *K. pneumoniae* which were not inhibited by neem oil. Besides antibacterial and anti-fungal activity, NIM-76 appreciably inhibited polio virus replication in vero cells as evidenced by the enhanced uptake of neutral red by the cells infected with NIM-76 treated virus compared to the cells infected with virus alone. The fact that NIM-76

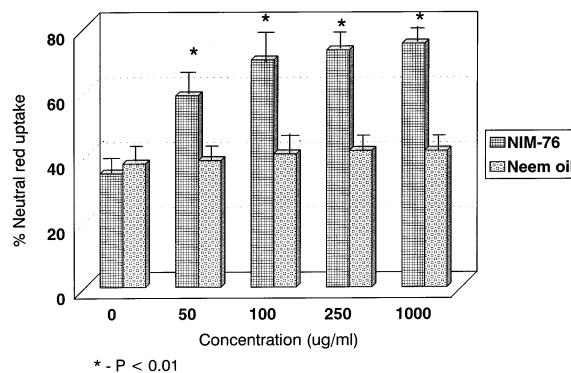


Fig. 1. Effect of NIM-76 and neem oil on polio virus replication in vero cell line.

Table 2

Effect of oral NIM-76 administration on TLC/DLC and mortality of mice during systemic candidiasis

	% Mortality	TLC	DLC	
			PMNs	Lymphocytes
Control	0	8560 ± 660	32 ± 1.6	64 ± 3.6
<i>C. albicans</i> infected	60	9150 ± 710	33 ± 1.8	62 ± 4.5
NIM-76 + <i>C. albicans</i>	30 ^a	9850 ± 750	39 ± 3.6	55 ± 3.5
Ketoconazole + <i>C. albicans</i>	40 ^a	7800 ± 620	30 ± 1.3	70 ± 5.9

^a Significantly different than *C. albicans* infected group. $P < 0.01$.

did not inhibit the viral multiplication when infection is already initiated indicated that the antiviral activity of NIM-76 is due to its direct inactivation of virus rather than inhibiting its replication.

The ability of NIM-76 to prevent the growth of *C. albicans* during systemic candidiasis and vaginal candidosis indicated that NIM-76 is very useful even as a therapeutic agent against candidial and possibly other infections. Although ketoconazole was able to inhibit *C. albicans* at much less concentrations in-vitro (50 µg/ml) than NIM-76 (250 µg/ml), NIM-76 was found to be more effective in-vivo at the same concentration (60 mg/kg) as revealed by % survival and c.f.u. of *C. albicans* in various tissues. We have reported earlier that NIM-76 stimulates cell mediated immunity especially macrophageal activity and lymphocyte proliferative response (Sai Ram et al., 1997). Therefore it is speculated that NIM-76 inhibits the growth of *C. albicans* due to its both anti-microbial and immunomodulatory activity.

NIM-76 isolated in our laboratory has a strong spermicidal activity. Repeated applications of NIM-76 in vagina of rabbits did not result in any significant irritation as evidenced by absence of oedematous thickening of submucosal layer, infiltration of leukocytes, vascular congestion etc (Riar et al., 1990). It kills the sperms by damaging the plasma membrane (Sharma et al., 1996). The ability of NIM-76 to prevent the growth of various pathogens such as *C. albicans* (which is one of the most common causes of vaginitis) and other opportunistic vaginal pathogens such as *E. coli*, makes it highly desirable vaginal contraceptive as it maintains the vaginal health besides serving the purpose of contraception in human beings.

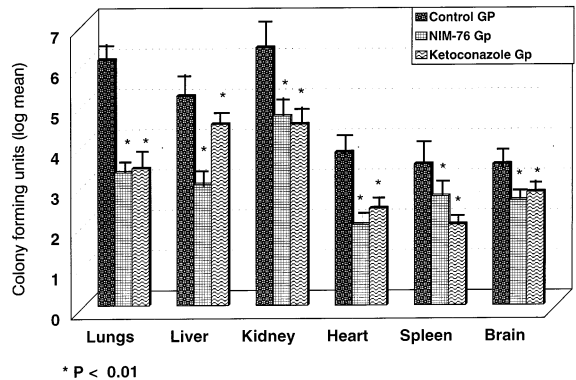


Fig. 2. Effect of NIM-76 on the growth of *C. albicans* in various tissues (ketoconazole was used as positive control).

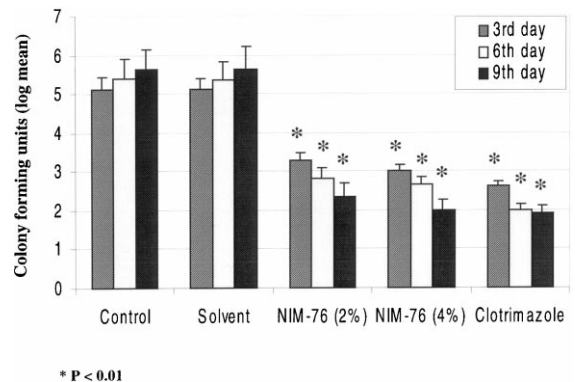


Fig. 3. Effect of NIM-76 on vaginal candidosis (clotrimazole was used as positive control).

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References

- Binde, N.K., Mehta, D.J., Lewis, R.J., 1958. Diuretic action of sodium nimbinate. *Indian Journal Medical Sciences* 12, 141–145.
- Chopra, R.N., Nayar, S.L., Chopra, J.L., 1956. *Glossary of Indian Medicinal Plants*. CSIR, New Delhi, India, pp. 31–32.
- Dhawan, B.N., Ratnaik, G.K., 1993. Pharmacological studies for therapeutic potential. In: Randhawa, N.S., Parmar, B.S. (Eds.), *Neem, Research and Development*. Society of Pesticide Science, India, pp. 242–249.
- Fuziwar, T., Takeda, T., Ogiwara, Y., Shimizu, M., Nornuru, T., Tomida, Y., 1982. Studies on the structure of polysaccharides from the bark of *Melia azadirachta*. *Chemical Pharmaceutical Bulletin* 30, 4025–4030.
- Irobi, O.N., Moo-Young, M., Anderson, W.A., 1996. Anti-microbial activity of Annatto (*Bixa orellana*) extract. *International Journal of Pharmacognosy* 34, 87–90.
- Kaii-a-Kamb, M., Amoral, M., Girre, L., 1992. Search for new anti-viral agents of plant origin. *Pharmacology Acta Helvetica* 67, 130–147.
- Khan, M., Wassilew, S.W., 1987. The effect of raw material from the neem tree, neem oil and neem extract. In: Schumutterer, H., Ascher, K.R.S. (Eds.), *Natural pesticides from the neem tree (Azadirachta indica A. Juss.) and other tropical plants*. GTZ, Eschborn, Germany, pp. 645–650.
- Kusamran, W.R., Tepsuwan, A., Kupradinun, P., 1998. Antimutagenic and anticarcinogenic potentials of some Thai vegetables. *Mutation Research* 402, 247–258.
- Murthy, K.S., Rao, D.N., Rao, D.K., Muthy, L.B.S., 1978. A preliminary study on hypoglycaemic and anti-hyperglycaemic effects of *Azadirachta indica*. *Indian Journal Pharmacology* 10, 247–250.
- OKpanyi, S.N., Ezeukwa, G.C., 1981. Anti-inflammatory and antipyretic activities of *Azadirachta indica*. *Planta Medica* 56, 111–115.
- Patel, R.P., Trivedi, B.M., 1962. In-vitro bacterial activity of some medicinal plants. *Indian Journal Medical Research* 5, 218–222.
- Rao, A.R., Kumar, S.S.V., Paramasivam, T.B., Kamalakshi, S., Parashuraman, A.R., Shanta, B., 1969. Study of antiviral activities of leaves of margosa tree on vaccinia and variola virus, a preliminary report. *Indian Journal Medical Research* 57, 495–502.
- Riar, S.S., Bardhan, J., Thomas, P., Kain, A.K., Prasad, R., 1984. Mechanism of action of anti-fertility action of neem oil. *Indian Journal Medical Research* 88, 339–342.
- Riar, S.S., Deva Kumar, C., Ilavazhagan, G., Bardhan, J., Kain, A.K., Thomas, P., et al., 1990. Volatile fraction of neem oil as a spermicide. *Contraception* 42, 479–487.
- Riar, S.S., Devakumar, C., Sawhney, R.C., Ilavazhagan, G., Kain, A.K., Bardhan, J., et al., 1991. Anti-fertility activity of volatile fraction of neem oil. *Contraception* 44, 319–326.
- Sai Ram, M., Sharma, S.K., Ilavazhagan, G., Devendra Kumar, Selvamurthy, W., 1997. Immunomodulatory effects of neem oil, a volatile fraction from neem oil. *Journal of Ethnopharmacology* 55, 133–139.
- Sharma, S.K., Sai Ram, M., Ilavazhagan, G., Devendra Kumar, Shivaji, S., Selvamurthy, W., 1996. Mechanism of action of NIM-76, a novel vaginal contraceptive from neem oil. *Contraception* 54, 373–378.
- Siddiqui, S., Faizi, S., Siddiqui, B.S., Ghiasuddin, S., 1992. Constituents of *Azadirachta indica*: isolation and structure elucidation of a new antibacterial tetranortriterpenoid, mahmoodin, and a new protolimonoid, naheed. *Journal Natural Products* 55, 303–310.
- Sinha, K.C., Riar, S.S., Tiwary, A.K., Dhawan, A.K., Bardhan, J., Thomas, P., et al., 1984a. Neem oil as a vaginal contraceptive. *Indian Journal Medical Research* 79, 131–136.
- Sinha, K.C., Riar, S.S., Bardhan, J., Thomas, P., Kain, A.K., Jain, R.K., 1984b. Antiimplantation effect of neem oil. *Indian Journal Medical Research* 80, 708–710.
- Udeinya, I.J., 1993. Anti-malarial activity of Nigerian neem leaves. *Transaction Royal Society Tropical Medicine Hygiene* 87 (4), 471.
- Upadhyay, S.N., Dhawan, S., Garg, S., Talwar, G.P., 1992. Immunomodulatory effects of neem. *International Journal Immunopharmacology* 14, 1187–1193.