Original Article

# Development and Evaluation of Novel Aqueous Neem Leaf Extract: A Potent Contraceptive Agent

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## Abstract

In the midst of the global epidemics of both unwanted pregnancies and sexually transmitted infections (STIs), possibilities that provide protection with minimal side effects are ideal. Therefore demand is increasing for vaginal contraceptives which protect from infection too. The spermicidal activity was determined using a modified method of Sander and Cramer. The extract was mixed with sperm suspension containing sperm. The mixture was mounted on the stage of Polarized microscope for 20 s at 100x (oil immersion) and observed for motile sperm. The spermicidal activity of novel aqueous neem extract on rat spermatozoa was same as in case of human spermatozoa. . The MEC of NANE was found to be 2.5 mg/ml and 5 mg/ml in rat and human spermatozoa respectively. The present study describes the simple, one step, novel method of extraction and evaluation of *in-vitro* spermicidal activity in rat spermatozoa and *in-vivo* contraceptive efficacy in female wistar rat. It may be concluded that prolonged and controllable drug delivery of neem constituents from mucoadhesive gel with various loading doses, is achievable and delivered dose in controlled manner for fertility control.

**Keywords:** Contraceptive; Neem leaf extract; Novel method of extraction; *In-vitro spermicidal activity in human spermatozoa.*

## Introduction

The population explosion is a global problem that poses significant threat to the quality of life, more particularly in the underdeveloped and developing countries. The key requirement to combat this grave situation is the availability of suitable contraceptive devices that people would adopt to control birth rate [1]. A number of contraceptive methods are available; however, acceptability of these methods has quite often been limited by their associated untoward side effects, failure rate or irreversibility. This prevailing situation demands the development of newer contraceptive options that should be simple, safe, reversible, cost - effective and, overall, that would be acceptable to the majority of the world population irrespective of culture, religion and race. Of the contraceptive strategies available, the barrier methods are the most commonly used option that has gained recent surge of interest because of its temporal use with possible avoidance of systemic effects, over-the-counter. availability and easy usage [2].Although male or female condoms used correctly and consistently is the only available method shown to be effective in preventing both unwanted pregnancies and ST/HIV infections, women often have little power to negotiate the use of condoms with their partners and are unable to protect themselves from nonconsensual coercive sex. The currently available spermicidal contraceptive formulations are effective, but their repeated use is

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associated with vaginal/cervical irritation or even ulceration and disturbance of the normal vaginal microflora that facilitates microbial infection and renders the subject susceptible to sexually transmitted diseases [3]. Therefore, development of safer spermicidal preparations is, at present, greatly needed. The contemporary trends of drug discovery including reproductive health care emphasize investigation of the terrestrial and marine environments for potent molecules, particularly in developed countries [4]. India, the center of mega-biodiversity with her varied climatic, altitudinal and soil conditions, possesses 3500 medicinal plants [5]. Efforts are being paid to look into the practicability of employing these herbs for fertility regulation.

The neem tree, *Azadirachta indica. A. Juss* (Nimba), is indigenous to the Indian subcontinent and its medicinal properties have been described in Ayurveda. The juice of fresh green leaves of *Azadirachta indica* was believed to suppress "Kam Vasana" (desire for sex). It was consumed by *sanyasees* in shrines and the pupils studying in *Gurukul* for the same purpose. Antifertility activity of *Azadirachta indica* leaves in male mice was studied. Freshly prepared water extract of crushed green leaves of *Azadirachta indica* was orally fed to mice every day for 1 month to study its effect on male reproduction function. It was observed that control mice showed 100% fertility rate. In *Azadirachta indica* treated animals, the antifertility effect was 80%. The study revealed that after 45 days of withdrawal of treatment, the percentage of pregnancies was 100%. Thus, *Azadirachta indica* leaves have shown reversible male antifertility activity [6]. Single Intra uterine neem treatment leads to a long-term pre-implantation block in fertility. This action obviously can not be due to any direct action of neem oil since the oil is cleared from the uterine lumen within 3-4 days, whereas the block of fertility lasts for 3-4 months in rats and 7-12 months in bonnet monkeys. Moreover, circulating progesterone levels and histological examination of the uterus does not indicate any anti-hormonal activity of neem oil [7] .Administration of praneem (purified neem seed extract) orally for six days after confirming pregnancy by the rising levels of CG in the blood, brought about termination of pregnancy; peanut oil given by the same route at the same dose did not show this effect. The effect was reversible and fertility was regained in the cycles subsequent to Praneem treatment. [8]. NIM-76 ( Hydro distillation fraction from neem seed) kills all sperm *in-vitro* in less than 20 set at a concentration of 5 mg and 25 mg/ml for rat and human sperm, respectively. In addition, NIM-76 did not possess abortifacient or antiimplantation activity [9]**.** Immunocontraceptive guided fraction from neem seed was identified to be a mixture of six components, which comprises of saturated, mono and di-unsaturated free fatty acids and their methyl esters. Dose response study was performed with the last active fractions. The antifertility activity with the active fraction was reversible in nature and it was completely active until 5% concentration. There was no systemic toxic effect following the administration of the active fraction [10].

Besides all the above preliminary studies, there is no documented evidence referring to the *in-vivo* contraceptive activity of the leaves extract of *Azadirachta indica* in rats. It was, therefore, our interest to investigate the spermicidal activity of the neem leaves in human spermatozoa and *in-vivo* contraceptive efficacy using Wistar rat with the final objective of using the prepared novel extract as a vaginal contraceptive. Study has been approved by the ethical committee i.e. CPCSEA.

## Materials and Methods

### Materials

#### *Animals*

Wistar rats of proven fertility were purchased from Yash farm, Pune, India, were used for all the experiments.

#### *Plant material*

Dried neem leaf was collected from Kothrud area, Pune, India. The leaf was identified and authenticated in the Department of Botany, Agarkar Research Institute, Pune, India.

#### *Lamb vaginal mucosa*

It was procured from slaughter house, Pune.

#### *Carbopol 934 P*

It was a gift sample from BF Goodrich.

#### *Chemicals and instruments*

Disodium hydrogen phosphate dihydrate, Sodium chloride and Potassium dihydrogen phosphate were obtained from merck ltd., Mumbai (India). Glucose was purchased from Poona Chemical Laboratory, Pune. Microscopy was done by Polarizing microscope: Nikon Eclipse, E600 Pol, Type 120. Micro centrifuge tube and Microtips were purchased from Tarson Pdts Pvt,Ltd., Kolkata, India. For viscometric test of prepared gel, Viscotech Rheometer: Rheologica Instruments.,AB,Lund, Sweden was used. For Mucoadhesion test, Ultra test: Force gauge instrument (Advanced Force Gauge, Mecmesin, West Sussex, England) was used.

### Methods

#### *Preparation of Neem Extract*

Hydrophilic lipid was taken in different proportions with neem leaf powder. Neem leaf powder and lipid mixture was heated in water bath at 50˚C. Then the mixture was stirred homogeneously and cooled.100 ml of water was added to it followed by filtration. filtrate was evaporated to 50˚C at 100 rpm on rotary evaporator to get dry extract. The paste was kept in desiccator till equilibrium moisture content was reached.

***Determination of in-vitro spermicidal activity using human spermatozoa***

***Semen samples***

The experiment was conducted on human sperm. Semen was collected from apparently normal male volunteers, who had 2–3 day's abstinence from sexual activity. Samples having a volume ≥ 2 ml, motility ≥ 50%, sperm concentration ≥20 million/ml, more than 50% with normal morphology, viability ≥ 50%, were used. Semen samples were collected in a dry and clean beaker and stored at 37°C in an incubator for half an hour.

*Test for spermicidal activity*

The spermicidal activity was determined using a modified version of the protocol originally described by Sander and Cramer, which measures the minimum concentration of spermicidal agent required to kill 100% sperm within 20 s. Different ratios of various concentrations of prepared extract were mixed with sperm suspension containing 1 million sperm. The mixture was mounted on the stage of Polarized microscope for 20 s at 100x (oil immersion) and observed for motile sperm. If any motile sperm were seen, the concentration was considered to be insufficient and test was recorded as a "fail." Two-hundred-fifty microliters of Baker's buffer was added to all the mixtures that passed the test and incubated at 37°C for at least 60 min. The solution was slowly vortexed using cyclone mixer and observed again after 60 min for presence of any motile sperm. The concentration at which it was tested was recorded as effective if both tests indicated absence of motile sperm. The end point was the lowest concentration of the extract that caused complete immobilization of all the sperm within 20 s of mixing.

***Determination of in-vivo contraceptive efficacy of NANE on female Wistar rat.***

***Animal husbandry***

Rats were housed in polypropylene cages. Maximum 5 animals were housed in one cage with stainless steel top. Well dried rice husk was used as bedding material with a 14 h light: 10 h dark cycle. Weekly twice change of bedding, cleaning and washing of cages and animal room was carried out. Rats were fed *ad libitum* with pelleted food. Fresh tap water was supplied in cleaned bottles. Standard laboratory environment was maintained.

***Determination of MEC in male Wistar rat***

***Isolation of rat sperm exudates***

Sperms were collected from adult male rats, which have been proven to be fertile, after sexual abstinence period for at least 3 days. The animals were sacrificed by cervical dislocation, and sperms were obtained from caudal epididymides. The epididymides were dissected out of the testis, and the caudal region was identified and pricked carefully with the aid of a sterilized needle (18-G) avoiding any blood vessel puncture. The exudates were collected and suspended in physiological saline. The sperms were allowed to swim up in the medium for 30 min in an incubator at $37^0$C. Cell count was performed using a Neubauers' haemocytometer on polarized microscope (100x oil immersion).

***Determination of spermicidal activity***

The spermicidal activity of novel aqueous neem extract on rat spermatozoa was same as in case of human spermatozoa (2.2.2.2.). The minimum effective concentration (MEC) of NANE (1:0.5) was obtained which was utilized for *in-vivo* contraceptive efficacy.

***Observation for regularity of estrous cycles***

All the procedures have been approved by the Ethical Committee for Animal Research i.e. CPCSCA. During fifteen days, every morning between 9:00 and 10:00 a.m., vaginal secretions were collected with an ear buds dipped with normal saline (Nacl 0.9%) by inserting the bud into the rat vagina, but not deeply. Vaginal fluid was placed on glass slides. A different glass slide was used for each cage of animals. Unstained material was observed under a polarized microscope, without the use of the condenser lens, with 10x and 40x objective lenses. Three types of cells could be recognized: round and nucleated ones are epithelial cells; irregular ones without nucleus are the cornified cells; and the little round ones are the leukocytes. The proportion among them was used for the determination of the estrous cycle phases.

The mean duration of the estrous cycle was 4-5 days for 60%-70% of female rats. However, some rats presented longer regular or irregular cycles. Irregular ones were characterized by keeping in the same phase during 4-5 days. Cycles, in which the alternation among the phases didn't follow the sequence proestrus, estrus, metestrus and diestrus (or intermediates), were also considered irregular [14].

***Intrauterine administration of NANE and control***

Regularly cycling adult Wistar rats (n =12) were subjected to light ether anesthesia on the day of proestrus phase of their cycle. The two uterine horns were gently pulled out through a small midventral abdominal incision. In one (right) uterine horn, 100 µL of NANE of 1:0:5 neem:lipid (MEC x 6 =15mg/ml in physiological saline) was introduced through a tuberculin syringe with a 24-G needle that penetrated through the cervical end (treated horn), while the contra lateral uterine horn received 100 µL of 5 mg/ml of lipid (control horn). The incision was closed by suture, and the animals, maintained in individual cages. There is no mortality due to surgical procedure and animals revived quickly in figure 1.

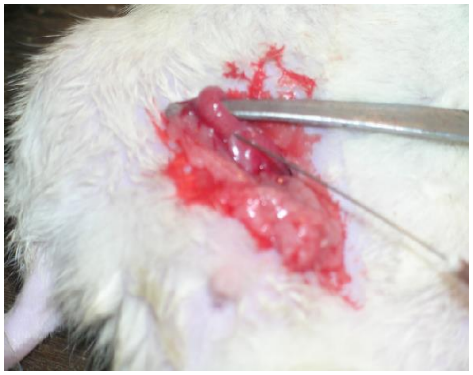

**Fig.1.** Intrauterine administration of NANE in to right uterine horn of regularly cycling female Wistar rat.

### *Mating of animals*

The female showing proestrous during day time undergoes mating at night and ovulation occurs early in the morning. The estrous is a period of desire, only when the female will receive the male. All females (n=12) were exposed to adult male rats that have been proven to be fertile in a 1:1 ratio. The following day, vaginal lavage was examined under microscope to check the presence of sperm.

### *Observations of uterus for implants*

The six rats were sacrificed on Day 8 following mating. The uterine horns were isolated and examined morphologically for the presence of implantation sites.

### *Determination of safety of NANE and reversibility of estrous cycle*

The isolated uterus (n=6) were subjected to histopathology using hematoxyline and eosin staining. Remaining six females were allowed to deliver pups and after delivering pups, they all were again checked for cycle regularity.

### *Formulation of gel*

Plain gels were formulated using various concentration of carbopol 934 P (i.e. 0.5 %, 1 %, 1.5 %, and 2 %) and gel with 5 % of loading dose of NANE was prepared using 2.5 % carbopol. The gel was subjected to viscometric test and mucoadhesive test. Based on the mucoadhesive strength of gel, the best concentration of polymer was decided. The pH of gel was maintained according to vaginal pH i.e. about 4.4.

### *Viscometric test*

The viscometric test was carried out for different concentration of plain gel as well as gel containing NANE. The test was carried out in the stress range of 0.1 Pa to 100 Pa. The flow behavior of the samples were measured by stress rheometer (Rheologica Instruments, AB, Lund, Sweden) using cone-plate geometry with the diameter of cone being 25mm,operating in the oscillation mode with a computer control. The gap was maintained at 0.5 mm and the viscosity was measured at $25^0$c which was maintained using cryostatic bath.

### *Mucoadhesive strength of polymer formulations*

The bioadhesive strength of prepared gels was estimated in terms of maximum adhesion force and work of adhesion shear required to separate the gel from lamb vaginal mucosa [15]. The method developed by Jimenez-Castellanos and co-workers was used [16]. The bioadhesive strength was investigated by using force gauge instrument (Advanced Force Gauge, Mecmesin, West Sussex, England) equipped with a 50 N load cell and metal probe (diameter, 5 mm; length, 15 cm) was then driven at a speed of 50 mm/min. In this study, two pieces of wooden block were bound to external surface of each piece of vaginal mucosa (1.8 cm X 1.8 cm). The lower wooden block was fixed to the flat surface and upper block was fixed to the metal probe. The formulation was adhered to the internal mucosal surface. The mucosa specimen and formulation were maintained in contact for 5 min, and then the probe was raised at a constant rate of 50 mm/min. Stress was thus imposed by linear displacement of the sample until fracture of the adhesive bond occurred, and the adhesion force Vs time profile was monitored. The maximum adhesion force and the area under the curve, which was the work of adhesion shear, were determined as a function of polymer concentration using the method developed by Lejoyeux and co-workers [17].

## Results

*In-vitro spermicidal activity in human spermatozoa*

The spermicidal activity of graded doses of NANE was studied *in-vitro* using human semen. The results of Sander–Cramer test showed potent spermicidal activity of *Azadirachta indica* leaf extract and it is presented in the form of MEC (table 1 and 2). The MEC was calculated in triplicate using the semen sample of three different volunteer (i.e. 50 µl, 65 µl and 71 µl) which indicate the volume of semen that contains one milion sperms. The photomicrograph of sperms is represented in Figure 2.

**Table 1:** Minimum effective concentration (MEC) of various neem leaf extracts.

| Sr No. | Extract Neem: Extracting lipid | Minimum Effective Concentration (Mean of triplicate) (mg/ml) | | |
|---|---|---|---|---|
| | | Gelucire® (44/14) | Gelucire® (50/13) | PEG 6000 |
| 1 | Without lipid(1:0) | 14.0 | 14.0 | 14.0 |
| 2 | 1:0.5 | 5.0 | 12.0 | 19.0 |
| 3 | 1:1 | 4.0 | 9.5 | 18.0 |
| 4 | Plain lipid (0:1) | 25.0 | 30.0 | Not observed |

### *In-vivo contraceptive efficacy of NANE on female Wistar rat*

### *MEC of NANE in male Wistar rat*

From the *in-vitro* spermicidal activity of neem leaf extract, it was concluded that NANE containing neem:lipid (1:0.5) has reasonably good spermicidal activity so this ratio was further evaluated for its spermicidal activity on isolated rat spermatozoa. The MEC for 100 % immobilization of sperms was found to be 2.5 mg/ml.

**Table 2:** MEC of different ratio of Gelucire® 44/14

| Sr No. | Extract<br>Neem: Gelucire® 44/14 | MEC (mg/ml)<br>(Mean of triplicates) |
|---|---|---|
| 1 | 1:0.25 | 8.5 |
| 2 | 1:0.5 | 5.0 |
| 3 | 1:0.75 | 6.5 |
| 4 | 1:1 | 4.0 |

### *Determination of cycle regularity*

Normal cyclic pattern was checked on the basis of vaginal cytology. About 30 female rat was observed for estrous cycle pattern and out of that 12 regularly cycling female rats were chosen for intrauterine administration of NANE.

Cytologically the cycle is divided in to four stages as diestrous, proestrous, estrous and metestrous (figure 3).

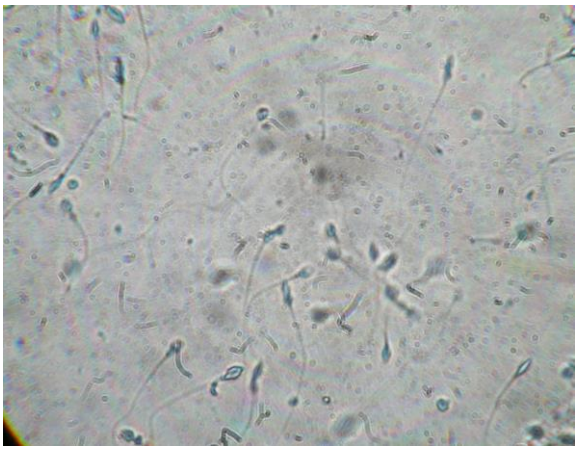

**Fig.2.** Photomicrograph of unstained human spermatozoa at 100x (oil immesrsion)

Diestrous phase is the longest phase characterized by a thick vaginal mucosa and the vaginal smear consists mainly of leucocytes mixed with few epithelial cells as shown in fig. 3 (a,b). The diestrous is also called anoestrous and is the resting asexual period. Proestrous phase is preparing phase preliminary to the next Estrous period, follows diestrous. It is characterized by large number of spherical nucleated epithelial cells in the vaginal smear as shown in fig. 3 (c,d). Cells occur either singly or in sheets. During proestrous phase stratum granulosum develops. The female showing proestrous during day time undergoes mating at night and ovulation occurs early in the morning. In estrous phase, the stratum granulosum of proestrous phase become changed in to statum corneum. The estrous phase is characterized by large number of keratinized epithelial cells as shown in fig. 3 (e,f). The estrous is a period of desire and maximum sexual receptivity, only when the female will receive the male. The last phase is metestrous phase which follows estrous and occur shortly after ovulation. During metestrous the exfoliate cytology of vaginal lumen is characterized by many leukocytes among a few cornified cells and keratinized epithelial cells as shown in fig. 3 (g,h). The time of ovulation is synchronized with estrous period to insure that the ova and sperm will be present at proper time and phase for fertilization.

The phenomenon of estrous is controlled by the estrogenic hormone estradiol 17β. Fertilization is a process of fusion of male and female gametes. Fertility of animal depends upon the co-ordination of many different processes and upon the normal functioning of both male and the female reproductive organs. Many compounds affect the fertility of animals. The time of ovulation is synchronized with estrous period to insure that both ova and sperm will be present at the proper time and phase for fertilization. This synchronization is essential for maintaining a high fertilization rate since the functional life of ovum and of the sperm in the female reproductive tract is limited to a shorter time period after ovulation.

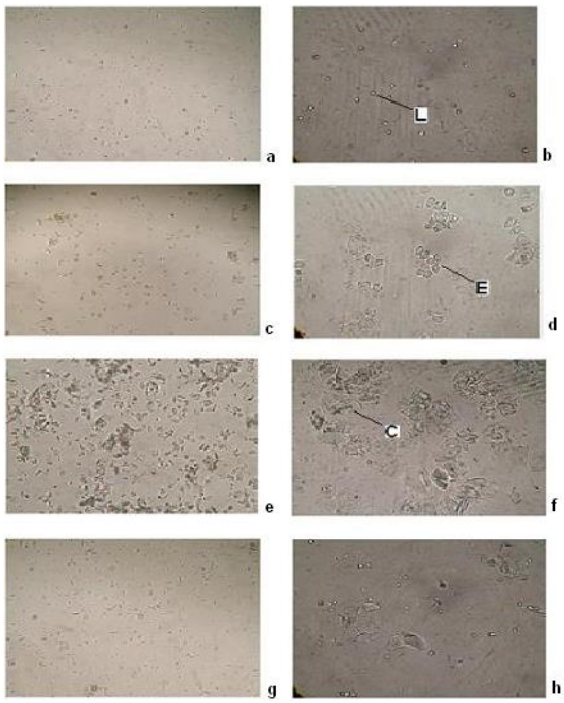


**Fig.3.** Photomicrograph of unstained vaginal smear from female rat at Diestrous (a,b), Proestrous (c,d), Estrous (e,f) and Metestrous (g,h). **Key:** Leucocytes (L), epithelial cells (E) and Cornified cells (C) are indicated. Vaginal smear was observed under polarized microscope at 10x (left panel) and 40 x (right panel) objective lenses.

### *Mating of rats*

After intrauterine administration of NANE (1:0.5) in one horn and control sample in other on the evening of proestrous phase, the animals were mated in 1:1 proportion and the mating was confirmed by presence of sperms in vaginal smear (figure 4) taken on the next morning.

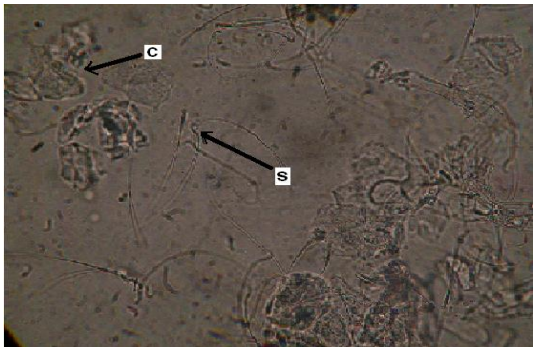

**Fig.4.** Observation of vaginal smear for the presence of sperms at 40 x. Cornified cells(C) and Sperms(S) are indicated.

### *Evaluation of in-vivo contraceptive efficacy*

The presence of sperms in vaginal smear following the mating night was designated day 1 of pregnancy (day 1 post coitum). On 8$^{th}$ day following mating, the uterus was isolated and checked for the presence of implants in both the horn. The horn treated with control shows the presence of implants whereas the horn treated with NANE didn't show the presence of any implants (figure 5).

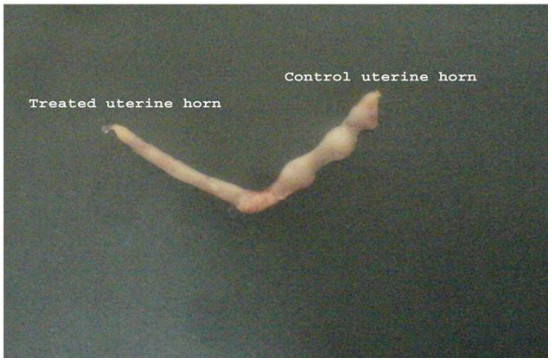


**Fig.5.** Female rat uterus showing 3 implants in control horn whereas no implant in test horn on 8$^{th}$ post-coitus day.

- Table value of $\chi^2$ for (2-1) (2-1) =1 degree of freedom at 5% level is 3.84.
- The calculated value of $\chi^2$ =8.572. So Calculated $\chi^2$ > table $\chi^2$
- So null hypothesis ($H_0$) is rejected. So extract is effective in preventing the unwanted pregnancy.

**Table 3**: No. of implants in treated and control horn

| Sr. No. | No. of implants in Treated horn | No. of implants in control horn |
|---|---|---|
| 1 | 6 | 0 |
| 2 | 5 | 0 |
| 3 | 6 | 1 |
| 4 | 4 | 0 |
| 5 | 3 | 0 |
| 6 | 4 | 0 |

### *Safety and reversibility of estrous cycle*

Histological examination of uterus isolated from six animals showed no inflammation or redness at the site of injection. No occurrence of necrosis was reported. Further, remaining six rats showed regular estrous cycle after delivering normal pups.

### *Viscometry*

Various plain carbopol gels (i.e. 0.5 %,1 %,1.5 %,2 %) and gel containing 5% NANE (1.5 %,2.5 %) was subjected to increasing stress of 0.1 to 100 Pascal (Pa) and strain (Pa. Sec.) was measured at 50 steps figure 6. Gels at all the percentage of carbopol showed Newtonian flow against applied stress. Plain gel containing 2% carbopol is having very high consistency, whereas gel containing 0.5 % carbopol has poor consistency. Although after loading of 5% NANE, consistency became less, 2.5 % carbopol with 5 % NANE had considerable viscosity and good syringability. So it can be reasonably selected for preparation of novel spermicidal gel.

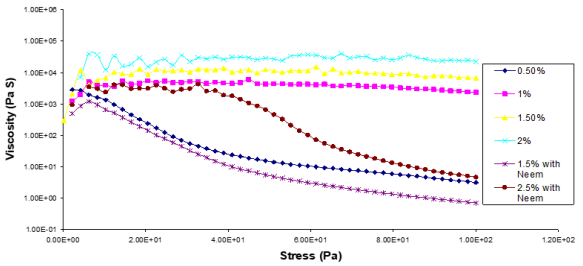


**Fig.6.** Viscometry of various carbopol gel.

### *Mucoadhesive strength of prepared gel*

The mucoadhesive strength of prepared gel was measured on lamb vaginal mucosa. The force of adhesion and work of adhesion shear were taken as a parameter for mucoadhesion. Maximum adhesion force and work of adhesion shear derived from mucin-polymer interaction between gel and lamb vaginal mucosa increased with increasing the carbopol 934P loading in gel. The result of the study (table 4) showed that in the presence of NANE, carbopol retains excellent mucoadhesion though it was required in little higher amount. The relation between work of adhesion shear and concentration of carbopol is shown graphically (figure 7).

**Table 4:** Mucoadhesive strength of various prepared gel.

| Concentration Of carbopol | Max. force of adhesion (in mN) | Avg. force of adhesion (in mN) | Distance of ditachment (in mm) | Work of adhesion shear(AUC) (mN.mm) |
|---|---|---|---|---|
| 0.5% | 830 | 587.42 | 3.1583 | 1877.42 |
| 1% | 960 | 621.62 | 3.8834 | 2446.58 |
| 1.5% | 700 | 603.57 | 5.1250 | 3112.83 |
| 2% | 1200 | 724.26 | 5.5837 | 4076.21 |
| 2.5% (with neem extract) | 1580 | 714.59 | 3.8917 | 2617.63 |

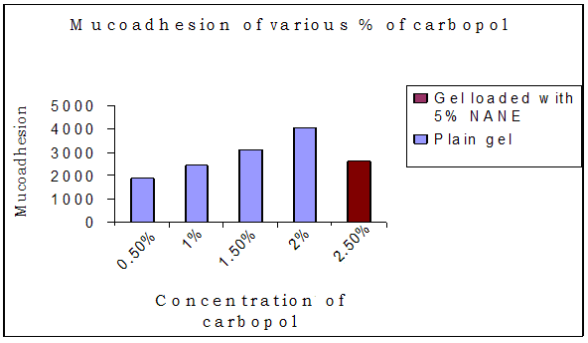


**Fig.7.**Work of adhesion shear of various concentration of carbopol.

## Discussion

With an overall objective to search for spermicidal agent that may effectively serve as the active constituent of vaginal contraceptives, the present investigation evaluated NANE for its sperm-immobilizing and contraceptive efficacy in a series of *in-vitro* and *in-vivo* experiments. The results documented that NANE exerted dose-dependent sperm

immobilizing effects. The MEC of NANE was found to be 2.5 mg/ml and 5 mg/ml in rat and human spermatozoa respectively and the effect was adjudged spermicidal because no revival of sperm motility was demonstrated after the NANE-exposed sperm were incubated in Baker's buffer for 60 minutes.

HIV infections that mainly occur through heterosexual contact challenge the global efforts to control the AIDS epidemic. Prevalence of other STDs is also on the rise. For women, only those techniques preventing contact of semen with the genital tract or methods that inactivate HIV and STD and spermatozoa after ejaculation can prevent both infection and unplanned pregnancies. A desperate need therefore exists for a vaginal contraceptive that would also prevent heterosexual transmission of infections. 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* [18] and against various pathogenic fungi belonging to the genera *Trichophyton*, *Epidermophyton*, *Microsporum*, *Geotrichium* and *Candida* [19]. In addition, NANE was found to be active against a number of viruses such as small pox, chicken pox, fowl pox, poliomyelitis, herpes viruses etc. [20] Moreover, fungal growth, which is generally observed in aqueous decoction of plant materials if exposed to ambient temperature in tropical climate for several days, was not observed in NANE even after exposure for several months. From this observation, it might be contemplated that NANE have antifungal properties which give additional credit to NANE's prospect as a future vaginal contraceptive.

The effectiveness of vaginally delivered spermicidal preparations may be affected by several factors, such as distribution of the preparation in the vagina, its removal or displacement by the coital act and effects of vaginal secretions on the spermicide. These factors cannot be successfully evaluated by a conventional visual observation test, such as the Sander–Cramer method. We therefore undertook some investigations that would test how far the sperm-immobilizing effects of NANE could effectively offer contraceptive effect *in-vivo*. We conducted the tests in rats. Taking into consideration that NANE would become diluted in the uterine or vaginal milieu, we administered NANE at doses five to six times that of *in-vitro* MEC [21].

The rat was used as a model for evaluation of efficacy of a vaginal contraceptive because in this species, mating involves deposition of semen over the cervix, not in the vagina [22]. Since the sperm passes by the vagina to reach the uterus, we administered NANE directly into the uterine horn. We took advantage of the bicornuate uterus of rat wherein we used one uterine horn as control and the other as experimental, while both horns are exposed to identical systemic milieu. It was observed that administration of NANE prevented establishment of pregnancy (as observed on Day 8 post coitus) in the treated side only, while fertilization and implantation occurred unhindered in the control horn. This observation suggests that even in the uterine milieu, NANE could effectively block sperm potential to reach and/or fertilize oocyte.

The prepared NANE was evaluated for any local toxic effect on uterine horn of treated as well as control of six rat which is statistically significant and remaining six rat were subjected to estrous cycle monitoring for further 14-15 days. It was observed that uterine tissues were completely devoid of any local irritation or inflammation. Remaining six rats showed normal estrous cycle.

A carbopol 934P based mucoadhesive drug delivery system developed in this study had a good bio adhesive property to lamb vaginal mucosa which could contribute extended duration of intimate contact with the mucosa and maintain effective drug concentration for a prolonged period of time [23]. It may be concluded that prolonged and controllable drug delivery of neem constituents from mucoadhesive gel with various loading doses, is achievable, and may lead to development of new bio adhesive controlled drug-delivery systems for fertility control.
Until now, neem seed extract or oil or oil components were studied as an effective contraceptive that is hydrophobic in nature. Further, during collection and processing of neem seed, they are contaminated with the aflatoxins so seed extract must be standardized for aflatoxin content. The lyophilized neem leaf extract which is hydrophilic in nature, mixes immediately with water as well as body fluids and kills sperm within 20s [24]. The NANE contains both hydrophilic as well as hydrophobic constituents, so with its use, a more potent vaginal contraceptive may be developed

## Conclusion

The present study describes the simple, one step, novel method of extraction and evaluation of *in-vitro* spermicidal activity in rat spermatozoa and *in-vivo* contraceptive efficacy in female wistar rat. It may be concluded that prolonged and controllable drug delivery of neem constituents from mucoadhesive gel with various loading doses, is achievable and delivered dose in controlled manner for fertility control. Further work needs to be done on anti-microbial activity of NANE to support dual function of NANE as contraceptive as well effective in STIs such as syphilis, gonorrhea and HIV infection.

### Conflict of interest

The authors declare that there are no conflicts of interest.


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