Antifertility potential of *Neem* flower extract on adult female Sprague-Dawley rats

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Abstract

Background: The search for a relatively cheap, widely available, widely accepted and effective contraceptive of plant origin; that is equally non-invasive in administration, non-hormonal in action, non-toxic and that is relatively long-acting, generated our interest in this study (in order to meet the increasing need for population control). The aim of this study was to determine the effects of alcoholic extract of *Neem* flowers on the estrous cycle, ovulation, fertility and foetal morphology of cyclic adult Sprague-Dawley rats.

Materials and Methods: Adult female Sprague-Dawley rats, weighing between 140-180g were used. There were 3 main experimental groups. Group 1 rats received 1 g/kg of alcoholic extract of *Neem* flower by gavage for 3 weeks and the effect on estrous cycle studied. Group 2 rats were administered 1 g/kg of *Neem* flower alcoholic extract at 9 a.m. and at 6 p.m. on proestrus and the effect on the number of ova shed on the morning of estrus observed. Rats in Group 3 were treated with 1 g/kg of alcoholic extract of *Neem* flower on days 1 to 5 *postcoitum*, and observation was made for anti-implantion / abortifacient effects and possible teratogenic effects on the foetuses. All the groups were control-matched.

Results: The estrous cycle of 80% of the rats was altered with a marked prolongation of the diestrus phase. *Neem* flower caused a statistically significant ($p < 0.05$) reduction in the number of ova shed in the morning of estrus in rats fed with the extract at 9 a.m. on proestrus. Neither anti-implantion / abortifacient nor teratogenic effect was observed in the rats treated with *Neem* flower.

Conclusion: Administration of alcoholic extract of *Neem* flower disrupted the estrous cycle in Sprague-Dawley rats and caused a partial block in ovulation and thus has the potential of being developed into a female contraceptive.

Keywords: *Neem* Flower, Ovulation, Estrous cycle, Fertility

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Introduction

Medicinal plants have increasingly become an integral part of the human society in combating various diseases, ranging from skin infection to gastrointestinal problems, since the dawn of civilization. The *Neem* tree (*Azadirachta indica* A. Juss) is one such medicinal plant, and symbolizes all that is wondrous in nature: for every part of the tree has been used as traditional medicine for household remedy against various human ailments from antiquity. In fact, it is considered to be the "village pharmacy" in many parts of India and has played a key role in Ayurvedic medicine and agriculture since time immemorial. It is a large evergreen tree growing 10 to 11 meters tall. The leaves are divided into numerous leaflets, each resembling a full-grown leaf[1-6]. The *Neem* tree flowers from the month of January through April in the Northern hemisphere. The flowers are pentamerous, small, whitish-pink and are borne on axillary cymose panicles. Flower buds open in the afternoon and evening producing a strong scent at night. The approximately 5 mm-long flowers have a sweet jasmine-like fragrance and produce ample quantities of nectar[7-9]. In traditional Ayurveda medicine a decoction made from the bark, leaf, root, fruits and flowers is used in the treatment of blood morbidity, biliary afflictions, itching, skin and peptic ulcers[10]. The bitter, astringent bark is applied as a decoction for haemorrhoids. The leaves are steeped for malaria. *Neem* juice (expressed from the leaves), infusion, or ointment is applied externally to wounds and carbuncles. The twigs are used to clean the teeth, firming up the gums and preventing gum disease. *Neem* oil, expressed from the seeds is commonly used for hair dressing and is believed to be strongly antifungal and antiviral. *Neem* oil has been used to treat leprosy and serves as a vehicle for other active ingredients[10].

Purohit and Daradka[11] reported that *Neem* flowers caused hypolipidaemic effects when administered on rabbits. Dietary *Neem* flowers caused a marked increase in glutathione S-transferase activity in the liver[12] and also possess chemopreventive potential on mammary and liver carcinogenesis[13]. 4 prenylated flavanones isolated from methanol extract of *Neem* flowers have been reported as potent antimutagens against heterocyclic amines[14]. Numerous investigators have reported that *Neem* leaves, bark, seeds and oils possess antifertility properties[15-20].

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However, no study has reported the use of *Neem* flower as an antifertility agent. In addition, it has been observed that during the rainy seasons (which also coincide with the period when the *Neem* tree flowers) the environment is usually littered by these flowers. This study was carried out with the overall aim of developing a female contraceptive that is cheap, non-hormonal, accessible and acceptable to most women in our environment. The specific objectives include: investigation of the effects of alcoholic *Neem* flower extracts on estrous cycle, ovulation; and determination of possible abortifacient and teratogenic effects of the extract in Sprague-Dawley rats. The outcome of such a study could also be of economic importance to the local community instead of the flowers merely littering the environment especially during the rainy season.

It is probable that an oral herbal contraceptive would allow couples control their fertility, which might in turn likely increase the number of couples practicing family planning. Other advantages of such a contraceptive would include the familiarity rural people have with herbal medicines, the probable fewer side effects associated with herbal preparations, their ready availability from local sources, and protection of privacy.

Materials and Methods

Animals

A total of 40 adult female Sprague-Dawley rats weighing between 140-180g were used for this experiment. They were procured from the Animal House of the College of Medicine, University of Lagos. The animals were housed in standard cages, five per cage, in a controlled temperature room (28°C), with a 12 h light: 12 h dark cycle, lights on at 6:00 a.m., in the Animal room of the Department of Anatomy, College of Medicine University of Lagos. Standard laboratory chow (obtained from Ladokun Feed Limited) and tap water were available *ad libitum*, and the animals were weighed daily. Vaginal smears were taken daily, and only animals displaying at least two consecutive 4-day estrous cycles were used. All animals were observed for clinical signs of drug toxicity (such as tremors, weakness, refusal of feeds, diarrhea, weight loss, hair-loss, coma and death) throughout the duration of the experiment. All procedures involving animals in this study conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals[21] and were approved by the Departmental Committee on the Use and Care of Animals.

Extract

The *Neem* tree on the main Campus of University of Lagos, Nigeria, was identified and authenticated by Professor J. D. Olowokudejo of the department of Botany of the University. Voucher specimen was deposited (accession no 636) in the herbarium of the Department of Botany. Fresh flowers were obtained from the same tree in the month of January, air-dried and reduced to coarse powder. The powdered plant material obtained was subjected to alcoholic extraction in the Pharmacognosy Department of the Faculty of Pharmacy, University of Lagos. Briefly, 50g of the crushed *Neem* flowers was mixed with alcohol and placed in the Soshlet apparatus. The mixture was heated at 60°C and the extract was obtained by distillation. The powder obtained (8.5 g, 17.0% yield) was stored at 4 °C before use and all dilutions of the extract were made in distilled water.

Effect on estrous cycle

The rats in this experiment were divided into two subgroups (1a and 1b) of 5 rats each. Rats in subgroup 1a received 1 g/kg body weight (22) of extract by gavage for 21 days, while those in subgroup 1b served as controls and received equivalent volume of distilled water. The four stages of the estrous cycle were defined by using the vaginal smear method. Vaginal smears were collected daily using a small suction pipette and normal saline (0.9% NaCl, w/v) between 9 a.m. and 10 a.m. The smear was placed on slide and examined using the light microscope. Rats exhibiting a 4-stage and 4-day estrous cycle of proestrus-estrus-metestrus-diestrus were classified as normal while any deviation from this pattern in terms of duration and sequence was categorized as abnormal.

Effect on ovulation

A total of 20 rats divided into 4 subgroups (2a - 2d) of 5 rats, each with a 4-day estrous cycle observed over a period of two weeks were used for this experiment. The effect of *Neem* flower alcoholic extract was observed on the number of ova shed with respect to the time of day the extract was administered. Rats in subgroup 2a received 1 mg/kg body weight of *Neem* flower alcoholic extract orally at 9 a.m. on proestrous. Subgroup 2b rats received 1 mg/kg body weight of *Neem* flower alcoholic extract orally at 6 p.m. on proestrous. Rats in subgroups 2c and 2d served as controls and received equivalent volume of distilled water at 9 a.m. and 6 p.m. respectively on proestrous. The rats were sacrificed the next day on estrous using chloroform anesthesia. At autopsy the oviducts of each rat were excised, placed between microscope slides, and examined at a magnification of

100× for the presence of ova (oocytes). Any ova that were found were counted.

Effects on the foetus

The third group of rats was randomly divided into two subgroups (groups 3a and 3b) of 5 rats each, comprising the treated (group 3a) and controls (group 3b). Group 3a rats had 1 mg/kg body weight of *Neem* flower alcoholic extract by gavage from day 1 to 5 post coitum to observe for possible abortifacient and teratogenic effects, while those in group 3b had equal volume of distilled water. The rats were placed in the cages of proven male breeders at a time between 2.00 and 4.00 p.m. on proestrus and left with the males until 10.00 a.m. the following day. Each female was checked on the day of estrus and the presence of one or more vaginal plugs or the presence of sperm in the vaginal smear or in the uterus was used as evidence that an animal had mated. A positive sperm plug was taken as the day 1 of pregnancy. After 20 days all the rats in each subgroup were sacrificed using chloroform. The number of foetuses were counted; weighed; sites of foetal resorption (if any) were recorded; placenti were weighed; the umbilical cord and crown-rump length were measured and foetuses were examined for any gross abnormalities.

Statistics

Results were expressed as means ± standard deviation (SD) and subjected to statistical analysis using one-way analysis of variance (ANOVA) and the Scheffe's post-hoc test. The significance level considered was $p < 0.05$.

Results

General effects

All rats fed with *Neem* had diarrhea, and there was a significant ($p = 0.025$) 6.46% reduction in the body weight of the rats treated with *Neem* flower alcoholic extract, while a 7.21% increase in body weight of the control rats was noted (Table 1).

Table 1: Effect of *Neem* flower alcoholic extract on the body weight of female Sprague-Dawley rats

T reatment group n = 40	Body weight (g)			
	Before Experiment	After Experiment	Weight Difference	%Weight Difference
Neem (n = 20)	174.34 ± 21.11[a]	163.08 ± 22.64	-11.26[b]	6.46
Control (DW) (n = 20)	170.28 ± 18.39	182.56 ± 18.74	12.28[b]	7.21

a: Mean ± SD

b: $p = 0.025$ (p value with respect to before the experiment)

Sprague-Dawley rats were weighed before and after being fed with alcoholic *Neem* flower extracts and distilled water (DW) for 21 days.

Effect on estrous cycle

Administration of 1 g/kg body weight of *Neem* flower alcoholic extract produced an irregular pattern in 80% of the rats (Table 2). These rats showed a prolonged diestrus pattern in each cycle.

Table 2: Effect of *Neem* flower alcoholic extract on the estrus cycle of Sprague-Dawley rats.

Treatment group n = 10	Estrous cycle			
	Normal		Irregular	
	N	%	*n*	%
Neem (*n* = 5)	1	20	4	80[b]
Control (DW) (*n* = 5)	0	100	0	0

b: $p = 0.001$ (p value with respect to the group that received distilled water)

The regularity of estrous cycle of Sprague-Dawley rats was assessed before and after being fed with alcoholic *Neem* flower extracts and distilled water (DW) for 21 days.

Effect on ovulation

Neem flower alcoholic extract administered at 9 a.m. on proestrus produced a statistically significant reduction ($p = 0.025$) in the number of ova shed in the oviduct in the morning of estrus when compared with the group administered distilled water. There was however no statistically significant difference in the number of ova shed between the rats treated with *Neem* flower alcoholic extract at 6 p.m. and the controls (Table 3).

Table 3: Effect of *Neem* flower alcoholic extract administered at 9 a.m. and at 6 p.m. proestrus on the number of ova shed on the morning of estrus.

Treatment group n = 20	Number of ova shed
Neem (9 a.m.) ($n = 5$)	6.60 ± 3.21^{ab}
Neem (6 p.m.) ($n = 5$)	12.80 ± 1.79
Control (Distilled Water) (9 a.m.) ($n = 5$)	13.50 ± 1.23
Control (Distilled Water) (6 p.m.) ($n = 5$)	13.60 ± 1.14

a: Mean ± SD

b: $p = 0.025$ (p value with respect to the group that received distilled water at the same time the extract was administered). The number of ova shed by Sprague-Dawley rats were enumerated after being fed with single doses of alcoholic *Neem* flower extracts and distilled water on proestrous.

Effects on the foetus

During and after the administration of the extract, there was no vaginal bleeding. Our study showed that *Neem* flower had no effect on implantation. No resorption site was observed. All foetuses were implanted and viable. There was no statistically significant difference in the number of foetuses, weight of foetuses, crown-rump lengths, umbilical cord lengths and weight of the foetal placenta of the *Neem*-treated and control rats. No gross external abnormality was observed (Table 4).

Table 4: Effects of *Neem* flower alcoholic extract on pregnancy and foetal parameters

Treatment group n = 10	Foetal parameters				
	Number of foetuses	Crown-rump length (cm)	Umbilical cord length (cm)	Foetal weight (g)	Placental weight (g)
Neem ($n = 5$)	6.75 ± 2.06^{a}	2.91±0.10	2.62 ± 0.36	2.82±0.10	0.55±0.10
Control (DW) ($n = 5$)	7.00±2.71	3.47±0.14	3.12±0.33	3.95±1.71	0.61±0.26

a: Mean ± SD

The fetuses and foetal parameters of Sprague-Dawley rats were assessed after pregnant rats were fed with alcoholic *Neem* flower extracts and distilled water (DW) for the first 5 days of gestation.

Discussion

Our study demonstrated that alcoholic extract of *Neem* flowers alters the estrous cycle, by prolonging the duration of the diestrus phase and subsequently lowering the frequency at which the estrus phase occurs. Consequently the frequency of ovulation is reduced and fertility may therefore be impaired. Our findings are in concert with those of some investigators in other parts of the world who have reported the antifertility property of *Neem* leaves, bark, seeds and oils[15-20]. However, our observations are in contrast to those of Upadhyay *et al.*, [17] who reported that *Neem* oil had no effect on ovarian function. The significant reduction in the number of normal follicles in the rats administered *Neem* flower alcoholic extract at 9 a.m. on proestrus in our study may have been due to disruption of the process of follicle selection due to atresia. Follicular growth is regulated by endocrine (follicle stimulating hormone, luteinizing hormone and prolactin) and local (paracrine and autocrine) factors. The latter include steroid hormones (such as progestins, estrogens and androgens) produced

by different cell types of the ovary and various non-steroidal regulators (such as oocyte maturation inhibitor, luteinization stimulator, luteinization inhibitor, follicle stimulating hormone inhibitor, insulin-like growth factors, transforming growth factors, epidermal growth factor, platelet-derived growth factor, inhibin and activin) [23-25].

Ovulation, the result of follicular growth, is a complex, multistep process that is triggered, in cycling rats, by the preovulatory luteinizing hormone surge on the evening of proestrus. This rapid surge of luteinizing hormone begins at about 2-3 p.m. on proestrus and ultimately reaches peak level at 5-7 p.m. on the same evening. Two investigators[26, 27] reported that the administration of chloroquine and sodium pentobarbital at 9 a.m. on proestrus blocked ovulation completely but when administered at 6 p.m. had no effect on ovulation. Gbotolorun *et al.*, [22] reported a partial block in ovulation at 9 a.m. and no effect on ovulation at 6 p.m. with the administration of *Neem* seeds. We observed a similar pattern with the use of *Neem* flower extract to the results obtained by these authors, suggesting a similar mechanism of blocking the rise in luteinizing hormone during early proestrus. Several reports cited in literature on the antifertility effect of *Neem* showed anti-implantation / abortifacient effect on rodents if administered early from day 2 to 7 postcoitum [28-30]. Also praneem (purified *Neem* extract) given orally from day 8 to 10 postcoitum resulted in complete resorption of embryos[31]. Their findings however are at variance with our present investigation in which we observed on autopsy on the 20th day, that there were no resorption sites; all the foetuses were alive and there were no gross external malformation. Analysis also revealed no statistically significant difference in the parameters (number of foetuses, crown-rump length, umbilical cord length, foetal and placental weight) compared in the foetuses of the treated and control rats. These differences observed between our findings and those of other authors could be attributed to the fact that there is variability in *Neem* with respect to azadirachtin content [32]. However, further studies need to be carried out to determine the azadirachtin content of the species that we used in order to substantiate our hypothesis.

In our present study, though we observed some level of toxicity in the treated rats evident by the diarrhea suffered by the rats, however we recorded no deaths. These observations are somewhat in contrast to previously reported findings that recorded both toxicity and mortality rates. Sadre *et al.*, reported the toxicity effect of *Neem* leaves on guinea pigs and rabbits with a mortality rate of 74.9% and 90% respectively[20] while Gbotolorun *et al.*, reported the toxicity effect of *Neem* seed on rats with a mortality rate of 40%[22].

Conclusions

Administration of alcoholic extract of *Neem* flower disrupts the estrous cycle in Sprague-Dawley rats and causes a partial block in ovulation and has the potential of an ideal antifertility agent. Further studies are needed in both primates and humans, to find out if *Neem* flower will have similar effects observed in the rodents.

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