Effect of *Azadirachta Indica* (neem) leaf aqueous extract on the functional parameters of human spermatozoa *in vitro*

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Summary

The neem leaf extract (NLE) has been reported to exhibit spermicidal and oocidal activities under *in vitro* cultured conditions but the effect of NLE on various sperm function parameters have not been studied. Therefore, in the present study we have assessed effect of different concentrations of NLE on various sperm functions characteristics to develop a water-based vaginal contraceptive. The human sperm (10 million sperm/mL), were exposed to different concentrations of NLE (3, 9, 15, and 30mg/mL) for 20 seconds at 37°C. Thereafter, sperm function characteristics such as motility, viability, hypo-osmotic swelling (HOS), nuclear decondensation (NDC), acrosome intactness (AI) and deoxyribonucleic acid (DNA) stability were analyzed. A dose-dependent inhibition of sperm motility was observed, while 15mg/mL dose immobilized 100% of spermatozoa. Whereas other sperm functional parameters like viability, DNA intactness, HOS, NDC and AI decreased from 94% to 9%, 71% to 29%, 77% to 25%, 62% to 29% and 53% to 25%, respectively. The data obtained in the present study reveal that treatment of NLE causes reduction in functional parameters to infertility range. Therefore, a preparation containing 15mg of NLE in 150 µl of 0.9% NaCl was tested in rat as a vaginal contraceptive, and the data revealed 100% efficacy in mating studies after 21 days of vaginal application. The findings indicate the potential use of NLE for developing water-based vaginal contraceptive.

Key words : *Azadirachta indica*, neem leaf extract (NLE), sperm function tests, DNA stability, vaginal contraceptive

Introduction

For contraceptive development many approaches, such as barrier, immunological and hormonal, were explored. Recently, considerable emphasis has been given to validate products of indigenous medicinal plants scientifically for their use in humans, using good study design with modern tools and techniques.

Neem plant (*Azadirachta indica*) has been extensively used in the Ayurvedic system of medicine for a long time. Various parts of this plant are used for the treatment of various diseases. The neem bark aqueous extract is reported to have therapeutic potential for controlling gastric hyper-secretion and gastro-duodenal ulcer (Bandopadhyay et al., 2004). A dental gel formulation containing neem extract has been reported to reduce oral infections, plaque index and bacterial count (Pai et al., 2004a; 2004b). The medicinal utilities have been described especially for neem leaf (Subapriya and Nagini, 2005). Neem leaf and its constituents are reported to exhibit immunomodulatory, anti-imflammatory, antihyperglycemic, antiulcer, antifungal, antibacterial, antimutagenic, anticarcinogenic, nematicidal, antimalarial, antiviral, insecticidal and antioxidant properties (Sharma et al., 2003; Wanderscheer et al., 2004; Udeinya et al., 2004; Siddiqui et al., 2004; Subapriya and Nagini, 2005; Sithisaran et al., 2005).

The role of neem products in male fertility regulation has been well studied. The ethereal extract of neem stem bark induced reversible reproductive endocrine malfunction in male rats (Raji et al., 2003). The ethanolic neem leaf extract (NLE) has been reported to induce abnormal head morphology and reduce mean sperm count in murine (Khan and Awasthi, 2003). The chloroform extract of neem oil inhibited spermatogenesis and sperm motility in mice (Yin et al., 2004). A single intra-vas administration of neem oil causes infertility in male rats' up to eight months without changing blood testosterone level and any inflammatory / obstructive changes in epididymis and vas deferens (Upadhyay et al., 1993). Volatile fraction of neem oil (NIM-76) inhibited spermatozoal activity *in vitro* and *in vivo* in rats, rabbits, monkeys and humans (Riar et al., 1990, 1991; Sharma et al., 1996). *In vivo* study in male rats with fresh green leaf extract caused reversible antifertility (Deshpande et al., 1980), while powdered neem leaves caused histological and biochemical changes in the testis (Joshi et al., 1996; Aladakatti et al., 2001). Aqueous leaf extract of *Azadirachta indica* caused reversible alterations in the male reproductive organs of Parkers strain of mice (Mishra and Singh, 2005). NLE is also reported to inhibit motility and viability of human spermatozoa treated *in vitro* (Khillare and Shrivastav, 2003).

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The neem products have also been studied for the regulation of female fertility. Neem oil is used as a vaginal contraceptive (Upadhyay et al., 1990) and its reversible antifertility effects have been reported in rats and bonnet monkeys (Upadhyay et al., 1990, 1994). A biologically active fraction of neem oil extract that has reversible anti-fertility effect was identified and characterized (Garg et al., 1998). Purified neem seed extract, called praneem, abrogates pregnancies (Mukharjee et al., 1996) and reduces progesterone levels in rats, baboons and monkeys (Talwar et al., 1997). Recently, it has been shown that neem oil inhibits total number of follicles and number of developing follicles in the rat ovary. It was hypothesized that the treatment of neem oil might have inhibited gonadotropin- dependent oocyte growth and maturation pathway, thereby reducing the number of developing follicles, degeneration of egg and inhibition of sperm-egg interaction (Juneja and Williams, 1993; Dhaliwal et al., 1998; Roop et al., 2005). A study by Chaube et al. (2006) indicated that neem leaf aqueous extract induced morphological and biochemical changes associated with apoptosis prior to degeneration of rat oocyte cultured *in vitro*. Another study by Gbotolorun et al. (2008) showed that administration of alocoholic 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. Owolabi et al. (2008) revealed that the methanolic extract of neem leaves may affect fertility adversely by reducing serum levels of LH and, subsequently, the release of ova during ovulation.

Ability of sperm to fertilize the ovum is not only dependent on motility but also on other functional characteristics. Therefore, besides motility, other parameters such as viability (World Health Organizationm, 1999), hypo-osmotic swelling (Jayendran et al., 1994) and nuclear decondensation (Rodriguez et al., 1985) are now being increasingly assessed to predict successful outcome in IVF settings. The present study was thus designed to evaluate the different sperm function characteristics after *in vitro* exposure to NLE with the aim to develop a vaginal contraceptive.

Materials and Methods

All procedures conform to the stipulation of the Institutional Clinical Ethical Committee's norms.

Semen samples

Semen samples were collected from the normal male volunteers with proven fertility, after 2-4 days sexual abstinence. After liquefaction for 30 minutes at 37° C, a basic semen analysis was performed. Samples having the semen volume e" 2 mL, motility e"50%, sperm concentration e" 15 million /mL, normal morphology e" 50%, viability e" 50%, and total leukocyte count < 1 million/mL were used.

Sample preparation

The highly motile spermatozoa were collected by swim-up technique in the Tyrode's solution (Sigma Chemical Co., St. Louis, MO, USA) with 3% BSA. The final sperm concentration was adjusted to 10 million sperm / mL.

Preparation of neem - leaf extract

The neem leaf extract was prepared as described by Khillare and Shrivastav (2003). The various amounts (3, 9, 15, 20 and 30 mg) of lyophilized NLE were added to the sperm suspension containing 10 million/mL and incubated at 37° C for 20 seconds. After 20 seconds of incubation various functional parameters of spermatozoa were checked.

Sperm motility

A drop of sperm suspension was examined at 400x in a phase-contrast microscope and per cent of spermatozoa with rapid linear progressive and sluggish linear progressive (a+b) type of motility was determined at every NLE treated concentration (Jayendran et al., 1994).

Sperm viability

Eosin-nigrosine staining (World Health Organization, 1999) was utilized for determining sperm viability in suspension. In brief, a drop of control or treated sperm suspension was mixed with two drops of 1% eosin Y. After 30 seconds three drops of 10% nigrosin were added and mixed well. A smear on a clean glass slide was made from the mixture and allowed to air-dry. Dead sperm took to pink while live sperm remained bluish fluorescent. The *per cent* live and dead spermatozoa were determined with each concentration of neem leaf extract using a phase-contrast microscope.

Hypo-osmotic swelling test (HOST)

A hypo-osmotic solution was prepared with 0.735% sodium citrate and 1.351% fructose in distilled water (Jayendran et al., 1994). To 1 mL of the pre-warmed hypo-osmotic solution 100 µL of control or treated sperm suspension was added and incubated at 37° C for half an hour. The *per cent* spermatozoon with tail coiling was determined.

Nuclear chromatin decondensation test (NCDT)

Control and treated spermatozoa were pelleted by centrifugation at 400x for 15 minutes and resuspended in 0.05M borate buffer containing dithiothreitol (DTT, 2 mM). After 30 minutes at room temperature, 1% lauryl sulphate (SDS) was added and mixed gently. The mixture was diluted with equal volume of borate buffer (0.05 M) containing 2.5% glutaraldehyde. An aliquot of the mixture was stained with Rose Bengal (0.8%) and was examined under a phase-contrast microscope. The *per cent* of decondensed spermatozoa was determined (Rodriguez et al., 1985).

Acrosome intactness test (AIT)

The AIT was carried out as described by Gopalkrishnan et al. (1995). To perform AIT test glass slides were coated with 5% gelatin and stored at 4° C after fixing in 1% glutaraldehyde in PBS solution for two minutes. The control or treated sperm suspension was diluted 1:20 with PBS-D-Glucose and kept at 37° C for thirty minutes. A drop of control or treated sperm suspension was smeared on gelatin-coated slides at room temperature for 5-10 minutes to evaporate excessive moisture, and thereafter slides were kept in moist petridish at 37° C for 2 hours. Slides were examined in a phase-contrast microscope at 400x. *Per cent* of spermatozoa with halos of the diameter 10 mm around the heads was determined.

DNA stability test

Acridine orange test was carried out as described by Tajeda et al. (1984) to determine the DNA stability. In brief, control and treated spermatozoa were smeared on the clean slides and air-dried for twenty minutes. The smeared slides were dipped in Carnoy Solution (3:1 methanol: glacial acetic acid) for two hours. The slides were then dried for a few minutes at room temperature and stained with 1% aqueous solution of acridine orange containing 0.1M citric acid and 0.3 M disodium hydrogen phosphate for five minutes. Slides were washed with distilled water and observed in a fluorescent microscope using 490 nm excitation filter and 530nm barrier filter. *Per cent* green colored spermatozoa (double-stranded normal DNA) and red colored spermatozoa (single stranded DNA) were determined.

Neem as vaginal contraceptive

Four adult female rats with proven fertility were administered with 15mg of neem extract dissolved in 150 µL of 0.9% NaCl into the vaginal cavity between 10-11 AM daily for 21 days. Equal no of female rats were given 150 µl of saline in a control group. Thereafter, rats in both the groups were kept for cohabitation with adult fertile males daily for five hours during this period. The mating behavior was observed throughout the study period. After the 21st day, the females were monitored for pregnancy.

Statistical analysis

The data are mean ± standard deviation (SD) of six replicates. All percentage data are converted through Arch-sine Square root transformation and then analyzed by one way analysis of variance (one way ANOVA) using SPSS software (11.5 version), Chicago, USA. The significance was tested at 0.05 level.

Results

Effect of NLE on motility of spermatozoa

The NLE treatment inhibited motility of spermatozoa in a dose-dependent manner (one way ANOVA; $F=208.924$, $p<0.0001$), while 15 mg/mL NLE induced 100% spermatozoa to become immotile (Table 1).

Effect of NLE on viability of spermatozoa

The NLE treatment inhibited sperm viability in a dose-dependent manner (one way ANOVA; $F=586.937$, $p<0.0001$), while at 15 mg/mL dose of NLE only 10% viable spermatozoa were observed (Table 1).

Effects of NLE on hypo-osmotic swelling of spermatozoa

The NLE treatment significantly inhibited functional integrity of plasma membrane of human spermatozoa in a dose-dependent manner (one way ANOVA; $F= 107.423$, $p< 0.0001$). However, at 15mg/mL dose of NLE, only thirty five percent spermatozoa showed the functional integrity (Table 1).

Effect of NLE on nuclear chromatin decondensation (NCD) of spermatozoa

The NLE treatment inhibited NCDT in a dose-dependent manner (one way ANOVA; $F= 51.518$, $p< 0.0001$). At 15 mg/mL concentration about 44% spermatozoa showed NCDT suggesting that the NLE does not much affect the chromatin decondensation (Table 1).

Effect of NLE on acrosome intactness of spermatozoa

NLE inhibited acrosome intactness in a dose-dependent manner (one way ANOVA; $F=26.747$, $p< 0.0001$). However, the acrosome intactness at 15mg/mL dose of NLE was reduced to around 37% from initial 53%, suggesting that loss of motility is not related to acrosome intactness of spermatozoa (Table 1).

Table1- Effect of various doses of neem leaf extract on the functional parameters of human spermatozoa after 20 seconds of incubation at 37º C (N=6). Values are expressed as mean (%) ± SD of 4 replicates. Data analyzed by one way ANOVA followed by multiple t-test.

	0mg	3mg	9mg	15mg	20mg	30mg	ANOVA Test
% Motility ± SD	88.5± 8.5	51 ± 3	13 ± 2	0	0	0	F=208.924 p<0.0001
%Viability ± SD	94 ± 4.4	61 ± 2	24 ± 3.6	9.5 ± 1.7	4.5 ± 1.7	0.5 ± 0.9	F=586.937 p<0.0001
% HOST ± SD	77 ± 4	66.5± 3.8	52 ± 2	35 ± 4.7	33 ± 1.5	25 ± 5.7	F=107.423 p< 0.0001
% NDT ± SD	62 ± 2.5	56.5± 1.6	50.5 ± 6.8	44 ± 3	29 ± 4.2	29 ± 3	F=51.518 p< 0.0001
% AIT ± SD	53.5 ± 3	50.5 ± 1	41 ± 4.7	37.5 ± 4.6	32.5 ± 5.5	25.5± 6.8	F=26.747 p< 0.0001
%DNA stability ± SD	71 ± 8.7	51.5± 5.8	40 ± 3.4	29 ± 2.8	27 ± 4	18 ± 2.2	F=58.467 p<0.0001

Effect of NLE on DNA stability of spermatozoa

NLE treatment induced DNA instability in a dose-dependent manner (one way ANOVA; F=58.467, p<0.0001) as evidenced by a reduction in number of green colored spermatozoa (double-stranded). The 15 mg/mL dose of NLE induced reduction in number of green sperm from 70% to 30% suggesting that the NLE acts at the level of DNA (Table 1).

Neem as vaginal contraceptive

The NLE treatment completely inhibited pregnancy rate and none of the treated rats was pregnant during the course of treatment, while control rats had pregnancy rate with an average of (9±2) litters per rat. The mating behavior during the course of treatment was found normal in treated as well as control rats.

Discussion

In the present study, we report specific alteration in sperm function parameters/characteristics after exposure to different doses of neem leaf aqueous extract. There is a dose-dependent reduction in sperm function characteristics like motility, viability, HOST, NDCT, AIT, and DNA stability. The parameter that was affected 100% at 15 mg dose was motility. Sperm, a type of specialized cells, were seen to be susceptible to relatively low doses of neem leaf aqueous extract. Loss of 100% forward and sluggish progressive motility, at the dose of 15 mg, within an hour, was observed. Sperm motility is directly dependent on the ATP supply from the sperm mitochondrial pool. It was reported that reactive oxygen species (ROS) action causes a decrease in the rate of glycolysis by inactivation of adenylate dehydrogenase and decrease in lactate content. Higher doses of ROS cause a decrease in intracellular ATP (Spragg et al., 1987) and the exhausted pool of ATP led to cell death (O' Donnell-Tormay et al., 1985) where in sperm motility decreased with time. It is, therefore, likely that the process of availability of ATP for metabolism was affected as a result of NLE treatment. This needs to be studied further to see the generation of ROS during NLE treatment or non-availability of ATP molecules for metabolism.

Similarly, the viability was also reduced in a dose-dependent manner; while at 15 mg dose the viability was only 9% in NLE-treated spermatozoa. This indicates that the biochemical pathway which is required for viability has been blocked after NLE treatment. The sperm plasma membrane integrity, as determined by the hypo-osmotic swelling test, decreased in a dose-dependent manner and only 35% spermatozoa responded to HOST. The other functional characteristics like NDCT, AIT and DNA stability were also affected in the order of 44%, 37%, and 29% respectively, at 15mg dose.

Application of 15 mg/day NLE for 21 days did not allow pregnancy in treated female rats, while control rats had 9 ± 2 pups per animal suggesting that this dose was sufficient to prevent conception in rats.

The data of the present study demonstrate that two sperm function parameters such as motility and viability were drastically affected, while other parameters such as HOST, NDT, AIT and DNA stability were not affected in a similar fashion but these functional parameters were brought down to infertility range when human spermatozoa were used *in vitro*. The same phenomenon might have occurred with rat spermatozoa due to intra-vaginal application of NLE and contributed to nil pregnancy in the present study. Further study is required to understand

the molecular mechanisms underlying NLE-induced changes in sperm function parameters in human.

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References

Aladakatti RH, Nazeer Ahmed R, Ahmed M, Ghosesawar MG (2001) Sperm parameters changes induced by *Azadirachta indica* in albino rats. *J Basic Clin Physiol Pharmacol.* 12:69-76.

Bondopadhyay U, Biswas K, Sengupta A, Moitra P, Sarkar D, et al. (2004) Clinical studies on the effect of neem (*Azadirachta indica*) bark extract on gastric secretion and gastroduodenal ulcer. *Life Sci* 75:2867-2878. Chaube SK, Prasad PV, Khillare B, Shrivastav TG (2006) Extract of *Azadirachta indica* (neem) leaf induces apoptosis in rat oocytes cultured *in vitro*. *Fertil Steril*. 85:**1223-1231.**

Deshpande VY, Mendulkar KN, Sarde NL (1980) Male antifertility activity of *Azardirachta indica* in mice. *J Postgrad Med.* **26:**167-170.

Dhaliwal PK, Roop JK ,Guraya SS (1998) Antifertility activity of neem-seed oil in cyclic female rats. In: Dhaliwal GS, Randhawa NS, Arora A & Dhawan AK (Eds), *Ecological Agriculture and Sustainable Development. Vol.2.* Indian Ecological Society and Centre for Research in Rural and Industrial Development, Chandigarh, India. pp 340-346.

Garg S, Taluja V, Talwar GP, Upadhyay SN (1998) Immunocontraceptive activity guided fractionation and characterization of active constituents of neem (*Azadirachta indica*) seed extracts. *J Ethnopharmacol.* **60:**235-246.

Gbotolorun SC, Osinubi AA, Noronha CC, Okanlawon AO (2008) Antifertility potential of neem flower extract on adult female Sprague-Dawley rats. *Afr Health Sci.* **8:**168-173.

Gopalkrishnan K (1995) *Laboratory manual for short term training course on techniques in human semenology*. Mumbai: IRR.

Jayendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zeneveld LJD (1994) Development of an assay to assess the functional integrity of human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil.* **70:** 219-228.

Joshi AR, Ahamed RN, Pathan KM, Manivannan B (1996) Effects of *Azadirachta indica* leaves on testis and its recovery in albino rats. *Ind J Exp Biol.* **34:**1091-1094.

Juneja SC, Williams RS (1993) Mouse sperm-egg interaction in vitro in the presence of neem oil. *Life Science* **53**:PL279-284.

Khan PK, and Awasthi KS (2003) Cytogenetic toxicity of neem. *Food Chem Toxicol.* **41:**1325-1328.

Khillare B and Shrivastav TG (2003) Spermicidal activity of *Azadirachta indica* (neem) leaf extract. *Contraception* **68:**225-229.

Mishra RK, and Singh SK (2005) Effect of aqueous leaf extract of *Azadirachta indica* on the reproductive organs in male mice. *Ind J Exp Biol.* **43:**1093-103.

Mukherjee S, Lohiya NK, Pal R, Sharma MG, Talwar GP (1996) Purified neem (*Azadirachta indica*) seed extract (Praneem) abrogates pregnancy in primates. *Contraception* **53:**375-378.

O' Donnell-Tormey J, De Boer CJ, Nathan CF (1985) Resistance of human tumor cells in vitro to oxidative cytolysis. *J Clin Invest.* **76:**80-86.

Owolabi LL, Gbotolorun SC, Akpantah AO, Ekong MO, Ekanem TB (2008) Effect of methanolic extract of neem leaf (*Azadirachta indica*) on ovarian histology and hormonal milleu. *Nig Q J Hosp Med.* **18:**194-197.

Pai MR, Acharya LD, Udupa N (2004a) The effect of two different dental gels and a mouthwash on plaque and gingival scores: a 6-week clinical study. *Int Dent J.* **54:** 219-223.

Pai MR, Acharya LD, Udupa N (2004b) Evaluation of antiplaque activity of *Azadirachta indica* leaf extract gel- a six week clinical study. *J Ethnopharmacol.* **90:**99-103.

Raji Y, Udoh US, Mewoyeka OO, Ononye FC, Bolarinwa AF (2003) Implication of reproductive endocrine malfunction in male infertility efficacy of *Azadirachta indica* extract in rats. *Afr J Med Med Sci.* **32:**159-165.

Riar SS, Devkumar C, Bardhan J et al. (1990) Volatile fraction of neem oil as spermicide. *Contraception* **42:**79-87.

Riar SS, Devkumar C, Sawhney RC, et al. (1991) Antifertility activity of volatile fraction of neem oil. *Contraception* **44:**319-327.

Rodriguez H, Ohanian C, Bustos-Obregon E (1985) Nuclear chromatin decondensation of spermatozoa *in vitro*: a method for evaluating the fertilizing ability of ovine semen. *Int J Androl.* **87:**147-158.

Roop JK, Dhaliwal PK, Guraya SS (2005) Extracts of *Azadirachta indica* and *Melia azedarach* seeds inhibit folliculogenesis in albino rats. *Braz J Med Biol Res.* **38**:943-947.

Sharma SK, Sairam M, Ilawazagan G, Kumar D, Shivaji SS, Selvamurthi W (1996) Mechnism of action of NIM-76. A novel vaginal contraceptive from neem oil. *Contraception* **54:**373-378.

Sharma V, Walia S, Kumar J, Nair MG, Parmar BS (2003) An efficient method for he purification and characterization of nematicidal azadirectins A, B, and H using MPLC and ESIMS. *J Agric Food Chem.* **51:**3966-3972.

Siddiqui BS, Rasheed M, Ilyas F, Gulzar T, Tariq RM, Naqvi SN (2004) Analysis of insecticidal *Azadirachta indica* Juss fractions. *Z Naturforsch.* **59:**104-112.

Sithisaran P, Supabphol R, Gritsanapan W (2005) Antioxidant activity of Siamese neem tree (VP 1209). *J Ethnopharmacol.* **99:**109-112.

Spragg KG, Schraufstatter IU, Hyslop PA, Heinshaw DS, Cochrane CG (1987) Oxidant injury of cultured cells: biochemical consequences**.** *Prog Clin Biol Res.* **236:**253-258.

Subapriya R, Nagini S (2005) Medicinal properties of neem leaves: a review. *Curr Med Chem - Anti-Cancer Agents* **5:**149-156.

Tajeda RI, Mitchell JC, Norman A, Marik JJ, Friendman SA (1984) Test for practical evaluation of male infertility by acridine orange (AO) fluorescence. *Fertil Steril.* **42:** 87-91.

Talwar GP, Shah S, Mukherjee S, Chabra R (1997) Induced termination of pregnancy by purified extract of *Azadirachta indica* (neem). *Am J Reprod Immunol***. 37:**485-4891.

Udeinya IJ, Mbah AU, Chijioke CP, Shu EN (2004) An antimalarial extract from neem leaves is antiretroviral. *Trans R Soc Trop Med Hyg.* **98:**435-437.

Upadhyay SN, Dhawan S, Talwar GP (1993) Effect of neem (*Azadirachta Indica*) oil in male rats by single intra-vas administration-an alternative approach to vasectomy. *J Androl.* **14:**275-281.

Upadhyay SN, Kaushik C, Talwar GP (1990) Antifertility effects of neem (*Azadirachta indica*) oil by single intrauterine administration: a novel method for contraception. *Proc Biol Sci.* **242:**175-179.

Upadhyay SN, Dhawan S, Sharma MG, Talwar GP (1994) Long term contraceptive effects of intrauterine neem treatment (IUNT) in bonnet monkeys: an alternate to intrauterine contraceptive devices (IUCD). *Contraception* **49:**161-169.

Wandscheer CB, Duque JE, da Silva MA, Fukuyama Y, Wohlke JL, Adelmann J, Fontana JD (2004) Larvicidal action of ethanolic extract from fruit endocarp of *Melia azedarach and Azadirachta indica* against the dengue mosquito *Aedes aegypti*. *Toxicon* **44:**829-835.

World Health Organization (1999*) Laboratory manual for the examination of human semen and sperm-cervical mucus interaction.* **4th Edition.** New York: Cambridge University Press.

Yin Z, Jia R, Gao P, Gao R, Jiang D, Liu K, Liu S (2004) Preparation of contraceptive pill microcapsule and its antifertility effect. *Sheng Wu Yi Xue Gong Cheng Xue Za Zhi.* **21:**979-982.