Exploring The Spermicidal Properties of Extracted Neem Seed Oil: An Ex-Vivo Study Investigation Based on Tribal Knowledge

Gayatri H. Tiwaskar, Pawan Mahure, Prajakta Mowade, Apurva Tiwari, Krutika Sawarkar, Ajay G. Pise*

Dadasaheb Balpande College of Pharmacy, Besa, Nagpur, Maharashtra, India

(E-mail: ajaygpise@gmail.com)

***Abstract*—** Neem oil is a natural extract from the neem tree that has been used for its medicinal properties for centuries. Among its various uses, neem oil is believed to have spermicidal activity, which has been a topic of interest for researchers. In this study, we investigated the spermicidal properties of neem oil using an ex-vivo model and evaluated its efficacy at different concentrations. Additionally, we explored the traditional tribal knowledge associated with the use of neem oil as a spermicide. The results of our study showed that neem oil has a dose-dependent effect on sperm viability, with higher concentrations leading to a greater reduction in sperm motility and viability. The traditional use of neem oil as a contraceptive in tribal communities provides anecdotal evidence of its effectiveness as a spermicide. Our findings suggest that neem oil can be a potential natural alternative to synthetic spermicides. Further research is required to investigate the safety and efficacy of neem oil as a spermicide in humans.

***Keywords*—** spermicidal, neem oil, natural contraceptive, traditional knowledge, ex-vivo study, sperm viability.

Introduction

In recent times, traditional medicines have gained popularity as patients become more concerned about the side effects of modern medications. Ayurvedic medicine, which uses plants as a source of most medicines. Each plant has specific pharmacological activity based on the active principles present in it, and the phytochemistry of the plant should be studied to gain knowledge about the class of chemical compounds present in it.

The neem plant is used in various traditional medicines in Ayurveda for its different pharmacological activities, such as antimicrobial, antiviral, antiulcer, antibacterial, and spermicidal properties. The pharmacological activities of neem are mainly due to the presence of different terpenoids in different parts of the plant. Neem oil, which is mainly isolated from neem seeds, is used for various purposes, including cosmetics, insecticides, pesticides, and antibacterial, antiviral, and spermicidal activity. The triterpenoid azadirachtin present in neem oil is a potent antimicrobial compound [1].

The potential of neem oil as a natural contraceptive has been a topic of research interest for several years. A number of studies have been conducted to investigate the spermicidal properties of neem oil and its potential as a contraceptive agent. One such study is an ex-vivo investigation of the spermicidal activity of neem oil, which was conducted by Patil et al. in 2016 [2]. In addition to this study, several other research articles have been published on the efficacy of neem oil as a contraceptive, including an in-vitro study conducted by Nath et al. in 1993 [3].

Furthermore, researchers have explored the use of neem oil as an active ingredient in spermicidal formulations. Singh and Srivastava (2010) filed a patent for a herbal composition for contraception that contains neem oil as a key ingredient [3]. Bansal et al. (2013) investigated the effectiveness and mechanism of action of Azadirachta indica leaf extract-based spermicidal formulations, which contain neem oil as the active ingredient [4]. Another study by Kumar et al. (2012) explored a novel herbal spermicidal formulation containing neem oil [5].

This paper provides an overview of the research on neem oil as a natural contraceptive, including ex-vivo and in-vitro studies, as well as the development of spermicidal formulations containing neem oil as an active ingredient [6].

The ex-vivo study by Patil et al. (2016) investigated the spermicidal activity of neem oil and found that neem oil had a significant effect on sperm motility, with increasing concentrations of neem oil leading to a decrease in sperm motility. Nath et al. (1993) conducted an in-vitro study to investigate the efficacy of neem oil as a postcoital contraceptive and found that neem oil exhibited significant spermicidal activity at high concentrations. Bansal et al. (2013) studied the effectiveness and mechanism of action of neem oil-based spermicidal formulations and found that the formulations had a significant spermicidal effect both in vitro and in vivo. The study by Kumar et al. (2012) explored a novel herbal spermicidal formulation containing neem oil, which was found to be effective in preventing pregnancy in female rats.

Research Methodology

A. Materials and methods:

1. Collection of Plants Azadirachta indica bark was collected from the botanical garden of Dadasaheb Balpande College of Pharmacy and authenticated from the Rashtrasant Tukdoji Maharaj University (RTMNU), Nagpur.
2. The steps involved in the collection of neem seeds are as follows:
   matured neem seeds were collected from the trees, cleaned to remove the dirt, and then dried by spreading them out in the sun. Foreign materials such as stones and dirt were removed by hand picking, and the cleaned seeds were crushed using a mortar and pestle to obtain coarse powder [7].
3. Extraction Procedure:
   40 gm of neem seed was weighed and placed into the thimble of a Soxhlet extractor. 300 ml of hexane was measured with a measuring cylinder and poured into the Soxhlet extractor. The apparatus was then coupled, and a condenser unit was connected to an overhead water tank to pull rising solvent vapor. The heat source was a heating mantle operating at a temperature of 68°C. The solvent evaporated during the distillation path, thimble, and the expansion adapter, after which it condensed at the condenser unit of the Soxhlet extractor. At this position, the condensed vapor returned to the thimble as liquid droplets and got in contact with the sample therein. After completion of the extraction process, the extractor was removed, and the extract was collected. After extraction, the liquid was discharged into a condenser to separate the solvent from the oil extract. The mixture was distilled at a temperature of 68°C until the neem oil extract was completely free of the solvent [8].
4. Sample Preparation:
   The sample was prepared by dissolving neem oil in methanol, and it was used for physicochemical, phytochemical, and microbial content evaluation. Physico-chemical Evaluation of Neem Seed Oil Different parameters such as solubility, density, height, etc., were evaluated [9].

B. Phytochemical analysis:

The tests and procedures used are described below.

1. Test for Carbohydrates: Molisch test: Aqueous extract of the bark was mixed with alpha-naphthol solution in alcohol and conc. H_2SO_4 was added from the sides of the test tube.
2. Test for Alkaloids: Mayer's test, Dragendroff's test, Wagner's test and Hager's test: Different filterates of the bark extract were mixed with the respective reagents.
3. Test for Tannins: Lead acetate solution: Aq. or alcoholic extract of the bark was mixed with lead acetate solution.
4. Test for Terpenoids: Solkowski test: Extract of the bark was mixed with chloroform and conc. H_2SO_4. The mixture was then shaken well.
5. Test for Saponins: Frothing test and Haemolytic test: Drug extract or dry powder of the bark was mixed with water and blood, respectively.
6. Test for Glycosides: Brontrager test: Extract of the bark was mixed with hydrochloric acid and boiled. The mixture was then filtered and equal volume of benzene or chloroform was added. After shaking, the organic solvent was separated and ammonia was added.

7. Test for Flavonoid: Shinoda test: Ethanolic extract of the bark was mixed with magnesium ribbon and concentrated hydrochloric acid.

The above tests were carried out to determine the presence of various phytochemical constituents in Azadirachta indica seeds oil[10].

C. Thin Layer Chromatography:

A chromatographic evaluation of neem seed oil was conducted using thin layer chromatography (TLC) method. The sample for TLC was prepared by dissolving neem oil in methanol. The mobile phase used for the TLC was a mixture of chloroform and acetone in the ratio of 8:2. Azadirachtin, a triterpenoid compound, was identified at an Rf value and was visualized under 366nm UV light[11].

D. Microbiological Content Testing:

In the laboratory, nutrient agar was prepared aseptically by dissolving 28g of dehydrated nutrient agar base medium in approximately 800mL of distilled water. The mixture was then heated in a water bath until the agar melted, and the resulting solution was sterilized in an autoclave. The prepared medium was utilized for plate preparation throughout the study. To test for microbial growth in the herbal extract, Staphylococcus aureus bacteria were cultured on nutrient agar[12].

The following testing methods were employed for microbial growth in herbal extract:

1. Negative control testing: Nutrient agar was weighed and dissolved in distilled water, and then poured into a petri plate.
2. Positive control testing: Nutrient agar was weighed and dissolved in distilled water, bacteria was added to the medium, and then poured into a petri plate.
3. Testing with extract: Nutrient agar was weighed and dissolved in distilled water, herbal extract was added to the medium, and then poured into a petri plate.

These methods were used to test for microbial growth using Staphylococcus aureus as the bacteria and nutrient agar as the culture media[13].

E. Sperm Morphological Testing:

A. Appearance of Sperm:

a. Normal: Sperm with a smooth, oval-shaped head that is 5-6 micrometers long and 2.5-3.5 micrometers wide and a well-defined cap (acrosome) that covers 40% to 70% of the sperm head .

b. Abnormal: Sperm with head or tail defects, such as a large or misshapen head or a crooked or double tail.

B. Liquefaction Time of Semen: Semen is liquefied within 20 minutes at room temperature.

C. pH: The pH of semen ranges from 7.2 to 8.0.

F. Preparation of Sperm Suspension:

Semen samples were collected after 72-96 hours of sexual abstinence, and routine semen analysis was performed after liquefaction of the semen at 37°C. The semen sample was collected and liquefied at 37°C for 20 minutes.

G. Immobilization Assay: The composite extract, prewarmed at 37°C, was added at concentrations of 0.1-0.5 ml to the ejaculates. A drop of the mixture was immediately placed on a glass slide, covered with a coverslip, and at least five fields were examined at x100 under a phase-contrast microscope to record sperm motility[14].

H. Sperm Viability Testing: One drop of semen was mixed with two drops of 1% eosin, and after 30 seconds, three drops of 10% nigrosin solution were added and mixed well. A smear was made by placing a drop of the mixture on a glass slide and allowing it to air dry. The prepared slide was examined using a biological microscope[15].

Results and Discussion:

A. Authentication:

Azadirachta indica was authenticated by Department of Botany, RTMNU, Nagpur.

Authentication no.: 10654

Fig. 1: Authentication of *Azadirachta indica* (seed)

B. Microscopical Evaluation:

Test	Observation	Inference
Phloroglucinol + conc. HCL	Pink – brown color observed	Endosperm in which parenchyma cells with oil granules.

Table 1: Shows the Microscopical Evaluation of Neem Seeds

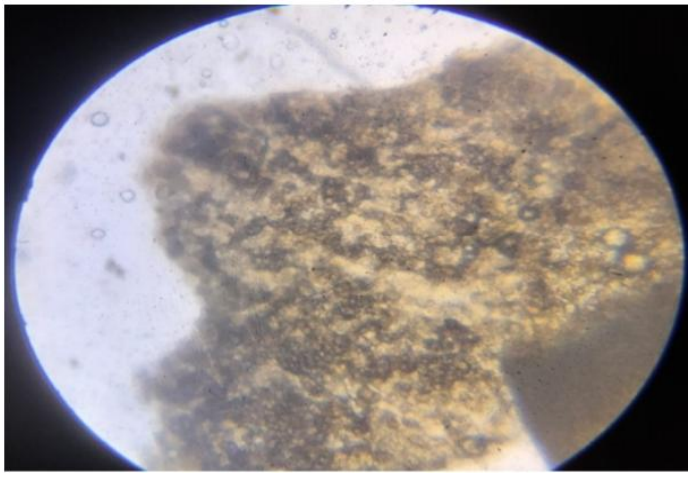

Fig. 2: Transverse section of *Azadirachata indica* Seed.

C. Chromatographic Evaluation of Neem Seed Oil:

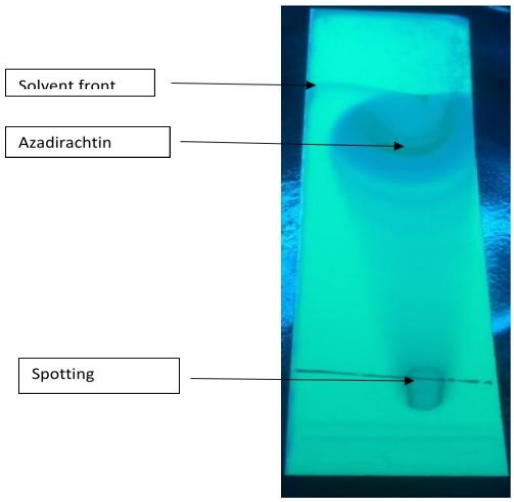

Fig. 3: TLC of Neem Seed Oil

Sr no.	Class of compounds	Test	Result
1.	carbohydrate	Molish test	-
		Fehling test	-
2.	Glycosides	Mayer test	-
		Wagner test	-
		Dragendroff test	-
		Hager's test	-
3.	Flavonoid.	Shinoda test	+
4.	Terpenoids	Salkowoski test	+
5.	Glycosides	Borntrager test	-
6.	Phenolic compounds	Ferric chloride test	+
		Lead acetate test	+
7.	Saponin	Frothing test	+
		Hemolytic test	+
8.	Tannin	Gelatin test	+

Table 2: Shows Phytochemical Evaluation of Extracted Neem Seed

Sample	Neem seed oil in methanol
Mobile Phase	Chloroform: Acetone (8:2)
Visualization	UV (366nm)
Rf Value	0.76

Table 3: Shows Mobile Phase and Its Rf Value of Chromatographic Evaluation

Sr. no.	**Methods**	**Results**
1.	Negative control testing (Nutrient Agar)	No growth
2.	Positive control testing (Nutrient Agar + Bacteria)	Growth Occurrence
3.	With extract testing (Nutrient Agar +extract)	No growth

Table 4: Microbiological Content Testing

D. Spermicidal Activity

1. Sperm Morphological testing:

A) Appearance of Sperm:

a. Normal:

A smooth, oval-shaped head that is 5-6 micrometers long and 2.5- 3.5 micrometers wide (less than the size of a needle point) A well-defined cap (acrosome) that covers 40% to 70% of the sperm head.

b. Abnormal:

Abnormal sperm have head or tail defects such as a large or misshapen head or a crooked or double tail.

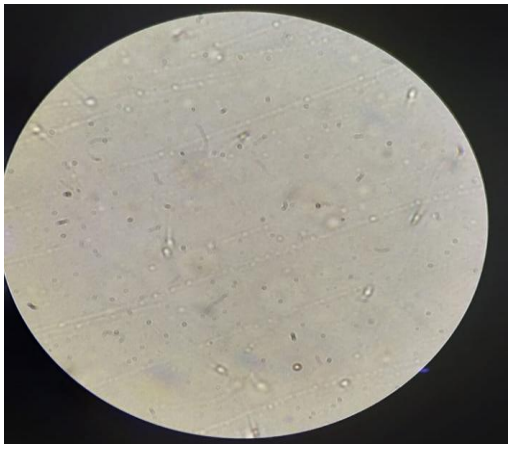

Fig. 4: a. Normal type

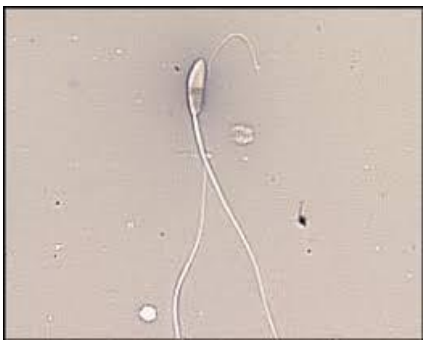

Fig. 5: b. Abnormal type

B. Liquefaction time of semen: Semen is liquified in 20 mins at room temperature.
C. pH: 7.2 to 8
2. Preparation of semen suspension:
Semen samples were collected and liquified at 37 °C in 20 minutes.

Viability of about 60%

3. Sperm count: 22 Millions/ml

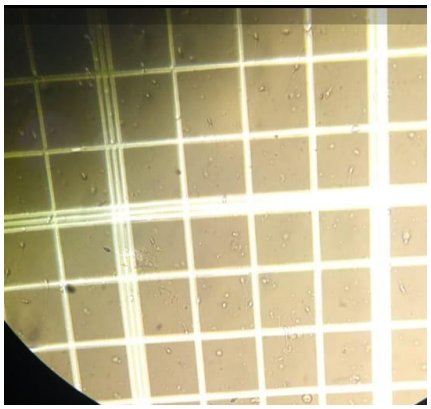

Fig.6: Sperm count in RBC chamber

E. Immobilization Assay:
Extract Concentration → 0.1 - 0.5 ml.

Drop → placed on pre-warmed slides

Under High power (x100) in Biological Microscope at the interval of time

Note: Above mention, the procedure shows the concentration range of 0.1 - 0.5 ml is already given in the literature. But here we considered the extracted neem seed oil concentration ranges from 1ml-5ml.

Sr. No.	Extract Concentration	Immobilization Time	Results
1.	1 ml	4 min	+
2.	2 ml	4 min	+
3.	3 ml	2.5 min	++

4.	4 ml	2 min	+++
5.	5 ml	1 min	++

Table 5: Evaluation of Immobilization Assay by Using Extracted Neem Seed Oil in Concentration Range of 1ml - 5 Ml. The Results Indicate (+) Poor (++) Good And (+++) Best Results with Respect to Time Interval.

F. Sperm Viability Testing:

Eosin-Nigrosin techniques:

- One drop of semen mixed with two drops of 1% eosin
- After 30 sec three drops of 10% nigrosin solution were added and mixed well
- A smear was made by placing a drop of the mixture on a glass slide and allowed to air dry.
- The prepared slide was examined using a Biological Microscope
- Results in pink-stained dead sperm were differentiated from unstained live sperm.[4]

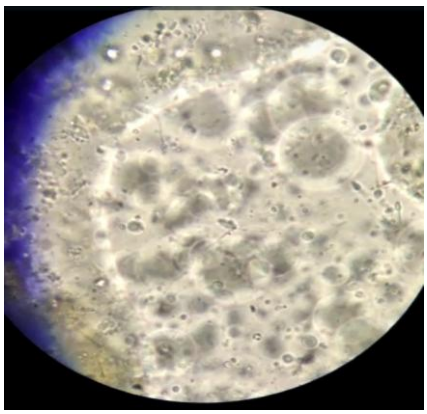

Fig.7: 5 ml concentration

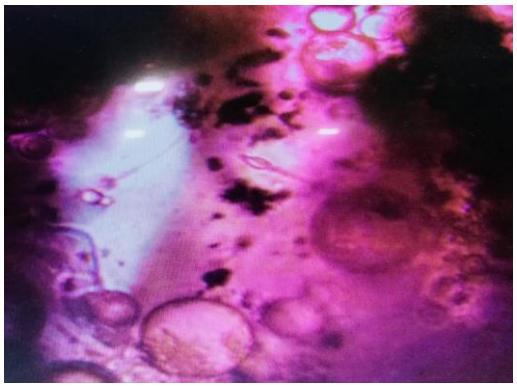

Fig. 8: 5 ml concentration

Conclusion:

In conclusion, the investigation of neem seed oil demonstrated its diverse chemical constituents including triterpenoids azadirachtin, and the presence of several phytochemical compounds. The microbiological evaluation revealed antibacterial activity against Staphylococcus aureus, and the spermicidal activity of the oil was shown to significantly affect sperm motility. Overall, these findings suggest that neem seed oil may be a potent agent for various biological applications. The Immobilization assay shows the best results in 5ml of concentration of Extracted Neem seed oil

with a contact time of 1 min. Table no. 5 indicates the results (+) poor (++) good and (+++) best results with respect to time interval. The procedure of Immobilization assay suggests a dilution procedure by using saline solution. In the case of Neem seed oil, it doesn't show any results due to its emulsive property. Therefore, this study suggested a pure sample of Extracted Neem seed oil for better results.

Acknowledgment

The authors are grateful to Dadasaheb Balpande College of Pharmacy, Besa, Nagpur for providing constant support and facilities.

References

1. Biswas, K., Chattopadhyay, I., Banerjee, R. K., & Bandyopadhyay, U. (2002). Biological activities and medicinal properties of Neem (Azadirachta indica). Current Science, 82(11), 1336-1345.
2. Patil, R., Jadhav, V., Patil, S., & Kadam, V. (2016). Spermicidal activity of neem oil: Ex-vivo study. Journal of Ayurveda and Integrative Medicine, 7(2), 83-85.
3. Kumar, V., Singh, N., & Singh, A. K. (2012). Novel herbal spermicidal formulation containing neem oil (Azadirachta indica). Indian Journal of Natural Products and Resources, 3(3), 387-391.
4. Nath, J., Gupta, S. K., & Gupta, S. (1993). In-vitro efficacy of neem oil as a postcoital contraceptive. Contraception, 48(5), 511-521.
5. Singh, K. K., & Srivastava, A. K. (2010). Herbal composition for contraception (No. WO2010075804A1). United States Patent Application Publication.
6. Bansal, R., Chauhan, A., & Singh, P. (2013). Azadirachta indica leaf extract-based spermicidal formulations: Effectiveness and mechanism of action. Pharmaceutical Biology, 51(10), 1302-1311.
7. Shanmugam, S. et al. Phytochemical and pharmacological activities of Azadirachta indica. Journal of Applied Pharmaceutical Science 01, 228–234 (2011).
8. Bhatnagar, P. et al. Review article : Neem seed oil - A potential spermicide. Contraception 82, 416–420 (2010).
9. Abdullahi, A. Y. et al. Extraction and Characterization of Oil from Neem Seeds Using Soxhlet Extraction Method. The Pacific Journal of Science and Technology 15, 193–199 (2014).
10. Tandel, K. R., & Thakurdesai, P. A. (2012). Phytochemical screening and antimicrobial activity of Azadirachta indica bark. Asian Journal of Pharmaceutical and Clinical Research, 5(Suppl 2), 89-92.
11. Bhattacharya, K., & Rana, S. (2016). Isolation and characterization of azadirachtin from Azadirachta indica (neem) leaves using column chromatography. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 7(5), 2205-2211
12. Basri, D. F., et al. "Antimicrobial activity of leaf extracts of tropical medicinal plants against Staphylococcus aureus, Escherichia coli and Candida albicans." Asian Pacific Journal of tropical medicine 7 (2014): S430-S433.
13. Naveena BM, Kulkarni VV. Antimicrobial activity of some medicinal plants against pathogenic microbes. Asian J. Plant Sci. Res. 2011;1(2):69-76.
14. World Health Organization. (2010). WHO laboratory manual for the examination and processing of human semen (5th ed.). World Health Organization.
15. Singh, R., & Nain, P. (2011). Antifertility activity of Aegle marmelos Correa in male albino rats. Advances in Bioresearch, 2(4), 157-161.