SPERMICIDAL ACTIVITY OF CHLOROFORM FRACTION OF HYDRO-METHANOL (2:3) EXTRACT OF CUMINUM CYMINUM: AN IN VITRO STUDY

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ABSTRACT
The chloroform fraction of hydro-methanol extract of *Cuminum cyminum* was examined for its effects on sperm motility in vitro at different concentration. Different doses of chloroform fraction were added to an amount of fresh semen, in a 1:1 volumic ratio. Motility and viability of cells, hypoosmotic swelling test and sperm revival tests were carried out. A dose and time dependent effect of this fraction on sperm motility and viability was observed. The maximal spermicidal effect was observed with a 2 mg/ml dose of the fraction. The effects were spermicidal but not spermiostatic as sperm immobilization effect was found to be irreversible. The maximum number of sperm failed to decondense when treated with 2 mg/ml chloroform fraction. The hypo-osmotic swelling of these sperm was reduced significantly at this highest concentration, indicating that the fraction may probably cause injury to the sperm plasma membrane. The present study indicates that chloroform fraction of hydro-methanol extract of *Cuminum cyminum* has potential spermicidal effect in vitro.

KEYWORDS: *Cuminum cyminum*; Male contraceptive; Spermicidal activity; Sperm viability.

INTRODUCTION
Male contraception is a worldwide interesting matter of concept and investigation. Even the World Health Organization (WHO) formed coalitions with governments and international...
agencies to support research and activities in this field.\(^1\) A lot of efforts have been made to find a safe and effective method with the least side effects but beyond condom and vasectomy there is no other choices for male contraception.\(^2\) Many hormonal, immunological and chemical substances are being investigated but none of them is completely desirable and without side effects.\(^3\) Magainin-A is a simple peptide isolated from the skin of African clawed frog Xenopus sp.\(^4\) and reported to have a wide spectrum of antimicrobial activity against Gram positive, Gram negative bacteria and fungi. It was also reported to be an effective vaginal contraceptive compound.\(^5\) Plants possess antifertility activity should be explored to develop novel and safe male contraceptive drugs. Neem oil extracted from the seeds of Indian neem tree Azadirachta indica has been widely studied for contraceptive potential \(^6\). During the last few decades, many medicinal plants reported in India and other countries have been assayed for antifertility/anti-implantation activity \(^7\). Organic extract of plants such as Celastrus paniculatus\(^8\) Catha edulis\(^9\) and Tripterygium wilfordii were well studied for its antifertility efficacy.\(^10\) Carica papaya seed extract in the in vitro condition has also been shown to possess sperm immobilizing effect on human spermatozoa.\(^11\) Allium sativum has also been reported to possess sperm immobilizing property.\(^12\) Allitridum, an active principal of A. sativum, showed spermicidal effect on rat and hamster spermatozoa.\(^13\) Most of the plant spermicidal compounds act on the sperm surface and disrupt the plasma membrane. Cuminum cyminum Cumin is a small, slender annual herb having seed like fruits locally known as jeera. The cumin seed is extensively used in mixed species and for flavoring curries, soups, sausages, bread, and cakes. It is an ingredient of curry powder and various pickle preparations. In indigenous medicine, cumin seeds have been used in realating sleepness, common cold and fever.\(^14\) Cumin has proven antibacterial\(^15\), hypolipidemic\(^16\) and anti hyperglycimic activities.\(^17\) The abortifacient activity of the seed has been investigated by fewer workers.\(^18\) Contraceptive effect of methanol extract of seed of Cuminum cyminum in adult male rats has been reported.\(^19\) Previously, we reported that the hydro-methanol extract of Cuminum cyminum inhibits the testicular activity.\(^20\) As a pilot study, we have screened different solvent fractions of hydro-methanol extract of Cuminum cyminum to determine their contraceptive spermicidal activity in a separate manner. The chloroform fraction of Cuminum cyminum showed promising effects. There is presently no information about the spermicidal action of this seed. For these reasons, the present investigation has been carried out to determine the spermicidal action and to evaluate different characteristics of sperm functions after in vitro exposure to a chloroform fraction of Cuminum cyminum with the aim to develop an effective herbal spermicidal agent.
MATERIAL METHODS

Plant material
*Cuminum cyminum* was collected from the local market of Midnapore town and authenticated by the Department of Botany and Forestry, Vidyasagar University, Midnapore-721102, West Bengal, India and the voucher specimen have been deposited in the Herbarium of the same Department. The seeds were washed under tap water and dried in an incubator thoroughly at 37°C for 2 days and grinded to fine powder utilizing blender.

Preparation of fraction
The powdered seeds were extracted with hydro-methanol at ambient temperature. The extract was concentrated under reduced pressure using a rotary evaporator at 45–50°C. The concentrated extract was partitioned between chloroform (GR grade, Merck India Ltd., Mumbai) and water. The organic fraction was evaporated to dryness under reduced pressure, resulting in a brownish residue. The different concentrations of fractions were prepared by dissolving the fraction in 0.1% dimethyl sulphoxide (DMSO), as 0.1% DMSO does not affect sperm parameters.[11]

Preparation of healthy human sperm for spermicidal study
Semen samples were obtained from normal 15 healthy volunteers and processed as per the guidelines detailed in the WHO [21]. Only the samples with >60×10⁶ sperm / ml normal morphology of more than 60% and grade A motility of more than 40%, with more than 70% sperm viability, were used for this study. Semen samples were collected after 72–96 h of sexual abstinence.

Immobilization assay
The chloroform fraction (prewarmed at 37°C) at concentrations of 2.0, 1.0, 0.5 and 0.25 mg/ml, and the human ejaculate collected from normal subjects were mixed thoroughly at a ratio of 1:1. For the control experiment, the ejaculates were mixed with physiological saline (pH 7.4) prewarmed to 37°C in the ratio 1:1. A drop of the mixture was immediately placed on a glass slide and covered with a cover slip, and at least five fields were examined at ×400 under a phase-contrast microscope to record sperm motility. The mixture was then incubated at 37°C. Immediately after incubation (within 20 s), within 2, 10, 20 min and within 30 min of incubation, sperm motility was recorded following the standard procedure.[22]
Fraction stability evaluation
To find out the effect of heat on the denaturing the structure of chloroform fraction, fraction was heated at 50°C, 70°C, 90°C and 100°C in a water bath for 30 min. The fraction was brought to 37°C, and sperm motility was tested in aliquots taken from the fraction heated at different temperatures.

EC50 determination
The effective concentration (EC50) for 50% immobilization of sperm by chloroform fraction was determined following the standard method. For this, different concentration of (2.0 to 0.25 mg/ml) chloroform fraction mixed with equal proportion (1 : 1) of human semen and kept for 20s. Motility was observed and counted immediately under light microscope.

Sperm revival test
The sperm and fraction mixture showing 100% inhibition of sperm motility was selected and rinsed twice with physiological saline and incubated once again in an extract-free media at 37°C for 30 min to observe a reversal of sperm motility. The above study was repeated three times using semen from the same individuals as well as from different individuals.

Sperm viability
Eosin nigrosin staining was performed to assess sperm viability. One drop of semen was mixed with two drops of 1% eosin Y. After 30 s, three drops of 10% nigrosin were added and mixed well. A smear was made by placing a drop of the mixture on a clean glass slide and allowed to air dry. One-hundred spermatozoa heads were counted and the number of unstained sperm was considered viable.

Hypooosmotic swelling test
The hypooosmotic swelling (HOS) test was performed to evaluate the effect of chloroform fraction on sperm membrane integrity. Control and chloroform fraction treated (at different concentrations as mentioned earlier) sperm suspensions were exposed to HOS solution (75 mM fructose and 20 mM sodium citrate) for at least 30 min at 37°C. Tail curling was examined using a phase-contrast microscope.
Statistical analysis
Data are expressed as mean ± SEM. For statistical analysis of data, ANOVA followed by “Multiple Comparison Student’s two tail t-test” was employed, and p<0.05 was considered significant.[25]

RESULTS
Sperm immobilization assay
All the sperms were immobilized at a concentration of 2 mg/ml within 20 sec. At a concentration of 1 mg/ml of fraction treatment, 24% of the sperm were in motile state when noted instantly (within 20 sec) after exposure to the fraction (Fig. 1). The sperm motility was 68% at a concentration of 0.5 mg/ml immediately (within 20 sec) after exposure to the fraction. However, at a concentration of 1 mg/ml all the sperm were immobilized within 20 min of exposure to the fraction. At a concentration of 0.25 mg/ml the fraction had no effect on sperm motility (Fig. 1).

EC_{50} determination
At a concentration of 0.75 mg/ml of the fraction, the motility of the sperm was found to be 50% when observed instantly (within 20 sec). So, the EC_{50} value of this fraction for human sperm is 0.75 mg/ml (Fig. 2).

Sperm revival test
None of the spermatozoa, once immobilized by the chloroform fraction, regained their after removing or washing the fraction and resuspending it in physiological saline followed by incubation at 37°C for 30 minutes.

Extract stability
The fraction (2.0 mg/ml) showed complete sperm immobilization within 2 min, when heated up to 90°C.

Viability of sperm
Sperm viability in the treated group at the dose of 2 mg/ml was found to reduce significantly than the control immediately (within 20 sec) after exposure to the fraction (Plate A to B). The viable count further declined with time and all the sperm in the treated group were found to be nonviable after 20 min (Fig. 3). At a concentration of 0.1 mg/ml, there was a reduction on the sperm viability immediately and after 20 min only 18% of sperm was found viable. At a
concentration of 0.5 mg/ml, the viability was decreased in duration dependent manner. After 30 min incubation, 42% of sperm was found viable. At a concentration of 0.25 mg/ml there was no significant change in sperm viability in comparison to the control (Fig. 3).

**Hypo-osmotic swelling of sperm**
The control showed a high percentage of tail curling while after treatment with fraction at the dose of 2 mg/ml, tail curling of spermatozoa was significantly reduced immediately after exposure to the fraction (Fig. 4). After 20 min of incubation no hypo-osmotic swelling was observed in the spermatozoa treated with 2 mg/ml of the fraction. At a concentration of 1 mg/ml and 0.5 mg/ml there was a significant reduction in tail curling in respect to the control however total failure to hypo-osmotic response was not noted even after incubation at 37° C for 30 min. At concentration of 0.25 mg/ml no significant change in hypo-osmotic response was observed throughout the duration of exposure when compare with the control (Fig. 4).

**Nuclear chromatin decondensation test**
Sperm nuclear chromatin decondensation was reduced significantly after immobilization of human sperm with 2 mg/ml of the fraction treatment in respect to the control group (Fig 5).

**RESULTS**

![Graph showing sperm viability](image)

Figure 1: Effect of chloroform fraction of hydro-methanol extract (2:3) of *C. cyminum* on motility of sperm. Data are expressed as mean ± SEM. ANOVA followed by “Multiple Comparison Student’s two tail t-test”. Bars with different superscripts (a, b, c, d) differ from each other significantly, p<0.05.
Figure 2  Determination of EC50 value of the chloroform fraction of hydro-methanol extract (2:3) of leaf of C. cyminum on the semen sample for reduction of sperm motility by 50% immediately (within 20 sec) on treatment with chloroform fraction at a concentration of 0.75 mg/ml.

Figure 3 Sperm viability of sperm after treatment of sperm with chloroform fraction of hydro-methanol extract (2:3) of C. cyminum. Bars showing the Mean ± SEM. ANOVA followed by “Multiple Comparison Student’s two tail t-test”. Bars with different superscripts (a, b, c, d) differ from each other significantly, p<0.05.
Figure 4 Effect of chloroform fraction of hydro-methanol extract (2:3) of *C. cyminum* on hypo-osmotic swelling response on sperm. Bars showing the Mean ± SEM. ANOVA followed by “Multiple Comparison Student’s two tail t-test”. Bars with different superscripts (a, b, c, d) differ from each other significantly, p<0.05.

Figure 5 Effect of chloroform fraction of hydro-methanol extract (2:3) of *C. cyminum* on nuclear chromatin decondensation in sperm. Bars showing the Mean ± SEM. Compare by “Student’s two tail t-test”. Bars with different superscripts (a, b) differ from each other significantly, p<0.05.
Plate-A Representative photograph of sperm viability from control sample immediately (within 20 sec) after mixing with the normal saline. A higher percentage of sperm was found viable (unstained) (×400).

Plate-B Magnified view (×400) of viability in treated (2 mg/ml) sperm immediately (within 20 sec) after exposure to the chloroform fraction of hydro-methanol extract (2:3) of *C. cyminum*. A higher percentage was found nonviable (stained) in compare to the control.

**DISCUSSION**

To find out whether the chloroform fraction of hydro-methanol (2:3) extract of the *C. cyminum* has any direct effect on human mature spermatozoa, the experiment has been designed.

In this experiment, human sperm mixed with chloroform fraction of hydro-methanol extract at the ratio of 1:1 and incubated at 37°C. Control was prepared by using pre-warmed DMSO. Sperm motility was affected remarkable by the fraction of this plant at a dose of 2 mg/ml. Fertilization ability of sperm is not only dependent on motility but also on other functional characteristics. Therefore, beside motility, other parameters such as viability [21], hypo-osmotic swelling [24] and nuclear chromatin decondensation are now being increasingly assessed to predict a successful outcome with different techniques followed in assisted reproductive technology to challenge the infertility. We found specific alteration in sperm function characteristic after exposure to different concentration of the fraction. The sperm immobilizing effect was dose-dependent. Fraction at a concentration of 0.5 mg/ml effect the sperm motility, however at this dose total immobilizing of sperm was not possible even after 30 min of incubation at 37°C. But at a dose of 2 mg/ml of the fraction, complete sperm immobilization was noted. Moreover, the motility of the sperm could not revived in the
fraction treated sperm followed by the washing or replacing the fraction with fresh media. This reveals that the fraction caused an irreversible damage to the sperm.

Most of the herbal spermicidal agents induce spermicidal effects by disrupting the plasma membrane as they act on sperm surface.\cite{26} Plant derivatives also resulted drastic inhibition in sperm membrane specific enzymes like acrosin and hyaluronidase, most important enzymes in the process of fertilization.\cite{12} From the sperm viability test it was indicated that the viability decreased with time and at a concentration of 2 mg/ml all the human sperm were found to be nonviable after 20 min indicating the damage of the sperm membrane integrity. Hypo-osmotic swelling test also supports the loss of sperm membrane integrity on treatment with the fraction. After 20 min exposure to the said fraction at a dose of 2 mg/ml no hypo osmotic swelling or tail curling observed in the human spermatozoa which may be due to damage in sperm membrane.\cite{27} The sperm membrane property is to allow transporting of ion and molecules selectively and this is essential for the normal sperm motility.

The successful fertilization and formation of pronucleus depends on the sperm NCD ability in the oocyte. The failure of sperm decondensation in the oocyte due to sperm abnormalities is unrecognizable by conventional semen analysis.\cite{28} The ability of the nuclear chromatin to decondence was affected by the chloroform fraction treatment as because after treatment with the fraction a significant decreased in nuclear chromatin decondensation was noted. The inability of the fraction treated sperm to decondence might be due to alteration in bonding pattern and cross-linking between DNAs.\cite{29}

It may be hypothesized that the sperm immobilizing activity of this fraction in the present study is caused by the disrupting the membrane architecture of sperm cell. The active ingredient(s) in the extract responsible for spermicidal activity will be delineated in future experiment.

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