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ABSTRACT
Different extracts (aqueous, 95% ethanolic and petroleum ether) prepared from fruits were used at different concentrations (10, 20, 40, 80 and 160 mg/ml) and alterations in motility, viability, plasma membrane integrity and morphology of cauda epididymal sperm were studied to evaluate the in vitro spermicidal potential of Mimusops elengi Linn. in wild mice. The results showed that in vitro treatment with ethanolic extract caused more severe reduction in motility, viability and plasma membrane integrity of sperm compared to aqueous and petroleum ether extracts of fruits of Mimusops elengi. Further, minimum effective concentration (MEC) of ethanolic fruit extract of Mimusops elengi that caused 100% immobilization with no revival of sperm after 15 sec was recorded as 80 mg/ml. Therefore, it is concluded from the present investigation that ethanolic extract in vitro is more potent in spermicidal action compared to aqueous and petroleum ether extracts of fruits of Mimusops elengi in wild mice.

Keywords: Mimusops elengi; Sapotaceae; Spermicidal; Contraception; Treatment.

1. INTRODUCTION
The fast increasing population is a problem of great concern in both underdeveloped and developing countries like India. The implication of effective family planning methods is the only solution to resolve this problem. Although there are several approaches of contraception in men and women viz. chemical, surgical, mechanical and immunological, but unfortunately none of these has emerged as an ideal one and suffer from one or more side effects. However, in the last few decades, the field of herbal contraceptives is getting popularized due to being cheap, easily available and natural with higher safety margins and lesser or no side effects. Several medicinal plants such as Azadiracta indica, Carica papaya, Hibiscus rosa-sinensis, Piper longum, Abrus precatorius, Aegele marmelos, Albizia
lebbeck, *Barleria prionitis*, *Cassia fistula*, *Terminalia chebula* etc. are known to be used since ancient time for the purpose of contraception both by male and female (Kaur et al., 2011; Gupta, 2012 a,b).

The plants used for contraception show anti-ovulatory, anti-implantation, embryotoxic, anti-spermatogetic, sperm immobilization and spermicidal activities. However, spermicidal properties of medicinal plants have been studied extensively (Chakrabarti et al., 2003; Silva-Torres et al., 2003; Paul et al., 2006; Souad et al., 2007; Harat et al., 2008; Hyacinth et al., 2012; Kumbar et al., 2012; Patil and Patil, 2012). This is because herbal spermicides commonly used in vaginal contraceptives do not lead to sterility and normal fertility is soon gained after discontinuation of treatment. Further, these are having no side-effects on the body of the user. Several medicinal plants are good source of spermicidal agents for example neem oil (Sinha et al., 1984b) and its derivatives viz. sodium nimbinate and sodium nimbistat (Sharma and Sakserna, 1959a,b) and NIM-76 (Sharma et al., 1996) from Indian neem tree Azadirachta indica (Meliaceae). Further, treatments with saponins isolated from Cyclomen persicum, Primula vulgaris and Gypsophyla paniculata (Prímorac et al., 1985), the purified fraction from the aqueous crude extract of Echeveria gibbiflora (Delgadó et al., 1999), seed extract of Carica papaya (Lohiya et al., 2000), the lyophilized leaf extract of Azadirachta indica (Khillare and Shrivastav, 2003), ethanolic extracts of Aegel marmelos leaves (Remya et al., 2009), aqueous fruit extract of Passiflora edulis (Alvarez-Gómez et al., 2010), crude extract from Polygala tenuifolia Wildl (Qui et al., 2011) result in in vitro immobilization of human sperm.

*Mimusops elengi* Linn. (M. elengi) is an evergreen ornamental tree of the family Sapotaceae in North and Peninsular India and in Andaman Islands. It carries a variety of names such as Bakul (Hindi and Bengali), Spanish cherry, West Indian Medlar or Bullet wood tree (English), Bakula (Sanskrit) etc. Almost all parts of this plant have been used in the indigenous system of medicine for various therapeutic purposes to cure various human ailments. *M. elengi* exhibits several pharmacological properties such as antiviral, antibacterial, antifungal, anthelmintic, antiacariogenic, antherlipidemic, antihyperglycemic, diuretic effects, free radical scavenging, antioxidant, cognitive enhancing, cytotoxic activities etc. (Gupta, 2013). However, there are only a few reports on spermicidal property of *M. elengi*.

Therefore, present study was undertaken to evaluate the in vitro spermicidal potential of *M. elengi* using aqueous, 95% ethanolic and petroleum ether extracts of fruits at different concentrations in wild mice.

2. MATERIAL AND METHODS

2.1. Preparation of plant extracts

The *M. elengi* plant was identified by Professor Ashwani Mishra and a voucher herbarium specimen was deposited in the Herbarium at Department of Botany for future record. The fruits of *M. elengi* were collected from the garden at Gyanpur, Bhadohi. *M. elengi* fruit extracts were prepared according to WHO protocols (WHO 1986, 1993). The fruits were washed properly with distilled water, shade dried for one week, and then grounded into fine powder using an electric grinder. The fruit powder (100 g) was extracted successively with 95% ethanol and petroleum ether (2000 ml, w/v 1:20) in a glass beaker at room temperature for three times of 24 hr duration each, with intermittent stirring. The extracts were then filtered with Whatman filter paper and the filtrates, thus obtained, were evaporated to dryness in an oven at 40°C to get brownish-blackish extracts (10-15 g). The crude extracts (aqueous, 95% ethanol and petroleum ether) of *M. elengi* fruit were stored at 18°C in a refrigerator for future use. Different concentrations (10, 20, 40, 80 and 160 mg/ml) of *M. elengi* fruit extracts were prepared in 0.9% physiological saline by serial dilution.

2.2. Animals and reagents

Wild house mice (*Mus musculus*) were caught near Gyanpur and were kept in the animal room for one week under standard conditions (temperature 23 ± 2°C and 12 hours photoperiod with 40-60% humidity and proper ventilation) in polypropylene cages having dry rice husk as the bedding material, following the guidelines of Laboratory Animal Care (NIH, 1985). Animals were given pelleted feed and fresh drinking tap water ad libitum. All chemical reagents were procured from either Sigma or Hi Media Lab Pvt. Ltd. (Mumbai, India).

2.3. Experimental design

Fifteen male wild house mice of 25-30 g weight were used in the present investigation. The male mice were randomly allocated into three treatment groups (aqueous, 95% ethanol and petroleum ether), each comprised of five animals. The mice were partially anaesthetized using diethyl ether and sacrificed by cervical dislocation. A scrotal incision was made to expose the testis and epididymis. For sperm analyses, cauda epididymis was dissected out, blotted free of blood and placed in a watch glass containing 0.5 ml of 0.9% physiological saline (pH = 7.4) maintained at 37°C on a hot plate (WHO 1999; Gupta, 2012c). The tissue was then minced carefully with the help of fine forceps and scissors to
ensure complete extrusion of spermatozoa from the cauda epididymidis. The tissue fraction was removed, and the sperm suspension was treated with M. elengi fruit extracts (aqueous, 95% ethanol and petroleum ether) at different concentrations (10, 20, 40, 80 and 160 mg/ml) and subsequently used for sperm analyses viz. motility, viability, abnormality and plasma membrane integrity. For the controls, sperm suspension was treated with 0.9% physiological saline instead of M. elengi fruit extracts (aqueous, 95% ethanol and petroleum ether).

2.4. Sperm analyses

The effects of M. elengi fruit extracts (aqueous, 95% ethanol and petroleum ether) at different concentrations (10, 20, 40, 80 and 160 mg/ml) on sperm functions such as motility, viability, abnormality and plasma membrane integrity were determined according to WHO Laboratory Protocol (WHO, 1999). Briefly, 10 μl of diluted M. elengi fruit extracts was mixed with 10 μl of epididymal sperm suspension (1:1 v/v) incubated at 37°C from each of five animals and tested for motility at various time intervals (15, 30, 60, 120 and 180 sec). For study of effects of M. elengi fruit extracts on viability, morphological abnormality and plasma membrane integrity, 10 μl of diluted M. elengi fruit extracts was mixed with 10 μl of epididymal sperm suspension (1:1 v/v) incubated for 30 min at 37°C from each of five animals. For the controls, 10 μl of 0.9% physiological saline was used instead of M. elengi fruit extracts (aqueous, 95% ethanol and petroleum ether).

2.4.1. Sperm immobilization test

Sperm motility was evaluated immediately after sacrificing the animal. A drop of sperm suspensions (control and M. elengi fruit extracts-treated) maintained at 37°C was placed on a clean glass slide, covered with a cover slip (20 mm X 20 mm) and then observed under a compound light microscope at 400 X. The motility was scored from randomly selected four different fields by counting more than hundred sperm (motile as well as non-motile) in each field. Sperm showing any degree of movement were considered to be motile. Sperm motility was calculated by using following formula as below:

\[
\text{Motility} (\%) = \frac{\text{Number of motile sperm}}{\text{Total number of sperm (Motile + immotile)}} \times 100
\]

2.4.2. Sperm revival test

The concentration of M. elengi fruit extracts at which sperm were completely immotile within 15 sec was subsequently tested for motility revival. The sperm suspension which showed 100% immobilization of sperm was washed twice with 0.9% physiological saline. Two hundred fifty micro liters of Baker's buffer (glucose 3%, NaHPO4 . 2 H2O 0.31%, NaCl 0.2%, KH2PO4 0.01%) was added to the above sperm suspension (which showed total immobilization), incubated at 37°C for 60 min and observed again for any motility. The minimum concentration of M. elengi fruit extract that caused 100% immobilization within 15 sec with no revival of motility in the buffer after 60-min incubation at 37°C was considered to be the minimum effective concentration (MEC). If reversal of motility in any sperm in test solution is observed the extract was considered to be failed for having spermicidal activity.

2.4.3. Sperm survival test

Sperm viability was assessed by using supra-vital staining technique. All glass wares as well as eosin-nigrosine stain were maintained at 37°C on a hot plate. A drop of sperm suspensions (control and M. elengi fruit extracts-treated) incubated for 30 min at 37°C was placed on a clean glass slide and mixed thoroughly with a drop of eosin-nigrosine stain (5% eosin Y + 10% nigrosine, 1:1) with the help of a fine glass rod. A part of this mixture was then transferred to a second glass slide and a thin film was prepared using another glass slide (WHO, 1999; Gupta, 2012). The smeared slide was then examined under a compound light microscope at 400X, and about one hundred sperm (both viable and dead) were counted from four different fields of the slide. Sperm which appeared pinkish (stained) were considered to be dead (non-viable), while those appeared colorless (unstained) as viable. This is based on the principle, that cells with damaged plasma membrane take up the stain, while the viable ones does not. Sperm viability was calculated by using the formula as below:

\[
\text{Viability} (\%) = \frac{\text{Number of viable sperm}}{\text{Total number of sperm (Viable + non-viable)}} \times 100
\]
2.4.4. Sperm abnormality test
The criteria of Wyrobek and Bruce (1975) and Zaneveld and Polakoski (1977) were employed for evaluation of sperm abnormalities in the control and *M. elengi* fruit extracts-treated (10, 20, 40, 80 and 160 mg/ml) sperm suspensions using supra-vital staining technique. Sperm were considered abnormal if they showed any of the following type of abnormalities such as atypical head (amorphous, oval, macrocephalic, microcephalic, and head without hook), absent head, bent/coiled neck, bent/coiled tail, broken tail, fragile tail, and immature sperm. About one hundred sperm both normal and abnormal were counted from four different fields of the smeared slide and the percent abnormality was calculated as below:

\[
\text{Abnormality (\%) = } \frac{\text{Number of abnormal spermatozoa}}{\text{Total number of spermatozoa}} \times 100
\]

2.4.5. Assessment of plasma membrane integrity
Hypo-osmotic swelling (HOS) test was performed to evaluate the effect of *M. elengi* fruit extracts on integrity of sperm plasma membrane (WHO, 1999). Control and *M. elengi* fruit extracts-treated (10, 20, 40, 80 and 160 mg/ml) sperm suspensions were exposed to HOS solution (75 mM fructose and 20 mM sodium citrate) for at least 30 min at 37°C. The sperm which showed characteristic swelling or tail coiling were considered to be normal and HOS-positive. The number of HOS-positive sperm was counted under a compound light microscope at 400 X. About one hundred sperm both HOS-positive and HOS-negative were counted from four different fields and the percent plasma membrane integrity was calculated as below:

\[
\text{Plasma membrane integrity (\%) = } \frac{\text{Number of HOS-positive sperm}}{\text{Total number of sperm}} \times 100
\]

2.5. Statistical analyses
All data were analyzed by Student’s t-test. Difference was considered significant at \( p < 0.05 \). Data was expressed as Mean ± SD.

3. RESULTS
3.1. Effect on sperm motility
Treatment with aqueous fruit extract of *M. elengi* at different doses (10, 20, 40, 80 and 160 mg/ml) for different durations (15, 30, 60, 120 and 180 sec), except at 10 mg/ml for 15 sec, brought significant reduction in motility of sperm compared to controls (see Table 1). The aqueous fruit extract of *M. elengi* caused reduction in sperm motility in a dose-dependent manner; however, there was no significant impact of duration of treatment (Table 1).

Further, treatment with petroleum ether fruit extract of *M. elengi* at different doses (10, 20, 40, 80 and 160 mg/ml) for different durations (15, 30, 60, 120 and 180 sec) brought significant reduction in motility of sperm in a dose-dependent manner compared to controls, though, there was no significant impact of duration of treatment (Table 1).

In contrast to aqueous and petroleum ether extracts, treatment with ethanolic fruit extract of *M. elengi* at different doses (10, 20, 40, 80 and 160 mg/ml) for different durations (15, 30, 60, 120 and 180 sec) caused significant reduction in motility of sperm strictly in a dose- and duration dependent manner compared to controls (see Table 1). Further, reduction in motility of sperm was more severe after treatment with ethanolic extract compared to aqueous and petroleum ether extracts of *M. elengi*. Furthermore, treatment with ethanolic extract at 80 and 160 mg/ml resulted in 100 % immobilization of sperm after 15 sec.

3.2. Effect on sperm revival
The sperm suspension which showed 100% immobilization of sperm after treatment with ethanolic fruit extract of *M. elengi* at 80 mg/ml was test for sperm revival. None of the sperm in the ethanol extract-treated sperm suspension gained motility after removal of the extract with physiological saline followed by incubation in the buffer at 37°C for 60-min (data not given).
3.3. Effect on sperm viability

Treatment with aqueous, ethanolic and petroleum ether fruit extracts (10, 20, 40, 80 and 160 mg /ml) of *M. elengi* caused significant reduction in viability of sperm compared to controls (Figure 1). Further, reduction in viability of sperm was more severe after treatment with ethanolic extract compared to aqueous and petroleum ether extracts (10, 20, 40, 80 and 160 mg /ml) of *M. elengi* (Figure 1), and the effect was dose-dependent.

3.4. Effect on sperm morphology

Treatment with aqueous, ethanolic and petroleum ether fruit extracts (10, 20, 40, 80 and 160 mg /ml) of *M. elengi* had no significant effect on morphology of sperm compared to controls (data not provided). No morphological changes were found in the head, mid-piece or tail of extracts-treated sperm compared to untreated sperm from controls.
3.5. Effect on integrity of sperm plasma membrane

A large fraction (88.40%) of sperm from controls responded positively to hypoosmotic solution by curling of the tail (HOS-positive). However, sperm treated with aqueous, ethanolic and petroleum ether fruit extracts (10, 20, 40, 80 and 160 mg/ml) of *M. elengi* showed significant reduction in integrity of plasma membrane compared to controls (Figure 2). Further, reduction in plasma membrane integrity of sperm was more prominent after treatment with ethanolic extract compared to aqueous and petroleum ether extracts (10, 20, 40, 80 and 160 mg/ml) of *M. elengi* (Figure 2), and the effect was dose-dependent.

4. DISCUSSION AND CONCLUSION

The sperm is a highly specialized cell which after formation in the testis and subsequent maturation in the epididymis gets two principal attributes, viz., motility and the fertilizing ability prerequisites for fertilization of the egg. Therefore, any negative impact on motility and fertilizing ability of sperm would seriously reduce the chance of fertilization of the egg. Forward motility is very essential for sperm to reach up in the female reproductive tract at the site of fertilization. On the other hand, the structural and functional modification of sperm plasma membrane which is a part of epididymal maturation is essential for attainment of fertilizing ability.

The results of the present investigation suggest that fruit extracts (aqueous, ethanolic and petroleum ether) of *M. elengi* reduced motility, viability and plasma membrane integrity of sperm collected from cauda epididymidis without any harmful impact on the morphology. The significant reduction in motility of sperm after in vitro treatment with fruit extracts clearly indicates the spermicidal potential of *M. elengi* (Khillare and Shrivastav, 2003). Further, reductions in motility, viability and plasma membrane integrity were more severe after treatment with ethanolic compared to aqueous and petroleum ether extracts of *M. elengi*. This probably suggests that the active ingredient responsible for spermicidal action of *M. elengi* is better extracted in the ethanolic solvent. The results also showed that treatment with ethanolic fruit extract of *M. elengi* caused reduction in motility, viability and plasma membrane integrity of sperm compared to controls (Figure 2).
integrity of sperm in a dose- and duration dependent manner. Similar results have been observed after *in vitro* treatment of sperm with *Cestrum parqui* (Souad et al., 2007) and *Hymenocardia acida* (Abu et al., 2011). Furthermore, treatment with ethanolic fruit extract of *M. elengi* at 80 and 160 mg/ml resulted in 100% immobilization of sperm after 15 sec with minimum effective concentration (MEC) of 80 mg/ml.

A number of spermicidal agents work through structural and functional modulation of the sperm plasma membrane (Sharma et al., 1996) by disruption of lipids, particularly, on the acrosome and midpiece resulting in rapid loss of motility (Khillare and Shrivastav, 2003; Garg et al., 1993, 1994). Therefore, the results of HOS test in the present investigation suggest that fruit extracts of *M. elengi* probably caused alteration in the plasma membrane resulting in significant reduction in sperm motility and viability. Similar results have been reported after incubation of motile sperm with different extract of *Ziziphus Mauritiana* (Dubey et al., 2011) and *Chenopodium album* (Kumara et al., 2007) *Lawsonia inermis* (Hyacinth et al., 2012). This is to be noted that plasma membrane plays a crucial role not only in the maintenance of sperm motility, but also in the induction of the acrosome reaction which is one of the key events in the fertilization of egg. Therefore, any alterations in the plasma membrane could also result into loss of fertilizing ability of sperm. Majority of plant derived spermicides have been isolated and characterized as triterpene saponins, flavonoids and phenol compounds (Farnsworth and Waller, 1982; Saha et al., 2007). Different saponins have been isolated and characterized in ethanolic extracts prepared from different parts of *M. elengi* which might be responsible for spermicidal potential of *M. elengi*. However, it is too early to reach a conclusion, and therefore, more work is needed to explore this plant. Further, a herbal constituent might be isolated from ethanolic fruit extract of *M. elengi* which could be used in the formulation of vaginal contraceptives for fertility control.

**Figure 2**
HOS test to show the effect of treatment with fruit extracts (10, 20, 40, 80 and 160 mg/ml) of *M. elengi* on integrity of sperm plasma membrane. Note the number of HOS-positive sperm is more severely reduced after treatment with ethanolic compared to other fruit extracts. Each point represents Mean ± SD for five animals. * Significantly (*p < 0.5*) different from controls by Student’s t-test.
REFERENCE


