STUDY OF CONTRACEPTIVE EFFECTS OF MEDICINAL PLANT *Artemisia vulgaris* BY *in vitro* MOUSE SPERM FUNCTIONS TEST AND *in vivo* FERTILIZATION TECHNIQUES

M.Sc.Thesis

(2020)

Submitted to:

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

For partial fulfillment of the requirement for the

Master of Science in Biotechnology

Submitted by:

Ms Sabina Bhandari

M.Sc. Biotechnology

Roll No: BT 417/073

T. U Regd. No: 5-2-19-757-2012

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Date: Jan 17, 2020

RECOMMENDATION

This is to certify that **Ms. Sabina Bhandari** has successfully completed her dissertation work entitled "**STUDY OF CONTRACEPTIVE EFFECTS OF MEDICINAL PLANT *Artemisia Vulgaris* BY *in vitro* MOUSE SPERM FUNCTIONS TEST AND *in vivo* FERTILIZATION TECHNIQUES** " under my supervision.

This thesis work was performed for the partial fulfillment for award of Master of Science in Biotechnology under the course code BT 621. The result presented here is her original findings. I, hereby, recommend this thesis for final evaluation.

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CERTIFICATE OF EVALUATION

This is to certify that this thesis entitled **"STUDY OF CONTRACEPTIVE EFFECTS OF MEDICINAL PLANT *Artemisia Vulgaris* BY *in vitro* MOUSE SPERM FUNCTIONS TEST AND *in vivo* FERTILIZATION TECHNIQUES"** presented to evaluation committee by **Ms. Sabina Bhandari** is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

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ACRONYMS

UN	United Nations
FDA	Food and Drug Administration
HOS	Hypo-Osmotic Swelling
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
FPNA	Fluorescein Isothiocyanate (FITC)-Peanut Agglutinin
GC-MS	Gas Chromatography- Mass Spectroscopy
HPLC	High Performance Liquid Chromatography
AD50	Anti-fertility Dose 50
LD50	Lethal Dose 50
l	Litre
g	Gram
µl	Microlitre
h	Hour
mM	Millimolar
DMEM	Dulbecco's Modified Eagle's Media
BSA	Bovine Serum Albumin
HEPES	(4-(2-hydroxyethyl)-1-piperazine-ethanosulfonic acid)
EtBr	Ethidium Bromide
g/l	Gram per liter
PBS	Phosphate Buffer Saline
mg	Milligram
ml	Milliliter
Tris	Tris (Hydroxymethyl) aminomethane
APS	Ammonium Per Sulfate
TEMED	(N, N, N, N-tetramethyl-ethylene diamine)
HCL	Hydrochloric acid
SGOT	Serum Glutamic-Oxaloacetic Transaminase

SGPT	Serum Glutamic Pyruvic Transaminase
ALP	Alkaline Phosphatase

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ABSTRACT

Several medicinal plants have been explored by many researchers regarding their anti-fertility effects. In the present study, we have assayed the effects of leaves of *Artemisia vulgaris* ethanol extracts on acrosome reaction, viability, sperm membrane integrity, protein profiling and *in vivo* fertilization. Mouse spermatozoa were incubated in DMEM medium with various concentrations of extract. The acrosome exocytosis was studied by labeling spermatozoa with FITC peanut agglutinin (FPNA) and staining with Coomassie. Sperm viability and sperm membrane integrity were studied by Trypan-blue staining and hypo-osmotic swelling test. *Artemisia* extract at very low concentration caused precocious acrosome reaction and loss of sperm viability. Acrosome integrity decreased exponentially from 81.85% to 14.09% and 77.36% to 11.58% in Coomassie and FPNA staining respectively, with increasing extract concentration up to 2000 µg/ml and an optimum effect at 1000 µg/ml in both staining techniques. However, the mean viability percentage of sperm sample was found to be decreased from 88.29±3.61 in control to 36.27±5.48 in treated sample with extract concentration of 2000 µg/ml evaluated by Trypan-blue staining and from 97.43±0.61 to 75.77±0.54 evaluated by hypo-osmotic swelling test. Sperm viability and membrane integrity was found to be affected less by plant extract compared to the acrosome integrity. These results indicate that *Artemisia* extract might effectively block fertilization by causing precocious acrosome exocytosis for spermatozoa, even without affecting their viability. A direct contraceptive effect was tested by injecting the plant extract into the vagina of female mice and allowing them to mate with normal males. The p-value of 0.0137 was obtained from the performed experiments, supporting the significant differences between extract treatment and control. However, the extract seemed to have no remarkable effect on major protein bands in SDS-PAGE of mouse spermatozoa.

Keywords: *Artemisia vulgaris*, spermatozoa, acrosome reaction, viability, *in vitro* sperm functions test, vaginal contraceptive

CHAPTER 1. INTRODUCTION

1.1 Background:

Human population is growing in a rapid pace, posing a major problem worldwide on all aspects of development. So there is an urgent necessity to control population explosion and to ensure better reproductive health to all. Half of the unplanned contraception and half of the resulting undesired pregnancies are due to the failure of contraception method. In this regard, WHO and other health organizations have put great notice on the search for a better form of contraception which will be safe, cheap, socially and economically acceptable and can interfere with natural process of reproduction (Luhadia *et al*., 2015, Madhukar, 2018).

Nearly 80% of the world population relies on traditional medicines for primary health care, most of which involve use of plant extracts (Singh & Hamal, 2013). On the basis of mode of actions, contraceptive medicinal plants can be divided into different categories such as anti-fertility plants, anti-ovulatory plants, anti-implantation plants, abortifacient plants etc. (Bala *et al.* 2014). These plants may act through impairment of fertilization, inhibition of implantation due to interruption in estrogen progesterone balance, fetal abortion, perhaps due to the lack supply of nutrients to the uterus and the embryo, and on the male by affecting sperm count, motility and viability (Madhukar, 2018, Shaik *et al*., 2014).

Despite tangible role of various hormonal contraceptives in controlling population growth, there has been a long debate on their uses because of their various undesirable side effects such as increased risk of cervical/breast cancers, heart attack, stroke, gall bladder disease and even infertility. There are other forms of non-hormonal contraceptives such as condoms, intrauterine implantation, vasectomy, etc. but these methods rely on physical interventions and may cause discomforts and physical impairments. Researches have been made for new types of non-hormonal contraceptives, but no tangible success has been achieved. One of the alternative approaches to such research could be the use of herbal extracts, which could be applied topically when required and would directly affect the gamete function without causing any hormonal imbalance (Peachman, 2018).

Nepal being rich in floral biodiversity, provides habitat to numerous medicinal plants. In Nepal, it has been reported that traditional healers use 1,792 plant species as medicine. Since many centuries, medicinal herbs have been used in successful management of various diseases like respiratory tract infection, gastrointestinal problems, dermatological disorders, etc. Researchers are also investigating various indigenous plants for their anti-fertility properties (Singh & Hamal, 2013).

Various plants and their extracts have been used for their anti-fertility properties. Plants with anti-fertility effects includes *Rubica cardiofolia, Artemisia vulgaris, Piper nigrum, Juniperus phoenica, Cuscuta reflexa, Leptadenia hastate, Tinospora cardiofolia*, *Acyranthes aspera* etc. (Daniyal & Akram, 2015). Medicinal plants contain various inherent active ingredients, mostly secondary metabolites, like alkaloids, flavonoids, terpenoids, phenolics, etc. which could directly or indirectly inhibit fertilization. With the advent of science and researches, several medicinal plants have been investigated and screening of various biochemical active components possessing contraceptive properties have been succeed. And many more others putative medicinal plants are left be investigated, screened and analysed (Daniyal & Akram, 2015).

*Artemisia vulgaris (*Titepati*),* belonging to the family Asteraceae is one of the important perennial and aromatic medicinal herb growing 1–2 m (rarely 2.5 m) tall and grows at the sides of paths and tracks, margins of cleared forests (Abiri *et al.,* 2018). This medicinal plant possess a broad spectrum of therapeutic properties like anti-malarial, antispasmodic, anti-steroidogenic, anti-fertility, antihypertensive, muscle relaxant, antiviral, antibacterial, antioxidant, cholinergic, diuretic etc. It is one of the important medicinal plant due to the presence of anti-malarial drug artemisinin, sesquiterpene lactones (derivative of artemisinin) and is usually known for its volatile oils (Trendafilova *et al.,* 2018)

(a) (b)

Figure 1.1 (a) *Artemisia vulgaris* whole plant growing wild around Central Department of Biotechnology, Tribhuvan University, Kritipur, Kathmandu; (b) Leaves of *A. vulgaris.*

1.2 Current Studies in Nepal

Nepal consists of 1,624 plant species with medicinal and aromatic values. For primary health care needs, 80% of the Nepalese population are dependent on the traditional plant-based medicines. Herbal Encyclopedia Bir Nighantu or Bir pharmacopia by Pandit Ghana Nath Devkota in 1969 is probably the first hand written effort towards medicinal

plants of Nepal covering 750 plants in detail (Shrestha, 2018). While work done by Banerji *et al.,* in 1955 is the earliest published work on medicinal plants in East Nepal. Research was done in Nepal by Nepalese researchers on medicinal plants like *Achyranthes aspera, Arnebia benthamii, Berberis aristata, Cissampelos pareira, Tinospora sinensis* etc. (Bhattarai *et al*., 2009). Different scientific research on medicinal plants are generally conducted by institutions like Kathmandu University, Tribhuvan University, Pokhara University, Nepal Academy of Science and Technology (NAST), Department of Plant Resources (DPR), National Ayurveda Research and Training Center (NARTC), Agriculture and Forest University and various other colleges and research institute in Nepal. Foreign researchers have also published articles based upon some Nepalese medicinal plants like *Centipeda minima, Drymaria diandra, Macaranga pustulata, Asparagus racemosus, Rhododendron anthopogon* etc. (Rajbhandari *et al*., 2001, Shrestha, 2018).

1.3 Statement of the Problem

Presently available allopathic contraceptives are endowed with multiples of problems.

- They cause hormonal imbalance, do not act directly on gametes.
- They cause serious side effects.
- They are costly.

So, the best alternatives to such problems is to explore the various locally available medicinal plants that would be safe and side-effect free contraceptive properties. An ideal contraceptive should be such that they act directly on gamete function, perturbing fertilization but not causing hormonal imbalance. Thus, screening method of putative contraceptive plants should be designed in such a way that the plant extract would act through non-hormonal pathway, and can be applied topically whenever required.

1.4 Research Hypothesis

The aim of the research is to investigate whether the ethanol extract of the plant *Artemisia vulgaris* possess contraceptives effects on various mouse sperm functions.

1.4.1 Null hypothesis

H_o1: Ethanol extract of *Artemisia vulgaris* does not inhibit various *in vitro* mouse sperm functions.

H_o2: Ethanol extract of *Artemisia vulgaris* has no effect on *in vivo* fertilization of mice.

1.4.2 Alternative hypothesis

$H_1$1: Ethanol extract of *Artemisia vulgaris* inhibits various *in vitro* mouse sperm functions.

$H_1$2: Ethanol extract of *Artemisia vulgaris* has effect on *in vivo* fertilization of mice.

1.5 Objectives

1.5.1 General Objectives

Study of contraceptive effects of ethanol extract of the plant *Artemisia vulgaris* by various *in vitro* sperm function tests and *in vivo* fertilization in mice.

1.5.2 Specific Objectives

i) Estimation of the dry material content of ethanol extract of *Artemisia vulgaris* and spectrophotometric analysis of the extract.
ii) To study the effect of extracts on acrosome reaction of mouse spermatozoa by Coomassie and FPNA staining methods.
iii) Viability testing of extract treated sperm cells by Trypan-blue staining method.
iv) Viability and membrane integrity testing of mouse spermatozoa treated with various concentrations of extract by hypo-osmotic swelling (HOS) test.
v) Protein profiling of extract treated sperm protein by SDS PAGE.
vi) Analyzing the effect of standardized concentration of plant extract on mouse *in vivo* fertilization.

1.6 Rationale of the study

Various forms of both hormonal and non-hormonal contraceptives are currently in use. But long term and continuous use of such contraceptives causes several undesirable side effects. Hormonal contraceptives predispose to increased risk of cervical/breast cancers, heart attack, stroke, migraine, higher blood pressure, infertility, liver tumors etc. Presently available non-hormonal contraceptives either require invasive procedures or cause physical discomfort or are unreliable. This calls for the development of alternative methods which have lesser side effects, self-administrable and economic. Much of these properties have been observed in medicinal plants. The anti-fertility properties of various medicinal plants have been discovered by many researchers from time to time. The major focus of our study was to study various contraceptive effects of locally available medicinal plant *Artemisia vulgaris.* The research was mainly focused on studying the effect of ethanol extract of plant leaves on mouse sperm acrosome, viability, membrane integrity and protein profiling. Also, this study was carried out to ascertain the contraceptive claim of *A. vulgaris* by assessing its effect on *in vivo* fertilization rate in mice. This research work

is only the preliminary attempt to validate the contraceptive effects of plant in laboratory set up.

1.7 Scope of the Study

1) The initial screening of *A. vulgaris* could suggest a necessity for further isolation of active compounds and their further analysis on a large scale.
2) In villages and rural areas, people cannot afford costly allopathic medicine, locally and easily available medicinal plants could be a better choice for solving and enhancing the health-related issues.
3) This research work will highlight the importance of *A. vulgaris*. If the plant is proved to be a potential contraceptive, people may be economically benefited by cultivating them or collecting from wild.
4) Novel information gathered from the current data will be important in preserving indigenous plants as well as in the discovery of novel compounds with promising anti-fertility potential.
5) The work will lay out a protocol for rapidly screening enormous store of putative contraceptive plants of Nepal.
6) The work will encourage to explore many more medicinal plants native to Nepal.

CHAPTER 2. LITERATURE REVIEW

According to UN report the world population was seven billion in 2011 and will reach to 8 billion in 2024, and 9.15 billion in 2050. Population growth is one of the main causes of poverty and pollution in developing countries. Modern medicines provided various methods for contraception but most have serious side effects. Most of the countries banned the use of hormonal contraceptive due to their carcinogenic effects (Zaman *et al*., 2019).

2.1 Anti-fertility agents or contraceptives

Rapid reduction of worldwide fertility rates from a total fertility rate (average number of births per woman) of 4-7 births in the early 1970s to 2-6 births in the late 2000s is predominantly credited to increased contraceptive use. Use of contraceptives has begun from the time when the first oral contraceptive pill was approved for public use by the FDA in 1960. Despite advances in contraceptive technology, the world population is still increasing at a tremendous rate, particularly in developing countries. Also, apart from this population growth, unintended pregnancies leading to elective abortions continue to be a major public health issue. This calls for a better method of contraception that is acceptable, effective, and available both in the developed and developing nations. It should be non-steroidal, non-barrier, non-surgical, intercourse independent, and reversible (Metchnikoff & Baskin, 2011, Ahmed *et al*., 2012).

Adolescent fertility regulation and pregnancy prevention is one of the most important health care issues of the twenty-first century. Reducing marriage before the age of 18 years by only 10% could contribute to a 70% reduction in maternal mortality rate. But due to various problems related, decline in child marriage rate could not be achieved in a fast track. So, to reduce pregnancy before the age of 20 years could be somehow achievable by development of contraceptives. Contraceptives use could control 2.1 million unplanned births, 3.2 million abortions, and 5,600 maternal deaths each year (Remsburg, 2014). But development of contraceptives is not fast as required. It could be due to that large pharmaceutical companies and research institutes of the developed countries have not included new contraceptive development as a priority (McLaughlin & Aitken, 2011).

Need and development of male contraceptives

Although several different choices and approaches are available for contraception in women, male involvement in the field of contraception has been low. The limited work on male contraception may be due to cultural background, social and economic condition, indifference and poor understanding of factors controlling male fertility. The only method available to men is still limited to condoms characterized by a high failure rate. The second popular method of male contraception is vasectomy, which is invasive and virtually not

reversible. These two methods account for only 8.9% of global contraceptive use. Many male hormonal contraceptives developed over the past several years have now advanced to clinical trials, and the outcome of these studies is yet far from realistic (Kogan & Wald, 2014, Rand *et al*., 2004, Dey *et al*., 2014) .

Infertility is frequently seen as a problem, with a 10-15% rate of incidence, and is associated with male factors in as many as 50% of the cases. A male infertility-associated factor is usually found together with abnormal semen parameters (Ovayolu *et al.,* 2016). Although female contraceptives are very effective to prevent unintended pregnancy, because of health conditions or side-effects, some women can not use them. The availability of male hormonal contraceptives would give men the chance to have control over their own fertility and to share the responsibility for family planning. Also, approximately 30% of couples currently rely on male contraceptive methods. Surveys have demonstrated that nearly 80% of men believe contraception is a shared responsibility and also, globally more than 50% of men showed interest in an alternative male contraceptive. So there is a high necessity for development of male contraceptives (Kogan & Wald, 2014, Amory, 2016).

Current studies

Many drugs have been investigated in male contraception. Development of one drug, Nifedipine, induces sterility by blocking calcium channels of sperm cell membranes which results in cholesterol deposition and membrane instability, rendering them incapable of fertilization. Herbal preparations have also been used as male contraceptives. Gossypol, a constituent of cottonseed oil, was found to be an effective male contraceptive in very large-scale experiments conducted in China. But gossypol cannot be used due to several drawbacks. It may cause respiratory distress, impaired immunity, body weight gain, anorexia, weakness, liver damage, apathy, and even death. Most recently, a glycopeptide from the outer coating of human eggs has been isolated that interacts with spermatozoa during fertilization process. One of the most researched methods of male contraception using drugs involves the use of hormones. Like female contraceptive pills, Male Hormone Contraceptives (MHCs) seek to stop the production of sperm by stopping the production of hormones that direct the development of sperm (Dey *et al.,* 2014).

Among the different approaches to control male fertility, hormonal contraception is the closest to possible clinical application. The support of government agencies such as the World Health Organization (WHO), Contraceptive Research and Development (CONRAD), and the National Institute of Child Health and Human Development has led to important progress in this field. However, progress in research continues to be slow and a marketable product is not on the horizon (Gava & Meriggiola, 2019).

2.2 Medicinal Plants

Medicinal plants are plants containing inherent active ingredients and are a great gift of nature as a cure for a plethora of human ailments. Approximately 75% of the population in developing nations receive herbal medical health care, compared with over half of the population in developed nations, mostly for lifestyle-related diseases (Daniyal & Akram, 2015). Evidences suggest that more than 70% of the current drugs that are in use have originated from nature (Gupta *et al.,* 2017). More than 25% of prescribed drugs are obtained from plants and their products. In the last few decades, researches on various medicinally important plants has progressed from simple documentation and screening process to modern day drugs (Zaman *et al*., 2019).

According to the Global Industry Analyst Market, the global herbal medicine sector was close to $115 billion profit in 2015, and the tremendous growth towards herbal and nature-based products was based on the presumption that these products cause fewer side effects than modern medicines (Daniyal & Akram, 2015). Medicinal plants could be a reliable source in development of non-toxic, orally effective, alternative and complementary medicine for preserving health and avoiding disease transmission (Liu *et al.*, 2013). The consumption pattern of medicinal plants have been started before the validation of so called “scientific drug therapy” or “modern medicine” (Abad *et al.,* 2012). The conceptual and practical knowledge of medicinal plants have varyingly considered as a cultural changes, function of plant habitat collection as well as biochemical and ecological aspects (Goulletquer, 2014).

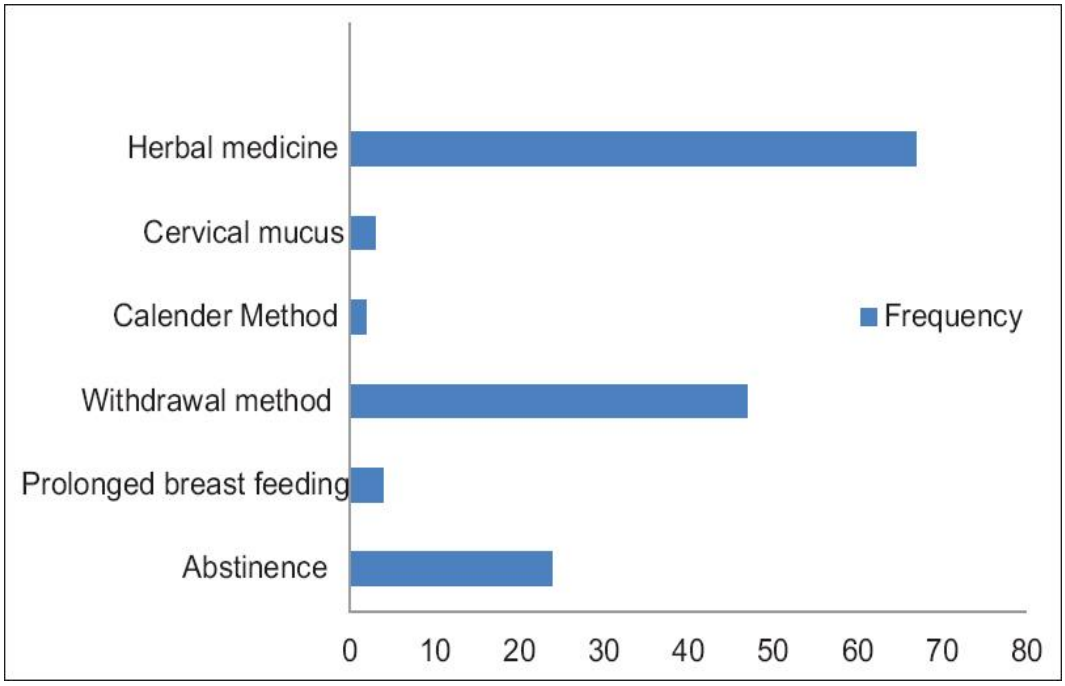


Figure 2.1 Frequency of the different types of contraceptive methods in family planning used by the respondents in Kano, Nigeria in which herbal medicine was the most commonly used method. (Source: Rabiu & Rufia, 2018).

2.2.1 Contraceptive Effects of Various Plants

Study of ethanol extract of *Tinospora cordifolia* was carried out for finding the reversible contraceptive effect and safety evaluation in animal model (Singh *et al*., 2011). In that research, the extract was found to be effective in producing reversible sterility. Weight reduction of the reproductive organ like testis, epididymis, seminal vesicle and ventral prostate of the treated male rats were observed. The treated rats also showed significant reduction in the sperm density, motility and fertility and also the induced infertility was completely reversed after withdrawal of treatment of another period of 60 days.

Anti-fertility effect of alcoholic extract of Iranian neem (*Azadirachta indica*) seeds was studies on mouse epididymal spermatozoa. Adult healthy mice were administered first 50 mg/kg body weight /day then 100 mg/kg body weight/day orally for 15 days where significant change in epididymal sperm motility was observed only in mice fed with 100 mg/kg body weight as compare to the control. Extract treated males when conjugated with normal cycling females, the fertility rate was also found to be decreased to 17% as compared to 97% in control animals ($p<0.001$) concluding that the extract could be as a potential anti-fertility agent (Dehghan *et al.,* 2005).

The aqueous extract of the leaf of *Aegle marmelos* (Bel) when fed at a dose of 50 mg/100 g body weight in male albino Wistar rats, resulted a significant reduction in the activities of testicular steroidogenic enzymes along with low levels of plasma testosterone and reduced weights of sex organs. The applied dose of the extract had no toxic effects on the

activities of transaminases and phosphatases in metabolic organs like liver and kidney. Hence, the plant was considered as a potent contraceptive (Das *et al.,* 2006).

Similarly ethanol extract of *Dioscorea esculenta* tuber resulted significant reduction in the weight of testes and epididymis in male albino rats. Ethanol extract was found to inhibit sperm concentration, motility, sperm abnormality and testosterone in treated rats but no significant changes were noted in the serum biochemical and liver marker enzymes (SGOT, SGPT and ALP), which might result in a male infertility (Shajeela *et al*., 2011).

To find out the contraceptive efficacy of neem leaf extract on female albino rats, an *in vivo* study was performed. Fifteen female albino rats were taken and divided into three groups of five female rats of each and after confirmation of estrus cycle, the extract was applied intravaginally at doses of 150 mg and 200 mg in the two groups of female rats of five numbers each in 0.9% saline solution. Five female rats were taken for control group and only 0.9% saline solution was applied intravaginally. And, immediately after application of the extract, the female rats were kept for mating with male rats in 2:1 ratio and after 24 h were checked for spermatozoa in the vaginal smears. At 200 mg dose all the spermatozoa were non-motile or dead and at 150 mg dose only 3-4% spermatozoa were sluggish and the rest were non-motile or dead. So, from this study it was found that 200 mg dose of the extract possessed 100% anti-fertility effect on implantation sites. In the control group of rats, 4-7 number of implantation sites were found in each (Suryawanshi, 2011).

Table 2.1: Some medicinal plants of Nepal, that have been surveyed for contraceptive actions

S.N.	Name of the plant	Parts of the plant used	Mode of action
1.	*Hyppophae rhamnoides* (Gautam *et al*., 2015	Fruit (Dose of 500mg/kg)	Shows 50% anti-fertility effect and 19.04% foetal loss.
2.	*Carica papaya* (Soni *et al.,* 2015)	Leaves (Methanolic extract)	Membrane damage in acrosome.
3.	*Dichranostigma lactucoides* (Gautam *et al*., 2015	Whole plant (Dose of 500 mg/kg and 1000mg/kg)	Shows 75% anti-fetility effect and 85% foetal loss in treatment with 500 mg/ml and 75% foetal loss in treatment with 1000 mg/ml of extract.

4.	*Azadirachta indica* (Dehghan *et al.,* 2001)	Seeds (Alcoholic extract)	Change in epididymal sperm motility.
5.	*Mamira teeta* (Gautam *et al*., 2015)	Whole plant (Dose of 200mg/kg)	Shows 50% anti-fertility effect.
6.	*Dioscorea esculenta* (Shajeela *et al*., 2011)	Tuber (Ethanolic extract)	Reduction in weight of testis and epididymis.

2.2.2 *Artemisia vulgaris*

Artemisia is a large and diverse genus of plants, belonging to the family Asteraceae. In the plant kingdom, family Asteraceae is endowed with essential oil-yielding plants, and among these plants, the genus *Artemisia* occupies top position for its bio-prospection and comprises over 500 species, mainly found in Asia, Europe and North America. Genus *Artemisia* comprises important medicinal plants which are currently the subject of phytochemical attention because of their biological and chemical diversity, and essential oil production (Pandey & Singh, 2017).

The name of genus *Artemisia* has come from the Greek word “goddess of hunting”. It is also known as mugwort, sailor's tobacco, common wormwood, wild wormwood, felon herb, old Uncle Henry, chrysanthemum weed, old man or St. John's plant and naughty man (Abiri *et al.,* 2018). It is known as ‘pati’ or ‘titepati (bitter-leaf plant)’ in Nepal. *A. vulgaris* is a perennial and aromatic herb growing 1–2 m (rarely 2.5 m) tall and grows at the sides of paths and tracks, margins of cleared forests. Leaves are 5-20 cm long, pinnate, sessile, alternate, simple and the margins are often rolled back. The upper surface is usually dark green and glabrous, occasionally pubescent while dense white tomentose hairs on the underside. The basal leaves are short petiole and lobbed with an end section and one to two pairs of small side leaflets. The rest of the leaves are sessile or almost sessile with a slit base. These plants are more common in Europe, Asia, Northern Africa, and Alaska and are naturalized in North America. The strong and aromatic smell of the plant is due mainly to high concentrations of volatile terpenes, constituents of their essential oils, especially in leaves and flowers (Abad *et al.,* 2012, Lian *et al*., 2018, Shaik *et al*., 2014).

Taxonomic Position:

Kingdom: Plantae

Class: Angiosperms

Order: Asterales

Family: Asteraceae

Genus: *Artemisia*

Species: *A. vulgaris*

Botanical name: *Artemisia vulgaris*

Common name: Mugwort, Common wormwood, Titepati, Sailor's tobacco etc.

Distribution in Nepal: 300 – 2500 meters

(Source: Plants profile for *Artemisia vulgaris* (common wormwood)". *PLANTS USDA.gov*)

Phytochemical Analysis and Screening of Metabolites of *A. vulgaris*

In phytochemical screening of methanol extract of leaves of *A. vulgaris*, showed the presence of high amount of alkaloids, flavonoids, and terpenoids, steroids and tannin along with several other phytonutrients. HPLC analysis of extract revealed the presence of two members of flavonoids, luteolin and morin (Pandey *et al.*, 2017).

Among various species of genus *Artemisia*, *Artemisia annua* is the mostly studied and researched species and the active ingredients, sesquiterpene lactone, artemisinin are the major components. Phytochemical analysis of *A. annua* resulted more than 80 natural products that include various flavonoids, coumarins and phenolic acids. Among many species of *Artemisia* genus, *A. vulgaris* is one of the important medicinal plant due to the presence of anti-malarial drug artemisinin, sesquiterpene lactones (derivative of artemesinin) (Abolaji *et al*., 2014, Trendafilova *et al.*, 2018). The 2015 Nobel Prize in Physiology or Medicine was awarded to Professor Youyou Tu for her key contributions to the discovery of artemisinin in treating malaria (Su & Miller, 2015).

A. vulgaris collected from Nepal was found to be rich in α-thujone (30.5%), 1, 8-cineole (12.4%), and camphor (10.3%), which were obtained by hydrodistillation and analysed by GC-MS (Satyal *et al.*, 2012). Artemisinin, discovered first time from *A. annua* was later reported from other species of *Artemisia* also including *A. vulgaris*. But the amount of artemisinin in this specimen was lower than the other *Artemisia* species. The content of artemisinin was found to be 50, 20 and 3 mg kg-1 dried weight for leaf, stem and root, respectively. Nepalese *A. vulgaris* has not been used as a commercial source of artemisinin but has number of other applications and such as ingredients in foods, pharmaceutics and cosmetics (Abiri *et al.,* 2018).

A study was carried out on ethanol extract from aerial parts of plant *Artemisia alba* Turra, by HPLC–MS and GC-MS analysis. The total amount of flavonoid was found to be 0.78 mg/mL while large amounts of chlorogenic acid were observed (4.66 mg/mL). Phytochemical screening of the methanol extract show the presence of flavonoids and saponins (Peron *et al*., 2017).

Contraceptive Properties and Studies of the Plant

In a study carried out by researchers for knowing the anti-fertility activity of *Artemisia vulgaris* leaves on female Wistar rats, the plant extract in 70 % methanol was fed to rats for 7 days. The extract was found to cause 50 % and 100 % anti-implantation effect with feeding extract at concentration 300 and 600 mg·kg−1 body weight respectively and strong estrogenic effects. It was shown that the plant extract inhibited implantation in a dose-dependent manner. The methanolic extract of the plant was also found to cause a significant ($P < 0.05$) increase in uterine weight in immature ovariectomised rats in dose-dependent manner (Shaik *et al.,* 2014).

A. vulgaris extract also showed anti-fertility effect on *Callosobruchus chinensis* (weevil). The study was performed to estimate the percentage of anti-fertility and toxicity (AD50 and LD50) of *A. vulgaris* in *C. chinensis* through the effect on hatchability rate of weevil. The findings showed that the plant extract is pollution-free biological anti-fertility agent, that can effectively control weevil reproduction (Pal & Pandey, 2011).

In another research, alcoholic extract of *A. vulgaris* was found to induce an irregular estrous cycle in female albino rats and caused 80% anti-implantation activity. The herbal extract even at the high dose did not cause gross malformations in pups delivered, proving its non-toxic nature (Narwaria *et al*., 1994). Similar anti-implantation or abortifacient effect was exhibited by extracts of related species like *A. kopetdaghensis and A. annua* on female rats (Abolaji *et al*., 2014). Decrease in litter size in all *A. annua* treated groups was observed, and the significant reductions in litter size were noted in the 100 and 300 mg/kg groups compared to the control. From this, it could be implied that *A. annua* might have prevented fertilization in rats. *A. annua* administered to pregnant rats during second and third trimesters of pregnancy, caused reductions in the levels of estrogen and litter size (Abolaji *et al*., 2014).

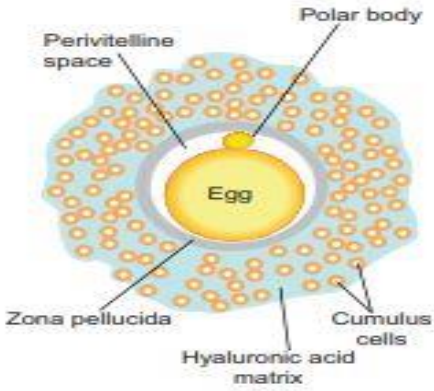
Studies in Nepal

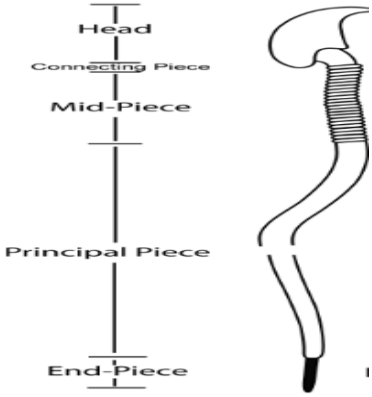
In spite of bearing numerous uses and advantages of *Artemisia*, only limited researches have been carried out on this plant in Nepal. In one such study, Pandey *et al.,* 2017 investigated chemical composition, antioxidant and antibacterial activities of essential oil of *A. vulgaris* extracted with methanol. Gas chromatography, mass spectroscopy and HPLC studies showed that the plant extract is rich in phenolic and flavonoids compounds.

2.3 Fertilization

Fertilization is a complex process of molecular events involving mature haploid male and female gametes and their mutual recognition and fusion to establish the genotype of a new individual. Fully grown, matured oocytes and normally differentiated haploid spermatozoa are the prerequisites for the success of the fertilization process (Kupker *et al.*, 1998).

Mammalian oocytes are released at the metaphase II arrested stage. Oocytes acquire the ability to fuse with sperm when they are fully mature and are arrested at the metaphase of meiosis II (Georgadaki *et al.*, 2016).





(2.2) (2.3)

Figure (2.2) The features of mammalian egg. Ovulated eggs (yellow) are surrounded by small cumulus cells embedded in extracellular material (blue), the cumulus layer is separated from the egg by the zona pellucida (gray), consisting of three glycoproteins (ZP1-3 in mouse and ZP1-4 in human) (Okabe, 2013); (2.3) Structure of a mouse spermatozoon, different regions of a spermatozoon are indicated (Manandhar & Sutovsky, 2007).

The distal segment of the caudal epididymis is the principal site for the storage of mature spermatozoa (Yanagimachi, 1994). Spermatozoa are small, asymmetrical and motile cells and is derived from the Greek word ‘sperma’ (meaning ‘seed’). These are the elongated motile cells specialized to deliver haploid male genomes to oocytes. The length of human and common domestic animal spermatozoa is about 50 µm, while rodent spermatozoa measure 150 µm to 250 µm. Homey opossum (*Tarsipens rostratus*) has the longest spermatozoa (350 µm) among mammals. Rodent spermatozoa have sickle-shape, falciform heads. Mammalian spermatozoa have three components; head, mid-piece and tail (Manandhar *et al.*, 2005, Georgadaki *et al.*, 2016).

The sperm head is the anterior-most part of spermatozoa, comprising the nucleus, perinuclear theca, acrosome, and plasma membrane. Its main function is to penetrate into the oocyte, deliver the haploid genome, and initiate embryonic development after fertilization. The central core of the sperm head is the nucleus that encloses a haploid set

of paternal chromosomes (Manandhar, *et al.,* 2005). The mammalian sperm nuclei are surrounded by a compact cytoskeletal structure, the perinuclear theca (PT) from all sides except the base. Topographically, the PT can be divided into three regions: sub-acrosomal layer, equatorial segment, and post-acrosomal sheath (Sutovsky *et al.,* 2003). The inner acrosomal membrane (IAM) overlying the sub-acrosomal PT is exposed after acrosome reaction, possibly playing roles in binding and penetrating through the zona pellucida (Manandhar & Toshimori, 2003).

Acrosome

The acrosome (Gr. akros = extreme or tip, soma = body) is a Golgi-derived cap-like vesicle located at the tip of the sperm head of many mammalian species (Buffone *et al*., 2008). Structurally and functionally it can be divided into two regions: (1) the anterior bulbous region that is exocytosed when spermatozoa pass through the cumulus cells or interact with the zona pellucida, and (2) a narrow pocket-like equatorial or posterior acrosome that is retained after acrosomal exocytosis and incorporated into an oocyte after fertilization (Manandhar & Toshimori 2001). Acrosome contains proteolytic enzymes, such as acrosin, trypsin, hyaluronidase and proteases, which are released by exocytosis during the acrosome reaction and required for the penetration of egg investments (cumulus cell layers and the zona pellucida) (Okabe, 2013).

Spermatozoa penetrate the cumulus oophorus by releasing hyaluronidase, breaking down hyaluronic acid, and dispersing the cumulus cells. During the acrosome reaction, fusion of the outer acrosomal membrane with the plasma membrane releases the contents of the acrosome and exposes the inner acrosomal membrane as the functional outer boundary of the sperm head (Georgadaki *et al.*, 2016, Manandhar & Toshimori, 2001).

Mammalian sperm must have properly formed acrosomes to be fully functional in the process of binding and penetrating the zona pellucida (ZP), the extracellular matrix surrounding the egg. Men or mice carrying mutations resulting defective acrosome are infertile or sub-fertile (Buffone *et al.*, 2008).

2.3.1 Events of fertilization

A successful fertilization comprises several steps of sequential events that are physiologically unique from somatic bodily functions (Georgadaki *et al.*, 2016).

Capacitation and Acrosome Reaction

Ejaculated sperm are not ready to fertilize an egg when they enter the vagina. During sperm maturation through the epididymis, spermatozoa obtain their fertility from the caput to corpus and motility from the corpus to caudal regions. In response to the dilution of semen in the vagina, they undergo several changes, which are collectively known as capacitation. This is the functional maturation of spermatozoa that takes place in the

uterus and fallopian tube during normal fertilization (Yanagimachi, 1994). After capacitation the spermatozoa becomes hyperactivated, enabling them to undergo acrosome reaction and penetrate through the zona pellucida. It is calcium-dependent and consists of a change in sperm membrane fluidity involving activation of adenosine triphosphatase (ATPase), and redistribution of mannose receptors, glycoproteins and glycolipids on the sperm surface with subsequent changes in the properties of the membranes (Kupker *et al*., 1998). Manandhar *et al*., 2009 reviewed that a very significant marker of capacitation could be ability to undergo acrosome exocytosis when spermatozoa allowed to interact with zona pellucida.

The sperm head binds the zona pellucida through specific ligand-receptor interaction and the binding process causes the acrosome to vesiculate and release enzymes on the zona pellucida by a process called acrosome reaction which was considered to assist spermatozoa to penetrate the egg investments. Spermatozoa that are unable to shed acrosome upon zona binding cannot penetrate the zona pellucida and cannot fertilize. However, recent observation indicates that fertilizing spermatozoa are acrosome reacted before contact with zona pellucida The percentage of acrosome reacted spermatozoa or the rate of successfully capacitated spermatozoa can be evaluated by briefly incubating the spermatozoa with denuded oocytes (cleaned from cumulus cells), fluorescent labeling sperm acrosome with FITC peanut agglutinin and then studying under fluorescent microscope (Llanos *et al.,* 1993, Castellini, 2007).

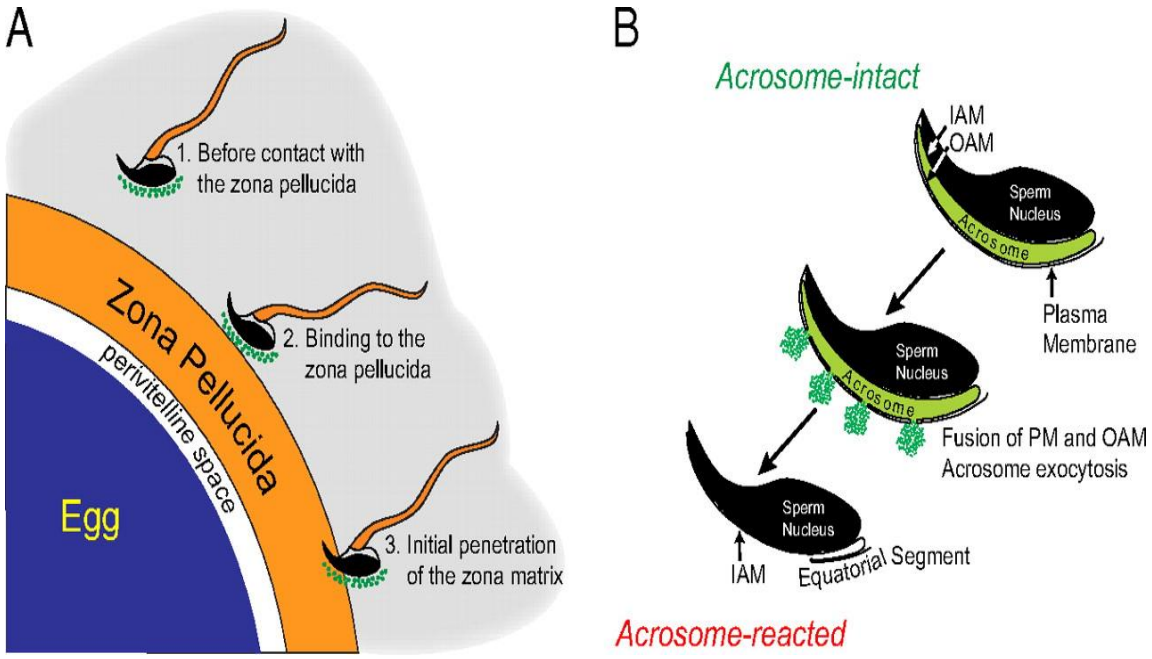


Figure 2. 4: (A) Sperm acrosome reaction. (B) The acrosome-intact sperm (Upper Right) the plasma membrane (PM) fuses with the outer acrosomal membrane (OAM), and acrosomal contents are released through resultant fenestrations (Center). Acrosome-reacted sperm within the perivitelline space have an exposed inner acrosomal membrane (IAM) and fuse to the egg via the residual equatorial segment of the plasma membrane (Lower Left) (Avella & Dean, 2011).

Zona Penetration and Sperm Oocyte Fusion

Spermatozoa initially interact with zona pellucida by loose binding which might be probably due to bonding between ZP3 proteins of zona pellucida and β-1, 4-galactosyltransferase of sperm surface (Miller *et al.*, 1992). The expression of ADAM3 on sperm surface was considered to be essential for sperm fertilizing ability in terms of enabling spermatozoa to bind to zona. After acrosome content has been shed, the inner acrosomal membrane of spermatozoa binding tightly with zona, penetrate the zona pellucida (Okabe, 2013) and then fuses with the oocyte plasma membrane. Quickly after sperm-oocyte fusion, the cortical granules of the oocytes are released, react with zona pellucida and thus make it impermeable to other spermatozoa.

Pronuclear Development

The third cytological observable event of fertilization is first embryonic cleavage and formation of 2-celled pro-embryos. After sperm-oocyte fusion, the oocyte is activated and completes the meiosis II division. Anaphase II takes place and the second polar body is extruded, and a haploid female pro-nucleus is formed. The sperm nucleus also form swollen male pro-nucleus. The percentage of two celled pre-embryos is the index of fertilization success.

Studies on Fertilization

Fertilization was originally studied for the purpose of developing an *in vitro* fertilization system. Initially, the role of female reproductive tract in capacitating spermatozoa was discovered by MC Chang in 1951. Soon a defined medium was developed in which spermatozoa could be capacitated, acrosome reacted and eggs fertilized. From then *in vitro* fertilization system was utilized by many researchers to analyze the mechanism of fertilization, with numerous factors contributing to sperm-egg interaction (Okabe, 2013). With the advent of *in vitro* fertilization (IVF) treatment programmes, a potential source of inseminated/unfertilized oocytes is now available for use in laboratory studies. Unfertilized oocytes from IVF cultures are typically discarded and offer the most abundant source of available oocytes for the sperm-zona binding assay (Graczykowski *et al.,* 1998, Hammitt *et al.,* 1993).

Fertilin is an ADAM1b/ ADAM2 heterodimer, a sperm-specific protein which was thought to be essential protein for sperm-egg fusion and ultimately success fertilization. So the elimination of Adam2 results in the loss of fertilin from spermatozoa, caused infertility in males. Therefore, researches are being proceeding by making fertilin null spermatozoa by disrupting the Adam1b gene. Also, infertility was success in mice by developing the Adam2-deficient/fertilin knockout mice as ADAM2 had a function in testis to form a dimer with testicular ADAM1a (Kim *et al*., 2006, Okabe, 2013).

2.4 Various *in vitro* Mouse Sperm Function Tests

2.4.1 Acrosome integrity test

After capacitation, only those spermatozoa that have undergone acrosome reaction (AR), are capable to fertilize. A sufficient number of motile acrosome-intact spermatozoa capable of fertilizing the oocyte at the appropriate time is a necessary prerequisite to ensure efficient fertilization of the oocyte (Reckova *et al*., 2015). Acrosome reaction is a modified exocytotic event in which the outer acrosomal membrane fuses with the plasma membrane of the spermatozoon at discrete points resulting in hybrid membrane vesicles. These vesicles then detach from the spermatozoa and finally lead to the complete loss of the acrosome with the release of the acrosomal enzymes which are thought to play a role in the penetration of spermatozoa throughout the outer oocyte vestments. Many studies have shown that infertility is also related to reduction of acrosin activity and/or reduced protamine content in the DNA in the acrosome (Tavalaee, *et al.*, 2007).

Many protocols of staining methods have been found to be useful for evaluation of acrosome integrity of semen samples of different animals. Methods include like the gelatin slide test, Coomassie staining, Chlortetracycline staining, phase-contrast microscopy, electron microscopy, monoclonal antibodies, FPNA etc. Among which only few methods are found to be appropriate for acrosomal assessment (Tavalaee, *et al*., 2007). Larson & Miller in 1999, studied acrosomes by using simple histochemical stain like Coomasse G-250 and by labeling with fluorescein conjugated Pisum sativum agglutinin (FITC-PSA) and found from statistical testing that there is no significant difference between either method.

Coomassie Staining

Acrosome integrity evaluated by Coomassie Blue G-250 staining method has become a reliable method for the assessment of acrosomal status in a variety of species including cattle and buffalo (Mehmood *et al*., 2009). 0.22% Coomassie Blue G-250 in 50% methanol and 10% glacial acetic acid was found to be simple and useful staining method for finding the acrosome integrity in semen sample and may be useful for studies of fertilization and as a part of a male fertility evaluation (Bendahmane *et al.,* 2002, De La Vega-Beltran *et al*., 2012, Oliveira *et al*., 2009). In humans, Coomassie staining has often been used to measure sperm concentration and morphology as indicators of fertility problems (Thiangtum *et al*., 2009).

In a study for the assessment of acrosomal status in rat spermatozoa, Coomassie brilliant blue (CBB G-250) dye staining procedure was followed and was found to be effective to assess *in vitro* capacitation and the status of the rat sperm acrosome. Researchers found that the majority of spermatozoa were either acrosome-intact or completely acrosome-

reacted; however, a small number of the spermatozoa appeared to be in the process of undergoing the Acrosome Reaction (AR) as evident by the presence of the stained membranous vesicles on the dorsal side of the sperm head after use of agonists that induces the AR (Bendahmane *et al*., 2002).

FITC Peanut Agglutinin (FPNA) Staining

In addition to the different acrosomal enzymes, acrosomal matrix of the spermatozoa also consists of glycoconjugates and variety of lectins have the ability to bind to those glycoconjugates. With the concept of this, different lectins have been developed and used to assess the sperm acrosomal status. The use of fluorescent labelled plant lectins or antibodies raised against acrosomal proteins are frequently described for the evaluation of the acrosomal status (Lybaert *et al*., 2009).

Fluorescein Isothiocyanate-*Pisum sativum* agglutinin (FITC-PSA) which binds to glycoconjugates in the acrosome and stains the acrosomal matrix had been used to detect the acrosomal loss of stallion spermatozoa (Cheng *et al.,* 1996). In that study, only two patterns of staining were observed, with intact acrosomes and missing acrosomes but not with acrosome reaction in progress. Peanut agglutinin (PNA) exclusively binds to the outer acrosomal membrane so, FITC-conjugated *Arachis hypogea* agglutinin (FITC-peanut agglutinin; FPNA) was used to assess the sperm acrosomal status and the acrosome reaction during equine gamete interaction (Mortimer *et al*., 1990). But among the two fluorescein-labelled (FITC) plant lectins, Pisum sativum (edible pea) agglutinin (PSA) and *Arachis hypogaea* (peanut) agglutinin (PNA), FPNA was found to be the most accurate and reliable method to experimentally detect and assess the acrosome reaction in mouse spermatozoa, as FPSA not only labelled the acrosome, but also the whole head and the flagellum. Also, combining of fluorescent antibody labelling other spermatozoa proteins is the major plus point of using this technique (Lybaert *et al*., 2009).

Several studies have been carried out using a variety of different fluorescent lectins to confirm the specificity and justify the use of the peanut lectin. These included lectins from wheat germ (*Triticum vulgaris*), soybean (*Glycine max*) and lentil (*Lens culinaris*) but all failed to bind exclusively to the acrosome. A study using fluorescein isothiocyanate (FITC)-labelled peanut lectin from *Arachis hypogaea* as a fluorescent acrosomal marker was able to find to differences in spermatozoa or sperm quality and was quantified by flow cytometry (Engh *et al.,* 1991). Srivastava *et al*., 2013 in their research, also checked for acrosome integrity of spermatozoa by FITC-PSA. A total of 200 spermatozoa were counted per slide and spermatozoa devoid of PSA staining were considered as fully acrosome reacted whereas PSA positive as acrosome intact live.

2.4.2 Viability and Sperm Membrane Integrity Testing

Sperm membrane integrity and viability are the most important measures of sperm

quality (Foster *et al.*, 2011). For normal fertility, about 60% or more sperm need to be viable. Spermatozoa viability was also evaluated using the LIVE⁄DEAD spermatozoa viability kit (L-7011; Molecular Probes, Eugene, OR, USA) using fluorescent microscopy (Thiangtum *et al*., 2009). For viability testing, various stains are being routingly used like Eosin-Nigrosin, Giemsa stain, Trypan-blue stain, etc. Trypan-blue staining is one of the cheap and rapid technique to determine sperm viability. The percentage of live (unstained) spermatozoa are expressed as viability. Principle behind this test is that viable spermatozoa with intact cell membranes remain colorless excluding the stain whereas, dead cells stain blue colored (Strober, 1997, Valle *et al*., 2008).

Hypo-Osmotic Swelling Test (HOS Test)

Sperm membrane integrity is an important measure of sperm quality. Different techniques like flow cytometry using SYBR-14/propidium iodide (PI) stain, an automated cell counting device using PI stain, eosin-nigroson stain, hypo-osmotic swelling test (HOS) etc. are frequently used to estimate sperm membrane integrity (Foster *et al*., 2011). Functional integrity of buffaloes and bulls spermatozoa is often evaluated by hypo-osmotic swelling test (Mehmood *et al.,* 2009). The principle of the HOS assay is based on fluid transport across the sperm tail membrane under hypo-osmotic conditions until equilibrium is reached and due to this influx of fluid, the tail expands and bulges in different patterns, considered as hypo-osmotic response which can be readily identified with a phase-contrast microscope. An undamaged sperm tail membrane permits one-way passage of fluid (water) into the cytoplasmic space by osmosis making such space swollen, and the generated pressure makes tail fibres curl, while the damaged or chemically inactive tail membrane allows fluid to pass in-and-out across the membrane freely without generating any pressure. Cytoplasmic swelling and curling of the tail do not occur. Thus, the resultant swelling of the tail means presumably normally functioning spermatozoa with an intact membrane (Hossain *et al*., 2010).

The advantage of the HOST is that it is very simple and repeatable (WHO, 1992). The test was first introduced to evaluate hypo-osmotic swellings for investigating membrane integrity of human spermatozoa (Hossain *et al*., 2010). And different patterns of tail swelling observed in this condition makes the HOS test useful in providing valuable information on sperm viability.

In a study, membrane functional integrity was assessed by the hypo-osmotic swelling test. The technique consisted of incubating 30 µl of diluted semen with 100 µl of hypo-osmotic solution (100 mOsm/Kg) at 37°C for 15 min. The samples were then fixed in 2% glutaraldehyde in buffer. One hundred cells were counted by sample for each parameter and the proportion of spermatozoa with swollen tails was considered as HOS positive (Monton *et al.*, 2015).

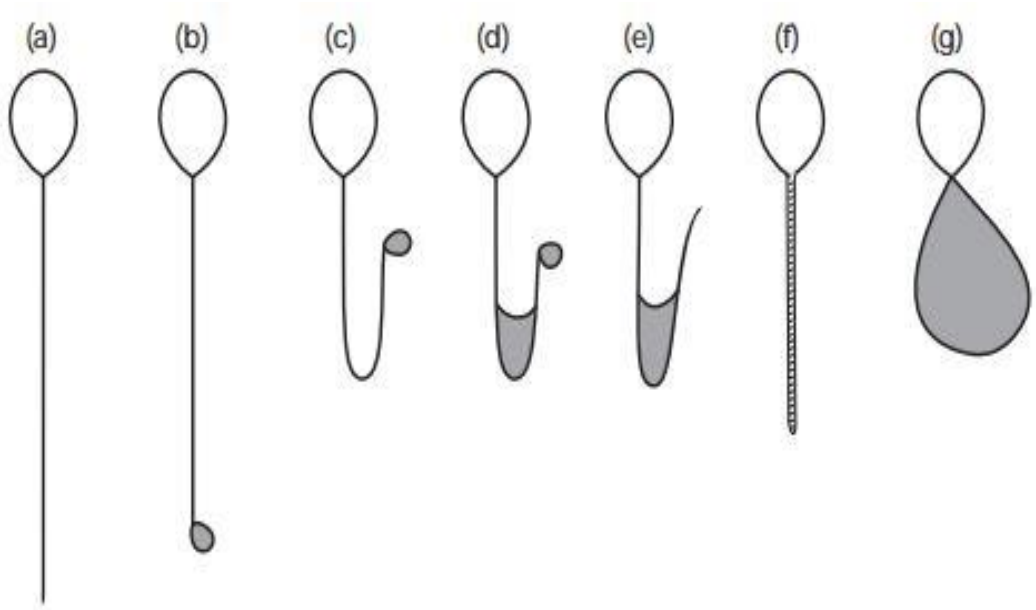


Figure 2. 5: Schematic representation of typical morphological changes of human spermatozoa subjected to hypo-osmotic stress: a = no change; b-g = various types of tail changes. Tail region showing swelling is indicated by the hatched area (Jeyendran *et al*., 1984).

2.5. Identification of Estrous Cycle of Mice for *in vivo* fertilization

For various research works *in vivo* fertilization, protocols are followed using mouse as an experiment model. The laboratory mice have been extensively used in many fields of medical and biological research because of their body size, ease of manipulations and breeding characteristics (Kashiwazaki *et al.,* 2009). Female mice attain sexual maturity by 5 to 8 weeks with their productive breeding life of 7-8 months. Gestation period is of 18-21 days and litter size vary from 2 to 12+ (Silver *et al*., 2000).

The reproductive cycle in rodents called estrous cycle is short, precise and lasts for 4-5 days comprising four phases namely proestrus, estrus, metestrus and diestrus. Estimation of the estrous cycle is crucial to assess the functioning status of the female reproductive system in mice. At around 26th day of birth, reproductive period of mice begins with the opening of vagina. Vaginal opening is an apoptosis mediated event and is an important secondary sexual character in mice and can be used as an external indicator for the puberty onset, identified by simple visual inspection (Ekambaram *et al.,* 2017).

Beginning of estrous cycle i.e. proestrus phase begins when a new batch of eggs reach maturity within ovarian follicles that are ripe and large. At this phase female usually show a bloated vulva with an open vagina. Estrus phase begins with the ovulation of fully mature oocytes in which mice remains in an extended state with an open vagina and lasts for 15 hours. This is the phase in which females are maximally receptive to male advances. Then follows the metestrus phase in which mature eggs move through the oviducts and into the uterus and the vagina is closed at this stage. At the end of metestrus, if there has

been a successful copulation, induces hormonal changes that prepare the uterus for a pregnancy. If pregnancy has not occurred, the metestrus phase is ultimately followed by the last phase of the estrous cycle, diestrus. At this phase unfertilized eggs are eliminated with the vagina at a minimum size, and new follicles begin to undergo a rapid growth for the next ovulation (Silver *et al*., 2000).

In the proestrus phase there is an increase in 17-β Estradiol levels and small surge in Prolactin and with the elevated Estradiol level, Luteinizing Hormone (LH) and Follicle Stimulating Hormones (FSH) are released from anterior pituitary into the circulation. And the peak in FSH levels signals ovulation and entry into the estrus stage, in which there is sharp decline of 17-β Estradiol levels and Prolactin levels peak. It is the period of heat or sexual receptivity. Metestrus and Diestrus are homologous to the human early and late secretory phases respectively in which progesterone levels peak (Ekambaram *et al.,* 2017).

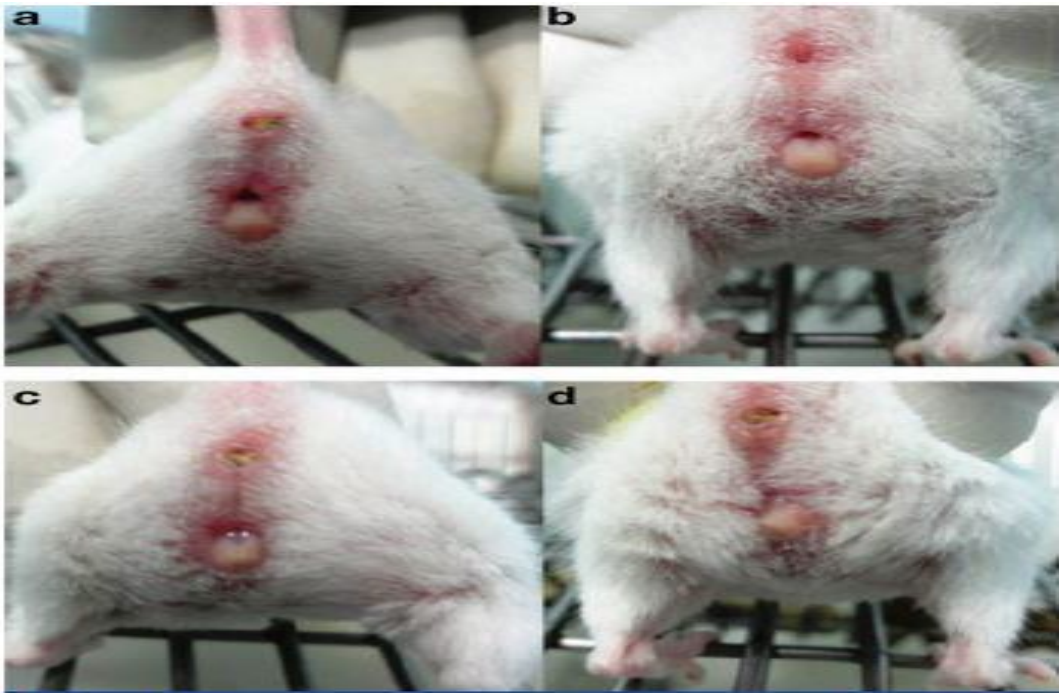


Figure 2. 6 (a-d): Appearance of vagina in different phases of estrous cycle. a-Proestrus; b-Estrus; c-Metestrus; d-Diestrus (Ekambaram *et al*., 2017).

2.6 Immunocontraceptives

Immunocontraception is non-hormonal form of contraception, based on the same principles as disease prevention through vaccination. The advent of "immunocontraception" represents the first true approach to the development of family planning methods. It involves the administration of contraceptive vaccines (CVs) or preformed antibodies that induces an adaptive immune response against some essential element of the reproductive process, thus preventing pregnancy or causing an animal to become temporarily infertile. There are three major targets involved in the development of CVs, namely, gonadotropin releasing hormone (GnRH), follicle stimulating hormone

(FSH), and luteinizing hormone (LH). Other targets of CV are, human chorionic gonadotropin hormone (hCG), zona pellucida (ZP) and sperm antigens. Immunocontraceptives may overcome the demerits of currently used hormonal contraceptives in terms of safety, target specificity, lack of endocrine metabolic side effects and no requirement of an implant or device or surgical intervention. Contraceptive of this type is not currently available for human use (Dwivedi & Yadav, 2012, Kaur & Prabha, 2014).

The history of immunocontraception started with the 'spermatozoa', as the first target. It started earlier from 1899 when Karl Landsteiner from Austria and Serge Metchnikoff from Russia, demonstrated that injection of sperm from heterospecies can produce antibody response. In 1929, Morris J. Baskin, a Denver-based surgeon and the clinical director of the Denver Maternal Hygiene Committee, used human sperm to produce reversible sterilization in fertile women. Subsequently, there are few preliminary reports that used bull sperm instead of human sperm to induce antibodies in women for contraception. Between 1950 and 1970, the sperm immunization studies regained impetus for fertility regulation. The recent Nobel Prize winner of Physiology and Medicine in 2010, Dr. Robert Edwards, was also very interested in immunocontraception (Metchnikoff & Baskin, 2011).

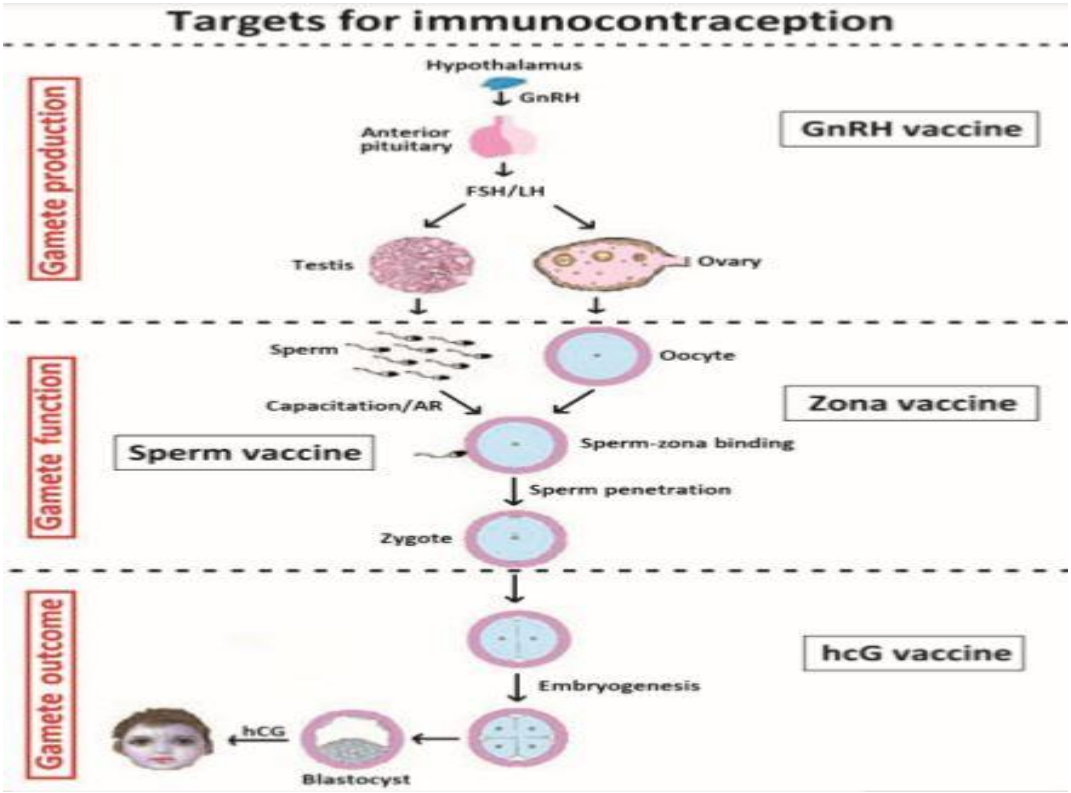


Figure 2.7: Schematic model indicating various targets that are being explored for the contraceptive vaccine development. These include targeting gamete production [gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH)], gamete function [zona pellucida (ZP) proteins of the oocytes and sperm antigens], and gamete outcome (human chorionic gonadotropin [hCG]) (Metchnikoff & Baskin, 2011).

CHAPTER 3. MATERIALS AND METHODS

3.1. Laboratory Setting and Study Site

All the works for the thesis was conducted in the Research Laboratory of Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

3.2 Collection of Plant Material and Extract Preparation

Leaves of *Artemisia vulgaris* (before the onset of flowering) were collected from plants growing wild around the Tribhuvan University, Kritipur, Kathmandu and only healthy leaves were selected. The selected leaves *of A. vulgaris* were subjected to washing under running tap water to remove the surface contamination. The plant material was dried in air in shade for overnight followed by oven drying at 60°C. After complete drying leaves were then grinded to fine powder form using a mechanical blender. Fine powdered form of leaves (approximately 100 g) was mixed with 80% ethanol as a solvent in 1:3 ratio in a dark bottle. Then the mixture was kept in shaker for three overnights and after incubation, the extract was filtered through 0.2 micron Whatman filter paper. The filtered extract was then preserved for further use in airtight dark bottle in refrigerator.

3.3 Plant Extract Concentration

For finding the concentration, 5 ml of extract was taken in a clean Petri plate and left for complete drying in hot air oven at 110°C.

Weight of empty plate = V1

Weight of plate+ 5 ml extract = V2

Weight of plate + extract after vaporization = V3

Net weight of extract = V3-V1

Concentration of extract = (V3-V1)/5 ml

And from this stock extract, different concentrations were prepared by diluting with DMEM medium.

3.4 Spectrophotometric Reading of the Extract

Scanning spectrophotometer (Thermo Scientific) was used for reading absorbance of the extract. Extract dilution of 1:50, 1:100 and 1:500 were prepared and absorbance noted with wavelength ranging from 200 nm- 800 nm. All those wavelength versus absorbance records were taken in photographs and also those data were recorded manually. Also, the peak points at which wavelength showed the maximum absorbance were noted.

3.5 Mice Purchasing and Handling

Swiss albino male and female mice of 8-12 weeks were purchased from the Department of Plant Resources (DPR), Thapathali, Kathmandu. They were housed in our animal lab

house in polypropylene cages and maintained on a 12 h light: 12 h dark cycle, controlled temperature of 24-28°C, with water and solid pellet food and were handled by following approved protocol.

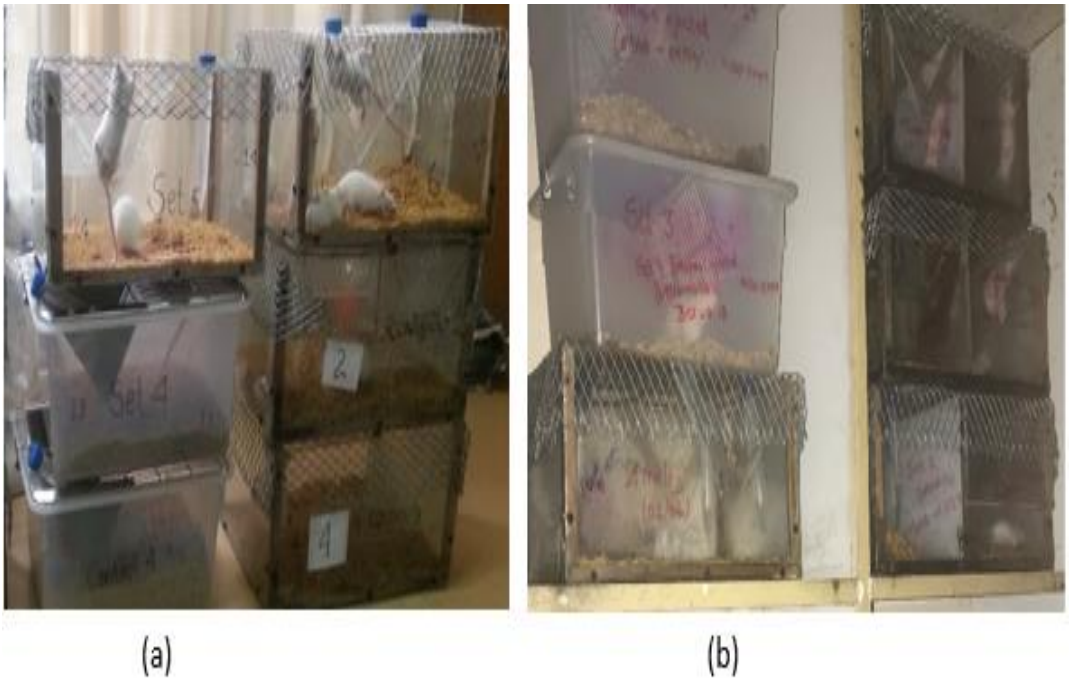


Figure 3. 1 (a) and (b) Mice housed in animal lab house of Central Department of Biotechnology.

3.6. Sperm Collection, Culture and Treatment

After one week of acclimatization, mice were used for the experiment. Mice were sacrificed using cervical dislocation method and dissection performed with sterile dissection equipment's. Testes were located and excluding fat bodies wrapped around it, both caudal epididymis were excised and immediately placed in 5 ml of DMEM (Dulbecco's Modified Eagle's Media) supplemented with 5 mg/ml BSA, 15-20 mM HEPES buffer. Epididymis were minced and sperm samples were collected. For all experiments the sperm concentration was adjusted to 10^6 cells/ml. For studying sperm function assays, treatment of spermatozoa with various concentrations of herbal extracts was done for duration of capacitation period (2 h).

Various concentrations of extract were prepared by mixing required volume of plant extract and making the final volume of 1 ml with media.

Table 3.1: Various concentrations of the extract with media and 80% ethanol.

S.N.	Extract concentration (µg/ml)	Extract volume (µl)	Media volume (µl) (DMEM+HEPES+BSA)
1	0	0	1000
2	25	0.61	999.39
3	100	2.5	997.5
4	400	9.85	990.15
5	500	12.32	987.68
6	800	19.70	980.29
7	1000	24.64	975.36
8	2000	49.26	950.74
9			80% ethanol volume (µl)
10	1000 (Vehicle control)	24.64	975.36
11	2000 (Vehicle control)	49.26	950.74

3.7 In-Vitro Sperm Function Tests

For studying the contraceptive effects of ethanol extracts of *Artemisia* leaves, *in vitro* cultured mouse spermatozoa were treated with various concentrations of extracts. The effects of the extract on various sperm functions were analyzed as described below:

3.7.1 Effect of Extract on Sperms Acrosome Integrity

To have idea on how extracts effect on mice spermatozoa, first of all effect was observed on acrosome of mice spermatozoa. Sperm samples were treated with various concentrations of extract and effect was observed. For finding extract effect on acrosome integrity, two staining techniques were followed.

3.7.1.1 Coomassie Staining of Mice Spermatozoa

Sperm Isolation, Treatment and Incubation

First of all, various concentrations of plant extract (25 µg/ml, 100 µg/ml, 400 µg/ml, 500 µg/ml, 800 µg/ml, 1000 µg/ml and 2000 µg/ml) were prepared by mixing required volume of extract on media (Table 3.1). Media without extract was prepared as a control and also replacing extract with 80% ethanol on media, (vehicle control) was prepared for checking the effect of ethanol on acrosomes. And then those prepared samples were incubated in incubator at 37°C for two hours for media stabilization with the plant extract. Within this

two hours of incubation, sperm sample was collected as described above. Prepared spermatozoa sample on media was centrifuged at 300 rpm for 1 minute just to sediment the unwanted tissue particles. Supernatant containing spermatozoa were collected. Then 100 µl of sperm samples were added to each prepared different concentration of extracts and mixed properly.

Incubation was performed for two hours at 37°C and then 50 µl of 40% formalin (final 4%) was added to each eppendrof tube and left at room temperature for 15 minute. Centrifugation was done at 12,000 rpm for 3 minute. Discarding the supernatant, pellet with around 100 µl of media was preserved and re-suspended. A thin layer of saliva was applied on coverslips to improve the sperm cell attachment. After complete drying, 50 µl of sperm suspension was applied on each coverslip. After 2 min of sedimentation the remaining fluid was removed and the coverslips were allowed to air dry. They were stained with Coomassie Brilliant Blue (CBB G-250) for 5 minutes and then washed properly. Finally after complete drying the coverslip were attached on slides with cells side up and then observed in microscope under 40X and 100X lens. Spermatozoa with acrosome and without acrosome were counted for finding the effect of extracts on acrosome integrity (Larson & Miller, 1999).

Acrosome integrity (%) = (Spermatozoa with intact acrosomes / Total Number of spermatozoa counted) x 100%

3.7.1.2. FITC Peanut Agglutinin (FPNA) Staining

Sperm isolation, treatment and incubation were done as described above.

Incubation was performed for two hours at 37°C followed by centrifugation at 12,000 rpm for 3 minute. Then clean coverslips were taken and thin smear of saliva was made for enhancement of attachment of sperm cells on coverslip. After complete drying of the coverslip, 50 µl of sperm suspension of fairly good concentration was added and allowed to stand for 3-5 minutes. Then the unattached spermatozoa were removed by washing with 1X PBS and coverslips were flooded with 4% formalin (made in PBS) for 30 minutes to 1 hour. After washing with PBS, the coverslips were treated with 1% Tween 20 (made in PBS) for 30 minutes at room temperature. Tween is poured off and then 50 µl of 250X diluted FPNA was added followed by incubation at 37°C for 1 hour (covered with aluminum foil to avoid light). After then removing FPNA, 50 µl of Ethidium Bromide (EtBr) (100 µg/ml) was added and incubation for 10 minutes at room temperature in dark. EtBr was removed and washed with PBS. Then a drop of 10% glycerol (in PBS) on a clean grease free slide was taken and the labelled coverslips were mounted on individual slides by inverting on the mounting drop. Flowingly the edges of coverslips was sealed with DPX (mordant). Observation of spermatozoa was done under blue channel of epifluorescent microscope and the sperm cells with acrosome and without acrosomes were counted.

Acrosomes appeared green as stained by FPNA and nucleus appeared red. Acrosome integrity were calculated as below:

Acrosome integrity (%) = (Spermatozoa with intact acrosomes / Total Number of spermatozoa counted) x 100%

3.7.2. Effect of Extracts on Spermatozoa Viability

Viability and membrane integrity of spermatozoa, were studied by staining with Trypan-blue staining and hypo-osmotic swelling test.

3.7.2.1. Trypan-blue Staining

Sperm isolation, treatment and incubation were done as described above.

Incubation was performed for two hours at 37°C followed by centrifugation at 12,000 rpm for 3 minute. Supernatant is discarded and the pellet with around 20-30 µl of media was preserved and immediately kept in ice bucket. Then from each tubes, 5 µl of sperm was dropped in a clean grease free slide and mixed with 5 µl of 0.4% Trypan-blue stain. The mixture was covered with the coverslip were observed under 100X objective of a microscope (Strober, 1997). The viability of sperm cells were calculated as:

Spermatozoa viability (%) = (Viable spermatozoa / Total Number of spermatozoa counted) x 100%

3.7.2.2. Hypo-Osmotic Swelling Test (HOS Test)

Preparation of HOS solution:

0.735 g Sodium citrate dehydrate and 1.351 g D-fructose were taken and mixed in 100 ml of distilled water.

Sperm isolation, treatment and incubation were done as described above.

After incubation, centrifugation was performed for 3 minute at 1,800 rpm and pellets with around 100 µl sample was collected. For control sample, sperm sample and hypo-osmotic solution was added in 1:10 ratio whereas for different concentrations of extracts, only 100 µl of sperm sample was added. Then 2 hours incubation was performed for extracts samples whereas only 1 hour incubation for control sample.

After 1 hour of incubation at 37°C, centrifugation was done at 2,000 rpm for 4 minute and leaving around 100 µl sample a bottom remaining supernatant was discarded. 1-2 drops of sperm sample was placed on slide, covered by coverslip, edges was sealed by DPX and then tail swellings of spermatozoa were observed under 40X microscope. For sperm samples treated with different concentrations of extracts, after 2 hours incubation, centrifugation was performed for 3 minute at 1,800 rpm and pellets with around 100 µl sample was kept. HOS solution was added as like for control in 1:10 ratio followed by

incubation for 1 hour followed by centrifugation at 2,000 rpm for 4 minute and similarly like that for control, slide was prepared and sperm cells were counted with straight or different types of tail curling (Jeyendran *et al.,* 1984).

Spermatozoa viability (%) = (Spermatozoa with swollen tails / Total Number of spermatozoa counted) x 100%

3.8 Protein Profiling by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For analyzing the effect of extract on sperm proteins, SDS-PAGE and silver staining was performed. 10% SDS polyacrylamide gel were casted in tray by following a standard method. The required glass plates were assembled and the prepared 12% resolving gels (22.2% Acrylamide, 0.6% Bis-acrylamide, 1M tris/HCL pH 8.8, 10% SDS, 10% APS, TEMED, distilled water) was poured between glass plates and left for polymerization. APS and TEMED were added just before pouring. As soon as the gels got polymerized, stacking gel (22.2% Acrylamide, 0.6% Bis-acrylamide, 1M tris/HCL pH 6.8, 10% SDS, 10% APS, TEMED, distilled water) was poured over resolving gel and again left for polymerization by keeping comb over.

Within this period, 30 µl of concentrated sperm suspension (treated with 1000 µg/ml of extract) was mixed with 8 µl of 5X sample buffer (1M Tris HCl, pH 6.8, 25% glycerol, 10% SDS, 5% 2-mercaptoethanol, 2% bromophenol blue in ethanol) and heated in a boiling water for 5 minutes. The protein extracts were spinned for 5 min. Then after polymerization of gels, 10 µl of samples and protein marker were loaded to 10% PAGE precast gels and electrophoresed with tris-glycine buffer. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (CBB) (2.5 g CBB R-250, 450 ml Methanol, 100 ml Glacial acetic acid and made volume of 1 liter with distilled water) and left overnight. On the next day, excess stain was removed by washing with destaining solution (300 ml Methanol, 100 ml Acetic acid and made volume of 1 liter with distilled water) and then gels were photographed.

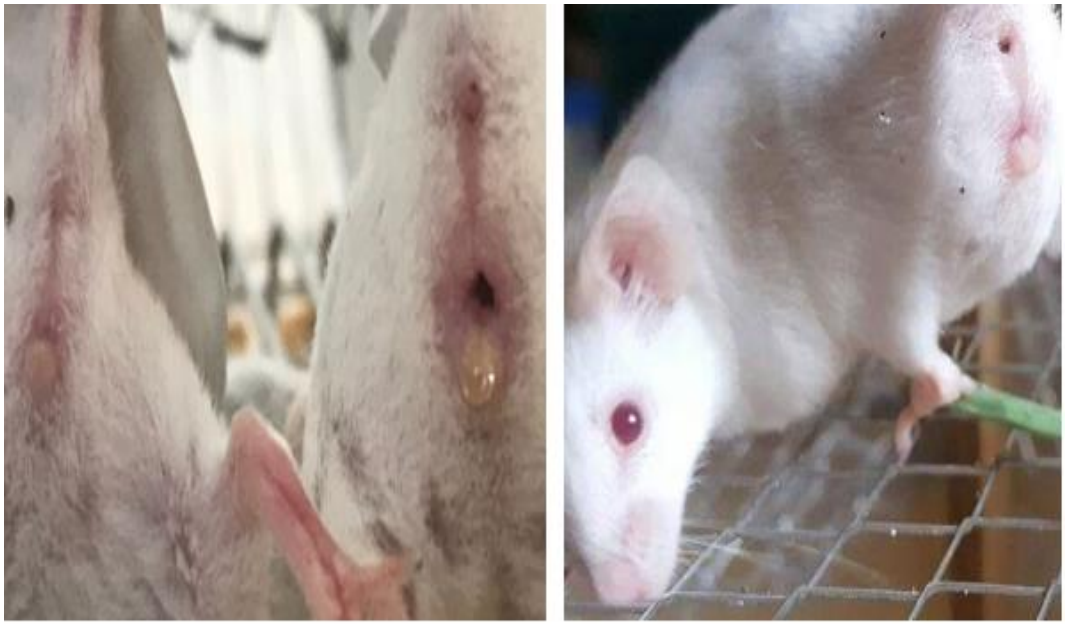
The gels were further processed for silver staining. The gel previously stained with Coomassie was used. So without fixation, Coomassie stain was removed as far as possible by destaining solution as described before. It was followed by washing the gel in H_2O for 30 minutes. Then the gel was sensitized in 0.02 % sodium thiosulfate (0.04 g $Na_2 S_2O_3$, 200 ml H_2O) for 1 min and washed for three times in water for 20 sec. Incubation of gel was performed for 20 min in 4°C cold 0.1% silver nitrate solution (0.2 g $AgNO_3$, 200 ml H_2O, 0.02% formaldehyde) and after washing in water for 20 seconds for three times was placed in a new staining tray. The gel was then developed in 3% sodium carbonate (7.5 g Na_2CO_3 in 250 ml H_2O) and 0.05% formaldehyde in which 125 µl of 35% formaldehyde was added just before use. The developer solution was changed immediately when it turns

yellow and was terminated when the staining was sufficient. Then after the gel was washed for 20 seconds in water followed by terminating the staining in 5 % acetic acid for 5 minutes. The gel was again washed in water for three times for 10 minutes to ensure complete removal of acetic acid and then was only visualized.

3.9 In-vivo Fertilization

To perform *in vivo* fertilization, female mice of 6-8 weeks old and male of 8-10 weeks old were used. Before experimentation, they were caged in our animal lab house for 1 weeks for acclimatization providing them with adequate foods and water. After 1 weeks of acclimatization, estrus cycle was checked each day in each female mice.

Experimentation was performed on five sets of mice. For each set of experiment five mice were used for extract treatment and five as a control. Estrus cycle was regularly monitored for female mice. On the day in which they were found to be in estrus cycle, 24.64 µl of 1000 µg/ml of *Artemisia* extract was injected through vagina while for control no any treatment. Immediately, extract injected female mice were caged with male mice in 2:1 ratio. Extract injection was regularly performed for three consecutive days on the evening time. On the following morning regularly for three days (within 10-12 hours post mating), the female mice were checked for the evidence of copulation. Mice with closed copulatory plug and waxy, whitish substances around vaginal area were found to be gone successful mating. Also microscopy was performed with the vaginal swab for the presence of spermatozoa on successful mated mice. And on the following morning of the last dose treatment i.e. fourth day morning, female mice were separated and that day was considered day one of pregnancy. Mice were routinely fed throughout the gestation period of 21 days and after delivery, number of pups delivered by control mice and extract injected mice were compared (Suryawanshi, 2011).



(3.2) (3.3)

Figure (3. 2) Mice in the right side with vaginal opening in estrus cycle, in the left side not in estrus cycle; (3.3) Mice with closing of copulatory plug and presence of waxy, fluidy substances outside vagina revealing successful mating.

3.10 Statistical Analysis

Each experiment were done in triplicate repeats. Statistical processing of data for the calculating mean, standard deviations, standard errors, and for drawing graphs and interrelationship curves, Microsoft Excel program was used. Data are expressed as Mean ± S.E. For statistical analysis of the data, two-tailed *t* test was employed wherever required. $p<0.05$ was considered significant and was calculated from the website given below.

https://www.medcalc.org/calc/comparison_of_means.php?fbclid=IwAR3Uo3Psut1u1k38yCOqwAp1mheuxrBIkaZnGY4Q24zv-cVCOgGohrY_kwQ

CHAPTER 4. RESULTS

4.1 Plant Extract Concentration

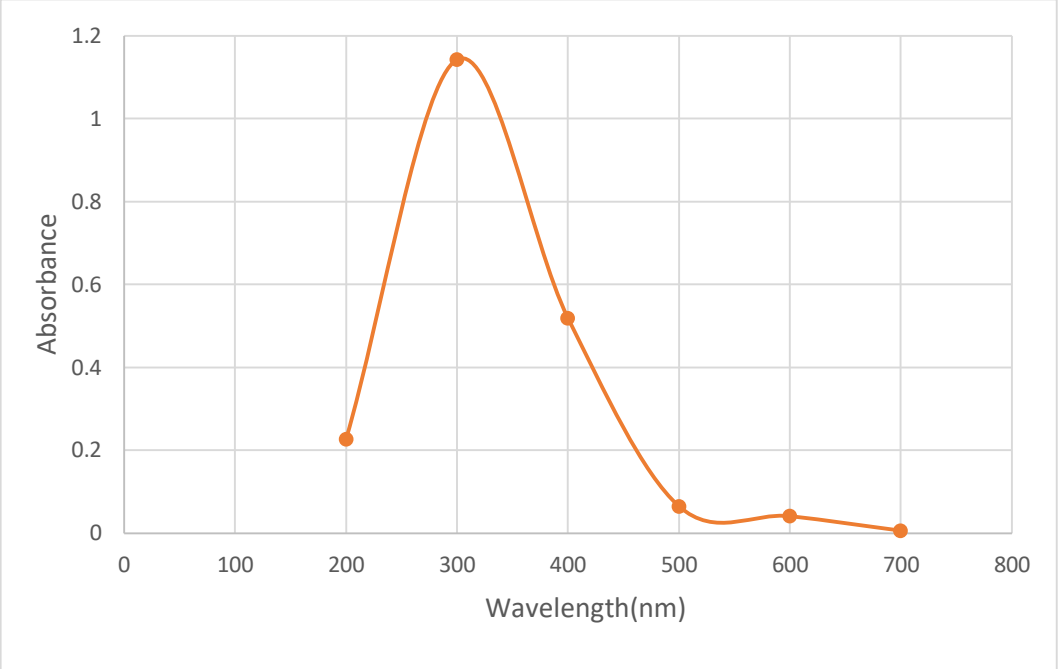
Powdered form of leaves sample of *Artemisia vulgaris* was extracted with 80% ethanol in 1:3 ratio and the concentration of the ethanol extract of the plant was found to be 40.6 mg/ml.

4.2 Spectrophotometric Reading of Ethanol Extract of *A. vulgaris*

Absorption spectrophotometer reading of ethanol extract was performed in scanning spectrophotometer with wavelength ranging from 200-800 nm. The extract was diluted with 80% ethanol in 1:50, 1:100 and 1:500 ratio with 80% ethanol and performed separately for all three dilutions. Absorption spectrum of ethanol extract shows that the peak value of the plant between 200-500 nm and the major peak was obtained at 300 nm with the absorbance 1.142 (Graph 4.1). The UV-visible spectroscopy was performed for general overview on presence of metabolites in the extract.

Table 4. 1. Absorbance of *A. vulgaris* extract sample of concentration 100 µg/ml.

Wavelength (nm)	Absorbance
0	-
100	-
200	0.226
300	1.142
400	0.519
500	0.065
600	0.041
700	0.006
800	0.000



Graph 4.1. Absorption spectrum of *Artemisia* leaf extract in 1:100 dilution with 80% ethanol.

4.3 Treatment with *Artemisia* Extract Causes Precocious Acrosome Exocytosis in Mouse Spermatozoa

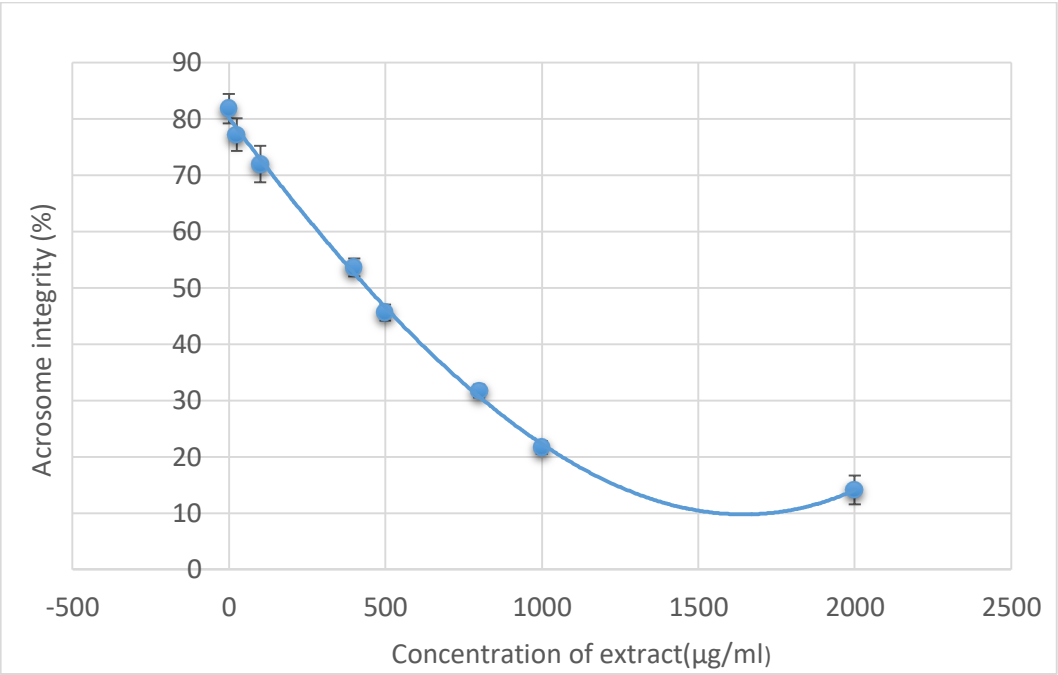
4.3.1. Coomassie Staining of Spermatozoa

Artemisia extract caused precocious acrosome reaction in mouse spermatozoa. The percentage of acrosome intact spermatozoa were estimated by counting 150-200 spermatozoa and acrosome integrity (%) was calculated in the control sample and treatment samples.

Effect of plant extract on acrosome integrity (%) of mouse spermatozoa was analyzed by staining with coomassie staining technique. In untreated control incubation for 2 h, the percentage of intact acrosome was 81.85% (Tab. 4.2). The plant extract caused high rate of acrosome reaction. The percentage of acrosome intact spermatozoa steeply decreased with increasing extract concentration from 25 µg/ml to 1000 µg/ml. At extract concentration 2000 µg/ml, only 14.09% spermtaoza showed intact acrosome was observed (Tab. 4.2). At higher concentration range of extract, beyond 1000 µg/ml, the percentage of acrosome reaction did not appreciably increase (Graph 4.2). Also compared to the control, integrity (%) was not found to be notably decreased in both vehicle controls (1000 µg/ml and 2000 µg/ml; Tab. 4.2).

Table 4.2. Acrosome integrity of mouse spermatozoa treated with different concentrations of *Artemisia vulgaris* extract.

S.N	Extract concentration (µg/ml)	Experiments (1) Acrosome integrity (%)	(2) Acrosome integrity (%)	(3) Acrosome integrity (%)	Mean	Standard Deviation	Standard Error
1	0	82.14	84.32	79.09	81.85	2.62	1.51
2	25	76.05	80.47	75.06	77.19	2.88	1.66
3	100	68.55	75	72.24	71.93	3.23	1.86
4	400	53.33	55.34	52.18	53.61	1.59	0.92
5	500	45.77	46.9	44.09	45.58	1.41	0.81
6	800	32.96	31.06	30.98	31.66	1.12	0.64
7	1000	20.5	21.68	22.73	21.63	1.11	0.64
8	2000	16.96	12.00	13.33	14.09	2.56	1.48
9	1000 (Vehicle control)	78.32					
10	2000 (Vehicle control)	75.90					



Graph 4.2. Acrosome integrity of mouse spermatozoa treated with various concentrations of *Artemisia* leaf extract. Each dotted point is a mean value from three readings taken. The vertical lines indicate the standard deviations.

Acrosome intact mouse spermatozoa displayed crescent-shape staining on the dorsal side of the head when stained with the protein dye Coomassie Brilliant Blue G-250 (Fig. 4.1, insert A) whereas spermatozoa with no or disintegrated acrosomes or spermatozoa that have undergone acrosomal exocytosis do not display such acrosomal crescent staining (Fig. 4.1, insert B). Around 80 % spermatozoa were found to be with intact acrosomes as observed in the control sample (Fig. 4.1). Similarly, in extract treated group, a greater number of spermatozoa displayed lack of acrosomal staining (Fig. 4.2 a and b).

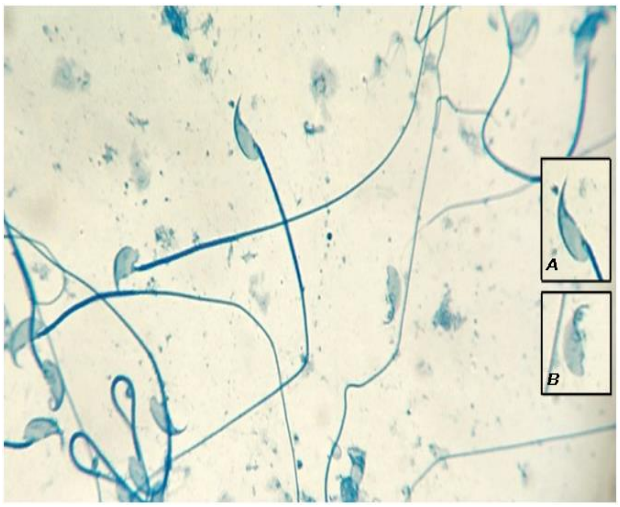
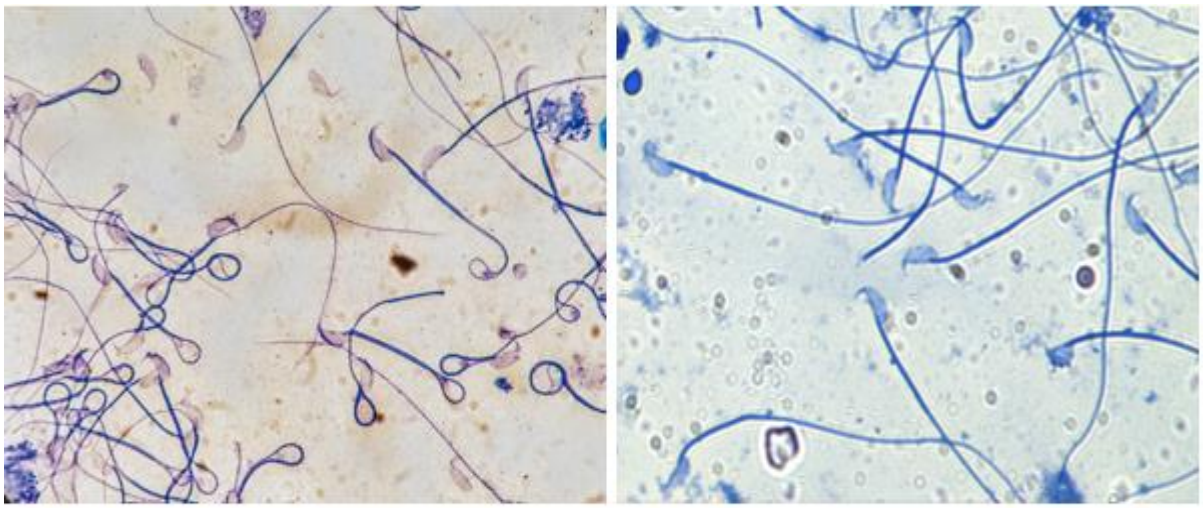


Figure 4. 1. Acrosome of mouse spermatozoa stained with Coomassie blue in control sample. Spermatozoa with intact acrosome show distinct blue-ridge on the dorsal (convex) surface of the head, at the curved tip and at the concave, upper head region (Insert A). Sperm head that were lacking in the acrosome did not show such staining (Insert B).



(a) (b)

Figure 4. 2 (a) Coomassie staining of spermatozoa treated with extract of concentration 500 µg/ml; (b) Spermatozoa treated with 2000 µg/ml concentration of extract.

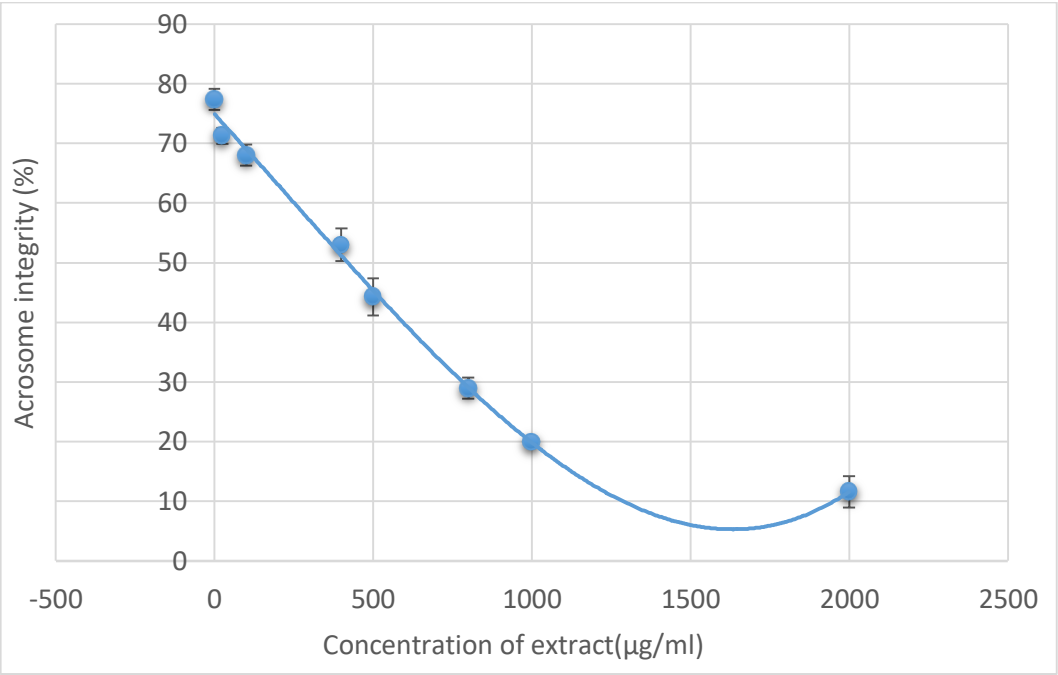
4.3.2. FPNA Labeling of Spermatozoa

Effect of extract on acrosome exocytosis in mouse spermatozoa was also studied by FPNA staining method. The results were very similar to those observed with coomassie staining. In untreated control sample, the percentage of intact acrosome was 77.36 % and was found to be steeply decreased with increasing extract concentration from 25 µg/ml to 1000 µg/ml. At extract concentration 2000 µg/ml, only 11.58% spermtaoza showed intact acrosome. Compared to the control, integrity (%) was not found to be significantly decreased in both vehicle controls (1000 µg/ml and 2000 µg/ml) having 75.67% and

71.28% respectively (Tab 4.3). But at concentration above 1000 µg/ml, the percentage of acrosome reaction did not appreciably increase (Graph 4.3).

Table 4.3: Acrosome integrity of mouse spermatozoa treated with different concentrations of *A. vulgaris* extract.

	Experiments					
	(1)	(2)	(3)			
Extract concentration (µg/ml)	Acrosome integrity (%)	Acrosome integrity (%)	Acrosome integrity (%)	Mean	Standard Deviation	Standard Error
0	77.46	79.08	75.55	77.3633	1.76	1.02
25	71.17	70.02	72.72	71.30	1.35	0.78
100	66.24	68.09	69.79	68.04	1.77	1.02
400	50.6	52.34	55.97	52.97	2.73	1.58
500	41.44	43.88	47.61	44.31	3.10	1.79
800	30.21	29.74	26.97	28.97	1.75	1.01
1000	19.66	20.4	19.59	19.88	0.44	0.25
2000	14.52	10.88	9.35	11.58	2.65	1.53
1000(Vehicle control	75.67					
2000(Vehicle control)	71.28					



Graph 4.3: Acrosome integrity of mouse spermatozoa treated with various concentrations of *Artemisia* leaf extract.

On staining of sperm cells with FPNA and ethidium bromide and observation under blue light of epifluorescence microscope, the spermatozoa were observed with green acrosome portion and red on nucleus. Control sperm sample without extract treatment were found to emit green fluorescence on the acrosome stained by FPNA stain and red fluorescent due to the staining of nucleus by Ethidium Bromide (Fig. 4.3 – 4.6). Acrosome loss was found to be increased in a dose-dependent manner of extract as number of fully or partially disintegrated acrosomes was found to be increased in extract treated with concentration of 2000 µg/ml (Fig. 4.6). Spermatozoa stained with FPNA were found to be very low whereas spermatozoa stained only with EtBr were found to be more in treatment with extract of 2000 µg/ml (Fig. 4.6).

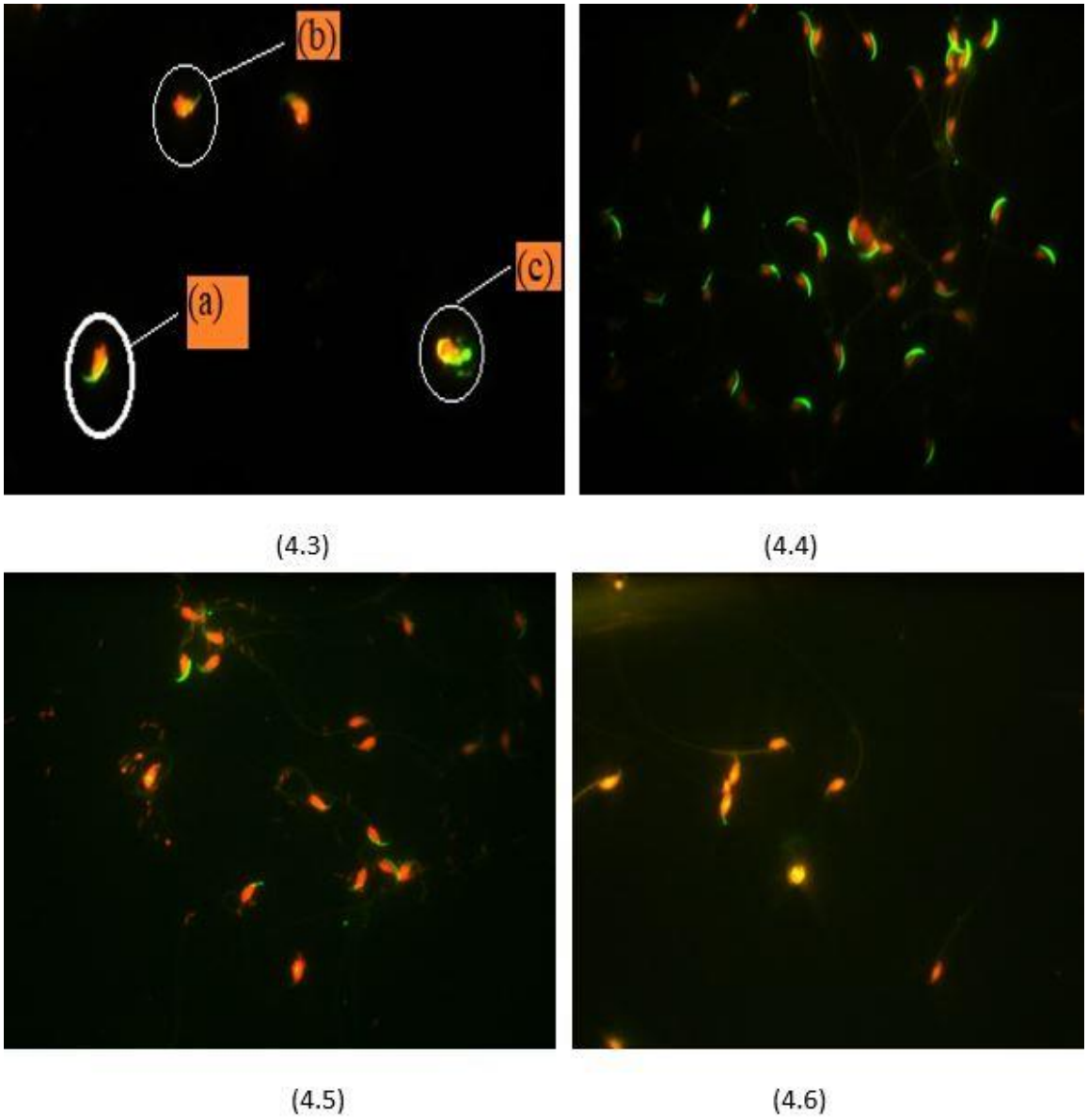


Figure (4.3) (a) Spermatozoa with intact acrosome on the dorsal part of the head; (b) Spermatozoa without acrosome and stained only by EtBr; (c) Spermatozoa with acrosome undergoing exocytosis due to treatment with plant extract; (4.4) FPNA staining of mouse spermatozoa without extract treatment (control) in which most of spermatozoa possess green, crescent shaped acrosome on the dorsal side and stained red on nuclei part with EtBr; (4.5) Spermatozoa treated with extract concentration of 400 µg/ml in which few are stained with FPNA and most of them are also with disintegrated acrosomes; (4.6) Spermatozoa treated with 2000 µg/ml concentration of extract.

The acrosome integrity of 81.85±1.51 in control untreated sample was found to decreased to 21.63±0.64 in spermatozoa sample treated with extract concentration of 1000 µg/ml and from 77.36±1.02 to 19.88±0.25 when stained with Coomassie and FPNA staining techniques respectively. Also as shown in the Table 4.4, p=0.0001 in comparison between control and extract treatment for both staining techniques, the extract showed significant effect on acrosome reaction.

Table 4.4. Prediction equations of acrosome assessment comparison between control and standardized concentration of extract by two staining techniques.

Parameters	Coomassie staining		FPNA staining	
	Control	Treatment (1000 µg/ml)	Control	Treatment (1000 µg/ml)
Acrosome integrity (Mean±S.E.)	81.85±1.51	21.63±0.64	77.36±1.02	19.88±0.25
p-value	0.0001		0.0001	

4.4 Treatment of spermatozoa with extract have minimal effects on viability and sperm membrane integrity

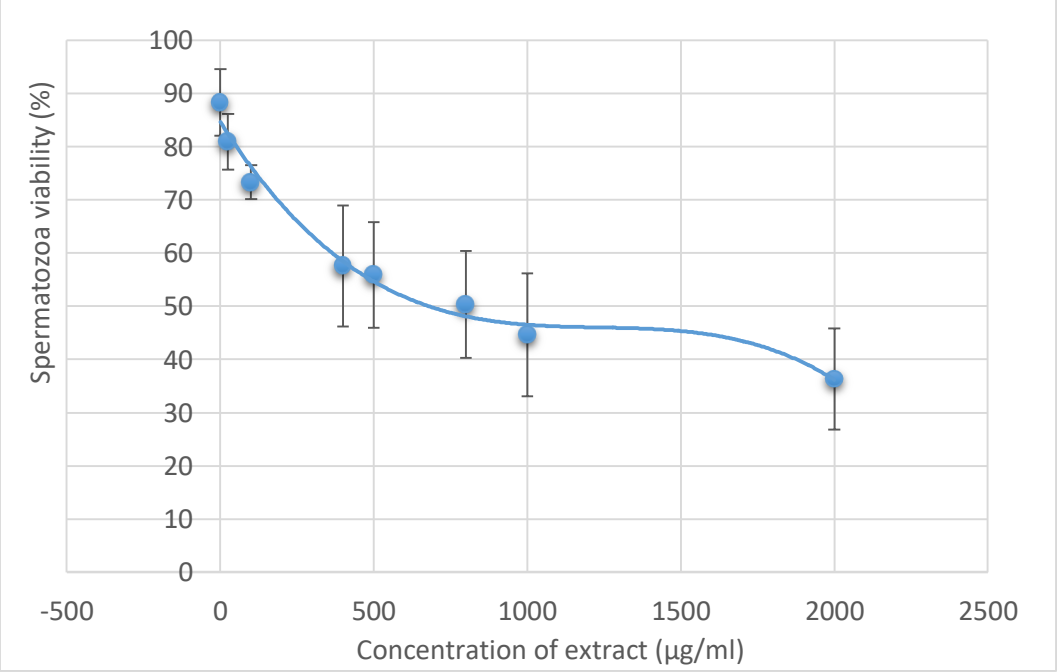
In comparison to the effect of ethanol extract of *A. vulgaris* on acrosome integrity, effects on sperms viability and membrane integrity were found to be low. In freshly isolated sample, almost 90% to 98% spermatozoa were viable in each experiments.

4.4.1 Trypan-blue staining

In untreated control sample after incubation for 2 h, the percentage of viable spermatozoa was found to be 88.29%. On treatment with different extract concentration, decrease in viability (%) of spermatozoa was found in a concentration dependent manner from 25 µg/ml to 2000 µg/ml. At extract concentration of 2000 µg/ml, only 36.27% spermtaoza were viable (Tab. 4.5). In spermatozoa treated with extract concentration of 1000 µg/ml, viability was found to be largely deviated from its mean with standard deviation value of 11.57 and also at concentration above 1000 µg/ml, viability was not found to be steeply decreased (Graph 4.4) signifying it the most effective concentration for effect on spermatozoa viability loss. Compared to the control, ethanol seemed to have minimal effects on spermatozoa viabilty as viability (%) was found to be decreased in both vehicle controls (1000 µg/ml and 2000 µg/ml) having 84.50% and 82.38% respectively (Tab. 4.5).

Table 4.5: Calculation of viability of spermatozoa treated with different concentration of extracts.

Extract concentration (µg/ml)	Experiments (1) Viability (%)	(2) Viability (%)	(3) Viability (%)	Mean	Standard Deviation	Standard Error
0	95.4	85.93	83.55	88.29	6.26	3.61
25	86.78	76.61	79.37	80.92	5.25	3.03
100	76.79	72.62	70.52	73.31	3.19	1.84
400	44.5	62.82	65.4	57.57	11.39	6.57
500	44.5	60.73	62.5	55.91	9.92	5.72
800	38.75	56.37	55.89	50.34	10.03	5.79
1000	31.29	52.23	50.32	44.61	11.57	6.68
2000	25.33	40.99	42.5	36.27	9.50	5.48
1000(Vehicle control, VC)	84.50					
2000(VC)	82.38					



Graph 4.4. Sperm viability against various concentrations of *Artemisia* leaf extract. The sperm viability has been estimated by Trypan-blue method.

On Trypan-blue staining, viable spermatozoa possessing intact cell membrane exclude stain and remained colorless (Fig. 4.7 a) whereas dead spermatozoa stained as blue color (Fig. 4.7 b). On control sample without extract treatment, most of the spermatozoa were viable as revealed by Coomassie staining. Most of the visualized spermatozoa were colorless (Fig. 4.8). On increasing the extract concentration on treatment, more spermatozoa were found to be stained by Trypan-blue as viability loss was increased due to effect of extract. In comparison to spermatozoa with extract treatment of 100 µg/ml concentration (Fig. 4.9), higher frequency of blue stained spermatozoa with Trypan-blue when they were treated with concentration of 2000 µg/ml (Fig. 4.10) signifying the effect of extract on viability loss in a concentration dependent manner.

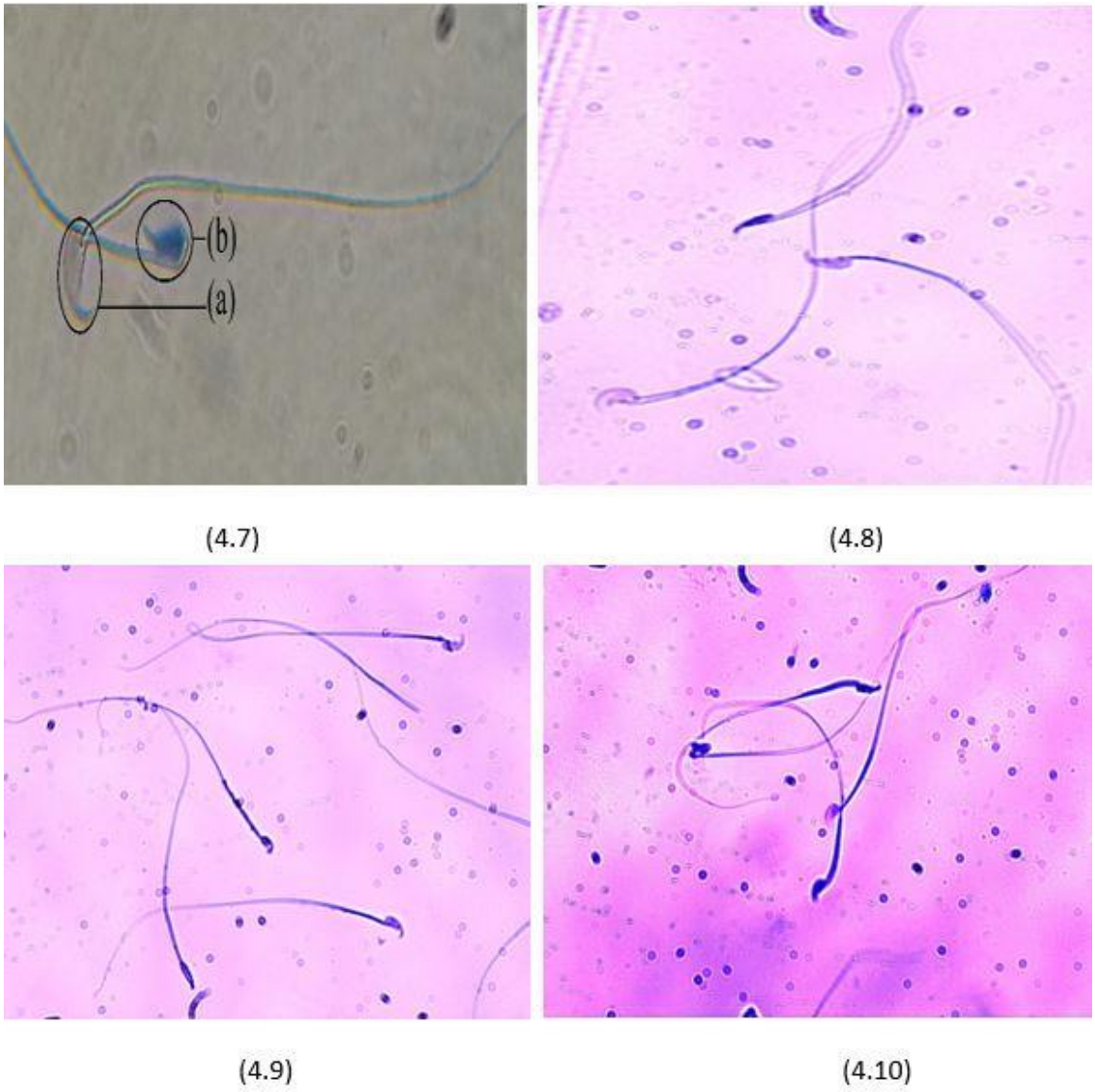


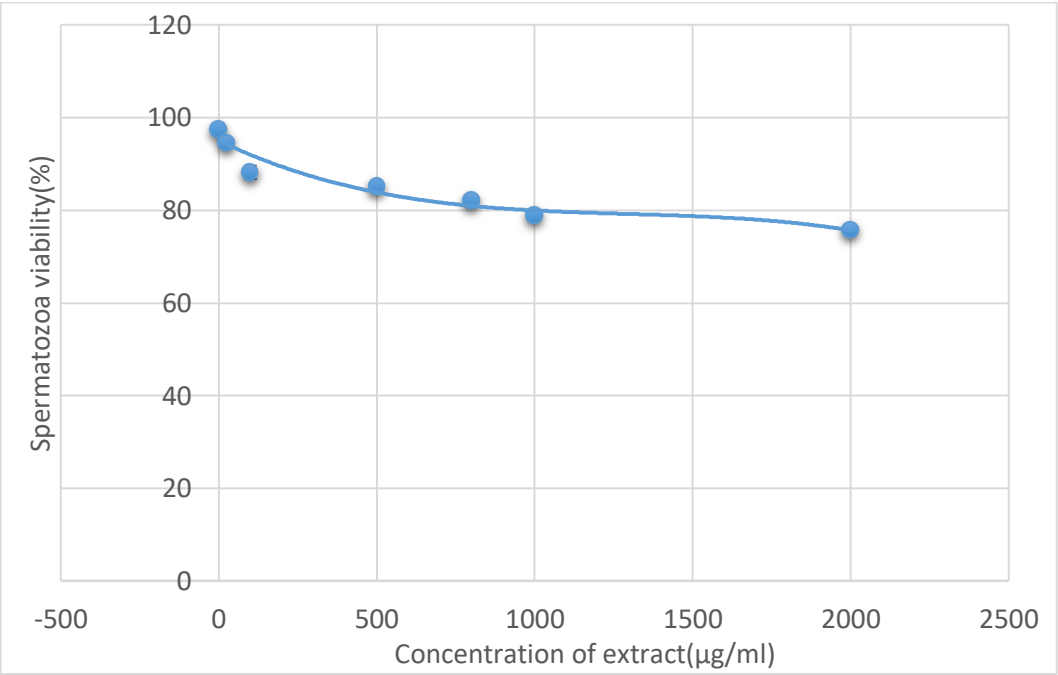
Figure (4.7-4.10). Trypan-blue staining of mouse spermatozoa (a) Viable spermatozoon with intact membrane unstained by Trypan-blue; (b) Dead spermatozoon stained blue by Trypan-blue; (4.8) Viability testing of spermatozoa without extract treatment, control sample; (4.9) Staining of spermatozoa treated with extract concentration of 100 µg/ml; (4.10) Viability test with extract treatment of 2000 µg/ml.

4.4.2 Hypo-osmotic swelling test

In addition to viability test, HOS test also determines membrane integrity of spermatozoa. In untreated control sample after incubation for 2 h, the mean percentage of viable spermatozoa with intact plasma membrane was found to be 97.43%. Extract concentration was ranged from 25 μg/ml to 2000 μg/ml in which the viability (%) was found to be 94.53% and 75.77% respectively (Tab. 4.6). With the increasing concentration of extract, viability was decreased in a similar trend for all treatment. But there was not a sharp decrease in viability with increasing concentration of the extract (Graph 4.5). And as the value did not differ much from the control, the extract is supposed to have minimal effect in the viability of spermatozoa.

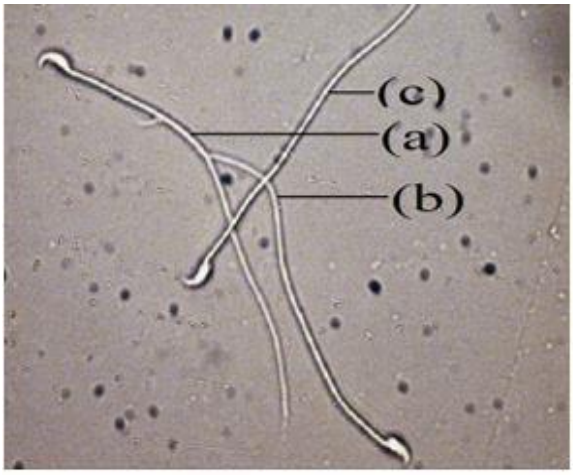
Table 4.6: Effect of Artemisia leaf extract on the membrane integrity of mouse spermatozoa, estimated by hypo-osmotic swelling test.

	Experiments					
Extract concentration (μg/ml)	(1) Viability (%)	(2) Viability (%)	(3) Viability (%)	Mean	Standard Deviation	Standard Error
0	98.49	97.45	96.36	97.43	1.0651	0.6149345
25	95.08	94.11	94.4	94.53	0.4979	0.2874601
100	89.81	87.69	87.06	88.18	1.44071	0.8317919
500	85.21	85.03	85	85.08	0.11358	0.0655743
800	80.9	81.66	83.46	82.00	1.31474	0.7590637
1000	79.81	78.7	77.98	78.83	0.9219	0.5322593
2000	76.7	74.81	75.8	75.77	0.94536	0.5458021



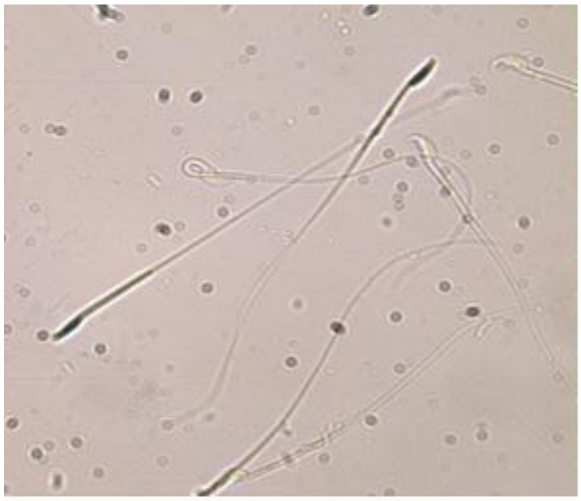
Graph 4.5. Sperm viability against various concentrations of *Artemisia* leaf extract by using hypo-osmotic swelling test. Curve plotted from the data shown in the Tab. 4.6.

In hypo-osmotic swelling test, viable spermatozoa with intact membrane when treated with hypo-osmotic solution, developed bent or different types of swollen tails (Fig. 4.11 a, b) but dead spermatozoa with damaged plasma membrane maintained straight middle-piece and tail (Fig. 4.11 c). In untreated control sample almost all spermatozoa were found to have different types of tail swelling (Fig. 4.12). On treatment with different extract concentration, viability percentage was found to be in decreasing trend with increasing number of dead spermatozoa with straight middle-piece or tail (Fig. 4.13 and 4.14).

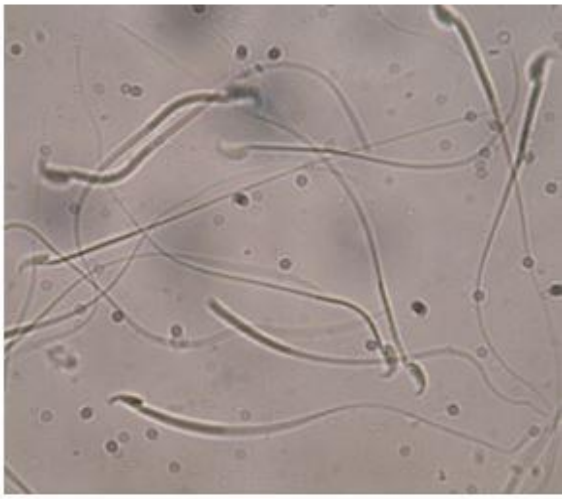


(4.11)

(4.12)



(4.13)



(4.14)

Figure 4.11. (a) and (b) Viable spermatozoa with swelling or bended middle-piece and tail; (c) dead sperm with straight body structure when kept in hypo-osmotic solution; (4.12) Different types of hypo-osmotic swelling of mice spermatozoa (control sample). (4.13) Hypo-osmotic swelling test of mice spermatozoa treated with 25 µg/ml concentration of plant extract; (4.14) Spermatozoa treatment with 2000 µg/ml) extract.

Spermatozoa viability was 88.29±3.61% in control untreated sample. This value was decreased to 44.61±6.68% in sperms sample treated with extract concentration of 1000 µg/ml (p=0.0045). Identical value was also obtained by Trypan-blue staining method. This signified that the extract has significant effect on sperms viability loss. In hypo-osmotic swelling test also spermatozoa viability was decreased from 97.43±0.61 to only 78.83±0.53 (p=0.0001) (Tab 4.7).

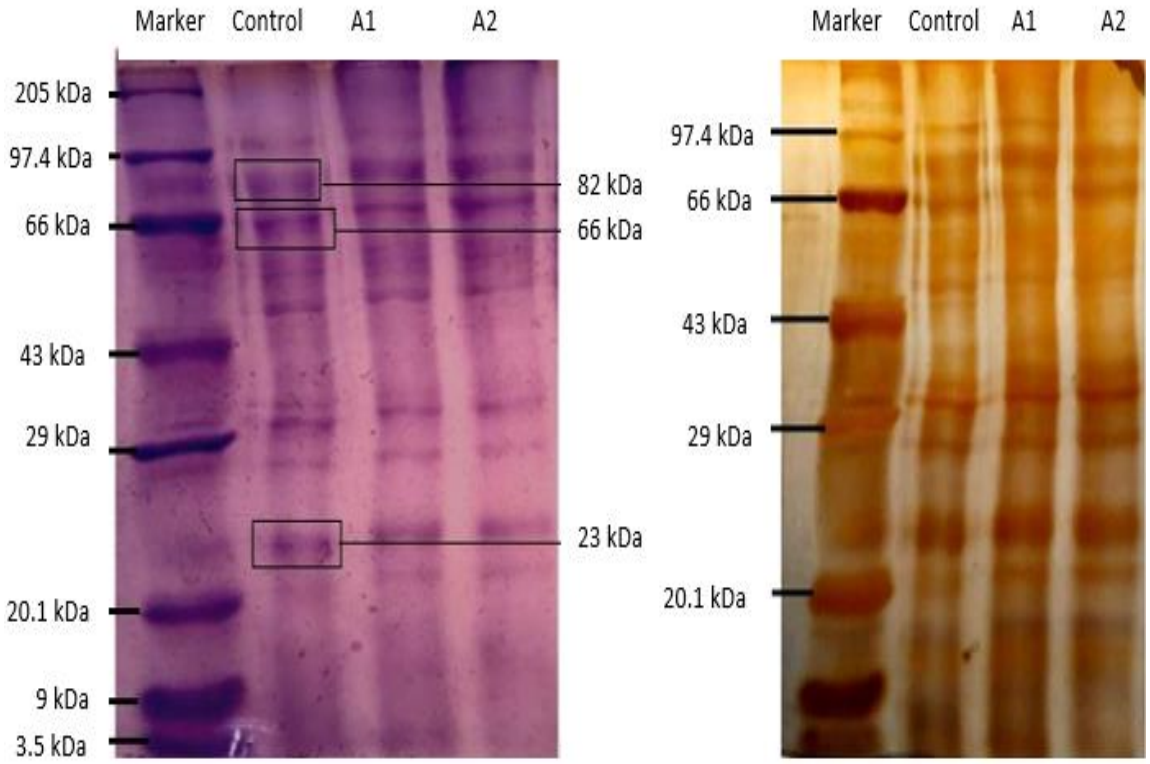
Table 4.7. Comparative spermatozoa viability loss due to extract treatment, evaluated by two staining techniques.

Parameters	Trypan-blue staining		Hypo-osmotic swelling test	
	Control	Treatment (1000 µg/ml)	Control	Treatment (1000 µg/ml)
Viability percentage effect (Mean±S.E.)	88.29±3.61	44.61±6.68	97.43±0.61	78.83±0.53
p-value	0.0045		0.0001	

4.5 Extract effect on spermatozoa proteins by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein expression pattern of sperms sample treated with two different concentrations of extract 500 µg/ml (A1) and 1000 µg/ml (A2) were compared with control sample without extract treatment. Fig. 4.15 a, showing the bands after Coomassie staining and Fig. 4.15 b, is of gel with bands visualized after performing silver staining. In both staining protocols GeNei Protein Molecular Weight Marker (3.5 kDa to 205 kDa) was used (Fig. 4.15 c). Protein marker was loaded on Lane 1, followed by extract untreated control sample on Lane 2 and Lane 3 and Lane 4 loaded with spermatozoa treated in DMEM media with extract concentration of 500 µg/ml (A1) and 1000 µg/ml (A2) respectively.

In both gels, there was observation of major 11 protein bands ranging between 23-82 kDa but most of the observed bands were not clear and distinct for their molecular weight. Among them also, protein bands of molecular weight 23, 66 and 82 kDa were observed to be most abundant proteins of the spermatozoa. And on comparison of bands between treated and control samples with marker, no distinct differences on expression pattern was observed. This might be due to changes in minor proteins may not be visible in total protein profile by both these performed staining techniques. But was seen some differences in protein intensities of some bands like as protein bands of molecular weight 30 kDa and 23 kDa in extract treated sample lanes which might be due to effect of extract treatment.



(a) (b)

Figure 4.15. Protein profiling of untreated control and treated mouse spermatozoa (a) PAGE electrophoresis stained with Coomassie staining and (b) Silver staining; (A1) spermatozoa treated with 500 µg/ml extract, and (A2) spermatozoa treated with 1000 µg/ml of extract.

4.6 Extract have significant effects on *in vivo* fertilization

The effect of the extract on *in vivo* fertilization rate of mice was performed in five sets of mice. After delivery, no. of the litters born were counted from each mice in each experiment and litter size was compared between control and extract treated mice. In all experiments in extract untreated control mice, number of litters born was found to be either 9 or 10 whereas number of litters delivered was reduced to 5 in extract treatment mice (Tab. 4.8). Among the five sets used for the experiment, sample injected mice of the first set did not give any birth.

Table 4.8. Comparison of number of mice births in control and extract treated mice (24.64 µl of 1000 µg/ml concentrate extract, intravaginally injected).

Set	Number of litters born(control)	Number of litters born (treatment)
1	9	0
2	10	8
3	9	7
4	10	5
5	9	5

The mean of number of pups' birth was found to be decreased from 9.4±0.21 in untreated control mice to 5±2.22 in treated mice. The p-value of 0.0137 was obtained from the performed experiments in five sets of mice which was less than chosen significance level (α) 0.05 suggesting that the observed data is sufficiently inconsistent with the null hypothesis and so null hypothesis was rejected. And also as t-value is greater than critical value (Tab. 4.9), there seems significant difference between control and plant extract treatment in mice in *in vivo* fertilization.

Table 4.9: Prediction equations of the effect of extract on *in vivo* fertilization of mice between control and standardized concentration of the extract.

Parameters	Control	Treatment (1000 µg/ml)
Mean± S.E.	9.4±0.21	5±2.22
t-value	3.143	
p-value	0.0137	
Critical value	2.30	

CHAPTER 5. DISCUSSION

There has been many researches on plant *Artemisia vulgaris* in their medicinal uses, but very few works were focused on the contraceptive use of the plant. The present study has shown that *Artemisia* leaf extract exerts significant effect on acrosome exocytosis and *in vivo* fertilization, minimal effects on viability and membrane integrity whereas not readily visible effect on protein profiling.

From spectrophotometric analysis of the extract, absorption spectrum was obtained with the highest peak at wavelength of 300 nm (Graph 4.1) that resembles absorbance of flavonoids in the extracts as UV–Vis absorption spectrum of flavonoids shows two peaks of maximum absorption at 240–280 nm and 300–550 nm (de Oliveira-Júnior *et al*., 2017). Presence of flavonoids in our extract could be supported by work done by Shaik *et al*., 2014 in which phytochemical examination of methanolic extract of the leaves of *A. vulgaris* in 70% methanol revealed the presence of steroids, flavonoids and saponins. Flavonoids have been reported to possess anti-fertility activities like anti-implantation and anti-estrogenic.

But from only absorption spectrum, various active metabolites of extract exhibiting their effects cannot be confirmed. So, further phytochemical analysis and HPLC-MS or GC-MS should be carried out. Phytochemical analysis of *A. annua* by Abolaji *et al*., 2014 resulted more than 80 natural products that include various flavonoids, coumarins and phenolic acids. Pandey *et al.,* 2017 showed the presence of high amount of alkaloids, flavonoids, and terpenoids, steroids and tannin along with several other phytonutrients in methanol extract of leaves.

The acrosome reaction is very important in the series of events leading to a successful fertilization of oocytes. Acrosome assessment is a fundamental tool for both research purpose and diagnosis of male infertility (Lybaert *et al.,* 2009). After capacitation i.e. the functional maturation of spermatozoa, spermatozoa are supposed to become fusion-competent only after they have undergone the acrosome reaction (Yanagimachi, 1994). During acrosome reaction, spermatozoa head binds the zona pellucida through specific ligand-receptor interaction causing the acrosome to vesiculate and release enzymes on the zona pellucida. Primary attachment is performed by either acrosome-intact or acrosome reacted spermatozoa but in the mouse, only acrosome-intact spermatozoa can initiate binding to the zona pellucida (Llanos *et al.,* 1993). Spermatozoa that are unable to shed acrosome upon zona binding cannot penetrate the zona pellucida and cannot fertilize. So, for successful fertilization spermatozoa must go acrosome reaction. But spermatozoa quickly die after acrosome reaction. That means, precociously acrosome

reacted spermatozoa cannot penetrate zona-pellucida or fertilize oocytes. Hence, here we investigate whether the extract could induce acrosome reaction and then block fertilization.

The products or extracts of different medicinal plants are known to exert their effects on spermatozoa by generating reactive oxygen species (ROS) or protect spermatozoa by scavenging free radicals of ROS. Oxidative stress induced by free radicals could in turn disturb the membrane structural components which lead to the sperm plasma membrane and acrosome dysfunction (Eskandari & Momeni, 2016). Spermatozoa with intact acrosomes display acrosome crescent intensely dark blue staining like shown in Fig. 4.1, (Insert A), whereas spermatozoa with disintegrated acrosomes remain unstained with the stain (Fig. 4.1, Insert B).

On treatment of spermatozoa with different concentrations of the extract, spermatozoa with intact acrosomes were found to be decreased with increase in the concentration of extract which might be due to increased oxidative stress on increased extract concentration leading to the more acrosome exocytosis. The acrosome loss occurred at an exponential rate from 0 to 1000 µg/ml concentration of extract and beyond 1000 µg/ml (i.e 2000 µg/ml), acrosomal loss was higher but did not appreciably increase (Graph 4.2). So, here we chose 1000 µg/ml as standardized concentration of the extract for protein profiling and *in vivo* fertilization experiment. Higher concentration of the extract means higher volume of the ethanol to be injected in mice which might possess more undefined toxic effects.

FPNA stains the acrosome matrix by binding to glycoconjugates in the acrosome and produces bright green fluorescence under fluorescence microscope. Hence FPNA labeling is a standard method to study presence or absence of acrosome in spermatozoa. Spermatozoa with disrupted acrosome do not label with FPNA (Fig. 4.5, 4.6) (Okabe, 2014, Bevers, 1996). In a study by Cheng *et al.,* 1996, Fluorescein isothiocynate (FITC-PNA) was followed to assess the acrosomal status and the zona-pellucida induced acrosome reaction in stallion spermatozoa. Spermatozoa sample were stained with FITC-PNA, and counterstained with the DNA dye ethidium homodimer. In the research, acrosome-intact spermatozoa were found to display intensively green fluorescence over the acrosomal cap, whereas reacting spermatozoa showed a patchy disrupted image of fluorescence.

In acrosome integrity test by both Coomassie and FPNA staining techniques, the mean integrity (%) was found to be 81.85±1.51 and 77.36±1.02 in untreated control sample and was decreased to 21.63±0.64 and 19.88±0.25 in treated sample with 1000 µg/ml respectively. And, as both staining methods showed no significant differences on their results both protocols were considered as effective. By both staining techniques the extract was found to have significant effect on acrosome integrity ($p= 0.0001$). In a study

by Larson & Miller, 1999, both methods were followed and were found to have very similar results on acrosome reaction frequencies. We have also found very similar results of acrosome staining with FPNA and Coomassie staining.

In a study carried out by Alvarez *et al.,* 1988, ethanol was found to possess effects on spermatozoa by two possible actions, one via inhibition of the acrosome reaction and another by accelerating loss of acrosomes accompanied by loss of other membrane structures or components needed for gamete fusion. So, we performed a vehical control experiment by replacing the extract by 80% ethanol. Spermatozoa with intact acrosomes was found to be 78.32% and 75.90% when treated with amount of ethanol equivalent to the extract concentration of 1000 µg/ml and 2000 µg/ml (vehicle controls) respectively. These values were not significantly different from the blank control (81.85%). Hence, absence of significant differences on intact acrosomes in blank control and vehicle controls proved that ethanol itself had no significant effect on acrosome status.

Viable spermatozoa possess intact cell membrane so, they exclude Trypan-blue stain remain colorless whereas dead cells with their damaged or injured membranes stain with blue color (Valle *et al*., 2008). On treatment of spermatozoa with different concentrations of the extract, viabiilty loss was found to be increased on increasing extract concentration. Extract may damage membranes of spermatozoa due to which number of dead spermatozoa increased and viability (%) decreased.

Different components of the extract may exert effects on spermatozoa by generating ROS. As spermatozoa membranes are rich in polyunsaturated fatty acids, they can easily undergo lipid peroxidation and significant damage to cell structure in the presence of ROS which may lead to change in membrane fluidity and results in loss of motility (Thiangtum *et al*., 2009). Membranes are the primary site for structural and functional injury in spermatozoa. Sperm membrane integrity is of fundamental importance in the fertilization process as fertilization will not occur if sperm membrane is biochemically inactive despite of being structurally intact also (Srivastava *et al*., 2013, Neild *et al.,* 1999). So, the effect of extract on sperm membrane integrity or viability was analyzed between control and treated spermatozoa by hypo-osmotic swelling test. Viability (%) of 97.43 ± 0.61 on untreated control sample was decreased to 78.83 ± 0.53 on treatment of spermatozoa with extract concentration of 1000 µg/ml ($p<0.05$) which signifies that there seemed significant effect of extract on spermatozoa viability loss but was minimal effect than on acrosome integrity.

Spermatozoa sample when exposed to a hypoosmotic solution, functional spermatozoa, water and small molecular-weight compounds and elements enter into the spermatozoa and will undergo swelling higher pressure. This results various types of tail bendings (Neild *et al.,* 1999). Effect of extract on viability analyzed by two different techniques

resulted different data. Trypan-blue method showed higher percentage of effect than HOS method (Tab. 4.7). This discrepancy might be due to two reasons. First, spermatozoa which have intact plasma membrane might be permeable to Trypan-blue. Second, Trypan-blue staining increases if spermatozoa are kept longer in Trypan-blue solution. Hence at present, we would consider only an overall effect, but not comparative effects.

Spermatozoa of mammals contain several proteins and during fertilization sperm-zona binding is mediated by several proteins and glycoproteins triggering the acrosome reaction and facilitating sperm-zona penetration. During this reaction, several proteins responsible for sperm-oolemma binding and penetration are exposed into the equatorial region of the sperm membrane (Ashrafzadeh, *et al*. 2013). So, a change in the expression of these sperm proteins may be a major cause of men's infertility or subfertility. The molecular mechanisms associated with spermatozoa functions, such as motility, capacitation, an acrosome reaction, and fertilization (spermatozoa–oocyte interaction), are reported to be altered by the sperms protein expression pattern (Selvam *et al.,* 2019). So, as protein expression pattern is crucial in fertilization here we checked out the protein expression pattern of untreated control and extract treatment sperms protein via SDS-PAGE electrophoresis and silver staining.

Compared to the control, as no remarkable difference on different lanes expression was observed (Fig. 4.15 a, b). In both gels, 11 protein bands were observed but were not clearly distinct which might be due to overlapping of one protein by others. The protein bands of molecular weight 23, 66 and 82 kDa were found to be more concentrated compared to others. There was seen some differences in protein intensities in both staining of some bands like as protein bands of molecular weight 30 kDa and 23 kDa in extract treated sample lanes which might be due to effect of extract or other unknown possible reasons. Protein bands of 23 kDa is supposed to be a major component of rat epididymal secretions and sperm plasma membranes (Jones and Hall, 1991) while the observed protein bands of 66 kDa to be of acrosomal protein proacrosin which is enriched in both the caput and cauda epididymis (Tanphaichitr *et al*., 2015). The protein bands of 82 kDa could be Fertilin beta (ADAM-2) proteins which are integral membrane proteins of the ADAM family and are highly produced in the testis, but also in the vas deferens and the epididymis. This protein is involved in key steps of the sperm-oocyte membrane interaction (Fàbrega *et al*., 2011). Reasonably the plant extract might exert effect on minor proteins which could be obscured by the other dominant protein bands in the total protein profile. To reveal such proteins differential extraction, preferentially membrane proteins must be pursued. Putative target proteins could be identified by Western blotting and/or Ms sequencing.

Standardized concentration of the extract (i.e. 1000 µg/ml) was injected intravaginally during estrus cycle of five sets of mice. Plant extract efficiency as a vaginal contraceptive would be reflected by the number of litters delivered. The present work has shown that

the plant extract effectively reduced the litter size ($p<0.05$) but the efficiency was not 100%. In estrus cycle, pro-estrus phase lasts for 13 h and estrus or ovulation phase for 15 h. After estrus it the pro-estrus phase in which females are able to conceive when mating. So, to have maximum chance of successful mating between mice, we put mating couples for three overnights with extract injection on each day.

Among the five sets of mice used for experiments, extract injected mice of first set did not give any birth. For reproductive success of mice to bring out different environmental factors play a crucial role like nutrition, light cycle and intensity (14h light/10h dark), stress, noise, vibrations, odors, over-handling, health status, seasonal effects etc. So one among these reasons could be the cause for unsuccessful breeding on first set of mice. Also, pseudo-pregnancy might have occurred as the presence of vaginal plug does not guarantee pregnancy (Silver *et al*., 2000). Here, we performed tests on only five sets of mice. The results provided a strong evidence that the extract could be an efficient contraceptive if applied intravaginally. For confirmative conclusion more experiments are needed to be performed with more sets of mice. Moreover several toxicological studies must be performed before it could be tested for human use. A similar study was carried out by Paul *et al*., 2010 in which hexane fraction (0.1 g/ml) of the two plants *Achyranthes aspera* and *Stephania hernandifolia*, was tested in rats as vaginal contraceptives. The results based on the number of litters delivered resulted in 100% contraceptive efficiency, indicating that the hexane fraction of the two plants can be used as an effective vaginal contraceptive (Paul *et al.*, 2010).

CHAPTER 6. SUMMARY

A more recent method of estimating fertility of spermatozoa is to assay their ability to undergo the acrosome reaction (Larson & Miller in 1999) when they bind zona pellucida. It is because spermatozoa must release acrosomal enzymes on zona surface to digest zona fibers and make passage through them. Spermatozoa soon die after acrosome reaction. Hence spermatozoa which lose acrosome precociously cannot penetrate zona or fertilize. The present study was done to investigate whether ethanol extract of *Artemisia vulgaris* leaves, could qualify as a potent contraceptive, by affecting sperm functions including acrosome exocytosis, viability and membrane permeability.

To know the effect of ethanolic extract of leaves of *A. vulgaris* on acrosome integrity, effect was analysed with the different concentrations of the extract (0 µg/ml to 2000 µg/ml). And the extract seemed to have significant effects on acrosomal disintegration which was in a dose-dependent manner. Acrosomal staining performed by both staining techniques, Coomassie and FPNA labeling was found to be almost comparable with the most significant effect on acrosome loss when treatment with extract concentration of 1000 µg/ml in both staining techniques. Then the effect of extract was analysed on viability of mouse spermatozoa. With the increased concentration of the extract treatment, spermatozoa viability loss (%) was in increasing trend in both techniques performed i. e. Trypan-blue staining and hypo-osmotic swelling test (p,0.05). Compared to the acrosomal integrity, the extract was found to be have significant but minimal effect on spermatozoa viability.

After performing some *in vitro* mice sperm functions test, effect of extract was checked on protein profiling of the spermatozoa sample prepared in DMEM media. Protein profiling was performed by SDS PAGE followed by Coomassie and silver staining techniques. Extract untreated control spermatozoa sample and standardized concentration of the extract (1000 µg/ml) were loaded on both gels. On comparison with the marker loaded, no significant differences on observed different bands in all lanes was visualized expect in some bands of extract treated lanes, in which intensities of staining of expressed protein in both gels was observed. Lastly but the most important, the effect of the extract was checked for its efficiency in *in-vivo* fertilization in mice. Experiment carried out in five different sets of mice was found to have significant effects ($p<0.01$) on the number of pup's delivered when comparison was made between intravaginally extract injected (1000 µg/ml) mice and control sets of mice.

CHAPTER 7. CONCLUSION

Plants have been used as a source of contraceptive agents to control fertility by human being to check population and maintain sustainable development. This study revealed strong evidence that ethanol extract of *A. vulgaris* can be a potent contraceptive. The present study has shown that the contraceptive effect is due to premature acrosomal exocytosis rather than damaging sperm membrane or killing spermatozoa. Low resolution SDS PAGE analysis could not distinctly reveal the protein changes in spermatozoa due to the treatment. The extract directly inhibits fertilization as shown by the fact that intravaginal application of the extract significantly reduces the fecundity.

This research is only the preliminary step. So, a much wider study is needed before using the plant extract as contraceptive in humans. More supportive evidence are needed from IVF experiments and toxicity studies before *Artemisia vulgaris* extract could be tested in humans.

RECOMMENDATIONS/ FUTURE PROSPECTS

- This is only the preliminary research. So, more analysis and experiments should be carried out for the confirmation of leaves of *A*. *vulgaris* as a potent anti-fertility agent.
- Phytochemical screening and isolation of the various metabolites possessing different contraceptive properties is recommended.
- To know the significant effect and mechanisms of extract on *in vivo* fertilization, experiments should be carried out in a large number of mouse sets. So in future, experiments in a large scale is expected to be carried out.
- Besides in vivo studies, in vitro fertilization experiment is a powerful technique to investigate various key processes of fertilization like capacitation, spontaneous/induced acrosome reactions, zona binding, zona penetration, ooctyte activation, cleavage etc. Direct effect of extract on gamete functions, fertilization and early embryonic developments can be effectively studied in *in vitro* system.
- As this research could lay out a protocol for researches on medicinal plants, many more are yet be carried out regarding various aspects of various still hidden plants also.

LIMITATIONS OF THE EXPERIMENT

- Our research lacks research on toxic effects of the plant extract which it could possess too.
- We were not able to carry out research for knowing the effect of extract on *in vivo* fertilization in sufficient sets of mice.
- We were not able to carry out the *in vitro* fertilization in the present study.

REFERENCES

Abad, M. J., Bedoya, L. M., Apaza, L., & Bermejo, P. (2012). The Artemisia L. genus: A review of bioactive essential oils. Molecules, 17(3), 2542–2566. https://doi.org/10.3390/molecules 17032542

Abiri, R., Silva, A. L. M., Mesquita, L. S. S. D., Mesquita, J. W. C. D., Atabaki, N., Almeida, E. B. D., … Malik, S. (2018). Towards a better understanding of *Artemisia vulgaris*: Botany, phytochemistry, pharmacological and biotechnological potential. Food Research International, 109, 403–415. https://doi: 10.1016/j.foodres.2018.03.072

Abolaji, A. O., Eteng, M. U., Ebong, P. E., Dar, A., Farombi, E. O., & Choudhary, M. I. (2014). Artemisia annua as a possible contraceptive agent: A clue from mammalian rat model. Natural Product Research, 28(24), 2342–2346. https://doi.org/10.1080/14786419.2014.936016

Ahmed, S., Li, Q., Liu, L., & Tsui, A. O. (2012). Maternal deaths averted by contraceptive use: An analysis of 172 countries. The Lancet, 380(9837), 111–125. https://doi.org/10.1016/S0140-6736(12)60478-4

Alvarez, J. G., Lee, M. A.,Iiozzo, R. V., Lopez, I., Touchstone, J. C., & Storey, B. T. (1988). Ethanol Accelerates Acrosomal Loss in Human Spermatozoa. Journal of Andrology, 9(6), 357–366. https://doi.org/10.1002/j.1939-4640.1988.tb01065.x

Amory, J. K. (2016). Male contraception. Fertility and Sterility, 106(6), 1303–1309. https://doi.org/10.1016/j.fertnstert.2016.08.036

Ashrafzadeh, A., Karsani, S. A., & Nathan, S. (2013). Mammalian sperm fertility related proteins. International Journal of Medical Sciences, 10(12), 1649–1657. https://doi.org//10.7150/ ijms.6395

Avella, M. A., & Dean, J. (2011). Fertilization with acrosome-reacted mouse sperm: Implications for the site of exocytosis. Proceedings of the National Academy of Sciences of the United States of America, 108(50), 19843–19844. https://doi.org/10.1073/pnas.1118234109

Bala, K., Arya, M., & Katare, D. (2014). Herbal Contraceptive: An Overview. World Journal of Pharmacy and Pharmaceutical Sciences, 3(8), 1305–1326.

Banerji, M. L. (1955). Some edible and medicinal plants from east Nepal. J. Bomb. Nat. Hist. Soc. 53: 153-155.

Bendahmane, M., Zeng, H., & Tulsiani, D. R. P. (2002). Assessment of acrosomal status in rat spermatozoa: studies on carbohydrate and non-carbohydrate agonists. 404, 38–47.

Bevers, M. (1996). Use of Peanut to Assess the Acrosomal. Society, 674–682

Bhattarai, S., Chaudhary, R. P., Taylor, R. S., & Ghimire, S. K. (2009). Biological Activities of Some Nepalese Medicinal Plants Used in Treating Bacterial Infections in Human Beings. Nepal Journal of Science and Technology, 10, 83–90. https://doi.org/10.3126/njst.v10i0.2830

Buffone, M. G., Foster, J. A., & Gerton, G. L. (2008). The role of the acrosomal matrix in fertilization. 522(July), 511–522. https://doi.org/10.1387/ijdb.072532mb

Castellini, C. (2007). Semen production and management of rabbit bucks. 265–278.

Cheng, F. P., Fazeli, A. R., Voorthout, W., Bevers, M.M & Colenbrander, B. (1996) Use of PNA (peanut agglutinin) to acess the acrosomal status and zona pellucida induced acrosome reaction in stallion spermatozoa. Journal of Andrology 17, 674-682.

Daniyal, M., & Akram, M. (2015). Anti-fertility activity of medicinal plants. Journal of the Chinese Medical Association, 78(7), 382–388. https://doi: 10.1016/j.jcma.2015.03.008

Das, U. K., Maiti, R., Jana, D., & Ghosh, D. (2006). Effect of aqueous extract of leaf of Aegle marmelos on testicular activities in rats. Iranian Journal of Pharmacology and Therapeutics, 5(1), 21–25.

Dehghan, M. H., Ph, D., Martin, T., Ph, D., & Dehghanan, R. (2005). Anti-fertility effect of Iranian neem seed alcoholic extract on epididymal sperm of mice. 3(2), 83–89.

de Oliveira-Júnior, R. G., Ferraz, C. A. A., Souza, G. R., Guimarães, A. L., de Oliveira, A. P., de Lima-Saraiva, S. R. G., … da Silva Almeida, J. R. G. (2017). Phytochemical analysis and evaluation of antioxidant and photoprotective activities of extracts from flowers of *Bromelia laciniosa* (Bromeliaceae). Biotechnology and Biotechnological Equipment, 31(3), 600–605. https://doi.org/10.1080/13102818.2017.1288073

Dey, D., Chatterjee, A., Banji, D., & Bhowmik, B. B. (n.d.). Current Status of Male Contraception.

De La Vega-Beltran, J. L., Sánchez-Cárdenas, C., Krapf, D., Hernandez-González, E. O., Wertheimer, E., Treviño, C. L., … Darszon, A. (2012). Mouse sperm membrane potential hyperpolarization is necessary and sufficient to prepare sperm for the acrosome reaction. Journal of Biological Chemistry, 287(53), 44384–44393. https://doi.org/10.1074/jbc.M112.393488

Dwivedi, J., & Yadav, S. (2012). Advents in Contraception : Immunocontraception. 1, 5–19.

Engh, E., Clausen, O. P. F., & Purvis, K. (1991). Acrosomal integrity assessed by flow cytometry in men with variable sperm quality. Human Reproduction, 6(8), 1129–1134. https://doi.org/10.1093/oxfordjournals.humrep.a137498

Eskandari, F., & Momeni, H. R. (2016). Silymarin protects plasma membrane and acrosome integrity in sperm treated with sodium arsenite. International Journal of Reproductive BioMedicine, 14(1), 47–52. https://doi.org/10.29252/ijrm.14.1.47

Ekambaram, G., Kumar, S. K. S., & Joseph, L. D. (2017). Comparative study on the estimation of estrous cycle in mice by visual and vaginal lavage method. Journal of Clinical and Diagnostic Research, 11(1), AC05–AC07. https://doi.org/10.7860/JCDR/2017/23977.9148

Fàbrega, A., Guyonnet, B., Dacheux, J. L., Gatti, J. L., Puigmulé, M., Bonet, S., & Pinart, E. (2011). Expression, immunolocalization and processing of fertilins ADAM-1 and ADAM-2 in the boar (sus domesticus) spermatozoa during epididymal maturation. Reproductive Biology and Endocrinology, 9, 1–13. https://doi.org/10.1186/1477-7827-9-96

Foster, M. L., Love, C. C., Varner, D. D., Brinsko, S. P., Hinrichs, K., & Teague, S. (2011). Comparison of methods for assessing integrity of equine sperm membranes. THE, 76(2), 334–341. https://doi.org/10.1016/j.theriogenology.2011.02.012

Gautam, L. N., Karanjit, N., Shakya, K., Pradhan, N., & Shakya, D. (2015, November 13). *A Compilation Report of Preliminary Phytochemical and Biological Screening of Some Medicinal Plants of Nepal*.

Gava, G., & Meriggiola, M. C. (2019). Update on male hormonal contraception. Therapeutic Advances in Endocrinology and Metabolism, 10, https://doi.org/10.1177/2042018819834846

Georgadaki, K., Khoury, N., Spandidos, D. A., & Zoumpourlis, V. (2016). The molecular basis of fertilization (Review). International Journal of Molecular Medicine, 38(4), 979–986. https://doi.org/10.3892/ijmm.2016.2723

Goulletquer, P. (2014). Biodiversity in the marine environment. Cham: Springer.

Graczykowski Jacek W, Mary M.Francis, Richard J.Paulson and Rebecca Z.Sokol (1998), Factors influencing sperm–zona pellucida binding in vitro using the intact zona model, Human Reproduction vol.13 no.3 pp.670–675

Gupta, S. C., Prasad, S., Tyagi, A. K., Kunnumakkara, A. B., & Aggarwal, B. B. (2017). Neem (Azadirachta indica): An Indian traditional panacea with modern molecular basis. Phytomedicine, 34(May), 14–20. https://doi.org/10.1016/j.phymed.2017.07.001

Hammitt, D. G., Syrop, C. H., Walker, D. L., & Bennett, M. R. (1993). Conditions of oocyte storage and use of noninseminated as compared with inseminated, nonfertilized oocytes for the hemizona assay. Fertility and Sterility, 60(1), 131–136. https://doi.org/10.1016/S0015-0282(16)56050-6

Hossain, A., Osuamkpe, C., Hossain, S., & Phelps, J. Y. (2010). Spontaneously developed tail swellings (SDTS) influence the accuracy of the hypo-osmotic swelling test (HOS-test) in determining membrane integrity and viability of human spermatozoa. Journal of Assisted Reproduction and Genetics, 27(2–3), 83–86. https://doi.org/10.1007/s10815-009-9375-x

Jeyendran, R. S., Ven, H. H. V. D., Perez-Pelaez, M., Crabo, B. G., & Zaneveld, L. J. D. (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. Reproduction, 70(1), 219–228. https://doi.org/10.1530/jrf.0.0700219

Jones, R., & Hall, L. (1991). A 23 kDa protein from rat sperm plasma membranes shows sequence similarity and phospholipid binding properties to a bovine brain cytosolic protein. Biochimica ET Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1080(1), 78–82.https://doi: 10.1016/0167-4838(91)90114-f

Kashiwazaki, N., Seita, Y., Takizawa, A., Maedomari, N., Ito, J., & Serikawa, T. (2009). Techniques for In vitro and In vivo Fertilization in the Rat. Methods in Molecular Biology Rat Genomics, 311–322. https://doi: 10.1007/978-1-60327-389-3_22

Kaur, K., & Prabha, V. (2014). Immunocontraceptives: New approaches to fertility control. BioMed Research International, 2014(July 2014). https://doi.org/10.1155/2014/868196

Kim, E., Yamashita, M., Nakanishi, T., Park, K., Kimura, M., KashiwaMbara, S., & Baba, T. (2006). Mouse Sperm Lacking ADAM1b / ADAM2 Fertilin Can Fuse with the Egg Plasma Membrane and Effect Fertilization *. 281(9), 5634–5639. https://doi.org/10.1074/jbc.M510558200

Kogan, P., & Wald, M. (2014). Male contraception: History and development. Urologic Clinics of North America, 41(1), 145–161. https://doi.org/10.1016/j.ucl.2013.08.012

Kupker, W., Diedrich, K., & Edwards, R. G. (1998). Principles of mammalian fertilization. Human Reproduction, 13(SUPPL. 1), 20–32. https://doi.org/10.1093/humrep/13.suppl_1.20

Larson, J. L., & Miller, D. J. (1999). Simple Histochemical Stain for Acrosomes. 449(June 1998), 445–449. https://doi.org/10.1002/(SICI)1098-2795(199904)52

Lian, G., Li, F., Yin, Y., Chen, L., & Yang, J. (2018). Herbal extract of *Artemisia vulgaris* (mugwort) induces antitumor effects in HCT-15 human colon cancer cells via autophagy induction, cell migration suppression and loss of mitochondrial membrane potential. Journal of B.U.ON., 23(1), 73–78

Liu, M., Fan, J., Wang, S., Wang, Z., Wang, C., Zuo, Z., Huang, Y. (2013). Transcriptional profiling of Chinese medicinal formula Si-Wu-Tang on breast cancer cells reveals phytoestrogenic activity. BMC Complementary and Alternative Medicine, 13. https://doi.org/10.1186/1472-6882-1311

Llanos, M., Vigil, P., Salgado, A. M., & Morales, P. (1993). Inhibition of the acrosome reaction by trypsin inhibitors and prevention of penetration of spermatozoa through the human zona pellucida. Reproduction, 97(1), 173–178. https://doi: 10.1530/jrf.0.0970173

Luhadia, G., Sharma, D. K., & Soni, P. K. (2015). Exploration of Traditional Medicinal Plants for Anti-fertility Effects : a Review. Adv. Pharmacol. Toxicol, 16(1), 2381. https://doi.org/10.13140/RG.2.1.5 151.6964

Lybaert, P., Danguy, A., Leleux, F., Meuris, S., & Lebrun, P. (2009). Improved methodology for the detection and quantification of the acrosome reaction in mouse spermatozoa. Histology and Histopathology, 24(8), 999–1007.

Madhukar B. Patil (2018) 'Ethnomedicines Used in Gynecological and Other Related Problems by Tribal Peoples of North Maharashtra, India', International Journal of Current Advanced Research, 07(5), pp. 12353-12360. http://dx.doi.org/10.24327/ijcar.2018.12360.2169

Manandhar, G., Miranda-Vizuete, A., Pedrajas, J. R., Krause, W. J., Zimmerman, S., Sutovsky, M., & Sutovsky, P. (2009). Peroxiredoxin 2 and Peroxidase Enzymatic Activity of Mammalian Spermatozoa1. Biology of Reproduction, 80(6), 1168–1177. https://doi.org/10.1095/biolreprod. 108 .071738

Manandhar, G., Schatten, H., & Sutovsky, P. (2005). Centrosome Reduction During Gametogenesis and Its Significance1. Biology of Reproduction, 72(1), 2–13. https://doi.org/10.1095/biolreprod. 104.031245

Manandhar, G., & Sutovsky, P. (2007). Comparative Histology and Subcellular Structure of Mammalian Spermatogenesis and Spermatozoa. Comparative Reproductive Biology, 81–98. https://doi: 10.1002/9780470390290.ch3c

Manandhar, G., & Toshimori, K. (2003). Fate of Postacrosomal Perinuclear Theca Recognized by Monoclonal Antibody MN13 after Sperm Head Microinjection and Its Role in Oocyte Activation in Mice1. Biology of Reproduction, 68(2), 655–663. https://doi.org/10.1095/biolreprod.102.006098

McLaughlin, E. A., & Aitken, R. J. (2011). Is there a role for immunocontraception? Molecular and Cellular Endocrinology, 335(1), 78–88. https://doi.org/10.1016/j.mce.2010.04.004

Mehmood, A., Anwar, M., & Naqvi, S. M. S. (2009). Motility, acrosome integrity, membrane integrity and oocyte cleavage rate of sperm separated by swim-up or Percoll gradient method from frozen-thawed buffalo semen. Animal Reproduction Science, 111(2–4), 141–148. https://doi.org/10.1016/j.anireprosci.2008.02.011

Metchnikoff, S., & Baskin, M. J. (2011). Contraceptive Vaccines : Success, Status and Future Perspective. 66, 2–4.

Miller, D. J., Macek, M. B., & Shur, B. D. (1992). Complementarity between sperm surface β-l, 4-galactosyl-transferase and egg-coat ZP3 mediates sperm–egg binding. Nature, 357(6379), 589–593. https://doi.org/10.1038/357589a0

Montón, A., Gil, L., Malo, C., Olaciregui, M., González, N., & de Blas, I. (2015). Sage (salvia officinalis) and fennel (foeniculum vulgare) improve cryopreserved boar epididymal semen quality study. Cryo-Letters, 36(2), 83–90.

Mortimer, D., Curtis, E., & Camenzind, A. (1990). Combined use of fluorescent peanut agglutinin lectin and Hoechst 33258 to monitor the acrosomal status and vitality of human spermatozoa. Human Reproduction, 5(1), 99–103.https://doi:10.1093/oxford journals. humrep.a137050

Narwaria, A., Khosa, R. L., & Dhar, S. K. (1994). Experimental studies on artemisia vulgaris - a possible anti-fertility drug. Ancient Science of Life, 14(1–2), 10–105.

Neild, D., Chaves, G., Flores, M., Mora, N., Beconi, M., & Agüero, A. (1999). Hypoosmotic test in equine spermatozoa. Theriogenology, 51(4), 721–727. https://doi.org/10.1016/S0093-691X(99)00021-7

Okabe, M. (2013). The cell biology of mammalian fertilization. Development (Cambridge), 140(22), 4471–4479. https://doi.org/10.1242/dev.090613

Oliveira, H., Spanò, M., Santos, C., & Pereira, M. de L. (2009). Adverse effects of cadmium exposure on mouse sperm. Reproductive Toxicology, 28(4), 550555. https://doi.org/10.1016/j.reprotox. 2009.08.001

Ovayolu, A., Arslanbuğa, C. Y., Gun, I., Devranoglu, B., Ozdemir, A., & Cakar, S. E. (2016). Can blood and semen presepsin levels in males predict pregnancy in couples undergoing intra-cytoplasmic sperm injection? Pakistan Journal of Medical Sciences, 32(5), 1116–1120. https://doi.org/10.12669/pjms.325.10610

Pal, M., & Pandey, S. K. (2011). Effect of *Artemisia vulgaris* isolated extract and determination of AD50and LD50for anti-fertility and mortality of *Callosobruchus chinensis* (L.). Journal of Ecophysiology and Occupational Health, 11(1–2), 101–106.

Pandey, B. P., Thapa, R., & Upreti, A. (2017). Chemical composition, antioxidant and antibacterial activities of essential oil and methanol extract of *Artemisia vulgaris* and *Gaultheria fragrantissima* collected from Nepal. Asian Pacific Journal of Tropical Medicine, 10(10), 952–959. https://doi.org/10.1016/j.apjtm.2017.09.005

Pandey, A. K., & Singh, P. (2017). The Genus Artemisia: a 2012–2017 Literature Review on Chemical Composition, Antimicrobial, Insecticidal and Antioxidant Activities of Essential Oils. Medicines, 4(3), 68. https://doi.org/10.3390/medicines4030068

Paul, D., De, D., Ali, K. M., Chatterjee, K., Nandi, D. K., & Ghosh, D. (2010). Comparative study on the spermicidal activity of organic solvent fractions from hydroethanolic extracts of *Achyranthes aspera* and *Stephania hernandifolia* in human and rat sperm. Contraception, 81(4), 355–361. https://doi.org/10.1016/j.contraception.2009.09.001

Peachman, R. R. (2018). Weighing the Risks and Benefits of Hormonal Contraception. Jama, 319(11), 1083. https://doi: 10.1001/jama.2018.0448

Peron, G., Baldan, V., Sut, S., Faggian, M., Roccabruna, L., Zanini, D., Dall'Acqua, S. (2017). Phytochemical investigations on *Artemisia Alba* Turra growing in the North-East of Italy. Natural Product Research, 31(16), 1861–1868. https://doi.org/10.1080/14786419.2016.1263845

Rajbhandari, M., Wegner, U., Jülich, M., Schöpke, T., & Mentel, R. (2001). Screening of Nepalese medicinal plants for antiviral activity. Journal of Ethnopharmacology, 74(3), 251–255. https://doi.org/10.1016/S0378-8741 (00)00374-3

Rand, M. G. O., Widgren, E. E., Sivashanmugam, P., Richardson, R. T., Hall, S. H., French, F. S., … Rao, A. J. (2004). Reversible Immunocontraception in Male Monkeys Immunized with Eppin A Cluster of Metabolic Defects Caused by Mutation in a Mitochondria tRNA. Science, 306, 1189–1190.

Rabiu, A., & Rufai, A. (2018). The role of traditional contraceptive methods in family planning among women attending primary health care centers in Kano. Annals of African Medicine, 17(4), 189. https://doi: 10.4103/aam.aam_60_17

Reckova, Z., Machatkova, M., Machal, L., & Jeseta, M. (2015). Relationship between acrosome integrity changes and in vitro fertilising ability of bovine spermatozoa. Veterinarni Medicina, 60(9), 469–475. https://doi.org/10.17221/8437-VETMED

Remsburg RE. (2014). WHO discussion papers on adolescence. Contraception, 36. https://doi.org/10.1590/S1413-81232010000700002

Selvam, Agarwal, Pushparaj, Baskaran, & Bendou. (2019). Sperm Proteome Analysis and Identification of Fertility-Associated Biomarkers in Unexplained Male Infertility. Genes, 10(7), 522. doi: 10.3390/genes10070522

Shaik, A., Kanhere, R. S., Cuddapah, R., S, N. K., Vara, P. R., & Sibyala, S. (2014). Natural Medicines, 12(3), 180–185. https://doi.org/10.1016/S1875-5364(14)60030-3

Shajeela, P. S., Mohan, V. R., Louis Jesudas, L., & Tresina Soris, P. (2011). Anti-fertility acitivity of ethanol extract of *Dioscorea esculenta* (L.) Schott on male albino rats. International Journal of PharmTech Research, 3(2), 946–954.

Shrestha, B. G. (2018). Status of Research on Medicinal Plants in Nepal : A Review. (April).

Silver, L. M. (2000). Mouse genetics: concepts and applications. Oxford: Oxford University Press.

Singh, A. G., & Hamal, J. P. (2013). Traditional Phytotherapy of Some Medicinal Plants Used by Tharu and Magar Communities of Western Nepal, Against Dermatological Disorders. Scientific World, 11(11), 81–89. https://doi.org/10.3126/sw.v11i11.8558

Singh, A., Kala, S., & Kapoor, D. N. (2011). Reversible contraceptive efficacy and safety evaluation of ethanolic extract of *Tinospora cordifolia* in animal model. Archives of Applied Science Research, 3(5), 587–592.

Soni, P. K., Luhadia, G., & Sharma, D. K. (2015). Anti-fertility Activates of Traditional Medicinal Plants in Male With Emphasis on Their Mode Action : a Review. Journal of Global Biosciences, 4(1), 1165–1179.

Srivastava, N., Srivastava, S. K., Ghosh, S. K., Kumar, A., Perumal, P., & Jerome, A. (2013). Acrosome membrane integrity and cryocapacitation are related to cholesterol content of bull

spermatozoa. Asian Pacific Journal of Reproduction, 2(2), 126–131. https://doi.org/10.1016/S2305-0500 (13)60132-3

Strober, W. (1997). Trypan Strober, W. 1997. Trypan-blue Exclusion Test of Cell Viability. Current Protocols in Immunology, 1–2. doi:10.1002/0471142735.ima03bs21Blue Exclusion Test of Cell Viability. Current Protocols in Immunology, 1–2. https://doi.org/10.1002/0471142735.ima03bs21

Suryawanshi, J. A. S. (2011). Neem - natural contraceptive for male and female - an overview. 1(2), 1–6.

Sutovsky, P., Manandhar, G., Wu, A., & Oko, R. (2003). Interactions of sperm perinuclear theca with the oocyte: Implications for oocyte activation, anti-polyspermy defense, and assisted reproduction. Microscopy Research and Technique, 61(4), 362–378. https://doi.org/10.1002/jemt.10350

Su, X.-Z., & Miller, L. H. (2015). The discovery of artemisinin and the Nobel Prize in Physiology or Medicine. *Science China Life Sciences*, *58*(11), 1175–1179. https://doi.org/ 10.1007/s11427-015-4948-7

Tanphaichitr, N., Kongmanas, K., Kruevaisayawan, H., Saewu, A., Sugeng, C., Fernandes, J., Baker, M. (2015). Remodeling of the plasma membrane in preparation for sperm-egg recognition: Roles of acrosomal proteins. Asian Journal of Andrology, 17(4), 574–582. https://doi.org/10.4103/1008-682X.152817

Tavalaee, M., Razavi, S., & Nasr-Esfahani, M. H. (2007). Effects of sperm acrosomal integrity and protamine deficiency on in vitro fertilization and pregnancy rate. Int J Fertil Steril, 1(1), 27–34.

Thiangtum, K., Pinyopummin, A., Hori, T., Kawakami, E., & Tsutsui, T. (2009). Effect of catalase and superoxide dismutase on motility, viability and acrosomal integrity of frozen-thawed cat spermatozoa. Reproduction in Domestic Animals, 44(SUPPL. 2), 369–372. https://doi.org/10.1111/j.1439-0531.2009.01420.x

Trendafilova, A., Todorova, M., Genova, V., Peter, S., Wolfram, E., Danova, K., & Evstatieva, L. (2018). Phenolic Profile of *Artemisia Alba* Turra. Chemistry and Biodiversity, 15(7). https://doi.org/10.1002/cbdv.201800109

Valle, R. R., Valle, C. M. R., Nichi, M., Muniz, J. A. P. C., Nayudu, P. L., & Guimarães, M. A. B. V. (2008). Validation of non-fluorescent methods to reliably detect acrosomal and plasma membrane integrity of common marmoset (Callithrix jacchus) sperm. Theriogenology, 70(1), 115–120. https://doi.org/10.1016/j.theriogenology.2008.03.011

Yanagimachi, R. (1994). Fertility of mammalian spermatozoa: Its development and relativity. Zygote, 2(4), 371–372. https://doi.org/10.1017/S0967199400002240

Zaman, W., Ahmad, M., Zafar, M., Amina, H., Lubna, Ullah, F., Jahan, S. (2019). The quest for some novel anti-fertility herbals used as male contraceptives in district Shangla, Pakistan

APPENDIX

DMEM (Dulbecco's Modified Eagle's Media)

DMEM powder	10 g/l
HEPES	15mM
BSA	5 mg/ml
PVP	0.5%
pH	7.2

Phosphate Buffer Saline 10X (PBS)

NaCl	8 g/l
KCl	0.2 g/l
Na2HPO4.2H2O	1.44 g/l
KH2PO4	0.24 g/l
pH	7.2

4% Formalin

40% Formaldehyde	1.25ml
1X PBS	5 ml

Coomassie (G-250) Stain (100ml)

Glacial acetic acid	10 ml
Coomassie G-250 powder	0.25 g

2 µg/ml Ethidium Bromide (EtBr)

Stock Solution

EtBr	50 mg
DMSO (100 mg/ml)	500 µl

Working Solution

Ethidium bromide (200 µg/m	2 µl
PBS	1 ml

Sample Buffer 5X

1M Tris/HCl pH=6.8	31.25 ml
Bromophenol Blue (2% in ethanol)	750 µl
SDS Powder	10 g
Glycerol	25 ml
2-mercaptoethanol	5ml
Distilled water	28 ml

FPNA Stain (250X)

PBS	990 µl
10 % NaN2	10 µl
FPNA (from stock)	4 µl

10 ml Stacking Gel Solution

22.2% Acrylamide/Bisacrylamide	2 ml
Distilled Water	6.6 ml

1M Tris/HCl, pH 6.8	1.25 ml
10% SDS	100 µl
10% Ammonium Persulfate	50 µl
TEMED	5 µl

20 ml Running Gel Solution

22.2% Acrylamide/0.6 % Bisacrylamide	9.01 ml
Distilled Water	7.5 ml
1M Tris/HCl, pH 8.8	3.18 ml
10% SDS	200 µl
10% Ammonium Persulfate	100 µl
TEMED	10 µl

10X PAGE Running Buffer

Tris base	30 g
Glycine	144 g
SDS	10 g
Distilled Water	1000 ml

1X PAGE Running Buffer

10X Stock	100 ml
Distilled Water	900 ml

Destaining Solution

Methanol	300 ml
Acetic Acid	100 ml
Distilled Water	600 m

Figure: Plant sample collection around locality of Tribhuvan University.

Figure: Food and water feeding to mice.

Figure: Swiss Albino mice dissection and sperm sample preparation from cauda epididymis in DMEM media.

Figure: International researchers of IVF visit to our Department.

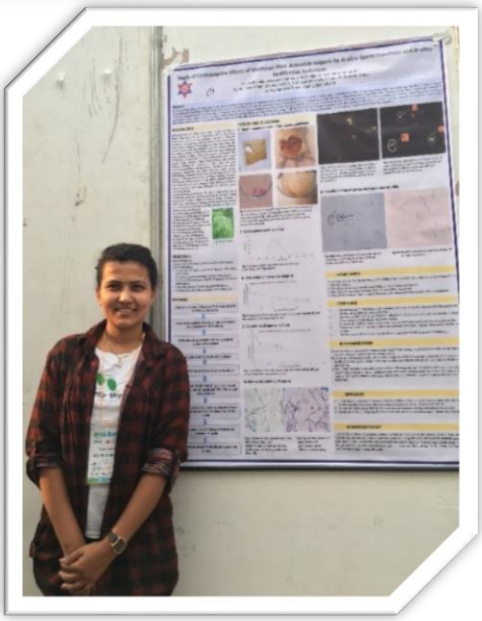


Figure: Poster presentation on World DNA Day, 2019.