Contraceptive Effect of *Tinospora cordifolia* Stem Extract in Mice

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SARBESH RIJAL

ACRONYMS

µl	Microlitre
µg	Microgram
mM	Millimolar
EtBr	Ethidium Bromide
g/L	Gram per liter
hr	Hour
M	Molar
mg	Miligram
ml	Milliliter
Tris	Tris-(Hydroxymethyl)-aminmethane
PAGE	Polyacrylamide gel electrophoresis
DMEM	Dulbecco's Modified Eagle's Media
BSA	Bovine Serum Albumin
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanosulfonic acid
PBS	Phosphate Buffer Saline
SDS	Sodium Dodecyl Sulfate
FPNA	Fluorescein Isothiocyanate (FITC) – Peanut Agglutinin
APS	Ammonium Per Sulfate
TEMED	(N,N,N,N-tetramethyl-ethylene diamine)

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ABSTRACT

Herbal plants have been used extensively for the development of male contraceptives. *Tinospora cordifolia* is one of the most explored plants by researchers for the study of anti-fertility effect in male. The current study deals with the effect of ethanolic extract of stem of *Tinospora cordifolia* on mouse spermatozoa. The acrosome integrity, sperm viability and sperm membrane integrity have been studied in the present experiment along with protein profiling of spermatozoa and *in vivo* fertilization. FITC peanut agglutinin (FPNA) labelling was used for the study of acrosome integrity of spermatozoa after being treated with *T. cordifolia* extract. The percentage of acrosome loss was proportional to the concentration of extract, 90.13% in control to 44.37% at 2000 µg/ml concentration of the extract. 1000 µg/ml concentration of the extract was found to have most significant effect. Similarly, viability of spermatozoa evaluated by trypan blue staining method, ranged from 94.40% in control to 73% at 2000 µg/ml concentration of extract treated with spermatozoa. By hypo-osmotic swelling test, viability of spermatozoa ranged from 97.48% in control to 84.50% at 2000 µg/ml concentration of extract treatment. Thus, the present study showed that sperm viability is less affected by *Tinospora cordifolia* extract than acrosome. Protein profiling of spermatozoa was studied by SDS PAGE followed by Coomassie and silver staining. Treatment with the plant extract did not cause significant changes in the bands of protein from that of control. The contraceptive effect of the plant stem was directly investigated by *in vivo* fertilization experiment in which female mice were intra-vaginally treated with the plant extract and allowed to copulate with male. The treated females showed low litter rate than control (p-value 0.1563). These observations indicate that *Tinospora cordifolia* could be a potential sperm targeted contraceptive.

Keywords: *Tinospora cordifolia,* spermatozoa, acrosome integrity, viability, *in vivo* fertilization, male contraceptive

CHAPTER 1: **INTRODUCTION**

1.1 Background

The rapid increase in population and unintended pregnancies remain major health issues throughout the world, despite the availability of the present methods of birth control. Half of all pregnancies in the United States are unintended, which results in over a million elective abortions annually (Naz, 2009). Hormonal contraceptives are recognized as a breakthrough in medicine. However, its risks and benefits have been evaluated by the researchers since its availability and has never been free of adverse effects. Studies have even found that the adverse outcomes include risk of breast cancer, depression, thrombosis and other cardiovascular effects in females (Mruk, 2007, Abderrahman, 2018).

As the population continues to soar, continued research for the development of safe, effective, affordable and reversible contraceptives is crucial. There have also been great advances in hormonal and non-hormonal male contraceptive studies. Different non-hormonal approaches are being investigated at present, which include interfering with sperm maturation in the testis and the epididymis, interfering with sperm morphology, motility, metabolism, as well as with sperm-egg fusion, and Sertoli-germ cell interactions (Mruk and Cheng, 2008).

Mankind started to use plants to cure diseases and relieve physical sufferings since the beginning of time. It is believed that nearly 80% of the world population rely on traditional medicines for primary health care, which mostly involves the use of plant extracts (Kaur and Sharma, 2011). Different national and international agencies in India and China screened and subsequently identified many plant species with anti-fertility effects. However, due to the side effects or incomplete inhibition, the search for an active, safe and effective herbal contraceptive still remains (Kaur and Sharma, 2011).

Herbal plants such as *Phytolacca dodecadra, Calendula officinalis, Acacia caesia, Gossypium spp., Carica papaya, Cannabis sativa, Dioscorea bulbifera, Artemisia vulgaris* are few plant species that have been extensively researched for the purpose of developing male contraceptives. Being rich in floral biodiversity, Nepal possesses numerous medicinal plants that have antifertility effect and has been catalogued based on ethnobotanical surveys (Watanabe et al, 2005; Shakya 2012). Most of these plants have been biochemically analyzed and found to possess various terpenoids, phenolics, flavonoids, alkaloids, fatty acids, glycosides and other metabolites. Assays of their antifertility property have been mostly based upon oral feeding of plant extract to animals and by

observing antiovulatory or anti-implantation effects. Antifertility effect is most often due to hormonal imbalance or other toxic effects in the body.

1.2 Importance of *Tinospora cordifolia* (Guduchi)

Tinospora cordifolia belongs to family Menispermaceae and is commonly known as "Guduchi" in Sanskrit. It is a large, deciduous climbing shrub found in tropical and sub-tropical regions. The root, stem and whole plant have been reported for medicinal properties like anti-diabetic, anti-periodic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-oxidant, anti-allergic, hepatoprotective and immunomodulatory (Saha and Ghosh, 2012). Recent studies have reported that methanolic extract of *Tinospora cordifolia* stem is capable of causing infertile state in male rats due to interference in the testicular androgen level, consequently altering the process of spermatogenesis (Gupta and Sharma, 2003). Interestingly, antioxidant property of *T. cordifolia* helps in protecting the structures and functions of spermatozoa, which may lead to enhancing the preservation qualities of semen during artificial insemination procedures (Jayaganthan et al., 2013).

1.3 Importance of Laboratory Mice in Biomedical Research

The important role of laboratory mouse in biomedical research has escalated its use for the study of mutagenesis, transgenic and gene-targeting technologies and so on. Due to low costs, room space and related resources needed to accommodate these mice, Swiss Albino Mice are mostly used for research purposes. The techniques of breeding have been revised for over 75 years and thus can be considered safe, reliable, economical and efficient.

For *in vivo* or *in vitro* experiments, the fundamentals of mouse reproductive biology and its handling is essential. Generally, laboratory mice become sexually mature by six weeks of age. Although females can conceive when they are as young as 23 days old, those usually produce small litters. Thus, mating is considered suitable when they are six to eight weeks old. The gestation period is 20-21 days for laboratory mouse (JAX, n.d.). The litter size is 8-14 pups for inbred strain and weaning period is 21 days (Mackay and Schroeder, 1935).

Identification of the stage of estrous is the most useful section for in-vivo experiments for choosing mice that will mate when paired with a male. The estrous cycle in mouse is divided into four stages (proestrus, estrus, metestrus and diestrus) and repeats every 4-5 days unless interrupted by pregnancy, pseudopregnancy or anestrus. Proestrus and estrus phases of estrous cycle are observed for timed mating which are useful when embryos of a precise stage are needed or to accurately predict the birth or pseudopregnancies for embryo transfer or artificial insemination (Byers et al., 2012).

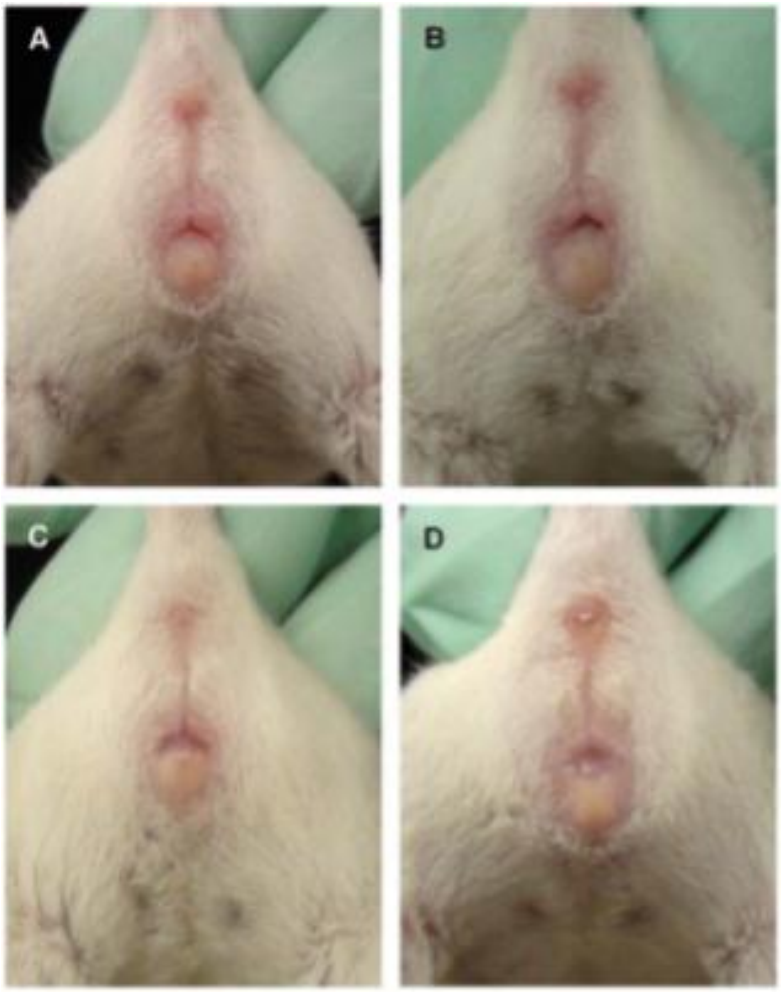

Figure 1.0.1: Four stages of estrous in swiss albino mice. A. Proestrus, B. Estrus, C. Metstrus and D. Diestrus (Adapted from Byers et al., 2012)

The changes occurring in the mouse estrous cycle can be detected using various methods, which includes evaluating vaginal cytology, measuring electrical impedance, biochemical analysis of urine, and visual observation of the external genitalia (Byers et al., 2012). The visual method is less stressful and faster for the experiments, as it eliminates the possibilities of mechanical manipulation of the vaginal tissues. The changes in the vagina, especially the size of the vaginal opening and the degree of coloration, occur during different estrous stages (Champlin, Dorr, & Gates, 1973). The proestrus stage in mice is characterized by swollen, moist, pink tissue in the vaginal opening. The opening is wide and often wrinkles or striations are observed along the dorsal and ventral edges. Similarly, during the estrus stage, the vaginal opening becomes less pink, less moist and less swollen. In metestrus, the vaginal opening is not open wide, not swollen, and white cellular debris may be visible. The vaginal opening is small and closed with no tissue swelling in diestrus stage of the cycle(Byers et al., 2012).

1.4 *in vivo* Fertilization

The mammalian reproduction is a complex process comprising several biological steps. The interaction of gametes and the subsequent fertilization are regulated by a series of events. The sperm selection during capacitation has been one of the focal points for the development of in vitro fertilization techniques. The study of oviduct microenvironment and the culture conditions have been important steps in in-vitro fertilization and have made progress over the years to unlock the complex series of events during the capacitation of mammalian sperm and the development of new sperm selection methods (Talevi and Gualtieri, 2004).

1.5 Statement of Problem

Birth control is one of the major health related problems in the developing countries. Nepal, being one of them, has been able to in bringing down the total fertility in the past few years using hormonal contraceptive. However, it has long been debated about the use of hormonal contraceptives and their 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 the innovation of non-hormonal contraceptives, but no tangible success has been achieved. So, in order to develop non hormonal contraceptives without any side effects, intensive research has been motivated towards herbal contraceptives. 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.

1.6 Significance/Rationale of the Study

Herbal plants are abundantly found in almost all parts of the earth, which can be a good source of medicine for various purpose. The metabolites present in the plants can be used for their immunological or antifertility properties. Most of the previous researches were based upon the screening of antifertility effects by hormonal changes. However, the problems caused by the changes in hormones in the body are more severe. *Tinospora cordifolia* is a plant of medicinal value, which stem is widely used for body resistance against infections and other immune functions. The plant has inhibitory effect on the testicular sperm production and epididymal sperm maturation. The methanolic extract of *Tinospora cordifolia* stem is found to be capable of causing infertile state in male rats due to interference in the testicular androgen level, resulting in the alteration of the process of spermatogenesis. On the contrary, the current research is targeted for the alteration of acrosome reaction without any interference in the hormonal levels. Acrosome reaction

is one of the most important steps in fertilization, triggered by sperm-zona binding. The use of *Tinospora cordifolia* extract disintegrates acrosome, reducing the chances of sperm-zona binding and thus the conception. Moreover, the topical use of the sample extract prevents any interference in the hormonal levels as compared to the previous studies. Thus, the main aim of present project is to research on the effect of ethanolic extract of *Tinospora cordifolia* for interfering the gamete function i.e.sperm without causing hormonal imbalance in the body.

1.7 Objective and research question

1.7.1 General objective

Investigating contraceptive effect of *Tinospora cordifolia* stem extract in mouse IVF system by assessing acrosome integrity, viability of spermatozoa and *in vivo* fertilization.

1.7.2 Specific objectives

- Extraction of plant sample and measurement of concentration.
- Measurement of absorbance of ethanolic extract of the sample by spectrophotometric reading.
- Analyzing effect of sample extract of different concentration on acrosome integrity of spermatozoa by FPNA staining method.
- Analyzing viability of spermatozoa with the treatment of different concentration of sample extract by using Trypan Blue Staining Method.
- Analyzing viability of spermatozoa with the treatment of different concentration of sample extract by using Hypo-osmotic Swelling Test.
- Investigating contraceptive effect of sample extract by *in vivo* fertilization, when applied intravaginally.

1.7.3 Research Question

The aim of the research is to determine whether “the plant *Tinospora cordifolia* has contraceptive effect in mice by acting on the integrity of acrosome without any effect on the viability of spermatozoa.” The present research is motivated towards development of male contraceptive by using medicinal plant *Tinospora cordifolia*.

Null hypothesis: Use of ethanolic extract of *Tinospora cordifolia* has no effect on the fertility of mice.

Alternative hypothesis: Use of ethanolic extract of *Tinospora cordifolia* reduces the fertility of mice.

CHAPTER 2: **LITERATURE REVIEW**

2.1 Birth Control Problems and Role of Medicinal Plants

In developing countries, men make most of the decision regarding family formation and their preferences regarding the contraceptive choices. Furthermore, most of the methods available are female based. According to the United Nations, in 2005, only 14% of married women in the developing regions were relying on the methods that require active male participation – vasectomy, condoms, periodic abstinence or withdrawal (Dahal 2008).

Sexually active women tend to face additional health problems with the increase in the prevalence of sexually transmitted diseases (STDs). Cervical cancer is one of the most frequent cause of cancer death in women (Naz, 2011b). A recent study at Copenhagen University Hospital suggested that there is 20% increase in relative risk of breast cancer in hormonal birth control users compared with nonusers (Abderrahman, 2018). Studies suggest that although only 5% of sexually active women do not use contraceptives, they account for 47% of all unintended pregnancies, while remainder being the result of incorrect or inconsistent contraceptive use, or method failure. Safety concerns have been one of the major issues for women with 76% citing health concerns as their primary reason for discontinuation of the pill or the IUD, a study of an ethnically diverse group of urban women suggested (Guendelman et al., 2000).

Contraceptive vaccines have been found to be one of the best alternatives for ideal contraceptive. The contraceptive vaccines (CV) investigated in various laboratories can be categorized into three main categories: CV targeting gamete production, gamete function and gamete outcome. The target molecules for gamete production include luteinizing hormone-releasing hormone (LHRH/GnRH) and Follicle Stimulating Hormone. The gamete function includes sperm antigens and oocyte zona pellucida (ZP). The gamete outcome targets primarily HCG molecule (Naz, 2009).

Although vaccines targeting gamete production have shown varied degrees of efficacy, they either affect sex steroids or show only a partial effect in inhibiting gametogenesis. Contraceptive vaccines targeting zona pellucida have a high contraceptive efficacy, but they cause oophoritis, affecting sex steroids. However, pharmaceutical companies have started developing vaccines based on LHRH/GnRH, which has clinical applications in inhibition of increased secretions of sex steroids, as well as substitutes for castration of animals (Naz, 2011a). Spermatozoa and sperm vaccine represent exciting prospect for contraception. Spermatozoa have both auto and isoantigenic potentials and can therefore form antibodies in both men and women. Fertilization and fertility can be affected both in vitro and in vivo by anti-sperm antibodies by several mechanisms that includes

inhibition of sperm capacitation, acrosome reaction and sperm-zona interaction and penetration (Naz, 2009).

In present day, women can choose among a spectrum of more than 11 different contraceptive methods, whereas only two methods have been developed for men: condom and dissection of the vas deferens (vasectomy). Despite these, the male contraceptive methods account for 14% of those used worldwide. A recent study suggests that more than 60% of men in Germany, Spain, Brazil and Mexico were willing to use a male contraceptive method.

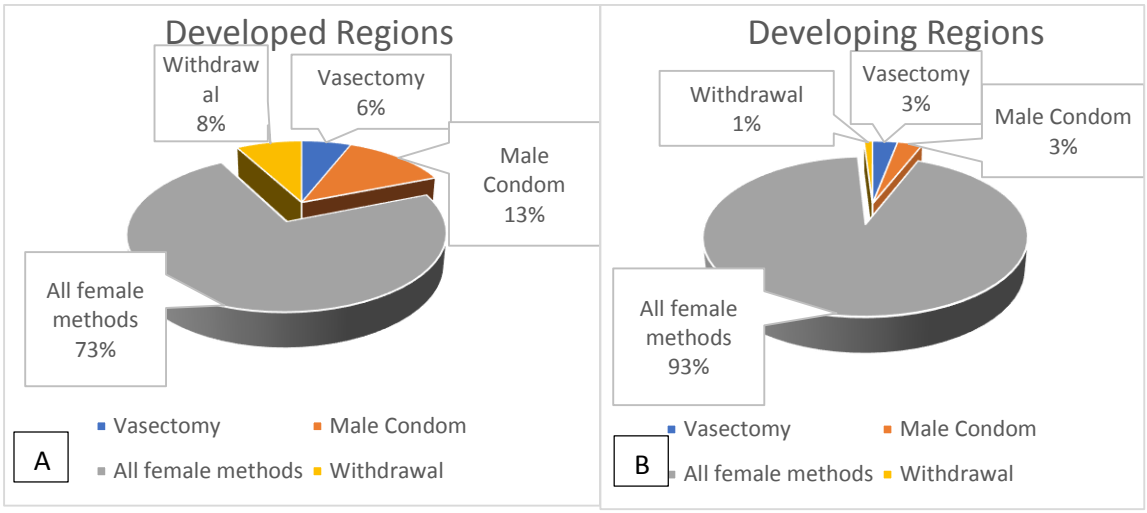

Figure 2.0.1: Use of existing male contraceptives in (A) developed regions; (B) developing regions. (Adopted from the United Nations Population Division World Contraceptive Use 2003)

The existing methods of male contraception are divided into three main categories, based on their target of action:

1. Methods that hinder the transport of sperm in the female reproductive system
2. Methods that suppress spermatogenesis
3. Methods that disrupt the maturation or fertilizing ability of spermatozoa (Kanakis and Goulis, 2015).

The functionality of the testis is regulated by the steroid and peptide hormones secreted by the hypothalamus and pituitary. Considering that, scientists have accentuated on introduction of exogenous multiple hormones. However, due to undesirable side effects, especially hormonal imbalances have concerned the scientists to draw any conclusions.

Non-hormonal male contraception approaches have also been made recently, one of which was injecting plugs into the vasa deferentia. The main component of these plugs, commonly medical-grade polyurethane or hydrated gel in fluid form, as injected into the vasa deferentia, would harden in place and block the flow of sperm. Although this method had been widely applied as a potential alternative to vasectomy in China, the published

clinical data is not enough to completely accept it compared to other alternatives (Zheng et al, 2013). Another interesting approach for male contraception was heat treatment. The method was designed for testing in monkeys by locally warming monkey testes at 43^{o}C water for continuous two days (30 minutes per day). The amount of sperm in the semen could decrease up to 80% after 28 days. It came even close to zero in 2 months on heat treatment in combination with testosterone implant, as administration of exogenous testosterone can reduce secretion of intratesticular androgen level. Moreover, the density of semen sperms recovered to normal levels after 2-3 months on withdrawing the testosterone implant. However, it is still limited to the animal trial stage at the moment (Liu, 2010).

Similarly, there have been approaches on multi-epitope vaccines and on sperm-specific antigens. Among the compounds derived from plants, only gossypol had been intensively studied for antifertility effects and then abandoned due to its slow recovery pattern and irreversible effect (Zheng et al., 2013). A research in China, supported by grants from World Health Organization and Rockefeller Foundation, showed that the men became infertile in 75 days if the pills of 20mg gossypol were orally taken every day. However, two kinds of side-effects, hypokalemic paralysis (0.75%) and irreversible infertility (9.9%), were observed. A low dose of gossypol in combination with testosterone and heat stress is suggested to be hopeful as a prospective male contraceptive and yet to be tested (Liu, 2010).

Studies on sperm-specific ion channels is one of the most recent studies for potential male contraceptives. There have been reports of attempts to control sperm fertility with ion channel blockers, with the calcium channel blocker nifedipine been utilized to cause reversible infertility. One of the Ca^{2+} channels, the voltage-gated Ca_v family is blocked by nifedipine. However, it also possesses the ability to block CatSper channel, which is the only mammalian sperm calcium channel till date, that can be directly detected by electrophysiological method and supported by gene manipulation. The un-specificity of the channels is a major limitation for the study (Zheng et al, 2013).

According to the Nepal Population and Housing Census 2011 report, 13.3% of the population use pill or injectable hormonal contraceptives. Though the use of hormonal contraceptive has substantially contributed in bringing down the total fertility rate to 2.6 and curtailing the population growth, many countries have even banned the use of hormonal contraceptives because of its carcinogenic effects (Umadevi et al, 2013). Likewise, 98% of women would trust their partner to use a male contraceptive (Nya-Ngatchou and Amory, 2013). Thus, it has become crucial for researchers to develop safe, effective, affordable and reversible male contraceptives. Since the long-term use of hormone-based male contraceptives might result in undesirable pathological

circumstances, for example, the functions of androgen-dependent organs, such as the prostate (Mruk, 2007), it has been understood that the non-hormonal male contraceptive approaches might be more appropriate for human use. Nevertheless, progress and possibilities on male contraceptives are still slow and limited. Contraceptive vaccines and inhibitors of spermatogenesis and sperm motility might deliver a great potential for non-hormonal male contraceptives. Therefore, it has become necessary to use fertility regulating bioactive agents of plant origin which are ecofriendly and interfere with the natural patterns of reproduction (Joshi et al., 2011).

More than 35,000 plant species around the world are being used for medicinal purposes (Kaur et al., 2011). Despite progress in synthetic chemistry, even today about 25% of prescription medicines are derived either directly or indirectly from plants. Over the last 20 years, almost half of the small molecules introduced as drugs were derived from plant sources and over 100 of them derived either directly or indirectly from plants are currently at some point in the clinical trials process (Barboza et al., 2009). Medicinal plants contain organic compounds such as tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids which provide certain physiological action on the human body (Vaghasiya et al., 2011).

The World Health Organization (WHO) has set up a Task Force on Plant Research for fertility regulation with primary objective of finding new orally active non-steroidal contraceptive compounds. Various medicinal plant extracts have already been tested for their antifertility activity, showing different mode of actions in both male and female (Joshi et al, 2011). These plants may act through rapid expulsion of the fertilized ova from the fallopian tube, inhibition of implantation due to a disturbance in estrogen-progesterone balance, fetal abortion, perhaps due to lack of supply of nutrients to the uterus and the embryo, and also on the male side through affecting sperm count, motility and viability (Soni, Luhadia and Sharma, 2015).

Herbal plants such as *Phytolacca dodecadra, Calendula officinalis* and *Acacia caesia* constitute of saponins showing spermicidal action. Saponin molecules interact with the common lipid bilayer of spermatozoa and affect the glycoproteins of the cellular membrane and ionic transport across the membrane. *Cannabis* extract also induces higher lipid peroxidation in the testis, along with concomitant decrease in the levels of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase. Similarly, gossypol of *Gossypium* species causes spermatogenesis inhibition, whereas, the chloroform extract of *Carica papaya* causes membrane damage in the acrosome, bent mid piece, coiled tail, detached head and arrest of spermatogenesis. Likewise, the alcoholic seed extracts of *Abrus precatorius* significantly decreases cauda epididymal sperm motility (Soni et al., 2015).

A study in *Piper betle* Linn. on Swiss albino male mice has shown positive results for reversible antifertility effect. The treatment with extract resulted in the decreasing weight of the reproductive organs leading to depletion of sperm count within 60 days. Similarly, the decrease in fructose level suggested the drug treatment hampered the glycolytic metabolism of spermatozoa resulting in infertility (Sarkar et al., 2000).

Similarly, ethanolic seed extract of *Abrus precatorius* has also shown to induce a highly significant decrease in testicular sperm count and daily sperm production. (Abu et al., 2012)

Table 2.0.1: List of plants found in Nepal, that have been investigated for contraceptive use and their mode of action

S.No.	Name of Plant	Mode of Action
1.	*Abrus precatorius* (Abu et al., 2012)	Decreases caudal epididymal sperm motility
2.	*Artemisia vulgaris* (Narwaria, 1994)	Anti-implantation activity in female albino rats
3.	*Cannabis sativa* (Soni et al., 2015)	Induces higher lipid peroxidation in the testis
4.	*Carica papaya* (Soni et al., 2015)	Causes membrane damage in the acrosome and arrest the spermatogenesis
5.	*Curcuma longa* (Naz, 2011b)	Affect sperm motility and function
6.	*Dioscorea esculenta* (Shajeela et al., 2011)	Reduce sperm count and motility
7.	*Gossypium spp.* (Soni et al., 2015)	Inhibition of spermatogenesis
8.	*Piper betle* (Sarkar et al., 2000)	Decrease in weight of reproductive organs leading to depletion of sperm count
9.	*Piper nigrum* (Mishra & Singh, 2009)	Alteration of reproductive organs
10.	*Phytolacca dodecadra* (Soni et al., 2015)	Spermicidal action by affecting the glycoproteins of the cellular membrane and ionic transport across the membrane

2.2 *Tinospora cordifolia* as Anti-Fertility Agent

2.2.1 Tinospora cordifolia

Tinospora cordifolia is identified as an important medicinal component of a majority of Ayurvedic preparations (Aranha et al., 2012). It is a large, glabrous, succulent and deciduous climbing shrub which belongs to the family menispermaceae. The stem is rather succulent with long filiform fleshy aerial roots from the branches. The bark of *Tinospora cordifolia* is creamy white to grey, consisting rosette like lenticels. It comprises of membranous and cordate leaves with small and yellow or greenish yellow flowers.

Tinospora cordifolia is commonly known as 'guduchi' and widely used in Ayurvedic system of medicine to enhance the immune system and the body resistance against infections. (Sivakumar et al., 2010). The plant also has anti-cancer, anti-diabetic, nerve cell and liver protective actions, with a wide variety of antioxidant properties (Jayaganthan et al., 2013). The constituents isolated from *Tinospora cordifolia* belongs to various classes of alkaloids, glycosides, lactones, steroids, diterpenoid, sesquiterpenoid, phenolics, aliphatic compounds, polysachharides and other compounds. These compounds alone or in combination may be responsible for the antioxidant and other medicinal properties of the plant (Khan et al., 2017).

2.2.2 Medicinal Value of *Tinospora cordifolia*

A study in BALB/c and Swiss albino mice provided scientific support to the alleged immunostimulatory property of *Tinospora cordifolia*, as the extract was found to stimulate the humoral immunity with increase in antibody-producing cells and circulating antibody titer. It was also found to reduce the solid tumor volume in mice significantly (Mathew and Kuttan, 1999). Similarly, *Tinospora cordifolia* also had effect on the functions of macrophages obtained from mice treated with the carcinogen ochratoxin A (OTA). The plant extract inhibited OTA-induced suppression of chemotactic activity and production of IL-1 and TNF-α by macrophages.

Moreover, antibacterial activity were observed against E.coli when treated with the alcoholic extract of the plant (Sinha et al., 2004). It has also been reported to have effect

in improved phagocytic and intracellular bactericidal capacities of neutrophils (Saha and Ghosh, 2012). The root extract of *T. cordifolia* in aqueous form was administered orally to alloxan induced diabetic rats, which resulted in significant reduction in blood glucose and brain lipids and also caused an increase in body weight, total haemoglobin and hepatic hexokinase (Sinha et al., 2004).

Oral administration of *Tinospora cordifolia* stem and leaves extract in Swiss albino male mice has shown hepatoprotective effect against lead nitrate induced toxicity, preventing liver damage. Similarly, it contains alkaloids such as acholine, tinosporin, isocolumbin, palmatine, tetrahydropalmatine and magnoflorine, which showed protection against aflatoxin-induced nephrotoxicity.

Tinospora cordifolia is also known for its anti-arthritic and anti-osteoporotic effects as it has been reported to affect the proliferation, differentiation and mineralization of bone like matrix on osteoblast model systems in vitro. The stimulation of the growth of osteoblasts, increase in the differentiation of cells into osteoblastic lineage and increase in the mineralization of bone like matrix have also been reported as the effect of alcoholic extract of *Tinospora cordifolia* (Saha and Ghosh, 2012).

Tinospora cordifolia has a possible role as a potential agent in male fertility regulation. Researches indicate an inhibitory effect of the plant on the testicular sperm production and epididymal sperm maturation consequently resulting in the gradual decline in the fertility rate (Gupta and Sharma, 2003). The decreased number of spermatozoa or reduced androgen production with the treatment of plant extract may affect the level of sialic acid. The reduced sialic acid might alter the structural integrity of acrosomal membrane and ultimately affect the metabolism, motility and fertilizing capacity of spermatozoa. Also, the reduced fertility was completely reversed after withdrawal of the treatment along with no apparent abnormality in the litters delivered by females mated with the males of recovery group (Singh et al., 2011).

On the contrary, a study also revealed that dietary supplementation of *Tinospora cordifolia* enhanced antioxidant enzyme amounts in ram semen resulting in the protection of structures and functions of spermatozoa. This property is supposed to enhance the quality of semen by enhancing preservation qualities during artificial insemination procedures. According to the study, *Tinospora cordifolia* supplementation reduced serum testosterone but increased the function of testes and accessory glands (Jayaganthan et al., 2013).

2.3 Mouse Reproductive System

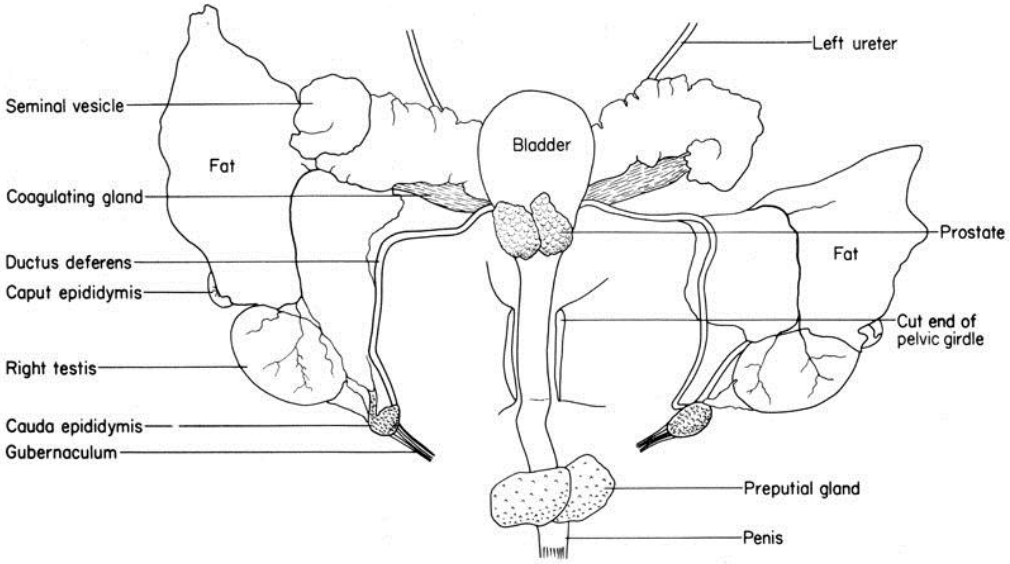

Figure 2.0.2: Reproductive System of male mouse (Adapted from The Anatomy of the Laboratory Mouse, Margaret J. Cook, 143 pages, 1965)

Mammalian reproduction is a complex process which is based upon a series of highly regulated and synchronized physiological events. Several aspects are responsible for the successful fertilization, which includes physical, biochemical, endocrine, behavioral and environmental factors. Besides these, it also depends on the morphological characteristics of the spermatozoan and the oocyte (Vigil et al., 2011). Sperm fertility refers to the ability of spermatozoa to fertilize physiologically normal and structurally intact oocytes either *in vivo* or *in vitro*. The study shows that the mouse spermatozoa collected from the corpus epididymis fertilize only 3% of zona-intact oocytes in vitro, compared to 51% if injected microsurgically into the perivitelline space (Yanagimachi, 1994).

Mammalian sperm are motile. The tail of the spermatozoa helps it propel forward; the acrosomal enzymes clear the way through cumulus cells and zona pellucida; whereas, the mitochondria of the midpiece supply energy (Manandhar and Toshimori, 2005; Piomboni et al., 2012). However, they do not possess the ability to fertilize an egg immediately upon ejaculation, unlike sperm of many lower species (Visconti et al., 1995). This ability is gained while passing through the female reproductive tract. This process is called as the capacitation. A number of changes occur in spermatozoa when deposited in the female reproductive tract. Those include the phosphorylation of various proteins, activation of PKA and PKC, removal of cholesterol from the membrane and elevation of the intracellular Ca^{2+}. In mammals, the spermatozoa acquire functional maturity in the proximal segment of the cauda epididymis where the spermatozoa become fertilization competent only in the uterus/fallopian tube of females. Similarly, the distal segment of the cauda epididymis

is the principal site for the storage of mature spermatozoa (Yanagimachi, 1994). Acrosome has been described as an organelle found in the apical region of spermatozoon covering the anterior extremity of the nucleus and functions as a secretory vesicle (Manandhar and Sutovsky 2007). The acrosomal vesicle is surrounded by two major membranes, the outer acrosomal membrane (OAM) and the inner acrosomal membrane (IAM) (Okabe, 2013). Acrosome consists of a number of hydrolytic enzymes such as glycohydrolases and proteinases. Besides these, it also contains esterases, sulfatases, phosphatases and phospholipases (Tulsianiet al., 1998). Equatorin is one of the few constitutive molecules found in the equatorial region of acrosome, as recognized by the monoclonal antibody (mAb) MN9 and revealed by immunofluorescent and electron microscopy (Manandhar and Toshimori, 2005).

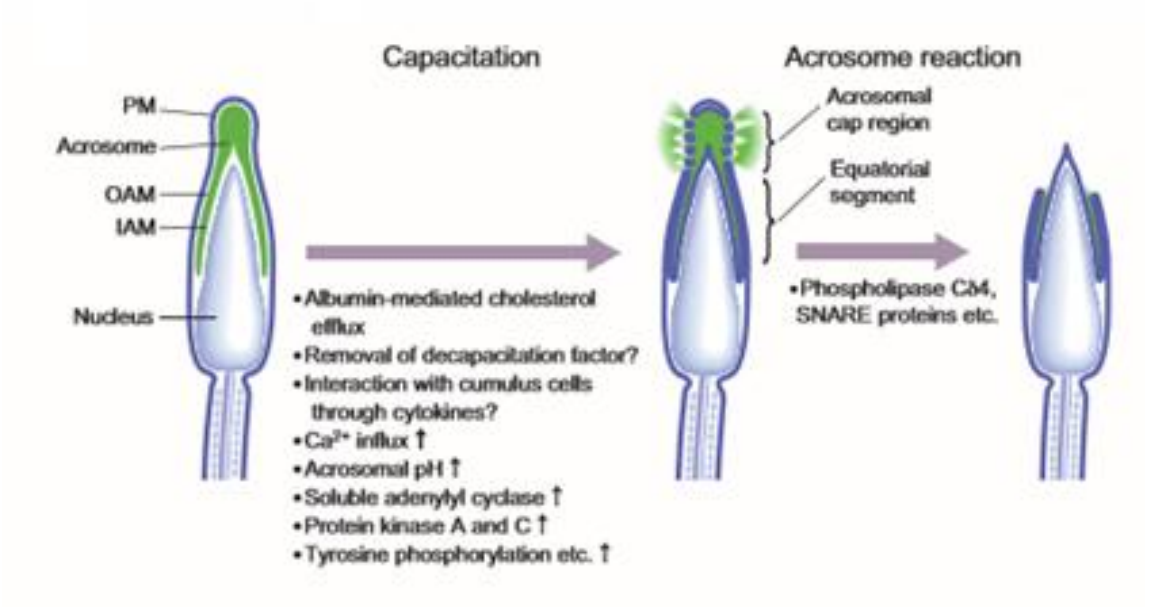

Figure 2.0.3: The features of Spermatozoa (Okabe, 2013)

The sperm cell is produced by spermatogenesis, a differentiation process involving extremely marked genetic, cellular, functional and chromatin changes. Recent proteonomics advances have made progress in the identification of the proteins that make up spermatozoa. However, due to the very peculiar chemical nature of the abundant protamines, it is not possible to resolve these extremely basic proteins in a conventional 2-D gel. Similarly, protamines are insoluble in the presence of SDS, which results in a certain amount of protein to enter the second SDS-PAGE dimension. Thus, a convenient option identified could be to process independent aliquots and to analyze them separately using a 1-D acidic PAGE for protamines and a conventional 2-D PAGE for the rest of the proteins (Oliva et al., 2009).

2.4 Molecular Biology of Fertilization

2.4.1. Species-Specific binding of sperms to eggs

It has been well known that binding of sperm to the egg zona pellucida (ZP) is most often species-specific, well supported by the *in vitro* experiments on different mammalian species. The ZP serves as a barrier to sperm from heterologous mammalian species, which indicates that the ZP possess receptors that are recognized by sperm from the same species and that sperm may possess egg-binding proteins compatible with eggs from the same species.

mZP3 is one of three mouse ZP glycoproteins, which has been identified as a sperm receptor. Studies suggest that acrosome-intact spermatozoa recognize and bind to specific O-linked oligosaccharides located on serine residues (serine 332 and 334) near the carboxy terminus of mZP3 polypeptide. There are some controversies in these studies because dozens of different sperm proteins have been implicated in species-specific binding of sperm to eggs.

2.4.2. mZP3, Ca^{2+}, G Proteins and Acrosome Reaction

As mZP3 is the natural agonist that initiates the acrosome reaction upon binding of sperm to eggs, several types of molecules participate in initiation of the acrosome reaction. These include several signal-transducing components, including G proteins, inositol – 3,4,5-triphosphase (IP3) and IP3 receptors, phospholipase C, Ca^{2+} and voltage-sensitive Ca^{2+} channels (Wassarman et al., 2001).

AR is triggered by sperm-zona binding and is an exocytotic secretory process that results in the release of proteolytic enzymes designed to facilitate both ZP penetration and remodeling of the sperm surface in preparation for oocyte fusion (Aitken et al., 2006). The preliminaries to normal fertilization are capacitation and the acrosome reaction.

Capacitation is the functional maturation of spermatozoa which takes place in the uterus and fallopian tube during normal fertilization (Yanagimachi, 1994). The female reproductive tract can be considered a microenvironment that supplies the conditions needed for the survival, capacitation and migration of spermatozoa required for subsequent fusion with the oocyte (Vigil et al., 2011). Cholesterol and other sterols are detached from the sperm surface, and non-covalently attached glycoproteins acquired in the epididymis are released from the sperm surface during capacitation (Ikawa et al., 2010).

2.4.3. Penetration of the Egg ZP by Sperm

The penetration of the ZP requires combination of sperm motility and enzymatic hydrolysis. The enzymatic hydrolysis is catalyzed by an acrosome serine protease called

acrosin (Wassarman et al., 2001). More recent findings indicate that sperm proteasomes are involved in zona penetration (Sutovsky et al., 2004).

2.4.4. ADAM, Integrins and Interaction of Sperm with Eggs

The fusion between acrosome-reacted sperm with eggs takes place by using plasma membrane at the postacrosomal region of the sperm, which is only possible after the acrosome reaction has taken place. The binding is thought to be mediated by a member of the ADAM family of trans-membrane proteins on sperm and integrin α6β1 receptors on eggs. The heterodimer fertilin (-α, ADAM-1; -β, ADAM-2) and cyritestin (ADAM-3) are the two mouse-sperm proteins that have been studied in some detail (Wassarman et al., 2001).

Oocyte activation involves a signal transduction cascade leading to the conversion of the oocyte to a diploid embryo (Anifandis et al., 2016). As spermatozoa activates the egg, it induces calcium oscillations and completion of the second meiotic cell division. It leads to exocytosis from peripherally located cortical granules. An enzyme, ovastacin, accumulates in cortical granules and results in cleavage of ZP2, which is considered to decrease the affinity to spermatozoa (Okabe, 2013). The release of intracellular calcium (Ca^{2+}) stored in the endoplasmic reticulum is apparently the necessary element that initiates oocyte activation. However, the exact mechanism via which Ca^{2+} is released within the oocyte is yet to be clarified (Anifandis et al., 2016).

A member of the tetraspan superfamily of integral plasma membrane proteins, CD9, in the egg plasma membrane has a vital function in sperm-egg fusion in mice. CD9 is intimately associated with integrin α6β1, to which fertilin-β binds, thus CD9 would regulate the interactions between integrin and fertilin that are ultimately responsible for sperm-egg fusion (Wassarman et al., 2001).

The first cleavage division in mouse separates the zygote into two halves. One of the 2-cell embryo blastomeres cleaves ahead of its sister and tends to contribute most of its descendants to the embryonic part of the blastocyst, whereas the other, later dividing one, contributes progeny predominantly to the abembryonic (Piotrowska and Zernicka-goetz, 2002).

2.5 Sperm Function Assays

Traditionally, the male infertility diagnosis depended upon a descriptive evaluation of semen which considered the number of spermatozoa present in the ejaculate, their motility and morphology. The modern concept and studies, however, demonstrated that it is not these fundamental parameters that determines fertility, but their functional competence (Aitken et al., 2006). The various sperm functions can be analyzed by using numerous assays. The usually considered assays include: (i) tests that examine defective

sperm function through the use of biochemical tests; (ii) bioassays of gamete interaction and induced-acrosome reaction scoring; (iii) computer-aided semen analysis (CASA) for the evaluation of sperm motion characteristics; and (iv) sperm-zona pellucida binding assay for IVF outcome (Oehninger et al., 2000).

2.5.1 Conventional Semen Profile

The relationship between the conventional criteria of semen quality and fertility has demonstrated that the probability of conception is related to factors like sperm count and morphology. However, this descriptive approach has been limited as it has been unable to detect the functional deficiencies responsible for the lack of fertility.

2.5.2 Cervical mucus penetration and motility

The ability of spermatozoa to penetrate hyaluronate polymers and cervical mucus is correlated with the capacity for sperm-oocyte fusion. Although hyaluronate penetration assays significance has not been fully studied *in vivo* for male fertility, studies have indicated that the direct quantitative assessment of sperm movement by CASA accurately reflects the fertilizing ability of human spermatozoa *in vitro*.

2.5.3 Hyperactivation and Capacitation

Hyperactivated motility is the signal for the attainment of a capacitated state, which is characterized by the development of high velocity, large amplitude, asymmetric flagellar waves, and is thought to facilitate detachment of spermatozoa from the oviductal epithelium and penetration of the zona pellucida. According to a recent analysis, hyperactivation has been identified as the most important attribute of movement in predicting the fertilizing potential of human spermatozoa (Aitken et al., 2006).

Hyperactivation is defined as a functional change in the sperm movement pattern which usually occurs during the capacitation process. The hyperactivated movement of spermatozoa varies between species due to interspecies differences in construction of flagellum. Hyperactivated motility was first evaluated by visual observations of the flagellar beat using descriptive criteria like ‘serpentine’, ‘whiplash’ and ‘figure-of-eight’. The measurement of the radius of curvature in rabbit spermatozoa and analysis of ‘flagellar curvature’ in mouse spermatozoa has been performed for the analysis of flagellar beat (Kay and Robertson, 1998).

Physiological factors such as Ca^{2+}, cAMP, bicarbonate and metabolic substrates have been found to be crucial for the initiation or maintenance of hyperactivated motility. Ca^{2+} is responsible for increasing flagellar asymmetry, a characteristic of hyperactivated motility. It is a critical element in the signaling pathway to convert symmetrical to the asymmetrical bending. Similarly, cAMP is responsible for initiation and maintenance of flagellar beating. It is involved in the regulation of flagellar waveform by maintaining Ca^{2+} at an appropriate

level. Bicarbonate supports hyperactivation in mouse sperm b modulating the activity of adenylyl cyclase in the flagellum (Suarez and Ho, 2003).

Computer-assisted sperm analysis (CASA) has been used for the objective evaluation of hyperactivation. A common procedure used for a particular species is to incubate sperm under capacitating conditions and compare swimming patterns with sperm that have not been exposed to capacitating conditions. A set of movement patterns seen to increase over time under capacitating conditions is then categorized as hyperactivate (Suarez and Ho, 2003). Similarly, manual measurements of head movements have been gradually superseded by automated measuring methods using video microscopy and computed image analysis. These instruments or analyzers reconstructs sperm trajectories from the position of the sperm head in successive video frames as defined by the user. Thus, the movement parameters are derived by calculating curvilinear velocity (VCL), straight-line velocity (VSL), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) (Kay and Robertson, 1998).

2.5.4 Sperm-zona interaction

The ZP is an acellular glycoprotein shell that surrounds the oocyte and serves as the site for both sperm-egg recognition and induction of the acrosome reaction. A unique zona glycoprotein, ZP3, appears to mediate the recognition and activation of spermatozoa at the zona surface, while a second glycoprotein, ZP2, facilitates the secondary binding of acrosome-reacted spermatozoa to the zona matrix during penetration. Quantification of sperm-zona binding using salt-stored, hemi zonae pellucidae has been utilized as a bioassay of sperm function (Aitken et al., 2006).

In most studies in vitro, mouse sperm-oocyte interaction is performed by the removal of cumulus oophorus, allowing direct sperm contact with the ZP upon insemination (Jin et al., 2011). Cumulus cells are involved in oocyte growth and maturation. The role of cumulus oophorus for *in vivo* fertilization is species dependent. Cumulus cells removal before *in vitro* fertilization has decreased sperm penetration in cattle and pigs. However, removal of cumulus in different mouse strains did not affect the fertilization rates, rather assisted to assess the presence of polar bodies, since extrusion of the first polar body is an indication of oocyte meiotic maturation. Similarly, assessment of cytoplasm vacuolization and granulation is very difficult in cumulus-intact oocytes. After its removal, the oocyte cytoplasm can be evaluated as even, dense and finely granulated or as coarse granular or with mixed light and dark areas. Mechanical forces or enzymatic treatment are used to artificially denude cumulus cells from oocyte (Van Soom et al., 2002). Hyaluronidase treatment before insemination is one of the common methods to remove cumulus oophorus for direct sperm-zona interaction (Jin et al., 2011).

2.5.5 Acrosome Reaction

The acrosomal status can be assessed using fluorescent lectins that bind to the outer acrosomal membrane (Arachis hypogaea agglutinin) or to the acrosomal contents (or Pisum sativum agglutinin). These probes yield a uniformly labelled acrosomal cap in non-acrosome-reacted cells and a flouorescent band running around the equatorial segment of the sperm head, when the AR has occurred (Aitken et al., 2006).

Capacitation has been shown to be involved in numerous changes in sperm plasma membrane, metabolism, motility and intracellular components (Yanagimachi, 1994). It is believed that capacitation can be achieved in vitro in balanced salt solutions with appropriate concentration of electrolytes, metabolic energy sources and serum albumin. A few works in different species has suggested that the presence of serum albumin, Ca^{2+} and $NaHCO_3$ are required for capacitation. However, Fraser (1985) has suggested that the albumin is required for the acrosome reaction and not capacitation, which is believed to be responsible for the removal of cholesterol from the sperm plasma membrane (Visconti et al., 1995). Capacitated sperm are supposed to become fusion-competent only after they have undergone the acrosome reaction.

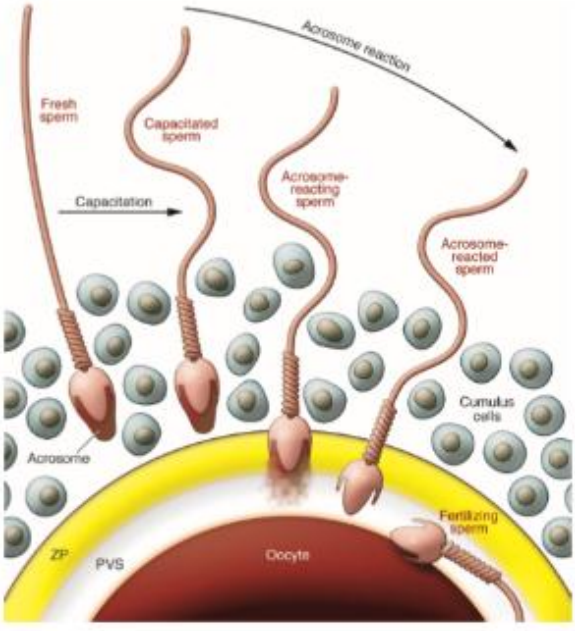

Figure 2.0.4: Schematic diagram representing capacitation and acrosome reaction during reproduction (Ikawa et al., 2010)

Acrosome reaction is activated by ligand-receptor interactions between zona glycoproteins and sperm membrane proteins. This reaction normally takes place on the surface of the zona pellucida, the egg's extracellular matrix (Yanagimachi, 1994). The rostral acrosomal content is dispersed during the reaction by a vesiculation process of the outer acrosomal and plasma membranes (Manandhar and Sutovsky, 2007). With the

conclusion of acrosomal reaction, the spermatozoon has suffered various physiological changes, namely: a) acrosome enzyme release favoring the passing of the spermatozoon through the zona pellucida; b) exposition of the inner acrosome membrane as a new cell surface domain; and c) in the case of the principal acrosome segment, acquisition of the fusogenic ability of the plasma membrane in the spermatozoon equatorial segment. These three important events are described to later determine the fertilization (Vigil et al., 2011).

2.6 FITC – Peanut Agglutinin (FPNA)

The visualization of sperm acrosomal status is an important aspect for the evaluation of semen quality, as male infertility may be caused by a lack of spermatozoa with intact acrosome at ejaculation. Electron microscopy can be utilized to accurately assess the acrosomal status. However, it is costly and highly laborious (Bevers, 1996). The acrosome can also be observed by phase contrast microscopy (Lybaert et al., 2009) but the major problem is that the acrosome loss cannot be observed on living spermatozoa by phase contrast or differential interference contrast microscopy (Mendoza et al., 1992). The triple stain technique (Talbot and Chacon, 1981) and Coomassie Blue labelling (Larson and Miller, 1999) are some of the other common techniques used for staining spermatozoa. However, combination of classical stains is extremely time consuming and difficult to run in parallel with other immunohistochemical techniques. In contrast, fluorescence techniques allow simultaneous acrosome detection and labelling of other spermatozoan structures (Lybaert et al., 2009).

The outer acrosomal membrane or the acrosomal matrix consists of glycoconjugates that bind to a variety of lectins. These lectins have been used to assess the sperm acrosomal status of man, mouse, boar and bull (Bevers, 1996). Peanut agglutinin, when applied on permeabilized spermatozoa, gives essentially the same staining patterns as an antiserum to the acrosomal enzyme acrosin. The lectin from edible pea shows an affinity for terminal α-D-glucosyl and α-D-mannosyl residues of glycoproteins (Mendoza et al., 1992). The acrosome reaction is an exocytotic event during which the process the sperm plasma membrane fuses with the outer acrosomal membrane, resulting in the release of the acrosomal contents and exposure of the inner acrosomal membrane. A research in the feasibility of using PNA (Peanut Agglutinin) in stallion sperm suggested that the FITC-PNA binding was mainly limited to the acrosomal cap (Bevers, 1996). In conclusion, FPNA labelling is restricted to the acrosome and is not influenced by the fixation procedure and is more accurate compared to other similar techniques (Lybaert et al., 2009). The simplicity of the staining protocol, the relatively low cost of reagents and wide range of applications warrant the use of this method for both clinical and research purposes (Mendoza et al., 1992).

2.7 Methods of Testing Viability of Spermatozoa

2.7.1 Hypo-osmotic Test

The integrity and functional activity of the sperm are important measures in the fertilization process, which can be used as an indicator of the fertilizing potential of spermatozoa (Ramu and Jeyendran, 2013). Non-viable sperm is not capable of fertilizing oocytes (Poe-Zeigler, 1997). The integrity and functional activity of the sperm membrane that determine viability is crucial for fertilization (Liu and Baker, 1992). The methods used for those purposes are based upon the supravital stains of dead cells and changes of morphology of sperm tail incubated in hypoosmotic condition. Normal mammalian spermatozoa when suspended in hypoosmotic solutions develop tail vesicles (Śliwa and Macura, 2005). Water and small molecular-weight compounds and elements will attempt to enter into the sperm to reach osmotic equilibrium. This inflow of fluids will increase sperm volume, and the plasma membrane will bulge to achieve a minimum surface-to volume ratio (Ramu and Jeyendran, 2013). This ability of the sperm tail to swell demonstrates that transport of water across the membrane occurs naturally and implies the presence of intact membrane (Śliwa and Macura, 2005). Different patterns of tail swelling are observed in this condition, which makes the hypo-osmotic swelling (HOS) test useful in providing valuable information on sperm viability (Ramu and Jeyendran, 2013).

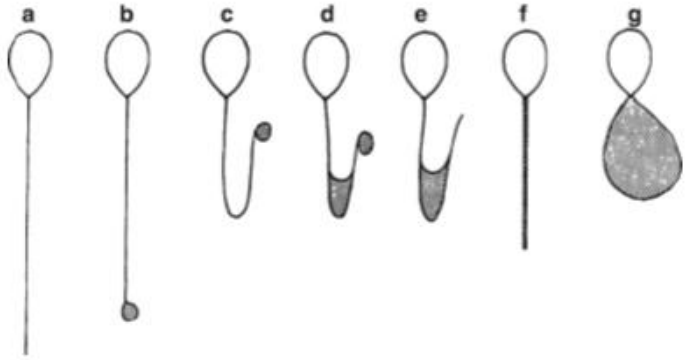

Figure 2.0.5: Schematic representation of various morphological changes of human spermatozoa exposed to hypo-osmotic stress. (a) Sperm with unaltered morphology; (b-g) Sperm with different types of tail swelling indicated by hatched area. (Adapted from Ramu & Jeyendran, 2013)

2.7.2 Dye Exclusion Test

Dye exclusion is a rapid technique to measure cell viability. It is used to determine the number of viable cells present in a cell suspension. The test is simply based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue or Eosin, whereas dead cells do not. Hence, dead cells show staining with these dyes. However, there is always possibility that a cell's viability may have been compromised even though its membrane integrity is maintained (Strober, 1997).

CHAPTER 3: **MATERIALS AND METHODS**

3.1 Laboratory setting and sample collection

All the works required for the research were conducted in Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Experimental Plant: *Tinospora cordifolia*

3.2 Sample Preparation

The *Tinospora cordifolia* stem sample was dried and grinded to powder form using a mechanical blender. The grinded sample was weighed and diluted with 80% ethanol in the ratio 1:10. It was then kept in orbital shaker for three overnights for extraction. The extract was then filtered through 0.2 micron Whatmann filter paper and stored in dark airtight bottle at $4^{o}C$ for further use (Singh et al., 2011). For determining the concentration of the extract, 5 ml extract was taken in a petri plate and completely dried at $110^{o}C$ in oven.

The calculation was done as follows:

Weight of empty plate = V1

Weight of empty plate + 5ml extract = V2

Weight of plate + extract after vaporization = V3

Net weight of extract = V3-V1

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

On the basis of concentration obtained from complete drying, various concentration of extract in media was prepared.

3.3 Spectrophotometric Reading

Scanning spectrophotometer was used from wavelength 300 λ to 800 λ by making 1:100 dilution as per required according to the color of sample. Record was taken in photograph with all data. The peak was observed and wavelength with maximum absorbance was noted.

3.4 Preparation of Media

Optimized DMEM (Dulbecco's Modified Eagle's Media) media 10 g/L, with addition of 15 mM HEPES buffer and 5 mg/ml BSA or 0.5% PVP was used for the incubation of sperm samples. A pH of 7.2 was maintained.

3.5 Mice Purchasing and Handling

Male and female swiss albino mice of 10-14 weeks and 8-12 weeks respectively, were purchased from the Department of Plant Resources (DPR), Thapathali, Kathmandu. Polypropylene cages were used to house the mice in our laboratory and maintained on a 12hrs light: 12hr dark period. The temperature was maintained by using light bulbs at 22-28^{o}C.

3.6 Preparation of Sperm Sample

The housed mice were used for experiment after 5-7 days of acclimatization. They were sacrificed by using cervical dislocation method and dissected using sterile dissection equipment. The testes with attached epididymis was taken off in media containing Petriplate, after dissection. The fat bodies were carefully removed and only epididymis was separated in a fresh media containing Petriplate. Caudal epididymis (tail epididymis) was extracted, from which the sperm sample was suspended in 500 µl of media. The extracted sample was centrifuged at 300 rpm for one minute to sediment the tissue particles. The supernatant was transferred in a clean Eppendorf tube and thus obtained sperm sample was ready for use.

Treatment of spermatozoa with various concentrations of plant extracts

Table 3.0.1: Various concentrations of plant extract made by dissolving with media and 80% ethanol for vehicle control

Sample	Concentration	Extract (µl)	Media(µl) (DMEM+HEPES+BSA)
T1	25 µg/ml	0.57	999.43
T2	100 µg/ml	2.27	997.73
T3	500 µg/ml	11.36	988.64
T4	1000 µg/ml	22.72	977.28
T5	2000 µg/ml	45.45	954.55
		Ethanol (µl)	
T4c	1000 µg/ml	22.72	977.28
T5c	2000 µg/ml	45.45	954.55

The prepared concentration of plant extract with media was incubated at 37^{o}C for two hours. The prepared sperm sample was then kept in equal volume in each Eppendorf tube (a control and various concentration of plant extract) and incubated again at 37^{o}C for two hours.

3.7 Acrosome Staining with FITC Peanut Agglutin (FPNA) for Acrosome Integrity Study

For the study of acrosome integrity of spermatozoa, various concentration of plant extract was made by dissolving media as follows and the volume was made upto 1ml. Treated and control spermatozoa were applied on clean and dry coverslips were taken and thin smear was made with saliva in each coverslip. It was air dried. 100 µl of sperm suspension of each concentration was added and allowed to stand for three minutes. The unattached sperm was removed by washing with PBS. It was then immersed in 4% formalin (in 1X PBS) for 30 minutes to one hour and washed with PBS. 100 µl of 1% Tween20 (in 1X PBS) was added and it was incubated for 30 minutes at room temperature. Tween20 was removed after 30 minutes and 50 µl of 500X diluted FPNA was added. It was then covered with aluminum foil to avoid light and incubated at 37^{o}C for one hour. FPNA was then removed and 100 µl of 2 µg/ml Ethidium Bromide (EtBr) was added. It was incubated for 10 minutes at room temperature in dark. After 10 minutes, Ethidium Bromide was washed off with PBS. A small drop of 10% glycerol (in 1X PBS) was taken on clean slide. The labeled coverslips were mounted by inverting on the mounting drop and edges were sealed with DPX. The slides were then observed under green and blue channel of epifluorescent microscope.

3.8 Sperm Viability Test

Sperm viability test was performed for determining the effect of extract on viability of spermatozoa. Two major techniques were used for the purpose; i) Trypan Blue Staining Method and, ii) Hypoosmotic Swelling Test

For the study of viability of spermatozoa, various concentration of plant extract was made by dissolving media as follows and the volume was made upto 1ml same as above Table 3.0.1.

3.8.1 Trypan Blue Staining Method

The prepared concentration of plant extract as mentioned in Table 3.0.1 with media was incubated at 37^{o}C for two hours. After two hours of incubation, 100µl sperm sample was added in each Eppendorf tube. It was again left for two hours incubation at 37^{o}C. The tubes were then centrifuged at 12,000 rpm for 3 minutes. The supernatant was discarded and pellet with about 20-30 µl of media was preserved and immediately kept in ice bucket. Clean grease free slides were taken and 5 µl each of the preserved sample was added to respective slide. It was followed by the addition of 5 µl of 0.8% trypan blue stain and mixed thoroughly. Coverslip was applied and the slides were observed under 40X and 100X objective lenses. Viable and dead sperm cells were counted by observing the staining (Strober, 1997).

3.8.2 Hypoosmotic Swelling Test

Hypoosmotic Solution consisted 1.5mM fructose, 1.5mM sodium citrate in distilled water. Solution A was prepared by mixing 2.7 g fructose with 100ml distilled water. Similarly, solution B was prepared by mixing 1.47 g sodium citrate with 100ml distilled water. Both solutions A and B were mixed in equal volume and stored in 1 ml aliquots at -20^{o}C.

The prepared concentration of plant extract as mentioned in Table 3.0.1 with media was incubated at 37^{o}C for two hours. For control, the sperm sample was mixed with hypoosmotic solution in the ratio 1:10. It was incubated at 37^{o}C for one hour and then centrifuged at 2000 rpm for 4 minutes. The supernatant was discarded leaving around 100µl pellet and the tube was kept immediately in an ice tray. The pellet was dissolved properly and 50 µl of the dissolved sample was kept in a clean and dry slide. It was sealed with DPX and the cells were counted by observing the swelling of the tail of spermatozoa.

Similarly, 100µl sperm sample was added in each Eppendorf tube which consisted of different concentration of the plant extract. The other tubes of various concentration were centrifuged at 1800 rpm for 3 minutes. The supernatant was discarded and hypoosmotic solution was added to each tube in the ratio 1:10. It was then incubated at 37^{o}C. After one hour of incubation, it was centrifuged at 2000 rpm for 4 minutes. By keeping around 100 µl pellet, the supernatant was discarded and the tube was kept immediately in an ice tray. A clean and dry slide was taken and 50 µl of the sample was kept and sealed with DPX. The viable and dead cells were counted by observing the swelling of the tail of spermatozoa (Ramu and Jeyendran, 2013).

The viability percentage of sperm cells were calculated as:

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

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

For Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) separation, direct heating method with 5X sample buffer was used. 50 µl sperm sample was mixed with 10 µl of 5X sample buffer (1M Tris HCl, pH 6.8, 25% glycerol, 10% SDS, 5% 2-mercaptoethanol, bromophenol blue - 2% in ethanol) in the ratio 1:4, as the sample had very low protein concentration, and heated in a boiling water for 5 minutes.

For preparing SDS polyacrylamide gel, the glass plates were assembled and 10% resolving gel was poured into the gap between the glass plates. The gel was placed in a vertical position at room temperature until polymerization. Since, polymerization began as soon as the TEMED was added in the gel, the gel was poured quickly after mixing with TEMED and APS. After polymerization, the stacking gel solution was poured directly onto the surface of the resolving gel and placed for polymerization at room temperature. 10 µl of

each of the samples were then loaded to gels and electrophoresed with tris-glycine buffer. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (CBB) for overnight. Destaining solution was applied for 3-4 hours and bands were visualized (Sambrook et al. 1989).

For silver staining, the Coomassie stain was first removed by using destaining solution for 3-4 hours. The gel was washed in H_2O for 30 minutes and sensitize in 0.02% sodium thiosulfate for one minute. The gel was then washed in water three times for 20 seconds and incubated for 20 minutes in 4^{o}C cold 0.1% silver nitrate solution. It was again washed in water three times for 20 seconds and placed in a new staining tray. The gel was developed in 3% sodium carbonate and 0.05% formaldehyde after washing in water for one minute. The developer was changed immediately as it turned yellow and terminated after sufficient staining. The gel was washed in water for 20 seconds and staining was terminated in 5% acetic acid for 5 minutes. It was then left at 4^{o}C in 1% acetic acid for storage and washed in water for three times for 10 minutes to ensure complete removal of acetic acid before analysis.

3.10 *in vivo* Fertilization

in vivo fertilization experiments were done after determining the optimum effective concentration of the extract. For acrosome reaction and viability effects the optimum concentration was found to be 1000 µg/ml (see below). The mice were noted and categorized in different cages for the experiment according to their ages. Five sets of mice were categorized with female of 8-10 weeks and male of 8-12 weeks. The estrus cycle was observed for female mice each day.

As a female mouse was found to be in the proestrus or estrus period, it was kept for mating with male mice in the evening. Before keeping it with male mice for mating, the sample extract was injected with micropipette through the vaginal opening of female mice. A control was used for each set with same procedure but without the injection of sample extract. The following morning, the female mouse was checked for the copulatory plug, a whitish, opaque, waxy structure within the vaginal opening, which acted as confirmation for successful mating. It was also cross-checked with swab of the vagina for semen sample in some mice for observing the presence of spermatozoa. The gestation period of swiss albino mice lasts for about 20-21 days. The mice were routinely fed and observed for pregnancy. General procedures for mice breeding were applied before, during and after pregnancy, with routine observation. The number of pupils born were counted and noted down (JAX, n.d.).

3.11 Statistical Methods

Mean, standard deviation and standard error of the respective data from acrosome integrity test and viability test were calculated using Microsoft Excel spreadsheet, along with respective scattered dot plots for further evaluation.

For the evaluation of *in vivo* results, p-value was calculated by computing the mean, standard deviation and the sample size by the help of the website below.

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

CHAPTER 4: **RESULTS**

4.1 Sample Extraction

4.1.1 Determination of Concentration of Plant Extract

Weight of empty plate = 29.504 gm

Weight of empty plate + 5ml extract = 33.733 gm

Weight of plate + extract after vaporization = 29.526 gm

Concentration of extract = 4.4 mg/ml

The concentration of *Tinospora cordifolia* stem extract in 80% ethanol was found to be 4.4 mg/ml. The sample was further dried to obtain concentration of 44 mg/ml.

4.2 Absorbance Spectrophotometer Reading of Ethanolic Extract of the Sample

Table 4.0.1: Absorbance of *Tinospora cordifolia* extract sample of concentration 500 µg/ml

Wavelength	**Absorbance**
0	
100	
200	0.054
250	0.138
300	0.09
350	0.044
400	0.012
450	0.006
500	0.001
550	0.002
600	0.003
650	0
700	0

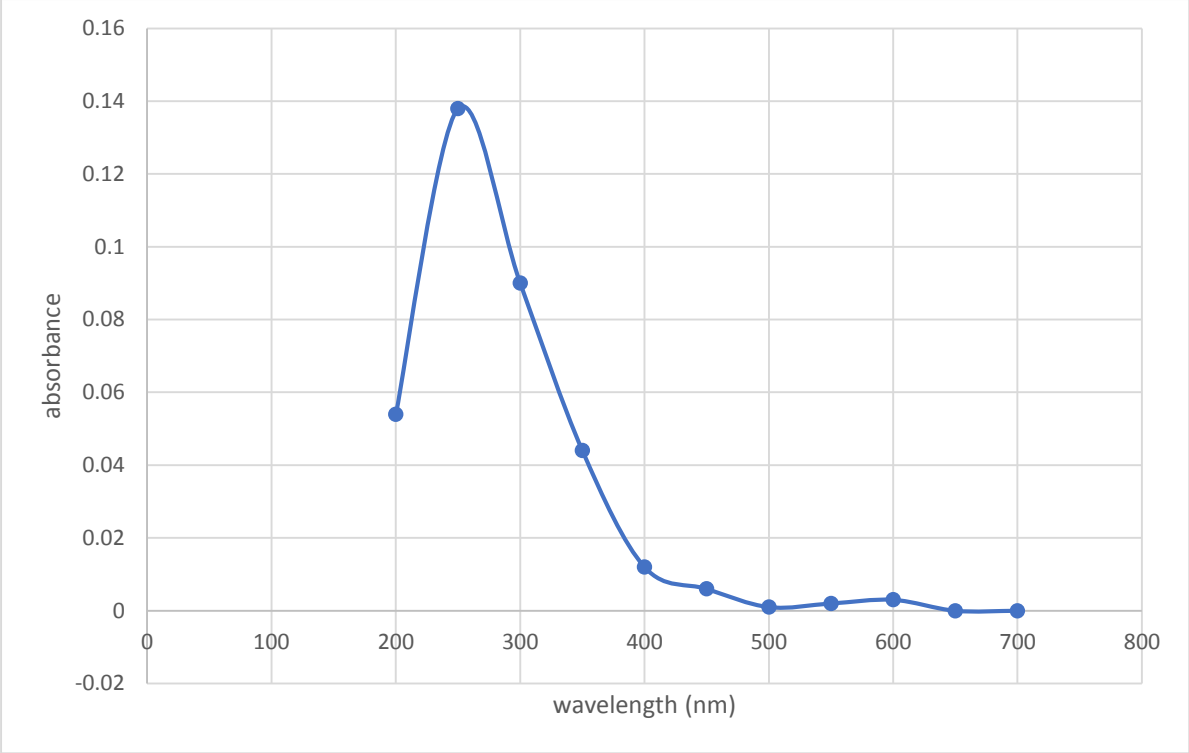

Figure 4.0.1: Absorbance spectrum of *Tinospora cordifolia* extract

From the absorbance spectrophotometer reading of the sample, one major peak was obtained. The UV spectrum peaks show the peak value between 200-300 (~245) with the absorption value of 0.14 (Fig. 4.0.1). The UV-visible spectroscopy interpretation was performed for the confirmation of presence of a major metabolite in the extract. The absorption peak is characteristic for phenolic compounds and its derivatives (Altemimi et al., 2017). These compounds are found to be responsible for the variation in the anti-oxidant activity of the plant (Ganie et al., 2013).

4.3 Effect on *Tinospora cordifola* extract Acrosome Integrity

The effect of extract on acrosome reaction by spermatozoa was tested by using FPNA method. Five different concentrations of *Tinospora cordifolia* extract, 25 µg/ml, 100 µg/ml, 500 µg/ml, 1000 µg/ml and 2000 µg/ml, were used with a negative control and a vehicle control each for 1000 µg/ml and 2000 µg/ml. The average percentage of intact acrosome in the control was observed to be 90.13%. The extract resulted in the decrease of integrity with the increase in the concentration of the sample. The acrosome integrity was found to be lowest in the extract of concentration 2000 µg/ml with 48.21%. However, it was largely deviated in the extract of concentration 1000 µg/ml shown by the standard deviation value of 11.91. Due to this large deviation in 1000 µg/ml and can be considered the most effective concentration for the sample to disintegrate the acrosome.

Reports have been found that ethanol might have effects on the spermatozoa that includes the damage of sperm chromatin and DNA, as well as higher rate of apoptosis (Rahimipour et al., 2013). In the present study, vehicle controls were used to assess if

ethanol exerts appreciable effect on the sperm morphology or function. The media was supplemented with 80% ethanol equivalent to the extract concentrations at 1000 µl/ml and 2000 µl/ml. At these concentrations, 97% and 89% of spermatozoa showed intact acrosomes respectively. These observations of vehicle control proved that the ethanol in the extract had minimum effect in the acrosome reaction.

Table 4.0.2: Effect of *Tinospora cordifolia* extract on Acrosome Integrity of mouse spermatozoa evaluated by FPNA staining method

Experiments							
		I	II	III			
S.No.	**Conc (µg/ml)**	**Integrity (%)**	**Integrity (%)**	**Integrity (%)**	**Mean**	**Standard Deviation**	**Standard Error**
1	0	87.6	90.5	92.3	90.13	± 2.37	1.36
2	25	83.05	81.53	84.54	83.04	± 1.50	0.86
3	100	73.15	79.28	79.47	77.3	± 3.59	2.07
4	500	63.2	70	76.85	70.01	± 6.82	3.94
5	1000	50.74	68.5	73.38	64.20	± 11.91	6.87
6	2000	42.3	42.6	48.21	44.37	± 3.32	1.92
7	1000 (VC)		97.01				
8	2000 (VC)		90.07	89			

Scattered dot plots of mean values of acrosome integrity of spermatozoa by using FPNA staining method were plotted with concentration of *Tinospora cordifolia* extract and acrosome integrity on X-axis and Y-axis respectively. The significant decrease in the frequency of spermatozoa with intact acrosome was observed up to 1000 µg/ml extract concentration in the plot trendline. Similarly, the error bars represented that there is much less likely that the acrosome integrity at the concentrations 500 µg/ml and 1000 µg/ml might differ significantly. However, significant difference was seen in the acrosome integrity of concentrations from control to 1000 µg/ml, and 1000 µg/ml to 2000 µg/ml. This suggested that the concentration 1000 µg/ml can be considered as the most effective concentration in the present experiment for the sample to disintegrate the acrosome and reduce the chances of conception.

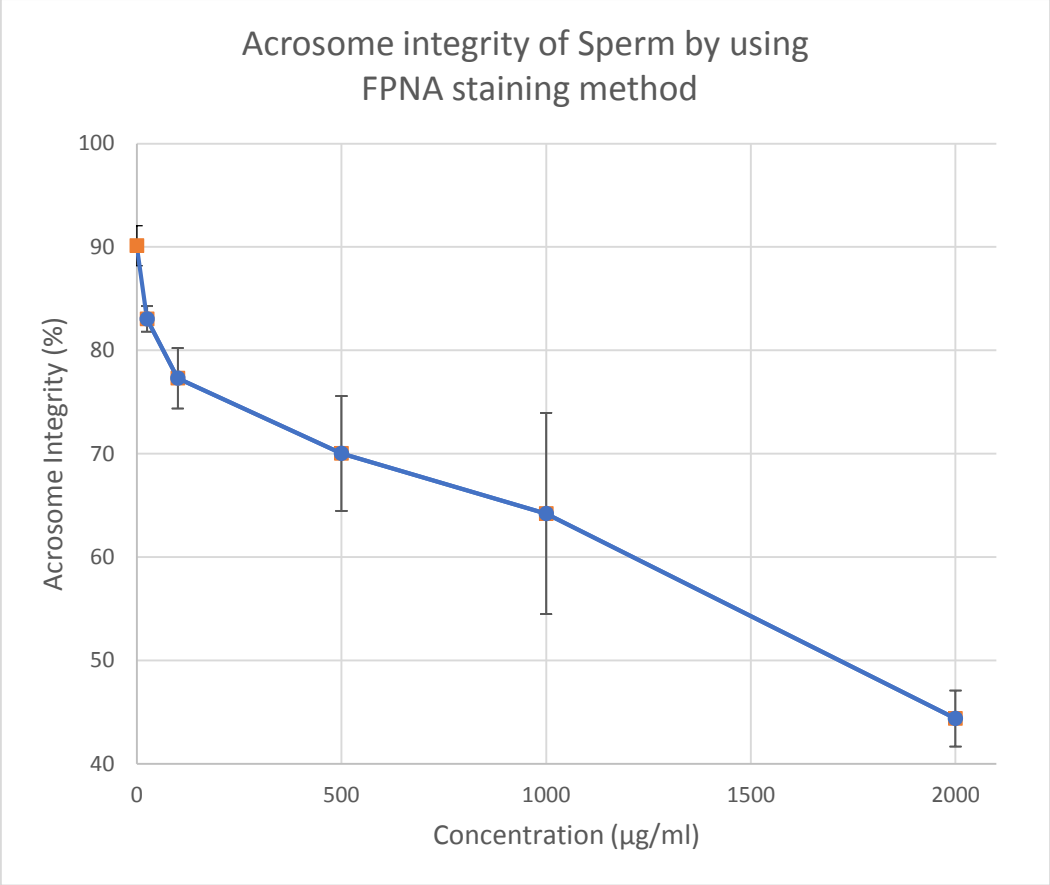

Figure 4.0.2: Acrosome Integrity of sperm against different concentration of *Tinospora cordifolia* extract evaluated by using FPNA staining method. Each point is a mean value from three estimations. The vertical bars represent the standard deviations.

FPNA staining method was used to stain spermatozoa treated with extract of different concentrations. Under fluorescent microscope the stained sperm cells displayed green fluorescence in acrosome and red fluorescence in the nucleus. The green fluorescent on the acrosome was due to the FPNA staining whereas the red fluorescent on the head of sperm was observed due to the staining of nucleic acid by Ethidium Bromide (Fig. 4.0.3). In untreated control spermatozoa, more than 90% spermatozoa with intact acrosome were observed in the control (Fig. 4.0.3 A). However, 10% spermatozoa with disintegrated acrosome were also observed. The green fluorescence was not observed in such spermatozoa as shown in Fig 4.0.3 (B). Similarly, in treated group, a greater portion of acrosome intact spermatozoa was observed in the concentration 25 µg/ml of extract compared to 100 µg/ml, 1000 µg/ml and 2000 µg/ml. A large proportion of fully or partially disintegrated acrosome was observed with the increase in concentration of the extract, as seen on Fig 4.0.3 (C,D,E and F).

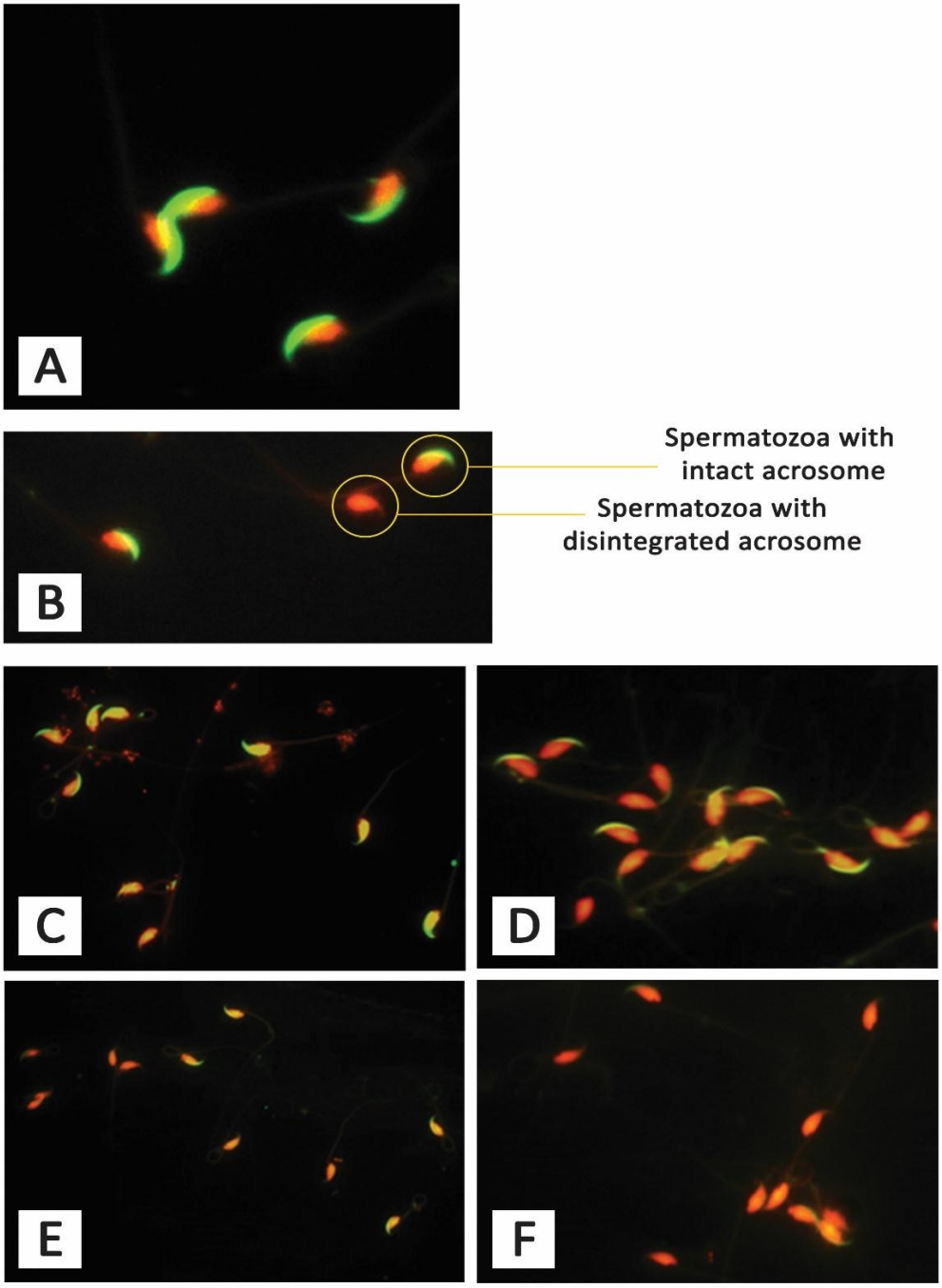

Figure 4.0.3: FPNA staining of spermatozoa (A) FPNA staining of untreated spermatozoa (control); (B) FPNA staining of spermatozoa treated with extract showing intact acrosome and disintegrated acrosomes; (C) Spermatozoa treated with 25 µg/ml concentration of extract, (D) Spermatozoa treated with 100 µg/ml concentration of extract, (E) Spermatozoa treated with 1000 µg/ml concentration of extract, (F) Spermatozoa treated with 2000 µg/ml concentration of extract

4.4 Effect on Sperm Viability after Treatment with *Tinospora cordifolia* Extract

Two different methods, namely trypan blue staining method and hypoosmotic swelling test, were used to test the effect on spermatozoa viability after treatment with *Tinospora cordifolia* extract. Five different concentrations of the extract, 25 µg/ml, 100 µg/ml, 500 µg/ml, 1000 µg/ml and 2000 µg/ml, were used in the experiment. The spermatozoa viability decreased with increase in concentration of extract but there was no significant difference in the viability as compared to the control. The p-value was found to be 0.0014 at 95% confidence level. The percentage of viable spermatozoa in control was similar for the both the methods with about 95% and 97% for trypan blue staining and hypoosmotic swelling test respectively. However, more viable cells were observed in hypoosmotic swelling test compared to trypan blue staining for each concentration of *Tinospora cordifolia* extract.

4.4.1 Sperm Viability Test by using Trypan Blue Method

Trypan blue method for sperm viability was performed by staining with trypan blue stain. The dead cells were stained blue by trypan blue stain whereas the stain was not able to enter the live spermatozoa cells and appeared transparent (Fig. 4.0.5 A). About 89% of the cells were counted to be viable in the experiment at 25 µg/ml concentration of *Tinospora cordifolia* extract. As the viability decreased with the increase in concentration of the extract, the lowest viable percentage of spermatozoa was found to be 73% at 2000 µg/ml concentration of the extract (Fig. 4.0.4). At this concentration, the viability percentage was largely deviated from its mean with Standard Deviation value of 6.87 (Tab. 4.0.3). Vehicle controls were also used in the study to assess if ethanol exerts appreciable effect on the sperm morphology or function. The media was supplemented with 80% ethanol at concentrations 1000 µl/ml and 2000 µl/ml and approximately 87% and 86% of spermatozoa were observed to be viable respectively (Tab. 4.0.3). These data did not differ much with the viability percentage of the control, indicating no significant effect of ethanol in the viability.

Table 4.0.3: Effect of *Tinospora cordifolia* extract on mouse sperm viability evaluated by using Trypan Blue Method

Experiments								
		I	**II**	**III**				
S.No.	**Conc (µg/ml)**	**Sperm Viability (%)**	**Sperm Viability (%)**	**Sperm Viability (%)**	**Mean**	**Standard Deviation**	**Standard Error**	**p-value**
1	0	92.95	94.3	95.96	94.40	1.50	0.87	
2	25	88.57	89.23	88.88	88.89	0.33	0.19	
3	100	83	81.15	81.81	81.98	0.93	0.54	
4	500	80.67	74.49	78.19	77.78	3.11	1.79	
5	1000	79.39	73.13	77.06	76.52	3.16	1.82	0.0014
6	2000	76.97	65.07	76.97	73.00	6.87	3.96	
7	1000 (VC)	90.66	83.74		87.20			
8	2000 (VC)	89.28	81.81		85.55			

Scattered dot plot of mean values of percentage of spermatozoa viability was plotted against different concentrations of *Tinospora cordifolia* extract. The plot was seen to be almost horizontal, indicating no significant decrease in spermatozoa viability after 100 µg/ml concentration of the extract. Slightly higher number of spermatozoa cells were found to be viable at 25 µg/ml concentration of the extract and the control. The error bars showed that the viability of spermatozoa was less likely to significantly differ in 500 µg/ml, 1000 µg/ml and 2000 µg/ml.

Calculation of Significance Values:

Difference	-14.766
Standard error	1.875
95% CI	-19.9712 to -9.5608
t-statistic	-7.876
DF	4
Significance level	P = 0.0014

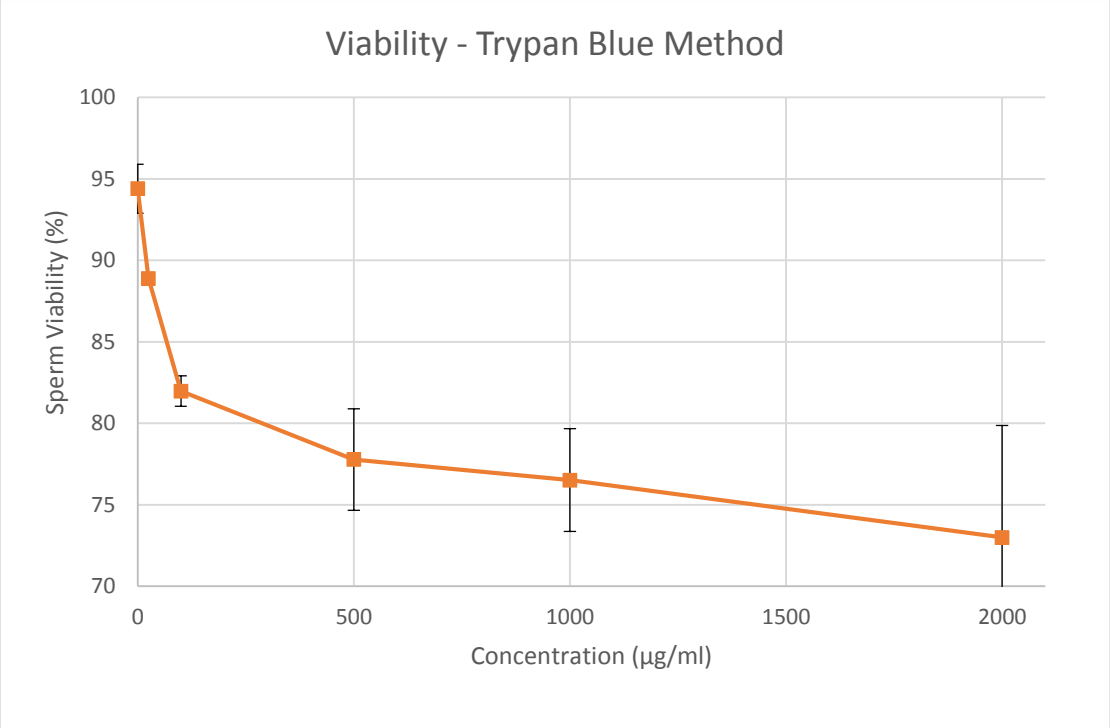

Figure 4.0.4: Sperm Viability against different concentration of *Tinospora cordifolia* extract sample by using Trypan Blue Method. Each value represents mean from three estimates and vertical bars represent standard deviations.

Trypan blue stain stains the dead spermatozoa as the stain cannot enter live cells with intact cell membrane. Therefore, the dead spermatozoa were stained dark blue, while the live were observed transparent or light blue in color as seen in fig. 4.0.5 (A). Control or spermatozoa without extract treatment can be seen in fig. 4.0.5 (B), where no distinct staining was observed. Fig. 4.0.5 (C) and (D) showed the viable and dead cells with treatment of extract. Similarly, only clearly observed spermatozoa in the respective optical field were counted during the experiment. The spermatozoa were counted using optical microscope and viability of spermatozoa at different concentrations of *Tinospora cordifolia* extract was calculated.

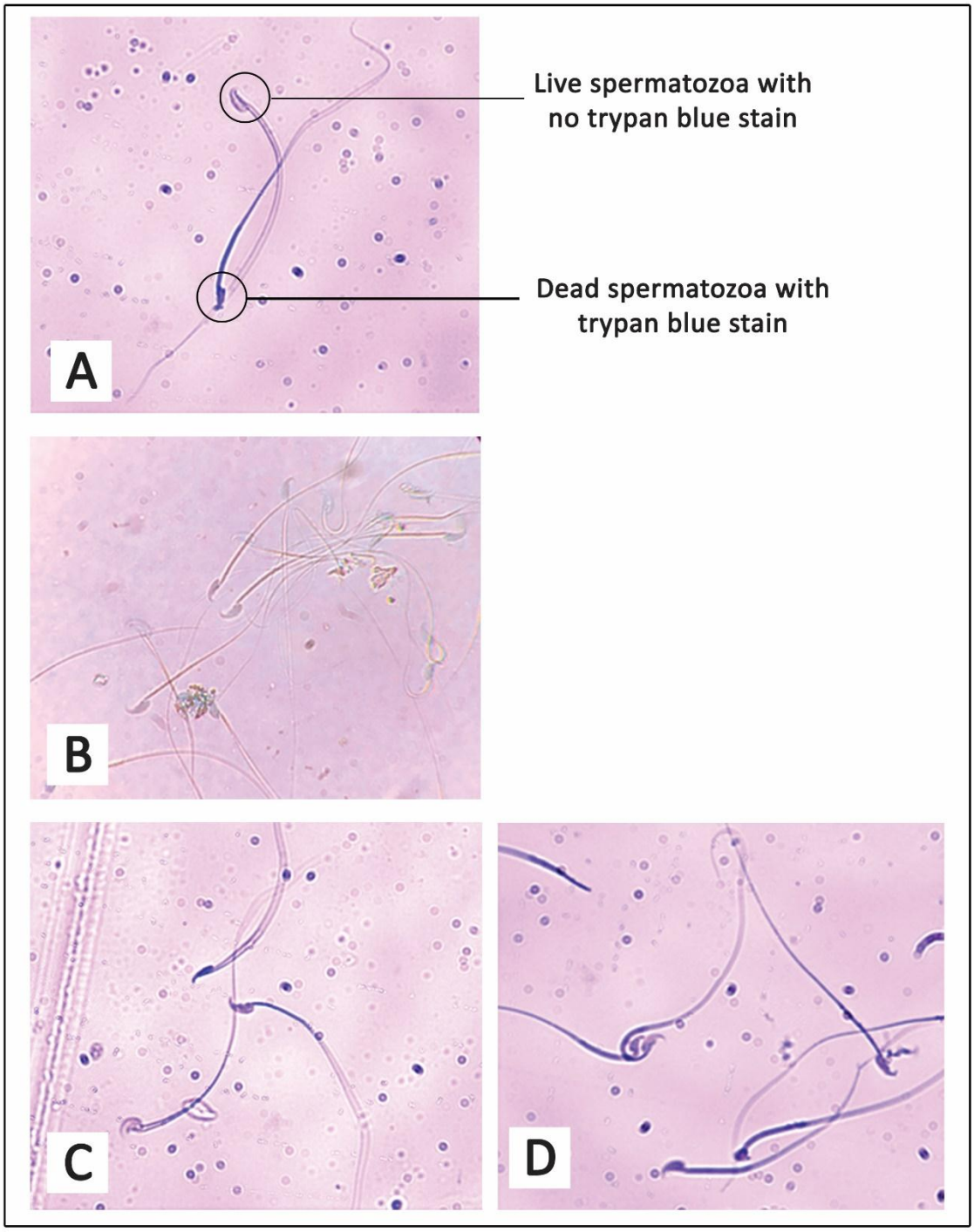

Figure 4.0.5: (A) Viable spermatozoa with intact membrane with no trypan blue staining and dead spermatozoa with trypan blue staining; (B) Trypan Blue staining of spermatozoa without treatment of extract (control); (C) Trypan Blue staining of spermatozoa treated with 500 µg/ml concentration of the extract; (D) Trypan Blue staining of spermatozoa treated with 2000 µg/ml concentration of the extract.

4.4.2 Sperm Viability Test by using Hypo-osmotic Swelling Test

Hypo-osmotic swelling test is another method for the study of viability of spermatozoa. It is based on the principle that the sperm cells when exposed to hypo-osmotic stress, water and small molecular-weight compounds and small molecules will enter into the sperm to reach osmotic equilibrium that results in different patterns of tail swelling, also known as ballooning effect. The swelling reflects structural and functional integrity of the membrane, which is the basis for its viability as well (Ramu and Jeyendran, 2013). In the present study, the percentage of sperm viability was found to be 97.48% in the control and viability decreased with the increase in concentration. The percentage of spermatozoa viable at 2000 µg/ml concentration of extract was found to be 84.50% (Tab. 4.0.4). Since, the value did not differ much from the control, the extract is supposed to have minimal or no effect in the viability of spermatozoa.

Table 4.0.4: Viability Test of spermatozoa with different concentrations of extract, by using Hypo-osmotic Swelling Test

Experiments								
		I	**II**	**III**				
S.No.	**Conc (µg/ml)**	**Sperm Viability (%)**	**Sperm Viability (%)**	**Sperm Viability (%)**	**Mean**	**Standard Deviation**	**Standard Error**	**p-value**
1	0	95.98	96.45	100.00	97.48	2.20	1.27	0.0572
2	25	89.76	94.31	97.64	93.90	3.95	2.28	
3	100	87.93	93.18	96.18	92.43	4.18	2.41	
4	500	83.45	89.12	93.10	88.56	4.85	2.80	
5	1000	79.26	87.30	90.84	85.80	5.93	3.43	
6	2000	77.17	87.20	89.15	84.50	6.43	3.71	

The scattered dot plot clearly showed no sharp decrease in the viability of spermatozoa with the increase in concentration of *Tinospora cordifolia* extract. Moreover, the overlapping error bars indicated that there is no significant difference in the viability percentage of spermatozoa at 25 µg/ml, 100 µg/ml, 500 µg/ml, 1000 µg/ml and 2000 µg/ml.

Difference	-8.440
Standard error	3.190
95% CI	-17.2963 to 0.4163
t-statistic	-2.646
DF	4
Significance level	P = 0.0572

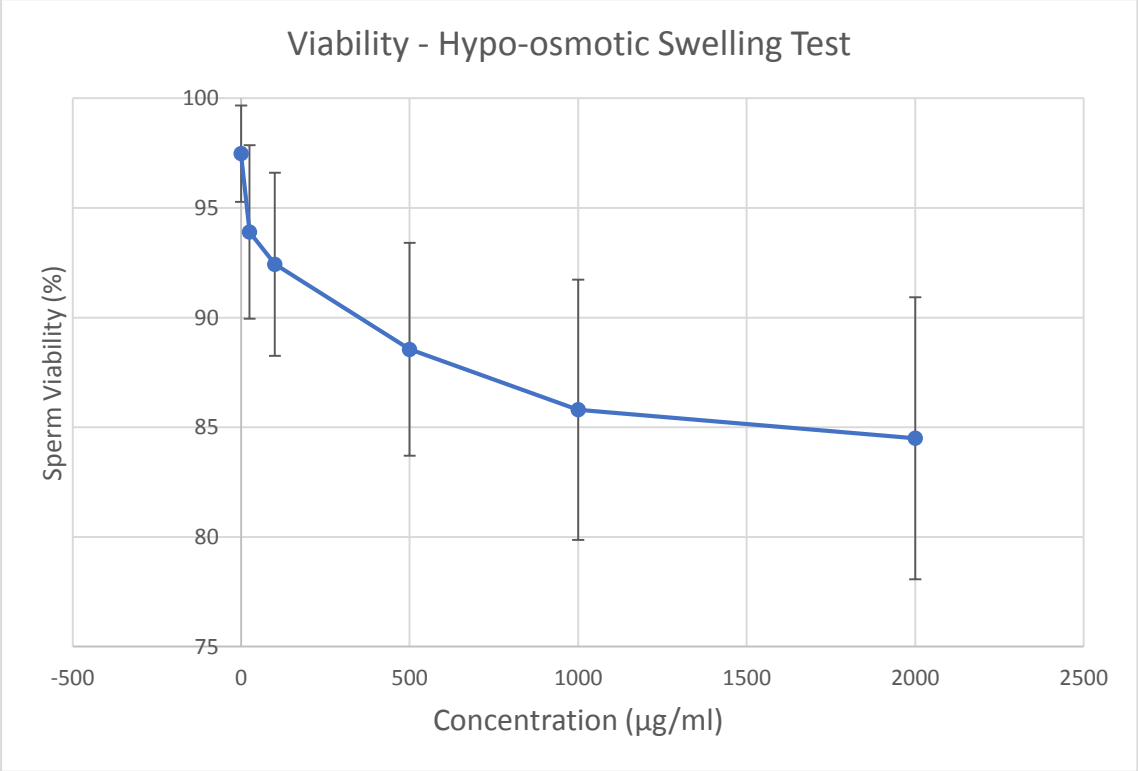

Figure 4.0.6: : Sperm Viability against different concentration of *Tinospora cordifolia* extract sample by using Hypo-osmotic Swelling Test

In hypo-osmotic swelling test, as the sperm cells were exposed to hypo-osmotic stress, different patterns of tail swelling were observed. The spermatozoa that had their tail swollen were counted to be live, whereas the spermatozoa with unaltered tail were counted as dead cells as shown in the fig. 4.0.7 (A). Various types of tail swelling were observed in this test. Fig 4.0.7 (B) showed the hypo-osmotic swelling test of control with different swelling patterns, whereas fig 4.0.7 (C-G) consisted of increasing number of unaltered spermatozoa alongside different patterns of tail swelling.

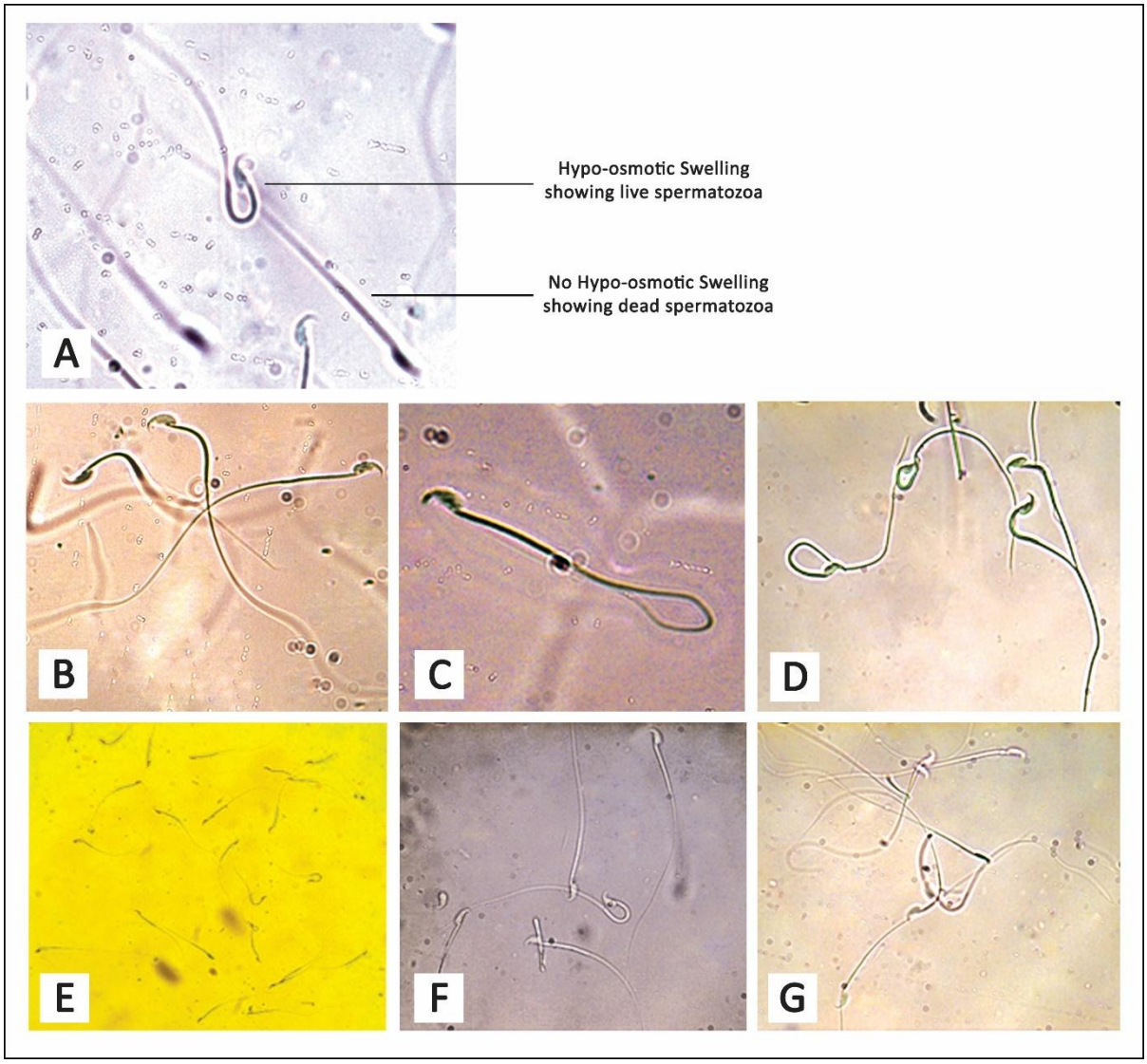

Figure 4.0.7: (A) Hypo-osmotic swelling test of spermatozoa treated with sample extract showing live and dead spermatozoa; (B) Hypo-osmotic swelling test of spermatozoa (control); (C) Hypo-osmotic swelling test of spermatozoa treated with 25 µg/ml concentration of extract; (D) Hypo-osmotic swelling test of spermatozoa treated with 100 µg/ml concentration of extract; (E) and (F) Hypo-osmotic swelling test of spermatozoa treated with 1000 µg/ml concentration of extract; (G) Hypo-osmotic swelling test of spermatozoa treated with 2000 µg/ml concentration of extract

4.5 Protein Profiling by SDS-PAGE

Changes in protein profile of spermatozoa due to extract treatment was studied by using SDS-PAGE. Total proteins of spermatozoa was extracted by direct boiling them in denaturing loading buffer. Lane 1 was loaded with GeNei Protein Molecular Weight Marker (3.5KDa to 205KDa), Lane 2 and Lane 5 with control consisting of spermatozoa in DMEM media and Lane 3 (T1) and Lane 4 (T2) consisted spermatozoa with extract at concentration 1000 µg/ml and 500 µg/ml respectively. Fig. 4.0.8 (A) showed the bands after Coomassie staining and Fig. 4.0.8 (B) after silver staining. According to the bands

observed, the spermatozoa proteins were found to be more concentrated around 66 KDa, supposed to be p67 proacrosin (ROSENFELD, 1966). The protein-banding pattern studied on SDS PAGE electrophoresis (Fig. 4.0.8) indicated that there was no significant difference in banding pattern of control and the samples.

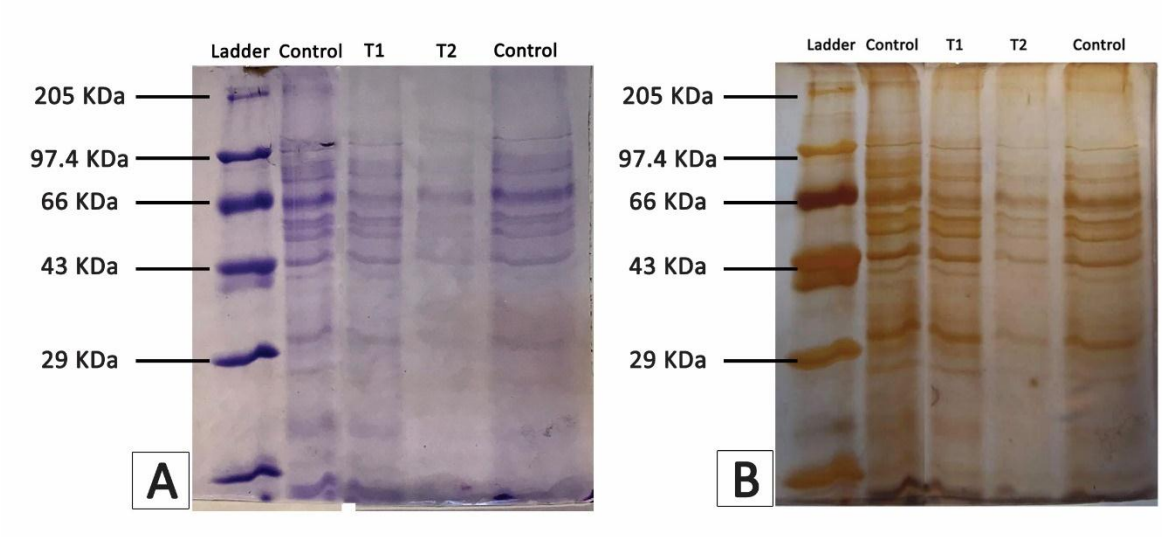

Figure 4.0.8: Protein Profiling by SDS-PAGE (A) Coomassie Staining; (B) With Silver Nitrate Staining

4.6 *in-vivo* Fertilization

Table 4.0.5: Number of births in control and extract (23µl, 1000 µg/ml) injected intravaginally into female mice

Set	**Control**	**Sample**
1	9	6
2	0	0
3	9	0
4	10	3
5	8	8
Mean	7.2	3.4
SD	±4.0865633	±3.5777088

Standard error = 2.429

95% CI = 9.4013 to 1.8013

t-statistic = -1.564

DF = 8

Significance level(P) = 0.1563

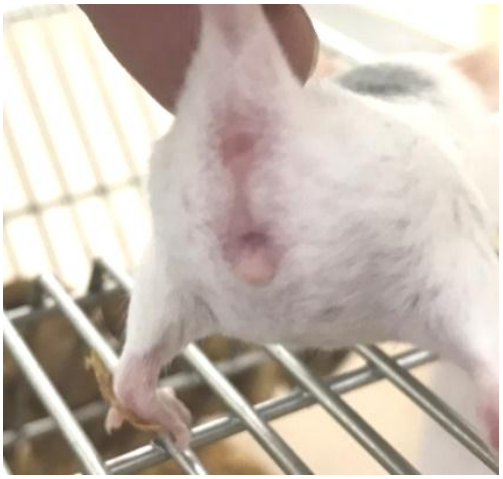

Figure 4.0.9: Identification of estrus phase in female swiss albino mice

In the present study, *in vivo* fertilization was performed to observe the effect of the sample extract directly on the litter size. A total of five sets of experiments were conducted, with a control. The 23 µl of sample extract of concentration 1000 µg/ml was injected topically through the vaginal opening of female mice before keeping it for mating, whereas the control mice were just checked for the estrous cycle and kept for mating with the same male mouse. 1000 µg/ml extract was used as it was found to be the most effective concentration to disintegrate acrosome, as shown by the results of FPNA staining test for acrosome integrity by use of *Tinospora cordifolia* extract. Among the five sets used for the experiment, both control and sample injected mice of the second did not give any birth. Similarly, sample injected mice in set 3 also did not give any birth, whereas all other control and sample injected mice were able to conceive. The average number of births in control was 7.2 and sample injected mice was 3.4 in five sets. In set 1 and set 4, the sample injected mice gave birth to less number of pupils than their control, whereas in set 5, both control and sample injected mice gave birth to 8 pupils each. The p-value 0.1563 was obtained from the observed results of total of five sets that failed to provide enough evidence that the difference between treatment and control is statistically significant. However, from the above experiment, it was clearly observed that a smaller number of pupils were given birth by the extract injected mice compared to the control, which gave a clear overview of the effectiveness of the sample extract.

CHAPTER 5: **DISCUSSION**

5.1 *Tinospora cordifolia* extract may contain various phenolic compounds

Tinospora cordifolia was selected for the experiment due to its accessibility as it is found at lower hills and mountains and capable of causing infertile state in male rats due to interference in the testicular androgen levels (Gupta and Sharma, 2003). 80% ethanol was used for the extraction purpose as it solubilizes most of the secondary metabolites. 44 mg/ml concentration of the sample extract was obtained from drying the sample at 110^{o}C resulting in complete removal of ethanol and water. The initial extraction process resulted in 4.4mg/ml concentration of the sample extract, which was further dried to concentrate to 44 mg/ml concentration.

The absorbance spectrophotometer reading showed the peak value between 200-300 with 0.14 absorption. Plant cells contain two types of metabolites, primary and secondary. Primary metabolites such as carbohydrates, lipids, vitamins, proteins, crude fiber and fats directly relate to the growth and metabolism of plants. Secondary metabolites like phenol, alkaloids, terpenoids, sterols, flavonoid, lignin and tannins etc. are considered as products of primary metabolite and are not usually involved in metabolic activity (Papitha et al., 2016). The absorption bands obtained confirmed the presence of phenolic compounds and its derivatives, which are primarily responsible for herbal properties in the plant (Altemimi et al., 2017). The precise composition of the extract can be investigated by biochemical analysis and HPLC that were not done in the present work.

5.2 *Tinospora cordifolia* extract induces Acrosome Integrity

The effect of *Tinospora cordifolia* extract on acrosome integrity of mice spermatozoa was studied. It was found to decrease with the increase in concentration of sample extract, with the percentage of acrosome integrity ranging from 90.13% in control to 44.37% at 2000 µg/ml of sample extract. The acrosome loss occurred at an exponential rate from 0 to 1000 µg/ml concentration of extract. The acrosome loss was further higher at 2000 µg/ml concentration of the sample extract, but we chose 1000 µg/ml for *in vivo* fertilization experiment due to two reasons. First, at higher concentration, there could be yet undefined toxic effect of the extract. Second, if higher volume of extract is injected to incur higher concentration, the mice will receive higher volume of ethanol (solvent), that may by itself pose harmful effect. It was not possible to further concentrate the extract due to precipitation problem. Acrosome evaluation is considered a valuable tool in the diagnosis of male infertility, as acrosome reaction is a major event for sperm penetration through zona pellucida. The use of lectins to label the acrosome for the same has been

identified as an effective method compared to others such as triple stains, chlortetracyclins staining, antibodies labelling, phase contras or electron microscopy. The combining of fluorescent antibody labelling with spermatozoal protein is the main advantage of using this technique (Lybaert et al., 2009).

Fluoresceinated Peanut Agglutinin (FITC-PNA) stains the acrosomal matrix by binding to glycoconjugates in the acrosome. The staining detected the loss of acrosomal matrix (Fig. 4.0.3 B) in mouse spermatozoa, thus confirming the effect of the sample extract in the experiment. The outer acrosomal membrane, as observed from electron microscope, has been found to be specific binding site of PNA and a reliable form of lectin. Therefore, a bright fluorescent over the acrosomal cap indicated the presence of the outer acrosomal membrane, representing acrosome intact sperm. However, a disrupted fluorescence or no fluorescence over the acrosomal cap represented the disruption of the membrane by the treatment of sample extract. This loss or degeneration of acrosomal membrane directly affects the fusion of spermatozoa with zona pellucida, thus inhibiting the process of zona binding (Bevers, 1996). The use of other lectins (PSA and PGA) have also been tested by Cunha, Carvalho and Dode (2015) and no difference in results have been observed regarding the acrosome integrity (Cunha, Carvalho, and Dode, 2015). Ethidium Bromide acts as an intercalating agent and interacts with nucleotides to emit fluorescence.

5.3 Effect on Sperm Viability after treatment with *Tinospora cordifolia* Extract

The effect on sperm viability was tested by using trypan blue method and hypo-osmotic swelling test, after treatment with *Tinospora cordifolia* extract and found to decrease with higher concentrations with no significant effect. The p-value 0.0014 showed that the difference between control and treated sample is statistically significant. However, the rate of sperm viability was not much lower. This value can only be confirmed by increasing the sample size. Ethanol is considered to have significant effect on the viability of spermatozoa (Rahimipour et al., 2013), but negligible effect was observed by using the vehicle control in which ethanol was supplemented in the media.

The trypan blue method is one of the most common dye exclusion test methods to determine the number of viable cells. According to this principle, the live cells possess intact cell membrane that exclude trypan blue, whereas dead cells do not. Other dyes which can be used in this test are Eosin or propidium. The method however has its own limitations as the study of viability solely depends on the integrity of cell membrane (Strober, 1997).

Hypo-osmotic swelling test is based on change of morphology of sperm tail when incubated in hypo-osmotic condition. It is due to the inflow of water and small molecular

-weight compounds and elements that enter the live sperm cell to reach osmotic equilibrium (Ramu and Jeyendran, 2013). Sperm cells treated with different concentrations of sample extract when treated with hypo-osmotic solution produced better results compared to the trypan blue method. The cells were difficult to observe as well by using trypan blue method, which might be due to the problem in stain or staining procedure.

5.4 Protein Profiling by SDS-PAGE

SDS-PAGE was performed by using direct heating method and the protein bands were observed, where there was no significant difference in the control and spermatozoa treated with *Tinospora cordifolia* extract. The protein profiling was done to see if there was any effect on sperm proteins by the use of *Tinospora cordifolia* extract. The bands were found to be concentrated more around 66KDa. The bands is supposed to be of acrosomal protein p67 proacrosin or mouse sperm protein Sp56. The Sp56 polypeptide is present in testes and epididymis but not in other mouse tissues and could be identified on the basis of its specificity, and high affinity for ZP3 protein recognized by sperm (Rosenfled, 1966). During the process, 5X sample buffer was used for the extraction as the sperm concentration was low. Silver nitrate staining was performed to enhance the visibility of the protein bands. In the present SDS-PAGE analysis, the entire proteins of spermatozoa were extracted. The effect of extract might be limited to few surface proteins which might be not revealed in the total protein profile. The difference in protein bands might be overshadowed by other unaffected major proteins. To reveal the affected target proteins, either differential extraction should be done or Western blotting with antibodies against putative proteins should be performed.

5.5 *in-vivo* Fertilization

The experiment of *in-vivo* fertilization resulted in lesser number of pupils in the mice injected with sample extract than that of control. As the process was manual and tedious, sample repeats and multiple experiments were performed to minimize the error or biosness. The optimal concentration, determined through the acrosome integrity test and viability test, was used for the *in vivo* experiment. However, sample loss might occur during the injection process. Female mice were first observed for their estrous cycle, which lasts for about 4-5 days normally. The cycle is continuous throughout the year and they are only receptive to males when they are in estrus. The sample injection was performed during the proestrus or estrus stage out of the four stages (diestrus, proestrus, estrus and metestrus) and left for 2-3 overnights for mating. The samples were injected each day for 3 days in the evening and kept for mating, as mating typically occurs at night. The mice were kept together consecutively for 3 days for mating to increase the probability of successful mating. As there is chance of loss of samples during that period,

the samples were injected each day. The vaginal plug was observed each morning for the confirmation of mating, after which the male and female mice were separated (Hill, M.A. 2019). The gestation period varied from 20-31 days for different sets of mice. As observed in the experiment, there was no certainty that all the female mice used for the experiment would conceive, which was seen from set 2. Thus, mice with lesser litter size is more preferred to mice with no litter. For better confirmation of the results and to minimize errors, sample size should be increased.

CHAPTER 6: **SUMMARY**

In this research, anti-fertility effect in mouse spermatozoa was tested by stem extract of *Tinospora cordifolia.* Ethanolic extract of the plant stem was prepared with different concentrations, the effect of which was studied on acrosome integrity and viability of spermatozoa. Optimized DMEM media was used for incubation, with optimum pH 7.2.

The acrosome integrity was studied by using FPNA staining method, which showed decrease in the acrosome integrity of mouse spermatozoa with the increase in concentration of sample extract. The acrosome integrity ranged from from 90.13% in control to 44.37% at 2000 µg/ml of sample extract. Similarly, trypan blue method and hypo-osmotic swelling method were used for the study of viability of spermatozoa. The p-value 0.0014 obtained from the viability test showed significant difference between the control and treated spermatozoa, statistically. However, negligible effect was seen practically. The percentage of spermatozoa viable at 2000 µg/ml concentration of extract was found to be 84.50%, which was the lowest compared to 97.48% of control.

There was no significant alteration in the protein structure of spermatozoa on treatment with *Tinospora cordifolia* extract on performing SDS-PAGE. From the *in vivo* experiment, p-value 0.1563 was obtained from the observed results of total of five sets that failed to provide enough evidence that the difference between treatment and control is statistically significant.

From above results, it is clear that *Tinospora cordifolia* stem extract was able to produce anti-fertility effect in mouse. However, sample size should be increased for further confirmation and to minimize errors.

CHAPTER 7: **CONCLUSION**

Tinospora cordifolia stem extract was used for the experiment to test its anti-fertility effect in mouse spermatozoa. The effect of the plant stem was successfully studied for its effect in anti-fertility, by studying the acrosome integrity and viability of spermatozoa treated with the sample extract and the optimum concentration of its effect was determined.

The acrosome integrity of mouse spermatozoa was found to decrease exponentially from 0 – 1000 µg/ml concentration of extract and optimum concentration of its effect was determined to be 1000 µg/ml. Negligible effect of the extract was observed in the viability of spermatozoa, studied by using two different methods: trypan blue method and hypo-osmotic swelling method. SDS-PAGE was performed to observe any alteration in the protein structure of spermatozoa on treatment with *Tinospora cordifolia* extract. However, no significant difference was observed from the SDS-PAGE bands.

The *in-vivo* experiment was performed in five different sets of mice which showed significant decrease in the number of pupils on treatment with sample extract. Thus, it can be concluded from the experiment that *Tinospora cordifolia* stem is capable of causing anti-fertility effect and reduce the chances of conception.

LIMITATIONS OF THE EXPERIMENT

1. During *in-vivo* experiment, a lot of variables are responsible for the birth of pupils and litter size. So, it is difficult to confirm that the effect in the fertility is due to the use of the sample extract. The only solution to this problem is to increase the sample size.

RECOMMENDATION/FUTURE PROSPECTS

1. Since, the *in-vivo* experiment depends on a lot of variables, both *in-vivo* and *in-vitro* experiments can be performed to confirm that the sample *Tinospora cordifolia* is capable of causing anti-fertility effect.
2. The positive and negative effects of the sample extract and its mechanisms are yet to be studied thoroughly, so there is still a lot of scope for further study in the topic.

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APPENDICES

Phosphate Buffer Saline 10X (PBS)

Sodium Chloride	8 g/L
Potassium Chloride	0.2 g/L
Disodium hydrogen phosphate	1.44 g/L
Potassium dihydrogen phosphate	0.24 g/L
Final pH	7.2

4% Formalin

40% Formaldehyde	1.25 ml
1X PBS	5 ml

2 µg/ml Ethidium Bromide (EtBr)

Stock Solution

EtBr	50 mg
DMSO (100 mg/ml)	500 µl

Working Solution

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

Sample Buffer 5X

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

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=6.8	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

Coomassie Staining Solution

Coomassie Brilliant Blue R-250	2.5 g
Methanol	450 ml
Glacial Acetic Acid	100 ml
Distilled Water	450 ml

Destaining Solution

Methanol	300 ml
Acetic Acid	100 ml
Distilled Water	600 ml