EVALUATION OF CONTRACEPTIVE PROPERTIES OF MEDICINAL PLANT *DIOSCOREA BULBIFERA* BY *IN VITRO* SPERM FUNCTION AND *IN VIVO* FERTILIZATION ASSAY TECHNIQUES

M.Sc. Thesis
(2020)

Submitted to:
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

For the Partial Fulfillment of the Requirement for the Award of Degree of Master of Science in Biotechnology

Submitted by:
Jayaswori Sharma
T.U. Regd. No.: 5-2-37-1152-2012

Supervised by:
Dr. Gaurishankar Manandhar
Central Department of Biotechnology

DECLARATION

I hereby declare that the thesis work entitled "Evaluation of Contraceptive Properties of Medicinal Plant *Dioscorea bulbifera* by *in vitro* Sperm Function and *in vivo* Fertilization Assay Techniques" submitted to the Central Department of Biotechnology, Kirtipur is a record of an original work done by me under the guidance of Prof. Dr. Gaurishankar Manandhar, Central Department of Biotechnology and this thesis work is submitted in the partial fulfillment of the requirements of the degree of Master of Science in Biotechnology. The results embodied in this thesis have not been submitted to any other University or Institute of any degree.

Jayaswori Sharma
Roll No.: 12/073

ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my supervisor Prof. Dr. Gaurishankar Manandhar, for the continuous support of my Masters' study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better supervisor and mentor for my thesis.

Besides my advisor, I would like to thank Head of Department Prof. Dr. Krishna Das Manandhar, Central Department of Biotechnology, Kirtipur, Kathmandu, Nepal for his encouragement, insightful comments and letting me conduct my research work.

I would also like to thank my senior PhD. Scholar Mr. Ramanuj Rauniyar, for his continuous guideline and support throughout the thesis work.

I would like to recognize all faculty members Prof. Dr. Rajani Malla, Prof. Dr. Tilak R. Shrestha, Prof. Dr. Mohan Kharel, Prof. Dr. Ganga Kharel, Mrs. Jarina Joshi, Mr. Balhari Poudel, Dr. Smita Shrestha, Mrs. Pragati Pradhan and Mrs. Alina Sapkota along with all teaching and non-teaching staffs of Central Department of Biotechnology.

I thank my fellow lab mates: Sabina Bhandari, Sarbesh Rijal, for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had in the last two years.

I am thankful to my friends along with my all Seniors and Juniors.

Last but not the least, I would like to thank my family, for giving birth to me at the first place and supporting me spiritually throughout my life. This accomplishment would not have been possible without them.

ACRONYMS

IUD	Intrauterine device
NDHS	Nepal Demographic and Health Survey
WHO	World Health Organization
LH	Luteinizing hormones
FSH	Follicle Stimulating hormones
COC	Combined oral contraceptives
POPs	Progestogen only pills
CIC	Combined injectable contraceptives
CVR	Contraceptives vaginal ring
LAM	Lactational amenorrhea method
ELFA	Enzyme linked fluorescent assay
PSA	Pisum sativum agglutinin
FITC	Fluorescein isothiocyanate
ELISA	Enzyme linked immunosorbent assay
SDS- PAGE	Sodium dodecyl Sulphate polyacrylamide gel electrophoresis
Gm	Gram
ml	milliliter
PVP	Polyvinyl pyrrolidone
BSA	Bovine serum albumin
µl	micro liter
µg/ml	micro gram per milliliter
DMEM	Dulbecco's Modified Eagle's Medium
HEPES	4-(2-hydroxyethyl)-1-piperazineetanesulfonic acid
TEMED	N, N, N', N'-tetramethylenediamine
CBB	Coomassie Brilliant Blue

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF GRAPHS

ABSTRACT

Dioscorea bulbifera is one of the medicinal plants which have shown to possess various medicinal properties like anti- tumor, anti - inflammatory, anti- arthritis, anti-fertility etc. *D. bulbifera* causes antifertility effect by suppressing ovulation and implantation of embryos in females. The main objective of the present study is to assess a direct contraceptive effect of *Dioscorea bulbifera* bulbil and tuber extracts by investigating *in vitro* sperm function assays and *in vivo* fertilization (IVF) techniques. Spectrometric analysis exhibited absorbance peak at 250 nm in both extracts. The tuber extract showed distinct shoulder peaks at 280 nm and 340 nm while such peaks were in conspicuous in the bulbil extract. Mouse epididymal spermatozoa were incubated in capacitation medium with various concentrations of extract. Spermatozoa were labeled with FITC peanut agglutinin (FPNA) or Coomassie to study extract induced acrosome reaction. The treated spermatozoa were stained with trypan blue or incubated with hypoosmotic medium to study viability and membrane damage. Both extracts caused significant acrosome exocytosis at very low concentrations. Comparatively, tuber extract resulted similar rate of acrosome loss as bulbil extract. *D. bulbifera* tuber extract also caused membrane damage, evaluated by trypan blue staining method and hypoosmotic swelling test. Viability loss was remarkably lesser than acrosome exocytosis. SDS PAGE was performed for protein profile analysis between the treated sperm sample and untreated control, from which we observed no remarkable change on sperm protein bands in the treated sperm cells. Finally, by performing *in vivo* fertilization test we have observed that the extract treated female mice had reduced litter size. These results suggest that *D. bulbifera* extract might effectively inhibit fertilization. The present research will help in characterizing potential contraceptive effect of *Dioscorea bulbifera* and in determining whether it could be used as a targeted and topical, contraceptive.

Keywords: *Dioscorea bulbifera,* anti- tumor, anti- inflammatory, anti-arthritis, anti-fertility, *in vivo* fertilization, FITC peanut agglutinin, acrosome exocytosis, contraceptive

Chapter I

INTRODUCTION

1.1. Background

1.1.1. Contraceptives

Birth control is the process to prevent pregnancy. It has become effective only from 20th century. The most common method of birth control is use of oral contraceptive pills, vaginal rings, patches and injections, sterilization by tubal ligation (in female) and vasectomy (in male). Other methods include condom, diaphragms, birth control sponges and fertility awareness methods (Hanson and Burke, 2010).

There are two types of contraceptives hormonal and non-hormonal. Birth control pills, vaginal rings, contraceptive skin patch and hormone releasing contraceptive coils are hormonal contraceptives (Contraception: hormonal contraceptives, 2008), whereas cervical cap, vasectomy, condom, diaphragm, contraceptive sponge, spermicide, copper intrauterine device act by inhibiting sperm cell to interact with the oocyte (Non-hormonal Birth Control Methods).

There is also another type of non-hormonal contraceptive which is known as immunocontraceptives. This type of contraceptive involves the interaction of antibody and antigen. One of the proteins that were targeted is fertilin α which plays an important role in sperm – egg adhesion during the fertilization. Antibody produced against fertilin α might inhibit sperm – egg adhesion during fertilization (Wong *et al*. 2001). After injecting recombinant fertilin subunit polyclonal anti fertilin IgG was seen in the blood but not in the vaginal lavages of test organism. These studies concluded that antibodies cannot pass into the inner lumen of uterus or fallopian tube to block fertilization (Hardy *et al*. 1997). Scientists have also shown interest in zona protein and the vaccine (Spay Vac) has been produced using antigen against porcine zona protein. The antibody produced by vaccination was found to be reactive towards the equine zona protein (Mask *et al*. 2015). But the zp vaccine immunocontraceptives have shown various side effect which can lead to permanent sterility. The use of such vaccines is not possible in humans because they cause ovarian dysfunction and oocyte degeneration (Joone *et al.* 2017). In addition to inflicting ovarian pathology in long term, they could cause hormonal imbalance as an immediate side effect (Joone *et al*. 2018).

The Nepal Demographic and Health Survey, 2011 has indicated that the Crude Birth Rate (CBR) is around 24.3 per thousand in Nepal. Nepal was one of the first countries of South Asia, where information about family planning was available through a non-

governmental program. The Fertility, Family Planning and Health Survey of 1991, Nepal Family Health Survey of 1996 and all rounds of NDHS (2001, 2006, 2011 and 2016) provide data about use of contraception. According the NDHS in 2016 the use of contraceptives is found to be 52.6% (modern method42.8%, female sterilization 14.7%, male sterilization 5.5%, pill 4.6%, injectables 8.9 %, condom 4.2%, Norplant 3.3% and IUD 1.4%) is the current percentage of population using contraceptives (National Population report, 2017).

With the report of National Population 2017 majority of females are using pill which is a hormonal contraceptive. Hormonal contraceptives are in the form of combined estrogen and progesterone pill and the first effective long-term, reversible method of birth control, hormonal contraception, which is used by an estimated 140 million women worldwide. Though with the high effectivity contraceptive pills have many adverse effects such as an increased risk of breast cancer, depression, thrombosis, and other cardiovascular events (Peachman 2018). The adverse effects of oral and injectable contraceptives are increased blood transaminase, cholesterol levels, indigestion, weight gain, headache, fatigue, hypermenorrhea and intermenorrhagial bleeding also disturb the metabolism of lipid, protein, carbohydrates, enzymes and vitamins (Unny *et al*.2003)

The target of male contraceptives is development of antispermatogenic agents to suppress sperm production, prevention of sperm maturation, prevention of sperm transport through vas deferens and prevention of sperm deposition (Sharma *et al*.2001). For the female contraceptives should be anti-ovulatory, affect the union of ova and sperm, be abortifacient, have effect on the uterus and show anti-implantation activity (Unny *et al*.2003).

1.1.2. Medicinal plants

Plants have been used worldwide as a safe natural source of medicines. From time immemorial, humans have relied on plants that could meet their basic necessities such as food, shelter, fuel and health. Out of all the important uses of plants, their therapeutic abilities played an inevitable part in the lives of primitive societies, as they relied on plants for healing ailments. The knowledge of healing powers of plants was initially passed down orally through generations, and as civilizations grew written records were prepared for the benefit of the population. Majority of herbal plants possess pharmacological principles, which has rendered them useful as curatives for numerous diseases. World Health Organization reports that 70% – 80% of the world population confide in traditional medicine for primary health care (Ogbuewu *et al.* 2011).

Medicinal plants have great importance in health sectors. We have been using medicinal plants as traditional treatment method to cure diseases and other health related problems. As medicinal plants consist of many phytochemical compounds which help in designing drugs (Ahn 2017). It has been found that compound isolated from plant have properties of antibiotics, anti-cancer agents, anti-inflammatory compounds, analgesics and antifertility activity. Out of 56% of currently prescribed drugs 24% drugs are derivative from plant species (Sen and Samanta, 2014).

Ayurveda, the science of traditional medicine in Hinduism remains to be the main source of medical knowledge and skill in most part of South Asia including Nepal. Vaidhyas and Kabirajs followed Ayurveda in their pursuit of knowledge and practice in medicine. It has been estimated that Ayurvedic knowledge was accessed by Nepali Vaidhyas as early as about 879 A.D. Nepal's wealth of Himalayan herb is reputed in Ayurvedic medicine all over the Indian sub-continent since time immemorable. Nepal has been supplying collected herbs as raw materials to Indian markets and manufacturing companies from ancient periods (National Registration of Medicinal Plants, 2000).

For the development of new drugs pharmaceutical companies are looking for more potent drugs with little toxic effect, self-administrable, less expensive and completely reversible, which qualities are observed in drugs of plant origin (Unny *et al.* 2003).

Many medicinal plants have shown various effects on male and female fertility due to which their extract can be used as contraceptives. Although many medicinal plants have been claimed to prevent fertility, only few plants were so far being investigated for their antifertility activity (Shaik *et al.* 2017).

Table 1.0.1: List of plants found in Nepal, that have been investigated for contraceptive use, their parts used, research done on animal model and mode of action

S. N	Name of the plants	Part used	Animal model	Mode of action
1	*Achyranthus aspera* Linn. (Anuja *et al*. 2011)	Root	Rat	Spermicidal action
2	*Artemisia vulgaris* Linn. (Shaik *et al.* 2014)	Leaves	Rat	Antiimplantation and estrogenic activity
3	*Curcuma longa* Linn. (Naz & Lough, 2014)	Root	Rat	Inferences with Spermatogenesis
4	*Dioscorrea bulbifera* (Rai & Nath, 2005)	Tuber		Contraceptives
5	*Dioscorea esculenta(l.) schott* (Shajeela et al.2011)	Tuber	Rat	Antiandrogenic effect
6	*Dioscorea villosa* (Atsukwei *et al*. 2015)	Tuber	Rat	Antiestrogenic effect
7	*Ocimum sanctum* Linn (Ahmed *et al*. 2002)	Leaves	Rat	Antiandrogenic property
8	*Piper longum* Linn. (Lakshmi *et al*. 2006)	Fruits	Rat	Antifertility activity
9	*Zinziber officinale Rosc*. (Afzali & Ghalehkandi, 2018)	Rhizome	Rat	Abortifacient
10	*Tinospora cordifolia* (Ittiavirah, 2013)	Stem	Rat	Reduction in testosterone

The World Health Organization (WHO) has established research regarding orally active non-steroidal contraceptive compounds from plants. It has been reported that there are many medicinal plants that show antifertility effect. One of the major actions is

their effect on sex hormones particularly for suppressing fertility, regularizing menstrual cycle, relieving dysmenorrhea, treating enlarged prostate, menopausal symptoms, breast pain etc. (Williamson *et al.* 1996). More over plants with estrogenic property can directly influence pituitary action by peripheral modulation of luteinizing hormone (LH) and follicle stimulating hormone (FSH), decreasing their secretions and blocking ovulation (Brinker. 1997). The plants with anti-estrogenic activities intercept in the process of development of ovum and endometrium and on the other hand, plants have abortifacient effects (Gark *et al*. 1975). The site of action of antifertility agents in females, comprises of the hypothalamus, the anterior pituitary, the ovary, the oviduct, the uterus and the vagina. The mammalian uterus is the main site of antifertility effects (Wiliamson *et al*. 1996). Estrogenic compounds possess ability to increase the uterine wet weigh and induce cornification and opening of vagina in immature rats which results anti-implantation effects (Turner, 1971). Plant extracts are also shown promising antifertility effects when administered to male rats. The various effects on male reproductive system to induce antifertility action shown by plants includes antispermatogenic effect, post-testicular antifertility effect, spermicidal, sperm immobilizing effect, antiandrogenic effect etc. (Shaik *et al*. 2017).

In an article "A comprehensive review of plant used as contraceptives Pradhan *et al.* in 2012 had enlisted 133 different types of medicinal plants with their parts to be used as well as their action in antifertility. In which there are medicinal plants which are *Achyranthes aspera, Artemisa* species, *Curcuma* species, *Dioscorea bulbifera, Hibiscus rosa-sinensis, Piper nigrum* and so many which are easily available in our surrounding environment.

Dioscorea bulbifera (*Vyakur*)

The classification of Dioscorea bulbifera is below:

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Monocotyledonae

Order: Dioscoreales

Family: Dioscoreaceae

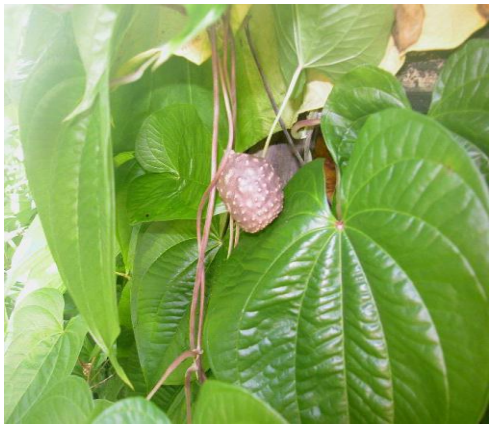
Genus: *Dioscorea*

Species: *Dioscorea bulbifera*. L

The genus Dioscorea was founded by Linnaeus in 1737 and, as created by him, included two of the more important food yams - *D. alata* and *D. bulbifera*. The genus is named in honor of the Greek physician Pedenios Dioscorides, a medical officer in the Roman army at the time of Nero (Coursey, 1967).

Dioscorea bulbifera is a monocotyledonous, dioecious, herbaceous, perennial, vigorous, twinning vine with non-spiny stems to 20 m or more in length, freely branching above, internodes around or slightly angled in cross section. Leaves are alternate, blades 9-12.5 × 5.5-11 cm, oval, chartaceous glabrous, with 9-11 parallel veins, the apex acuminate to caudate, the base cordate, the margins slightly undulate, petioles (4) 12-15 cm long, winged and projecting as a part of pseudo stipules surrounding the stem at base, bulbils axillary, rounded, 5-6cm wide. Plants inflorescences are axillary and simple fasciculate. Flowers are white or pinkish tinged, diminutive and sessile, staminate flowers with perianth approximately 1.2 mm long and 6 fertile stamens, pistillate flowers with perianth approximately 1.4mm long and a hypanthium approximately 2 mm long fruits are capsules 3-winged, 2.5 cm long (Sandoval and Rodriguez, n. d). Tubers are toxic or edible according to the variety; they are renewed annually (Mbiantcha *et al.*2010). The Nepali name for *Dioscorea bulbifera* is Githha and Vyakur. It is one species out of 13 found in Nepal and are commercially used in trade. It is distributed from 150 to 2000 m, especially in Chure and Mahabharat of Nepal and harvested from December to February (NEHHPA, 2018).

a) Plant of *Dioscorea bulbifera*



b) *Dioscorea bulbifera* with bulbil

c) Subterranean tuber

Figure 1.0.1: *Dioscorea bulbifer* (Air potato management plan, 2008).

a) b)

Figure1.0.2: a) Bulbils of *Dioscorea bulbifera f*rom Dharding District. b) Dried tuber *of Dioscorea bulbifera.*

The wild *Dioscorea bulbifera* contain 18 compounds of pharmaceutical values including the steroid saponin and diosgenin (Liu *et al*. 2011). Diosgenin is similar to the adrenal hormone DHEA and the ovarian hormone progesterone. Hence, it is widely used in the synthesis of corticosteroids, estrogen, and contraceptives (Liu *et al*. 2011). Phytochemical screening of aqueous and methanol extracts revealed the presence of flavonoids, furanoids and saponins (Mbiantcha *et al.* 2010). *Dioscorea bulbifera* is one of the unique medicinal plants among 600 species in the family Dioscoreaceae which has found its importance in traditional medicine throughout the world. Due to which it has emerged as a promising complementary and alternative source of traditional medicine attracting prime attention towards spectacular scientific advancement in exploration of new biomolecules (Ghosh *et al.* 2015).

1.1.3. Fertilization

Fertilization is the process by which eggs and spermatozoa interact, achieve mutual recognition, and fuse to create a zygote, which then develops to form a new individual, thus allowing for the continuity of a species (Okabe, 2013).

There are three stages of fertilization, a) Capacitation and acrosome reaction; b) Sperm-oocyte binding and fusion; and c) Cortical reaction, meiosis resumption of oocyte and cleavage of the zygote (Georgadaki *et al*. 2016).

a) Capacitation and acrosome reaction: The phase of sperm functional maturation is known as sperm capacitation. It occurs in the genital tract of the female, and acts as a preparatory step for the acrosome reaction (Yanagimachi, 1994). Capacitation involves a number of processes, such as functional coupling of the signal transduction pathways that enable spermatozoa to undergo acrosome reactions induced by ZP3; alterations in flagellar motility that may be required to penetrate the zona pellucida; and the development of the capacity to fuse with eggs (Evans & Florman, 2002).

The acrosome reaction of the sperm is caused by the core protein of ZP3. The outer membrane of the acrosome fuses at multiple sites with the plasma membrane and the contents of the acrosome are released. After the acrosome reaction, it is believed that the protein pre‐acrosin of the acrosomal vesicle binds to ZP2, and becomes activated, forming the enzyme acrosin which digests glycoproteins of zona pellucida. Digestion of the zona pellucida is followed by the fusion of the membrane of the sperm to the egg membrane, which seems to be caused by the PH‐30 sperm protein (Blobel *et al*. 1990).

b) Sperm- egg binding and fusion: Sperm oocyte fusion involves sperm attachment to the oocyte membrane by cell‐cell adhesion, leading to membrane fusion of the two gametes (Evans, 2002). The inner acrosomal membrane of the sperm, being exposed following the acrosome reaction, comes into contact with the oocyte membrane (Huang & Yanagimachi, 1985). The equatorial segment adheres to the posterior head of the sperm and fuses with the oocyte membrane (Yanagimachi & Noda, 1970). Several molecules have been identified in sperm and oocytes, with a crucial role in gamete binding. Fertilin α, fertilin β and cyritestin are also known as ADAM1, ADAM2 and ADAM3, respectively (Evans, 2002). Two molecules, trypsin‐like acrosin and spermosin proteases have been proposed to be involved in the first physical contact of the two gametes (Rubinstein, *et al*. 2006).

c) Cortical reaction, meiosis resumption of oocyte and cleavage of the zygote: Once the sperm fuses with the oocyte, the beating of the tail stops immediately. The fusion of sperm and the oocyte membrane appears to cause the polymerization of actin and

microvilli extension. The sperm instead is drawn into the oocyte by elongation and fusion of the microvilli of the egg. As a result, the sperm nucleus and other organelles are incorporated into the oocyte cytoplasm (Georgadaki *et al*. 2016).

Seconds after fertilization, the membrane potential of the oocyte undergoes a large depolarization via a massive influx of Na+ ions. The depolarization of the egg takes about a minute to repolarize via K+ leakage. This is the fast block to polyspermy: sperm cannot fuse to a membrane that is not ‑ 70 mV (Georgadaki *et al*. 2016).

The cortical reaction is a process through which cortical granules from the oocyte are released preventing polyspermy. The fast block of polyspermy immediately prevents additional sperm getting attached to the oocyte. On the other hand, the cortical reaction establishes a permanent barrier to sperm entry and functions as the main part of the slow block of polyspermy in many animals. The cortical reaction is propagated over the surface of the egg by a wave of Ca++ (Georgadaki *et al*. 2016).

The sperm nucleus undergoes a series of changes, including chromatin decondensation and the formation of a new nuclear envelope, to form the male pronucleus. The latter uses microtubules to migrate to the center of the cell. Other sperm organelles persist during the early cleavage stages of the embryo and they may play a role in development (Evans & Florman, 2002).

The last phase of oocyte activation is the resumption and completion of meiosis. This leads to 2ndpolar body extrusion, cleavage of the zygote and embryonic cell divisions (Krauchunas & Wolfner, 2013).

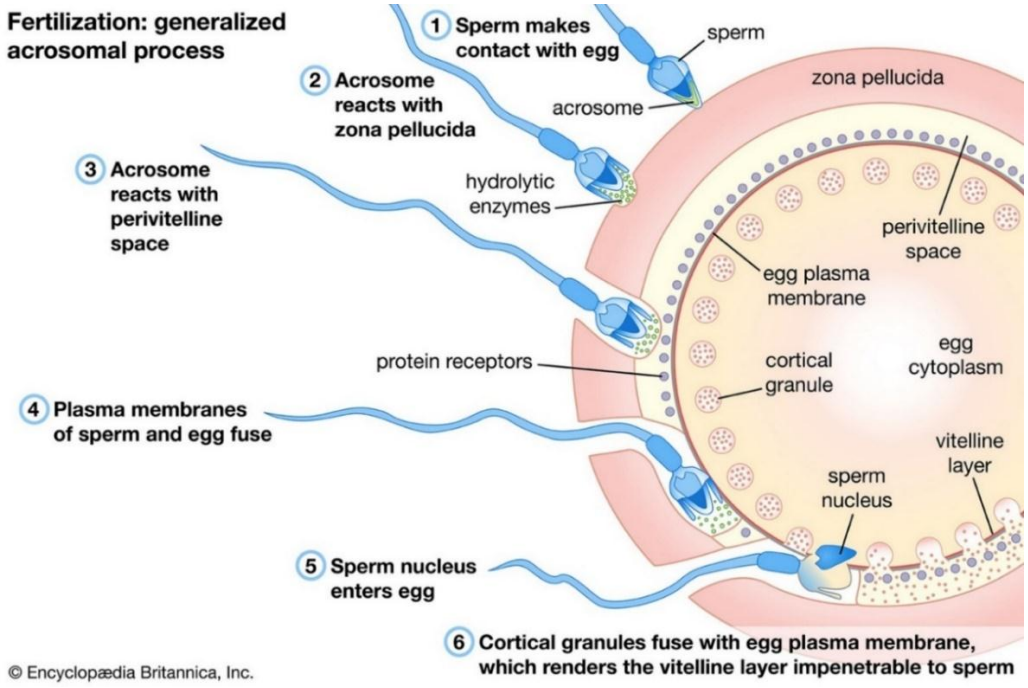


Figure1.0.3: Stages of sperm penetration through zona pellucida (Monroy, 2018).

1.1.4. ESTROUS CYCLE

The estrous cycle is the main reproductive cycle of non-primate vertebrate's female. The time duration of these cycle varies in different animals. In polyestrous animals (rats, mice) estrus cycles take place throughout the year, in seasonally polyestrous animals (horses, sheep) multiple estrus cycles take plays only during certain periods of the years. In monoestrous animals (dogs, wolves) only one estrous cycle occurs per year (Hill, 2019).

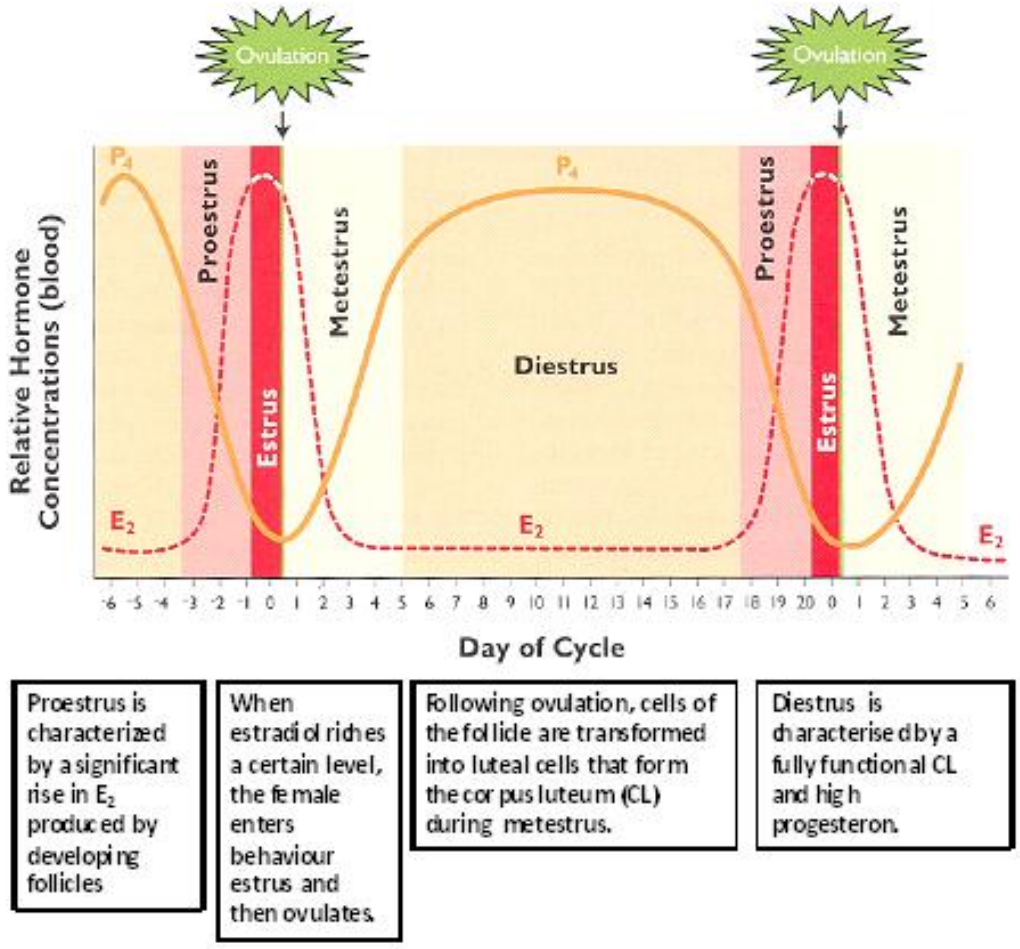


Figure1.0.4: Stages of estrous cycle (E_2: estradiol, P_4: progesterone); Senger (2004)

Mouse is a polyestrous mammal which estrus cycle repeats after every 4 or 5 days until and unless interrupted by pregnancy, pseudopregnancy and other events. Estrus cycle is very complex where there is rhythmic interaction of pituitary and ovarian hormones. The periodicity of estrus observed in mature females is a direct result of cyclic changes that occur in the ovary, which, in turn, reflects altered hypothalamic activity and changes in gonadotropin secretion. The reason of the basic responsibility for such rhythmicity is still not thoroughly understood. The key to cyclic reproductive

activity apparently lies in the hypothalamus, which communicates with the anterior pituitary by way of a portal system. FSH, a protein, is released by the anterior pituitary and acts to promote follicle growth. Another anterior pituitary protein, LH, aids in the final development of the mature follicle and facilitates production of estrogens by the theca interna cells of the FSH-primed follicle. Further release of LH by the hypophysis results in rupture of the follicle and ovulation. Increasing titers of estrogen during the later phases of follicular growth are thought to act, by way of the hypothalamus, both to suppress further release of FSH and to favor release of more LH. Progesterone, another gonadal steroid, is also produced in the ovary in small quantities during the follicular growth phases. Progesterone, in small doses, promotes ovulation be enhancing LH release. Thus, the gonadal hormones produced during follicular growth act on the hypothalamus to suppress further release of FSH while promoting release of LH and hence ovulation. Functional development of the corpus luteum in the mouse is induced by mating and, when this does not occur, gonadal hormone titers decrease allowing succession of a new cycle. The prime factor allowing for the periodicity of estrous phenomena is thought to be cyclic activity in the hypothalamus which is reflected in LH release (Allen, 1922).

Divisions of the estrous cycle

The mouse estrous cycle has been divided into as few as four phases: diestrus, proestrus, estrus, and metestrus; or as many as 13 (Thung *et al.* 1956). The latter system consists of a single stage of diestrus, four of proestrous, two of estrus, and six of metestrus. Since the cycle is continuous and a division into so many stages of the cycle would rarely be used, this section will rely on a five-stage description of the cycle (Allen, 1922). The first two stages (proestrus and estrus) are anabolic stages during which active growth is in progress in various parts of the genital tract. They culminate in ovulation and, where mating occurs, in fertilization. The third and fourth stages, metestrus-1 and metestrus-2, are catabolic stages characterized by degenerative changes in the genital tract. The last (or first) stages, diestrus, is a period of quiescence or slow growth.

1.2. Current studies

There has been a lot option for female contraceptive uses. But there are still some women who want to use contraceptives but they could not due to the side effects of hormonal contraceptives as well as economical condition and other problems. There are not many choices for male contraceptives available. Although the male reproductive system is complex and it is very difficult to find out the effective

compound for the intervention. This study is focused on developing non- hormonal and topical usable female contraceptives that block fertilization inhibiting sperm functions in the female reproductive milieu.

1.3. Research hypothesis

This research is aimed to evaluate the contraceptive effects of medicinal plant *Dioscorea bulbifera.*

Null hypothesis: *Dioscorea bulbifera* does not shows effective contraceptive effects on mice.

Alternative hypothesis: *Dioscorea bulbifera* shows effective contraceptive effects on mice

1.4. Objectives

1.4.1. General objectives

To evaluate contraceptive effects of tuber and bulb extracts of *Dioscorea bulbifera.*

1.4.2. Specific objectives

To investigate contraceptive effects of *Dioscorea bulbifera* tuber and bulbil by *in vitro* sperm functioning assays.

- To investigate effect of plant extract on sperm viability.
- To investigate effect of plant extract on sperm acrosome reaction.
- To investigate the effects of plant extract on sperm proteins.

To investigate effect of plant extract in *in vivo* fertilization.

1.5. Rationale

As we know that our country is one of the developing countries which is suffering from problems related to health, environment and economics. One of the factors that combine all the three things together is population growth. The main target of our project is to identify the medicinal plants that would directly inhibit gamete functions and fertilization process. The putative contraceptive plant product will not affect other bodily physiology and hence will not pose deleterious side effect. On one hand, discovery of such plants would bring enormous economic benefit to the rural people as they can cultivate such plants or collect them from wild and sell them to make money. In the meantime, availability of locally available safe contraceptive plant will ensure substantial health benefit to the people.

Chapter II

LITERATURE REVIEW

2.1. Medicinal plants

Nepal is ranked as 9th among the Asian countries for its floral content with estimation of 9000 species of flowering plants (Bhattarai *et al.* 2006) among which 1,792 to 2,331 are categorized as useful medicinal and aromatic plants that are able to as traditional therapies. It was found that the traditional usage of medicinal plant for various disorders or diseases are clinically tested and proven (Etuk & Mohammed, 2009). Therefore, medicinal plants are used for antidiarrhea, antidysentery, treatment of toothache, anti-inflammatory anti-bacterial, antimicrobial, anesthetic, antihypertension, pigment inducer, dandruff treatment, jaundice and many more (Kumar *et al.* 2013). A large number of plant species with antifertility effect have been screened in China and India from 50 years ago which were subsequently fortified by national and international agencies (Kaur *et al.* 2011).

Plant parts that are used for medicinal purposes are bark, fruit, flower, inflorescence, leaf, root, rhizome, stem, seed, wood, and the whole plant. The most frequently utilized plant parts were roots and rhizomes of 38 species, followed by fruits of 26 species, leaves of 22 species, etc. Underground parts were frequently used, and this was attributed to presence of bioactive compounds. This was the result of study done by Kumar *et al*. in 2013 where they have recorded 238 species of plant from Darchula, Baitadi and Dadeldhura and out of which 132 species of plants that is 55% of plants had ethnomedicinal uses.

2.2. Medicinal plants as contraceptives

Medicinal plants have been used since time immemorial for their effect upon reproductive health mainly for suppressing fertility, regularizing menstrual cycle, relieving dysmenorrhea, treating enlarged prostate, menopausal symptoms, breast pain and labor- pain during and after childbirth. The action of herbal contraceptives may vary according to the plants. Their use may lead to rapid expulsion of the fertilized ova from the fallopian tube, inhibition of implantation due to disturbance of estrogen-progesterone balance, fetal abortion perhaps due to lack of supply of nutrients to uterus and the embryo. Also, on the male side through affecting sperm count, motility and viability.

The use of herbal preparation may vary. Some might be needed to take daily to maintain the contraceptive effect like wild yam (*Dioscorea villosa),* while other might be needed to take during fertile period (Anandel *et al.* 2015).

Pradhan *et al* in 2012 have enlisted 133 indigenous medicinal plants found in India. Likewise, Shaik *et al.* in 2017 have enlisted 233 medicinal plants in India. In both paper various plants had shown various antifertility effects like ant implantation, abortification activity, antioestrogenic activity, contraception activity, anti-gonadotropic activity, block oestrous cycle, anti-fertility, spermicidal, inhibit fusion of sperm and ovum, arrest oogenesis and depletes estrogen level, effecting spermatogenesis, antiovulatory activity, antiandrogenic activity and so on.

The site of action for antifertility agents in females comprises hypothalamus, anterior pituitary, ovary, oviduct, uterus and vagina. The hypothalamus produces two types of hormones follicle stimulating hormone (FSH) and Luteinizing hormone (LH) which controls the action of the uterus. Therefore, antifertility agents may act by disrupting hormonal function of the hypothalamus or the pituitary, or by interrupting the neural pathway to the hypothalamus that finally leads to liberation of gonadotrophin releasing hormones. Phytoestrogens are the compound of plants which are derivatives of oestrogen and progesterone found in ovary and placenta which imbalance the hormones and finally acts as contraceptives. Some of the compounds of plants which acts as phytoestrogens are isoflavones in soy, lignans of grains, stilbenes in skin of grapes. Also, some compounds like 2 triterpenic saponins called ardisiacrispin A and B isolated from *Ardisia crispa* root have shown uterocontracting properties (Pradhan *et al.* 2012).

It has been reported that antifertility agents found in plants act in various ways like Sdisrupting estrus cycle, showing antiestrogenic activity, anti-implantation activity and abortifacient. As indicated above, the plant extract shows various imbalance in the hormone which leads to disrupting the estrus cycles. The ethanolic extract of *Rivea hypocrateriformis* resulted in an irregular estrous cycle with shortened estrus and metestrus while with extended proestrus. *Cuminum cyminum* have shown antiestrogenic activity by higher activation of Sodium Potassium pump which increases transport of fluid into uterus lumen and lead to increase weight of uterine (Malini and Kumari,1987). *Sesbania sesban* seed powder caused reduction in endometrial height, shrunken the uterine glands, poor vascularity in the compact stroma and prevent the implantation causing abortion (Raj *et al.* 2011). Tape-vine leaves have shown anti-implantation effect by causing hypoglycemic effect which causes toxicity leading in reduction of corpus lutea due to increase in progesterone level (Mukherjee *et al.* 2006).

Male contraceptive agents may work as antispermatogenic agents to suppress sperm productions, prevent sperm maturation, prevent sperm transport through vas deferens and prevent sperm deposition. Plants bioactive products are reported to target the testes at the hormone level or spermatogenesis or both (Soni *et al.* 2015).

Action of plant extract as spermicidal has been seen in various plants extract like lemon juice which acidity leads to denaturation of dyein ATPase leading the sperm death, in the same way saponin found in some plants like *Acacia caesia*, *Phytolacca dodecadra* seems to modify the ion transport of cell membrane by attacking lipid bilayer (Dhar *et al*. 1989). The production of mature sperm cells is known as spermatogenesis. The *Cannabis* extract increases lipid peroxidation in the testes leading to arrest spermatogenesis (Soni et al. 2015) Similarly, Sertoli cell and Leydig cell plays important role in spermatogenesis. They are affected by *Tinospora cordifolia* (Gurav *et al*. 2017) *and Ocimum sanctum* (Aladakatti & Ahamed, 2005) extract respectively. For normal fertilization transport of sperm cell and its motility should be high. Sperm motility is also found to be affected by some plants like *Gossypum herbaceum, Carica papaya*and so on. Some plants affect spermatogenesis and other processes occurring in males (Soni *et al*. 2015).

Dioscorea bulbifera

Dioscorea bulbifera one of the medicinal plants which have anti-fertility activity as enlisted by both Pradhan *et al.* 2012 and Shaik *et al.* 2017.

Roasted tuber of *Dioscorea bulbifera* is used in leprosy, asthma, cough, cold, tuberculosis, contraceptive, constipation, indigestion, abdominal pain, dysentery, sore throat, Struma, wounds, boils, cuts, injury, carbuncle, tumor, tuberculosis, also used as refrigerant to reduce body heat during summer and to maintain kidney function. Also used in diseases of lungs, spleen, diarrhea, improving digestion and metabolism. Bulbils are used in treating children with typhoid. Fresh tuber is used to cure laryngitis in children, insect bite, ring worm, goiter, and fever. Also, tubers are used for the treatment of purgative, aphrodisiac, rejuvenating and tonic anthelmintic, hemorrhoids, scrofula, worm infestations, general debility and polyuric (Dutta, 2014).

The main components in *Dioscorea* species are Diosgenin (Asha and Nair, 2005), Sapogenin (Martin, 1969), Saponin (Nayaboga *et al.* 2014), Cyanidin (Hov *et al.* 2000), Flavonoids (Poornima and Ravishankar, 2007), Allantoin (Fujihara and Yamaguchi, 1978), Dioscorine (Adetoun and Ikotun, 1989), Phenolic compounds (Liu *et al.* 2011).

It has been found that the component of *Dioscorea* species have been used for its anti-oxidant activity (Hou *et al.* 2001), cytotoxic activity (Dong *et al*. 2004), anticancer

effects (Yu *et al.* 2004), antibacterial (Seetharam *et al.* 2003), antifungal, antimutagenic, hypoglycemic and immunomodulary effects (Son *et al.* 2007).

Among the components of *Dioscorea bulbifera* aglycons of steroids saponins like diosgenin have been used as precursor in the synthesis of sex hormones, cardiotonic glucosides, fertility control compounds, corticosteroids and anabolic agents (Bajaj, 1999).

Antifertility activity of *Dioscorea esculenta* have been tested on male albino rats (Shajeela *et al.* 2011) in the same way antifertility activity of *Dioscorea villosa* on reproductive hormones of female Wistar rats have been tested (Atsukwei *et al.* 2014). Antifertility effect of *Dioscorea bulbifera* has not published till now.

2.3. Methods used for contraceptives testing

As reviewed in Mishra *et al* 2017, there are various testing done for evaluating antifertility effects of mainly four plants *Azadirachta indica, Curcuma longa, Allamanda cathartica* and *Bacopa monnieri*. The antifertility testing was done in animal model Parkes male mice by using aqueous extract of all four plant (Upadhyay *et al.* 1993) Administration of 50 µl of *Azardirachta indica* extract on each side of the lumen of the vas deferens induced a long-term inhibition of fertility (Upadhyay *et al*. 1993). Similarly, Joshi *et al* (1996) found that the treatment in mouse caused a decrease in diameter of the seminiferous tubules with atrophy of the spermatogenic elements and the Leydig cells. Hoffer (1983) have found that in *Azadirachta indica* treated mice infertility effect is due to vacuoles formation in the seminiferous tubules and Sertoli cells which suppresses spermatogenesis.

Mice that have been treated with *Curcuma longa* rhizome extract with dose of 500 mg/kg body weight of mouse for 60 days, weight of epididymis, seminal vesicle, ventral prostate and testis was found to be decreased. Also, there was reduction in sperm count and motility. The number of germ cells was reduced and Leydig cells were also affected by the treatment (Ashok and Menakshi, 2004).

The aqueous root extract of *Allamanda cathartica* with the dose of 150mg/kg weight of mouse was given for varying days (14, 28 and 42 days) it did not affect the body weight, weights of testis and seminal vesicle but the weight of epididymis weight was decreased also sperm numbers, viability and motility in caudal epididymis were affected (Singh and Singh, 2008).

The aqueous extract of *Bacopa monnieri* with the doses of 250 mg/kg of mouse was treated to the mice for 28 and 56 days. It showed spermatogenesis and spermiogenesis the similar result like in case of *Allamanda cathartica* and also found

that when a female was mated with extract treated males there was no live implantation (Singh and Singh, 2009).

Another different approach was done by Paul *et al.* in 2005. They had used 50% ethanolic extract of *Stephania hermandifolia* and *Achyranthes aspera* on the freshly collected semen by invitro testing to determine the spermicidal effect of plant extract. In the experiment they had performed immobilization assay by using varying concentration of plant extract on semen sample and noticing the motility of the sperm; extract stability evaluation by changing the temperature following the sperm motility testing, EC_{50} (effective concentration of extract which causes 50% immobilization of highly motile sperms) determination, sperm revival test, sperm viability test using eosin and nigrosine stain, acrosome status and function test and hypoosmotic swelling test. From all the test and with their result they came to the conclusion that this composite plant extract possesses potential spermicidal activity in vitro.

In the paper written by Elvis-offiah *et al.* 2016 he had done his contraceptive experiments on female albino mice using *Emilia coccinea.* Two types of experiment were done one was to determine the hormonal changes and toxicity study and next is determine estrus cycle effect. In the first experiment various doses of methanolic extract of plant were given orally ranging from 100 mg/kg of body weight to 2000 mg/kg of body weight. Afterward the mice were killed after 24 hrs. and their various bio chemical analyses were done. It was found that up to 2000 mg/kg of body weight it was safe to use. In the second experiment the mice were observed to determine their estrus cycle and various doses of plant extract was given up to 6 days and their uterine weight was taken as well as hormonal assay was done using Minividas analyzer, Enzyme linked fluorescent assay (ELFA). It was found that high dose caused prolonged estrus cycle, increase progesterone level and enlargement of uterine finally ensuing anti-implantation effect.

Similarly, Shah *et al.* 2018 conducted an experiment by using female Wistar rats which were feed with the aqueous and alcoholic *Piper betle* extract with the doses of 500 mg per kg of body weight per day for 30 days. In this experiment various parameters were studied including effect of reproductive outcome, anti-implantation, abortifacient and anti-estrogenic activity. They had found up to 37.2% antifertility activity.

Two species of *Dioscorea* have been screened for contraceptive properties out of which one was *Dioscorea esculenta* and another was *Dioscorea villosa.* Shajeela *et al*.2011 had screened the contraceptive properties of *Dioscorea esculenta* using its ethanolic extract and animal model as male albino rats. In their experiment they had used the tuber ethanolic extract of plant with varying doses from 100 mg per kg of

body weight to 600 mg per kg body weight for 14 days. They had performed acute toxicity study, various biochemical assay, changes in weight of testes, epididymis, vas deference and ventral prostrate. Also, investigator performed sperm count, sperm motility and abnormality, serum biochemical analysis and hormonal assays (mainly LH and FSH by Enzyme Immunoassay Method (EIA)). Their study showed that the plant extract inhibited sperm concentration, motility and testosterone.

Atsukwei *et al*.2015 had studied in *Dioscorea villosa*, they had used ethanolic extract of plant's tuber. They had used female Wistar rats and experimented by oral administration of plant extract using doses 100, 200 and 200 mg/kg body weight. They had done only the phytochemical screening of plant extract and serum hormone concentration. They had found that the ethanolic plant extract altered the reproductive hormone and hence effected on reproduction process.

Scientist have studied acrosome by various methods as it plays key role in fertilization. Conventional method of determining the acrosome intactness was done by Gopalkrishna *et al*. (1995) in which the semen was first diluted with phosphate buffered-saline-d-glucose solution and left for incubation at 37°c. Then the sample were smeared on a gelatin coated slides and incubated in room temperature for 5-15 min and transferred to the moist chamber and incubated at 37°c for 2 hrs. The slides were observed under bright field microscope with 40X magnification. Observation of halo indicate intact acrosome.

Another novel method studied by Margalit *et al*. 1997 was using Pisum sativum agglutinin (PSA) which has the ability to bind specifically to glycoprotein of the acrosome matrix released during the acrosome reaction. One of the glycoproteins is acrosin protein. This technique was found correlated with the result obtained in conventional methods for determining acrosome status (Esteves *et al*. 2007). He had used Fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) in conjugation with hypoosmotic swelling test and supra vital staining Hoechst 33258 to monitor sperm viability. The staining used was observed using Phase contrast and Fluorescence epi-illumination module with 1000X magnification. Cells with green staining over the acrosomal cap were considered acrosome intact; equatorial red staining or no staining at all were considered acrosome reacted; 200 cells were counted per each slide. This experiment was simple but effective for establishing the state of acrosomal membrane to viability (Margalit *et al*. 1997)

In case of fertilization process acrosome reaction is the main step that take place, which involves lots of proteins (enzymes). In the study performed by Nagdas *et al*. 2013 have performed SDS-PAGE and $^{45}Ca^{2+}$- blot overall assay to identify the acrosomal membrane proteins. Using SDS-PAGE and a $^{45}Ca^{2+}$-blot overlay assay,

calcium-binding proteins of 64, 45, 43, and 39 kDa were identified in the AM enriched fraction. Phase separation analysis with Triton X-114 identified the 64 kDa polypeptide as an integral membrane protein. Rajeev *et al*.2004 had performed SDS- PAGE along with other five protocols for sperm protein analysis and he found that 57kDa protein was responsible for binding of sperm to the oocyte and known as sperm membrane protein.

Chapter III

MATERIALS AND METHODS

This study was performed in Central Department of Biotechnology (CDBT), Institute of Science and Technology, Tribhuvan University, Nepal. Major work of this study was wet lab based and some of the study were dry lab based experimental study.

3.1. Plant extract preparation

3.1.1. Pre extraction preparation

Dioscorea bulbifera bulbil was collected from Dhading area and tuber was obtained in dried form from Kathmandu area. After the collection of plant parts, they were washed properly and chopped into small pieces to increase the surface area to dry them properly. They were dried at room temperature for one day, afterwards dried in oven at 60°C for three days or more until the plant parts were completely dried. After that the plant parts were grinded into powder form by using a grinder.

3.1.2. Extraction

Plant extract was then extracted by soaking 100 gm of powder in 300 ml of 80% ethanol (1:3) by the process called maceration and left for three days in the shaker. Then, it was filtered by using Whatman's filter paper. After that the filtrate was stored in room temperature and ready to use for further experiments.

3.2. Determination of dry weight of plant extract

Firstly, weight of a glass Petri plate was taken in which 5 ml of ethanolic extract of plant part were added and dried at the temperature at 110°C. After complete evaporation of ethanol, the weight of the plate was taken again and was subtracted the value from the previous blank plate which gives g/5ml dry weight of plant extract.

Calculation

Let, W1= weight of blank Petri plate

W2= weight of Petri plate with dried 5 ml plant extract

Dry weight of plant extract = (W2-W1) g/ 5ml

Dry weight of plant extract= ((W2-W1)/5) g/ ml

3.3. Spectrophotometry analysis of plant extract

The ethanolic extract of plants parts were 100 times diluted with 80% ethanol so that the color density of the plant extract will not affect the absorbance of secondary

metabolites. Graphical representation of absorbance of plant extract from 190 to 490 nm was plotted with the help of spectrophotometer.

3.4. Cell culture media preparation

DMEM (Dulbecco's Modified Eagle's Medium, D5523, 10 gm/ L) was taken to which either sodium bicarbonate ($NaHCO_3$, 3.7mg/ml) or HEPES (4-(2-hydroxyethyl)-1-piperazineetanesulfonic acid, 25mM) was added to balance the pH in CO_2 incubator or normal incubator respectively. After adding the buffering salt and maintaining pH BSA (Bovine serum albumin, 5mg/ml) was added and volume was made up to required. Also, in case of viability testing we had used PVP (Polyvinyl pyrrolidone, 0.1 %) instead of BSA because BSA also takes the color of trypan blue and which makes the slide view inappropriate to count number of sperm cells.

3.5. Mouse 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: 12hrs dark period. The temperature was maintained at 22-28°C by using light bulbs.

3.6. Sperm sample preparation

Before dissecting the mice, the media was kept in the incubator for 30mins to 1hr so that the temperature of the media was maintained for the incubation of sperm sample. Mice were taken and then anaesthetized using chloroform. After that cervical dislocation was done to kill them. The abdominal part was swiped with 70% ethanol for sterilization then its outer skin was removed following the removal of inner abdominal peritoneum. Fat bodies were isolated which is attached to the testes of the mouse and carefully testes with complete epididymis parts were taken out and kept in the Petri plate containing media. Carefully all the fat bodies were removed almost not even small drop of fat should be left if possible. Then the epididymis was taken carefully from the testes and kept in separate plate with media from where the caudal epididymis part was extracted and kept in separate plate containing known volume of media (usually 500µl or more according to the requirement). With the help of forceps, scalpel and dissecting microscope the caudal part was punctured and the sperm content was pushed out of the epididymis on to the media.

After that the extracted sperm sample was taken in the 1.5 ml Eppendorf tube and centrifuged at 800 rpm for 1 min to remove the other unnecessary tissue part. The supernatant was taken in the next Eppendorf tube and pellet was discarded. Then the

sperm sample in the second Eppendorf was ready to use for further processing. Before using the sperm sample its motility should be observed in the microscope. This method was modified from the method used by Paul *et al*. 2006.

3.7. Treatment of sperm sample with various concentrations of plant extract

For these 6 Eppendorf tubes were taken and labelled as C (control), D1 (25 µg/ ml), D2 (100 µg/ ml), D3 (500 µg/ ml), D4 (1000 µg/ ml) and D5 (2000 µg/ ml) respectively. In the respected labelled Eppendorf tubes, the various concentrations of plant extract were prepared by using formula S1*V1=S2*V2, the final volume was made 1ml by adding stable media as per required. Also, in the control Eppendorf there was only media. The prepared varying concentration of plant extract were incubated in the incubator either CO_2 (lid should be open and buffer salt of the media should be sodium bicarbonate) or normal incubator (lid should be closed and buffer salt should be HEPES) at 37°c for 2hrs.

Table 3.0.1: Various concentration of plant extract made by dissolving tuber extract with concentration of 47.8mg/ml in media and 80% ethanol for vehicle control.

S. N	Concentration (µg/ ml)	Volume of extract (µl)	Volume of media (µl)
1	Control (0)	0	1000
2	25	0.5	999.5
3	100	2.1	997.9
4	500	10.5	989.5
5	1000	20.9	979.1
6	2000	41.8	958.2
		Volume of ethanol (µl)	Volume of media (µl)
7	1000 (vehicle control)	20.9	979.1
S8	2000 (Vehicle control)	41.8	958.2

Table 3.0.2: Various concentration of plant extract made by dissolving bulbil extract with concentration of 20.6mg/ml in media

S. N	Concentration (µg /ml)	Volume of extract (µl)	Volume of media (µl)
1	Control (0)	0	1000
2	25	1.2	998.8
3	100	4.9	995.1
4	500	24.3	975.7
5	1000	48.5	951.5
6	2000	97.1	902.9

After that equal volume freshly extracted sperm sample was added to all 6 Eppendorf tube with various concentration of plant extract prepared above and was incubated in the incubator at 37°C for 2 hrs.

After the completion of incubation time period Eppendorf tubes were centrifuged at 1200 rpm for 3mins. Then the supernatant was discarded leaving 100µl of solution. The pellet was dissolved with the remaining supernatant, this was done to concentrate the sperm sample I the form of pellet. Now these samples were ready to perform following experiments.

3.8. Determination of effect of plant extract on acrosome integrity of sperm cell by using various staining methods.

The obtained sperm sample after treating with various concentration of plant extract was used. In this experiment the saliva was spread over a clean coverslip to increase the attachment of sperm cells. Coverslips with saliva attached were kept in the labelled area. To the coverslips the treated sperm samples were spread according to the label. It was left for 3 to 5 mins and the unattached sperm samples were removed. The attached sperm samples were fixed by using 4% formaldehyde and left for 30 mins to 1 hr. in room temperature. After the fixation of sperm cells, the acrosome integrity was determined by using two type of staining, Coomassie staining and FPNA staining.

3.8.1. Determining acrosome integrity by using Coomassie staining

In the coverslips with fixed sperm cells Coomassie stain (0.25 g Coomassie G-250, 10 ml of glacial acetic acid and 90 ml of 25% methanol) was added like was done by Eskandari and Momeni in 2016 and left for 5 mins and washed as soon as possible with 1x PBS and left to dry. Then the coverslip was attached to the slide using DPX. The slides were observed under light microscope where the number of sperm cells with

and without intact acrosome was counted. The blue coloration on the dorsal side of the sperm cells indicate sperm cells with intact acrosome whereas the absence of blue color on the dorsal side indicates sperm cells without intact acrosome. From which acrosome integrity percentage was obtained as follows:

$$\text{Acrosome Intregity of sperm cell}(\%) = \frac{\text{Number of sperm cells with intact}}{\text{Total number of sperm cells}} * 100$$

Graph of acrosome integrity percentage versus concentration of plant extract and other statistical values were obtained.

3.8.2. Determining acrosome integrity by using FPNA (FITC peanut agglutinin) staining

After fixing the plant extract treated sperm cells with formaldehyde it was washed with PBS. Then 100µl of 1% tween 20 in PBS was added to the coverslip and incubated for 30 mins in room temperature. Tween 20 was removed and then plates were prepared for FPNA staining as FPNA solution should not get dried. Petri plate was taken and divided into four parts and labelled properly from the outer part. Blotting paper was cut in a circular size to fix it in the plate so that it could absorb water and could make the plate moist. Also, parafilm paper was cut to the same size and placed over the blotting paper so that the moisture will not affect the coverslip with sample to be stained. 500X diluted FPNA (990 µl of PBS and 10 µl of 10%sodium azide was used in which 2µl of FPNA was added) was prepared. 50 ml of 500X FPNA was added on the slide and kept in the plate and covered with aluminum foil then the plate was incubated in incubator at 37°C for 1 hr. After that FPNA was removed then 50 µl of EtBr (100 µg/ ml) was added and left for 5 mins and removed and washed with PBS. Finally, 10% of glycerol was added in a clean slide and the coverslip was mount on the slide, after that the edge of coverslip was sealed by using DPX. The procedure was done to each and every treated sperm samples with various concentration of plant extract. After the completion of slide preparation, the slides were observed under fluorescence microscope under blue light where sperm cell with acrosome will be seen with green color on the dorsal side of sperm cell due to FPNA staining. The nuclei will be stained red due to EtBr staining. The sperm cell stained with both FPNA and EtBr on the resected parts will be counted as sperm cell with intact acrosome and sperm cell with only EtBr staining indicated sperm cell without intact acrosome. This method was modified from the method performed by Rajabi-Toustani *et al*.2019. Acrosome integrity percentage was calculated like in the Coomassie staining and other statistical values will be obtained.

3.9. Determination of effect of plant extract on viability of sperm cell by various staining methods.

After the treatment of sperm cells with various concentration of plant extract, two types of staining were used to determine the viability of sperm sample namely, trypan blue staining and hypoosmotic swelling test.

3.9.1. Determining viability of sperm cell by trypan blue staining

For trypan blue staining, the procedure was followed from Kovacs and Foote in 1993, prepared solution containing plant extract treated sperm sample were kept in ice container to stop further action. Then slide was prepared by adding equal volume of 0.4% of trypan blue to the sperm sample prepared by treating with various concentration of plant extract and covering it by using coverslip. The number of viable sperm cells were counted which do not take the blue color of trypan blue, in the same way dead cells which takes the blue color in different field in light microscope. The viability percentage of sperm sample were calculated as:

$$\text{Viability of sperm cells}(\%) = \frac{\text{Number of viable sperm cells}}{\text{Total number of sperm cells}} * 100$$

After obtaining the viability percentage of respected sperm sample treated with various concentration of plant extract graph was plotted as concentration versus viability percentage.

3.9.2. Determining viability of sperm cells by using hypoosmotic swelling test

For hypoosmotic swelling test the protocol was followed given by Agrawal *et al*.2016, the sperm cell treated with various concentration of plant extract was further incubated in hypoosmotic solution (prepared from 0.735 g sodium citrate dehydrated and 1.351 g fructose in 100ml of distilled water) in 1:10 ratio i.e. 100µl of sperm sample in 1 ml of hypoosmotic solution and incubated at 37°C for 30 mins to 1hr. After the incubation the solution was centrifuged at 2000rpm for 5 mins. The supernatant was discarded leaving 100 µl behind in which pellet was dissolved. Then slides were prepared by directly adding the solution to the slide and covering it with coverslip and observing in light microscope. The sperm cells were observed for swollen tails which indicates viable cell which have intact membrane and the number of live and dead cell in various field was counted and the viability percentage were calculated like in case of trypan blue.

3.10. Detecting the effect of plant extract on sperm protein

For the detection of effect of plant extract on sperm protein SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was done by slightly modifying the protocol done by Matsumalo *et al*.2012. The instrument was used which was produced by Bio-Rad. The glass plate was assembled according to the manufacturer's instructions. The space bar was properly sealed and was checked using distilled water. In the glass plate line was marked approximately 1.5 cm from the top of the glass slide. 12% acryl amide solution was prepared for resolving gel and was poured into the gap between the glass plate up to marked line as soon as polymerization occurs after adding TEMED (N, N, N', N'-tetramethylenediamine). Propanol was used to remove the bubbles and maintained the level of resolving gel. Then the gel was let to dry. After the complete drying of the resolving gel the propanol was soaked out by using bloating paper. Staking gel was prepared which was 5% acrylamide solution and was poured above the resolving gel. Teflon comb was inserted soon avoiding air bubbles. After polymerization the comb was removed and propanol was again added to remove the bubbles and then was soaked using bloating paper. In this way gel was prepared. Afterward the slab was mounted into electrophoresis apparatus and tris-glycine electrophoresis buffer was added in the tank.

Then the prepared sperm sample treated with plant extract was washed with PBS for several times (3 or 4, by adding 1 ml of PBS and centrifuging the sample at 2000rpm) to remove the BSA present in the media while sample preparation. Then the samples and ladder were mixed with equal volume of sample loading buffer and was boiled for 3-5 mins.

The samples and ladder were loaded on to the well of the gel which was marked with the respected sperm samples and ladder. Gel was run by applying constant current at 25mA for 60 mins until dye run near the end of gel.

The glass plate was removed with care and was stained by keeping it in CBB (Coomassie Brilliant Blue R-250) staining solution for 15 to 30 mins. Then the gel was washed with distilled water for 2 or 3 times. Afterward the gel was kept in destaining solution until the color was disappeared and band was obtained. Finally, the gel was again washed with distilled water and prepared for silver staining.

For PAGE silver staining the Coomassie stain was removed from gel as much as possible either by keeping the gel in destaining solution for overnight or by distilled water. The gel was then sanitized in 0.02% sodium thiosulfate (0.0 4 g $Na_2S_2O_3$, 200 ml H_2O) for only 1 min. After that the gel was washed by distilled water for several times and gel was incubated in 4° C 0.1% silver nitrate solution (0.2 g $AgNO_3$, 200 ml

H_2O and 40 µl 0f 35% formaldehyde just before use) for 20 mins. The gel was washed for several times and the gel was transferred to a new staining tray where the gel was developed in 3% sodium carbonate (7.5 g Na_2CO_3 in 250 ml H_2O and 125 µl of 35% formaldehyde just before use). Staining was terminated by using 5% acetic acid for 5 mins according to the needed clarity of the gel. Then the gel was preserved in in 1% acetic acid for 5 mins. Finally, the effect of protein in the sperm sample due to plant extract treatment was evaluated.

3.11. Determination of effect of plant extract on *in vivo* fertilization

From the obtained data the concentration of plant extract which gives highest precocious effect to the acrosome was used for *in vivo* injection. For this experiment to perform female mice of age 10 weeks and male mice of age 12 weeks were brought from DPR. Those mice were left for three days so that they could adopt in the environment of animal warehouse in our department. Then everyday the vaginal opening was examined to find out the estrus cycle. After onwards the experiment was performed using two labelled mice where one is control while other will be plant extract injected mouse.

In this experiment for one set the male used was same but in case of female mice two female mice were used where one was control (no plant extract treated) whereas another was female injected with plant extract. The control was left for mating for three continuous days after identifying the estrus period of female mouse. Then the female mouse was separated and kept in different cage. The date was noted and observed up to 30+1 days. The number of birth of pupils was counted and noted. Similarly, for test female mouse in estrus phage was injected with 1000 µg/ ml concentration of plant extract into the vaginal opening like was done by Naz, 2011 in every evening continuously for three days and left for mating. In the same way date of mating was noted and test mice were observed for 30+1 days. The number of birth pupils were counted. Three sets of mice were used. For vehicle control the female mice was injected with 80% ethanol such that the volume of ethanol used was equal to the volume of plant extract used in case of test female mice and was observed for 30+1 days.

3.12. Statistical analysis

From the data obtained from various experiment mean, standard deviation, standard error was calculated using Microsoft Excel spreadsheet along with their scattering dot plots. Also, p- value was calculated using mean and standard deviation using the website below:

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

Chapter IV

RESULTS

4.1. Concentration of plant extract

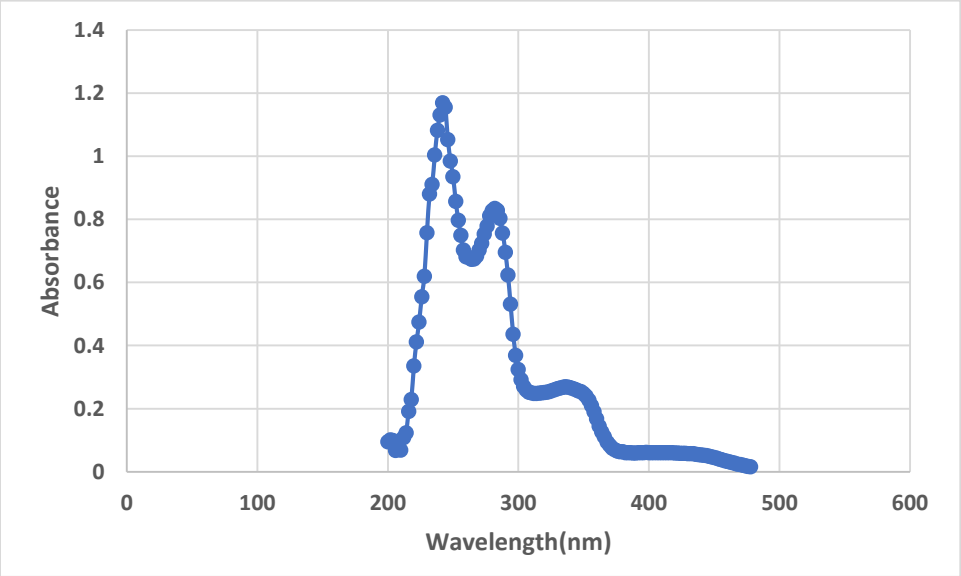
The concentration of plant extract was calculated by following the protocol explained in the methodology. It is found that

a) The concentration of tuber extract was found to be 47.8 mg/ ml.

b) The concentration of bulbil extract was found to be 20.6 mg/ ml.

4.2. Absorbance spectrophotometry reading of ethanolic extract of plant extract

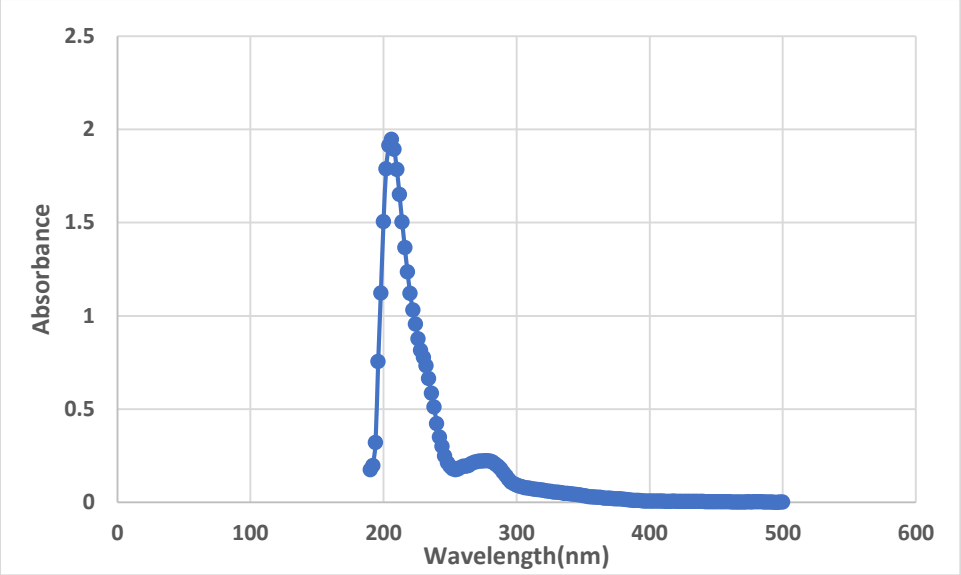
As the absorbance of ethanolic extraction of plant extract was done by taking the wavelength ranging from 190 to 490 nm using scanning spectrophotometer, the absorbance spectrophotometry reading of ethanolic extract of of *D.bulbifera* tuber is shown in the figure below.



Graph 4.0.1: Absorbance of ethanolic extract of *Dioscorea bulbifera* tuber.

From the absorbance reading three peaks were observed in the graph plotted as absorbances versus wavelength. The highest peak was seen in the wavelength of 200-250 similarly second peak was seen in the wavelength of 250-300 and third in the wavelength of 300-400.

Similarly, the absorbance spectrophotometry reading of ethanolic extract of *D.bulbifera* bulbil is shown in the figure below:



Graph 4.0.2: Absorbance of ethanolic extract of *Dioscorea bulbifera* bulbil.

Again, from the graph two types of peaks were seen; one was seen in the range of 200-250 and another in the range of 250-300. But the third peak which was seen in the absorbance of tuber extract is missing in case of bulbil extract.

4.3. *Dioscorea bulbifera* tuber and bulbil extracts cause precocious acrosome reaction

After treating the sperm sample with various concentration of plant extract, slide using the sperm sample was prepared. Two type of staining process was applied as explained in the methodology.

4.3.1. Precocious acrosome damage caused by *D.bulbifera* tuber

a) Acrosome reaction seen by using Coomassie staining

The data was obtained by counting the sperm cell with and without acrosome which was indicated by the microscopic examination of slide and pictures shown as bellow:

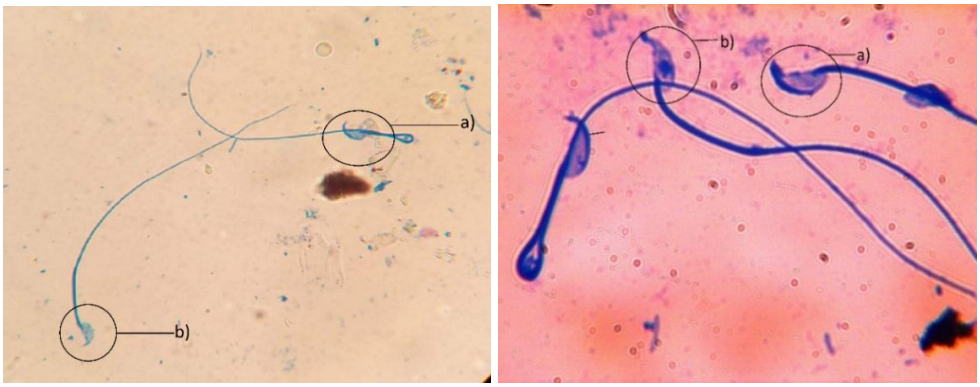


Figure 4.0.1: Coomassie staining a) Sperm cell with intact acrosome shown by thick blue color on dorsal side b) Sperm cell with detached acrorsome.

The sperm cell stained with Coomassie stain as shown in above figure are usually of two types which is with a thick blue color on the dorsal side and another with out thick color in the dorsal side. The coloration of the sperm cell on the dorsal side is due to ability of binding of Coomassie stain on the acrosomal region. There was also appearance of slightly degenerating acrosome which neither contain thick blue coloration on the dorsal side nor absence of blue coloration. The result of the sperm count with acrosome and without acrosome, percentage of intact acrosome their mean standard deviation and standard error is shown below.

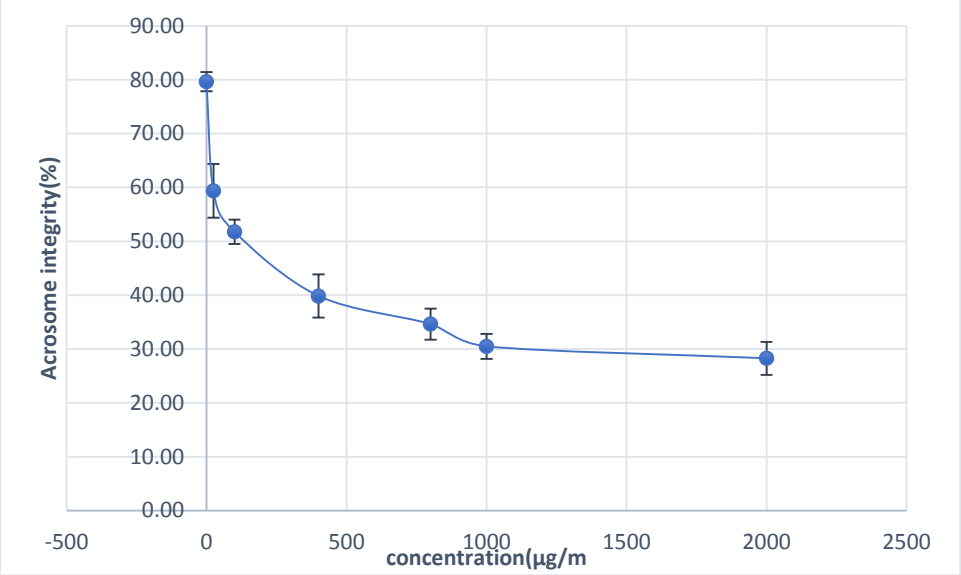
Table 4.0.1: Acrosome integrity of spermatozoa after treating with various concentration of *D.bulbifera* tuber extract using Coomassie stain including the standard deviation and standard deviation error of mean.

SD = Standard deviation; SE Standard Error

		Experiment 1	Experiment 2	Experiment 3			
S. N	Concentration (µg/ ml)	Acrosome Integrity (%)	Acrosome Integrity (%)	Acrosome Integrity (%)	Mean	SD	SE
1	Control	77.65	84	77.29	79.65	3.08	1.78
2	25	50.55	71.17	56.38	59.37	8.68	5.01
3	100	48.63	57.27	49.42	51.77	3.90	2.25
4	400	44.09		35.59	39.84	4.25	4.02
5	800	40.31		28.93	34.62	5.69	2.87
6	1000	27.64	36.22	27.59	30.48	4.06	2.34
7	2000	34.93	27.95	21.91	28.26	5.32	3.07

(P=0.0001); p value was calculated from the acrosome integrity percentage of mean of control and acrosome integrity percentage of mean of 1000 µg/ ml.

From the data in above figure graph was plotted using concentration versus the mean values of acrosome integrity.



Graph 4.0.3: Showing acrosome integrity of mouse spermatozoa treated with various concentration of *D.bulbifera* tuber extract using Coomassie stain

The above graph indicates decrease in spermatozoa population with acrosome loss due to extract treatment up to the concentration of 1000 µg/ ml.

4.3.2. Precocious acrosome reaction caused by *D.bulbifera* bulbil

a) Acrosome reaction seen by using FPNA staining

In FPNA staining the observed microscopic view of the prepared slide is shown below:

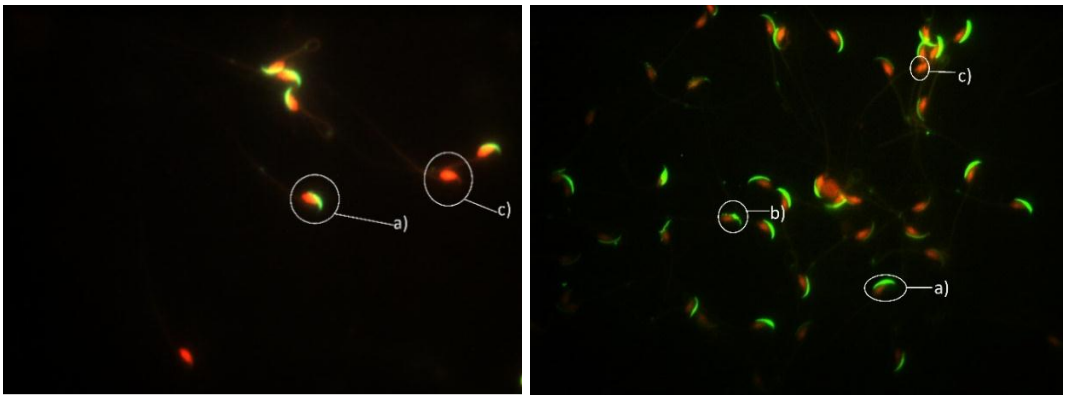


Figure 4.0.2: FPNA and EtBr staining of mouse spermatozoa. a) Sperm cell with intact acrosome shown by green fluorescence, b) Sperm cell with partly degraded acrosome and c) sperm cell with completely degraded acrosome shown by presence of only red fluorescence given by EtBr staining.

As in the figure above we can see two type of coloration in the sperm cell in which there is green color on the dorsal head of sperm cell which was given by FPNA stain whereas the red coloration on the other parts of sperm cell is given by EtBr. The sperm cell with both red and green color present showing the complete thick shape of

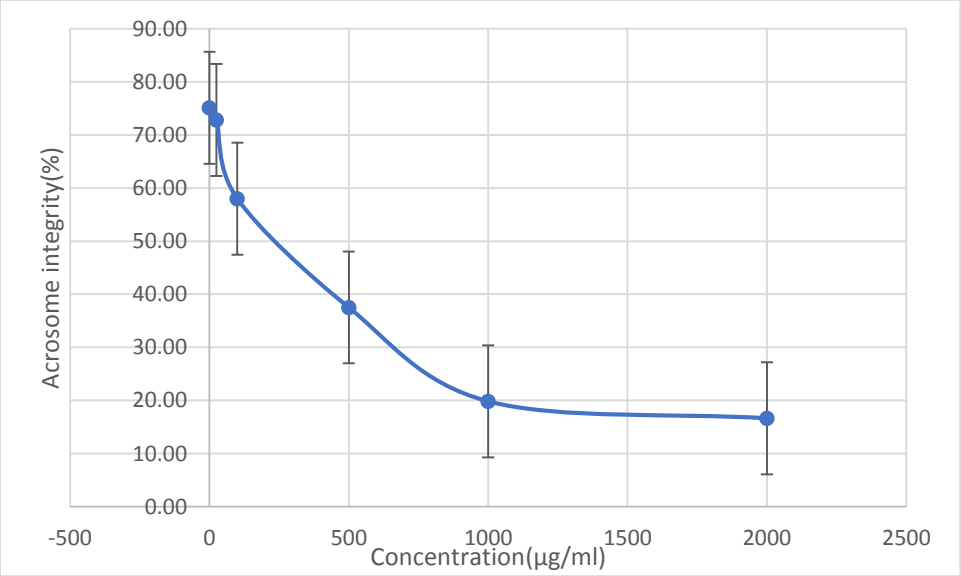
acrosome was counted as sperm cell with acrosome where as others are counted as sperm cell without acrosome. On the basis of which following data was obtained.

Table 4.0.2: Acrosome integrity of spermatozoa after treating with various concentration of *D.bulbifera* bulbil extract. The spermatozoa were labeled with FPNA and EtBr.SD, standard deviation;SE, standard deviation error of mean.

		Experiment 1	Experiment 2	Experiment3			
S. N	Concentration (µg/ml)	Acrosome integrity (%)	Acrosome integrity (%)	Acrosome integrity (%)	Mean	SD	SE
1	0	73.26	80.09	71.97	75.11	3.56	2.06
2	25	69.59	68.2	65.74	67.84	1.59	0.92
3	100	64.91	52.35	56.71	57.99	5.21	3.01
4	500	45.73	33.62	33.18	37.51	5.82	3.36
5	1000	19.2	25.41	14.87	19.83	4.33	2.50
6	2000	13.25	20		16.63	3.38	2.39

(P=0.0001); p value was calculated from the acrosome integrity percentage of mean of control and acrosome integrity percentage of mean of 1000 µg/ ml.

From the data obtained in above table graph was plotted using concentration versus acrosome integrity mean which is shown below:



Graph 4.0.4: Showing acrosome integrity of mouse spermatozoa treated with various concentration of *D.bulbifera bulbil* extract using FPNA stain with the error bar.

On the contrary, the curve is nearly flat beyond 1000µg/ml. This may hypothesize that the 1000µg/ml concentration of the plant extract can reduce the chances of conception as it decreases the acrosome integrity down to 30%.

b) Acrosome reaction seen by using Coomassie staining

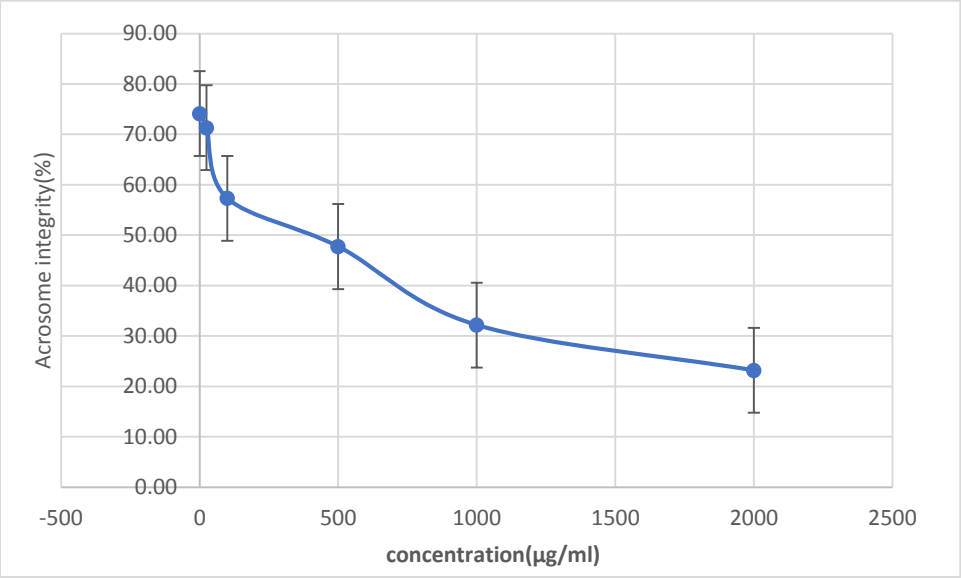
Similarly, like above in the table 4.0.1: on the basis of the figure 4.0.1: the data was obtained for the testing using bulbil of *D. bulbifera.* The acrosome reaction data obtained by using Coomassie staining is shown below:

Table 4.0.3: Acrosome integrity of spermatozoa after treating with various concentration of D.bulbifera bulbil extract. The spermatozoa were stained with Coomassie blue.

		Experiment 1	Experiment 2	Experiment 3			
S. N	Concentration (µg/ml)	Acrosome integrity (%)	Acrosome integrity (%)	Acrosome integrity (%)	Mean	SD	SE
1	0	79.12	73.36	73.52	74.10	2.75	1.59
2	25	74.89	56.42	69.83	71.31	8.08	4.67
3	100	66.2	53.49	65.69	57.26	6.30	3.64
4	500	62.32	39.11	49.16	47.73	9.58	5.53
5	1000	57.35	31.49	27.37	32.15	13.67	7.89
6	2000	19.69	26.63		23.16	3.47	2.45

(P=0.0065); p value was calculated from the acrosome integrity percentage of mean of control and acrosome integrity percentage of mean of 1000 µg/ ml.

From the data shown in the table, graph was plotted using concentration versus the mean value of acrosome integrity.



Graph 4.0.5: Showing acrosome integrity of mouse spermatozoa treated with various concentration of *D.bulbifera bulbil* extract using Coomassie stain with the error bar.

Further, to investigate whether presence of ethanol affects acrosome integrity or not we have used ethanol instead of plant extract and obtained the following data.

Table 4.0.4: Acrosome integrity of spermatozoa after treating with various volume of ethanol replaced from the plant extract.

		Experiment 1	Experiment 2
S. N	Concentration (µg/ml)	Acrosome Integrity (%)	Acrosome Integrity (%)
1	Control	74.66	90.5
2	1000 Vehicle control	74.38	90.2
3	2000 Vehicle control	73.25	89.94

This data indicates there are not much significance effect on acrosome integrity due to presence of ethanol.

4.4. Viability testing of spermatozoa after treatment with plant extract

For the viability testing two types of experiment were performed, which are trypan blue staining method and hypoosmotic testing method.

a) Viability testing using trypan blue stain

In this testing the spermatozoa were treated with various concentration of *Dioscorea bulbifera* tuber extract and their viability percentage was calculated by using trypan blue staining method and observed under microscope. The microscopic examination gave following pictures in which the sperm cell which is dead is permeable so trypan blue stain goes inside the cell and the sperm cell appears to be blue and counted as dead cell whereas the live sperm cell is very lightly stained and counted as viable cell.

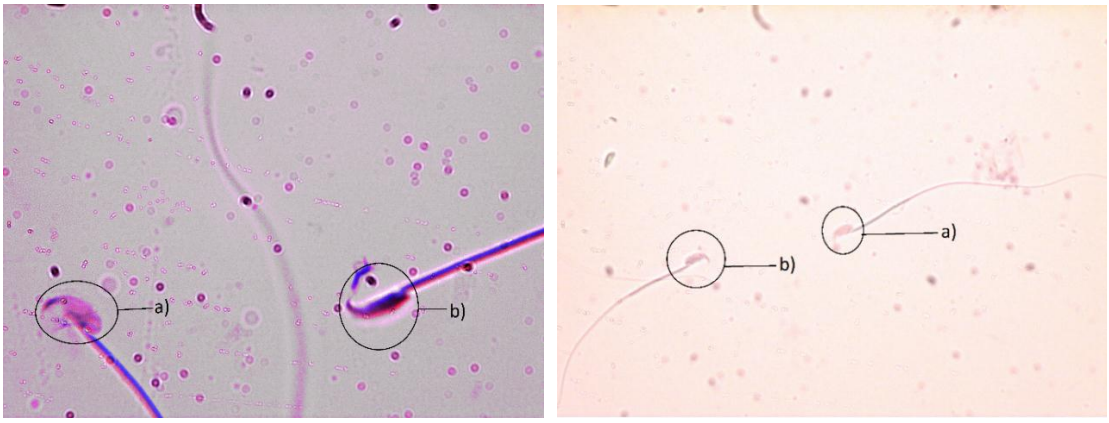


Figure 4.0.3: Trypan blue staining a) Viable cell with light blue color b) Dead cell with dark blue color.

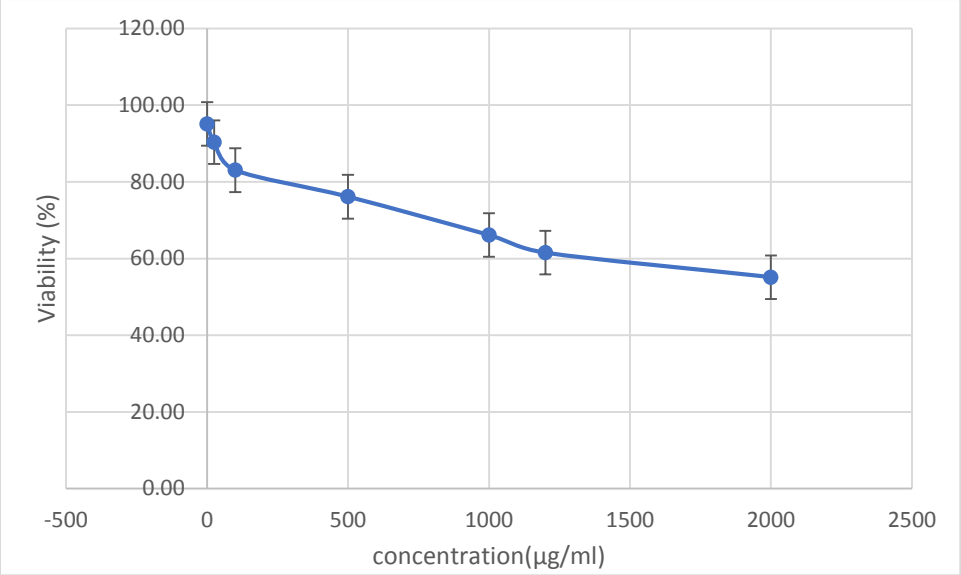
From the count following data were obtained;

Table 4.0.5: Viability testing of spermatozoa after treating with various concentration of *D. bulbifera* tuber extract and staining with trypan blue including standard deviation of mean and standard deviation error.

		Experiment 1	Experiment 2	Experiment 3			
S. N	Concentration (µg/ml)	Viability (%)	Viability (%)	Viability (%)	Mean	SD	SE
1	0	87.50	100.00	97.92	95.14	5.47	4.56
2	25	79.65	98.53	92.81	90.33	7.91	4.64
3	100	73.23	92.92	82.99	83.05	8.04	5.13
4	500	64.80	86.52	77.05	76.12	8.89	7.04
5	1000	50.98	80.80	67.35	66.38	12.19	9.37
6	2000	32.41	75.71	57.31	55.14	17.74	13.83

(P=0.0203); p value was calculated from Viability percentage of mean of control and Viability percentage of mean of 1000 µg/ ml.

From the data obtained graph of concentration and viability percentage was plotted which is shown below:



Graph 4.0.6: Viability of mouse spermatozoa incubated at various concentration of *D. bulbifera* tuber using trypan blue with the error bar.

The line in the graph indicates the minimal effect on the viability of sperm cell on the application of plant extract. Up to the concentration of 2000µg/ml the viability percentage was found to be 57.31%.

b) Viability testing using hypoosmotic testing: In this test the result of treatment of hypoosmotic solution on sperm cells were viewed under microscope and the picture obtained are as follows:

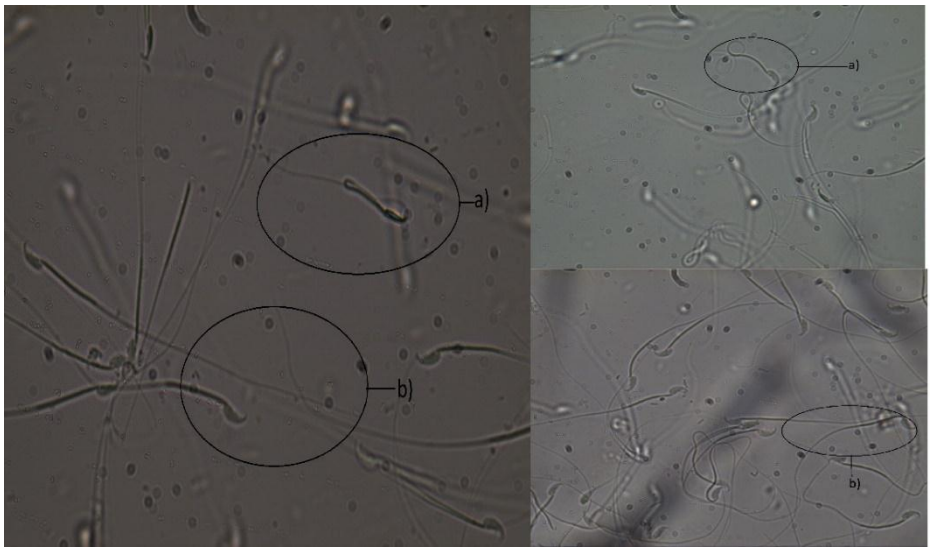


Figure 4.0.4: Hypoosmotic sweeling test a) Viable sperm cell with swollen tail b) Dead sperm cell with almost straight tail.

In above figure the bent tail of sperm cell indicates the intact membrane due to which when the cell are kept in hypoosmotic solution they swells up indicated by bendind of tail. In dead cell's which have lost the membrane, the tail remains straight.

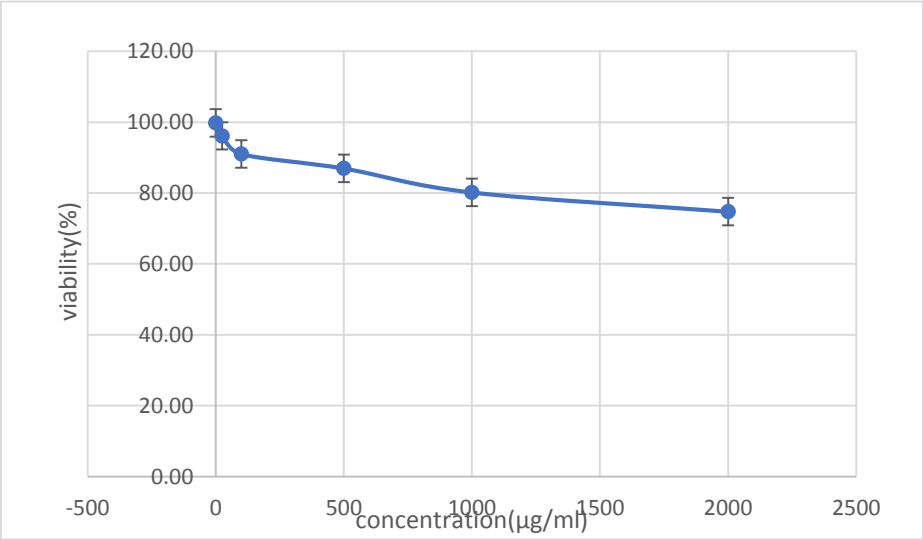
After treatment with various concentration of plant extract, the spermatozoa were further incubated in hypoosmotic media and Viability data was obtained by counting the sperm cell which have various deformed tail to the effect of hypoosmotic solution as observed in the figures above. The data obtained are below:

Table: 4.0.6 Viability testing of spermatozoa after treating with various concentration of D. bulbifera tuber extract and hypoosmotic staining including standard deviation of mean and standard deviation error.

		Experiment1	Experiment 2	Experiment 3			
S. N	Concentration (µg/ml)	Viability (%)	Viability (%)	Viability (%)	mean	S. D	S. E
1	0	99.34	100.00	100.00	99.78	0.31	0.18
2	25	94.12	98.12	96.15	96.13	1.63	0.94
3	100	87.59	92.40	93.02	91.00	2.43	1.40
4	500	84.51	87.57	88.76	86.95	1.79	1.03
5	1000	78.87	82.21	79.45	80.18	1.46	0.84
6	2000	72.00	78.26	74.03	74.76	2.61	1.51

(P<0.001) p value was calculated from Viability percentage of mean of control and Viability percentage of mean of 1000 µg/ ml.

From the data above the graph was plotted between the mean viability percentages versus concentration of plant extract used which is given below:



Graph: 4.0.7 Viability of mouse spermatozoa incubated at various concentration of *D. bulbifera* tuber evaluated by hypoosmotic testing. Vertical bars indicate the error bar.

The hypoosmotic swelling test also indicates that with highest concentration applied on sperm cell the viability percentage is still 74.76%.

4.5. Protein profiling by SDS-PAGE

SDS PAGE was performed to determine the effect of plant extract on the sperm proteins. After treatment and SDS PAGE was performed and result obtained is shown in the figure 4.0.5 below:

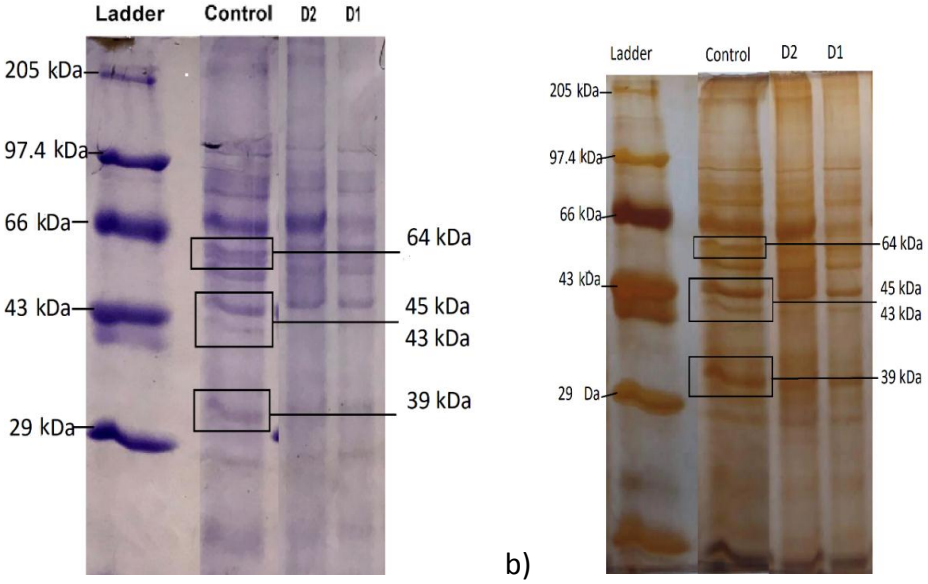


Figure 4.0.5. Protein profiling of Sperm cell by SDS-PAGE. Lane 1 - GeNei Protein Molecular Weight Marker (3.5 KDa to 205 KDa); Lane 2 - control sample; Lane 3- sperm sample treated with 2000µg/ml concentration of plant extract) on lane 3 and D_1 sample (sperm sample treated with 1000µg/ml concentration of plant extract) on lane 4. Further for observation of the protein band Coomassie staining was done as shown in the Figure 4.0.5 a) and silver staining was done as shown in Figure 4.0.5 (b).

4.6. Observation for Estrus stage

The vaginal opening of mice was observed on a daily basis to evaluate the estrus stage. The stages of vaginal opened and closed stages are as shown in the figure 4.0.6 (a). The closed condition corresponds to proestrus stage. On the next day, the vaginal opening was found to be open indicating estrus as seen in the figure 4.0.6 (b). The day after estrus stage the vaginal opening was found to be in the stage of closing which is metestrus as seen in the figure 4.0.6 (c). On the fourth day the vagina was completely closed called diestrus as seen in the figure 4.0.6(d). The estrus stages were properly recorded in order to determine the suitable period for mating the mice. As estrus stage is the best stage to perform mating between the mice so the treatment of plant

extract was also started at the estrus phase up to the third days after the phase and mice were left for mating as included in methodology above.

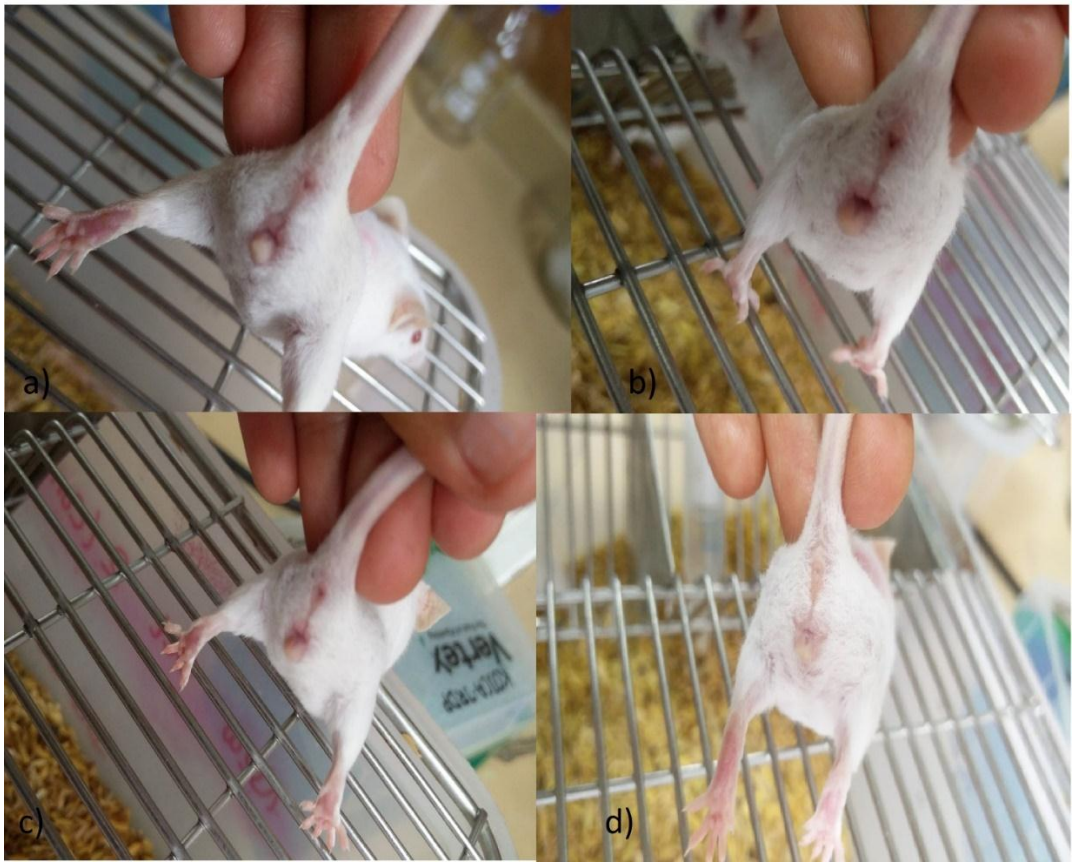


Figure 4.0.6: Four stages of estrus cycle observed in Swiss albino mice a) Proestrus, b) Estrus, c) Metestrus, d) Diestrus.

4.7 Results from *in vivo* fertilization

As the result obtained from precocious acrosome damage there was sharp decrease in acrosome integrity at the concentration of 1000 µg/ml. Further testing was done by performing in vivo experiment which was performed by injecting the plant extract from the vaginal opening continuously for 3 days and leaving the mice for mating at the same time as explained in the methodology above. After 30 days of observation on the test as well as control female mice the number of litters born were noted which is given in the box below:

Table 4.0.7: Litter sizeof mice intravaginally injected with *D. bulbifera* extract.

		No. of litters born		
S. N	Set	Control	Extract treated	Vehicle control
1	1	10	6	8
2	2	9	0	
3	3	9	0	
4	4	10	0	
5	5	9	0	
	Mean	9.4	1.2	
	S. D	±0.4199	±1.3765	

Difference	-8.200
Standard error	0.644
95% CI	-9.6841 to -6.7159
t-statistic	-12.741
DF	8
Significance level	P < 0.0001

Chapter V

DISCUSSION

Earlier studies of *Dioscorea* species have confirmed the presence of flavonoids, terpenoids, saponin, steroid and cardiac glycosides (Sheikh *et al*. 2013). Among all the secondary metabolites *D. bulbifera* yam also contains the steroidal saponin diosgenin, the principal material for the synthesis of corticosteroids, estrogen, and contraceptives. Diosgenin is among the ten most important sources of steroids and the most often known medicine of plant origin (Jayachandran et al. 2016). The concentration of *Dioscorea bulbifera* tuber and bulbil extract were found to be 47.8 mg/ml and 20.6 mg/ml respectively. This might indicate that the presence of soluble compounds in higher amount in tubers compared to bulbils. In the absorbance spectrophotometry of *Dioscorea bulbifera* tuber we can see three peaks in which first peak is seen in the wavelength around 250 nm second around 280 nm and third around 360 nm. According to Altemimi *et al.* (2017) the absorbance at 280 nm indicates the presence of phenolics compound where as at 360 nm indicates presence of phenolic acid whereas peak at 250 might be due to the alteration in the side chain of phenolic compound. The variation in the absorbance spectra between the tuber and bulbil of D. bulbifera may indicates the presence of different secondary metabolites.

In case of sperm acrosome integrity study, two types of stain were used which were Coomassie and FPNA. The principle of Coomassie staining is that the dye binds to the Sulphite groups in the acrosomal matrix as the acrosomal membrane became permeable after fixation and drying so that only the acrosome gives thick blue color (Brum *et al*. 2006). In case of tuber only Coomassie staining was done where the acrosome integrity percentage was found to be decrease from 77.29% for control to 30.48% for 1000 µg/ml. In the graph 4.0.3, there was exponential decrease in the acrosome integrity up to 1000 µg/ml concentration and in 2000 µg/ml the acrosome integrity was decreased more up to 28.26%.

In case of bulbil both types of staining method were used where the acrosome integrity percentage of sperm cell varies for Coomassie staining from control 75.11% to 19.83% for 1000 µg/ml and for FPNA staining from control 74.10% to 32.15% for 1000 µg/ml. In both cases the acrosome integrity percentage was exponentially decrease up to the concentration of 1000µg/ml. The principle for FPNA staining is that it specifically binds to the sugar Galactosyl β-1,3 N-acetylgalactosamine in acrosomal membranes (Rajabi-Toustani *et al.* 2018). From the result obtained using both the stain it indicates that there was not much difference in using either of the staining

methods. But talking about acrosome integrity percentage the effect using tuber was comparatively more than bulbil, seen by comparing the scattered dot plot of both results. So further testing was carried out using tuber.

In the graph of acrosome integrity 4.0.3 using tuber extract, it has found sharp exponential decrease in acrosome integrity compared to graph of acrosome integrity 4.0.5 using bulbil extract. Due to which further experiment was done using tuber extract of *D.bulbifera.*

As the ethanolic extract of plant parts was used, the test was also performed for whether ethanol directly affects acrosome integrity percentage or not. So, we pursued vehicle control experiment by using equivalent volume of ethanol as in the experiment of 1000 and 2000 µg/ml extracts treatments. In this experiment, we have found that there was not much effect of ethanol on sperm acrosome as shown by table 4.0.4.

Similarly, the p value obtained from acrosome integrity percentage table using Coomassie stain for tuber (Table 4.0.1), for bulbil (Table 4.0.3) and FPNA stain for bulbil (Table 4.0.2) was found to be less than 0.01. This indicates that the alternative hypothesis that have assumed was found to be correct as the p value obtained is significant.

For the viability testing two types of methods were used which were trypan blue staining and hypoosmotic treatment methods. The principle behind the trypan blue staining is that as the dead cells lose their membrane integrity because of which the dye can easily enter the cell and give blue coloration (Strober, 2001). In case of trypan blue staining the microscopic view was found to be somewhat not clear. Although the viability data obtained was found to be satisfying the hypothesis as the viability percentage of control was found to be 95.14% which exponentially decrease to 83.05% at 25 µg/ ml and the viability dropped to 55.14% at 2000 µg/ml. The principle behind the hypoosmotic testing for viability is that if the cell is viable it tries to maintained its membrane integrity during treatment with hypoosmotic solution, by passing the solution in through the membrane which leads in swelling of cell indicated by bending of tails (Ramu & Jeyendran, 2012). Similarly, viability result using hypoosmotic testing was also found to be positive towards hypothesis of the experiment. The result shows that only 25% change in the viability percentage, in between the control and 2000 µg/ml concentration treatment samples.

Also, in the viability percentage table 4.0.4 for trypan blue staining and Table 4.0.5 for hypoosmotic swelling test the p value obtained is also significant that is less than 0.01. This result verifies our hypothesis.

Combining the result of acrosome integrity and viability using both method Coomassie staining and FPNA for acrosome integrity and Trypan blue staining and Hypoosmotic swelling test for viability it indicates that the plant extract used for testing effect the acrosome integrity but give very less effect or negligible effect to the viability of sperm cells. Hence if the plant extract especially tuber extract of *Dioscorea bulbifera* is use as topical contraceptives it will be effecting the acrosomal part of the sperm cell with minimal side effect to other cells leading to lesser side effect comparatively given by hormonal contraceptives and other means.

As shown in the figure 4.0.5. (a) and (b) in the first lane the bands indicate the size of proteins from 29 kDa, 43 kDa, 66 kDa, 97.4 kDa and 205 kDa moving from bottom to top as indicated in the figure. We can see in all the three lane the protein band present were found to be similar with thick band near the marker band of 66 kDa also bands were seen near 45 kDa, 43 kDa and 39 kDa as indicated by Nagdas *et al* in 2013. This indicates that the plant extract treated of sperm cells does not cause change in the major protein bands. But each protein bands in the Coomassie stained SDS-PAGE would comprise several proteins and changes in some of them, particularly in the minor proteins may not be visible. To reveal such proteins, differential extraction, column fractionation, or Western blotting should be done.

For *in vivo* fertilization studies, the extract concentration at which the effect on acrosome integrity was found to decrease exponentially was chosen. The female mice were checked daily for their estrus cycle as indicated by Byers *et al.* 2012. On the day of estrus stage, mice were injected with the plant extract of volume 21 µl with 1000 µg/ ml concentration in the evening and left for mating. As from the acrosome integrity and viability data from the same concentration (1000 µg/ ml) we have seen that the plant extract had cause exponential decrease in acrosome integrity without causing much effect on sperm viability. We have chosen to mate the mice for three continuous day because as the mice estrus cycle have the time length of four days, excluding the period from pre estrus stage the chances of conception increases.

In the result of *in vivo* fertilization, five set of mice were used out of which only in first set the female mice treated with plant extract gave birth to 6 litters where as in other sets, the plant extract treated female were not found to be pregnant for 30+1 days of observation. In every set control gave birth to 9 or 10 litters. The obtained p value from the data was found to be less than 0.0001 which indicates that the result obtained was significant. This indicates that when the plant extract was injected in the anterior vaginal opening there was maximum reduce in the chances of conception resulting in zero litter born. Also, the experiment for vehicle control was performed by injecting 80% of ethanol through the vagina as explained in the methodology. The

obtained litter size was 8 which was very close to that of control litter size. This result can conclude that there was no effect of solvent (ethanol) which was used for the extraction of plant extract. Similar result had been reported in case of intravaginal treatment of curcumin extract on female mice which caused reduction in fertility significantly where p value ranges from $p<0.001$ to 0.0001 (Naz, 2011). Out of five experiment in four experiment there was 100% reduction in conception this may conclude that the plant *Dioscorea bulbifera* can be used as the potent medicinal contraceptive plant.

Chapter VI
SUMMARY

Contraceptives are the means of birth control. Nowadays the most commonly used contraceptives are hormonal contraceptives used by women. There are various side effects hormonal contraceptives out of which risk of breast cancer is serious.

In nature, there are various types of medicinal plants found which have been used by our ancestors for treating various diseases. Many plants are still needed to explore for their medicinal values. One of the medicinal plants that have been used by our ancestor is *Dioscorea bulbifera.* It is used in leprosy, asthma, cough, cold, tuberculosis, contraceptive, constipation, indigestion, abdominal pain, dysentery, sore throat, Struma, wounds, boils, cuts, injury, carbuncle, tumor, tuberculosis, also used as refrigerant to reduce body heat during summer and to maintain kidney function.

In the present study, we have investigated *Dioscorea bulbifera* for its contraceptive properties *using in vitro* sperm functioning assay and *in vivo* fertilization assay. The *in vitro* sperm functioning tests were done by two ways. First by testing the acrosome integrity of sperm cells by using Coomassie staining and by FPNA staining. Second by viability testing which was done by trypan blue staining method and hypoosmotic swelling test. In both cases, the sperm sample were treated with a various concentrations of plant extract (25 µg/ml to 2000 µg/ml) following the staining process. Cell count was done to determine the acrosome integrity percentage and viability percentage. On the basis of which effect of plant extract on sperm function was determined. The acrosome integrity percentage was decreased up to 28.26% using Coomassie staining for tuber extract, 16.63% and 23.16% for bulbil extract using FPNA staining and Coomassie staining respectively for final concentration (2000 µg/ ml). The Viability percentage deceased only up to 55.14% and 74.76% for tuber extract using trypan blue staining and hypoosmotic swelling test respectively for final concentration 2000 µg/ ml.

To observe the effect of plant extract on sperm protein SDS-PAGE was performed in which we had found that the plant extract does not show remarkable effect on the sperm protein bands.

In *in vivo* fertilization, the test female mice were injected with the plant extract into vagina and were allowed to mate and the number of litters born were observed to reduce to zero in all set except set 1.

Chapter VII

CONCLUSION

The contraceptive effect of *Dioscorea bulbifera* plant extract was evaluated. The extract caused loss of acrosome in mouse spermatozoa in concentration dependent manner. However, the viability percentage did not decrease as compared to acrosome integrity. It indicated that the plant extract is responsible for damaging the acrosome rather than the whole cell. These observations suggested that the plant extract affects the fertilizing ability of spermatozoa. In case of protein profiling of sperm cells, there was negligible effect due to which we have to find the mode of action of the plant extract. *in vivo* fertilization experiments showed remarkable decrease in the litter size. As both control and vehicle control were used, it can be concluded that the decrease in the litter size was due to the effect of plant extract.

LIMITATIONS OF THE EXPERIMENT

- It was difficult to maintain the suitable environment for mice due to improper housing.
- As FPNA stain was very much fragile so the counting of sperm sample should be done as soon as possible.
- To verify the *in vivo* result as well as to know the mode of action of plant in vitro fertilization should have done which was not done.

RECOMMENDATIONS/FUTURE PROSPECTS

- it is necessary to identify the specific compound in the extract which is showing antifertility effect. the active principle should be evaluated for contraceptive effect and toxicity.
- *In vivo* fertilization process should be used to identify the mode of action of plant extract.
- The number of sets in *in vivo* fertilization should be increase.

REFERENCES

Adetoun, A., and Ikotun, T. (1989). Antifungal activity of dihydrodioscorine extracted from a wild variety of *Dioscorea bulbifera* L. J. Basic Microbiol. 29, 265–267. doi: 10.1002/jobm.3620290504

Afzali, A., & Ghalehkandi, J. G. (2018). Effect of ginger, Zingiber officinale on sex hormones and certain biochemical parameters of male Wistar rats. *Bioscience Biotechnology Research Communications*, *11*(1), 181–186. doi: 10.21786/bbrc/11.1/25

Agarwal, A., Gupta, S., & Sharma, R. (2016). Hypoosmotic Swelling Test (HOS). *Andrological Evaluation of Male Infertility*, 93–96. doi: 10.1007/978-3-319-26797-5_12

Ahmed, M., Ahamed, R. N., Aladakatti, R., & Ghosesawar, M. (2002). Reversible Anti-Fertility Effect of Benzene Extract of Ocimum Sanctum Leaves on Sperm Parameters and Fructose Content In Rats. *Journal of Basic and Clinical Physiology and Pharmacology*, *13*(1). doi: 10.1515/jbcpp.2002.13.1.51

Ahn, K. (2017). The worldwide trend of using botanical drugs and strategies for developing global drugs. *BMB Reports*, *50*(3), 111–116. doi: 10.5483/bmbrep.2017.50.3.221

Air Potato Management Plan - FLEPPC - Florida Exotic Pest; 12-14. Retrieved from https://fliphtml5.com/blbz/vnpd/basic

Aladakatti, R., & Ahamed, R. N. (2005). CHANGES IN SERTOLI CELLS OF ALBINO RATS INDUCED BY Azadirachta indica A. Juss LEAVES. *Journal of Basic and Clinical Physiology and Pharmacology*, *16*(1). doi: 10.1515/jbcpp.2005.16.1.67

Allen, E. (1922) The oestrous cycle in the mouse. American Journal of Anatomy 30: 297–371.

Anand, A., Prasad, V., & Alam, M. (2015). Herbal or modern methods of contraception! choice is yours. *International Journal of Reproduction, Contraception, Obstetrics and Gynecology*, 947–953. doi: 10.18203/2320-1770.ijrcog20150405

Anuja, M., Nithya, R., Swathy, S., Rajamanickam, C., & Indira, M. (2011). Spermicidal action of a protein isolated from ethanolic root extracts of Achyranthes aspera: An in vitro study. *Phytomedicine*, *18*(8-9), 776–782. doi: 10.1016/j.phymed.2011.01.007

Asha, K. I., and Nair, G. M. (2005). Screening of *Dioscorea* species for diosgenin from southern western Ghats of India. Indian J. Plant Genet. Resour. 18, 227–230.

Ashok, P. & Meenakshi, B. (2004). Contraceptive effect of *Curcuma longa* (L.) in male albino rat. Asian Jornal Androl; 6:71–4. PMID:15064838

Atsukwei, D., Daniel. E., Adams, M., Tende. J., Tope, O., & Danmallam, L. (2015). Contraceptive Effect of Ethanolic Extract of *Dioscorea villosa* Tuber on Reproductive Hormones of Female Wistar Rats. *International Journal of Biochemistry Research & Review*, *5*(2), 135–144. doi: 10.9734/ijbcrr/2015/13433

Bajaj, Y. P. S. (1999) Dioscorea balcanica Kosanin and D. caucasica Lipsky: *In vitro* culture and production of diosgenin. In: Biotechnology in agriculture and forestry 43Medicinal and Aromatic Plants XI," Springer, Berlin Heidelberg, New York, 85-102

Bhattarai, K. R., & Ghimire, M. (2006). Commercially important medicinal and aromatic plants of Nepal and their distribution pattern and conservation measure along the elevation gradient of the Himalayas. *Banko Janakari*, *16*(1), 3–13. doi: 10.3126/banko.v16i1.357

Blobel, C. P., Myles, D. G., Primakoff, P., & White, J. M. (1990). Proteolytic processing of a protein involved in sperm-egg fusion correlates with acquisition of fertilization competence. *The Journal of Cell Biology*, *111*(1), 69–78. doi: 10.1083/jcb.111.1.69

Brinker, F. (1997) Inhibition of endocrine function by botanical agents, antigonadotropic activity. Br J Phytother.Vol.4:123-145.

Brum, A. M., Thomas, A. D., Sabeur, K., & Ball, B. A. (2006). Evaluation of Coomassie blue staining of the acrosome of equine and canine spermatozoa. American Journal of Veterinary Research, 67(2), 358–362. doi:10.2460/ajvr.67.2.358

Byers, S.L., Wiles, M.V., Dunn, S.L., & Taft, R.A. (2012) Mouse Estrous Cycle Identification Tool and Images. PLoS ONE 7(4): e35538. doi:10.1371/ journal.pone.0035538

CABI (2019). Dioscorea bulbifera (Air potato). In: Invasive Species Compendium. Wallingford, UK: CAB International. Retrieved from https://www.cabi.org/isc/datasheet/19295.

Coursey, D. G. (1967). Yams: An Account of the Nature, Origins, Cultivation and Utilization of the Useful Members of the Dioscoreaceae. London: Longmans, Greens and Co. Ltd.

Chevallet, M., Luche, S., & Rabilloud, T. (2006). Silver staining of proteins in polyacrylamide gels. Nature Protocols, 1(4), 1852–1858. doi: 10.1038/nprot.2006.288

Contraception: Hormonal contraceptives. (2017, June 29). Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK441576/.

Dhar, J., Bajpai, V., Setty, B., & Kamboj, V. (1989). Morphological changes in human spermatozoa as examined under scanning electron microscope after exposure to saponins isolated from. *Contraception*, *39*(5), 563–568. doi: 10.1016/0010-7824(89)90111-x

Dong, M., Feng, X. Z., Wang, B. X., Ikejima, T., and Wu, L. J. (2004). Steroidal saponins from Dioscorea panthaica and their cytotoxic activity. Die Pharmazie Int. J. Pharma. Sci. 59, 294–296. doi: 10.1002/chin.200431154

Dutt, B. (2014). Food and medicinal values of certain species of Dioscorea with special reference to Assam. Journal of Pharmacognosy and Phytochemistry. 3(4): 15-18. E-ISSN: 2278-4136

De Moreno, M. R., Smith, J. F., & Smith, R. V. (1986). Mechanism Studies of Coomassie Blue and Silver Staining of Proteins. Journal of Pharmaceutical Sciences, 75(9), 907–911. doi:10.1002/jps.2600750919

Elvis-Offiah, U. B., Bafor, E. E., Eze, G. I., Igbinumwen, O., Viegelmann, C., & Edrada-Ebel, R. (2016). In vivo investigation of female reproductive functions and parameters in nonpregnant mice models and mass spectrometric analysis of the methanol leaf extract ofEmilia Coccinea (Sims) G Dons. *Physiological Reports*, *4*(23). doi: 10.14814/phy2.13047

Esteves, S. C., Sharma, R. K., Jr, A. J. T., & Agarwal, A. (2007). Evaluation of acrosomal status and sperm viability in fresh and cryopreserved specimens by the use of fluorescent peanut agglutinin lectin in conjunction with hypo-osmotic swelling test. *International Braz j Urol*, *33*(3), 364–376. doi: 10.1590/s1677-55382007000300009

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

Etuk, E.U., & Mohammed, B.J. (2009). Informant consensus selection method: A reliability assessment on medicinal plants used in north western Nigeria for the treatment of diabetes mellitus. African Journal of Pharmacy and Pharmacology, 3(10):496–500. http://www.academicjournals.org/ajpp/abstracts/abstracts/abstracts2009/October/Etuk%20amd%20Mohammed.htm

Evans, J. P. (2002). The molecular basis of sperm-oocyte membrane interactions during mammalian fertilization. *Human Reproduction Update*, *8*(4), 297–311. doi: 10.1093/humupd/8.4.297

Evans, J. P., & Florman, H. M. (2002). The state of the union: the cell biology of fertilization. *Nature Cell Biology*, *4*. doi: 10.1038/ncb-nm-fertilitys57

Family Planning/ Contraception. (2018). Retrieved from https://www.who.int/news-room/fact-sheets/detail/family-planning-contraception

Fujihara, S., and Yamaguchi, M. (1978). Effects of allopurinol [4-hydroxyprazolo (3, 4-d) pyrimidine] on the metabolism of allantoin in soybean plants. Plant Physiol. 62, 134–138. doi: 10.1104/pp.62.1.134

Gark, S.K., Mathur, V.S., Chaudhury, R.R. (1978). Screening of Indian plants for anti-fertility activity. Indian J Exp Biol.Vol.16(10):1077-1079.

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

Ghosh, S. (2015). Phytochemsitry and Therapeutic Potential of Medicinal Plant: Dioscorea bulbifera. *Medicinal Chemistry*, *5*(4). doi: 10.4172/2161-0444.1000259

Gopalkrishnan, K. (1995). Standardized procedures in human semen analysis. *Current Science* Vol. 68, No. 4, Special issue on Reproductive Biology, pp. 353-362

Gurav, A., Nabi, S., Vijayakumar, H., & Mondal, D. (2017). Evaluation of hepatoprotective and antidiarrhoeal activity of guduchi, Tinospora cordifolia (Willd.) Miers ex Hook. f. & Thoms. in experimental rats. *Annals of Phytomedicine: An International Journal*, *6*(2). doi: 10.21276/ap.2017.6.2.16

Hanson, S.J.; Burke, Anne E. (2010). "Fertility control: contraception, sterilization, and abortion". In Hurt, K. Joseph; Guile, Matthew W.; Bienstock, Jessica L.; Fox, Harold E.; Wallach, Edward E. (eds.). *The Johns Hopkins manual of gynecology and obstetrics* (4th ed.). Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins. pp. 382–395. ISBN 978-1-60547-433-5.

Hardy, C. M., Clarke, H. G., Nixon, B., Grigg, J. A., Hinds, L. A., & Holland, M. K. (1997). Examination of the Immunocontraceptive Potential of Recombinant Rabbit Fertilin Subunits in Rabbit. *Biology of Reproduction*, *57*(4), 879–886. doi: 10.1095/biolreprod57.4.879

Hill, M.A. (2019) Embryology *Estrous Cycle*. Retrieved from https://embryology.med.unsw.edu.au/embryology/index.php/Estrous_Cycle

Hoffer. A. P. (1983). Effects of gossypol on the seminiferous epithelium in the rat: A light and electron microscope study. Biol Reprod.;28:1007–20. PMID:6860736. https://doi.org/10.1095/biolreprod28.4.1007

Hou, W. C., Lee, M. H., Chen, H. J., Liang, W. L., Han, C. H., Liu, Y. W., et al. (2001). Antioxidant activities of dioscorine, the storage protein of Yam (Dioscorea batatas Decne) tuber. J. Agric. Food Chem. 49, 4956–4960. doi: 10.1021/jf010606m

Huang, T. T. F., & Yanagimachi, R. (1985). Inner acrosomal membrane of mammalian spermatozoa: Its properties and possible functions in fertilization. *American Journal of Anatomy*, *174*(3), 249–268. doi: 10.1002/aja.1001740307

Ittiavirah, P. (2013). Evaluation of spermicidal and antiandrogenic activities of aqueous extract of Tinospora cordifolia (Willd.) stem. *African Journal of Pharmacy and Pharmacology*, *7*(34), 2392–2396. doi: 10.5897/ajpp2013.3625

IUCN Nepal. 2000. National Register of Medicinal Plants. Kathmandu: IUCN Nepal. ix+ 163 pp.

Joonè, C. J., Schulman, M. L., & Bertschinger, H. J. (2017). Ovarian dysfunction associated with zona pellucida–based immunocontraceptive vaccines. *Theriogenology*, *89*, 329–337. doi: 10.1016/j.theriogenology.2016.09.018

Joshi. A. R, Ahamed. R. N, Pathan. K. M, Manivannan. B. (1996). Effect of *Azadirachta indica* leaves on testis and its recovery in albino rats. Indian J Exp Biol; 34:1091–4. PMID:9055629

Kaur. R, Sharma. A, Kumar. R and Kharb. R. (2011). Rising Trends towards Herbal Contraceptives. Scholars Research Library. ISSN: 2231 – 3184.

Kovács, A., & Foote, R. H. (1992). Viability and Acrosome Staining of Bull, Boar and Rabbit Spermatozoa. *Biotechnic & Histochemistry*, *67*(3), 119–124. doi: 10.3109/10520299209110020

Krauchunas, A. R., & Wolfner, M. F. (2013). Molecular Changes During Egg Activation. *Current Topics in Developmental Biology Gametogenesis*, 267–292. doi: 10.1016/b978-0-12-416024-8.00010-6

Kunwar, R. M., Mahat, L., Acharya, R. P., & Bussmann, R. W. (2013, April 12). Medicinal plants, traditional medicine, markets and management in far-west Nepal. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3643841/

Kumar, M., Sheikh, M. A., & Bussmann, R. W. (2011). Ethnomedicinal and ecological status of plants in Garhwal Himalaya, India. *Journal of Ethnobiology and Ethnomedicine*, *7*(1), 32. doi: 10.1186/1746-4269-7-32

Lakshmi, V., Kumar, R., Agarwal, S., & Dhar, J. (2006). Antifertility activity of Piper longum Linn. in female rats. *Natural Product Research*, *20*(3), 235–239. doi: 10.1080/14786410500045465

Liu, H., Tsim, K. W. K., Chou, G.-X., Wang, J.-M., Ji, L.-L., & Wang, Z.-T. (2011). Phenolic Compounds from the Rhizomes of Dioscorea bulbifera. *Chemistry & Biodiversity*, *8*(11), 2110–2116. doi: 10.1002/cbdv.201000279

Malini, T., and Kumari, G.V. (1987). Estrogenic activity of Cuminum cyminum in Rats. Indian J. Exp. Biol., 25: 442-444.

Margalit, I., Rubinstein, S., & Breitbart, H. (1997). A Novel Method for Evaluating the Acrosomal Status of Mammalian Spermatozoa. *Archives of Andrology*, *39*(2), 87–99. doi: 10.3109/01485019708987906

Martin, F.W. (1969). The species of Dioscorea containing sapogenin. Econom. Bot. 23, 373–379. doi: 10.1007/BF02860683

Matsumoto, H., Haniu, H., Kurien, B. T., & Komori, N. (2012). Two-Dimensional Gel Electrophoresis: Glass Tube-Based IEF Followed by SDS-PAGE. *Methods in Molecular Biology Protein Electrophoresis*, 267–273. doi: 10.1007/978-1-61779-821-4_22

Mbiantcha, M., Kamanyi, A., Teponno, R. B., Tapondjou, A. L., Watcho, P., & Nguelefack, T. B. (2011). Analgesic and Anti-Inflammatory Properties of Extracts from the Bulbils ofDioscorea bulbiferaL. varsativa(Dioscoreaceae) in Mice and Rats. *Evidence-Based Complementary and Alternative Medicine*, *2011*, 1–9. doi: 10.1155/2011/912935

McCarthy.J.(2016). Why birth control could be the best weapon against climate change; Global Poverty Project, All Right Reserved; Retrieved from Inc

https://www.globalcitizen.org/en/content/climate-change-overpopulation-birth-control-sustai/

Mcleskey, S., Dowds, C., Carballada, R., White, R., & Saling, P. (1997). Molecules Involved in Mammalian Sperm-Egg Interaction. *International Review of Cytology*, 57–113. doi: 10.1016/s0074-7696(08)62231-7

Mishra, R. K., Singh, S., & Singh, S. K. (2018). Natural products in regulation of male fertility. *The Indian journal of medical research*, *148*(Suppl), S107–S114. doi:10.4103/ijmr.IJMR_1968_17

Monroy, A. (2018). Fertilization. Retrieved from https://www.britannica.com/science/fertilization-reproduction

Mukherjee. S., Banerjee. R., Upadhyay, S. N., Hazra, J., Poddar, K. N., Mukherjee, A., & Saha, A. (2006). Reproductive Effects of Ethnomedicinal Formulation of Tape-Vine Leaves in Female Rats. *Biological & Pharmaceutical Bulletin*, *29*(9), 1916–1922. doi: 10.1248/bpb.29.1916

Nagdas, S. K., Buchanan, T., Mccaskill, S., Mackey, J., Alvarez, G. E., & Raychoudhury, S. (2013). Isolation of a calcium-binding protein of the acrosomal membrane of bovine spermatozoa. *The International Journal of Biochemistry & Cell Biology*, *45*(4), 876–884. doi: 10.1016/j.biocel.2013.01.016

National Population Report 2017. (2017). Retrieved from https://www.mope.gov.np/downloadfile/National Population Report 2017_1515491881.pdf.

Naz, R. K. (2011). Can curcumin provide an ideal contraceptive? Molecular Reproduction and Development, 78(2), 116–123. doi: 10.1002/mrd.21276

Naz, R., & Lough, M. (2014). Curcumin as a potential non-steroidal contraceptive with spermicidal and microbicidal properties. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *176*, 142–148. doi: 10.1016/j.ejogrb.2014.01.024

Nepal Herbs & herbal products Association (NEHHPA), 2018. Retrived from nehhpa.org.np/herbs-directory/Dioscorea-bulbiferra-l-dioscorea-deltoidea-wall-ex-griseb.

Non-hormonal Birth Control Methods. (n.d.). Retrieved from https://www.drugs.com/article/non-hormonal-birth-control.html.

Ogbuewu, I., Unamba-Oparah, I., Odoemenam, V., Etuk, I., & Okoli, I. (2011). The potentiality of medicinal plants as the source of new contraceptive principles in

males. *North American Journal of Medical Sciences*, 255–263. doi: 10.4297/najms.2011.3250

Okabe, M. (2018). Beware of memes in the interpretation of your results - lessons from gene-disrupted mice in fertilization research. *FEBS Letters*, *592*(16), 2673–2679. doi: 10.1002/1873-3468.13101

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

Paul, D., Bera, S., Jana, D., Maiti, R., & Ghosh, D. (2006). In vitro determination of the contraceptive spermicidal activity of a composite extract of Achyranthes aspera and Stephania hernandifolia on human semen. *Contraception*, *73*(3), 284–288. doi:10.1016/j.contraception.2005.07.014

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

Phillips, O., & Gentry, A. H. (1993). The useful plants of Tambopata, Peru: II. Additional hypothesis testing in quantitative ethnobotany. *Economic Botany*, *47*(1), 33–43. doi: 10.1007/bf02862204

Poornima, G. N., and Ravishankar, R. V. (2007). In vitro propagation of wild yams, Dioscorea oppositifolia (Linn.) and Dioscorea pentaphylla (Linn.). Afr. J. Biotechnol. 6, 2348–2352. doi: 10.5897/AJB2007.000-2368

Pradhan DK, Mishra MN, Mishra A, Panda AK, Behera RK, Jha S and Choudhury S. (2013). A Comprehensive Review of Plants used as Contraceptives. *Int J Pharm Sci Res*. 2013; 4(1); 148-155.

Rai R, Nath V. (2005). Some lesser known oral herbal contraceptives in folk claims as antifertility and fertility induced plants in Bastar region of Chhattisgarh. J Nat Rem; 5(2):153-159.

Raj, A., Singh, A., Sharma, A., Singh, N., Kumar, P. & Bhatia, V. (2011). Antifertility Activity of Medicinal Plants on Reproductive system of Female Rat. International Journal of Bio-Engineering Sciences & Technology-IJBEST. Vol 02, Issue 03; ISSN: 2249-6483

Rajabi-Toustani, R., Akter, Q. S., Almadaly, E. A., Hoshino, Y., Adachi, H., Mukoujima, K., & Murase, T. (2019). Methodological improvement of fluorescein isothiocyanate peanut agglutinin (FITC-PNA) acrosomal integrity staining for

frozen-thawed Japanese Black bull spermatozoa. *Journal of Veterinary Medical Science*, *81*(5), 694–702. doi: 10.1292/jvms.18-0560

Rajeev, S. (2004). Sperm membrane protein profiles of fertile and infertile men: identification and characterization of fertility-associated sperm antigen. *Human Reproduction*, *19*(2), 234–242. doi: 10.1093/humrep/deh066

Ramu, S., & Jeyendran, R. S. (2012). The Hypo-osmotic Swelling Test for Evaluation of Sperm Membrane Integrity. Me Nagdas, S. K., Buchanan, T., Mccaskill, S., Mackey, J., Alvarez, G. E., & Raychoudhury, S. (2013). Isolation of a calcium-binding protein of the acrosomal membrane of bovine spermatozoa. The International Journal of Biochemistry & Cell Biology, 45(4), 876–884. doi: 10.1016/j.biocel.2013.01.016

Rubinstein, E., Ziyyat, A., Prenant, M., Wrobel, E., Wolf, J.-P., Levy, S., … Boucheix, C. (2006). Reduced fertility of female mice lacking CD81. *Developmental Biology*, *290*(2), 351–358. doi: 10.1016/j.ydbio.2005.11.031

Sen, T., & Samanta, S. K. (2014). Medicinal Plants, Human Health and Biodiversity: A Broad Review. *Biotechnological Applications of Biodiversity Advances in Biochemical Engineering/Biotechnology*, 59–110. doi: 10.1007/10_2014_273

Seetharam, Y. N., Jyothishwaran, G., Sujeeth, H., Barad, A., Sharanabasappa, G., and Shivkumar, D. (2003). Antimicrobial activity of Dioscorea bulbifera bulbils. Indian J. Pharm. Sci. 65, 195–196.

Senger, P. L. (2004). Pathways to Pregnancy and Parturition, 2nd edition, Current Conceptions, Inc., Washington

Shah, S. K., & Jhade, D. (2018). Evaluation of antifertility potential of Piper betle (Petiole) on female wistar rats "rising approaches of herbal contraception.'' *Biochemistry and Biophysics Reports*, *15*, 97–102. doi: 10.1016/j.bbrep.2018.08.001

Shajeela, P.S., Mohan, V.R., Louis Jesudas, L & Tresina Soris, P. (2011). Antifertility acitivity of ethanol extract of *Dioscorea esculenta* (L.) Schott on male albino rats. International Journal of PharmTech Research. Vol. 3, No.2, pp 946-954, ISSN: 0974-4304.

Sharma, N., & Jocob, D. (2001). Antifertility investigation and toxicological screening of the petroleum ether extract of the leaves of Mentha arvensis L. in male albino mice. *Journal of Ethnopharmacology*, *75*(1), 5–12. doi: 10.1016/s0378-8741(00)00362-7

Shaik, A., Yalavarthi, P., & Bannoth, C. (2017). Role of Anti-fertility Medicinal Plants on Male & Female Reproduction. *Journal of Complementary and Alternative Medical Research*, *3*(2), 1–22. doi: 10.9734/jocamr/2017/34632

Shaik, A., Kanhere, R. S., Cuddapah, R., Nelson, K. S., Vara, P. R., & Sibyala, S. (2014). Antifertility activity of Artemisia vulgaris leaves on female Wistar rats. *Chinese Journal of Natural Medicines*, *12*(3), 180–185. doi: 10.1016/s1875-5364(14)60030-3

Singh N, Singh SK. (2006). Aqueous fruit extract of *Mimus.ops elengi* causes reversible suppression of spermatogenesis and fertility in male mice. Andrologia; 48:807–16. PMID:27489141. DOI: 10.1111/and.12516

Son, I. S., Lee, J. S., Lee, J. Y., and Kwon, C. S. (2014). Antioxidant and anti-inflammatory effects of yam (Dioscorea batatas Decne.) on azoxymethane induced colonic aberrant crypt foci in F344 rats. Prev. Nutr. Food Sci. 19, 82–88. doi: 10.3746/pnf.2014.19.2.082

Soni, P.K., Luhadia, G., Sharma, D.K., and Mali, P. C. (2015). Antifertility Activates of Traditional Medicinal Plants in Male with Emphasis on Their Mode Action: A Review. Journal of Global Biosciences. ISSN 2320-1355. Volume 4, Number 1, pp. 1165-1179

Strober, W. (2001). Trypan Blue Exclusion Test of Cell Viability. Current Protocols in Immunology. doi: 10.1002/0471142735.ima03bs21

Tambe, A. S., Kaore, S. B., Sawane, M.V., and Gosavi, G.B. (2001). Acrosome intactness and seminal hyaluronidase activity: Relationship with conventional seminal parameters. Indian J Med Sci; 55:125-32. PMID: 11482165.

Thung, P. J., Boot, L. M., and Mu¨hlbock, O. (1956). Senile changes in the oestrous cycle and in ovarian structure in some inbred strains of mice. Acta Endocrinol 23, 8–32.

Turner, D.C. (1971). General endocrinology. 4th ed. Tokyo, WB Saunders Company, Topan Company Ltd.

Toda, S., Kimura, M., & Ohnishi, M. (1991). Effects of Phenolcarboxylic Acids on Superoxide Anion and Lipid Peroxidation Induced by Superoxide Anion. *Planta Medica*, *57*(01), 8–10. doi: 10.1055/s-2006-960005

Unny, R., Chauhan, A., Joshi, Y., Dobhal, M., & Gupta, R. (2003). A review on potentiality of medicinal plants as the source of new contraceptive principles. *Phytomedicine*, *10*(2-3), 233–260. doi: 10.1078/094471103321659997

Upadhyay. S.N, Dhawan. S and Talwar. G. P. (1993). Antifertility effects of neem (Azadirachta indica) oil in male rats by single intra-vas administration: an alternate approach to vasectomy. Indian J Exp Biol; 14:275–81. PMID:8226307

Williamson, E. M., Okpako, D. T., & Evans, F. J. (1996). *Selection, preparation and pharmacological evaluation of plant material*. Chichester, (Vol.1., pp. 191-212) Sussex: John Wiley & Sons.

Wong, G. E., Zhu, X., Prater, C. E., Oh, E., & Evans, J. P. (2001). Analysis of Fertilin α (ADAM1)-mediated Sperm-Egg Cell Adhesion during Fertilization and Identification of an Adhesion-mediating Sequence in the Disintegrin-like Domain. *Journal of Biological Chemistry*, *276*(27), 24937–24945. doi: 10.1074/jbc.m101637200

Yanagimachi R (1994) Mammalian fertilization. In: Knobil, E, Neill JD, editors, The Physiology of Reproduction. 2nd edn. New York: Raven Press Ltd; pp. 189–317.

Yanagimachi, R., & Noda, Y. (1970). Physiological changes in the postnuclear cap region of mammalian spermatozoa: A necessary preliminary to the membrane fusion between sperm and egg cells. *Journal of Ultrastructure Research*, *31*(5-6), 486–493. doi: 10.1016/s0022-5320(70)90164-4

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

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	5ml

DMEM (Dulbecco's Modified Eagle's Media)

DMEM powder	10 g/l
BSA	5 mg/ml
HEPES	3.7 g/l
PVP	0.5%
pH 7.2	

FPNA Stain (250X)

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

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-2	2.5 g
Methanol	450 ml
Glacial Acetic Acid	100 ml
Distilled Water	450 ml

Destaining Solution

Methanol	300 ml
Acetic Acid	100 ml