Comprehensive Assessment of Traditionally Used Medicinal Plants as Male Contraceptive: A Quest for Novel Herbal Contraceptives

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Comprehensive Assessment of Traditionally Used Medicinal Plants as Male Contraceptive: A Quest for Novel Herbal Contraceptives

ATHESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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“In the Name of ALLAH, the most Beneficent, the most Merciful”

Dedicated to My Parents

Who gave me the opportunity to

Study from the best institutions and

My Husband who supported me

Throughout the time.

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and the material contained in this thesis is original work. I have not previously presented any part of this work elsewhere for any other degree.

Qurat-ul-Ain

CERTIFICATE

This dissertation **"Comprehensive Assessment of Traditionally Used Medicinal Plants as Male Contraceptive: A Quest for Novel Herbal Contraceptives"** by **Ms. Qurat-ul-Ain** is accepted in its present form by the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Doctor of Philosophy in Reproductive Physiology.

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List of Abbreviations

DPPH	2, 2-diphenyl 1-picrylhydrazyl
BIRC3	Baculoviral IAP repeat-containing protein 3
BTB	Blood testes barrier
BSLA	Brine shrimp lethality assay
CAT	Catalase
ICCR	Committee for Contraception Research
CVR	Contraceptive vaginal ring
COCP	Combined oral contraceptive pill
Cu^{2+}	Cuprous ion
DSP	Daily sperm production
dL	Deciliter
EIA	Enzyme immunoassay
Fe^{2+}	Ferrous ion
$FeSO_4$	Ferrous sulphate
FSH	Follicle stimulating hormone
HRP	Horse radish peroxide
H_2O_2	Hydrogen peroxide
ITT	Intratesticular testosterone
IUC	Intrauterine contraception

LARC	Long-acting reversible contraceptives
LH	Luteinizing hormone
ME	Methanolic Extract
µmol	Micro molar
mmol	Mili molar
mM	Milimolar
DEPPD	N, N-Diethyl-p-phenylenediamine sulphate salt
nM	Nano molar
Nm	Nanometer
NADH	Nicotine adenine dinucleotide
POD	Peroxidase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RISUG	Reversible Inhibition of Sperm Under Guidance
NaCl	Sodium chloride
SOD	Superoxide dimutase
TMB	Tetra methyl benzidin
TBARs	Thiobarbituric acid reactive substances
WHO	World Health Organization

GENERAL ABSTRACT

Background: Population explosion is worldwide problem that possess significant threat to quality of life. The only solution to this problem is availability of suitable contraceptive. Number of medicinal plants at global level has been explored in this regard. Several studies have been carried out to explore the antifertility effect of medicinal plants in male animal models. Some of these plants are recognized for their spermicidal activities, anti-spermatogenic effects, reduce sperm count, affect the mobility and viability of sperms, change testicular morphology, cause alterations in serum testosterone level and some alter antioxidant defense mechanism. More than fifty plants had been reported to possess anti-fertility agents with anti-spermatogenetic, anti-androgenic or sperm immobilization activities. Research for the development of male contraceptives is important for the equitability of male and female in birth control program thus sorting the issue of population growth.

Objectives: Current study was designed for the comprehensive assessment of contraceptive efficacy of novel herbal plants traditionally used in male fertility regulation. The objectives of the study include:

- Comparative analysis of biological activities of four selected medicinal plants.
- Biological evaluation of designated methanolic leaf extracts of medicinal plants.
- To determine the male contraceptive potential of selected medicinal plants using *in vitro* technique to target the process of androgenesis and oxidative stress in testis.
- To evaluate the efficacy of selected medicinal plants *in vivo* to inhibit spermatogenesis or disrupt epididymal and testicular function, the perturbation of which results in reversible infertility.
- To analyze the processes of sperm production, its maturation and function with the aim of regulating or inhibiting specific targets that is involved in reducing the male fertility.
- To study the possible effect of medicinal plants on hypothalamus- pituitary-testicular axis (HPT) by determining plasma Testosterone, Luteinizing hormone and Follicle stimulating hormone concentrations.

Materials and Methods: Four plants belonging to different families were selected named as *Chenopodium ambrosioides, Ajuga integrifolia, Rumex hastatus* and *Hedera nepalensis*. Methanolic leaf extracts were prepared by soaking leaf powder in 99.9% methanol (leaves to solvent ratio 1:10) for seven days. After that, contents were filtered, concentrated under pressure, dried at room temperature and placed at 4ºC until further analysis.

In first study, methanolic leaf extracts of selected plants were screened for their phytochemical analysis, antioxidant potential, protein kinase inhibition activity, cytotoxic activity against brine shrimp nauplii (BSLA) and phytotoxic potential against *Lamna minor*. In addition, gas chromatography mass spectrometry (GC-MS) analysis was carried out to investigate bioactive phyto-constituents present in the methanolic extracts of selected plants.

In second set of experiment, testes and sperms of adult male rats were incubated with media having different concentrations (0, 1, 10, 100, 1000 µg/mL) of plant extracts at 37 ℃ in CO_2 incubator under 5% CO_2 and 95% air (v/v) for 2 hours. Oxidative stress in testis was determined through assessment of antioxidant enzymes activity and generation of reactive oxygen species (ROS). In addition, testicular testosterone levels were measured by Enzyme Linked Immuno Sorbant assay (ELISA) while sperm DNA damage was assessed through comet assay.

On the basis of *in vitro* study, acute toxicity study and *in vivo* study was conducted. For this, stock solution of plant extract was prepared in methanol and further diluted with normal saline. Final methanol concentration was 0.5-1% in saline. In the next series of experiments, adult male rats were given different concentrations of selected plants extracts (0, 50, 100 and 150 mg/kg/day) orally for twenty eight consecutive days. At end of experiments, fertility test was performed to check the reversibility of treatment by pairing three animals from each group with two untreated fertility proven female rats individually. Pregnancy outcome and litter size was noted. Remaining animals were decapitated, trunk blood was collected, plasma was separated and used for hormonal analysis (Testosterone, Luteinizing hormone, Follicle stimulating hormone). While testicular and epididymal tissues were used for the evaluation of alteration in sperm parameters, histological changes and determination of oxidative stress.

Results: Experimental findings of phytochemical analysis showed presence of total flavonoid contents (TFC) in methanolic extracts ranging from *R. hastatus* (242 µg QE/mg) to *H. nepalensis* (216 µg QE/mg), *A. integrifolia* (170 µg QE/mg) and *C. ambrosioides* (146 µg QE/mg). Similarly, total phenolic contents (TPC) were abundant in *R. hastatus* (308 µg GAE/mg) followed by *A. integrifolia (*302 µg/mg), *C. ambrosoides* (291 µg/mg) and *H. nepalensis* (277 µg/mg). In current study, maximum antioxidant capacity was shown by methanolic extract of *R. hastatus* with 309 µg/mg value followed by *A. integrifolia*, *C. ambrosoides* and *H. nepalensis* with 302, 291 and 277 µg/mg respectively. While, *A. integrifolia* possess highest free radical scavenging activity with inhibitory concentration (IC_{50} value) of 17 µg/mL followed by *C. ambrosioides*, *R. hastatus* and *H. nepalensis* with IC_{50} values of 56, 67 and 71 µg/mL respectively. In our study, methanolic extract of *H. nepalensis* showed high protein kinase inhibition against Streptomyces growth with 27.6 mm zone of inhibition/cytotoxicity at highest concentration, followed by *A. integrifolia* (20.7 mm), *R. hastatus* (14.7 mm) and *C. ambrosioides* (7.6 mm) indicating their cytotoxic potential. Results of cytotoxicity assay showed maximum percent mortality of 50, 54, 60 and 74 % at 1000 µg/mL with methanolic leaf extract of *R. hastatus*, *A. integrifolia*, *C. ambrosioides* and *H. nepalensis* respectively. However, phytotoxic activity revealed relatively moderate activity by *C. ambrosioides* (45%), *A. integrifolia* (38%) and *R. hastatus* (49%) at highest concentration (1000 µg/ml) while, *H. nepalensis* showed highest % inhibition (51%) towards *Lamna minor* at 1000 µg/mL. GC-MS analysis of methanolic leaf extracts indicated presence of thirteen compounds in *C. ambroisoides*, eleven in *A. integrifolia*, fifteen in *R. hastatus* and only three compounds were identified in *H. nepalensis*.

In the *in vitro* study, significantly increased oxidative stress with reduced antioxidant activity was observed in highest dose regimen (1000 µg/mL) of methanolic leaf extract of *C. ambrosioides*, *A. integrifolia* and *H. nepalensis*. However, *in vitro* exposure to *R. hastatus* caused slight increase in reactive oxygen species (ROS) production at high dose without significantly affecting antioxidant enzymes status. Increased ROS generation and lipid peroxidation lead to DNA damage in rat sperm. Similarly, decline in testicular testosterone level was noticed after two hours incubation with all the doses of *C. ambrosioides* and *A. integrifolia* leaf extract,

however, *R. hastatus* and *H. nepalensis* showed reduction only at higher doses regimens (100 and 1000 μg/mL).

Results of *in vivo* experiment using *C. ambrosioides* and *A. integrifolia* extracts showed that rate of sperm motility (%) and viability (%) as well as daily sperm production (DSP) were reduced in dose dependent manner. A marked reduction in concentration of antioxidant enzymes was noted in 100mg/kg and 150mg/kg dose treated rats while increase in levels of ROS and thiobarbituric acid reactive substances (TBARS) was also quite evident only in high dose exposure. Histopathological observations of testis exposed to *C. ambrosioides* extract for 28 days revealed significant decrease in seminiferous tubule diameter, lumen diameter and epithelial height in treated animals. Epididymal histology showed a little alteration in tubular and luminal diameter while epithelial height in both caput and caudal epididymis was significantly declined dose dependently. A significant reduction in levels of plasma testosterone, Follicle stimulating hormone (FSH) was recorded while plasma Luteinizing hormone (LH) was reduced non-significantly. *A. integrifolia* extract exposure caused histological alterations including sloughing of epithelium with wider lumen in high extract treated groups. In addition, plasma concentrations of Testosterone, LH and FSH were also reduced significantly in the treated groups. Fertility test of rats exposed to *C. ambrosioides* and *A. integrifolia* extract exhibited reduced pregnancy outcome in the females paired with treated male rats. Litter size was significantly reduced in 50 mg/kg and 150 mg/kg of *C. ambrosioides* extract treatment, however, this reduction was not significant in *A. integrifolia* extract treated rats.

R. hastatus extract exposure indicated no significant change in DSP, epididymal sperm number and antioxidant enzymes concentration, however, dose dependent decrease in sperm motility and viability while increase in levels of ROS and TBARS were seen only in high dose regimen. No histomorphometric changes in testis and epididymis were reported except wider lumen with slightly reduced epithelial height in high dose treated groups. A significant reduction in concentrations of plasma testosterone, LH and FSH was also recorded only in high extract treated animals. Percentage fertility and number of pups born per female paired with *R. hastatus* extract treated male rats were reduced non-significantly in high dose treated groups as compared to control.

Exposure of adult male rats to different doses of *H. nepalensis* resulted in dose dependent decrease in sperm motility, viability and DSP. A marked reduction in antioxidant enzymes levels and rise in levels of ROS and TBARS was noted in high dose treated rats. Histopathological observations of testis revealed normal tubular arrangement with reduced epithelial height and wider lumen in 100mg/kg and 150mg/kg dose treated groups. Similarly, epididymal histology showed normally arranged epididymis with slightly narrow lumen and relatively reduced number of sperms in plant extract treated (100mg/kg and 150mg/kg) groups. A significant reduction in plasma Testosterone, LH and FSH concentrations were observed in extract treated groups when comparison was made with control group. Pregnancy outcome and litter size was significantly reduced in 150mg/kg dose treatment group, but no signs of morbidity or mortality were observed in resultant pups.

Conclusion: It is concluded that although *R. hastatus* affect reproduction to some extant but it is not capable of suppressing fertility in male rats. On the other hand, *H. neplensis* have the potential to suppress fertility either by disturbing sperm parameters or interfering with hypothalamus- pituitary- gonadal axis (HPG). *C. ambrosioides* and *A. integrifolia* caused partial male sterility by disturbing spermatogenic cycle, inducing oxidative stress and hormonal imbalance. Although, fertility was compromised, but fertility suppression was reversible after cessation of treatment and no fetal mortality or morbidity was evident by fertility test.

GENERAL INTRODUCTION

Introduction

World population has been increasing with much higher rate and it is estimated to reach 8.9 billion by the year 2050 (Segal and Segal, 2004). This alarmingly high rate of population growth has detrimental affect on individual and social environment contributing to maternal and child morbidity and mortality. About 81 million pregnancies occurring each year are unplanned and unattended in both developing and under developed countries (Daulaire *et al*., 2002; Speidel *et al*., 2008). Although progress has been made regarding fertility control, yet, 10%–12% married couples remain unsatisfied (Townsend *et al*., 2011).

Millennium Development Goals (MDG) target of universal access to reproductive health reaffirms the need for contraceptive options as well as access to other key reproductive health services, including safe abortion, to reduce maternal mortality (MDG 5) and achieve gender equity (MDG 3).

To attain these targets, fertility regulation including contraception and infertility management formulates an important component of reproductive health for both genders (Allag and Rangari, 2002). On account of exceeding arguments, scientists have initiated to confront this severe problem by formulating active contraceptive methods (Unny *et al*., 2003) and there is an ultimate need to develop safe, cheap, effective and reversible contraceptive methods accessible by people of all religion, race and culture.

History

A scattered number of historical records are available regarding contraceptive methods and abortion practices; endure from the available literature of Latin (Pliny the Elder, 23 to 79 AD), Egyptian (Ebers Papyrus, 1550 BC) and the Greek (Soranus, 100 AD) (Potts and Campbell, 2002). Withdrawal before ejaculation, use of herbal abortifacient and other barrier methods including vaginal lubricants like beeswax and oil/honey were common contraceptive approaches used by Egyptian and Asian women (Azamthulla *et al*., 2015).

A sturdy progress in the field of fertility control has been made in past 50 years, starting with introduction of the first oral contraceptive (OC) pill in 1960s and

emerging techniques of spacing and birth control including contraceptive vaginal ring (CVR) and transdermal patch (Sitruk-Ware *et al*., 2013). In 1970 "International Committee for Contraception Research (ICCR)" was established which open up new gateways of research for development of innovative contraceptive methods. With the collaboration of industrial sector, ICCR and Population Council designed and manufactured different devices including intrauterine contraception (IUC) devices (Paragard®), subdermal implants (Norplant® and Jadelle®), and vaginal rings (Sitruk-Ware *et al*., 2013). These methods have been used by millions of women and readily available worldwide (UN, 2008).

Regardless of the convenient availability of contraceptive methods, lack of their practice is continued due to possible side effects and failure to achieve required outcomes. It is reported that from 50% unplanned pregnancies, 10% occurs due to proper method failure endorsed to the adherent use of inappropriate method of contraception (Frost *et al*., 2008). An ultimate consideration of processes involved in successful reproduction is crucial for the development of effective contraceptives and plays major role in the area of reproductive health sciences (Giudice, 2011).

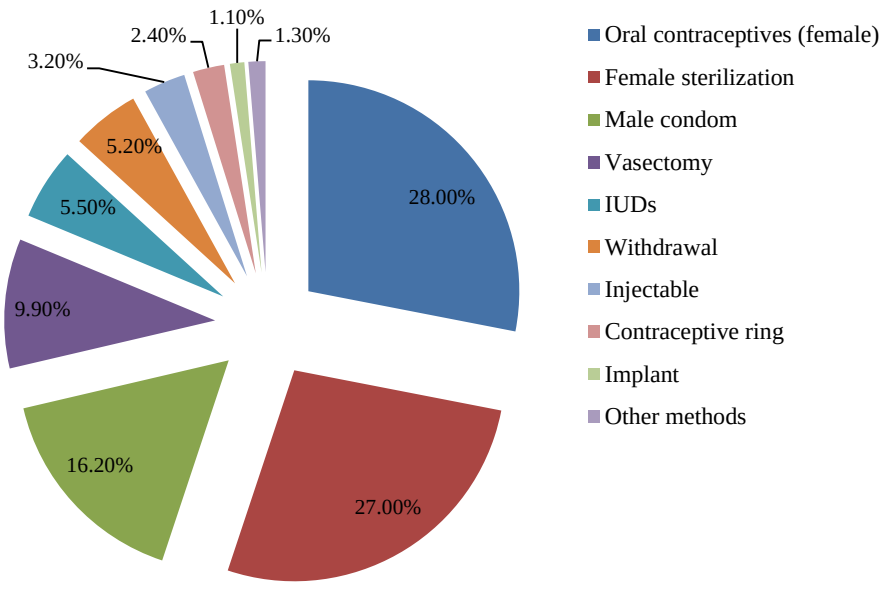


Figure 1. Available methods for contraception.

It is reported that 63% women of reproductive age are contraceptive users (UN, 2008). Women have wide variety of contraceptive choices ranging from daily oral pills to IUD and complete sterilization. For the past several years, research has been focused on female contraceptives lagging behind the male, as females have to endure childbearing and nurturing at the expense of their health.

Female contraception

Various effective methods comprising hormonal and chemical approaches for the fertility control have been explored over a long period of time (Gupta and Sharma, 2006). Currently, amongst the three means of monitoring population growth i.e abortion, sterilization and contraception, the contraceptive mode of birth control is the most popular approach. Various categories of contraceptive devices being used include physiological, mechanical and surgical devices (Azamthulla *et al*., 2015).

Female contraceptive methods practiced nowadays include:

1. Oral hormonal contraceptives (steroidal oral contraceptive pills) (Mishell, 1991).

2. Non-oral hormonal contraceptives

- Vaginal rings (Alvarez-Sanchez *et al*., 1992; Timmer and Mulders, 2000)
- Transdermal delivery system; (patches, skin spray, gels) (Díaz *et al*., 1991)
- "On-demand" contraception for occasional use (Nallasamy *et al*., 2013)
- Long-acting reversible contraceptives (LARCs)
- IUC, IUDs and intrauterine systems (IUSs) (Thonneau and Almont, 2008)
- Subdermal implants (Norplant, 2001)
- Injectable (norethisterone enanthate (NET-EN), medroxyprogesterone acetate acetate (DMPA)) (Draper, 2006)

3. Non-hormonal contraceptives

- Cyclooxygenase-2 (COX-2) inhibitors (Jesam *et al*., 2009)

The chemical and steroidal approaches possess numerous side effects like obesity, gastric trouble and carcinoma of cervix and breast, thromboembolism and asthma which drop their effectiveness and popularity among women. In addition, oral contraceptives are associated with neoplastic and cardiovascular disease, decreasing their usage (Mishell, 1991; Sitruk-Ware *et al*., 2013). The hormonal contraceptives also have detrimental effects on metabolic factors including lipid profile, and

homeostasis (Dinger *et al*., 2007; Lidegaard *et al*., 2009; Lidegaard *et al*., 2012). Greater use of contraceptive approaches is a shortest measure of health, development of population and women empowerment (Agrawal *et al*., 2012). The adverse effects originated by oral and injectable contraceptives are increased weight gain, headache, blood transminase, depression, cholesterol levels, indigestion, fatigue, inter-menorrheal bleeding and hypermenorrhea (not exceeding by 8%) (Bingel and Benoit, 1973; Spellacy, 1974).

Male contraception

So far, contraceptive methods available for men are either hormonal based or non-hormonal including vasectomy, condom and withdrawal before ejaculation during sexual intercourse (Bremner, 2012). Male contraceptives are mainly focused on prevention of sperm maturation, suppression of sperm production, alteration in sperm motility and transport through vas deferens, prevention of sperm deposition and interruption of sperm-egg interaction (Sharma and Jocob, 2001). Current situation demands development of male contraceptives with simplicity, reversibility and safety as preferred features.

Hormonal methods

Male hormonal contraception involves exogenous administration of androgen either alone or in combination with a progestin or GnRH analogue to suppress gonadotropins (LH and FSH) secretion so that process of spermatogenesis can be inhibited. High level of intratesticular testosterone (ITT) and action of FSH on Sertoli cells in necessary for successful spermatogenesis. Reduction in LH and FSH production lead to decline in ITT by Leydig cells and decrease in Sertoli cell functions essential for germ cell survival and maturation. This situation inhibits proliferation of spermatogonia, impaire spermition or cause release of immature sperms and induces germ cell apoptosis. Treatment with exogenous androgen impairs spermatogenesis without affecting normal sexual activities (Amory and Bremner, 2002; Wang and Swerdloff, 2004). Thus enhancement of feedback mechanism between hypothalamus-pituitary-testis axis and suppression of gonadotropin is vital for the effectiveness of male hormonal contraception. This hormonal method have shown 95% efficacy with minimum side effects and reversible as sperm number can be improved after 4 months of treatment withdrawal (Wang and Swerdloff, 2010).

Non-hormonal methods

Traditional male contraceptive methods such as periodic abstinence or *coitus interrupts* (withdrawal of penis from vagina before ejaculation) are accompanied with high rates of undesirable pregnancies. An ideal male contraceptive should be acceptable by both partners, not affecting libido and sexual activity, have no short or long term toxicity, safe and reversible without affecting offspring when reverted and as effective as female contraception methods (Nieschlag *et al*., 2003; Kogan and Wald, 2014).

Condoms: This is the oldest external barrier method and has been used for the prevention of pregnancy as well as sexually transmitted diseases for at least 400 years (Page *et al*., 2008). Most common types include Latex and Natural or "lambskin" condoms, though latter type may not provide protection against STDs. Relatively high rate of conception has been reported during first year of usage (Nieschlag, 2013). In addition to this, many men do not prefer condoms because they feel condoms lessen sexual pleasure (Grady *et al*., 1993), again hindering its consistent practice. They are used only for once and failure rate is 18%.

Vasectomy: It is a safe and permanent contraceptive method performed by surgical procedure in which the vas deferens is cut and the ends are joined by small scrotal incision. It is greatly effective procedure used by over 40 million men worldwide (Glasier *et al*., 2000). It is reported that about 10% of contraceptive users depend on this method with less complications and only 1% chances of failure (Nieschlag *et al*., 2013). Despite of their effectiveness, rare shortcomings that make them unappealing to many males as a choice for contraception are the psychological component regarding surgery for vasectomy and irreversibility.

Reversible Inhibition of Sperm Under Guidance (RISUG): Alternative to vasectomy, another barrier method has been developed to block vas deference either by injectable biomaterials, that form polymer when mixed, or silicone implants. RISUG is similar to vasectomy but more significant due to reversibility (Payne and Goldberg, 2014). It is made up of powdered styrene maleic anhydride (SMA) mixed with dimethyl sulfoxide (DMSO) to make injectable gel. Ongoing research on RISUG involves injecting polymer into vas deferens for blockage and elimination of polymer by another injection to retrieve fertility.

The chemical or synthetic based approaches are directly linked with disruption of endocrine functions leading to reproductive and metabolic impairments, either by interfering with hormonal production or inhibiting hormonal action. (Schug *et al*., 2011).

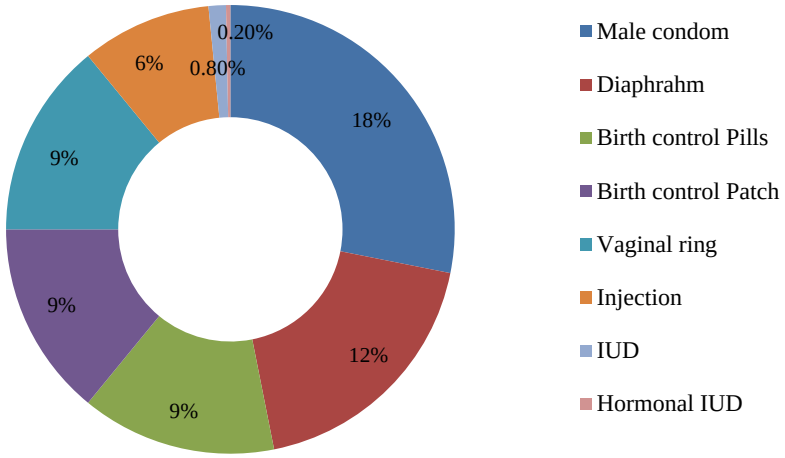


Figure 2. Contraceptive failure rate (Mosher and Jones, 2010)

Medicinal plants

The most interesting domain of pharmacological and therapeutic sciences is the quest for innovative drugs with minimum side effects, less expensive and more effective with complete reversibility. Therefore, plants grabbed the attention of many scientists as a key source of naturally occurring fertility regulating agents due to their minimum side effects (Umadevi *et al*., 2013). World Health Organization (WHO) estimates that in developing countries, 80% of the world's population depends principally on plants for their key healthcare due to shortage and lack of access to current medicine (WHO, 2000). Ethnobotanical awareness consists of herbs used in the old therapeutic systems such as folklore and herbalism. History of human civilization exhibits dependence of man on plants and plant products for food, shelter and for cure from physical sufferings. Since ancient times, mankind has used plant products to cure various diseases because of its better compatibility with the human body, religious and cultural acceptability and lesser side effects. The knowledge about herbal therapies was delivered orally from generation to generation. Subsequently, records were

established with the advancement of civilizations for future benefits (D'cruz *et al*., 2010).

Herbal contraceptives

Numerous medicinal plants have been screened for their antifertility potential and data has been collected for the development of effective male and female contraceptives (Qureshi *et al*., 2006). In recent years, research has been reported regarding extraction of different bioactive components from plants and evaluation of their bioactivity (Chauhan *et al*., 2010; Pokharkar *et al*., 2010). Most of the contraceptive techniques used in family planning program are for female as male have less options for reversible and effective contraceptives. Different herbs have been reported to possess abortifacient as well as spermicidal activities thus can be used to inhibit both male and female fertility (Sur *et al*., 2002; Dehghan *et al*., 2005; Hoesl *et al*., 2005; Gupta and Sharma, 2006). Natural plant products have slight characteristic androgenic and anti-androgenic properties and offer themselves as effective nonconventional sources of contraception with less harmful side-effects (Revathi *et al*., 2010). Although various indigenous medicinal plants possess ability to inhibit birth, only few have been explored for antifertility activity. Among them, some have spermicidal activity while other interferes with hormonal functions thus impairing spermatogenesis (Ahmad *et al*., 2012). Hence there is utmost need to develop plant and plant product based contraceptives with lower side effects and increased efficacy.

Herbal female contraceptives

Plants may induce infertility in females either by interfering hormonal production and action or by inhibiting implantation. On the basis of their mode of action, plants can be categorized into antiovulatory (inhibit ovulation), anti-implantation (inhibit implantation of fertilized egg into uterus), abortifacients (abort fetus) and antifertility plants (Raj *et al*., 2011; Priya *et al*., 2012; Kabra *et al*., 2013). Some of the active antifertility agents from the plants exert their functions by disrupting hypothalamus-pituitary-gonadal axis. As hypothalamus control the secretion of FSH and LH releasing hormones from anterior pituitary, necessary for successful fertilization, and these active agents halt the process either at hypothalamus or pituitary level.

Different antifertility plants have been reported previously. Some of the plants with antiovulatory activity includes *Aspilia africana* (Priya *et al*., 2012), *Butea*

monosperma Lam (Haloi *et al*., 2010) and *Ficus religiosa* (Pokharkar *et al*., 2010). While *Abrus precatorius* (Azmeera *et al*., 2012; Priya *et al*., 2012), *Aegle Marmelos Corr* (Gangadhar and Lalithakumari, 1995), *Ananas comosus* (Shah *et al*., 2009), *Derris coriacea* (Raj *et al*., 2011), *Jatropha curcus* (Pathak *et al*., 2005), *Rotalaria juncea* (Priya *et al*., 2012), *Tanacetum vulgare* (Kaur *et al*., 2011) and *Taxus wallichiana* (Ahmad *et al*., 2011) are reported to possess abortifacient activities. Some other plants with anti-implantation, anti-androgenic, antioestrogenic and contraceptive activities include *Allium cepa* (Pokharkar *et al*., 2010), *Biophytum sanctivum* (Shweta *et al*., 2011), *Crotalaria juncea* (Priya *et al*., 2012), *Dendrophthoe falcate* (Pokharkar *et al*., 2010), *Foeniculum vulgare* (Kalita *et al*., 2011), *Nelumbo nucifera* (Pokharkar *et al*., 2010), *Strychnos potatorum* (Shah *et al*., 2009). *Tripterygium wilfordi* (Kaur *et al*., 2011) and *Zizyphus jujuba* (Shah *et al*., 2009).

Herbal male contraceptives

Products obtained from plants having spermicidal activity are now current interest of the day (Khillare and Shrivastav, 2003; Souad *et al*., 2007). Many studies have been conducted to explore the antifertility effect of medicinal plants in male animal models (Deshpande *et al*., 1980; Zhen *et al*., 1995; Lohiya *et al*., 2000; Coutinho, 2002). The world health organization (WHO) has designed a method on plant research for fertility guidelines with an aim to invent orally active non-steroidal contraceptives. During the last few years, a vast number of plants have been screened and examined for their anti-fertility activity in males. Some of these plants are recognized for their spermicidal activities (Álvarez-Gómez *et al*., 2007a; Álvarez-Gómez *et al*., 2007b; Souad *et al*., 2007), antispermatogenic effect (Bhogaonkar *et al*.; Verma *et al*., 2002; Mishra and Singh, 2009b), spermatotoxic effects (Akbarsha and Murugaian, 2000), some of them reduce sperm count; others may affect the mobility and viability of sperms; some may change testicular morphology (Bhargava, 1989), some cause alteration in serum testosterone level (Vijaykumar *et al*., 2004; Jahan *et al*., 2009b; Mishra *et al*., 2009; Wahab *et al*., 2010; Ekaluo *et al*., 2011; Onyeka *et al*., 2012), poor sperm quality (Gupta *et al*., 2004; Anitha and Indira, 2006; Morey and KHANDAGLE, 2011; Sathiyaraj *et al*., 2011) and some alter antioxidant defense mechanism (Krishnamoorthy *et al*., 2007). Some of these plants includ, *Abrus precatorius* (Jahan *et al*., 2009a), *Allium sativum* (Chakrabarti *et al*., 2003), *Azadirachta Indica* (Kabeh and Jalingo, 2007), *Cananga odorata* (Pankajakshy and

Madambath, 2009), *Lagenaria breviflora* (Saba *et al*., 2009), *Garcinia cambogia* (Oluyemi *et al*., 2007), *Mollugo pentaphylla* (Rajasekaran *et al*., 1993), *Leptadenia hastate* (Bayala *et al*., 2011), *Martynia annua* (Mali *et al*., 2002), *Ocimum sanctum* (Leigh and Fayemi, 2008), *Piper nigrum* (Mishra and Singh, 2009a), *Primula vulgaris* (Primorac *et al*., 1985), *Ruta graveolens* (Khouri and El-Akawi, 2005), *Terminalia chebula* (Srivastav *et al*., 2010), *Tripterygium wilfordii* (Zi-Jian *et al*., 1992).

Medicinal plants are rich in several phyto-constituents. They show suppressive and inhibitory effects on male fertility. Numerous plants containing flavonoids, tannins, terpenes, quinines, diterpenoid lactones have been described to induce male antifertility properties by different mechanism (Reddy *et al*., 2003; Rao *et al*., 2004; Joshi *et al*., 2011). In recent years, for the discovery of innovative pharmacological compounds, various drugs are being derived from plants either directly or indirectly (Newman and Cragg, 2007; Li, 2010). Two important plant components saponins and terpenoids possess various biological activities like sperm immobilization (Rajasekaran *et al*., 1993). Saponins are the important component of popular spermicidal preparations currently available in the form of gels, creams or part of lubricated condoms (Garg *et al*., 1994).

In regards of health care, most of the available resources benefit middle class people while it has always been difficult to reach poor people with development support. Thus outmoded medicine is often the only inexpensive and manageable form of health care (Thirumalai *et al*., 2010). More than 50 plants had been reported to possess anti-fertility agents with anti-spermatogenetic, anti-androgenic or sperm immobilization activities (Khillare and Shrivastav, 2003; Unny *et al*., 2003). Research for the development of male contraceptives is important for the equitability of male and female in birth control program thus sorting the issue of population growth.

Based on the above literature review, current study was designed for the assessment of contraceptive efficacy of novel herbal plants traditionally used in male fertility regulation. Initially, a detailed survey was performed and total 48 species belonging to 28 families and 41 genera were recorded. For current study, four plants were screened on the basis of their accessibility to indigenous communities and frequent use for birth control by traditional health practitioners and local inhabitants. These plants were further progressed to biological and phytochemical studies to evaluate their possible effect on male reproduction as well as to report active chemical constituents which

may be used by pharmaceutical industries for making herbal contraceptive drugs. Moreover, novel information gathered from current data could be used by doctors/clinical practitioners and researchers which could be of great benefaction to society.

Aims and Objectives

The purpose of this research is to identify new agents that could lead to the development of safe and more potent contraceptive products for men that are involved in regulation of germ cell apoptosis, regulation of spermatogenesis, testicular or epididymis function, etc. There is no safe, efficient, and reversible male contraceptive drug available in the market. Therefore, present study was designed to identify biologically active medicinal plant and to evaluate their contraceptive potential.

The objectives of the study include:

- Comparative analysis of biological activities of four selected medicinal plants.
- Biological evaluation of designated methanolic leaf extracts of medicinal plants.
- To determine the male contraceptive potential of selected medicinal plants using *in vitro* technique to target the process of androgenesis and oxidative stress in testis.
- To evaluate the efficacy of selected medicinal plants *in vivo* to inhibit spermatogenesis or disrupt epididymal and testicular function, the perturbation of which results in reversible infertility.
- To analyze the processes of sperm production, its maturation and function with the aim of regulating or inhibiting specific targets that is involved in reducing the male fertility.
- To study the possible effect of medicinal plants on HPT axis by determining plasma Testosterone, Luteinizing hormone and Follicle stimulating hormone concentrations.

CHAPTER 1:

Screening and characterization of methanolic leaf extract of plants against biological activities.

ABSTRACT

Background: Medicinal plants are important component of flora widely distributed throughout the world. Plants possess a variety of bioactive compounds, biological evaluation and identification of which is necessary for the development of novel, safe and effective medicines. Based on the ethno-pharmacological literature and traditional knowledge, four plant species belonging to different families were selected.

Objectives: The present study was designed for the biological evaluation of methanolic leaf extracts of selected plants including *C. ambroisioides*, *A. integrifolia*, *R. hastatus* and *H. nepalensis.*

Materials and Methods: Methanolic extract was prepared by soaking leaf powder in 99.9% methanol (leaves to solvent ratio 1:10) for seven days. After that, contents were filtered, concentrated under pressure, dried at room temperature and placed at 4ºC until further analysis. The methanolic leaf extracts of selected plants were screened for their phytochemical analysis, antioxidant potential, protein kinase inhibition activity, cytotoxic activity against brine shrimp nauplii (BSLA) and phytotoxic potential against Lamna minor. In addition, GC-MS analysis was carried out to investigate bioactive phyto-constituents present in the methanolic extracts of selected plant extracts.

Results: Experimental findings of phytochemical analysis revealed highest amount of quercitin equivalents total flavonoids components (242 µg QE/mg extract), gallic acid equivalents total phenolic contents (308 µg GAE/mg extract) and total antioxidant capacity (309 µg/mg) were quantified by methanolic leaf extract of *R. hastatus.* While, highest 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity was shown by methanolic leaf extract of *A. integrifolia* with 17 µg/mL IC_{50} value. In our study, methanolic extract of *H. nepalensis* showed high protein kinase inhibition against Streptomyces growth with 27.6 mm zone of inhibition/cytotoxicity at highest concentration, followed by *A. integrifolia* (20.7 mm), *R. hastatus* (14.7) and *C. ambrosioides* (7.6 mm) indicating their cytotoxic potential. Cytotoxicity assay showed maximum percent mortality of 50, 54, 60 and 74 % at 1000 µg/ml with methanolic leaf extract of *R. hastatus*, *A. integrifolia*, *C. ambrosioides* and *H. nepalensis* respectively. Phytotoxic activity study revealed relatively moderate activity by *C. ambrosioides* (45%), *A. integrifolia* (38%) and *R. hastatus* (49%) at highest concentration (1000µg/ml) while, *H. nepalensis* showed highest % inhibition

(51%) towards *Lamna minor* at 1000µg/ml. Furthermore, GC-MS analysis indicated presence of thirteen compounds in *C. ambrosioides*, eleven in *A. integrifolia,* fifteen in *R. hastatus* and only three compounds were identified in *H. nepalensis*.

Conclusion: Results obtained from the current study support their use in traditional medicine as all the plants are rich with phenols, flavonoids and possess antioxidant activity. In addition, this study provides basis and direction for the further pharmacological investigations.

INTRODUCTION

Plants have always been considered as safe, probable substitute and rich source of new medications to help meet health maintenance. According to the World Health Organization (WHO), about 80% people use plants for their basic health need (Okigbo *et al*., 2008) and about 70% of the available drugs are derived from natural and botanicals products (Newman and Cragg, 2016). During the last few decades, use of medicinal plants and herbal products is considered as one of the most reliable and hot topic among researchers around the globe. A large number of study groups are engaged in investigating the medicinal properties of plants. Pakistan is the country rich with medicinal flora but extensive and laborious work is required to explore and investigate its pharmacological importance (Ahmad *et al*., 2012).

Many active components used in drug formation are natural products, most of them have unknown biological activities (Konan *et al*., 2007). However, no reliable scientific data concerning phyto-medicinal potential of plant is available in literature. Many researchers are engaged in directing their studies for the determination of toxic potential of medicinal plants (Badakhshan *et al*., 2009). Brine Shrimp Lethality Assay (BSLA) is a general bioassay with potential of detecting a broad spectrum of bioactivity in plant extracts (Pisutthanan *et al*., 2013). This assay was developed for screening of cytotoxic bioactive components of plant extracts as it is simple, inexpensive and time saving technique (Kivcak *et al*., 2002). Lemna minor are monocot plants that, in normal conditions, reproduce with budding (Ullah *et al*., 2012). As antitumor and cytotoxic components in plant extracts can inhibit Lamna minor growth, this assay provides base line screening for more specific bioassays (Pisutthanan *et al*., 2013). In the recent years, research on antioxidant activity of medicinal plant extracts has been increased as antioxidants are essential for scavenge reactive oxygen species (ROS) thus combating diseased condition (Fernandes *et al*., 2007; Sharififar *et al*., 2007; Andrade *et al*., 2009).

In current study, four medicinal plants belonging to different families were selected namely *C. ambrosioides, A. integrifolia, R. hastatus* and *H. nepalensis*. *C. ambrosioides* (goosefoot) is a perennial, aromatic herbaceous shrub of family Chenopodiaceae. It is pharmacologically used as chemopreventive agent (Kiuchi *et al*., 2002; Liu, 2004) and known for antibacterial (Sousa *et al*., 2012), antiviral (Zanon *et al*., 1999) and antifungal (Prasad *et al*., 2010) properties due to abundance of

flavonoids and terpenoids compounds. *Ajuga integrifolia* is a species of annual and perennial herbaceous plants belonging family Lamiaceae. Some of the pharmacological properties include antimalarial (Kuria *et al*., 2001; Cocquyt *et al*., 2011), antiplasmodial (Asnake *et al*., 2015) antifungal (Kariba, 2001) and anti-inflammatory (Gautam *et al*., 2011) activities. Additionally, *Rumex hastatus* belongs to family Polygonaceae. Its leaves are rich with phenolic components and considered as good source of antioxidant (Sahreen *et al*., 2011). *H. nepalensis* is species of flowering plants belonging to the family Araliaceae. This plant has also been evaluated for different biological activities including phytotoxic, antitumor, cytotoxic and antifungal activities (Inayatullah *et al*., 2007; Meng and Wang, 2010).

In present study, methanolic leaf extracts of selected plants were screened for their phytochemical analysis, antioxidant potential determination, protein kinase inhibition activity, cytotoxicity against brine shrimp nauplii and phytotoxic potential against *Lamna minor*. In addition, GC-MS analysis was conducted to investigate bioactive phyto-constituents present in the methanolic extracts of selected plant leaves. The findings of current study provide baseline data on the most promising plant species that could be utilized as a source for the discovery of pharmacologically important therapeutic tools as well as in contraceptive drug development.

MATERIALS AND METHODS

Plant material

Fresh leaves of *C. ambrosioides*, *A. integrifolia*, *R. hastatus* and *H. nepalensis* were obtained from agricultural and cultivated fields of Abbottabad, Mansehra, Dir and Galyat respectively. These plants were selected on the basis of their extensive medicinal use in respective site of collection and also in different areas of Pakistan. The botanical identification was done by Dr. Mushtaq Ahmad at Plant Systematics and Biodiversity Lab and voucher specimen were deposited in the herbarium of Plant Sciences Department, Quaid-i-Azam University Islamabad, Pakistan.

Plant extract preparation

Leaves of selected plants were separated from stem and air-dried. These air dried leaves, weighing almost 2 kg were stored until the preparation of the extract. Leaves were ground in waring blender and then sieved. Methanolic extract was prepared by soaking leaf powder in 99.9% methanol (leaves to solvent ratio 1:10) for seven days. After that, contents were filtered using Whattman filter paper and concentrated under pressure using Rotary evaporator (Model: Hei-VAP Heidolph, Germany). The filtrate mass was dried at room temperature and placed at 4ºC (Gulfraz *et al*., 2007) (Figure 3).

Plant Extract Preparation

C. ambosioides *A. integrifolia* *R. hastatus* *H. nepalensis*

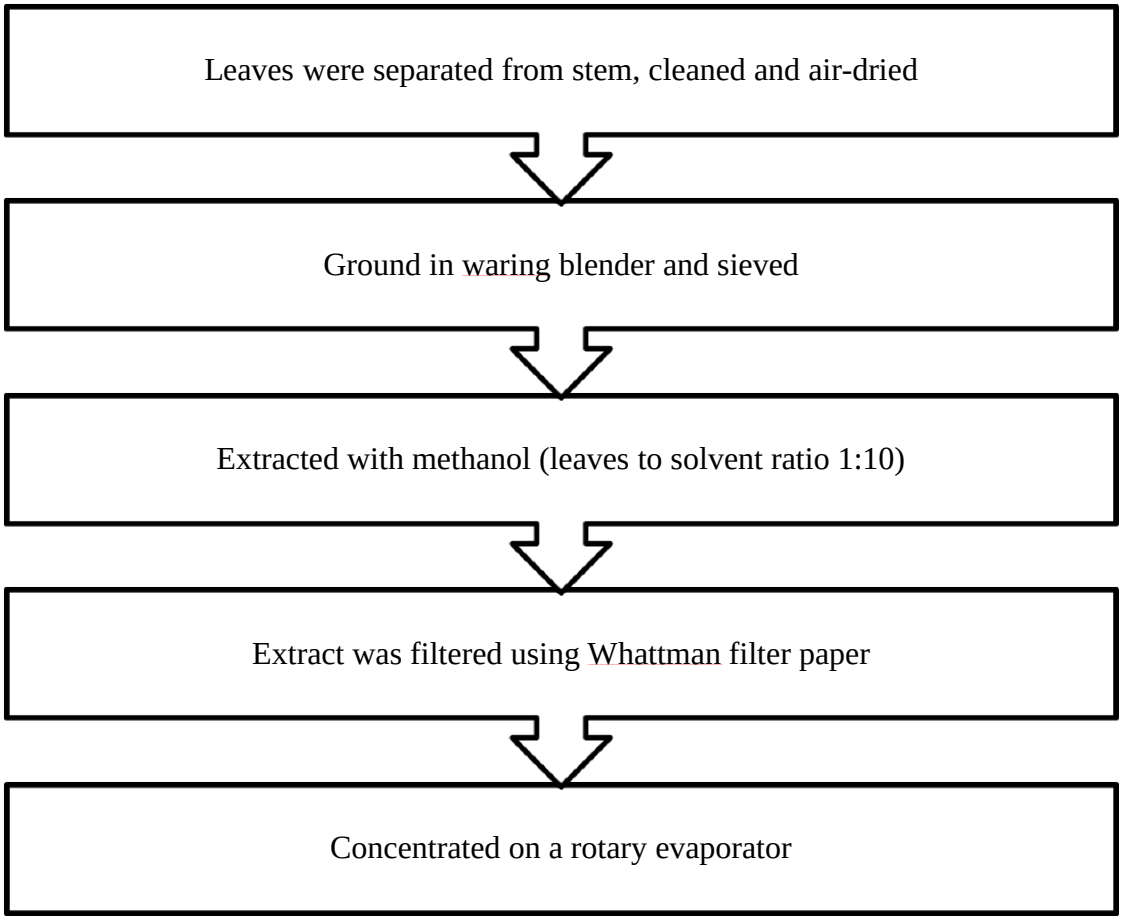


Figure: 3. Methanolic leaf extract preparation of selected plants.

Biological Evaluation

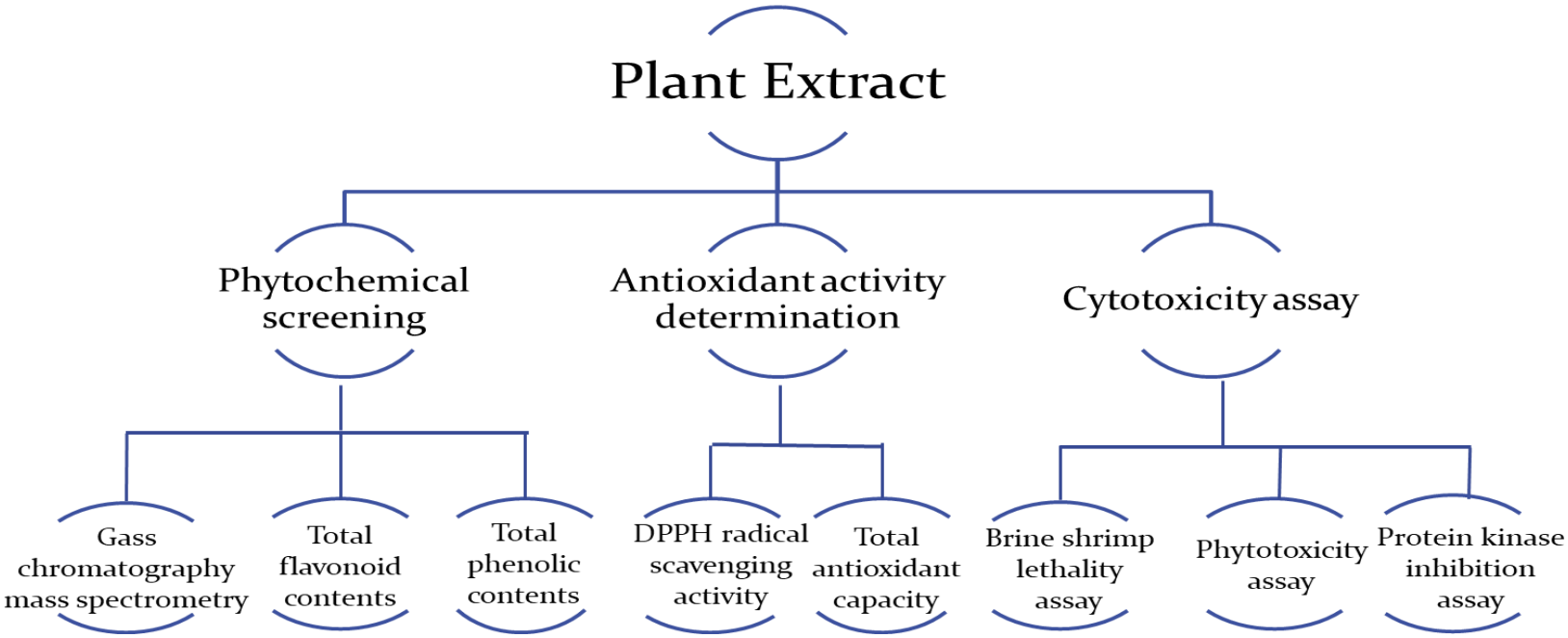


Figure: 4. Summary of biological evaluation of selected plant extracts.

Phytochemical analysis

Total flavonoid contents estimation (TFC)

For the determination of total flavonoids content, aluminum trichloride (AlCl3) colorimetric method was used as described earlier with slight modifications Quettire-Deleu *et al*. (Quettier-Deleu *et al*., 2000). From 4 mg/mL stock solution, test sample of 20 µl was taken in 96-well plate. DMSO as positive and quercetin as negative control was used. Whole mixture was incubated at 37 °C for 30 min. After incubation, aluminum chloride and potassium acetate were added (10 µl of each) along with distilled water to elevate the level up to 200 µl. Absorbance was recorded at 405 nm using micro plate reader. Results were analyzed thrice as µg QE/mg extracts.

Total phenolic contents estimation (TPC)

Total phenolic contents were assessed by using Folin-Ciocalteu method as described earlier by Jagadish *et al*. (Jagadish *et al*., 2009) with slight modifications. In the 96 well plates, an aliquot of 20 µl from stock solution (4 mg/mL) and 90 µl of Folin-Ciocalteu reagent was dispensed and incubated. Followed by 5 min of incubation, sodium chloride (6%) was dispensed in every well and again incubated for 90 min at room temperature. Gallic acid as positive and DMSO as negative control was used. Absorbance was recorded at 630 nm and results were analyzed thrice.

A**ntioxidant potential estimation**

DPPH assay

Free radical scavenging activity was assessed through DPPH assay using method described by Tai *et al*. in 2011 with slight modifications. Antioxidant activity of selected plants extracts was evaluated by their capability to reduce the stable 2, 2-diphenyl 1-picrylhydrazyl (DPPH). For this, selected doses of plant sample were taken in 96 well plate along with DPPH to get final concentration of 200 µl. The plate was incubated at room temperature for 60 min. DMSO as negative and ascorbic acid as positive control was used. After incubation, the absorbance was noted at 630 nm wavelength using microplate reader. There is inverse relationship between absorbance of DPPH solution and radical scavenging activity i.e. decrease in DPPH absorbance indicates increase in radical scavenging activity of tested sample. IC_{50} values were

calculated and expressed as µg AAE/mg of extract. Percent radical scavenging activity was determined by following formula:

%RSA = [1 − (OD of Extract)/(OD of Control)] × 100

Total antioxidant capacity determination

To determine total antioxidant capacity of the plants extracts, phosphomolybdenum method was used (Prieto *et al*., 1999). In this method, green colored phosphate/Mo (V) complex is made when Mo (VI) is reduced to Mo (V) (Jafri *et al*., 2014). From the stock solution, an aliquot of 20 µl of test sample was mixed with phosphomolybdenum reagent (180 µl) and reaction mixture was incubated in water bath 95 °C for 90 min. After incubation, sample was shifted to 96 well plates. DMSO as negative and ascorbic acid was considered as positive control. Absorbance was recorded at 630 nm wavelength using microplate reader. Results were analyzed thrice.

Protein kinase inhibition assay

In this assay, 85E strain of Streptomyces were used according to previously reported method (Yao *et al*., 2011). The prepared ISP4 media was autoclaved at 121 °C for 20 min, dispensed in the culture plates and allowed to solidify. Streptomyces culture was inoculated into the media plate with the help of cotton swab. Disc diffusion method was used in which Whattmann filter paper discs were impregnated with an aliquot of 20 mg/mL test sample and retained on media plates. DMSO and Streptomycin sulphate infused discs were considered as negative and positive controls. All the experiment was performed in Lamina flow chamber so that contamination could be evaded. All the plates were incubated for 24–48 h at room temperature to let the development of hyphae. The formation of clear zone indicated cytotoxicity while bald zone exhibited protein kinase inhibition (Franco-Correa *et al*., 2010).

Cytotoxicity assay

Brine shrimp lethality assay (BSLA)

This assay is widely used to determine cytotoxicity of bioactive components from the natural compounds. In current experiment, method described by Mclaughlin was used with slight modifications to conduct this assay (McLaughlin *et al*., 1991). A plastic dish containing sea water (3.8 g salt/L of water) was taken and partitioned. 50 mg of brine shrimp (*Artemia salina)* eggs were placed at one side of the dish and allowed to hatch at room temperature (26±1 °C) for 48 h under constant illumination and

aeration to ensure survival and maturity of nauplii. Stock solution (10mg/ml) of plants extract was prepared and transferred to clean vials with selected doses of test samples (10, 100 and 1000 µg/mL). Experiment was performed in triplicate. With the help of pipette, 10 shrimp's nauplii were inoculated in each vial and final volume was maintained upto 10ml by using sea water. Etoposide was used as positive control while nauplii in sea water without plant extract were considered as negative control. The vials were incubated at 25 °C for 24 h and number of dead larvae was counted by using magnifying lens. Percent mortality was assessed by using following formula:

Percent mortality = (number of dead shrimps nauplii/ total number of shrimps nauplii) × 100

Phytotoxicity assay

In vitro phytotoxic bioassay was performed to screen phytotoxicity of plants extract against *Lemna minor* using method reported by McLaughlin *et al*., (1991). From the stock solution (10mg/ml), 5, 50 and 100 µl test sample was transferred into separate petri plates to obtain 10, 100 and 1000 µg/mL of final concentrations respectively (3 plates per concentration). The solvent was allowed to evaporate by incubating the plates overnight under sterile conditions. Followed by incubation, 20ml of E-media (pH 5.5-5.6) and 15 *Lemna minor* fronds were transferred in petri plates. Paraquate along with solvent was considered as negative control. All the plates were incubated at 28±1 ºC for about seven days. On the seventh day, number of fronds per plates was noted and percentage growth regulation/inhibition was determined by using given equation:

Growth regulation (%) = 100 - (Number of fronds in test/Number of fronds in control × 100)

Gas chromatography-mass spectrometry analysis (GCMS)

The GC–MS analysis was carried out by using GCMS-QP5050 Shimadzu, Japan. The capillary column used was DB-5/RTX-MS (30 m length and 0.25 mm diameter consisting of 95% dimethyl polysiloxane). Helium was used as carrier gas at flow rate of 1 ml/min with linear velocity of 37.2 cm/sec and 1 µL injection volume. For sample analysis, column temperature was maintained at 90°C for 1 min after injection. Injector temperature was elevated to 200°C with 10°C increase in every minute. Final temperature was elevated to 250°C with 10°C increase in

temperature/min for 15 min. Temperature of detector was maintained at 300°C while that of injector at 250°C. An election ionization system was used for detection with ionization energy of 70 eV. Pressure was maintained at 60.0 kPa and sample was run for 60 min. A scan rate of 0.50 s (cycle time: 0.2 s) was applied, which covers a mass ranging 35 to 600 amu.

RESULTS

Determination of total flavonoid and total phenolic contents

Total flavonoid content (TFC)

The methanolic leaf extracts of selected plants possess wide range of flavonoid contents as shown in table 1. Among them, *R. hastatus* showed higher contents of 242 µg QE/mg of extract followed by *H. nepalensis* with 216 µg QE/mg of extract, *A. integrifolia* with 170 µg QE/mg of extract and *C. ambrosioides* with 146 µg QE/mg of extract as given in (Figure 5 & 7).

Total phenolic contents (TPC)

A wide range of total phenolic contents were found in methanolic extracts of selected plants as shown in table 1. In current study, the highest contents of Gallic acid equivalent phenols were observed in methanolic extract of *R hastatus* with 308 µg GAE/mg extract. Whereas values of total phenols varied from 286 µg GAE/mg in *H. nepalensis*, 261 µg GAE/mg in *A. integrifolia* and 227 µg GAE/mg in *C. ambrosioides* plant extracts as given in (Figure 6 & 7).

Table 1: Total flavonoid (TFC) and total phenolic contents (TPC) of methanolic leaf extract of four selected plants.

Plant Extract	TFC (µg QE/mg)	TPC (µg GAE/mg)
C. ambrosioides	146.42±0.95	227.12±1.76
A. integrifolia	170.51±1.08	261.60±1.16
R. hastatus	242.18±0.86	308.31±1.62
H. nepalensis	216.35±0.99	286.34±1.04
Quercetin	290.41±0.77	------
Gallic acid	------	389.36±1.22

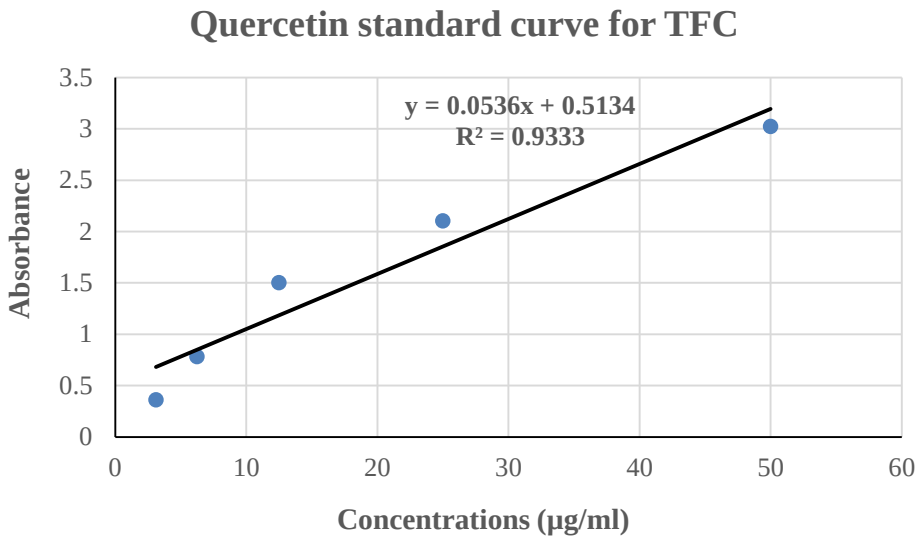


Figure 5: Quercetin standard curve for total flavonoids contents determination.

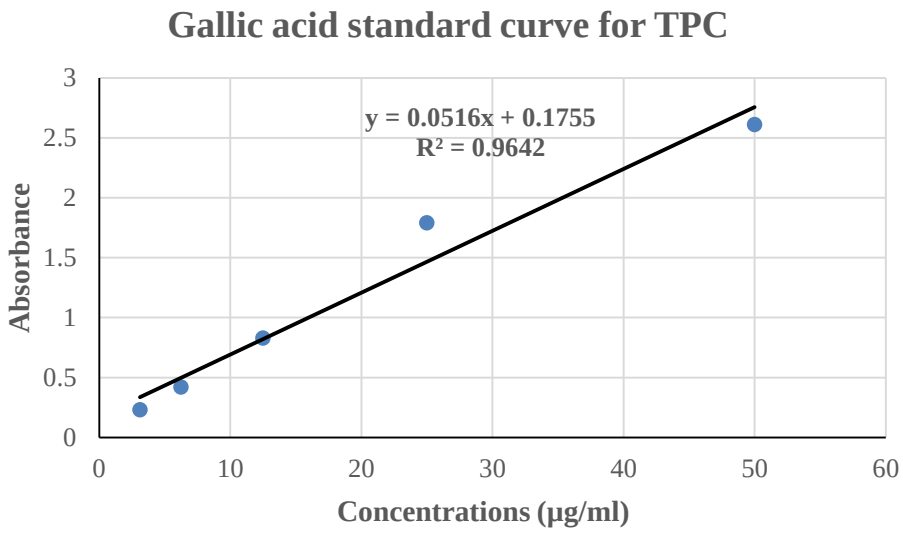


Figure 6: Gallic acid standard curve for total phenolic contents determination

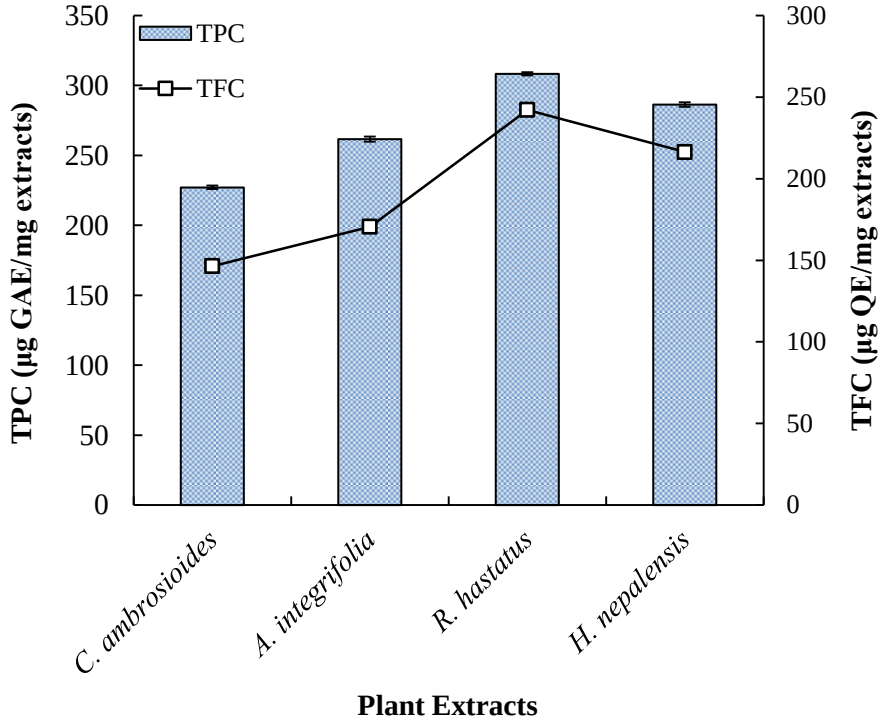


Figure 7: Total phenolic contents and total flavonoid contents estimation in methanolic leaf extract of *C. ambrosioides, A. integrifolia, R. hastatus* and *H. nepalensis*.

Antioxidant potential Estimation

DPPH assay

In current study, free radical scavenging activity of the methanolic leaf extracts of selected plants was estimated by using DPPH assay. The principal of assay comprises reduction of purple colored DPPH into yellow colored 2, 2-diphenyl-1-picrylhydrazyl, which is a stable compound. IC_{50} value was calculated for evaluation of results. It was observed that that methanolic leaf extract of *A. integrifolia* possess highest free radical scavenging activity with inhibitory concentration (IC_{50} value) of 17 µg/mL followed by *C. ambrosioides, R. hastatus* and *H. nepalensis* with IC_{50} values of 56, 67 and 71 µg/mL respectively (Table 2, Figure 8).

Total antioxidant capacity (TAC)

In current study, total antioxidant activity of methanolic leaf extract of four different plants was investigated. As summarized in table 3, maximum antioxidant activity was shown by methanolic leaf extract of *R. hastatus* with 309 µg/mg value followed by *A. integrifolia, C. ambrosoides and H. nepalensis* with 302, 291 and 277 µg/mg total antioxidant capacity as shown in figure 9 & 10.

Table 2: DPPH free radical scavenging activity of methanolic leaf extracts of selected plants with IC_{50} values.

Plant Extract	400 µg/mL	300 µg/mL	200 µg/mL	100 µg/mL	IC_{50} values
C. ambrosioides	88.90052	85.44503	77.8534	60.87543	56ug/ml
A. integrifolia	87.43455	86.02094	84.45026	70.12987	17 ug/ml
R. hastatus	84.7644	79.94764	67.48691	58.90052	67ug/ml
H. nepalensis	83.03665	80.68063	70.20942	56.43979	71ug/ml

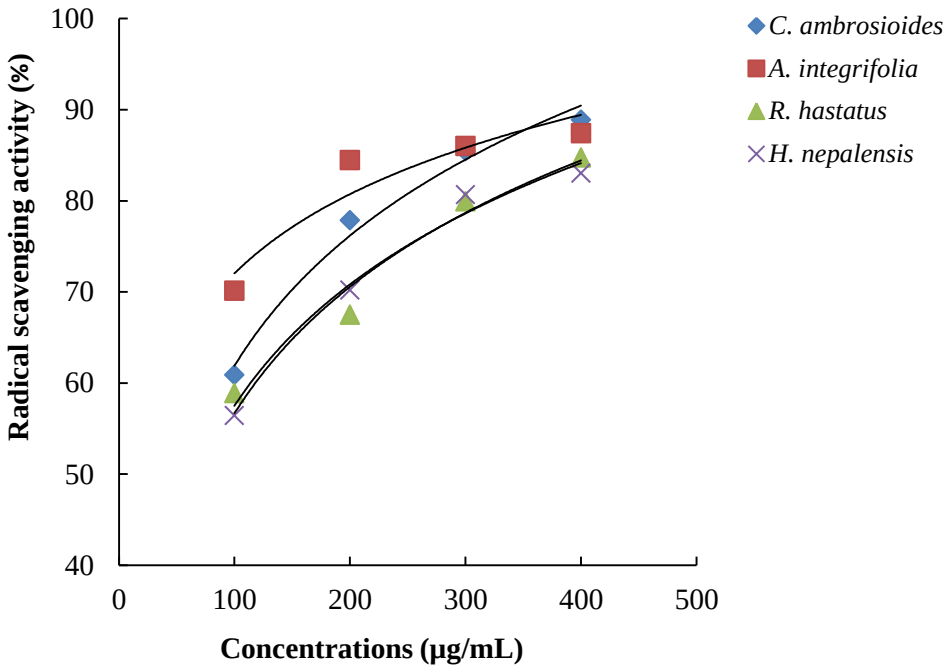


Figure 8: DPPH free radical scavenging activity (%) of methanolic leaf extracts of *C. ambrosioides, A. integrifolia, R. hastatus* and *H. nepalensis*.

Table 3: Total antioxidant capacity of methanolic leaf extract of four selected plants.

Plant Extract	TAC (µg AAE/mg)
C. ambrosioides	291.3795±1.13
A. integrifolia	302.9314±1.34
R. hastatus	309.4127±0.79
H. nepalensis	277.626±0.97

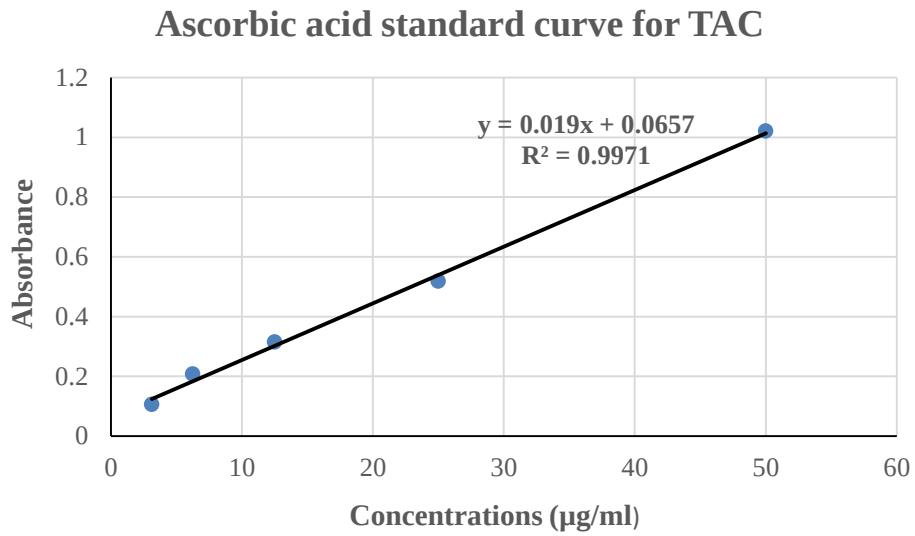


Figure 9: Ascorbic acid standard curve for total antioxidant capacity estimation.

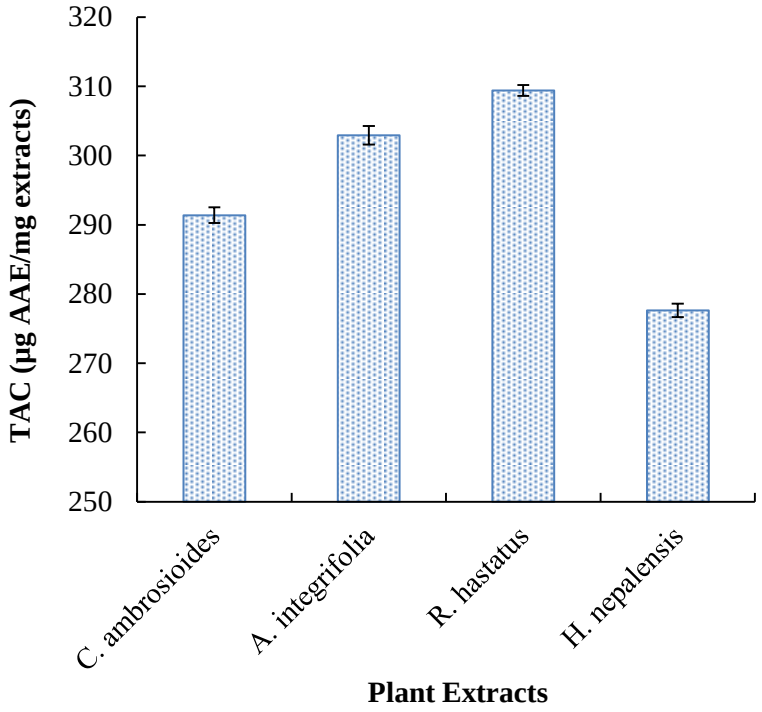


Figure 10: Total antioxidant capacity determination in methanolic leaf extract of *C. ambrosioides, A. integrifolia, R. hastatus* and *H. nepalensis*.

Protein kinase inhibition assay

Methanolic leaf extract of selected plants were screened for the protein kinases inhibition by using Streptomyces strain. In our study, methanolic extract of *H. nepalensis* showed high cytotoxicity value against *Streptomyces* growth with 27.6 mm zone of inhibition/cytotoxicity at highest concentration, followed by *A. integrifolia* (20.7 mm), *R. hastatus* (14.7 mm) *and C. ambrosioides* (7.6 mm). DMSO was used as negative control with no zone formation, hence exhibited no signs of cytotoxicity while Streptomycin sulphate was used as positive control and showed the clear zone formation with 20.92 ± 1.02 mm (Table 4, Figure 11 & 12).

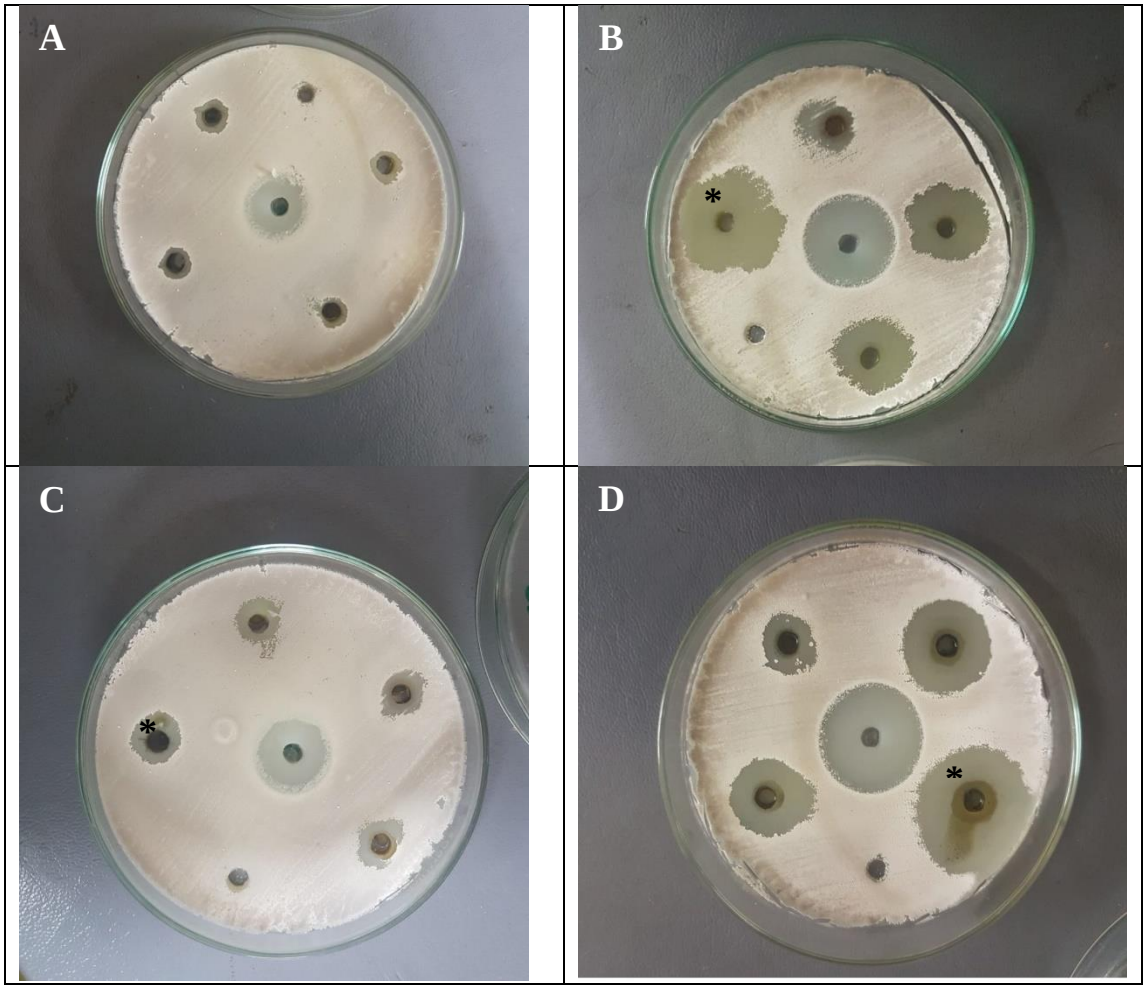


Figure 11: Protein kinase inhibition activity of selected plant extracts using Streptomyces strain (* represent clear zone). (A) *C. ambrosioides,* (B) *A. integrifolia,* (C) *R. hastatus,* (D) *H. nepalensis.*

Table 4: Mean ± SEM of diameter of zones in Protein kinase inhibition assay of selected plant extracts.

Plant extract	Diameter of PKI zones ± SEM (mm)			
	5mg	**4mg**	**3mg**	**2mg**
C. ambrosioides	7.86±0.08	7.56±0.14	7.43±0.08	-
A. integrifolia	26.06±0.14	21.53±0.12	20.1±0.34	14.7±0.31
R. hastatus	14.73±0.31	13.93±0.31	11.76±0.26	11.1±0.56
H. nepalensis	27.73±0.24	22.16±0.37	20.76±0.43	14.4±0.26
Streptomycin sulphate	20.76±0.17			

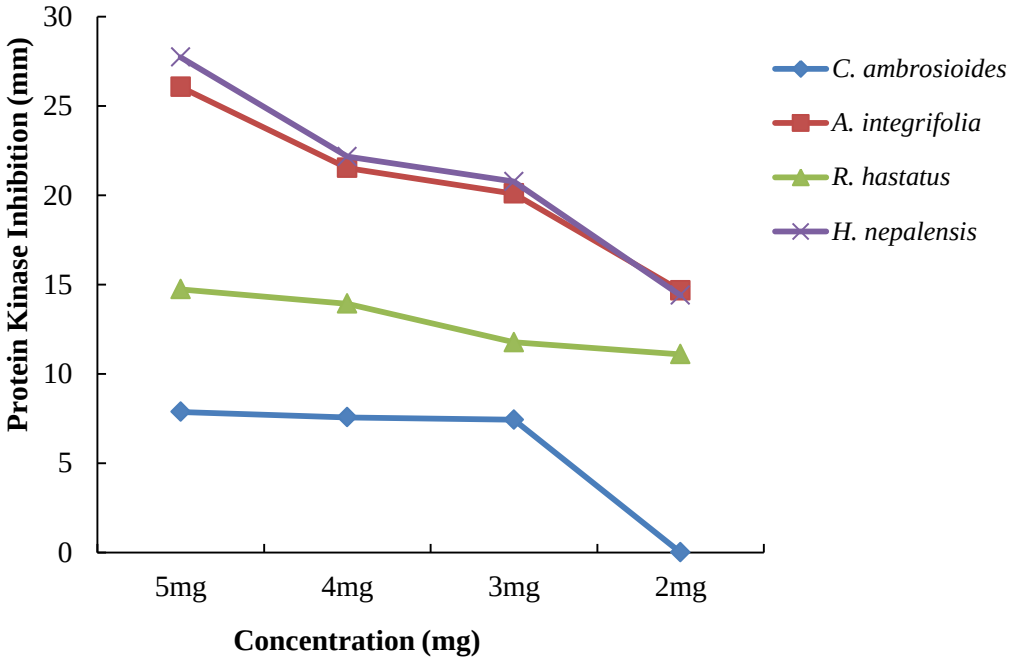


Figure 12: Protein kinase (PK) inhibition during *Streptomyces* hyphae formation of selected plant extracts.

Cytotoxicity assay

Brine shrimp lethality assay

The findings of brine shrimp lethality bioassay of methanolic extract of four selected plants are given in table 5. A dose dependent activity of all the plant extracts was seen with maximum mortality of shrimp's larvae at 1000 µg/ml dose. Experimental findings showed maximum percent mortality of 50, 54, 60 and 74 % at 1000 µg/ml with methanolic leaf extract of *R. hastatus, A. integrifolia, C. ambrosioides* and *H. nepalensis* respectively (Table 5, Figure 13).

Table 5: % mortality and number of survived shrimp nauplii after exposure with selected plant extracts.

Plant Extract	Dose (µg/ml)	No. of Shrimps	No. of Survivors	% Mortality
C. ambrosioides	10	30	30	0
	100	30	24	20
	1000	30	12	60
A. integrifolia	10	30	29	04
	100	30	26	14
	1000	30	14	54
R. hastatus	10	30	30	0
	100	30	22	27
	1000	30	15	50
H. nepalensis	10	30	26	14
	100	30	20	34
	1000	30	08	74

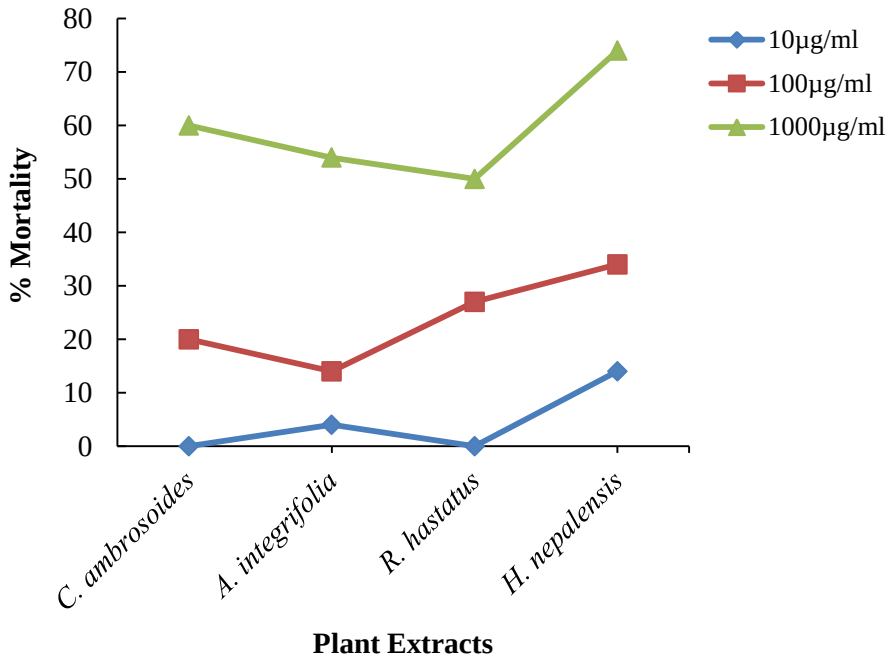


Figure 13: Percentage mortality (%) of Brine shrimp nauplii after treatment with various doses of methanolic leaf extracts of *C. ambrosioides, A. integrifolia, R. hastatus* and *H. nepalensis.*

In vitro phytotoxic bioassay

To evaluate the phytotoxic potential of methanolic leaf extract of selected plants, *Lemna minor* bioassay was performed. A dose dependent phytotoxicity was observed by all the tested plant extracts as low (% inhibition≤40%), moderate (% inhibition= 40-50%) and high (% inhibition≥50%) activities. At concentrations 10 and100 µg/ml, low phytotoxic activity was seen in all the tested samples. While moderate activity was observed by *C. ambrosioides* (45%), *A. integrifolia* (38%) and *R. hastatus* (49%) at highest concentration (1000 µg/ml). *H. nepalensis* showed highest % inhibition (51%) towards *Lamna minor* at 1000 µg/ml (Table 6, Figure 14).

Table 6: Phytotoxicity of four selected plants against *Lamna minor*.

Plant Extract	Dose (µg/ml)	No. of Fronds		% Growth Regulation/Inhibition
		Sample	Control	
C. ambrosioides	10	45	45	0
	100	38		15
	1000	25		45
A. integrifolia	10	40	45	11
	100	39		13
	1000	28		38
R. hastatus	10	45	45	0
	100	32		29
	1000	23		49
H. nepalensis	10	42	45	07
	100	35		22
	1000	22		51

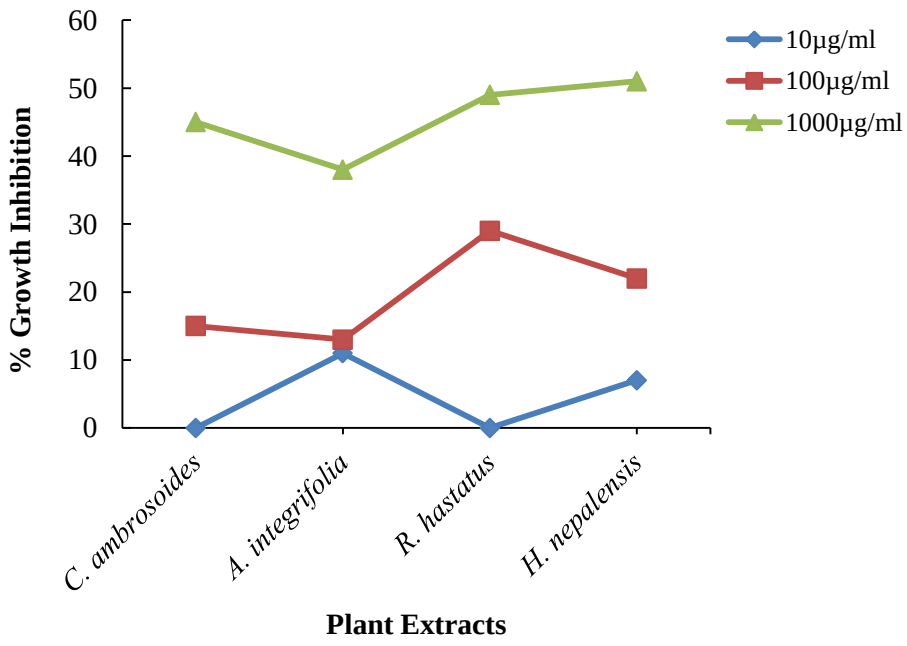


Figure 14: Percentage growth inhibition (%) of *Lemna minor* after treatment with various doses of methanolic leaf extracts of *C. ambrosioides, A. integrifolia, R. hastatus* and *H. nepalensis.*

Gas chromatography-Mass spectrometry (GC-MS) analysis

The details of different compounds identified by GC-MS analysis of methanolic leaf extract of *C. ambrosioides* are given in table 7 and GC-MS chromatogram is given in figure 15. These compounds are of different nature including fatty acid methyl ester, amino acids and phytol. The most abundant compounds found in present study were Arginine with percent area of 9.19% followed by Benzeneacetic acid,2,5-dihydroxy (8.02%), 9-Eicosene (5.91%), 4-Nonenoic acid (4.54%), 1-Heptadecene (3.29%), 1-Tridecene (3.24%) and Tetradecanoic acid (2.48%) along with minor constituents.

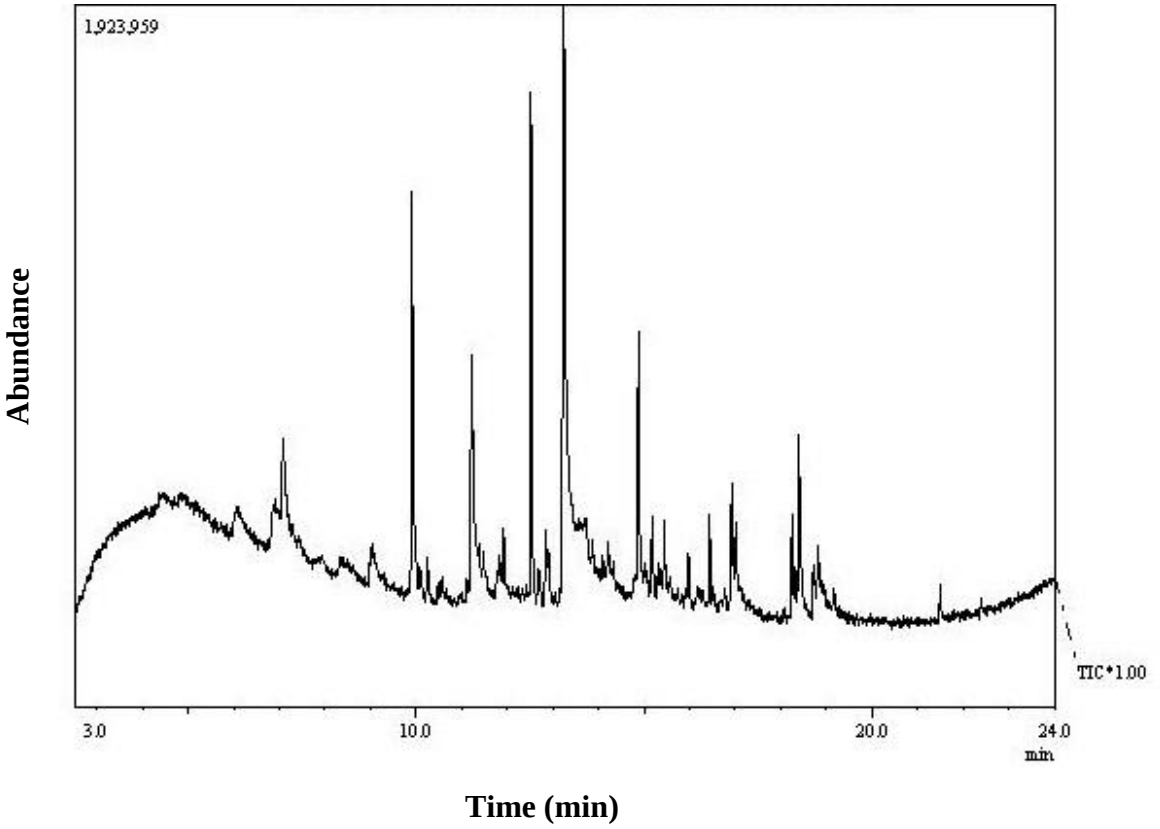


Figure 15. GC/MS chromatogram of methanolic leaf extract of *C. ambrosioides.*

Table 7: Compounds identified from methanolic leaf extract of *C. abroisoides* through GC-MS analysis.

Retention Time (min)	Name of Compound	Molecular formula	Molecular weight	% Area	% Height	Cas #
7.083	Arginine	C6H14N4O2	174	9.19	5.45	74-79-3
9.908	1-Tridecene	C13H26	182	3.24	7.31	2437-56-1
11.217	4-Nonenoic acid	C10H18O2	170	4.54	8.09	20731-19-5
12.517	1-Heptadecene	C17H34	238	3.29	12.62	6765-39-5
13.233	Benzeneacetic acid,2,5-dihydroxy	C8H8O4	168	8.02	17.81	451-13-8
14.875	9-Eicosene	C20H40	280	5.91	7.12	74685-29-23
15.183	Pterin-6-carboxylic acid	C7H5N5O3	207	-	-	948-60-7
15.450	Hexadecanal	C16H32O	240	-	-	629-80-1
15.975	Pterin-6-carboxylic acid	C7H5N5O3	207	0.29	0.21	948-60-7
16.433	Hexadecanoic acid, methyl ester	C17H34O2	270	0.68	2.66	112-39-0
16.925	Tetradecanoic acid	C14H28O2	228	2.48	3.67	544-63-8
18.250	11-Octadecenoic acid, methyl ester	C19H36O2	296	2.19	2.14	56554-45-1
18.400	Phytol	C20H40O	296	1.95	1.72	150-86-7

The GC-MS analysis of the methanolic leaf extract of *A. integrifolia* showed the presence of eleven phytocomponents presented in table 8 and the GC-MS chromatogram is also given in figure 16. Major constituents were Furan,2,3-dihydro-4-(1-methylethyl) (13.77%), 9,12-Octadecadienoic acid, methyl ester (9.96%), 2H-Pyran-3-ol, tetrhydro-3-methyl-6-propyl-,acetate (5.46%), 1-Pentacecene (4.25%), Phytol (4.14%), 7-Oxabicyclo[4,1,0]heptane, 1-methyl-4-(2-methyloxiranyl (4.04%), 1-(+)-Ascorbic acid2,6-dihexadecanoate (3.43%) and other minor constituents were reported.

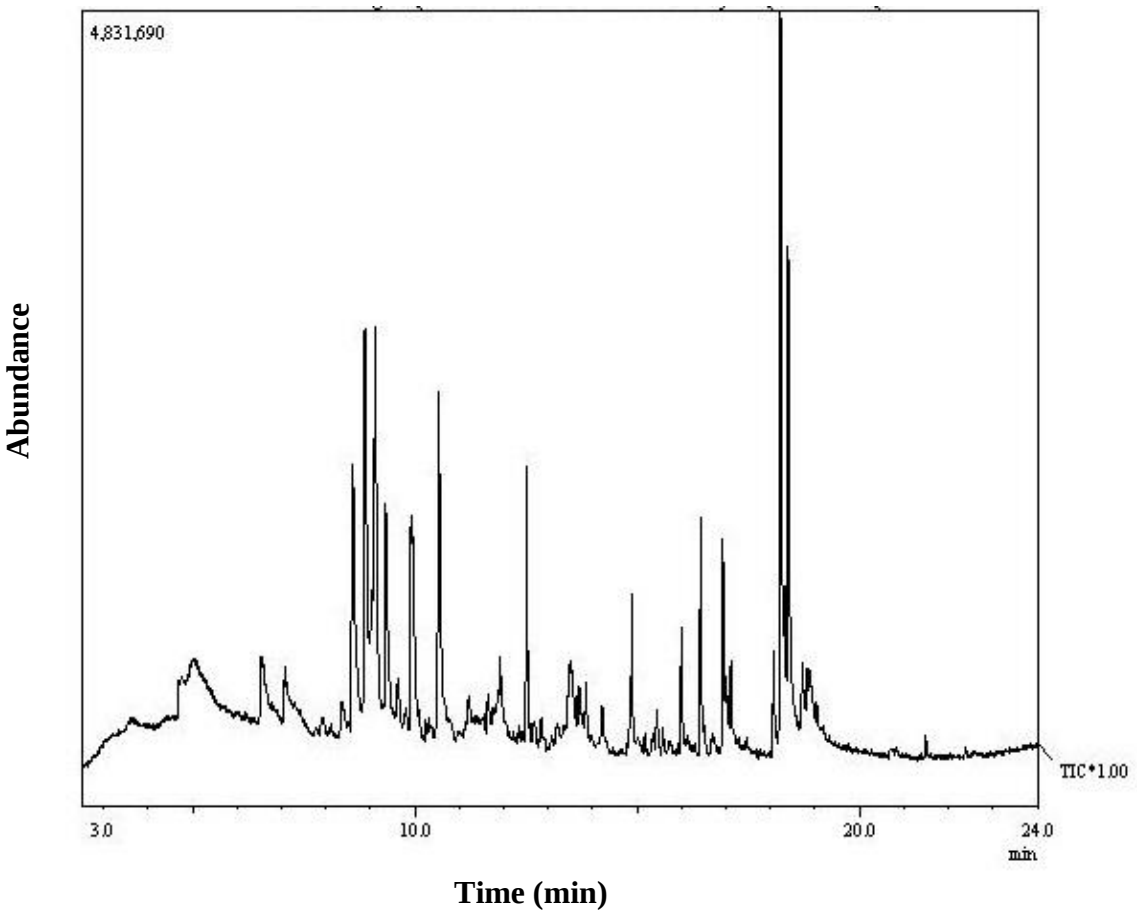


Figure 16. GC/MS chromatogram of methanolic leaf extract of *A. integrifolia*

Table 8: Compounds identified from methanolic leaf extract of *A. integrifolia* through GC-MS analysis.

Retention Time (min)	Name of compound	Molecular formula	Molecular weight	% Area	% Height	Cas #
8.608	Cyclohexanol,3,3,5-trimethyl (3,3,5-trimethylcyclohexan-1-ol)	C9H18O	142	1.21	1.78	116-2-9
9.108	7-Oxabicyclo[4,1,0]heptane, 1-methyl-4-(2-methyloxiranyl)	C10H16O2	168	4.04	8.13	96-8-2
9.350	Furan,2,3-dihydro-4-(1-methylethyl)	C7H12O	112	13.77	10.43	34314-84-6
9.917	1-Pentacecene	C15H30	210	4.25	7.00	13360-61-7
10.533	2H-Pyran-3-ol, tetrhydro-3-methyl-6-propyl-,acetate	C11H20O3	200	5.46	8.24	34647-45-5
12.517	1-Heptadecene	C17H34	238	2.31	0.84	6765-39-5
14.875	9-Eicosene	C20H40	280	1.60	3.36	74685-29-3
16.433	Hexadecanoic acid, methyl ester	C17H34O2	270	1.42	5.18	112-39-0
16.933	1-(+)-Ascorbic acid2,6-dihexadecanoate	C38H68O8	652	3.43	4.76	28474-90-0
18.225	9,12-Octadecadienoic acid, methyl ester	C19H34O2	294	9.96	18.69	2462-85-3
18.392	Phytol	C20H40O	296	4.14	2.66	150-86-7

Fifteen compounds were identified by GC-MS analysis of methanolic leaf extract of *R. hastatus*. These compounds included 1-Tetradecanol (13.71%), Acetic acid, trifluoro-, tetradecyl ester (9.59%), 1-Pentadecene (8.88%),7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl) (6.28%),1-Heptadecane (6.25%), Naphthalene (6.01%), 1-Hexadecanol, 2-methyl- (3.51%) and other minor constituents. The details of compounds are presented in table 9. While GC-MS chromatogram is given in figure 17.

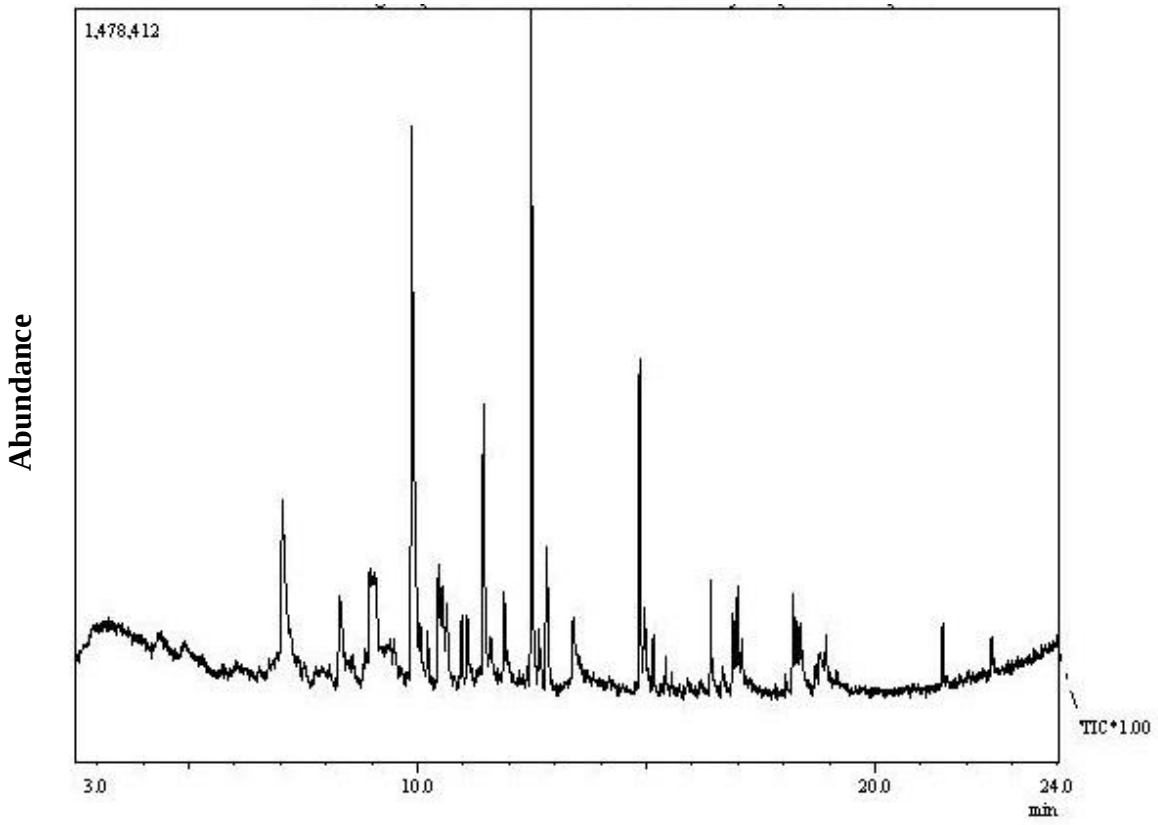


Figure 5. GC/MS chromatogram of methanolic leaf extract of *R. hastatus*.

Table 9: Compounds identified from methanolic leaf extract of *R. hastatus* through GC-MS analysis.

Retention time (min)	Name of Compound	Molecular Formula	Molecular Weight	% Area	% Height	Cas #
7.050	Acetic acid, trifluoro-, tetradecyl ester	C16H29F3O2	310	9.59	5.77	6222-2-2
9.875	1-Tetradecanol	C14H30O	214	13.71	17.72	112-72-1
10.467	Naphthalene, decahydro-1,4-dimethoxy-	C12H22O2	198	6.01	3.89	42068-24-6
10.967	Decanoic acid, 3-methyl-	C11H22O2	186	2.51	2.42	60308-82-9
11.083	7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl)	C10H16O2	168	6.28	7.46	96-8-2
11.650	(Z)-4-Chloro-2,3-dimethyl-1,3-hexadiene	C8H13Cl	144	2.53	2.52	105977-13-7
12.492	1-Pentadecene	C15H30	210	8.88	15.67	13360-61-7
14.858	1-Heptadecane	C17H34	238	6.25	10.13	6765-39-5
16.417	Pentadecanoic acid, 14-methyl-, ester	C17H34O2	270	0.34	0.22	5129-60-2
16.900	Undecanoic acid	C11H22O2	186	0.57	0.28	112-37-8
16.992	1-Hexadecanol, 2-methyl-	C17H36O	256	3.51	2.21	2490-48-4
18.217	Oleic acid	C18H34O2	282	1.73	2.85	112-80-1
18.308	6-Hexedecen-4-yne	C16H28	220	2.66	2.34	74744-52-8
18.375	3,4-Dimethylcyclohexanol	C8H160	128	2.57	1.02	5715-23-1
21.492	Pterin-6-carboxylic acid	C7H5N5O3	207	0.83	1.89	948-60-7

GC-MS analysis of methanolic leaf extract of *H. nepalensis* identified only three compounds including Hexanal (11.80%), Glycidol (9.74%) and 1-Methyldecylamine (1.67%). Name of compounds, retention time, molecular formula, % area, % height and cas # is given in table 10 while GC-MS chromatogram is presented in figure 18.

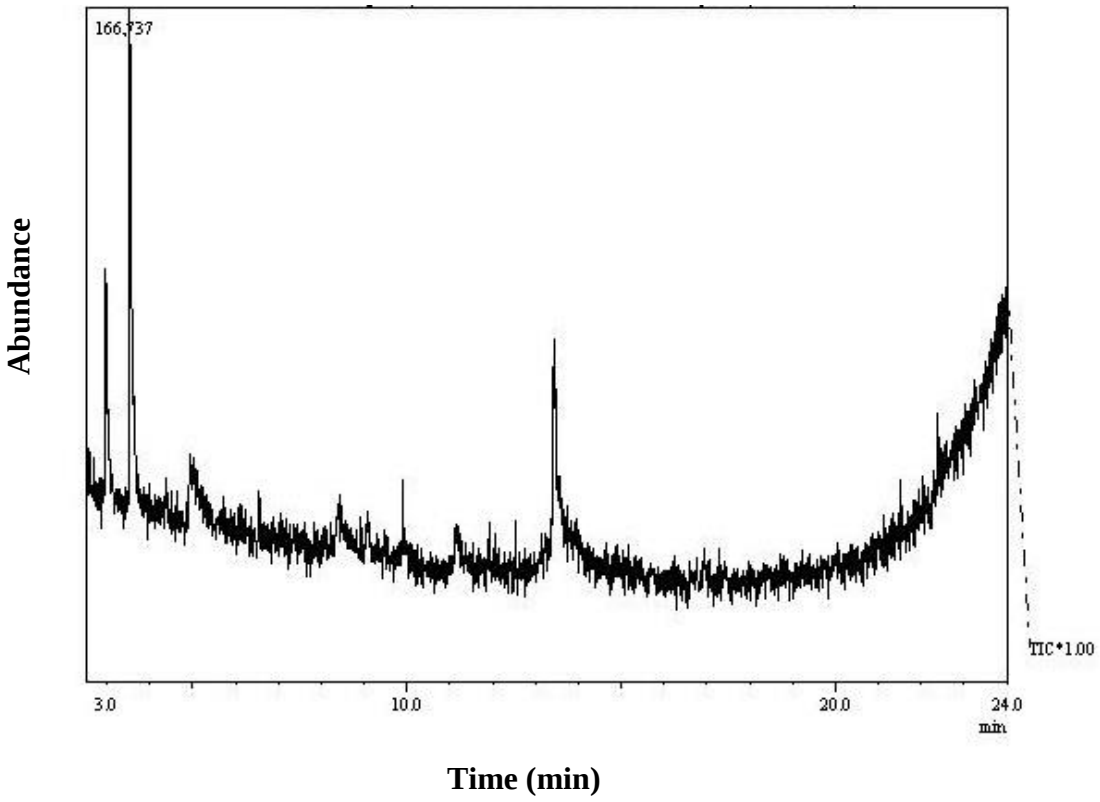


Figure 18. GC/MS chromatogram of methanolic leaf extract of *H. nepalensis.*

Table 10: Compounds identified from methanolic leaf extract of *H. nepalensis* through GC-MS analysis.

Retention time (min)	Name of Compound	Molecular formula	Molecular weight	% Area	% Height	Cas #
2.967	Glycidol	C3H6O2	74	9.74	9.72	556-52-5
9.9172	1-Methyldecylamine	C11H25N	171	1.67	3.27	13205-56-6
13.425	Hexanal	C6H12O	100	11.80	8.82	66-25-1

DISCUSSION

A total four selected plants were used in current study and their methanolic leaf extracts were analyzed to evaluate phytochemical fingerprinting, cytotoxic and antioxidant potential. Flavonoids and phenols are strongly associated with antioxidant activity of biological system and play important role to stabilize lipid oxidation (Rahman *et al*., 2017). In present study, highest total flavonoid contents in term of quercetin equivalent and total phenolic contents in term of gallic acid were found in methanolic extract of *R. hastatus* i.e. 242 µg QE/mg of extract and 308 µg GAE/mg extract respectively. Flavonoids and phenols are strongly associated with antioxidant activity of biological system and play important role to stabilize lipid oxidation (Rahman *et al*., 2017). Previously, high contents of polyphenols and flavonoid have been reported in various fractions of *R. hastatus* (Sahreen *et al*., 2011) exhibiting high antioxidant potential.

It is well known that oxidative stress induced by ROS and RNS disturb cellular homeostasis, dysregulates signaling network, damage proteins, nucleic acids and lipids leading to genomic instability (Weyemi and Dupuy, 2012). Excessive accumulation of ROS/RNS, lead to various diseases including cardiovascular diseases, neurodegeneration, hypertension, diabetes, atherosclerosis and rheumatoid arthritis (Phaniendra *et al*., 2015). Determination of free radical DPPH scavenging activity is simple method for the assessment of antioxidant potential. This process is relied on reduction of purple colored DPPH into yellow colored 2,2-diphenyl-1-picryl-hydrazyl by accepting electron from antioxidants. This change in color can be measured by recording absorbance. Another parameter for determination of free radical scavenging potential is IC_{50} value. Low IC_{50} specifies substantial free radical scavenging activities (Rahman *et al*., 2017). In our study, among all the selected plant extracts, *R. hastatus* exhibited highest total antioxidant capacity with 309 µg/mg followed by *A. integrifolia (*302 µg/mg), *C. ambrosoides* (291 µg/mg) *and H. nepalensis* (277 µg/mg). Previously, *H. nepalensis* exhibited antioxidant activity by scavenging H_2O_2 and reducing it into water (Jafri *et al*., 2017).

While lowest IC_{50} value was shown by methanolic leaf extract of *A. integrifolia* with highest free radical scavenging activity with inhibitory concentration (IC_{50} value) of 17 µg/mL followed by *C. ambrosioides* (56 µg/mL), *R. hastatus* (67 µg/mL) and *H.*

nepalensis (71 µg/mL). In present study, positive correlation between TFC, TPC and radical scavenging activity is related to electron donating ability of phenols.

Protein kinase inhibition activity of selected plants extracts was determined by inhibition of hyphae formation by using Streptomyces strain. In current study, methanolic extract of *H. nepalensis* showed high protein kinase inhibition against Streptomyces growth with 27.6 mm zone of inhibition/cytotoxicity at highest concentration followed by *A. integrifolia* (20.7 mm), *R. hastatus* (14.7 mm) and *C. ambrosioides* (7.6 mm) indicating their cytotoxic potential. As phosphorylation is necessary for the development and growth of Streptomyces hyphae, so this parameter was used in current study to evaluate the extracts for their protein kinase inhibiton activity to assess their cytotoxicity potential.

The brine shrimp lethality assay (BSLA) is a simple, rapid and inexpensive assay to check activity of plant extracts. Although, mechanism of action of active components in plant extract cannot be determined by BSLA but it can provide pilot screening for more specific cytotoxic bioassays (Ogbole *et al*., 2017). In current study, methanolic extracts of four selected plants were examined for cytotoxicity while using Brine shrimps lethality assay. A significant response of shrimp larvae towards different concentration of test sample (0, 10, 100 & 1000 µg/ml) was observed with maximum mortality at highest concentration. The experimental findings are supported by previous studies in which significant cytotoxic effect of methanolic crude extract of *Ajuga parviflora* was observed with percent mortality of 80% at 1000 µg/ml with (Rahman *et al*., 2013). Similarly, Ahmed *et al*., 2012 tested various fractions of *H. neplensis* by BSLA and reported that crude methanolic extract showed 50% cytotoxicity at 1000 µg/ml (Ahmad *et al*., 2012). The significant mortality of shrimp's larvae after exposure to tested plant extracts revealed that these plants may have cytotoxic components that should be isolated and investigated to support their traditional use.

Lamna minor bioassay was performed to find out phytotoxic potential of methanolic leaf extract of four selected plants. *Lemna minor* is common duckweed and aquatic freshwater monocot plant. Because of its sensitivity towards bioactive compounds, it is used as model for detection of phytotoxic components of tested plant (del Carmen Flores-Miranda *et al*., 2015). Results of current study exhibited low activity by all the tested plant extracts at concentrations of 10 and 100 µg/ml. At concentration of 1000

µg/ml, moderate activity by *C. ambrosioides*, *A. integrifolia* and *R. hastatus* while significantly high % inhibition by *H. nepalensis* was observed. Previously, Ullah *et al*., 2012 evaluated the toxic potential of the crude methanolic extract of *Calendula arvensis* (Ullah *et al*., 2012). Dose depent phytotoxicity was reported with low activity at 10 & 100 µg/ml dose while moderate activity at 1000 µg/ml concentrations. In another study, phytotoxic potential of *R. nepalensis*, *R. dentatus*, *P. persicaria*, *R. hastatus* and *P. plebejum* against *Lamna minor* was studied. Previous results revealed moderate % inhibition by *R. nepalensis*, *R. austral* and *P. persicaria* at 100 µg/ml concentration (Hussain *et al*., 2010) and provide support to our findings.

In present study, phytochemical components of methanolic leaf extract of four different plants namely *C. ambosioides*, *A. integrifolia*, *R. hastatus* and *H. nepalensis* were analyzed by GC-MS. Some identified compounds present in methanolic leaf extract of *C. ambrosioides* include many saturated fatty acids, lipids, amino acids and phytol. Most of the compounds are known for anticancer, antioxidant, antimicrobial and anti-inflammatory activities (Pandey and Gupta, 2014). Phytols have cholesterol lowering activity and used as a precursor for manufacturing synthetic form of vitamin E and K (Gabay *et al*., 2010).

In current study, methanolic leaf extract of *A. integrifolia* revealed eleven phytoconstituents when analyzed by GC-MS. Most of the components are used in food as flavoring agent. As reported earlier, 3,3,5-trimethylcyclohexan-1-can is used in food to add flavor but its excessive amount lead to kidney, liver and lung impairment (Pohanish, 2017). In methanolic leaf extract of *R. hastatus*, fifteen compounds were identified including carboxylic acids, straight-chain saturated and unsaturated fatty acids. 1-Tetradecanol is a major component used in cosmetics, soaps, ointments and as surfactant (Lang *et al*., 2002). Acetic Acid is another component identified in methanolic leaf extract of *R. hastatus*. It is well known for its antifungal and antibacterial properties (Nyeem *et al*., 2006).

GC-MS analysis of methanolic leaf extract of *H. nepalensis* identified only three compounds including Hexanal, Glycidol and 1-Methyldecylamine. Hexanal is a breakdown product of linoleic acid naturally present in human biofluids. Linoleic acid is essential for normal growth of mammary tissue but excessive accumulation is associated with gestational diabetes (Min *et al*., 2004) Glycidol, another major

component of methanolic extract of *H. nepalensis*, is known to be carcinogenic and involved in induction of neoplasms in humans or animals (El Ramy *et al*., 2007).

Conclusion

Reviewing the available literature, no clear data was found related to qualitative and quantitative analysis of methanolic leaf extracts of *C. ambrosioides, A. integrifolia, R. hastatus* and *H. nepalensis*. The findings of current study revealed that methanolic leaf extract of *R. hastatus* possess high flavonoids and phenolic contents with highest antioxidant potential followed by *A. integrifolia*, *C. ambosioides* and *H. nepalensis*. Moreover, methanolic extract of *H. nepalensis* is quite effective against brine shrimp and *Lemna minor* revealing its cytotoxic potential. These reported plant extracts possess variety of components, as identified by GC-MS analysis, which could be used as novel scaffolds in contraceptive drug discovery research. This study provides basis and direction for the further pharmacological investigations of these plants.

CHAPTER 2:

In vitro assessment of different concentrations of selected plant extracts on antioxidant status and DNA integrity.

ABSTRACT

Back ground: The use of medicinal plants and herbs for fertility regulation has been prevalent worldwide for many centuries. They possess natural substances having anti-androgenic properties and can be used as source of contraception. For the screening of plant based antifertility agents, in vitro approach can be practiced as it is simple, time saving and inexpensive.

Materials and methods: In this study, *in vitro* experimental approach was used in which testis and sperm of adult male rats were incubated with media having different concentrations (0, 1, 10, 100 & 1000 µg/mL) of plant extract at 37 ℃ in 5% CO_2 for 2 hours. After incubation, direct effect of selected plants extracts on testicular antioxidant status and testosterone secretion, as well as on sperm DNA integrity was evaluated.

Results: Significantly increased oxidative stress with reduced antioxidant activity was observed in highest dose regimen (1000 µg/mL) of methanolic leaf extract of *C. ambrosioides*, *A. integrifolia* and *H. nepalensis.* However, in vitro exposure to *R. hastatus* caused slight increase in ROS production at high dose without significantly affecting antioxidant enzymes status. Increased ROS generation and lipid peroxidation lead to DNA damage in rat sperm. Similarly, decline in testicular testosterone level was noticed after two hours incubation with all the doses of *C. ambrosioides* and *A. integrifolia* leaf extract, however, *R. hastatus* and *H. nepalensis* showed reduction only at higher doses regimens (100 and 1000 µg/mL).

Conclusion: The findings of current study recommend that aforementioned plants have the potential to disturb male fertility by inducing oxidative stress and hormonal imbalance in rat testis and disrupting sperm DNA integrity when exposed to higher concentrations of extracts.

INTRODUCTION

The use of plants and herbs for fertility regulation has been prevalent worldwide for many centuries. They possess natural substances having androgenic and anti-androgenic properties and can be used as source of contraception with fewer side effects. Many synthetic drugs are commercially available but herbal medicines are considered to be safer and more effective than their synthetic counterparts (Priya *et al*., 2012). Development of safe, effective and reversible male contraceptive with no effect on sexual activities or libido is challenging. This can be achieved either by interfering sperm production, affecting sperm morphology, sperm transportation or inhibiting interaction of sperm with egg (Mishra and Singh, 2009; SHARANGOUDA *et al*., 2010). Numerous indigenous plants are known for spermicidal activities hence used for induction of male antifertility (Sathiyaraj *et al*., 2010; Dubey *et al*., 2011).

For the screening of plant based antifertility agents, *in vitro* approach can be practiced as it is simple, time saving and inexpensive. Many scientists have conducted respective studies in this regard and reported their findings. An *in vitro* approach was used to test spermicidal activity of 160 different plant extracts. Results reported 30 plant extracts with spermicidal activity while 16 with sperm immobilization properties (Setty *et al*., 1977). In another study, 48 out of 72 plants traditionally used for contraceptive purpose, have revealed their antifertility potential (Maurya *et al*., 2004).

Lohiya *et al*., 2000 documented *in vitro* study in which *Carica papaya* seed extract arrested human sperm in a dose-dependent manner (Lohiya *et al*., 2000). In another study, aqueous seed extract of *Chenopodium Album* immobilized spermatozoa and disrupted sperm plasma membrane in rats and rabbits when exposed to varied concentrations of extract by using in vitro approach (Kumar *et al*., 2007). Similarly, various plant extracts including *Eurycoma longifolia* (Erasmus *et al*., 2012), *Rhynchosia volubilis Lour* (Guan *et al*., 2014), *Asplenium dalhousiae* (Radhika *et al*., 2010) and *Hibiscus macranthus* (Moundipa *et al*., 2005) have showed *in vitro* detrimental effects on spermatogenesis, DNA damage, testosterone production and anti-oxidant enzyme levels.

Antioxidant defense system is crucial for body to perform normal biological functions. Oxidative stress caused by formation of free radicals i.e. ROS and RNS damage biologically important molecules including proteins, lipids and DNA.

Antioxidants possess free radical scavenging and metal chelating properties, which are important to reduce oxidative stress (Aitken and Roman, 2008). Assessment of DNA damage caused by traditional medicinal plants is for sure an imperative issue as any change in hereditary material may lead to acute mutations thus increasing risk of malignancy and other illness (Demma *et al*., 2009). Numerous medicinal plants have been reported in literature with genotoxic potential (Sohni *et al*., 1994; Basaran *et al*., 1996; Romero-Jiménez *et al*., 2005).

Although little is known about toxicological profiles of selected plants, but the plant extracts used in the current study have long been used traditionally. *C. ambrosioides* have been used for many centuries as a dietary condiment and in folk medicine. Aqueous extract of *C. ambrosioides* have been known to negatively affect reproduction in Drosophila melanogaster (Wohlenberg and Lopes-da-Silva, 2009). *A. integrifolia* is the herb traditionally used as male contraceptive in different areas of Pakistan. It is found to have antiplasmodial as well as antimalarial activity (Asnake *et al*., 2015) *R. hastatus* is commonly known as "khatimal." Its leaves are good source of antioxidant because of presence of phenolic component (Sahreen *et al*., 2011). Seven phenolic compounds have been isolated from the roots of this plant (Zhang *et al*., 2009). *H. nepalensis* is locally called as Arbambal (Shah *et al*., 2009). This plant is traditionally used in folk medicines for the treatment of many diseases including diabetes (Frohne and Pfander, 2004), fever and rheumatism (Shah, 2006). Previously, inflorescence of *H. nepalensis* has been reported to halt male reproduction by immobilization of human spermatozoa (Pant *et al*., 1988).

Given the lack of literature and knowledge along with their extensive and widespread traditional use, the purpose of current study was to assess the effect of aforementioned plants on testicular antioxidant status and testosterone production as well as DNA integrity in rat spermatozoa by using *in vitro* experimental approach.

MATERIAL AND METHOD

Animals

Twenty four adult male Sprague Dawley rats (70 - 90 days old) were obtained from the primate facility of Animal Sciences Department, Quaid-i-Azam University. Animals were randomly kept in stainless steel cages in well ventilated zone at temperature of 26±1 ºC and 10 /14 h of dark and light cycles respectively. Animals were provided with standard laboratory feed and water was available in plastic bottles. All the experimentation and protocols were ratified by ethical committee of Animal Sciences department, QAU.

Plant collection and extract preparation

Fresh leaves of *C. ambrosioides*, *A. integrifolia*, *R. hastatus* and *H. nepalensis* were obtained from agricultural and cultivated fields of different areas of Pakistan. The botanical identification of plants was done by Dr. Mushtaq Ahmad at Plant Systematics and Biodiversity Lab and voucher specimen were deposited in the herbarium of Plant Sciences Department, QAU.

Methanolic extract of selected plants was used in this study. The detail of plant extract preparation is given in chapter 1. Leaves of selected plants were separated from stem, air-dried, ground in waring blender and then sieved. Leaf powder was soaked in 99.9% methanol (leaves to solvent ratio 1:10) for seven days to prepare methanolic extract. The extracts were filtered using Whattman filter paper and concentrated on a rotary evaporator according to the method described by Gulfraz *et al*., 2007 (Gulfraz *et al*., 2007). The filtrate mass was dried at room temperature and placed at 4ºC.

In vitro experiment

Twenty four adult male Sprague Dawley rats were used in this experiment. Experimental design was planned according to Moundipa *et al*. (2006) study with slight modifications (Moundipa *et al*., 2005). To best of my knowledge, there are no reported *in vitro* studies using methanolic extracts of these plants, so the range of doses were selected according to OECD protocols and previous *in vitro* studies using plant extracts as recommended by Srivastav *et al*. (2010) in their respective study (Srivastav *et al*., 2010). Five different doses (0, 1, 10, 100 and 1000 µg/mL) were selected for this study. Stock solution of plant extracts were prepared in methanol and

was further mixed with cell culture media. Methanol concentration was kept less than 0.5% in prepared media.

In vitro culturing of testis was executed as described earlier by Moundipa *et al*. (2006), Erasmus *et al*. (2012) and Srivastav *et al*. (2010) with little modifications (Moundipa *et al*., 2005; Srivastav *et al*., 2010; Erasmus *et al*., 2012). Rats were decapitated. Both testes were obtained and washed with cold physiological saline. After decapsulation, each testis was sliced into six fragments having almost equal. In autoclaved culture tubes, testicular slices were added along with 2 ml of culture media (DMEM/Ham F12 mixture medium 1:1 ratio, Gibco, USA), sodium bicarbonate (1.2 g/L) and antibiotics i.e. penicillin (50 IU/mL) and streptomycin (50 µg/mL). Subsequently, selected doses of plants extracts (0, 1, 10, 100, 1000 µg/mL of each plant extract) were added in each culture tube. Tubes were incubated for 2 hours at 37 ℃ in CO_2 incubator (with 5% CO_2 and 95% air (v/v)). After incubation, tissues were separated from media and washed with normal physiological saline. Tissues were homogenized in 1 mL of PBS (pH= 7.4), centrifugation was done at 4 ℃ at 30,000 rpm for 30 min. Supernatant was separated and stored at -80 ℃ until further analysis of biochemical and hormonal assays.

For the *in vitro* culturing of sperm, epididymis was separated immediately after dissection and washed with saline. With the help of sharp scissor, cauda part was crushed and homogenized in 3 mL of a buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Tris base, 10% glycerol with pH=7.4). The homogenates were centrifuged for 10 minutes at 4 ºC and supernatant was removed. Ham's F12 media (Sigma-Aldrich, Taufkirchen, Germany) alng with bovine serum albumen (Sigma-Aldrich) was added in sperm pellets. Culture tubes, containing 2 ml of sperm suspension and 2 ml of media with 0, 1, 10, 100 & 1000 µg/mL of each plant extract, were incubated in CO_2 incubator for 2 hours. Temperature was maintained at 37 ºC and 5% CO_2 was set. After 2 hours of incubation, centrifugation was done for 15 minutes at 1000 rpm and supernatant was removed leaving behind sperm pallet. The sperm pellets were diluted with 1 mL of phosphate buffer saline and used for comet assay.

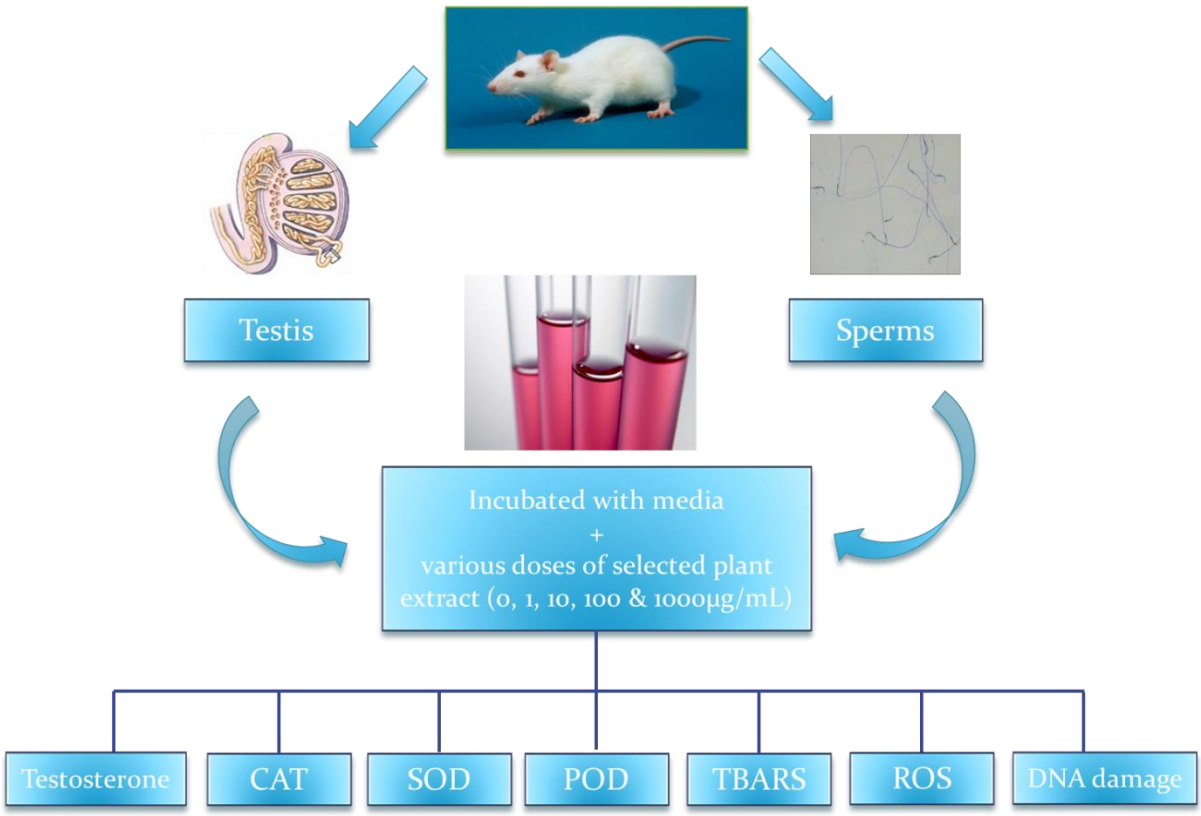


Figure 19: Schematic representation of experimental design of *in vitro* exposure of rat's testis and epididymal sperm to selected plant extracts.

Antioxidant enzyme status

Following centrifugation, supernatant of testicular tissues was used for determination of antioxidant enzymes status.

Superoxidase dismutase assay (SOD)

Superoxidase dismutase activity was evaluated by previously reported procedure by Kakkar *et al.* (1984) (Kakkar *et al*., 1984).

Procedure

A reaction mixture was prepared by adding about 0.1 ml of phenazinemethosulphate (186 uM), 1.2 ml of sodium pyrophosphate buffer (0.052 mM, pH 7.0) and 0.3ml of homogenate. Reaction was started by addition of 0.2 ml NADH (780 uM), and stopped after 60 sec by the addition of 1 ml of glacial acetic acid. Change in absorbance of reaction mixture was related as to change in colour intensity of chromogen formed. Absorbance was noted at 560 nm wavelength by using by spectrophotometer. Value was given in units/mg protein.

Peroxidase assay (POD)

Peroxidase (POD) activity was assessed by previously recommended procedures of Chance and Maehly, (1955) in their respective studies (Chance and Maehly, 1955).

Procedure

Reaction mixture was prepared in cuvette containing 1000µl of homogenate, 2.5 ml of phosphate buffer (50 mM, pH= 5.0), 0.1 ml of guaiacol (20 mM) and 0.3 ml of H_2O_2 (40 mM). After one minute, absorbance was recorded at 470 nm. Change in absorbance of 0.01 units/minute was considered as one unit of POD activity. The value of POD was expressed in mU/mg protein.

Catalase assay (CAT):

Catalase activity was assessed by already reported method of Chance and Maehly (1955) with some amendments (Chance and Maehly, 1955).

Procedure

In the cuvette, reaction mixture was made by adding 2.5 ml of phosphate buffer (50mM, pH 5.0), supernatant (0.1 ml) and 0.4 ml of H_2O_2 (5.9 mM). After one

minute, absorbance of reaction mixture was recorded at 240 nm by using spectrophotometer. Absorbance change of 0.01 unit/minute was called one unit of CAT activity.

Estimation of lipid peroxidation assay (TBARS):

Lipid peroxidation in testicular homogenate was estimated by using procedure given by Wright *et al.,* (1981) as adapted by Iqbal *et al.* (1996) (Wright *et al*., 1981; Iqbal *et al*., 1996).

Procedure

In the test tube, 0.58 ml phosphate buffer (0.1M pH 7.4) was dispensed along with 0.2 ml ascorbic acid (100mM) and 0.02 ml ferric chloride (100mM). After that 0.2 ml homogenate was incorporated in the mixture and incubated in water bath at 37 ºC for 1 hour. Following incubation, reaction was stopped by addition of 1.0 ml of trichloroacetic acid (10%). After that 1.0 ml of thiobarbituric acid (0.67%) was dispensed in all the tubes and again incubated in boiling water for about 30 min before moving to crushed ice. After that, samples were centrifuged at 2500 × g for 10 min. Absorbance of mixture was recorded by using spectrophotometer at the wavelength of 535 nm. The values were given as nM TBARS/min/mg tissue using molar extinction coefficient of 1.56×10^5 /M cm.

Estimation of Reactive Oxygen Species (ROS)

Estimation of reactive oxygen species (ROS) was carried out in testicular tissue by using procedure reported previously by Hayashi *et al.* (2007).

Procedure

Standards of H_2O_2 (30 % w/w, Sigma Aldrich) were prepared by dilutions and 5 µL of standard and homogenate were dispensed in 96 well plate along with 140 µL of sodium acetate buffer (0.1 M, pH= 4.8) and incubated at room temperature for 5 min. A solution of N, N-diethyl-para-phenylenediamine (DEPPD) and ferrous sulphate (1:25) was prepared and incubated in dark for 20 min. 100 µL of this solution was dispensed in well and mixed thoroughly. Absorbance was recorded for 90 s with 15 s interval by using microplate reader at 505 nm. One unit of ROS was equal to hydrogen peroxide concentration in the sample.

Estimation of DNA damage

DNA damage of each spermatozoa was determined by using a neutral single cell electrophoresis (SCGE / comet assay) using procedure described by Donnelly *et al.,* (1999) and Boe-hensen *et al.,* (2007) (Donnelly *et al*., 1999; Boe-Hansen *et al*., 2006). The comet assay (SCGE) is a simple approach used to reveal single and double strand DNA breaks and alkali-labile sites in DNA. Sperm samples were centrifuged at 1000 rpm for 15 min and pellets were diluted with phosphate buffered saline.

Slide Preparation

Frosted microscopic slides were coated with 100µL of 1% regular melting point agarose (RMPA) and instantly covered with large (22 x 50mm) coverslip. The slides were left at 4ºC until the solidification of agarose. After that, a mixture of 20 µl of sperm homogenate and 65µl of 1% low melting point agarose (LMPA) was prepared and spread over the first one. The slides were covered and let the agarose to solidify.

Lysis

In this step, slides were arranged in histology jars containing freshly prepared lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mMTris base, 1% (w/v) Triton X-100, pH =10.3) (Villani *et al*., 2012). After 24 hours of incubation at room temperature, slides were removed from the jars and washed with saline thrice with 20 min gap, so that any salt or chemical traces can be washed away

Neutral electrophoresis

In the electrophoresis tank, electrophoresis buffer (54 g/L Tris base, 0.5 M EDTA, 27.5 g/L boric acid, pH=8.0) was poured and slides were orderly arranged. Electrophoresis was conducted for 20 min at 25V (0.714 V/ cm). After the completion of procedure, slides were removed from the tank, air dried and covered in dark to avoid the light. Tank was emptied by draining the buffer and removing the tray. Slides were stored at 5°C overnight.

Analysis and scoring of the slides

Before analysing, the slides were rehydrated for about 60 min with distilled water and stained with acridine orange (200- 300 µl of 20µg/ml). Slides were examined under fluorescent microscope (40 X, AFX - 1 Optiphot, Nikon, Tokyo, Japan) and digital

photographs were taken for further analyses by using comet assay software Casplab, V. 1.2.3b2. The numbers of comets/100 spermatozoa were counted and head length (HL, µm), Tail length (TL, µm), head DNA (%), Tail DNA (%) and tail moment (µm) were noted.

Hormonal Analysis

Testosterone level was determined quantitatively in testicular homogenate by Enzyme Linked Immuno Sorbant Assay (ELISA) kits (Biocheck Inc, USA) by using the manufacturers instructions.

Principle of the Test

The principle of ELIZA was based on the competitive binding for a constant amount of rabbit anti-testosterone between testosterone present in the test sample and Testosterone-HRP (horse radish peroxidase) conjugate.

Procedure

For quantitative determination of testosterone concentrations in testicular homogenate, 10µl of standards, samples and controls were incubated in goat anti-rabbit IgG - antibody coated wells. Then 50µl of rabbit anti-testosterone was added, followed by the addition of 100µl testosterone-HRP conjugate in each well and mixed well for 30 seconds with slight shaking. After that the wells were incubated at 37°C for 90 minutes. During this process of incubation, a fixed amount of HRP-labeled testosterone competes either with endogenous testosterone in the standard, specimen or quality-specific testosterone antibody. Followed by incubation, all the unbound testosterone peroxidase conjugate was removed by washing the wells 5 times with distilled water. After that, 100µl of TMB-reagent was added in all the wells and mixed for 5-10 seconds. The wells were again incubated about 20 minutes at 37 ºC. Subsequently, 100µl of stop solution of was dispensed in all the wells and thoroughly mixed. The absorbance was recorded at 450nm by using micro plate reader. The values were expressed in ng/ml.

Statistical analysis

Data was analyzed by one way analysis of variance (ANOVA) followed by Dunnet's multiple comparison test to compare different groups with control by using Graph Pad Prism 5 software. Level of significance was set at $p<0.05$.

RESULTS

Effect of *C. ambrosioides* on testicular antioxidant status and testosterone secretion

Biochemical and hormonal profile of testicular tissue of rat was determined after incubation for two hours with different concentrations of methanolic leaf extract of *C. ambrosioides*. A dose dependent reduction in catalase activity was seen from 4.46±0.60 in control to 1.25±0.32 U/mg protein in high dose treated group. The reduction was significant in 10 μg/mL ($p<0.01$) and 1000 μg/mL ($p<0.01$) dose treatment groups as compared to control (Table 11, Figure 20). Similarly, SOD and POD levels were reduced in dose dependent manner in treated groups, but reduction was not significant within groups as well as with control (Figure 21 & 22). A dose dependent increase in TBARS and ROS levels were recorded in all the treated groups in comparison with control (Figure 23 & 24). However, significant increase in ROS concentration was observed in 100 μg/mL ($p<0.01$) and 1000 μg/mL ($p<0.001$) dose treated groups as compared to control group. Highly significant decrease in testicular testosterone ($p<0.001$) in 10, 100 and 1000 μg/mL extract treated testis was observed as compared to control group (Table 11, Figure 25).

Table 11: *In vitro* effect of methanolic leaf extract of *C. ambrosioides* on testicular antioxidants, ROS and Testosterone secretion.

Parameters	Extract Treatment (µg/mL)				
	Control	**1**	**10**	**100**	**1000**
CAT (U/mg protein)	4.46±0.60	4.33±0.53	2.19±0.70*	2.76±0.55	1.25±0.32**
SOD (U/mg protein)	20.37±2.38	15.12±2.32	19.68±2.38	16.71±3.28	14.63±1.98
POD (nmole)	11.54±1.08	12.31±1.27	10.55±1.54	12.56±1.28	6.84±1.48
TBARS (nM/mg tissue)	19.32±1.81	18.68±1.72	20.67±3.07	22.01±3.04	25.37±2.92
ROS (U/g tissue)	13.51±1.15	14.09±1.79	22.05±3.41	29.39±4.87**	34.85±3.41***
Testosterone (ng/ml)	1.14±0.02	0.95±0.04*	0.82±0.01***	0.46±0.07***	0.36±0.04***

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test)

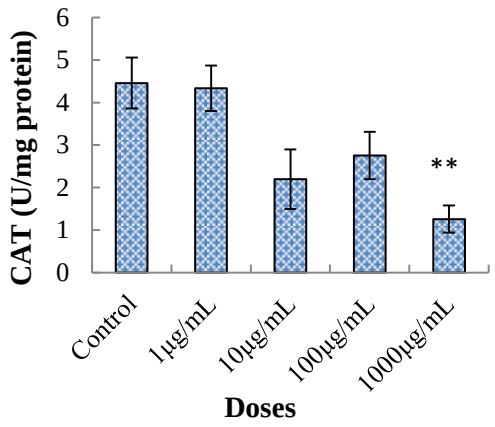


Figure 20: *In vitro* effect of methanolic leaf extract *C. ambrosioides* on testicular CAT activity in control and treated groups.

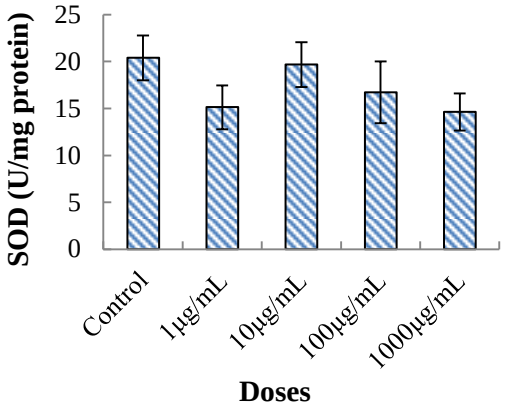


Figure 21: *In vitro* effect of methanolic leaf extract of *C. ambrosioides* on testicular SOD activity in control and treated groups.

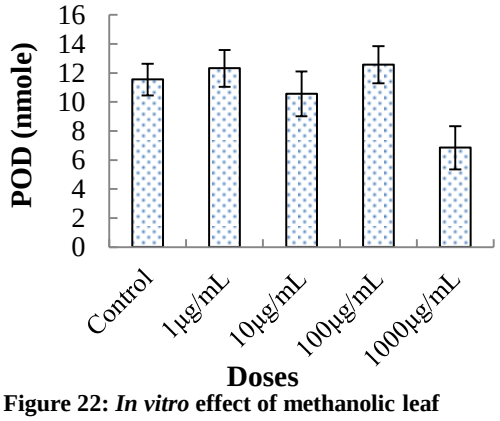


Figure 22: *In vitro* effect of methanolic leaf extract of *C. ambrosioides* on testicular POD activity in control and treated groups.

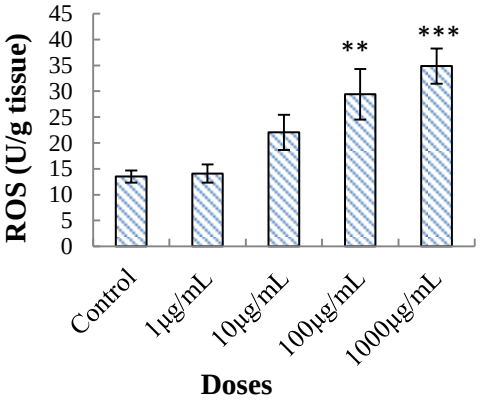


Figure 23: *In vitro* effect of methanolic leaf extract of *C. ambrosioides* on testicular ROS oxygen species in control and treated groups.

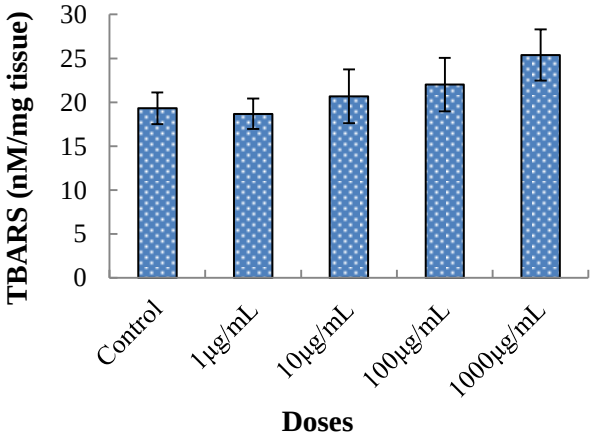


Figure 24: *In vitro* effect of methanolic leaf extract of *C. ambrosioides* on testicular TBARS in control and treated groups.

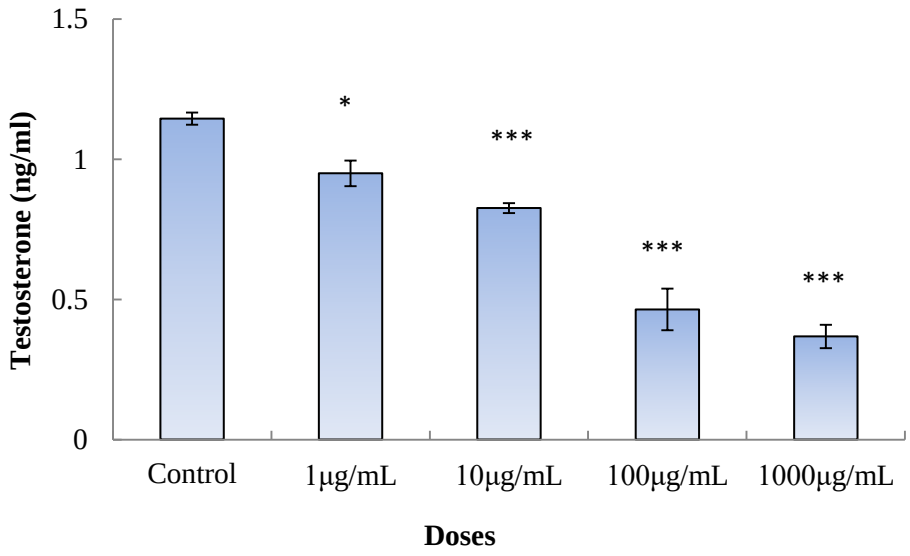


Figure 25: *In vitro* effect of methanolic leaf extract of *C. ambrosioides* on testicular Testosterone secretion in control and treated groups.

Effect of *A. integrifolia* on testicular antioxidant status and testosterone secretion

Antioxidant status and oxidative stress markers in rat testis were determined after incubation for two hours with different concentrations of methanolic extract of *A. integrifolia*. A dose dependent reduction in catalase activity was seen from 4.46±0.60 in control to 1.54±0.36 U/mg protein in high dose treated group. The reduction was significant in 1 μg/mL ($p<0.05$), 10 μg/mL ($p<0.01$) and 1000 μg/mL ($p<0.01$) dose treatment groups when compared with control group (Table 12, Figure 26). Similarly, SOD and POD levels were reduced dose dependently in all the treated groups, but reduction was significant ($p<0.05$) only in high dose treated testis when comparison was made with control group (Table 12, Figure 26). Increasing trend in levels of oxidative stress markers was observed in all testis incubated with different concentration of plant extract. Statistically significant increase ($p<0.001$) in TBARS level was noted in 100 and 1000 μg/mL extract treated testis when compared to control. Similarly, ROS concentration was increased significantly from 13.51±1.15 U/g tissue in control to 24.72±3.28 U/g tissue in 100 μg/mL ($p<0.05$) and 30.60±3.71 U/g tissue in 1000 μg/mL ($p<0.001$) (Figure 27). Other treated groups presented non significant increase in ROS levels when compared to control. Testosterone concentration was measured in testicular tissues incubated with different doses of plant extract for two hours, as presented in table 12, figure 28. Significant decrease in 10 ($p<0.01$), 100 and 1000 μg/mL ($p<0.001$) extract treated testis was observed as compared to the control group but the reduction in 1 μg/mL was non-significant.

Table 12: *In vitro* effect of methanolic leaf extract of *A. integrifolia* on testicular antioxidants, ROS and Testosterone secretion.

Parameters	Extract Treatment (µg/mL)				
	Control	1	10	100	1000
CAT (U/mg protein)	4.46±0.60	2.35±0.43*	1.75±0.26**	3.09±0.67	1.54±0.36**
SOD (U/mg protein)	20.37±2.38	14.70±1.60	21.64±1.62	17.00±1.80	12.30±1.67*
POD (nmole)	11.54±1.10	11.64±1.57	8.85±2.23	9.43±0.94	5.21±0.68*
TBARS (nM/mg tissue)	19.32±1.81	20.55±0.71	22.86±1.65	33.98±2.54***	37.28±3.53***
ROS (U/g tissue)	13.51±1.15	15.84±1.39	15.50±1.15	24.72±3.28*	30.60±3.71***
Testosterone (ng/ml)	1.15±0.02	1.09±0.03	0.89±0.07**	0.52±0.08***	0.51±0.03***

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

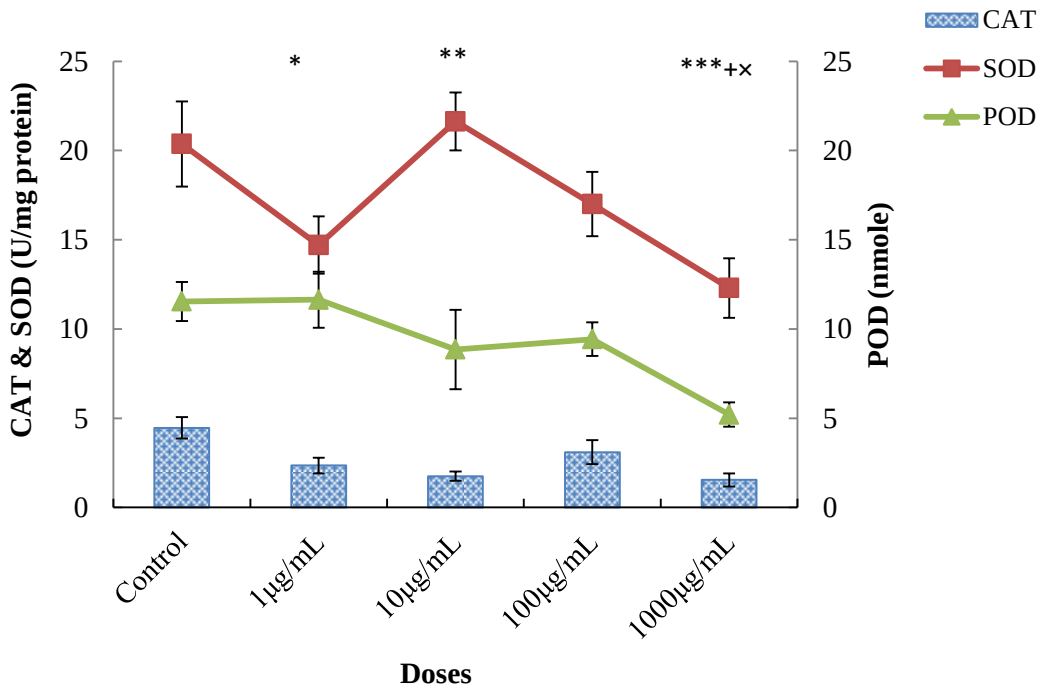


Figure 26: *In vitro* effect of methanolic leaf extract of *A. integrifolia* on testicular CAT, SOD and POD activity in control and treated groups.

*,**,***= indicates significant difference of CAT at probability of $p<0.05$, $p<0.01$ and $p<0.001$ compared to control.

+= indicates significant difference of SOD at probability of $p<0.05$ compared to control.

×= indicates significant difference of POD at probability of $p<0.05$ compared to control.

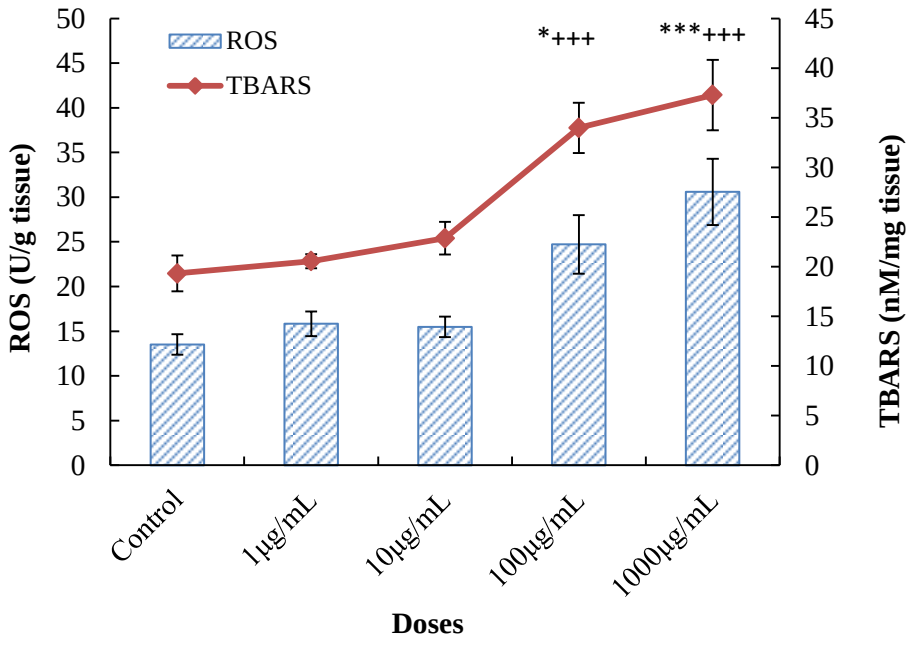


Figure 27: *In vitro* effect of methanolic leaf extract of *A. integrifolia* on testicular ROS and TBARS in control and treated groups.

*,***= indicates significant difference of ROS at probability of $p<0.05$ and $p<0.001$ compared to control.

+++= indicates significant difference of TBARS at probability of $p<0.001$ compared to control.

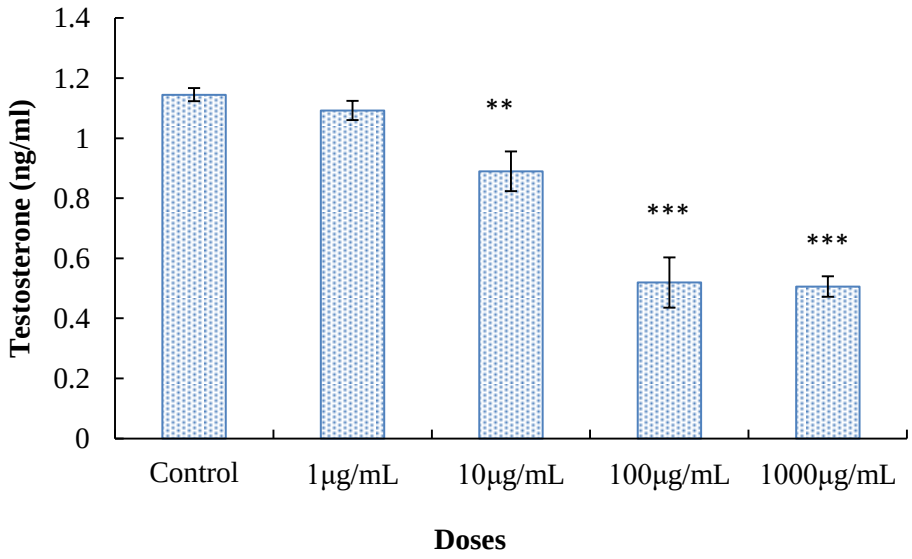


Figure 28: *In vitro* effect of methanolic leaf extract of *A. integrifolia* on testicular Testosterone secretion in control and treated groups.

Effect of *R. Hastatus* on testicular antioxidant status testosterone secretion

Treatment of testicular tissues of adult rat incubated for two hours with different concentrations of methanolic leaf extract of *R. hastatus* was done in order to determine antioxidant status and oxidative stress markers. There was observed a significant reduction ($p<0.05$) in 1 μg/mL (1.92±0.27) and 10 μg/mL (1.63±0.43) treated group as compared to control (5.59±0.93) (Table 13). No remarkable change in 100 μg/mL treated groups was observed as compared to control. The 1000 μg/mL treated group (5.15±1.64) presented no comparable change with control (5.59±0.93). Superoxidase dismutase (SOD) concentration lowered from 11.18±0.82 in control to 7.28±0.74 in high dose treated group. Similarly, POD level reduced from 9.76±0.64 in control to 7.51±1.26 in highest dose treatment group. However, 10 and 100 μg/mL treated groups showed increase in POD level as compared to control (Figure 29). Both SOD and POD enzymes did not showed any significant change within groups as well as with control group. Increase in levels of oxidative stress markers was observed in all testis incubated with different concentrations of plant extract (Table 13). A dose dependent increase in TBARs was observed in high dose treated group (30.32±3.23) when compared to control (21.94±1.91), however, this increase was not significant. An increase in ROS concentration was perceived in a dose dependent manner as compared to control, but, the significant change ($p<0.05$) was only seen in 1000 μg/mL treated group (Figure 30). Testosterone level was measured in testicular tissues incubated with various concentrations of plant extract for 2 hours, as shown in table 3. Highly significant decrease ($p<0.001$) in 100 and 1000 μg/mL extract treated testis was observed when compared with control group. Other groups (1 and 10 μg/mL) showed no significant change when comparison was made with control group (Table 13, Figure 31).

Table 13: *In vitro* effect of methanolic leaf extract of *R. hastatus* on testicular antioxidants, ROS and Testosterone secretion.

Parameters	Extract Treatment (µg/mL)				
	Control	1	10	100	1000
CAT (U/mg protein)	5.59±0.93	1.92±0.27*	1.63±0.43*	3.62±0.89	5.15±1.64
SOD (U/mg protein)	11.18±0.82	9.21±0.57	13.15±1.77	10.12±1.18	7.28±0.74
POD (nmole)	9.76±0.64	8.69±1.32	10.92±1.41	10.57±0.58	7.51±1.26
TBARS (nM/mg tissue)	21.94±1.91	24.95±2.08	26.05±2.84	31.03±3.61	30.32±3.23
ROS (U/g tissue)	12.64±0.85	13.23±0.50	13.07±0.80	19.66±1.17	20.82±4.12*
Testosterone (ng/ml)	1.13±0.02	1.07±0.05	1.00±0.02	0.89±0.04***	0.9±0.04***

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

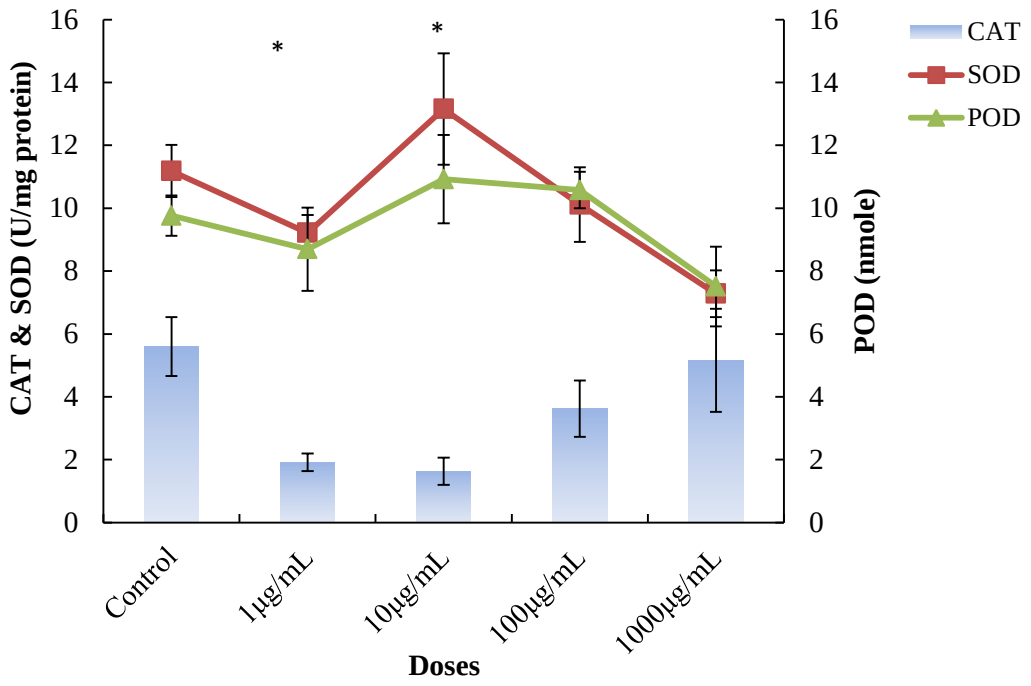


Figure 29: *In vitro* effect of methanolic leaf extract of *R. hastatus* on testicular CAT, SOD and POD activity in control and treated groups.

*= indicates significant difference of CAT at probability of $p<0.05$ compared to control.

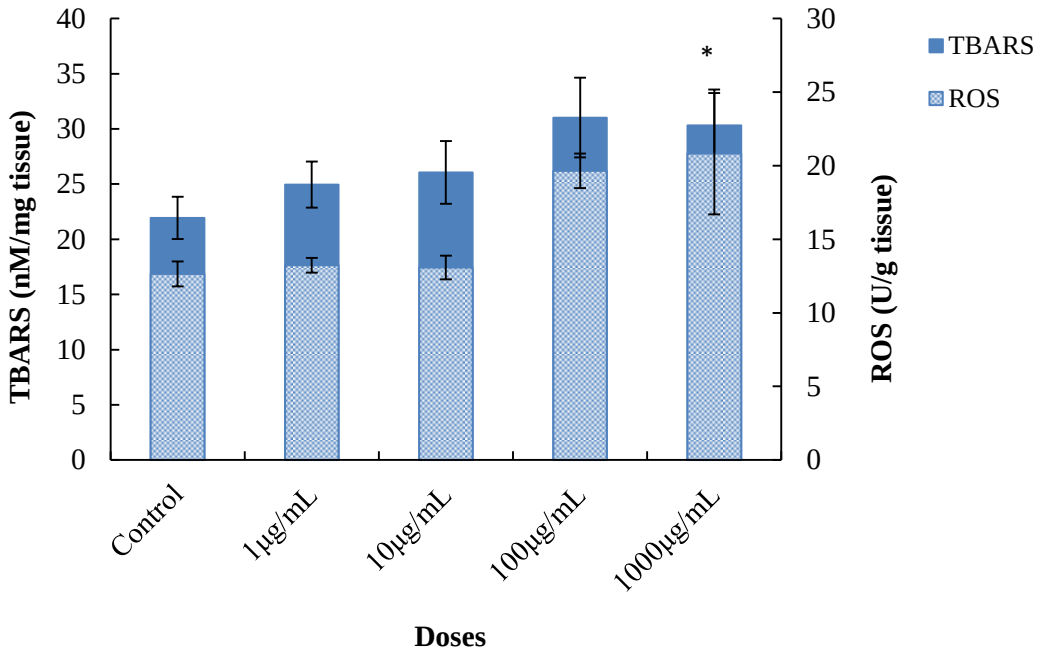


Figure 30: *In vitro* effect of methanolic leaf extract of *R. hastatus* on testicular ROS and TBARS in control and treated groups.

*= indicates significant difference of ROS at probability of $p<0.05$ compared to control.

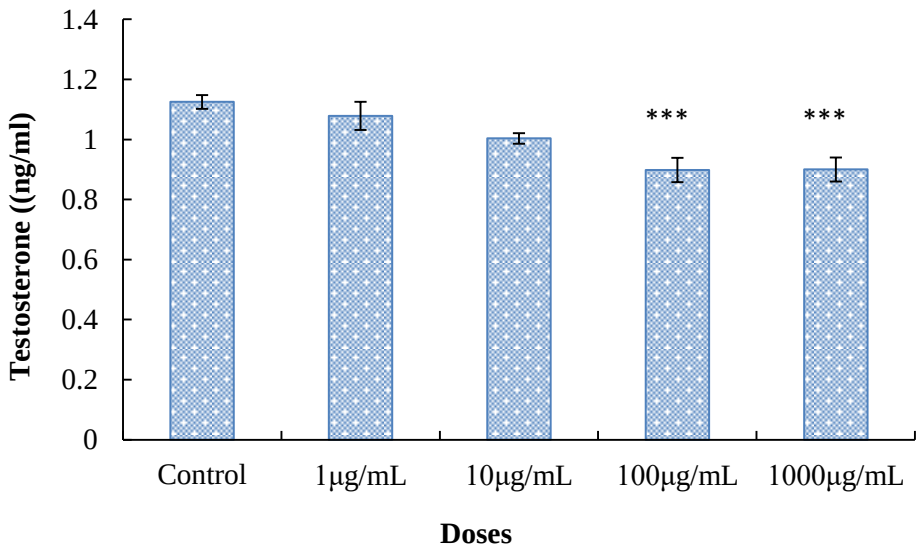


Figure 31: *In vitro* effect of methanolic leaf extract of *R. hastatus* on testicular Testosterone secretion in control and treated groups.

***= indicates significant difference of Testosterone concentration at probability of $p<0.001$ compared to control.

Effect of *H. nepalensis* on testicular antioxidants, ROS and testosterone secretion in rat testis

Table 14 presents the antioxidant enzyme status, measured after incubation of rat testis for two hours with different concentrations of methanolic extract of *H. nepalensis.* A dose dependent reduction in catalase activity was seen from 12.27±2.17 in control to 9.20±0.36 U/mg protein in high dose treated group; however, this change was not significant (Figure 32). A dose dependent decrease in SOD (9.33±1.75) and POD (6.88±0.14) levels in high dose treated group (1000 µg/mL) were evident as compared to control (13.08±1.83 and 11.67±1.35) but this reduction was significant ($p<0.05$) only in case of POD (Figure 33). Similarly, ROS and TBARS levels were increased in dose dependent manner but only high dose treated group presented significant change ($p<0.05$) (Figure 34). Testosterone concentration was also measured in testicular tissues incubated with different doses of plant extract for two hours and is presented in table 11. Decrease in all the extract treated groups was observed as compared to control group but this decrease was significant ($p<0.05$) in 100 and 1000 µg/mL extract treated groups (Table 14, Figure 35).

Table 14: *In vitro* effect of methanolic leaf extract of *H. nepalensis* on testicular antioxidants, ROS and Testosterone secretion.

Parameters	Extract Treatment (µg/mL)				
	Control	**1**	**10**	**100**	**1000**
CAT (U/mg protein)	12.27±2.17	9.69±1.25	10.08±0.99	11.69±1.28	9.20±1.06
SOD (U/mg protein)	13.08±1.83	13.95±3.61	8.67±1.31	10.96±2.09	9.33±1.75
POD (nmole)	11.67±1.35	7.46±1.55	6.08±0.99	7.55±1.38	6.88±0.14*
TBARS (nM/mg tissue)	14.95±1.35	18.55±1.70	17.66±1.84	18.77±1.19	21.06±2.35*
ROS (U/g tissue)	27.09±3.00	25.47±3.03	28.03±2.06	32.50±1.59	38.13±2.11*
Testosterone (ng/ml)	1.26±0.01	1.14±0.17	1.04±0.18	0.84±0.01*	0.89±0.007*

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

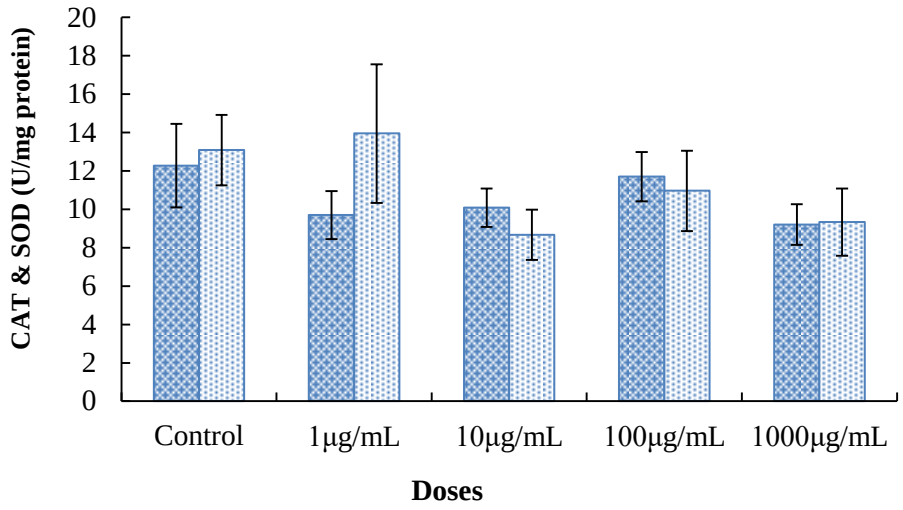


Figure 32: *In vitro* effect of methanolic leaf extract of *H. nepalensis* on testicular CAT and SOD activity in control and treated groups.

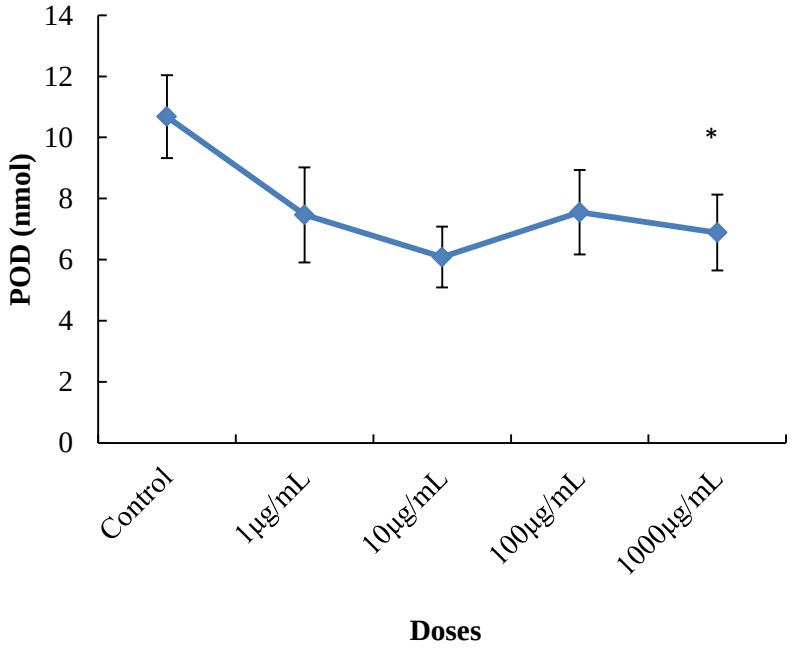


Figure 33: *In vitro* effect of methanolic leaf extract of *H. nepalensis* on testicular POD activity in control and treated groups.

*= indicates significant difference of POD at probability of $p<0.05$ compared to control.

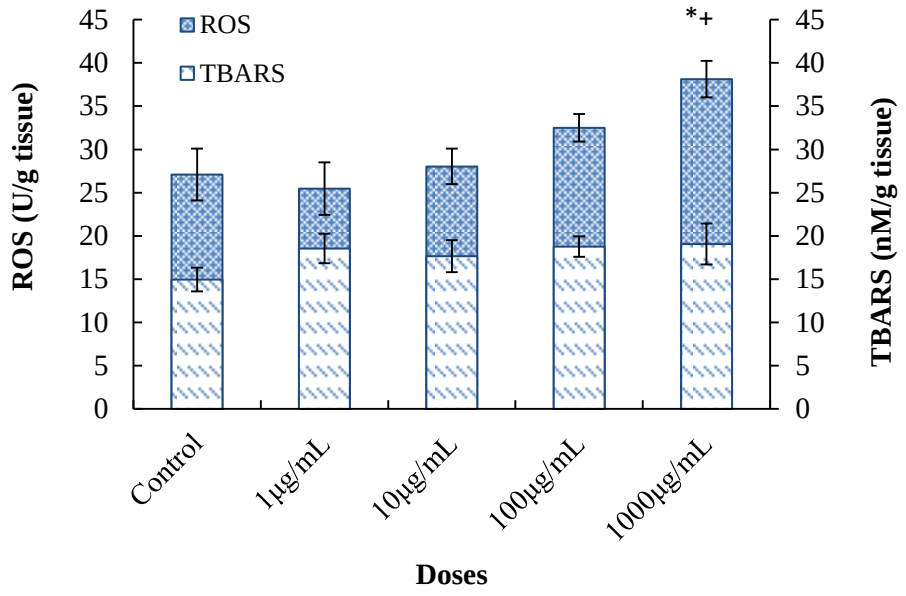


Figure 34: *In vitro* effect of methanolic leaf extract of *H. nepalensis* on testicular ROS and TBARS in control and treated groups.

*= indicates significant difference of ROS at probability of $p<0.05$ compared to control.

+= indicates significant difference of TBARS at probability of $p<0.05$ compared to control.

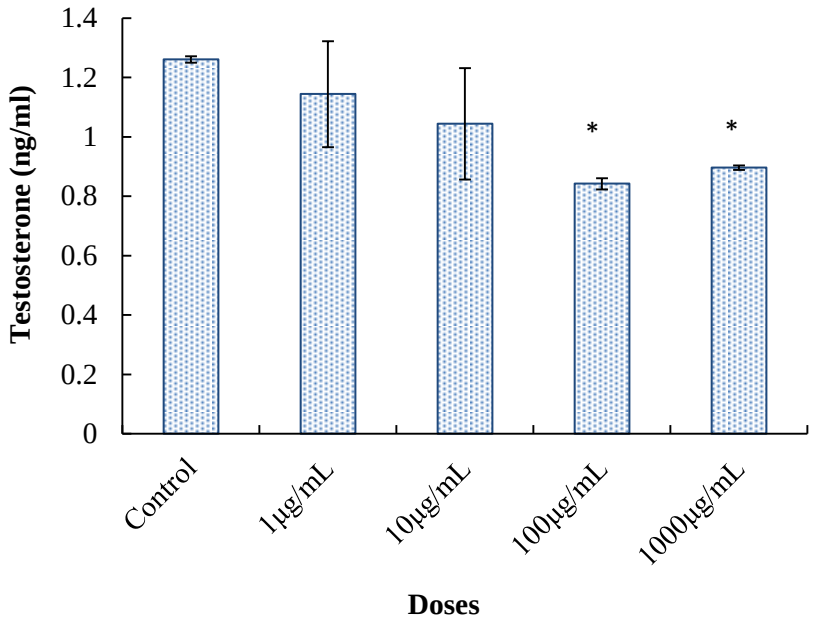


Figure 35: *In vitro* effect of methanolic leaf extract of *H. nepalensis* on testicular Testosterone secretion in control and treated groups.

*= indicates significant difference of Testosterone concentration at probability of $p<0.05$ compared to control.

In vitro effect of *C. ambrosioides* on Sperm DNA

Comet analysis of sperm DNA after two hours incubation with different concentrations of methanolic leaf extract of *C. ambrosioides* is given in table 15, figure 36. Statistically significant ($p<0.01$) reduction in the mean value of head length was seen in 100 and 1000 µg/mL extract treated groups when comparison was made with control. While, tail length was increased significantly ($p<0.05$) in high dose treated groups. In addition, exposure to *C. ambrosioides* extract caused significant ($p<0.001$) decline in percentage DNA in head and tail moment while increase in percentage DNA in tail was noted only in high extract treated groups when comparison was made to control sperms. However, other doses of extract did not caused distinct sperm DNA damage (Figure 37 & 38).

Table 15: *In vitro* effect of *C. ambrosioides* on sperm DNA in control and treated groups.

Parameters	Extract Treatment (µg/mL)				
	Control	1	10	100	1000
Head length (µm)	163.50± 4.47	159.20±3.95	156.80±5.22	144.90±2.77**	142.90±3.30**
Tail length (µm)	26.40±2.61	27.50±3.04	31.50±2.15	39±1.87*	41±5.79*
DNA in head (%)	90.10±1.04	87.15±1.76	86.18±1.08	74.27±3.30***	69.82±4.81***
DNA in tail (%)	9.90±1.04	12.85±1.76	13.82±1.08	25.73±3.30***	30.18±4.81***
Tail moment (µm)	2.73±0.56	3.14±0.22	3.66±0.53	6.56±0.85***	5.94±0.95**

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

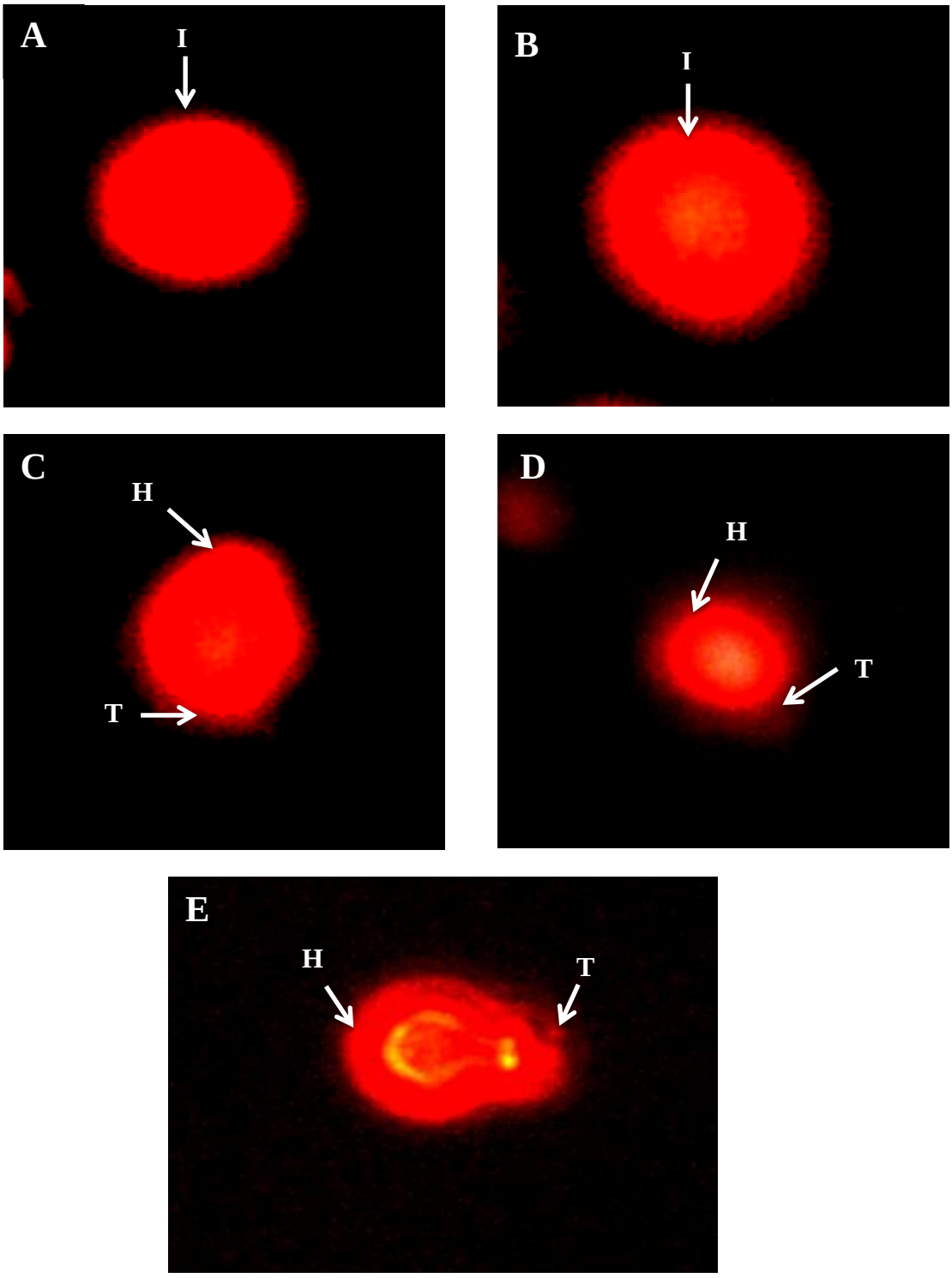


Figure 36: *In vitro* effect of methanolic leaf extract of *C. ambrosioides* on total length of chromatin dispersion in the sperm structure treated with (A) control (0 µg/mL), (B) 1 µg/mL, (C) 10 µg/mL, (D) 100 µg/mL and (E) 1000 µg/mL. 40 X. Head (H), Tail (T), Intact (I).

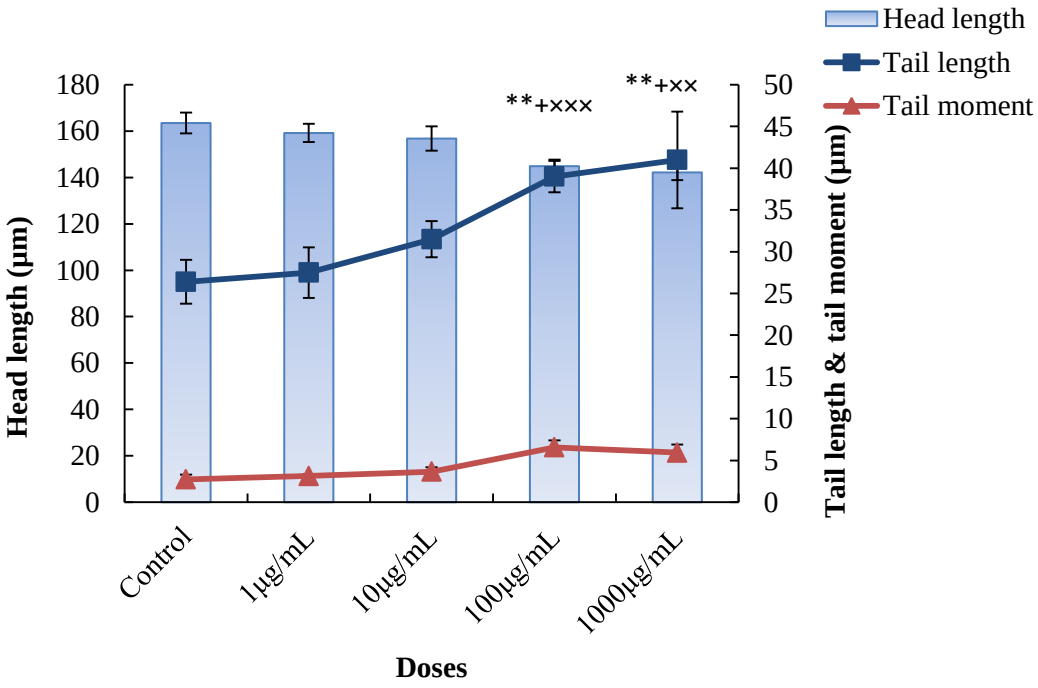


Figure 37: *In vitro* effect of methanolic leaf extract of *C. ambrosioides* on sperm DNA head length, tail length and tail moment in control and treated groups.

**= indicates significant difference of sperm DNA head length at probability of $p<0.01$ compared to control.

+= indicates significant difference of tail length at probability of $p<0.05$ compared to control.

××,×××= indicates significant difference of tail moment at probability of $p<0.01$ and $p<0.001$ compared to control.

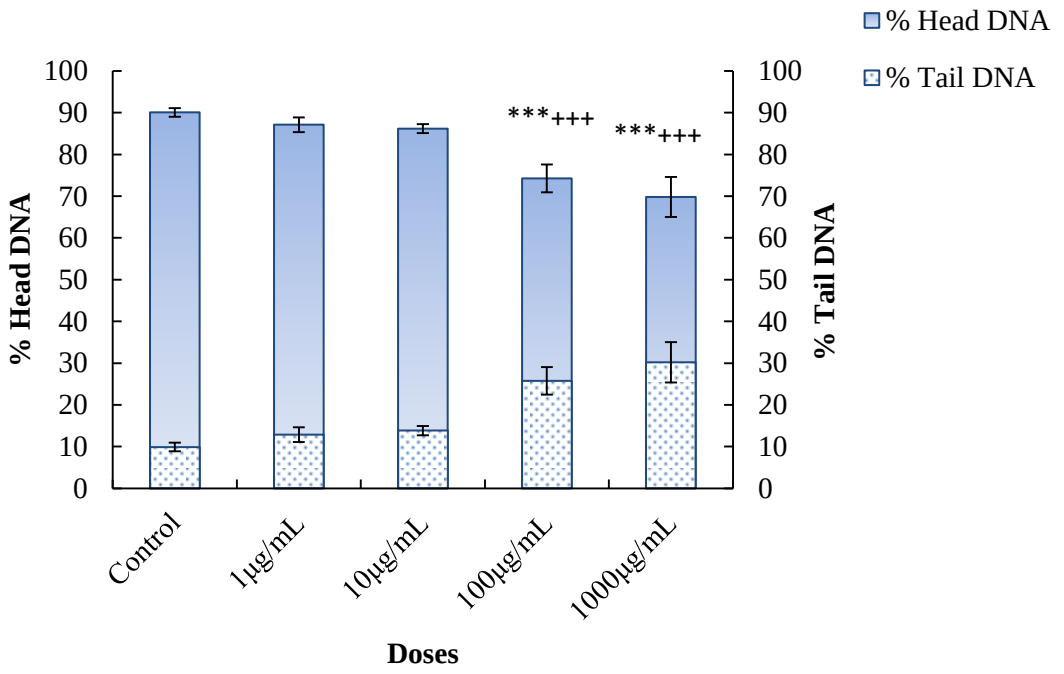


Figure 38: *In vitro* effect of methanolic leaf extract of *C. ambrosioides* on % head DNA and % tail DNA of sperm in control and treated groups.

***= indicates significant difference of % DNA in head of sperm at probability of $p<0.001$ compared to control.

+= indicates significant difference of % DNA in tail of sperm at probability of $p<0.05$ compared to control.

***In vitro* effect of *A. integrifolia* on Sperm DNA**

Incubation of epididymis sperms of adult male rats with various concentrations of methanolic extract of *A. integrifolia* was done for the evaluation of sperm DNA damage. A significant decrease in head length ($p<0.05$) and increase in tail length ($p<0.01$) was reported in 1000 µg/mL extract treated group, however, other doses did not markedly affect these parameters when compared to control group. Incubation to 10, 100 and 1000 µg/mL extract resulted significant ($p<0.001$) reduction in percentage DNA in head and increase in percentage DNA in tail (Figure 40 & 41). While, dose dependent change in mean values of tail moment was noted when compared to control (Table 16, Figure 39).

Table 16: *In vitro* effect of *A. integrifolia* on sperm DNA in control and treated groups.

Parameters	Extract Treatment (µg/mL)				
	Control	1	10	100	1000
Head length (µm)	180±3.63	182.33±7.37	177.11±6.33	161.22±4.05	160.33±4.16*
Tail length (µm)	26.89±2.32	26.11±1.69	28.89±2.50	33.22±2.95	39.44±3.58**
DNA in head (%)	92±0.69	88.73±1.17	80.59±2.74***	82.13±2.06**	79.76±1.39***
DNA in tail (%)	8±0.69	11.27±1.17	19.41±2.74***	17.87±2.06**	20.24±1.39***
Tail moment (µm)	3.99±0.34	3.08±0.38	4.11±0.48	5.68±0.91	5.77±0.89

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

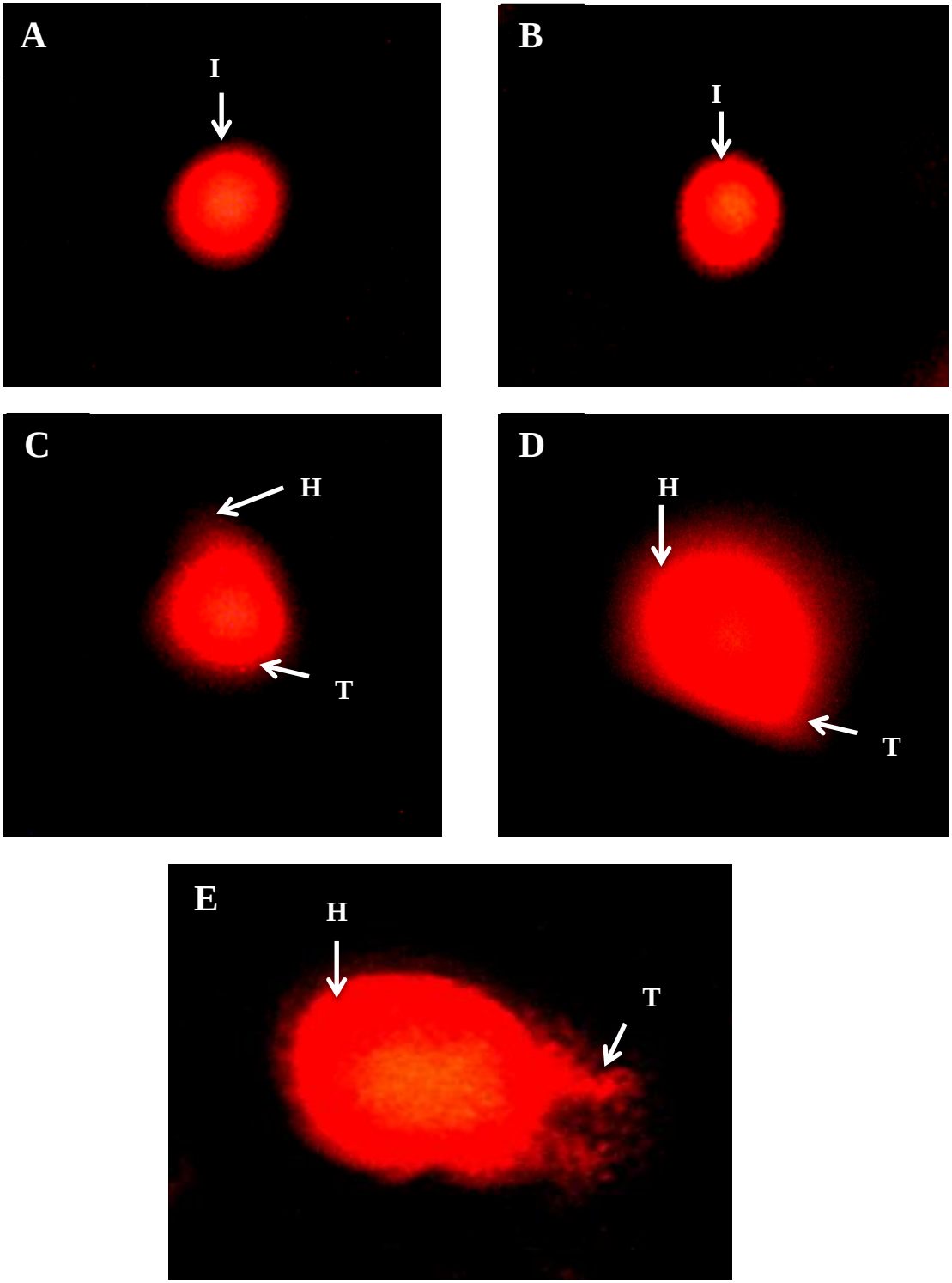


Figure 39: *In vitro* effect of methanolic leaf extract of *A. integrifolia* on total length of chromatin dispersion in the sperm structure treated with (A) control (0 µg/mL), (B) 1 µg/mL, (C) 10 µg/mL, (D) 100 µg/mL and (E) 1000 µg/mL. 40 X. Head (H), Tail (T), Intact (I).

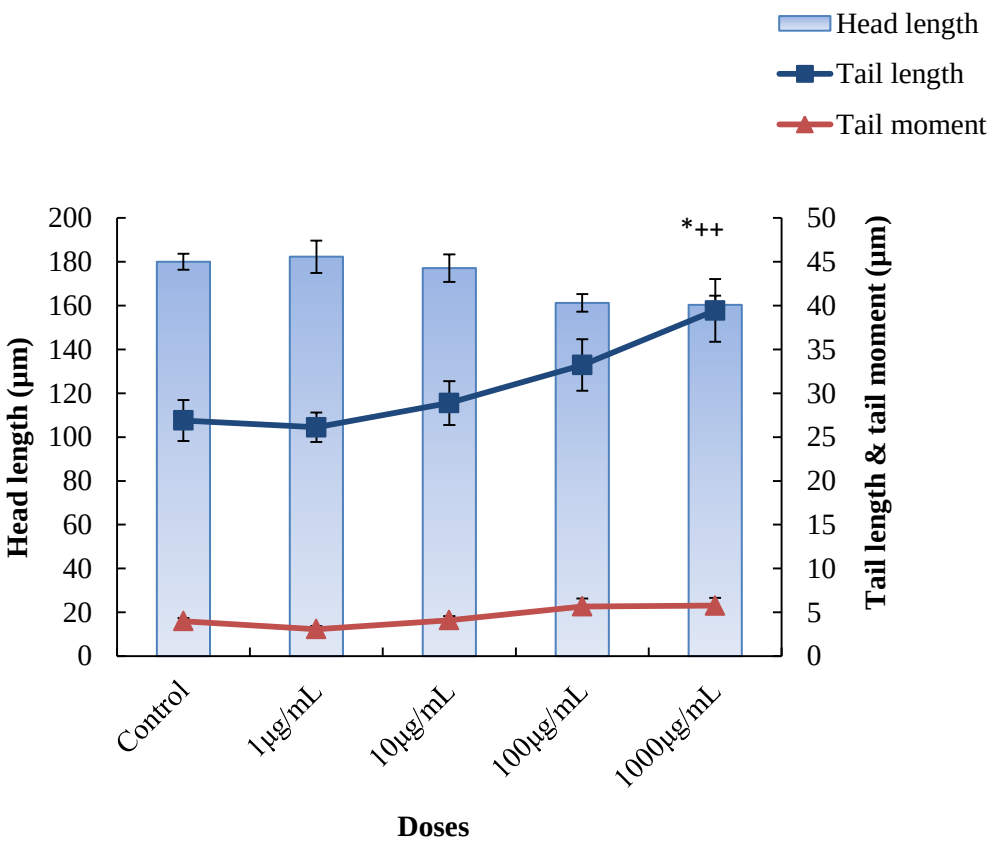


Figure 40: *In vitro* effect of methanolic leaf extract of *A. integrifolia* on sperm DNA head length, tail length and tail moment in control and treated groups.

*= indicates significant difference of sperm DNA head length at probability of $p<0.05$ compared to control.

++= indicates significant difference of sperm DNA tail length at probability of $p<0.01$ compared to control.

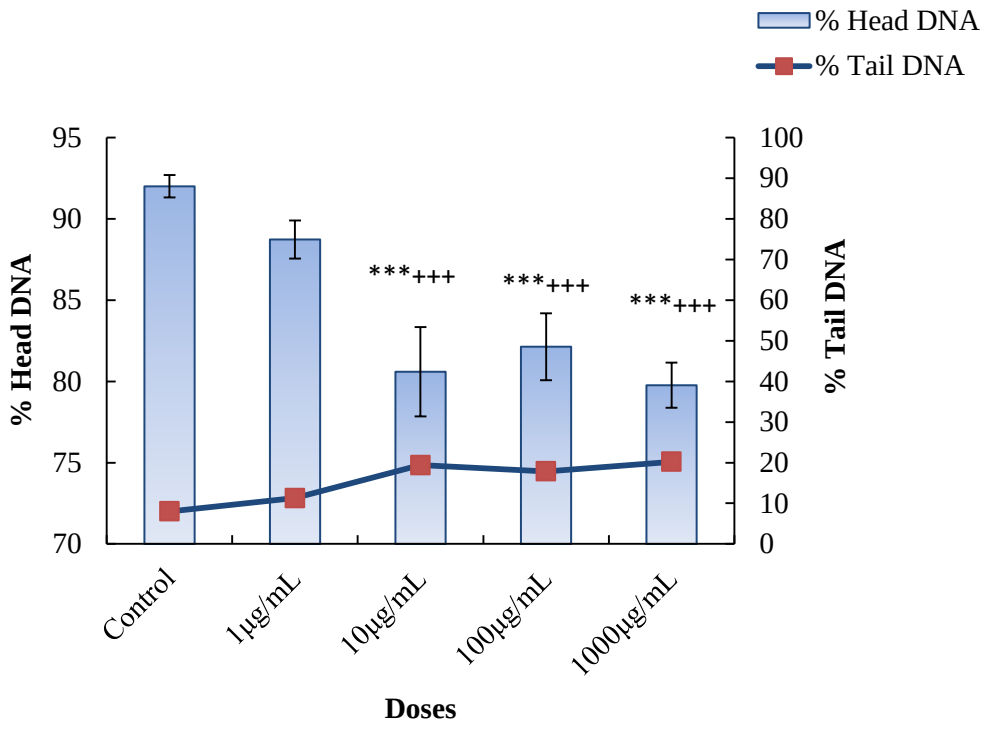


Figure 41: *In vitro* effect of methanolic leaf extract of *A. integrifolia* on % head DNA and % tail DNA of sperm in control and treated groups.

***= indicates significant difference of % head DNA at probability of p<0.001 compared to control.

+++= indicates significant difference of % tail DNA at probability of p<0.001 compared to control.

In vitro effect of *R. Hastatus* on Sperm DNA

Results of comet analysis of sperm DNA after two hours incubation with different concentrations of *R. Hastatus* extract of are presented in table 17 and figure 42. A non-significant change in head length from 155.10±4.22 µm in control to 160.20±4.60 µm in highest dose was reported when compared to control. Mean values of tail length, percentage DNA in tail and tail moment was increased dose dependently in 1, 10 and 100 µg/mL extract treated groups but change was significant ($p<0.05$) in 1000 µg/mL extract treated sperms. On the other hand, percentage DNA in head was reduced from 86.95±1.99% in control to 80±1.41% in highest dose treated group but reduction was significant ($p<0.05$) only in high dose treated sperms (1000 µg/mL) when comparison was made to control (Figure 43 & 44).

Table 17: *In vitro* effect of *R. hastatus* on sperm DNA in control and treated groups.

Parameters	Extract Treatment (µg/mL)				
	Control	1	10	100	1000
Head length (µm)	155.10±4.22	157.30±3.99	158.50±5.37	154.50±6.68	160.20±4.60
Tail length (µm)	30.40±1.85	30.10±1.64	33.70±1.54	35.60±1.80	36.30±1.33*
DNA in head (%)	86.95±1.99	86.42±1.02	82.26±1.63	80.84±2.26	80±1.41*
DNA in tail (%)	13.05±1.99	13.58±1.02	17.74±1.63	19.16±2.26	20±1.41*
Tail moment (µm)	2.74±0.25	2.78±0.33	3.10±0.41	3.72±0.35	4.38±0.55*

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

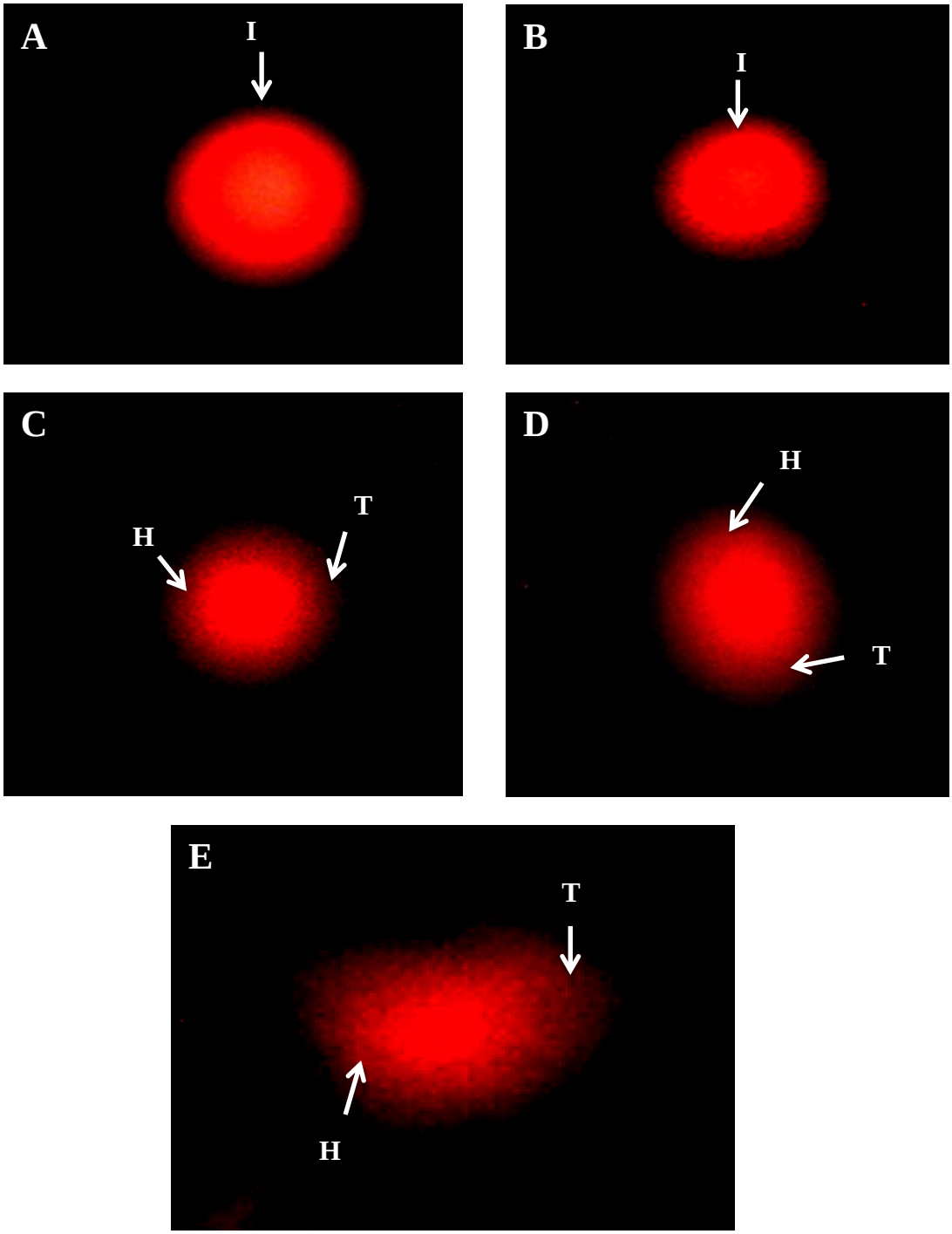


Figure 42: *In vitro* effect of methanolic leaf extract of *R. hatatus* on total length of chromatin dispersion in the sperm structure treated with (A) control (0 µg/mL), (B) 1 µg/mL, (C) 10 µg/mL, (D) 100 µg/mL and (E) 1000 µg/mL. 40 X. Head (H), Tail (T), Intact (I).

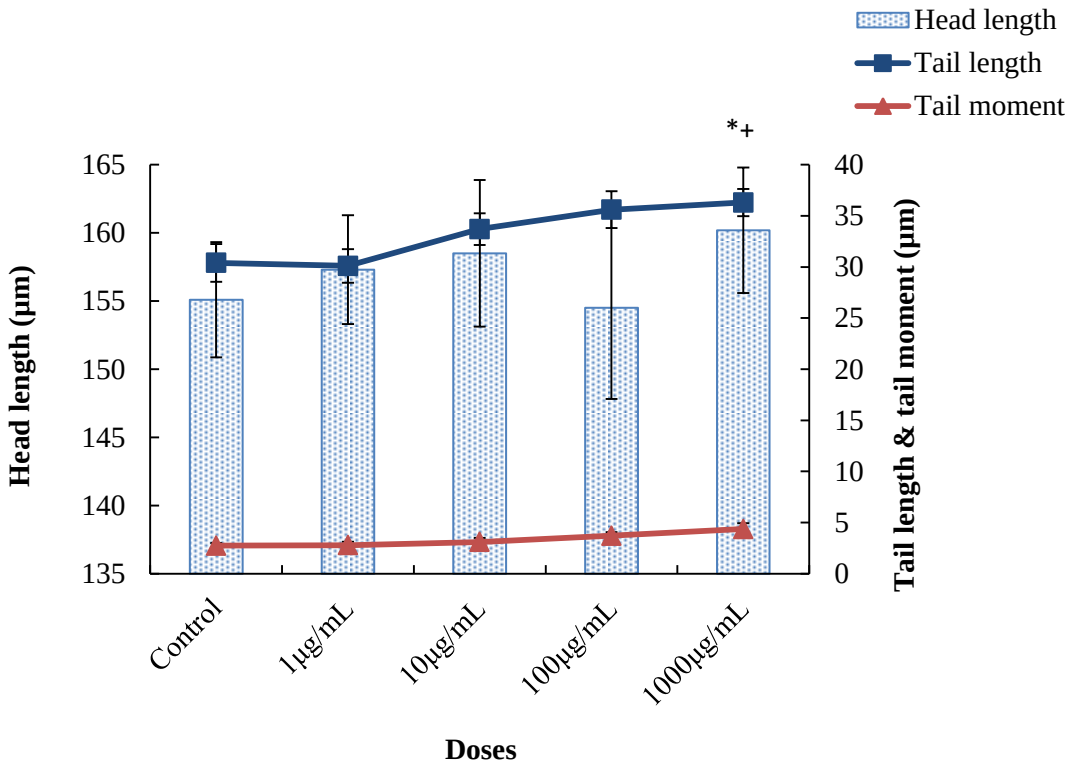


Figure 43: *In vitro* effect of methanolic leaf extract of *R. hastatus* on sperm DNA head length, tail length and tail moment in control and treated groups.

*= indicates significant difference of sperm DNA tail length at probability of $p<0.05$ compared to control.

+= indicates significant difference of sperm DNA tail moment at probability of $p<0.05$ compared to control.

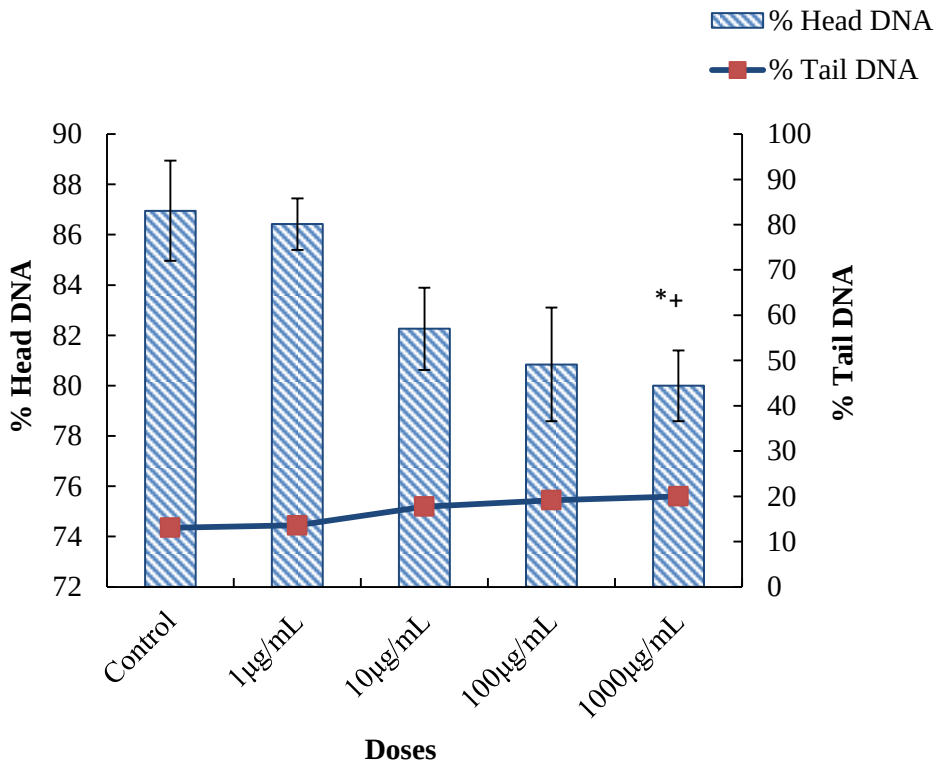


Figure 44: *In vitro* effect of methanolic leaf extract of *R. hastatus* on % head DNA and % tail DNA of sperm in control and treated groups.

*= indicates significant difference of % head DNA at probability of $p<0.05$ compared to control.

+= indicates significant difference of % tail DNA at probability of $p<0.05$ compared to control.

In vitro effect of *H. nepalensis* on Sperm DNA

Results of comet assay revealed significant reduction in head length of sperm after incubation with 10 µg/ml ($p<0.05$) and 1000 µg/mL ($p<0.01$) *H. nepalensis* extract. Additionally, exposure to 10 µg/ml lead to significant increase ($p<0.05$) in tail length, percentage DNA in tail and tail moment ($p<0.01$), while, mean value of percentage DNA in head was reduced significantly ($p<0.05$) when comparison was made with control group (Figure 46 & 47). Incubation of sperm with higher doses (100 and 1000 µg/mL) resulted significant ($p<0.001$) change in sperm parameters (increase in tail length, percentage DNA in tail and tail moment and decrease in percentage DNA in head) leading to sperm DNA damage (Table 18, Figure 45).

Table 18: *In vitro* effect of *H. nepalensis* on sperm DNA in control and treated groups.

Parameters	Extract Treatment (µg/mL)				
	Control	1	10	100	1000
Head length (µm)	190.55±6.32	187.91±5.25	174±4.52*	175.18±2.89	170.27±3.87**
Tail length (µm)	34.09±1.39	37.36±2.92	42.18±2.06*	51.27±1.87***	56.55±1.93***
DNA in head (%)	91.30±1.43	88.34±0.96	84.22±1.22*	75.99±2.92***	66.79±2.75***
DNA in tail (%)	8.70±1.43	11.66±0.96	15.78±1.22*	24.01±2.92***	33.21±2.75***
Tail moment (µm)	4.18±0.38	5.63±0.59	8.36±0.85***	10.35±0.79***	11.13±0.70***

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

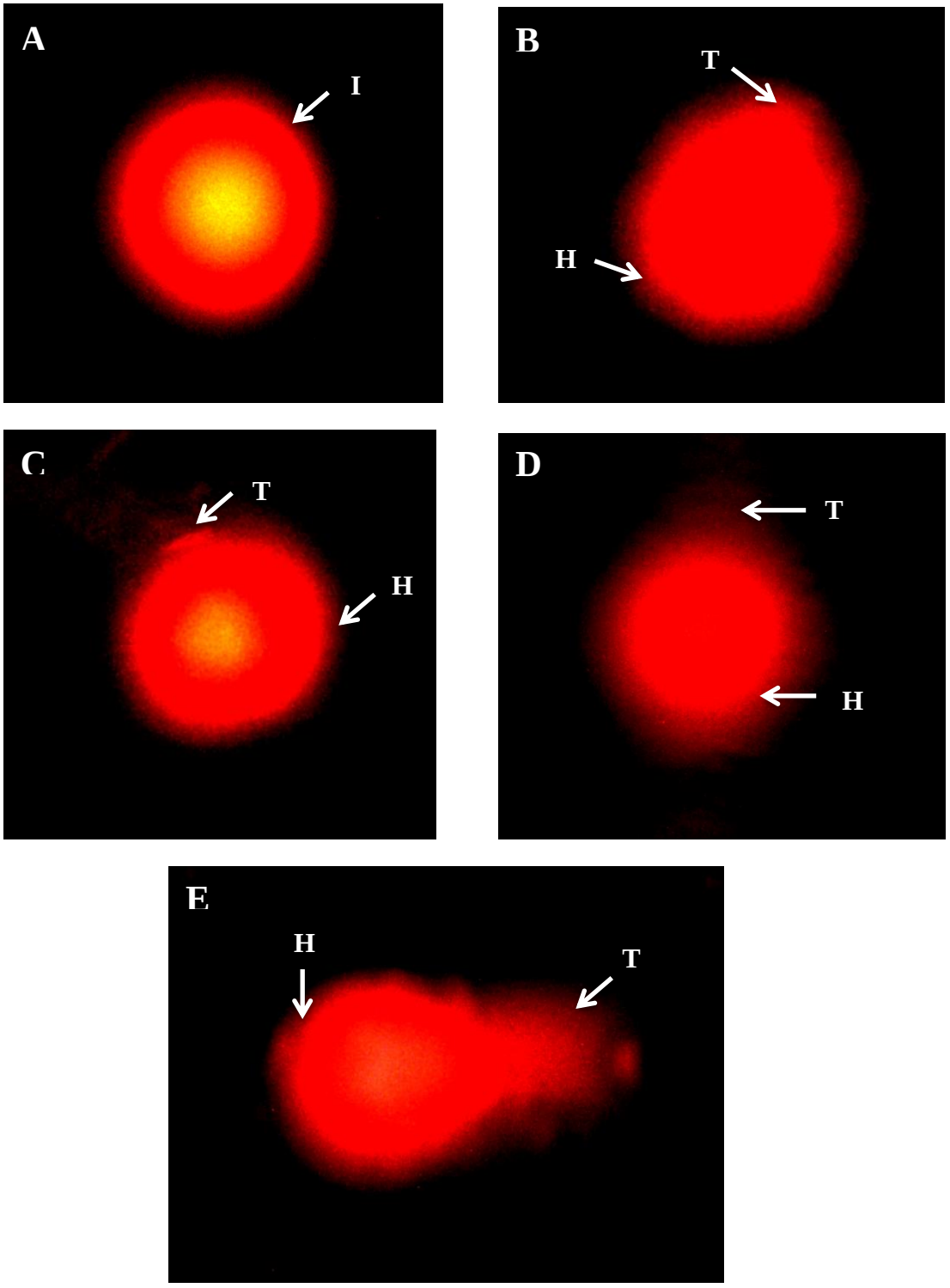


Figure 45: *In vitro* effect of methanolic leaf extract of *H. nepalensis* on total length of chromatin dispersion in the sperm structure treated with (A) control (0 µg/mL), (B) 1 µg/mL, (C) 10 µg/mL, (D) 100 µg/mL and (E) 1000 µg/mL. 40 X. Head (H), Tail (T), Intact (I).

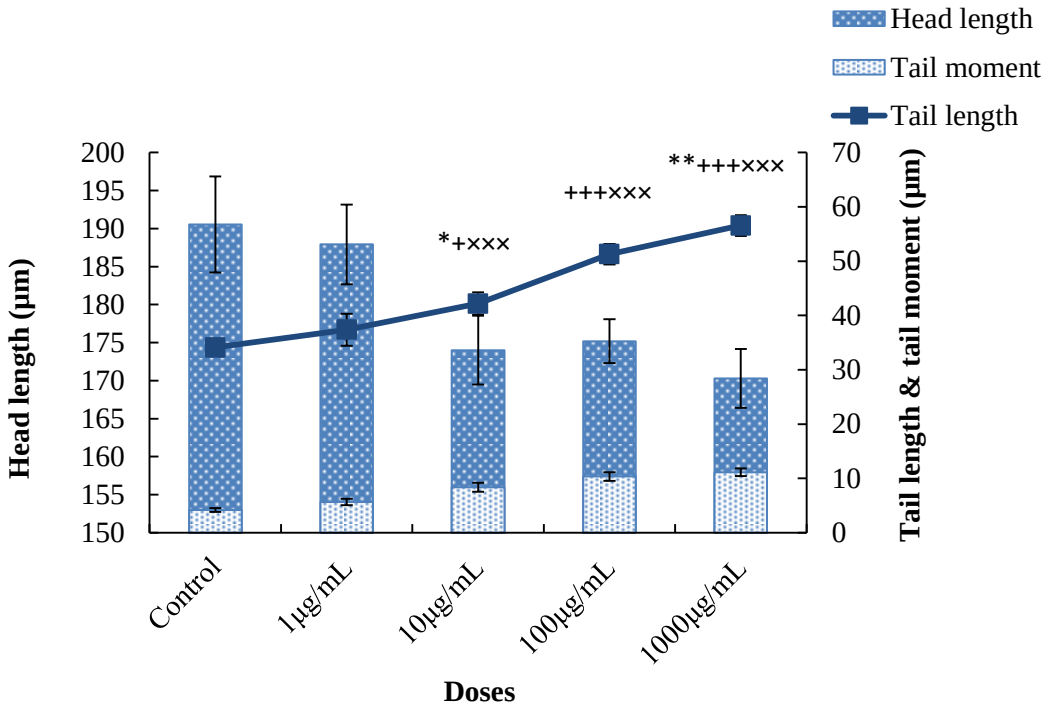


Figure 46: *In vitro* effect of methanolic leaf extract of *H. nepalnesis* on sperm DNA head length, tail length and tail moment in control and treated groups.

*,**= indicates significant difference of sperm DNA head length at probability of $p<0.05$ and $p<0.01$ as compare with control.

+,+++= indicates significant difference of tail length at probability of $p<0.05$ and $p<0.001$ compared to control.

×××= indicates significant difference of tail moment at probability of $p<0.001$ compared to control.

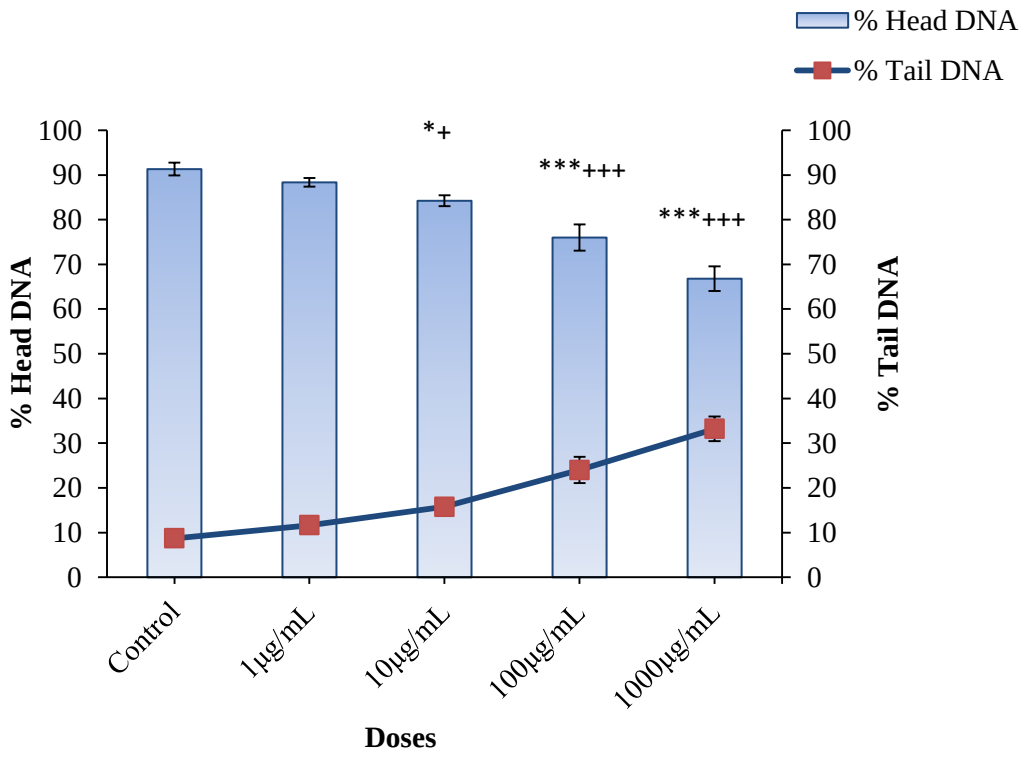


Figure 47: *In vitro* effect of methanolic leaf extract of *H. nepalensis* on % head DNA and % tail DNA of sperm in control and treated groups.

*,***= indicates significant difference of % head DNA at probability of $p<0.05$ and $p<0.001$ compared to control.

+,+++= indicates significant difference of % tail DNA at probability of $p<0.05$ and $p<0.001$compared to control.

DISCUSSION

During recent decades, there has been a rising concern in pharmaceutical plants, as well as their phytochemicals properties, and a number of traditional remedies have been brought together in several fields of medicine even in western societies as an increasing number of people are in search for alternative therapeutic methods. Particularly in the field of andrology, very limited traditional remedies have been presented into conventional medicine, mainly in Asian countries, and very little effort has been performed on this topic (Henkel *et al*., 2012). A large number of plants are known to have contraceptive effects in males disturbing process of spermatogenesis, steroidogenesis, sperm maturation and sperm transport (Soni *et al*., 2015). The present study purposed to evaluate the antifertility efficacy of methanolic leaf extract of *C. ambrosioides, A. integrifolia, R. hastatus* and *H. nepalensis* on the reproductive system of male mice by using *in vitro* experimental approach.

Testes are known to be more profound to oxidative stress because of presence of reactive oxygen species and unsaturated fatty acids responsible for lipid peroxidation (Aitken and Roman, 2008). The findings of this study showed that exposure of testis to different concentrations of selected plant extracts caused reduction in testicular antioxidant levels (SOD, POD, CAT) and increase in ROS and TBARS. However, this oxidative stress was more prominent in tissues incubated with methanolic leaf extract of *A. integrifolia* and *C.ambrosioides* than *H. nepalensis* and *R. hastatus*. This plant extract induced oxidative damage can impair spermatogenesis and steroidogenesis as reported earlier in Coccinia treated mice (Maneesh and Jayalekshmi, 2006; Reddy *et al*., 2006).

Results of comet assay in current study indicated that methanolic extract of *C.ambrosioides* affect sperm DNA after exposure with higher doses (100 and 1000 µg/mL). However, *H. nepalensis* and *A. integrifolia* have the potential to damage sperm DNA even at low dose i.e 10 µg/mL as well as at higher doses (100 and 1000 µg/mL). On the other hand, *R. hastatus* can't disrupt sperm DNA at lower concentrations except when sperms were incubated with 1000 µg/mL extract. Previously, various *in vitro* and *in vivo* studies have shown association of oxidative stress with testicular dysfunction and sperm DNA damage (Lucesoli and Fraga, 1995; Lucesoli *et al*., 1999; Kumar *et al*., 2002). Similar results were reported earlier, in which administration of fenugreek seed to mice reduced fertility in dose dependent

manner by affecting sperm parameters and producing abnormal sperms with DNA damage (Al-Yahya, 2013). Kumar *et al*. (2002) proposed association between DNA damage and oxidative stress leading to infertility in male mice (Kumar *et al*., 2002).

In present study, incubation of testicular tissues with methanolic leaf extract of selected plants reduced testicular testosterone concentrations. Normal circulating levels of testosterone are necessary for spermatogenesis and fertility regulation. Increased oxidative stress in testicular tissue might disrupt steroidogenesis by Leydig cells reducing hormonal levels (Hales *et al*., 2005).

Conclusion

In conclusion, methanolic leaf extract of selected plant has the potential to induce oxidative stress in testicular tissue associated with reduction in testicular testosterone and sperm DNA damage. It is suggested that *H. nepalensis, A. integrifolia* and *C.ambrosioides* plant extracts might have stage specific genotoxic effect on germ cells and can be used to suppress fertility by producing oxidative stress and damaging sperm DNA.

CHAPTER 3:

Evaluation of contraceptive efficacy of methanolic leaf extract of *Chenopodium ambrosioides* Hook. in male Sprague Dawley rats.

ABSTRACT

On the basis of *in vitro* results, current *in vivo* study was designed to evaluate antifertility efficacy of methanolic leaf extract of *C. ambrosioides* on male rats. For this, selected doses of extract (0, 50, 100 and 150 mg/kg) were given to rats via oral gavage for 28 consecutive days. Comparative to control group, significant increase in body weight and decrease in testicular weight was seen in extract treated groups while no change was evident in the weight of accessory sex organs. Results of sperm parameters showed reduction in sperm motility, viability and daily sperm production (DSP) in dose dependent manner. A marked reduction in concentration of CAT, SOD and POD was noted in 100 mg/kg and 150 mg/kg extract treated rats while increase in levels of ROS and TBARS was also quite evident only in high dose regimen. Histopathological observations revealed adverse degenerative changes in testicular tissues including remarkable reduction in tubule diameter, lumen diameter, epithelial height. Additionally, significant decrease in spermatogonial populations and number of mature spermatids was also noted. Epididymal histology showed a little change in tubular and lumen diameter while epithelial height in both caput and caudal epididymis was significantly reduced in dose dependent manner. A significant decrease in levels of plasma Testosterone and Follicle stimulating hormone (FSH) was also recorded. No distinct change in plasma Luteinizing hormone (LH) was observed. Fertility test of present study also found reduced pregnancy outcome in the females paired with treated male rats as compared to those mated with control animals after 60 days of treatment withdrawn. Litter size was significantly reduced in 50 mg/kg and 150 mg/kg dose treatment, however, this reduction was not significant in case of 100 mg/kg extract treated rats. In conclusion, it is suggested that the methanolic extract of *C. ambrosioides* leaf is quite effective in reversible suppression of male fertility.

INTRODUCTION

World population has been tremendously increasing day by day with its undesirable consequences on environmental and economic growth in poor-developed countries (Thakur *et al*., 2010). This situation would intensify the need for effective measures in order to control the birth rate. Subsequently, several attempts have been made to control the birth rate by various means. At the beginning, several synthetic compounds (Chemical nature) were used as oral contraceptives and very little attention was paid to the plant kingdom. However, use of these synthetic agents was associated with several deleterious side effects such as hormonal imbalance, increased risk of cancer, hypertension, and weight gain (McNamara, 1996). Therefore, there is a necessity to replace these substances by safe and effective alternative like plant-based contraceptive agents. Now a day, almost 25% of medicinal prescriptions constitute active principle (s) derived from plants. Therefore, medicinal plants, holds a great promise for the discovery of new antifertility agents.

Several medicinal plants have been shown to be associated with antifertility properties (Garg *et al*., 1998; Hiremath *et al*., 1999; Hiremath *et al*., 2000). Various herbal remedies are traditionally used as contraceptives abortifacients and emmenagogue agents (Ritchie, 2001). Numerous plants containing flavonoids, tannins, terpenes, quinines and diterpenoid lactones have been shown to induce male antifertility through different mechanism (Reddy *et al*., 2003; Rao *et al*., 2004; Joshi *et al*., 2011). Alkaloids, flavonoids, steroids, saponins and phenols found in Chenopodiaceae plants have been identified (Ibrahim *et al*., 2007; Kokanova-Nedialkova *et al*., 2009).

Chenopodium is a genus of numerous perennial or annual herbaceous flowering plants species which are cosmopolitan in distribution (Gelin *et al*., 2003). *C. ambrosioides* is a perennial, aromatic herbaceous shrub of family Chenopodiaceae that is extensively cultivated in Eastern and Central USA, Europe, Maryland, Mexico and Canada. Common name of *C. ambrosioides* is ‘mastruz’ or ‘erva-deSanta-Maria’ in Brazil, whereas in other American countries it is commonly known as goosefoot, epazote and paico (Ososki *et al*., 2002; Cruz *et al*., 2007). Decoctions and infusions of inflorescences, roots and leaves of *C. ambrosioides* have been used for many centuries as a dietary condiment and in folk medicine.

Because *C. ambrosioides* is plentiful in flavonoids and terpenoids compounds, it is pharmacologically used as cancer chemopreventive agent (Kiuchi *et al*., 2002; Liu, 2004). Other therapeutic roles of *C. ambrosioides* include anthelmintic (Giove, 1996), antiviral (Zanon *et al*., 1999), antibacterial (Sousa *et al*., 2012), antifungal (Kishore *et al*., 1993; Prasad *et al*., 2010), antipyretic (Bum *et al*., 2011) and analgesic applications. They are widely used for the treatment of several metabolic disorders such as diabetes, hypercholesterolemia along with other digestive, urogenital, respiratory, and nervous disorders (De Pascual *et al*., 1980). Few cases of intoxication have been studied in human beings and rat's species which is accompanied with the consumption of essential oil of *C. ambrosioides* in fairly large amounts (Chevallier, 1996; Ruffa *et al*., 2002). These toxic effects are more likely due to the presence of terpenoids that despite of diverse pharmacological properties, also have toxic aspects (Kiuchi *et al*., 2002; Liu, 2004). Moreover, *C. ambrosioides* is also toxic for several insects and potentially used as a botanical insecticide (Gadano *et al*., 2002; Tavares and Vendramim, 2005). The most bioactive component of *C. ambrosioides* in this aspect is the ascaridole, an essential oil which is a well-known worm repellent substance (Gadano *et al*., 2002). It was previously shown that aqueous extract of *C. ambrosioides* negatively influence the reproduction in Drosophila melanogaster (Wohlenberg and Lopes-da-Silva, 2009). So it can be hypothesized that *C. ambosioides* can be used to suppress fertility.

Figure 48. **Plant *Chenopodium ambrosioides* L. in its natural habitat.**

Indigenous use for antifertility: Powder of plant leaf is prepared and 4 tea spoons are used daily three times for 13- 16 days.

Taxonomy

The taxonomy of the plant according to National Plant Data Center, NRCS, USDA is as follows

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Caryophyllidae

Order: Caryophyllales

Family: Chenopodiaceae

Genus: *Chenopodium* L.

Specie: *Chenopodium ambrosioides* L.

However, no study yet was found in literature about the reproductive toxicity of this preparation in males. Considering this aspect, the aim of this work was to investigate if the methanolic extract obtained from fresh leaves of *C. ambrosioides* is able to induce antifertility effects and cause reversible reproductive impairment in male rats.

MATERIALS AND METHODS

Plant material and extraction

Chenopodium ambrosioides L. locally called as "Jungli Bathua" is found in agricultural and cultivated fields of Shangla Alpuria, Shahpur, Lilowni, Chakesar, Hayatabad, Ajaori and Kass, Attock, Swat, Parachinar and Abbotabad, Pakistan. It was collected from cultivated fields of Abbottabad (Hazara) located at altitude of 1256 m and was identified by Dr. Mushtaq Ahmad at Plant Systematics and Biodiversity Lab (QAU) and the voucher specimen was deposited in herbarium under accession number #14226.

Methanolic leaf extract of *C. ambrosioides* was prepared as described in chapter 1. Shortly air dried leaves were ground in waring blender and then sieved. The dried powder of leaves was extracted with ethanol (leaves to solvent ratio 1:10). The extracts were filtered using Whattman filter paper and concentrated on a rotary evaporator (Gulfraz *et al*., 2007).

Animals

Adult male Sprague Dawley rats (90 days old) were taken from Primate colony of Animal Sciences Department, Quaid-i-Azam University Islamabad. Animals were housed in well ventilated room with 12 h light/12 h dark cycle and room temperature was maintained at 20-25 ˚C. They were provided with standard laboratory diet and tap water ad libitum. All the animals were randomized into various groups of ten animals per cage and acclimatized for one week before initiation of experiment. All the animal handling and procedures were ratified by the ethical committee of animal sciences department.

Experimental design

Experiment was designed to assess the effect of methanolic extract of *C. ambrosioides* leaves on reproductive functions of male rats. Acute oral toxicity study and in vivo study was conducted on the basis of *in vitro* results.

Acute toxicity study

Acute oral toxicity study was performed according to OECD protocol 423 (acute toxicity class method). Fifteen adult Sprague Dawley rats (80-90 days old) were selected randomly and distributed into 5 groups each having 3 animals. Control group

was given 1% methanol. While the animals of first group were administered with 5mg/kg body weight of plant extract via oral gavage and observed for 14 days. If none of the animals showed mortality, then dose was assigned as nontoxic. Similar procedure was performed with higher doses 50, 300 and 2000mg/kg of plant extract. Behavioral changes and signs of mortality and morbidity were noted till the completion of experiment at day 14.

In vivo exposure study

In this experiment, forty male animals were randomly placed in four groups each having ten animals (n=10). First group was considered as control group and was adminstered with 0.9% saline via oral gavage. Animals in the remaining groups were treated with various doses of leaf extract (50, 100 and 150mg/kg respectively). All the doses were administered orally for period of 28 consecutive days. Doses were selected according to previous studies (Pereira *et al*., 2010) and OECD guideline # 408 (use doses according to the relevant substance if doses are not previously reported). Animals were monitored throughout experiment carefully to see any morphological or physical changes.

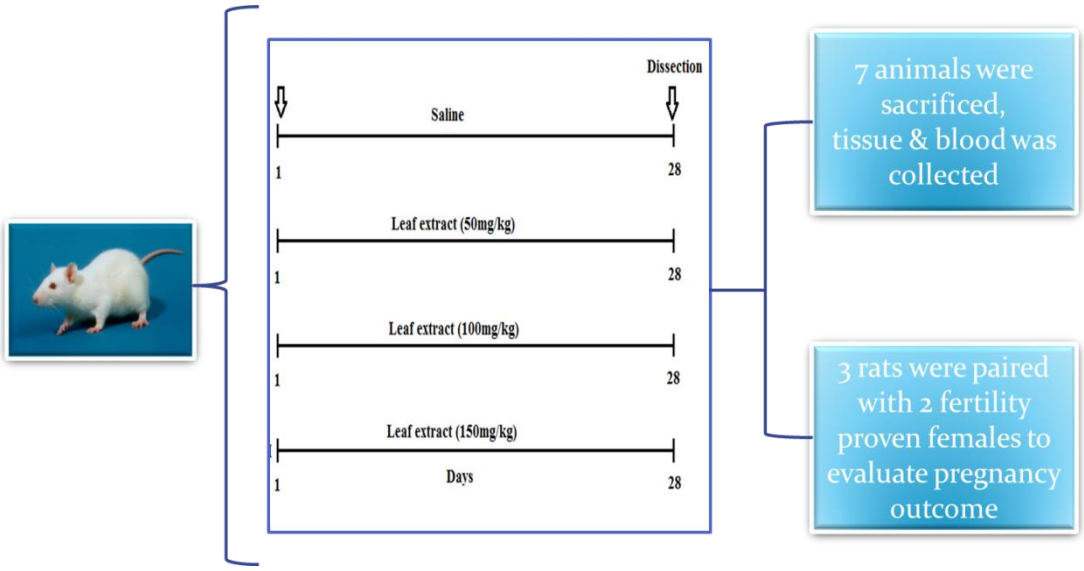


Figure 49: Schematic representation of experimental design of *in vivo* exposure to *C. ambrosioides* methanolic leaf extract.

Blood and tissue collection

On day twenty ninth, seven animals from each group were weighed and killed by decapitation. Trunk blood was drawn and kept in heparinized tubes. All the blood was centrifuged at 3000 rpm for 15 min, plasma was separated and stored at -20 ºC until hormonal analysis. Tissues of reproductive tract (testis and epididymus) were dissected out; washed with saline and weighed. One of the testes was kept in freezer for analysis of daily sperm production and epididymis was minced for further assessment of epididymal sperm count and other sperm parameters. Other testicular and epididymis were fixed in 10% formalin for histological studies.

Fertility test:

At the end of experiment, three animals from each methanolic extract treated group were paired with two untreated fertility proven female rats individually. Vaginal smear from each matted rat was prepared for three consecutive days and number of spermatozoa in the smear was counted. Their litter size, morbidity and mortality if any, were noted.

Effect on Sperm Motility

For the assessment of sperm motility, small portion of cauda epididymis was cut and crushed in 1 ml of pre-warmed (at 37 ºC) phosphate buffer saline (pH 7.3) having a drop of nigrosin stain to make homogeneous mixture. 50 µl of homogenate sample was taken with help of pipette and placed on a pre-warmed slide. A minimum of 10 fields were observed and 100 spermatozoa per sample were calculated for motility via high power microscopy at 40x magnification (Halvaei *et al*., 2012).

Evaluation of Sperm Viability

Eosin-nigrosin staining test was used for the assessment of sperm viability. Semen samples were mixed with dye (eosin-nigrosin). With the help of a pipette, 15 µl droplet of this mixture was placed on a glass slide. Smear was prepared and air dried at room temperature. These slides were examined under a light microscope at 40x resolution. Alive spermatozoa remained unstained (white) whereas, dead cells were stained red. The percentage of dead and alive spermatozoa was calculated by counting at least 100 sperm cells (Halvaei *et al*., 2012).

Epididymal Sperm Count

For the determination of epididymal sperm count, small portion of caput, corpus and cauda epididymis was cut, placed and crushed in 1 ml of 37 °C normal saline solution. 2-3 drops of nigrosine stain was also added into this mixture. With the help of a pipette, 50 µl of this homogenate sample was taken and placed on a pre-warmed glass slide. A minimum of 10 fields were observed and spermatozoa were counted in each part of epididymis through high power microscopy at 40x magnification.

Daily Sperm Production (DSP)

Frozen testicular tissues of all animals were thawed at room temperature for few minutes before homogenization. Tunica albuginea was removed; parenchyma was weighed and homogenized in 3ml of 0.9% sodium chloride (NaCl) solution containing 0.5% triton X-100 in rotor-stator homogenizer (IKA-Werke GmnH. Co. KG, Staufen, Germany) (Robb *et al*., 1978). The homogenate thus prepared was diluted upto 5 folds. 20µl of this homogenate sample was deposited on Neubauer chambers and late spermatids were calculated under microscope at 40x magnification. Readings were taken in triplicate. Later on, this reading was then used to assess the total number of spermatids per testis. For the calculation of DSP, following formula was used.

Daily sperm production (DSP) = Y / 6.3

Antioxidant enzymes

Antioxidant enzymes were estimated in testicular tissue of control and treated animals. The stored testicular samples were thawed and homogenate was prepared in 3 ml of PBS having pH 7.4. Centrifugation was done at 30,000 rpm for 30 minutes at 4 °C. Supernatant was collected for determination of antioxidant status of testicular tissue.

Superoxide Dismutase (SOD)

By using the protocol of Kekkar *et al*. (1984), activity of SOD in testicular homogenate was determined (Kakkar *et al*., 1984). The detailed procedure is given in chapter 2.

Peroxidase (POD) activity

Peroxidase (POD) activity was assessed by using procedure as recommended by Chance and Maehly, 1955 (Chance and Maehly, 1955). Detailed procedure is given in chapter 2.

Catalase (CAT)

CAT activity was assessed by using method proposed by Chance and Maehly, (1955) (Chance and Maehly, 1955) explained in detail previously in chapter 2.

Thiobarbituric acid reactive substances (TBARS)

Estimation of lipid peroxidation was done by using method reported elswhere (Wright *et al*., 1981) as given in chapter 2.

Estimation of Reactive Oxygen Species Assay (ROS)

Estimation of Reactive Oxygen Species (ROS) was carried out in testicular homogenate of control and treated animals by using method reported by Hayashi *et al.* (2007) and detailed procedure is given in chapter 2 (Hayashi *et al*., 2007).

Tissue Histology

Histology of testicular and epididymal tissues was performed in order to determin the potential antifertility effects of methanolic extract of *C. abrosoides*. Following collection of testis and epididymis, following procedure was carried out.

Fixation

Tissues were fixed in 10% formaldehyde for 24-48 hours

Dehydration

After fixation, tissues were dehydrated at room temperature in ascending grades of alcohol as given below.

70% Ethanol ----------120 min

80% Ethanol ----------120 min

90% Ethanol ----------120 min

100% Ethanol ----------120 min

100% Ethanol ----------120 min

100% Ethanol ----------120 min

Following fixation, tissues were passed through xylene to become clear and transparent

Xylene I ----------120 min

Xylene II ----------120 min

Embedding

Following steps were taken for embedding of tissues in paraffin wax.

Xylene + Paraffin (1:1) (62 °C) ----------120 min

Paraffin I (62 °C) ---------- 4 hrs

Paraffin II (62 °C) ---------- 4 hrs

Afterwards, tissues were transferred in melted wax within a boat. After removing bubbles from wax, it was allowed to solidify. Paraffin wax blocks with tissues in them were trimmed and mounted on wooden blocks for section cutting by using knife or scalpel.

Microtomy

In this step tissue was cut into seven µm thin sections using microtome in which wooden blocks were placed (Thermo, Shandon finesse 325, UK). The long ribbons having tissue section were placed in hot water for stretching followed by fixation to previously clean albumenized glass slides. These slides were kept on Fischer slide warmer, that were later shifted in incubator (60 ºC) for the complete stretching for a night.

Staining

Slides were deparaffinized in xylene and tissue sections were rehydrated in descending grades of alcohol. Following steps were performed.

Xylene I ---------- 10 min

Xylene II ---------- 10 min

100% alcohol I ---------- 3 min

100% alcohol II ---------- 3 min

90% alcohol ---------- 3 min

70% alcohol ---------- 3 min

50% alcohol ---------- 3 min

Water ---------- 3 min

Hematoxylin ---------- 8 min

Wash with tape water ---------- 5-10 min

95 % Acidified alcohol ---------- 2 min

Washed with tape water ---------- 2 min

Bluing solution ---------- 2 min

Washed with tape water ---------- 2 min

70% alcohol ---------- 2 min

Eosin ---------- 2 min

Washed with tape water ---------- 2 min

90% alcohol ---------- 5 min

100% alcohol ---------- 5 min

Xylene I ---------- 5 min

Xylene II ---------- 5 min

Permount - 3 small drops/slide of DPX were added and then cover slip was carefully placed before the permount dried.

Microscopy

By using Leica LB microscope (Germany) equipped with cannon digital camera (Japan), observation and microphotography of prepared slides was done. Images were taken at 40 X and histomorphometric analysis was done by using image J2x software. Seminiferous tubule diameter, luminal diameter and epithelial height of seminiferous tubules while diameter of tubules, lumen and epithelial height of epididymal tissue were reported.

Hormonal Analysis

Testosterone

Plasma testosterone concentration was measured quantitatively using Enzyme Linked Immuno Sorbant Assay (ELISA) kits (Biocheck Inc, USA). Detailed procedure is described in chapter 2.

Luteinizing hormone (LH)

Plasma LH concentration was determined quantitatively by luteinizing hormone ELISA kit (Reddot biotech INC).

Principle:

The principal of Luteinizing hormone enzyme linked immuno sorbent assay is based on the competitive inhibition binding with LH specific antibody, between biotin labeled LH and LH present in the test samples.

Procedure:

For the measurement of LH concentration in tested samples, 50µl of standards, blank and test plasma samples were dispensed in the LH antibody coated wells. Afterward, 50 µl of detection reagent A was added in each well immediately. And reaction mixture was shaken slightly by shaking the micro plate so that the mixture became cleared. Then plate was enclosed in the plate sealer given with kit. Micro plate were allowed to incubate for 60 minutes at 37˚ C. after the completion of incubation, wells were washed by auto washer 3 times thoroughly and dried by tapping gently on filter paper. Followed by washing, 100 µl of detection reagent B was dispensed in each well. Again micro plate was sealed and left at 37˚ C for incubated of 60 minutes. The plate was washed again 5 times. Subsequently, 90 µl of Horseradish Peroxidase (HRP) was dispensed in every well, sealed and let the plate to incubate at 37˚ for 15-25 minutes. This step was performed in dark to avoid the light exposure. Blue color was rendered after the addition of HRP. The inverse relation is present between the amount of bounded HRP conjugate and LH concentration in the sample. The color intensity is inversely proportional to the amount of LH in sample. Followed by incubation with HRP, 50 µl of stop solution was dispensed in the entire wells changing the color from blue to yellow. Plate was shaken gently for mixing

thoroughly. Plate was carefully cleaned to get rid of any fingerprints and air bubbles from liquid if present were removed. Absorbance was measured at 450nm wavelength. Minimum detection limit of the kit was 0.11 ng/ml.

Follicle Stimulating Hormone (FSH)

Plasma FSH level was determined quantitatively by Follicle stimulating hormone ELISA kit (Reddot biotech INC).

Principle:

Follicle stimulating hormone enzyme linked immune sorbent assay is principally based on the competitive inhibition binding with the FSH specific antibody, between biotin labeled FSH and FSH in the plasma test samples,.

Procedure:

For the measurement of FSH concentration in tested samples, 50µl of standards, blank and test plasma samples were dispensed in the FSH antibody coated wells. Afterward, 50 µl of detection reagent A was added in each well immediately. And reaction mixture was shaken slightly by shaking the micro plate so that the mixture became cleared. Then plate was enclosed in the plate sealer given with kit. Micro plate were allowed to incubate for 60 minutes at 37˚ C. after the completion of incubation, wells were washed by auto washer 3 times thoroughly and dried by tapping gently on filter paper. Followed by washing, 100 µl of detection reagent B was dispensed in each well. Again micro plate was sealed and left at 37˚ C for incubated of 60 minutes. The plate was washed again 5 times. Subsequently, 90 µl of Horseradish Peroxidase (HRP) was dispensed in every well, sealed and let the plate to incubate at 37˚ for 15-25 minutes. This step was performed in dark to avoid the light exposure. Blue color was rendered after the addition of HRP. The inverse relation is present between the amount of bounded HRP conjugate and FSH concentration in the sample. The color intensity is inversely proportional to the amount of FSH in sample. Followed by incubation with HRP, 50 µl of stop solution was dispensed in the entire wells changing the color from blue to yellow. Plate was shaken gently for mixing thoroughly. Plate was carefully cleaned to get rid of any fingerprints and air bubbles from liquid if present were removed. Absorbance was measured at 450nm wavelength. Minimum detection limit of the kit was 0.1 mIU/ml.

Statistical analysis

Values were expressed as Mean ± SEM. Data was analyzed by one way analysis of variance (ANOVA) followed by Dunnet's multiple comparison test to compare different groups with control by using Graph Pad Prism 5 software. Level of significance was set at $p<0.05$.

RESULTS

Acute toxicity

Acute toxicity of methanolic leaf extract of *C. ambrosioides* in rats after single oral dose ranging from 5 - 2000 mg/kg body weight is given in table 19. After 14 days, no symptoms of mortality or morbidity were observed at all the tested doses. There was no obvious difference in rat's behavior or eating and drinking habit.

Fertility Test:

Effect of *C. ambrosioides* leaf extract on the fertility and mean number of pups born per female after 60 days of treatment withdrawal is given in table 20, figure 50. Low number of sperms was observed in vaginal smear of untreated female rats mated with treated male rats. A dose dependent decrease in percentage fertility and number of pups born per female were noted in all dose regimens when compared with control group. In group treated with 50 mg/kg methanolic extract, the fertility rate was 83%. However, in 100 mg/kg treated group, 66% of females were observed to be conceived while only 50% females conceived in 150 mg/kg leaf extract treated group. The no of pups born per female was also found to be decreased dose dependently in all treatment groups when compare with control group. However, no signs of morbidity or mortality were observed in resultant pups.

Gravimetric analysis

A significant increase in body weight was detected in animals of all the *C. ambrosioides* leaf extract treated groups in comparison to control group (Table 21, Figure 51). A prominent decrease ($p<0.001$) in both right and left testicular weight was observed in 50 mg/kg dose treated group while no change was seen in other extract treated groups (Figure 52). Similarly, no significant change was seen in epididymal and prostate weight of all the extract treated groups. However, weight of seminal vesicle was reduced ($p<0.01$) in 50 mg/kg group when compared to control.

Table 19. Results of acute toxicity study in rats after single oral dose of *C. ambrosioides* leaf extract.

Dose (mg/kg body weight)	Number of animals	Short-term result (24 h)	Long-term results (14 days)
0	3	Survival	Survival
5	3	Survival	Survival
50	3	Survival	Survival
300	3	Survival	Survival
2000	3	Survival	Survival

Table 20: Effect of *C. ambrosioides* leaf extract on the fertility and the Mean ± SEM number of pups born per female after 60 days of treatment withdrawal.

Groups	**Percent fertility**	**Number of pups born/female**
Control	100	8.5 ± 0.83
50mg/kg	83.33	6.16 ± 1.76
100mg/kg	66.66	5 ± 1.77
150mg/kg	50	3.16 ± 1.55*

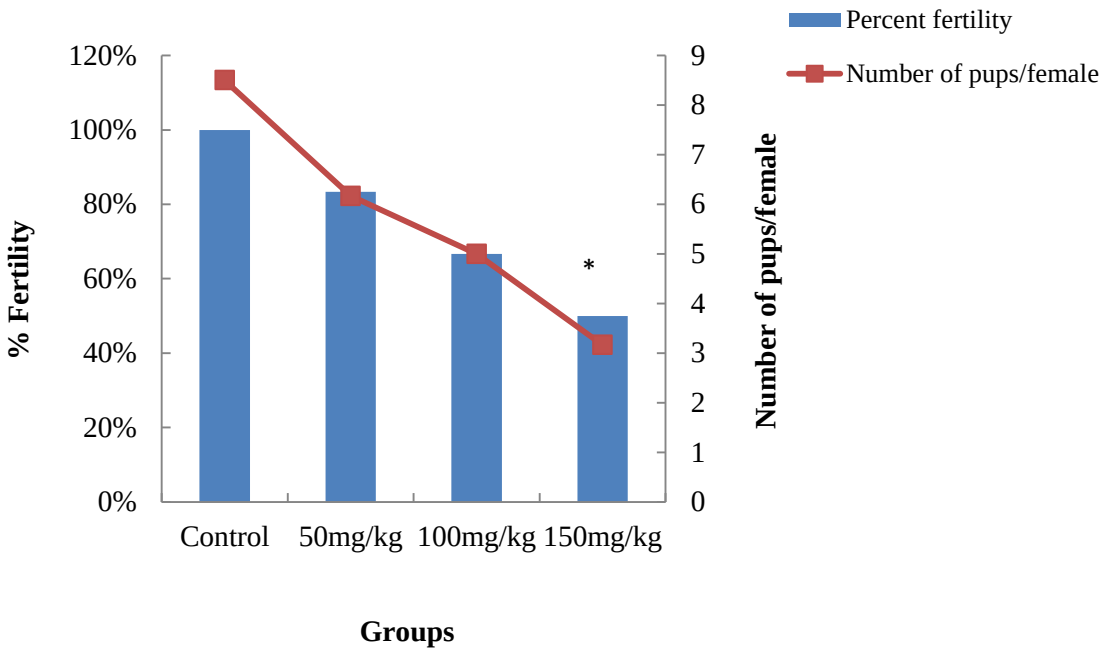


Figure 50: Effect of *C. ambrosioides* leaf extract on % fertility and the number of pups born per female after 60 days of treatment withdrawal.

*= indicates significant difference of number of pups / female at probability of $p<0.05$ compared to control.

Table 21: Effect of *C. ambrosioides* leaf extract on mean ± SEM body weight gain (g), testicular weight (g) and reproductive organs weight (g) of control and treated groups.

Groups	Body weight gain (g)	Testicular weights (g)		Epididymus weight (g)		Seminal vesicle weight (g)	Prostate weight (g)
		Right	Left	Left	Right		
Control	80.2 ± 6.92	1.41 ± 0.07	1.43 ± 0.07	0.51 ± 0.03	0.54 ± 0.02	1.33 ± 0.17	0.53 ± 0.02
50mg/kg	51.4 ± 10.13	1.24 ± 0.04	1.27 ± 0.05	0.55 ± 0.02	0.51 ± 0.02	1.02 ± 0.09	0.65 ± 0.07
100mg/kg	74 ± 7.22	1.33 ± 0.04	1.35 ± 0.03	0.51 ± 0.02	0.54 ± 0.03	1.17 ± 0.12	0.73 ± 0.05
150mg/kg	58.02 ± 8.37	1.37 ± 0.08	1.36 ± 0.08	0.53 ± 0.02	0.53 ± 0.02	1.44 ± 0.08	0.70 ± 0.07

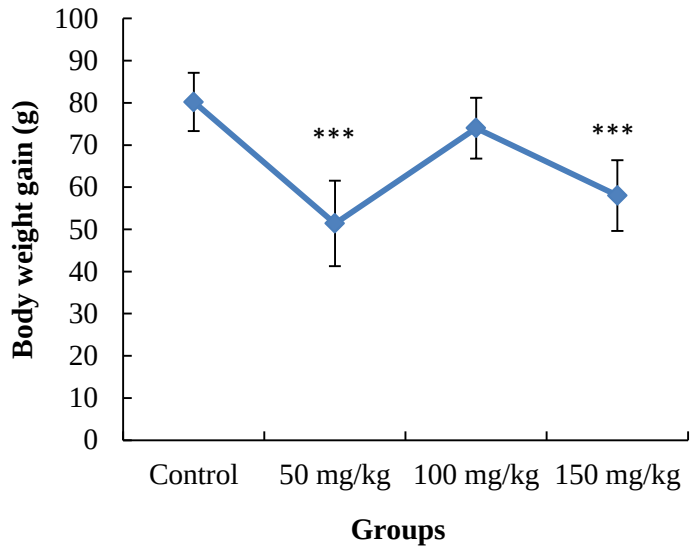


Figure 51: Mean ± SEM body weight gain (g) of control and treated groups of rats, receiving methanolic leaf extract of *C. ambosioides* for 28 days.

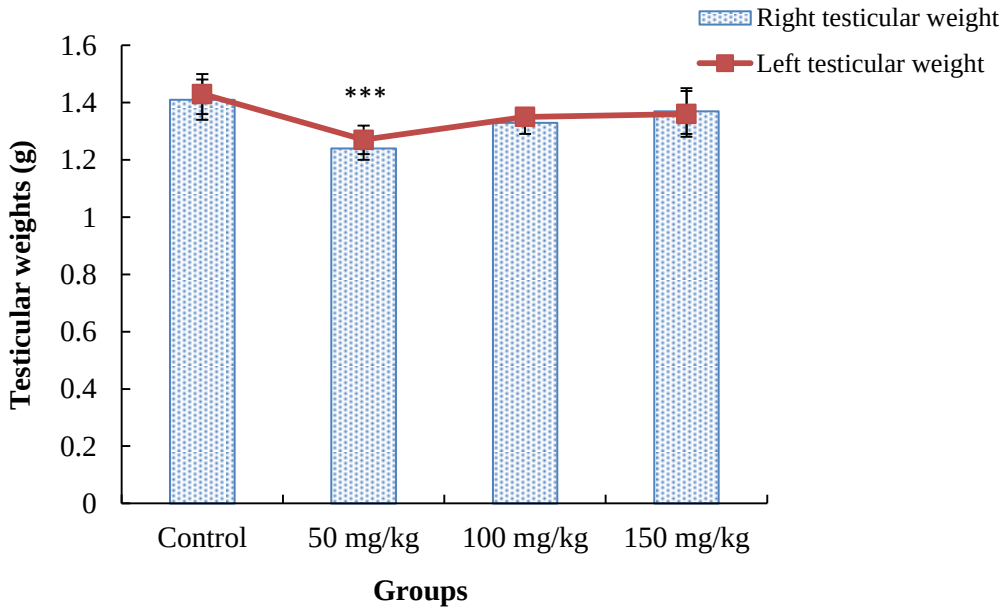


Figure 52: Mean ± SEM of Testicular weight gain (g) of control and treated groups of rats, receiving methanolic leaf extract of *C. ambosioides* for 28 days.

***= indicates significant difference of % at p<0.001 compared to control.

Sperm Motility and Viability

Mean ± SEM of sperm motility and viability rate of control and treated groups are shown in Table 22. A significant reduction ($p<0.05$) in sperm motility rate was seen in dose dependent manner. There was non significant reduction in sperm viability rate observed in all the extract treated groups when compared to control group (Table 22, Figure 53).

Epididymal Sperm Count

A significant reduction ($p<0.01$) in caput sperm number was noticed in high and medium dose treated groups as compared to control. While non significant decrease was seen in 50 mg/kg group. A significant decrease in the corpus sperm count was observed in 50 mg/kg ($p<0.01$) and 150 mg/kg ($p<0.001$) groups when compared to control. Cauda sperm count was significantly reduced ($p<0.001$) in all the treated groups when compared to control group (Table 22, Figure 54).

Daily Sperm Production

Mean ± SEM of daily sperm production in control and treated groups are shown in Table 22 & figure 55. A dose dependent decrease ($p<0.001$) in daily sperm production was observed in all extract treated groups.

Table 22: Effect of *C. ambrosides* leaf extract on mean ± SEM of % sperm motility, viability rate, DSP and epididymal sperm count among control and treated groups.

Groups	Motility rate (%)	Viability rate (%)	DSP × 10^6 /testis	Epididymal sperm count		
				Caput	Corpus	Cauda
Control	75.50±2.25	80.00±7.07	2.47±0.19	1616.25±6.72	1436.25±15.32	1993.75±16.43
50mg/kg	72.50±4.79	76.50±4.19	1.20±0.14***	1552.5±15.36	1280±11.22**	1818.75±7.86**
100mg/kg	50.00±5.77*	62.50±4.79	0.79±0.10***	1481.25±14.77*	1346.25±11.79	1775±18.40***
150mg/kg	55.00±6.45*	60.00±7.07*	0.58±0.06***	1486.25±15.66*	1227.5±12.77***	1775±16.13***

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

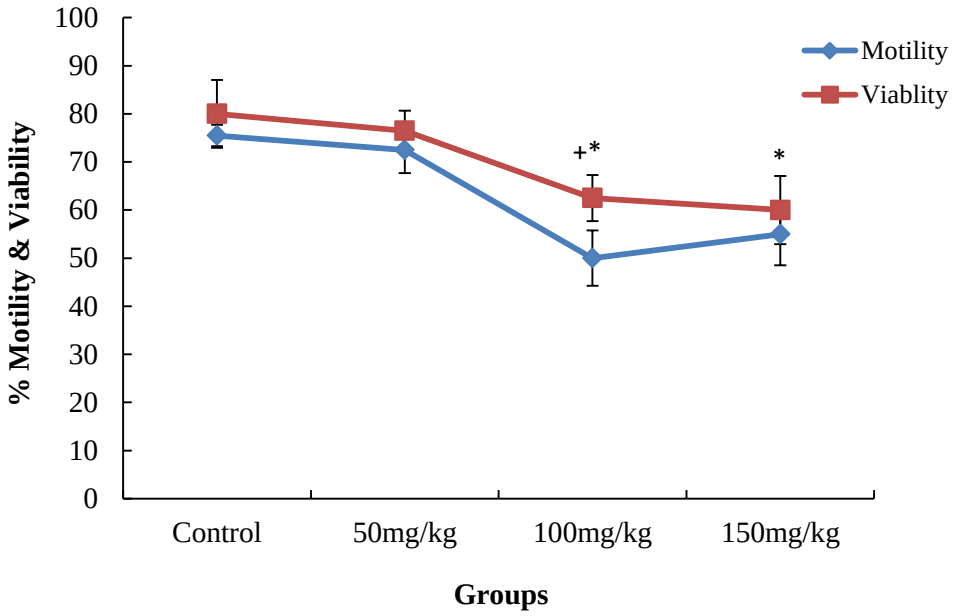


Figure 53: Mean ± SEM of % sperm motility & viability rate in control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

*= indicates significant difference of % motility at probability of $p<0.05$ compared to control.

+= indicates significant difference of % viability at probability of $p<0.05$ compared to control.

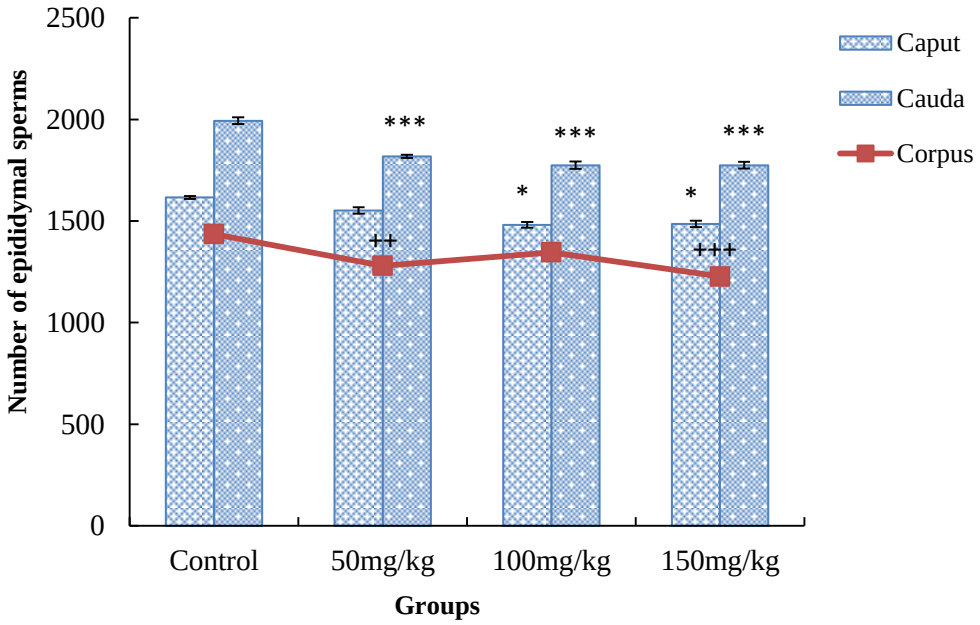


Figure 54: Mean ± SEM of epididymal sperm count in control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

*, ***= indicates significant difference of number of caput and cauda sperm at probability of $p<0.05$ and $p<0.001$ as compared to control.

++, +++= indicates significant difference of number % viability at probability of $p<0.05$ compared to control.

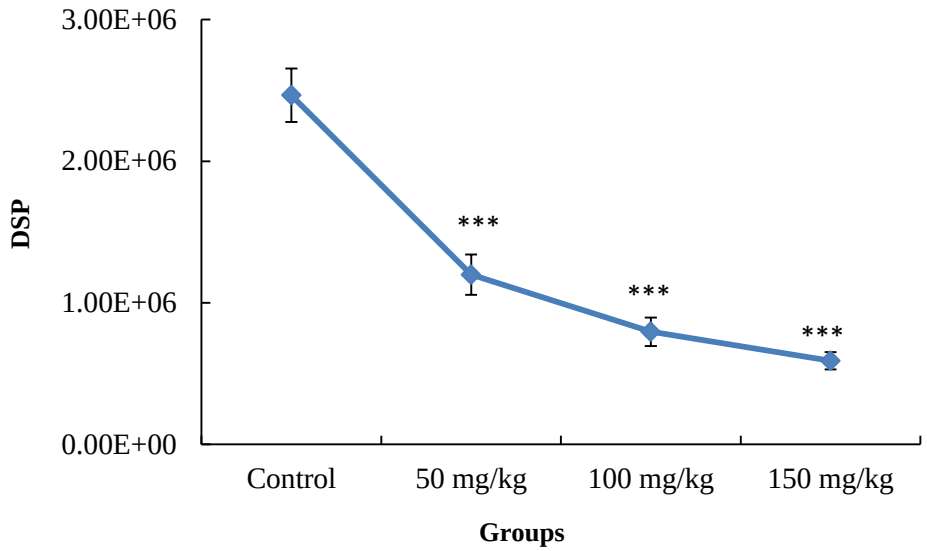


Figure 55: Mean ± SEM of Daily sperm production in control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

***= indicates significant change at probability of p<0.001 compared to control.

Antioxidant Enzymes

Exposure to methanolic extract of *C. ambrosioides* resulted in significant decrease ($p<0.001$) in catalase activity within testis of all the treated groups when compared to control group (Figure 56). Similarly, the activity of SOD was decreased significantly ($p<0.05$) in 150 mg/kg group when compared to that of control group. However non significant change was seen when comparison was made between low dose treated and control group (Figure 57). Peroxidase activity was found to be significantly reduced ($p<0.001$) in 100 mg/kg and 150 mg/kg groups as compared to control group, whereas no significant change was observed in 50 mg/kg group ((Figure 58). The value of TBARS was elevated significantly ($p<0.01$) in 150 mg/kg group as compared to control while no significant change was seen in all other treated groups (Figure 59).

In all the extract treated groups, an increase in ROS value was observed in comparison to control group, given in table 20. A significant increase ($p<0.05$) in ROS value was seen in 150 mg/kg group while other treated groups showed non significant change in ROS value when comparison was made with control group (Table 23, Figure 60).

Table 23: Effect of *C. ambrosioides* leaf extract on mean ± SEM antioxidant activity of testicular tissues of control and treated groups.

Groups	CAT (U/mg)	SOD (U/mg)	POD (nmole)	ROS (µmol/min)	TBARS (nM/mg)
Control	41.30±2.41	49.41±4.45	5.88±0.54	0.77±0.23	2.01±0.41
50mg/kg	31.02±2.044**	47.60±6.29	4.16±0.67	1.03±0.02	2.35±0.5
100mg/kg	11.83±2.29***	33.205±2.97	1.48±0.44***	1.50±0.39	3.47±0.42
150mg/kg	5.52±0.84***	31.49±2.93*	1.43±0.46***	1.97±0.26*	4.59±0.47**

Values are expressed as mean ± SEM

*,**,*** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

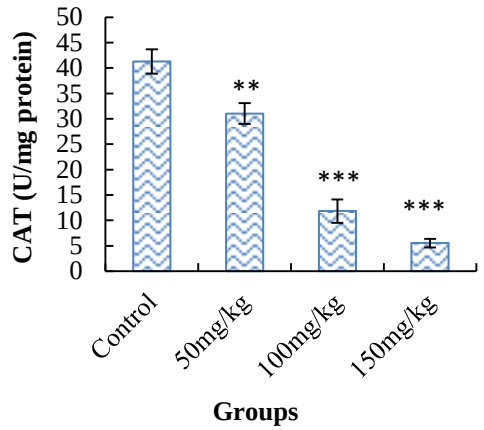


Figure 56: Specific activity of CAT in testis of control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

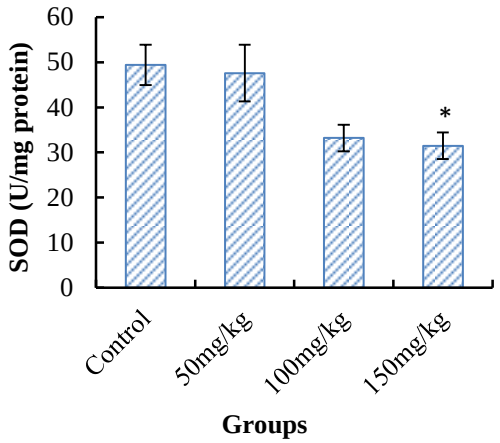


Figure 57: Specific activity of SOD in testis of control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

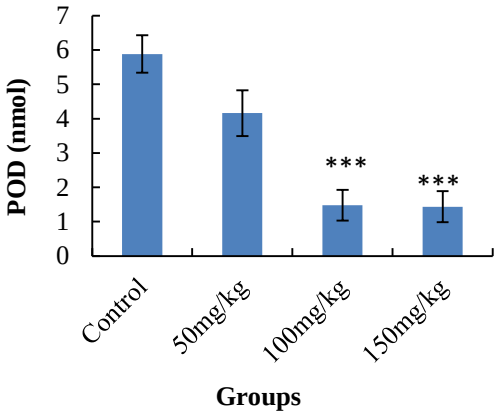


Figure 58: Specific activity of POD in testis of control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

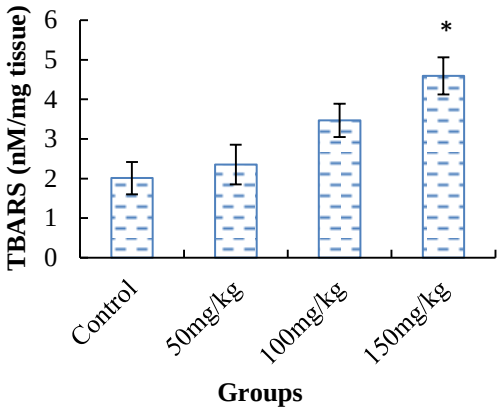


Figure 59: Specific activity of TBARS in testis of control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

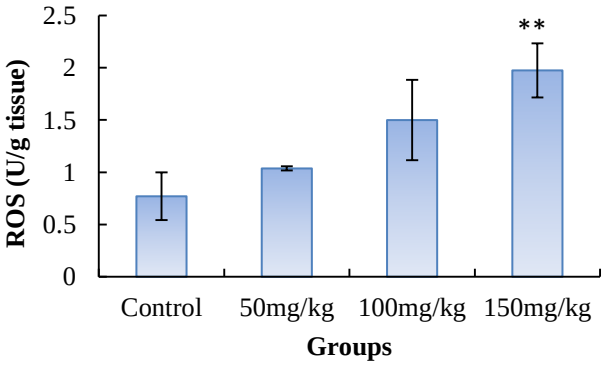


Figure 60: Specific activity of ROS in testis of control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

Histomorphometric Analysis

Histomorphometric findings of testicular and epididymal tissues are given in table 24 and 61. The diameter of seminiferous tubule and lumen, epithelial height and tallness of tunica albugenia of testicular tissues were considered, whereas, in case of epididymis, diameter of lumen and duct along with height of epithelium was considered.

Testis

No significant change was detected in lumen diameter of 50 mg/kg and 100 mg/kg treated groups when comparison was made with control group. However, significant increase ($p<0.05$) was seen in 150 mg/kg extract treated group. Epithelial height was found to be little effected by dose administration. However, irregular arrangement of seminiferous tubules with interstitial spaces and low number of spermatozoa in epithelium were observed in higher dose regimens. The unremarkable change was seen among low and medium dose treated groups (Figure 62). There was a substantial Change in area of seminiferous tubule and interstitium (%) in 10 0mg/kg ($p<0.05$) and 150 mg/kg ($p<0.01$) treated groups as compared with the control one (Table 24, Figure 63).

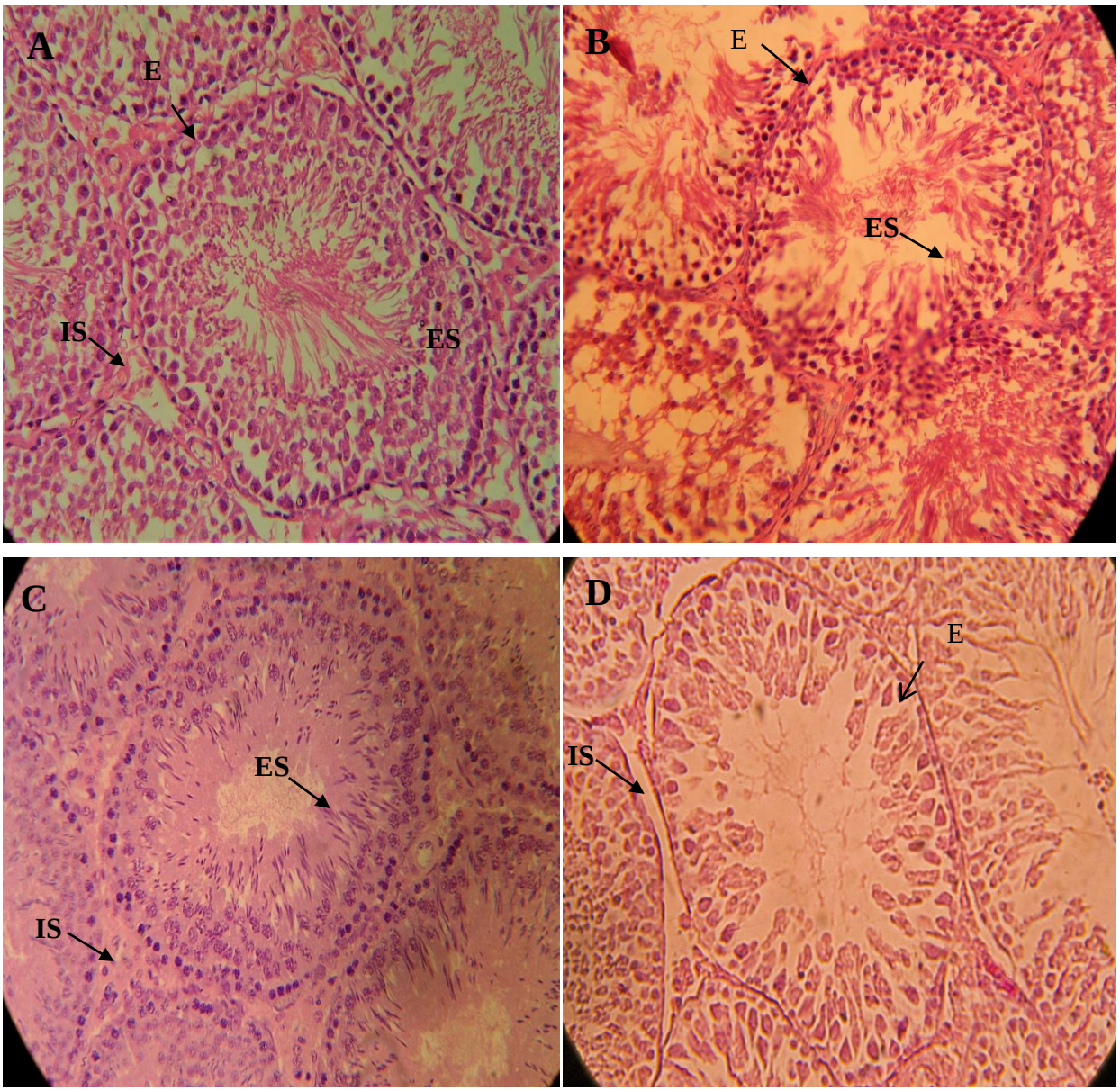


Figure 61: Photomicrograph of seminiferous tubules of male rats receiving doses of *C. ambrosoides.* Regularly arranged tubules, lumen filled with spermatids with normal germ cells shown in (A) Control; represents normal process of spermatogenesis, lumen filled with mature spermatozoa, (B) low dose treated groups; showing tubules with immature spermatids released in lumen and degenerated epithelial layer, (C) medium dose treated group; showing increased lumen diameter and amplified epithelial height, tubules with interstitial spaces are present, (D) high dose treated group; thin epithelium, lumen is empty, large interstitial space are present. Magnification 40X.

Table 24: Effect of *C. ambrosides* leaf extract on mean ± SEM of seminiferous tubule diameter (µm), tubular lumen diameter (µm), seminiferous tubule epithelial height (µm), area of seminiferous tubule (%) and interstitial space (%) in control and treated groups.

Groups	Seminiferous tubule diameter (µm)	Tubular lumen diameter (µm)	Epithelial height (µm)	Area of seminiferous tubule (%)	Interstitial space (%)
Control	218.09±7.23	33.10±1.10	83.38±2.01	86.38±0.58	13.62±0.58
50mg/kg	212.02±7.90	35.36±1.05	78.48±2.16	86.02±0.58	13.98±0.58
100mg/kg	203.45±5.80	40.15±3.72	79.87±3.56	84.14±0.49*	15.86±0.49*
150mg/kg	211.71±7.31	39.54±2.25*	75.25±3.37	83.92±0.36**	16.08±0.36**

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

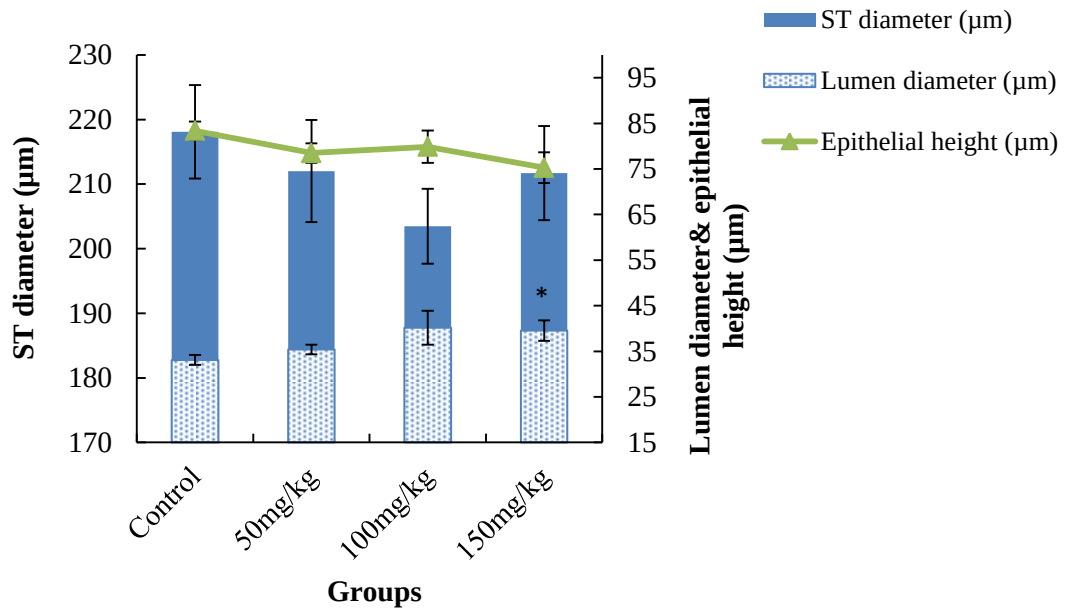


Figure 62: Mean ± SEM of seminiferous tubule diameter (µm), tubular lumen diameter (µm) and epithelial height (µm) in testes of control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

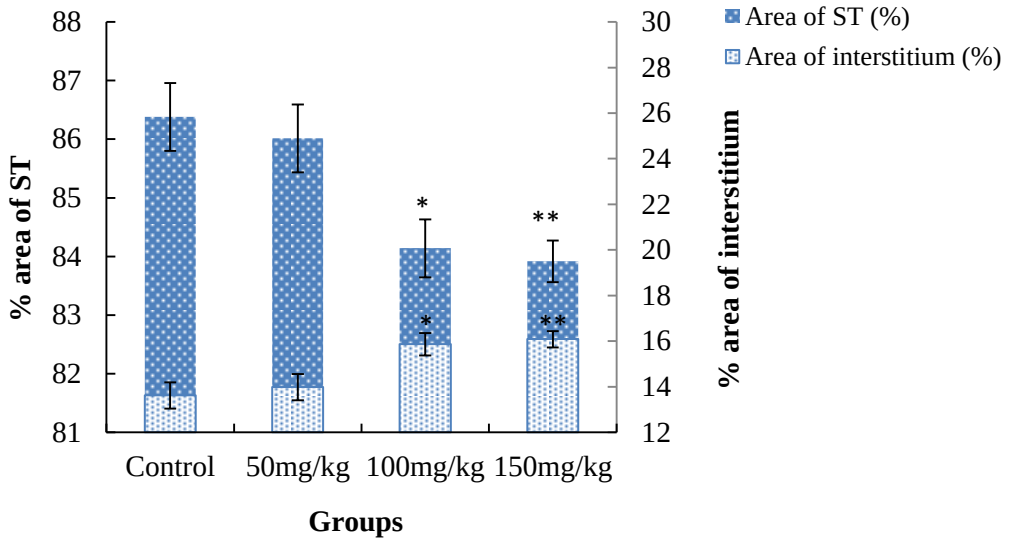


Figure 63: Mean ± SEM % area of seminiferous tubule and % area of interstitium in testes of control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

*,**= indicates significant change at probability of $p<0.05$ and $p<0.01$ compared to control.

Epididymis:

Table 25 & figure 64 and 65 shows histomorphometric results for caput and cauda epididymis.

Caput epididymis

No noteworthy change was observed in ductular diameter of 50 mg/kg and 150 mg/kg dose treated groups as compared to control. However, remarkable decrease ($p<0.05$) was seen in 100 mg/kg extract treated group. A little change in luminal diameter was observed in all dose treated group when compared with control, though, the change was not significant. The epithelial height was found to be reduced in dose dependent manner in all the dose treated groups. A significant change was evident in 100 mg/kg and 150 mg/kg dose treated groups (Table 25, Figure 66).

Cauda Epididymis:

The ductular and lumen diameter of caudal epididymis was reduced in all the dose treated groups as compared to control group but the change was non significant. Epithelial cell height of cauda epididymis was found to be significantly reduced ($p<0.005$) in 100 mg/kg dose treated group as compared to control. Non significant change was seen in low and high dose groups (Table 25, Figure 67).

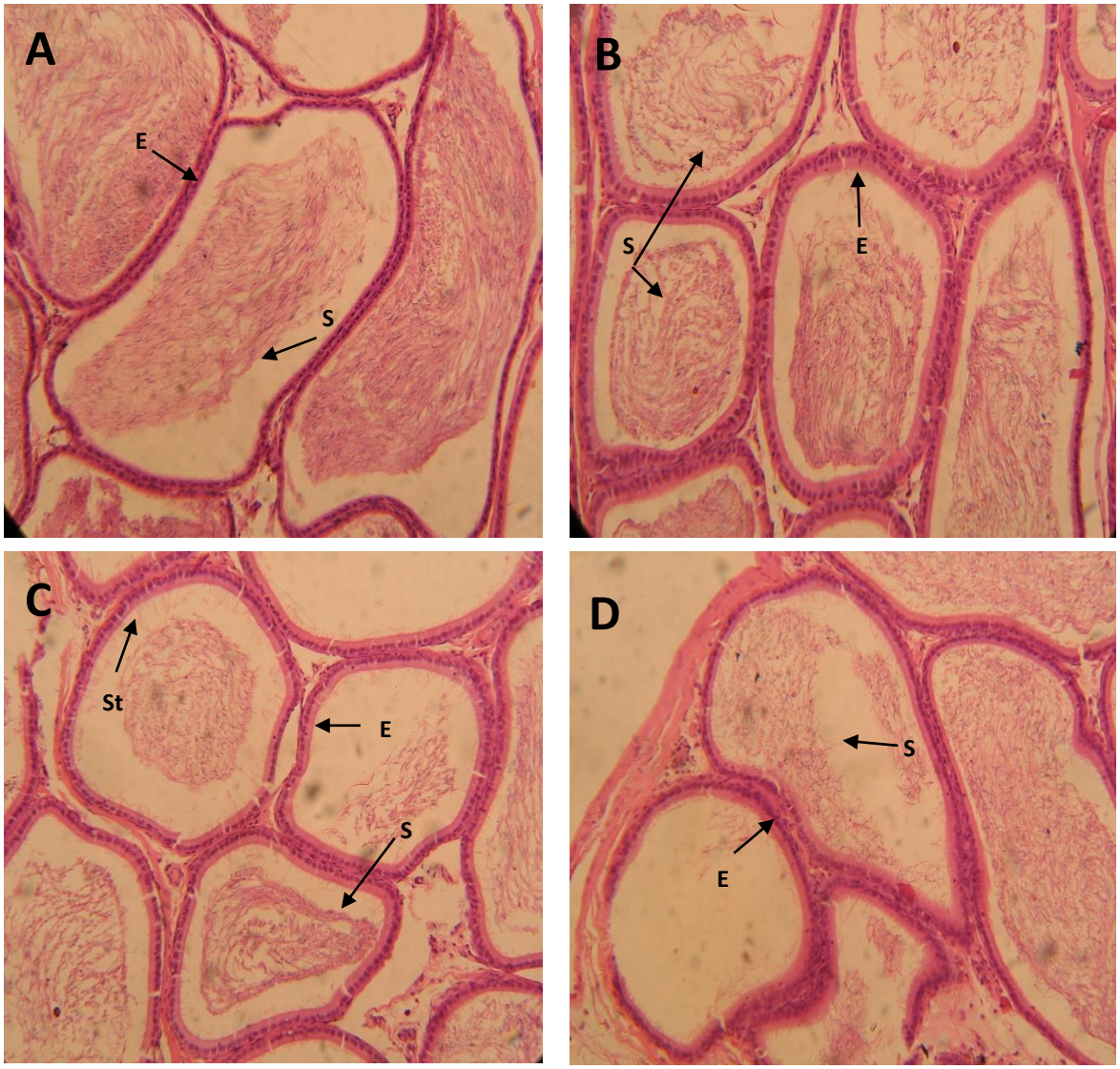


Figure 64: Photomicrograph of caput epididymis of male rats receiving doses of *C. ambrosoides* from: (A) Control group; with normal morphology of caput epididymal cells having thin pseudostratified epithelium lined with stereocilia. Lumen is heavily filled with spermatozoa, (B) low dose treatment group; reduced pseudostratified epithelium with lumen filled with spermatozoa, (C) medium dose treatment group; showing reduction in number of spermatozoa, low number of spermatozoa in lumen and quite thick epithelium, (D) high dose treatment group; showing increase in pseudostratified epithelium and very little concentration of spermatozoa as compared to other two groups group. Stereocilia (St), Epithelium (E), Spermatozoa (S). Magnification 40X.

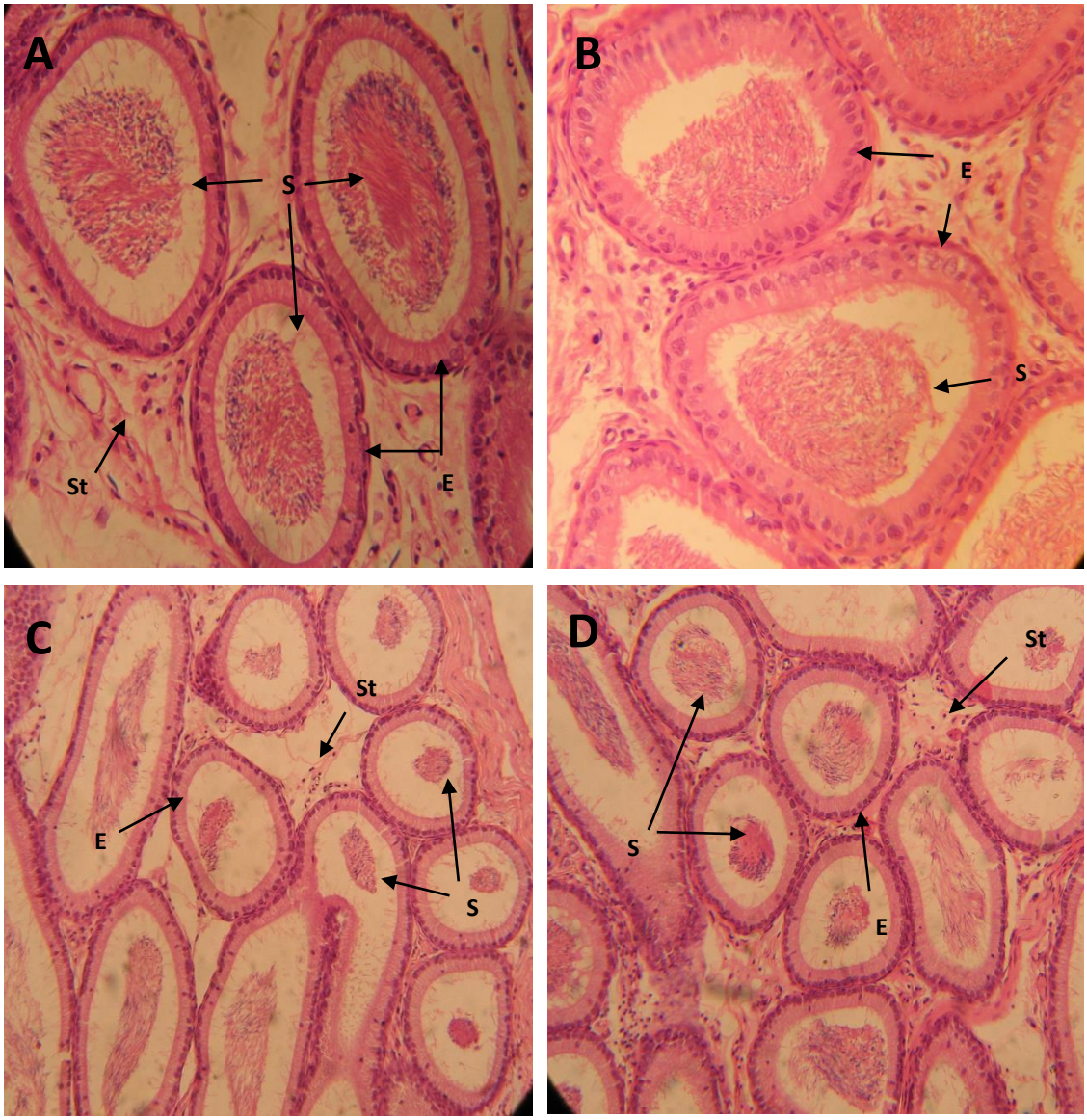


Figure 65: Photomicrograph of cauda epididymis of adult male rats receiving doses of *C. ambrosoides* showing (A) Control; exhibiting normal morphology of cauda epididymis; compactly arranged tubules with thick epithelium, lumen filled with mature sperms, (B) low dose treated group; showing marked changes in structure of tubule with thick epithelium while lumen filled with sperms, (C) medium dose treated group; showing irregular arrangement of tubules surrounded by stroma, lumen has very little number of spermatozoa, (D) High dose treated group; showing further maximum increase in epithelial height and a little in lumen sperm concentration. Spermatozoa (S), Epithelium (E), Stroma (St). Magnification 40X.

Table 25: Effect of *C. ambrosides* leaf extract on mean ± SEM of ductular diameter (µm), luminal diameter (µm) and epithelial cell height (µm) of epididymis in control and treated groups.

Groups	Ductular diameter (µm)		Lumen diameter (µm)		Epithelial height (µm)	
	Caput	Cauda	Caput	Cauda	Caput	Cauda
Control	353.58±8.14	451.44±15.48	228.12±19.47	410.78±12.94	31.36±2.30	36.90±1.79
50 mg/kg	323.93±18.49	446.86±9.44	226.65±11.55	411.57±9.01	26.70±0.99	35.58±1.74
100 mg/kg	290.77±11.92*	447.40±10.02	234.67±8.77	399.18±8.57	25.81±1.04*	30.33±1.36*
150 mg/kg	273.07±21.04	442.46±15.10	235.70±27.15	403.95±13.80	21.45±1.61*	32.81±2.28

Values are expressed as mean ± SEM

*,**,*** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

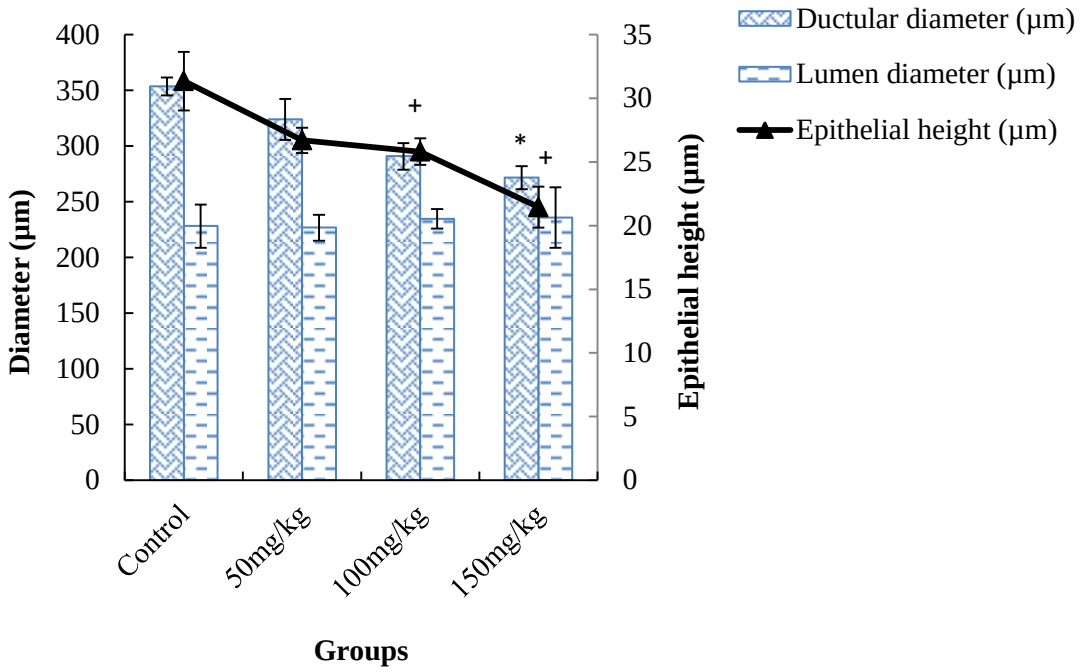


Figure 66: Mean ± SEM of ductular diameter (µm), lumen diameter (µm) and epithelial height (µm) in caput of control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

*= indicates significant difference of ductular diameter at probability of $p<0.05$ as compared to control.

+= indicates significant change of epithelial height at probability of $p<0.05$ compared to control.

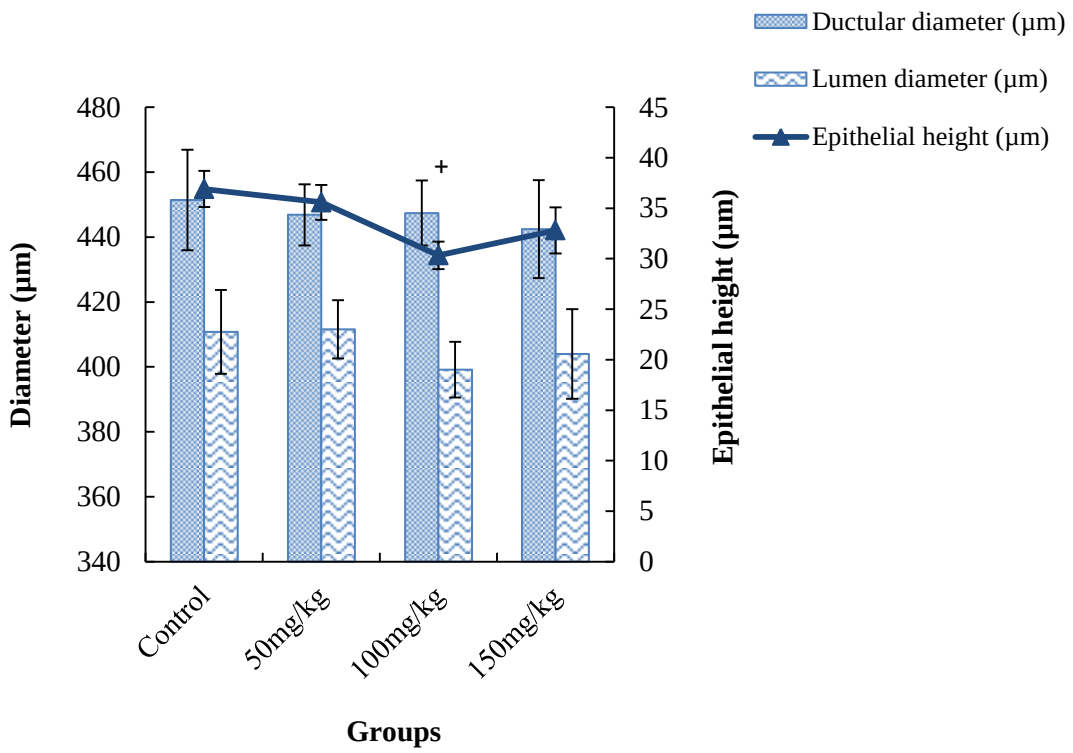


Figure 67: Mean ± SEM of ductular diameter (µm), lumen diameter (µm) and epithelial height (µm) in cauda of control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

+= indicates significant difference of epithelial height at probability of $p<0.05$ compared to control.

Hormonal Analysis

The concentrations of plasma Testosterone (ng/ml), LH and FSH in adult male rats following 28 days of treatment has been given in Table 26 & figure 67. Plasma testosterone levels were reduced significantly ($p<0.05$) in 50 mg/kg and 100 mg/kg dose treated groups when compared with control group. Similarly, a significant reduction ($p<0.001$) was also seen in 150 mg/kg extract treated group when it was compared to control group.

Plasma LH concentration (IU) was decreased in all extract treated groups when compared to control group but this reduction was not significant. Similarly, plasma FSH levels were decreased in all extract treated groups when compared to control. A significant decrease in plasma FSH concentration (IU) was observed in 50 mg/kg and 150 mg/kg extract treated rats ($p<0.05$ and $p<0.01$ respectively) while non-significant change in 100 mg/kg dose treated groups was seen in comparison to control group (Table 26, Figure 68).

Table 26: Effect of *C. ambrosides* leaf extract on mean ± SEM plasma Testosterone (ng/ml), LH (IU) and FSH (IU) concentrations in control and treated groups.

Groups	Plasma Testosterone concentration (ng/ml)	Plasma LH concentration (IU)	Plasma FSH concentration (IU)
Control	8.58±0.39	1.53±0.17	2.38±0.16
50mg/kg	5.83±0.92*	1.06±0.06	1.14±0.32*
100mg/kg	5.52±0.35*	1.25±0.12	1.24±0.40
150mg/kg	3.20±0.61***	1.24±0.20	0.47±0.09**

Values are expressed as mean ± SEM

*,**,*** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

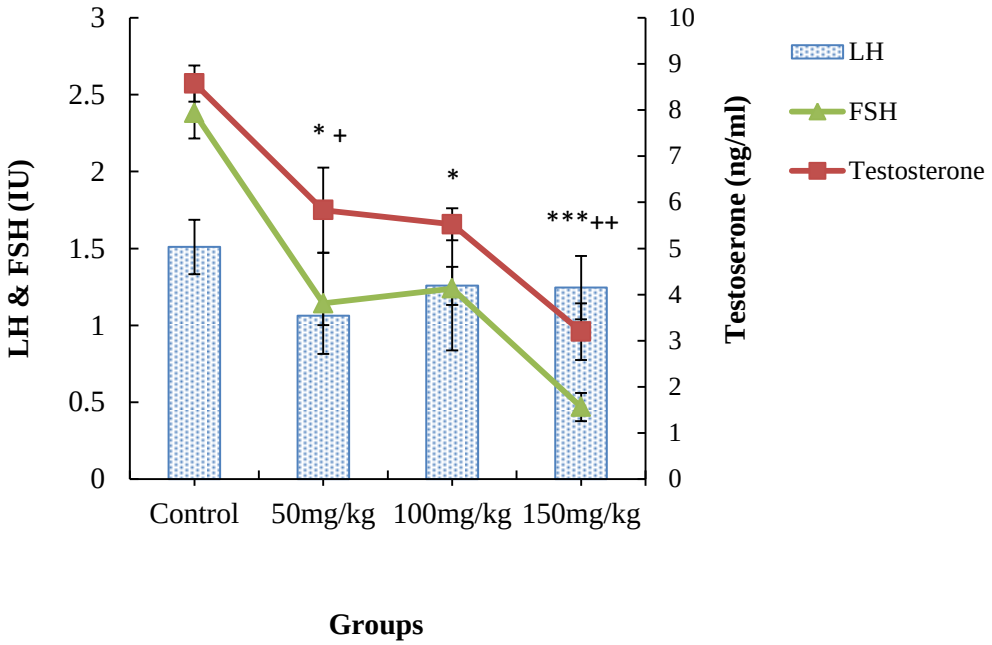


Figure 68: Mean ± SEM of plasma Testosterone, LH and FSH concentration in control and treated rats receiving methanolic leaf extract of *C. ambosioides* for 28 days.

*,***= indicates significant difference of Testosterone at probability of $p<0.05$ and $p<0.001$ as compared to control.

+,++= indicates significant difference of FSH at probability of $p<0.05$ and $p<0.01$ compared to control.

DISCUSSION

In a developing country, involvement of males in family planning is restricted due to lack of literacy, education and access to healthcare facilities. One basic aim of research is to develop herbal contraceptive drugs for men that are more suitable for protective motives and monetary reasons (Naik *et al*., 2016). Numerous therapeutic plants are known to own wide-ranging antifertility properties in male, usually affecting spermatogenesis/steroidogenesis in the testis (Brandão-Costa *et al*., 2016; Naik *et al*., 2016; Patel *et al*., 2017) . The medicinal significance of wormseed or Mexican tea, *C. ambrosioides* is commonly acknowledged in various regions of the world where several studies have testified that the plant possess antileishmanial, antifungal, nematicidal, antischistosomial properties, antiaflatoxigenic and antioxidant activity (Kumar *et al*., 2007; Chekem *et al*., 2010; Bai *et al*., 2011; Moilo *et al*., 2014; Monzote *et al*., 2014). Previously no study has been carried out to reveal the contraceptive potential of this plant, therefore, the present studies was conducted and the results showed that methanolic leaf extract of *C. ambrosioides* exerts antifertility effects on male reproductive system of adult rats as depicted by significant reduction in fertility rate.

In present study, administration of *C. ambrosioides* leaf extract for 28 days in adult male rats induced highly significant reduction in sperm motility and viability among all the extract treated groups I, II, III (50mg/kg, 100mg/kg, 150mg/kg) as compared to control group (0mg/kg). Reduced fertility rate and pregnancy outcomes in females mated with treated males were also observed. Complete spermatogenic arrest is not necessary for male contraception; fertility can be eliminated by altering structure or function of spermatozoa (Dwivedi *et al*., 1990). Many studies have related the plant based male sterility with decreased sperm number and sperm motility (Watcho *et al*., 2001; Thanga *et al*., 2012). The results were in line with studies of (Ramya and Shivanandappa, 2017). This reduction might be due to capability of plant extract to cross blood testes barrier (BTB) and either hinder with normal process of spermatogenesis in seminiferous tubules either by effecting sperm proteins and sertoli cells, altering epididymal function and effecting motility of mature spermatozoa, or altering androgen synthesis and feedback regulation of HPG axis shifting spermatogenesis as suggested by Naik *et al.* (2016) in their respective study (Naik *et al*., 2016).

The sperm count and motility rate determine the fertility status of an individual (Ghosh *et al*., 2015). A marked decline in epididymal sperm count was observed in dose dependent manner in our study when adult male rats were exposed to varied doses (50, 100, and 150mg/kg) of methanolic extract of *C. ambrosioides* for 28 days. Similar findings were also previously reported by Ramya and Shivanandappa, (2017) in their studies where treatment of male rats with methanol fruit extract (300, 900 mg/kg bw) for 60 days resulted in reduced sperm number in the epididymis (Ramya and Shivanandappa, 2017).

A significant reduction in daily sperm production was also detected in all the extract treated (50, 100, and 150 mg/kg) groups as compared to control group. The exact mechanism by which the methanolic extracts decreased sperm count is not known, but it has been suggested that the plant components may have crossed the blood testes barrier to exert detrimental effects on seminiferous tubules of the testes. Reduction in sperm count indicates alteration in normal steroidogenesis and disruption of morphological organization in testes, compromising semen quality as suggested by Ghosh *et al*. in their respective work (Ghosh *et al*., 2015).

A balance between oxidants/antioxidants is necessary to be maintained to prevent body from oxidative stress and reproductive impairments (Ghosh *et al*., 2015). The current study presented that oral administration of adult male Sprague Dawley rats with different concentrations of *C. ambrosioides* leaf extract for 28 days of trial shifted normal oxidative/antioxidant status of body and induced production ROS. The antioxidant enzyme activity was measured for two important enzymes, CAT and SOD whose levels were found significantly reduced in a dose dependent manner in testes. Both are found to have free radical scavenging activity in male reproductive organs. POD is another enzyme involved in cell defense mechanism which converts hydrogen peroxide into water through oxidation-reduction reaction and prevents cell damage and death. Its levels were also significantly reduced in present study suggesting increased production of free radical byproduct such as TBARs and reactive oxygen species. The results of our study are in agreement with that of Ghosh *et al*. (2015) where increased level of free radical by-products such as CD and TBARS in the testicular tissues reinforced the generation of ROS, when Aqueous-ethanolic (1:1) extract of *T. chebula* fruit was orally administered at a dose of 60 mg/0.5 mL distilled water/day to adult fertile male rats for 28 days of experiment (Ghosh *et al*., 2015).

Histopathological studies of testes showed methanolic extract of *C. ambrosioides* caused a drastic and dose dependent reduction in number of spermatogonia, spermatocytes and mature spermatozoa as well as sloughing off of germinal epithelium in all the dose treated groups (50, 100, and 150 mg/kg). Seminiferous tubules were visible with empty lumen, degenerated epithelial layer and increased interstitial space in 50 mg/kg group with increased tubular diameter and wider lumen spaces. With increase in dose regime (100 mg/kg of plant extract) reduced lumen diameter and amplified epithelial height was evident. Here, compact tubules with little interstitial space were present. The high dose treated group (150 mg/kg) showed maximum damage to epithelium, empty lumen and large interstitial spaces present.

The development and maturation of spermatozoa together with normal process of steroidogenesis is the key to determine prime male fertility. Both events of spermatogenesis and steroidogenesis within testes are controlled by normal production and release of gonadotropins LH and FSH coming from anterior pituitary (Naik *et al*., 2016). Testosterone is produced from two sources, first from Leydig cells as a result of positive induction with LH, secondly from medullary region of kidney. The appropriate levels of testosterone are necessary to carry out normal functions of testes. The present findings showed that methanolic leaf extract of *C. ambrosioides* affected the normal function of Leydig cells resulting in significantly reduced production of plasma and intratesticular testosterone levels in a dose dependent manner. Reduced testosterone concentrations are contributed by disturbed functioning of Leydig cells in male rats thus encouraging spermatogenic arrest and infertility.

Conclusion

Thus, it is concluded that methanolic leaf extract of *C. ambrosioides* caused partial male sterility by disturbing spermatogenic cycle, inducing oxidative stress and hormonal imbalance. Although, sperm motility and density was also reduced but fertility suppression was reversible after cessation of treatment. As no fetal mortality or morbidity was evident, so it is suggested that *C. ambrosioides* is capable to suppress male fertility without adverse toxicity.

CHAPTER 4:

Possible antifertility effects of methanolic leaf extract of *Ajuga integrifolia* Buch. Ham- An *in vivo* approach.

ABSTRACT

Contraception is a matter of worldwide and nationwide public health concern. The practice of herbalism has become conventional these days. *Ajuga integrifolia* (Lamiaceae) is the herb traditionally used for contraceptive purposes in men in different regions of the world. However, scientific knowledge about the contraceptive potential of this plant is unknown. The current study was designed to assess antifertility activity of methanolic leaf extract of *A. integrifolia* on adult male rats. In this study, in vivo experimental approach was used to see effect of different concentrations of plant extract on reproduction. Rats were exposed to different doses of plant extract for twenty-eight consecutive days. Sperm parameters (motility, viability, daily sperm production, epididymal sperm count), biochemical (CAT, SOD, POD, ROS and TBARS) and plasma hormonal concentrations (Testosterone, LH and FSH) were evaluated. In addition, histology of testicular and epididymal (caput and cauda) tissues was performed. Fertility test was performed to check reversibility of the plant extract activity. Significant reduction in sperm motility, viability and daily sperm production was evident in high dose treated groups. Similarly, plant extract exposure caused reduction in epididymal sperm production exhibiting spermicidal activity of this plant. Significant decrease in antioxidant activity while increase in reactive oxygen species was observed in high dose treated groups as compared to control. Histological results of tests showed normal arrangement of seminiferous tubules while sloughing of epithelium with wider lumen was observed in high dose treated groups. Similarly, in epididymal tissue, thin epithelium with empty lumen was seen only in high dose treated group compared to control. In addition, plasma concentration of Testosterone, LH and FSH was also reduced significantly in the treated groups. Fertility test of present study also found reduced pregnancy outcome in the females paired with treated male rats. While number of pups were reduced non significantly in high dose treated animals. Findings of current study recommend that methanolic leaf extract of *A. integrifolia* has the capacity to halt normal process of spermatogenesis by reducing sperm production and hormonal secretion, and inducing oxidative stress in the testis of adult rats.

INTRODUCTION

During the last few decades, use of medicinal plants and herbal products is considered as one of the most reliable and hot topic among researchers around the globe. A large number of study groups are engaged in investigating the medicinal properties of plants. Contraception simply means the inhibition of conception or use of those chemicals or drugs which prevent pregnancy. Contraception is a matter of worldwide and nationwide public health concern (Sharma *et al*., 2013). For this purpose, numerous approaches are being practiced to reduce total fertility rate in both men and women especially in emerging countries (Lampiao, 2011). However, male contraception has gain attention and requires further investigation. Although a large number of immunological, hormonal and chemical substances have been assessed for male contraception but none of them is totally desirable and free of side effects (Harat *et al*., 2008). Several side effects associated with these synthetic agents include hypertension, weight gain, hormonal imbalance and increased risk of cancers (Kumar *et al*., 2012). Although, it is usually accepted that plant derived medicines are more effective and dependable than their synthetic counterparts, however, the toxicity profile of most therapeutic plants has not been completely assessed (Vongtau *et al*., 2005; Jahan *et al*., 2009b). The practice of herbalism has become these days conventional worldwide.

Although extensive progress has been made in the development of highly safe, effective and acceptable approaches of birth control in females, contraceptive options are limited and slow in men. On a global scale, practicing male based contraceptive approaches accounts for about 14% contraceptives (Jain *et al*., 2012). In this regard, many researchers have shown quite interested to discover plants having contraceptive potential to regulate male fertility (Chowdhury *et al*., 2001; Kamal *et al*., 2003; Jahan *et al*., 2009b). This would certainly address the issues of population explosion and its negative effects on natural resources.

Ajuga L. is a genus of annual and perennial herbaceous flowering plants belonging to mint family Lamiaceae. Members of this family are being used in traditional medicine and in insect control (Kew *et al*., 2010). Most of its species are native to Africa and Asia. Some of the pharmacological properties possessed by different species of Ajuga include antimalarial (Kuria *et al*., 2001; Cocquyt *et al*., 2011), antiplasmodial (Asnake

et al., 2015) antifungal (Kariba, 2001) and anti-inflammatory (Gautam *et al*., 2011) activities. It is also used in treatment of skin diseases, fever, toothache, dysentery, headache, and high blood pressure (Kariba, 2001).

Ajuga integrifolia is low growing, erect and perennial herb, harvested for local medicinal use and potential source of essential oil. It is found in Northeast and east tropical Africa, Indian Subcontinent, Afghanistan, China, Indonesia, Philippines and New Guinea. In Pakistan, it is located in Murree, Swat, Shangla, Dir and many regions of Punjab province. The specie *A. integrifolia* is found to have anti-HIV activity (Asres *et al*., 2001), antimalarial as well as antiplasmodial activity (Asnake *et al*., 2015). Traditionally, decoction of the fresh leaves is drunk by Ethopian people to treat diarrhoea and malaria while, paste made of leaves is applied on the affected part for treating wound (Gedif and Hahn, 2003). Another study supported its use to cure stomach disorders (Abate, 1989), high blood pressure and diabetes (Jansen, 1981). The herb is traditionally used among Pakhtun population of Shangrilla, Dir and its locality for contraceptive purposes in men.

Figure 69. Plant *Ajuga integrifolia* Buch. Ham in its natural habitat.

Indigenous uses for Antifertility: Fresh plant is powdered and then extract is used before meal twice daily.

Taxonomy

The taxonomy of the plant according to National Plant Data Center, NRCS, USDA is as follows

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Lamiales

Family: Lamiaceae

Genus: *Ajuga* L.

Specie: *Ajuga integrifolia* Buch. Ham.

However, the knowledge about the contraceptive potential of the plant is unknown. The current investigation reports the contraceptive efficacy and toxicity if any, of the methanolic leaf extract of *A. integrifolia*.

MATERIALS AND METHODS

Plant collection and extract preparation

Ajuga integrifolia Buch. - Ham, locally called as 'Kohri booti' is the herb found in many regions of Northern areas of Pakistan including Margalla Hills, Sawat, Shangrilla, Mansehra, Kaghan and Naran valley. It was collected from agricultural fields of District Mansehra (Hazara), Pakistan at elevation of 1088 meters. The botanical identification was done by Dr. Mushtaq Ahmad at Plant Systematics and Biodiversity Lab and sample was deposited in the herbarium of Quaid-i-Azam University Islamabad, under the accession number # 77695. Methanolic leaf extract of *A. integrifolia* was prepared according to method reported by elswhere (Gulfraz *et al*., 2007). Detailed procedure is described in chapter 1.

Animals

Forty adult male Sprague Dawley rats weighing 150±10g were got from rodent colony of Animal Sciences Department, Quaid-i-Azam university Islamabad. Rats were housed in separate stainless steel cages in well ventilated rooms one week prior to start of experiment for acclimatization. Room temperature of 20-26 ºC and 12 h dark/12 h light cycle was maintained. Rats were provided with standard laboratory feed and water ad labitum. All the animal handling and protocols were ratified by ethical committee of Animal Sciences department, QAU.

Experimental Design

This study was conducted to find out antifertility effect of methanolic extract of *A. integrifolia* on adult male rats. For this, acute toxicity and in vivo approaches were used. In the in vivo experiment, effect of oral administration of plant extract for 28 days on reproduction of male rats was evaluated.

Acute oral toxicity

Acute oral toxicity study of methalonic leaf extract of *A. integrifolia* was carried out according to method described in chapter 3.

In vivo experiment

Forty adult male Sprage Dawley rats (80-90 days old) were randomly placed into four study groups (n=10 rats/group). Rats were administered with varied concentrations of

plant extract (0, 50, 100 and 150 mg/kg/day) orally for twenty-eight consecutive days. Stock solution of plant extract was made in methanol and further diluted with normal saline. Final methanol concentration was 0.5-1% in saline. Different doses used in this study were selected according to previously reported studies (El Hilaly *et al*., 2004) and OECD guideline # 408.

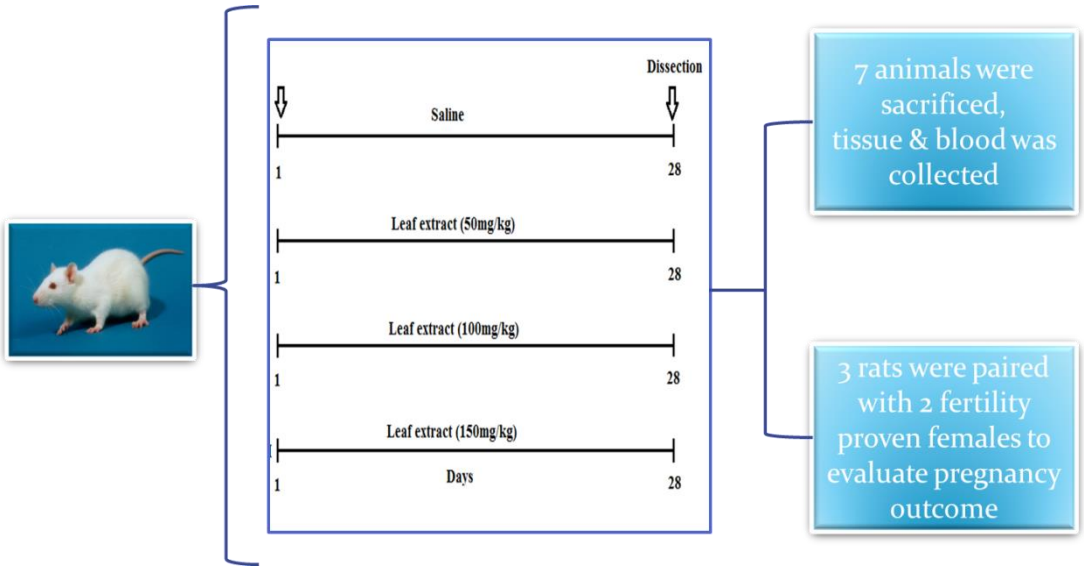


Figure 70: Schematic representation of experimental design of *in vivo* exposure to *A. integrifolia* methanolic leaf extract.

At day 29, seven animals from each group were weighed and decapitated. Blood was immediately taken in heparinized syringes and centrifugation was done at 3000 rpm for 15 minutes. Plasma was separated and kept at -20ºC until analysed. Both testicular and epididymal tissues were removed and washed with physiological saline. Left testes was stored in freezer for analysis of daily sperm production and biochemical investigation while, left epididymis was minced for further assessment of epididymal sperm count and other sperm parameters. Right testicular and epididymal tissue were fixed in 10% formalin for histological studies.

Fertility test:

Fertility test was done according to method described in previous chapter.

Assessment of Sperm Motility and Viability

Sperm motility and viability was determined as explained in chapter 3. (Halvaei *et al*., 2012).

DSP and Epididymal sperm count

Epididymal sperm count was done by using method described by Thanga *et al*. 2012 (Thanga *et al*., 2012). While DSP was performed according to protocol given by (Robb *et al*., 1978). Detailed methodology of epididymal sperm count and DSP is explained in chapter 3.

Biochemical Analysis

Antioxidant enzymes were estimated in testicular tissues. The stored samples were thawed and homogenized in 3 ml of phosphate buffer saline (pH 7.4). The homogenate was processed through centrifugation at 30,000 rpm for 30 minutes at 4 °C. Supernatant was collected for determination of antioxidant status of SOD, POD, CAT, TBARS and ROS according to previously reported methods (Chance and Maehly, 1955; Wright *et al*., 1981; Kakkar *et al*., 1984; Iqbal *et al*., 1996; Hayashi *et al*., 2007) described in previous chapter.

Histopathological analysis

Histological analysis was performed according to method described in chapter 1. Testicular and epididymal tissues fixed in 10% formalin were dehydrated in ascending alcoholic grades. After that, tissues were washed with xylene, embedded in paraffin wax and mounted on wooden blocks with the help of melted wax. Seven µm thin sections were cut using microtome. The ribbons were stretched in warm water followed by fixation in clean albumenized glass slides and placed in incubator (40 ºC) over night for complete deparafenizationt. Tissues were rehydrating by descending alcoholic grades followed by Hematoxylene and Eosin staining and permount with DPX. Microphotography was done by using high magnification microscope equipped with digital camera. Image J2x software was used for morphometric analysis.

Hormonal Analysis

Plasma Testosterone concentration was measured quantitatively by using enzyme linked immune sorbent assay (ELISA) kit of Biocheck Inc, USA. While Plasma LH

and FSH concentrations were measured by ELISA kit of Reddot biotech INC. Detailed procedure is given in previous chapter.

Statistical analysis

All observed data was subjected to one-way analysis of variance (ANOVA) followed by Dunnet test for comparison of different groups with control group using Graph Pad Prism 5 software. All the results are shown in Mean ± SEM. Significance level was set at $p< 0.05$.

RESULTS

Acute toxicity

Acute toxicity of methanolic leaf extract of *A. integrifolia* in rats after single dose administered orally ranging from 5 - 2000 mg/kg body weight is given in table 27. No mortality or symptoms of morbidity were observed at all the tested doses. Furthermore, no prominent variation in behavior or eating habit were noted in animals.

Fertility Test:

Effect of *A. integrifolia* leaf extract on the fertility and mean number of pups born per female after 60 days of treatment withdrawal is given in table 28. Vaginal smear collected from untreated female rats mated with high dose treated males showed reduced number of sperm. Percent fertility 66%, 50% and 33% was noted in 50 mg/kg, 100 mg/kg and 150 mg/kg extract treated groups respectively. While number of pups were reduced non significantly in high dose treated animals (Figure 71).

Table 27. Results of acute toxicity study in rats after single oral dose of methanolic leaf extract of *A. integrifolia.*

Dose (mg/kg body weight)	Number of animals	Short-term result (24 h)	Long-term results (14 days)
0	3	Survival	Survival
5	3	Survival	Survival
50	3	Survival	Survival
300	3	Survival	Survival
2000	3	Survival	Survival

Table 28: Effect of *A. integrifolia* leaf extract on the fertility and the Mean ± SEM number of pups born per female after 60 days of treatment withdrawal.

Groups	**Percent fertility**	**Number of pups born/female**
Control	100	7.5 ± 1.06
50mg/kg	66.66	4.83 ± 1.60
100mg/kg	50	3.83 ± 1.74
150mg/kg	33.33	2.83 ± 1.80

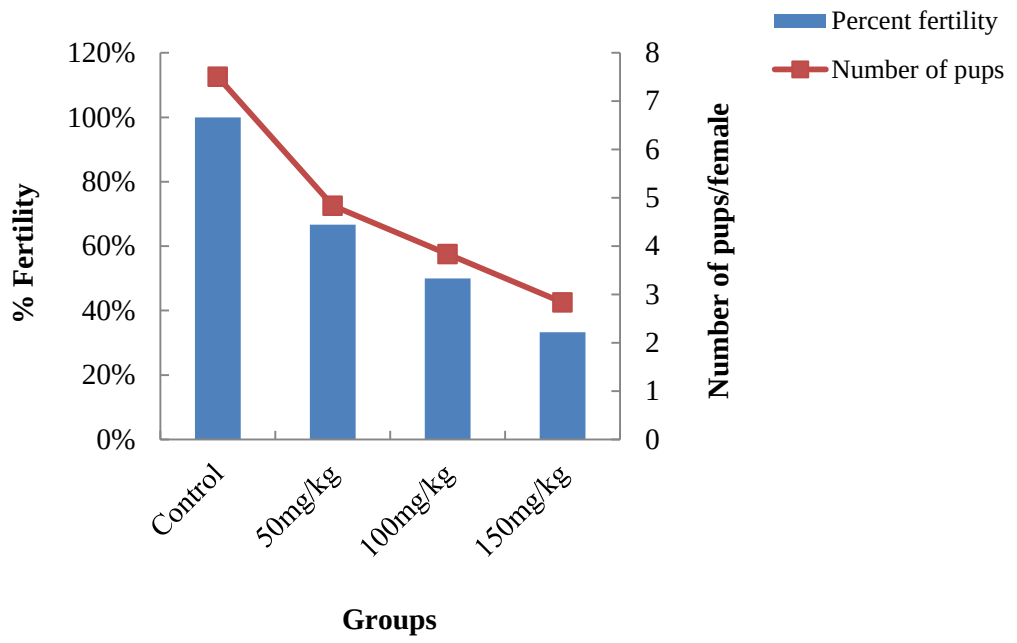


Figure 71: Effect of *A. integrifolia* leaf extract on % fertility and the number of pups born per female after 60 days of treatment withdrawal.

Effect of extract on body weight gain and reproductive organ weight

Body weight gain was significantly changed in all extract treated groups when comparison was made with control group animals (Table 29, Figure 72). Similarly, both left and right testicular weight in 50 mg/kg and 150 mg/kg was decreased significantly ($p<0.05$ & $p<0.01$ respectively) when compared with control group (Table 29, Figure 73).

Animals of 50 mg/kg and 100 mg/kg extract treated groups displayed significant ($p<0.05$) reduction in epididymal weight while non-significant difference was noted in 100 mg/kg treated group when compared with control group. In addition, Similarly, seminal vesicle weight was also decreased significantly ($p<0.001$) in all dose treated groups. While no change was noted in prostate weight in all extract treated groups (Table 29).

Table 29: Effect of *A. integrifolia* leaf extract on mean ± SEM body weight gain (g) and reproductive organ weight (g) of control and treated groups.

Parameters	Groups			
	Control	**50mg/kg**	**100mg/kg**	**150mg/kg**
Body weight gain (g)	61.6 ± 2.46	40.6 ± 2.48**	29.4 ± 2.00***	39.0 ± 5.55***
Right testicular weight (g)	1.31 ± 0.03	1.18 ± 0.02*	1.21 ± 0.02	1.10 ± 0.03***
Left testicular weight (g)	1.29 ± 0.01	1.13 ± 0.05*	1.23 ± 0.03	1.09 ± 0.03**
Epididymus weight (g)	1.17±0.03	0.98±0.04*	0.93±0.04*	1.01±0.04
Seminal vesicle weight (g)	1.15 ±0.05	0.84±0.07**	0.73±0.07***	0.71±0.03***
Prostate weight (g)	0.77±0.04	0.67±0.03	0.61±0.04	0.63±0.04

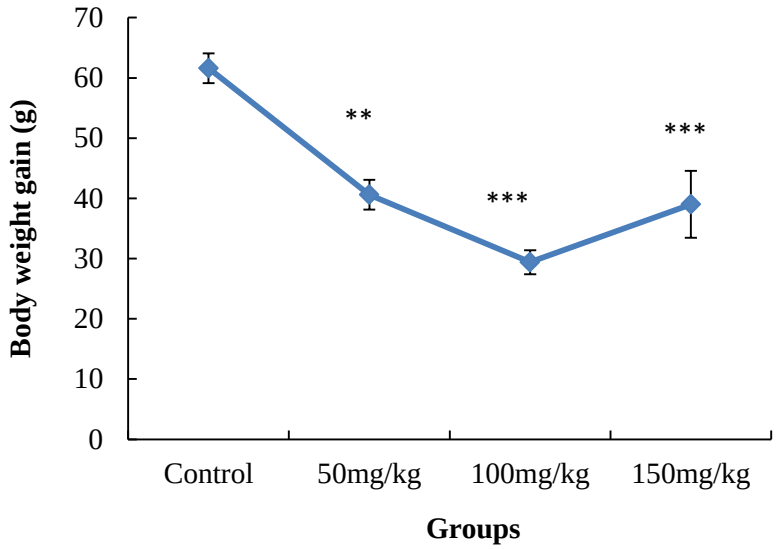


Figure 72: Mean ± SEM body weight gain (g) of control and treated groups of rats, receiving methanolic leaf extract of *A. integrifolia* for 28 days.

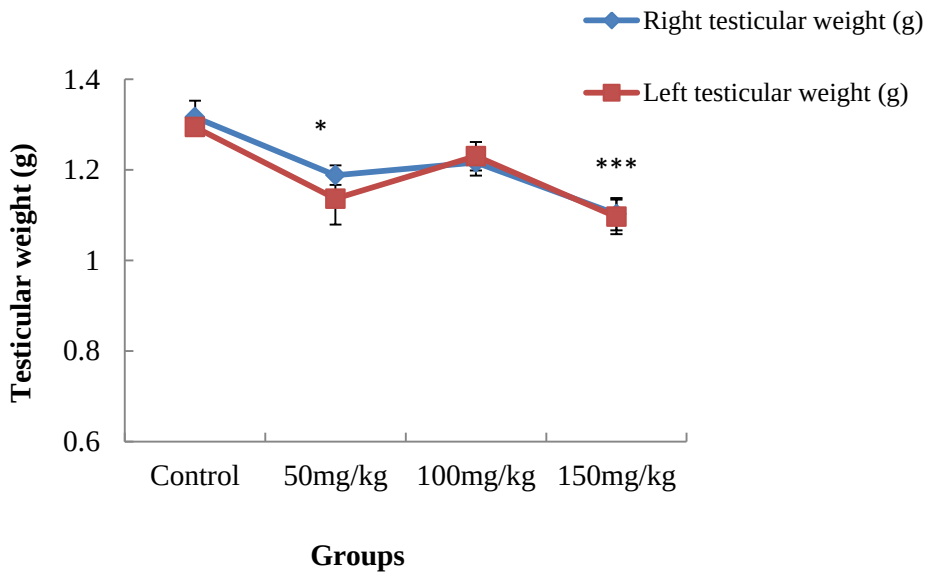


Figure 73: Mean ± SEM of Testicular weight gain (g) of control and treated groups of rats, receiving methanolic leaf extract of *A. integrifolia* for 28 days.

*,**,***= indicates significant difference at probability of $p<0.05$, $p<0.01$ and $p<0.001$ compared to control.

Effect of extract on sperm parameters

A significant reduction in sperm motility ($p<0.001$) and viability rate ($p<0.05$) was seen in 100 mg/kg group while 150 mg/kg ($p<0.001$) treated group caused reduction in both parameters at probability value of $p<0.001$. No prominent change was noted in 50 mg/kg treated rats as compared with control group (Table 30, Figure 74).

Mean ± SEM of epididymal sperm count in control and treated groups are shown in Table 3. Non significant change was seen in epididymal sperm count of 50 mg/kg group as compared to control. However, significant reduction in sperm number of caput ($p<0.01$), cauda ($p<0.01$) and corpus ($p<0.05$) was observed in 100 mg/kg dose treatment group. Similarly, 150 mg/kg treated rats exhibited significant decrease in caput ($p<0.05$), corpus and cauda ($p<0.001$) sperm count when compared with control group animals (Table 30, Figure 75).

A remarkable decrease in daily sperm count was evident in all the experimental groups exposed to methanolic leaf extract of *A. integrifolia*, as compared to control group. However, highly significant decrease ($p<0.001$) was only noticed in 150 mg/kg treated group as compared to control (Table 30, Figure 76).

Table 30: Effect of *A. integrifolia* leaf extract on mean ± SEM sperm parameters of control and treated groups.

Parameters	Groups			
	Control	**50mg/kg**	**100mg/kg**	**150mg/kg**
Motility rate (%)	77.2±1.64	72.8±2.80	61.4±2.36***	56.4±2.42***
Viability rate (%)	79.2±2.83	74.0±4.20	64.4±2.82*	55.8±2.72***
DSP × 10^5 /testis	8.85±0.59	7.62±0.87	6.84±0.56	3.54±0.39***
Caput sperm count	1687.50±43.89	1637.50±68.08	1543.75±37.32	1525.00±48.94
Corpus sperm count	1487.50±29.75	1418.75±37.32	1343.75±51.41	1256.25±97.56*
Cauda sperm count	1956.25±38.69	1806.25±27.71	1731.25±21.34**	1643.75±59.83***

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

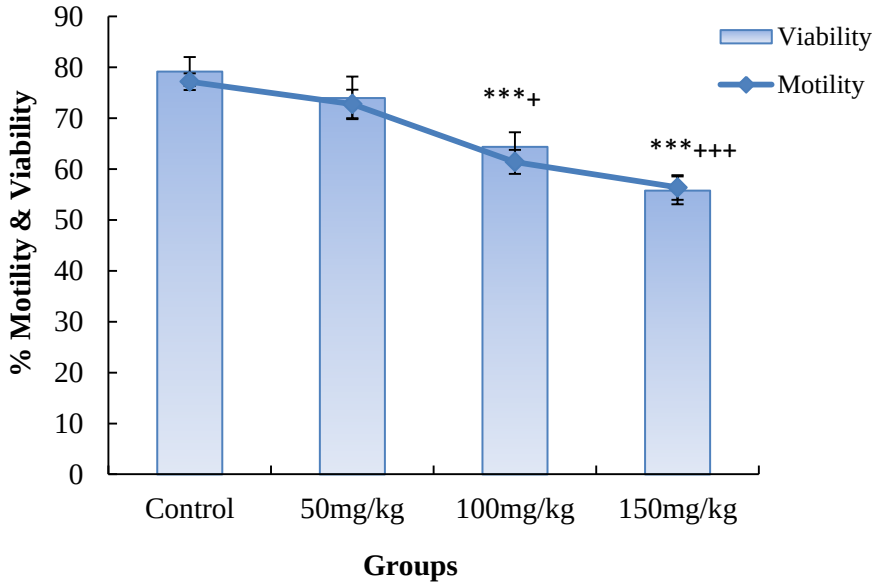


Figure 74: Mean ± SEM of % sperm motility & viability rate in control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

***= indicates significant difference of % motility at probability of $p<0.001$ compared to control.

+,+++= indicates significant difference of % viability at probability of $p<0.05$ and $p<0.001$ compared to control.

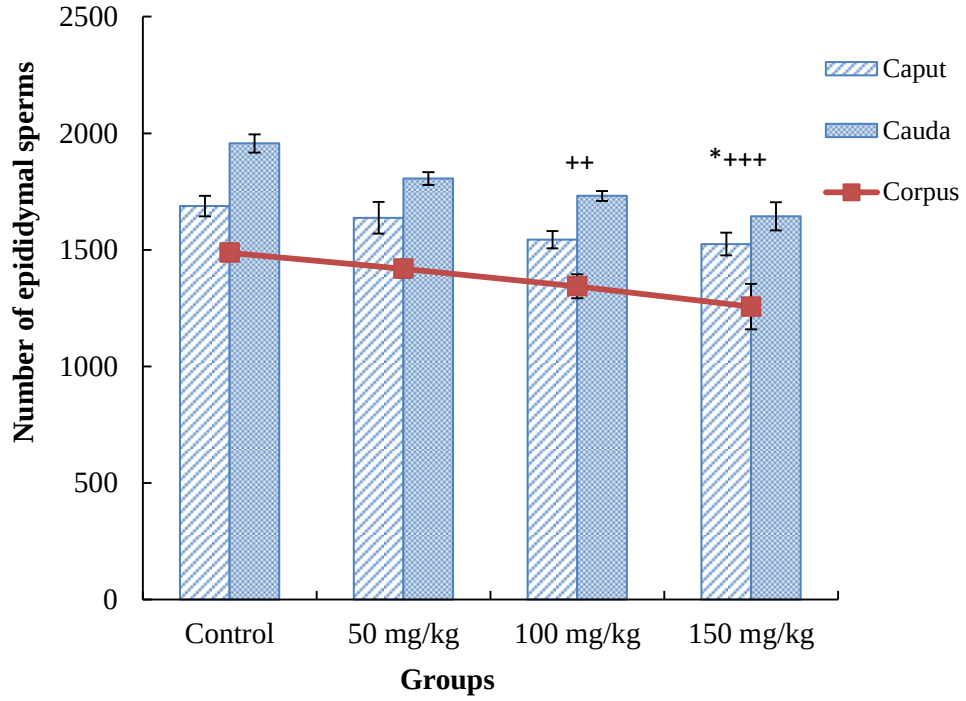


Figure 75: Mean ± SEM of epididymal sperm count in control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

*= indicates significant difference of number of corpus sperm at probability of $p<0.05$ compared to control.

++,+++= indicates significant difference of number of cauda sperm at probability of $p<0.01$ and $p<0.001$ compared to control.

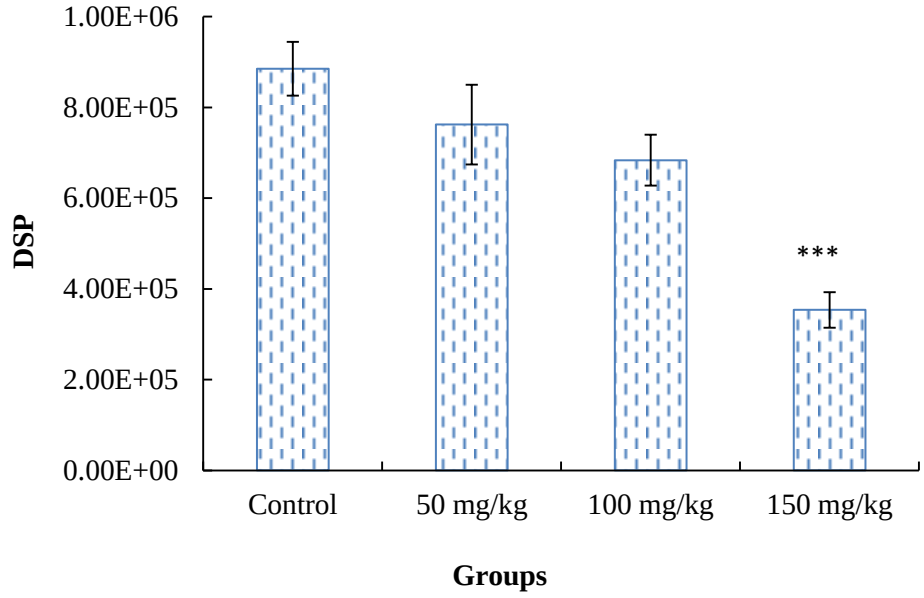


Figure 76: Mean ± SEM of DSP in control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

***= indicates significant difference at probability of p<0.001 compared to control.

Effect of extract on biochemical parameters of rat testis

A non-significant change was evident in levels of SOD while a significant decrease ($p<0.05$) in catalase enzyme within testis was seen among all the three dose (50 mg/kg, 100 mg/kg and 150 mg/kg) treated groups as compared to control (Table 31, Figure 77 & 78). Similarly, a substantial reduction ($p<0.01$) in POD level was evident only in 150 mg/kg treated group (Figure 79).

The concentrations of TBARS were gained in all the treated groups as compared to control group but significant change ($p<0.05$) was only found in 150 mg/kg treated group (Figure 80). On the other hand, number of reactive oxygen species was gradually increased in all experimental groups with increase in concentration of plant extract; however, the change was not statistically significant (Table 31, Figure 81).

Table 31: Effect of *A. integrifolia* leaf extract on mean ± SEM specific activity of antioxidant enzymes, ROS and TBARS in the testis of control and treated groups.

Groups	CAT (U/mg)	SOD (U/mg)	POD (nmole)	ROS (µmol/min)	TBARS (nM/mg)
Control	39.69±3.66	53.93±3.44	18.77±2.53	0.96±0.09	0.17±0.06
50mg/kg	21.22±4.22*	48.92±5.28	14.69±1.77	0.99±0.04	1.16±0.39
100mg/kg	22.24±4.24*	47.26±3.28	13.14±2.40	1.02±0.05	1.04±0.59
150mg/kg	20.93±3.11*	44.29±8.34	7.13±1.99 **	1.06±0.02	2.25±0.57*

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

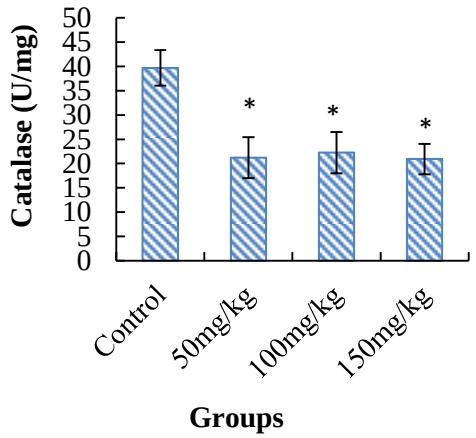


Figure 77: Specific activity of CAT in testis of control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

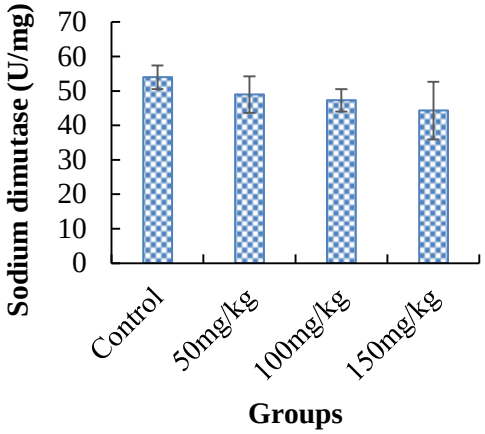


Figure 78: Specific activity of SOD in testis of control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

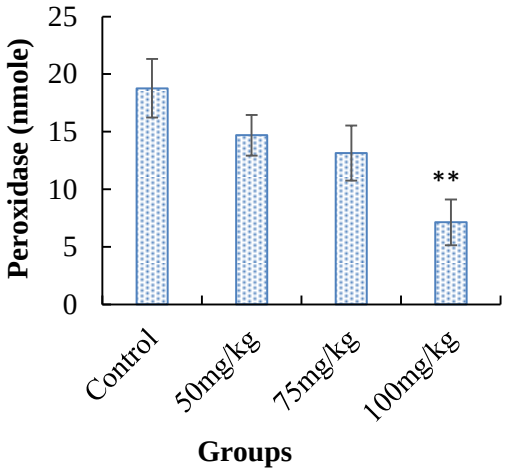


Figure 79: Specific activity of POD in testis of control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

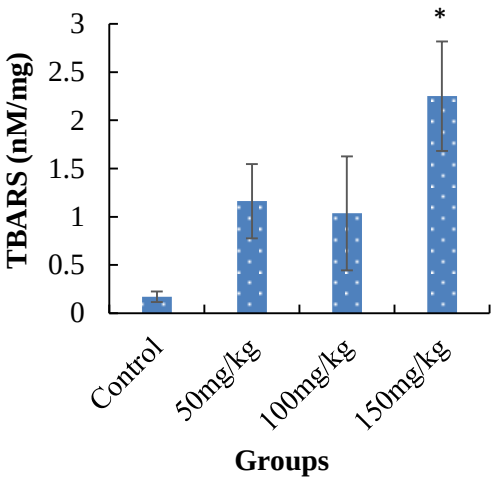


Figure 80: Specific activity of TBARS in testis of control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

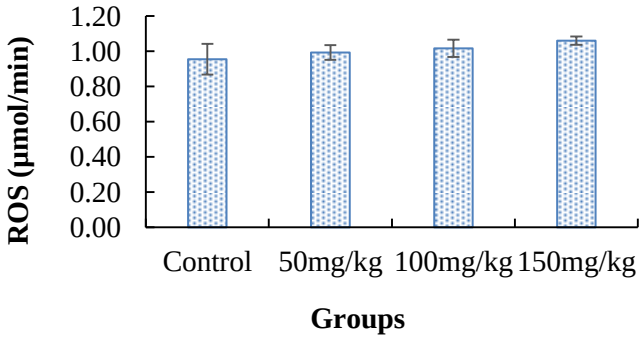


Figure 81: Specific activity of ROS in testis of control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

Effect of extract on morphological changes in rat testis and epididymis

Testis of control group showed compactly arranged seminiferous tubules with thick stratified epithelium and narrow lumen filled with round and elongated sperms. A little to no change in seminiferous tubule structure was seen in all treatment groups when compared with control. However, lumen was slightly widened with reduced number of mature sperms and thin epithelium was detected in high extract treated groups (100 mg/kg and 150 mg/kg) (Figure 82).

Similarly, tubules in caput and cauda epididymis of control group exhibited larger diameter surrounded by stroma, wider lumen and thin pseudostratified epithelium lined with sterocilia. No change in tubular arrangement was noted except widened empty lumen and sloughing in epithelium of high dose extract treated group (150 mg/kg) (Figure 83 & 84).

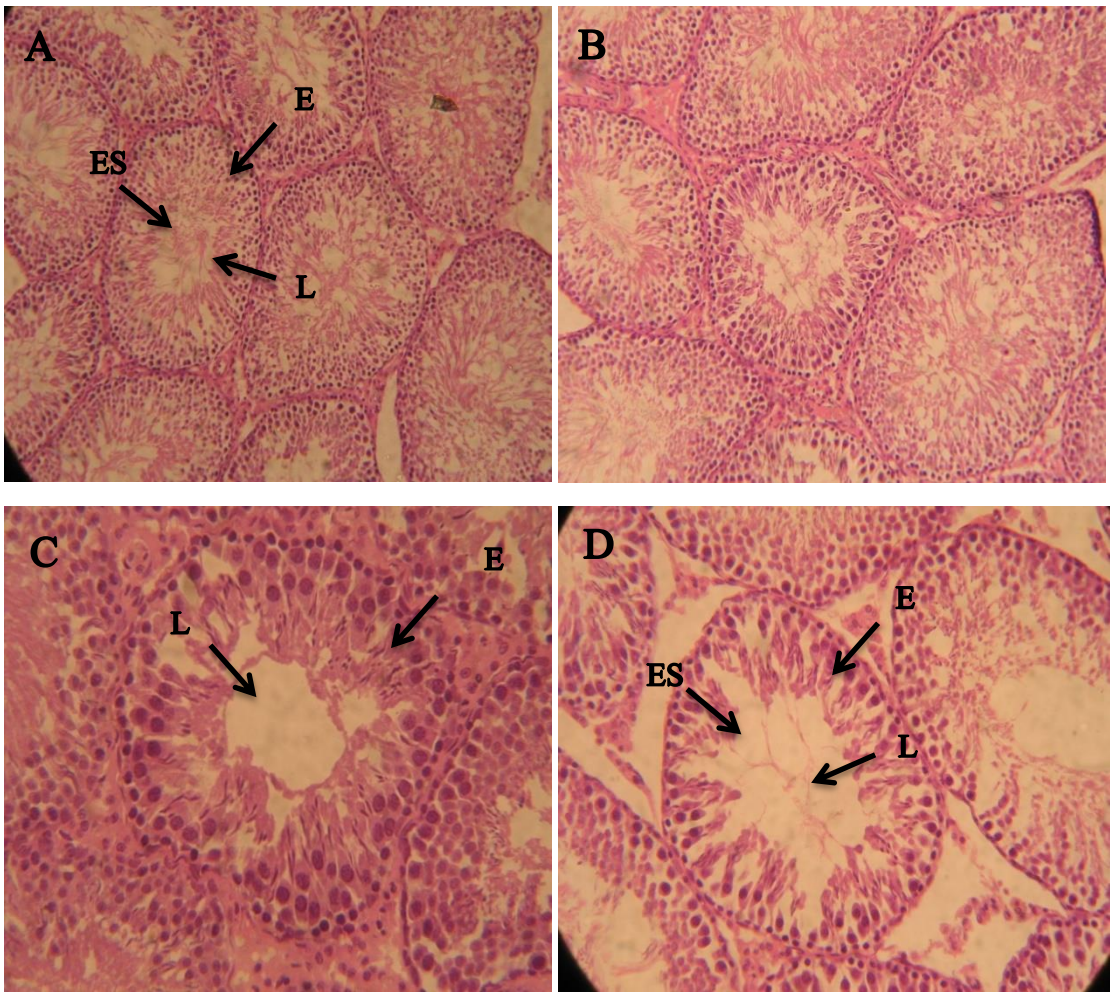


Figure 82: Photomicrograph of seminiferous tubules of adult male rats treated with methanolic extract of *A. integrifolia* (A) Control; showing compact arrangement of seminiferous tubules, thick epithelium and lumen filled with spermatids, (B) 50mg/kg group; showing normal tubules with thick epithelium and sperm filled lumen, (C) 100mg/kg group; showing sloughing of epithelium and empty lumen, (D) 150mg/kg group; showing thin epithelium, wider lumen with less number of spermatids. Lumen (L), Elongated spermatids (ES), Epithelium (E). Magnification 40X.

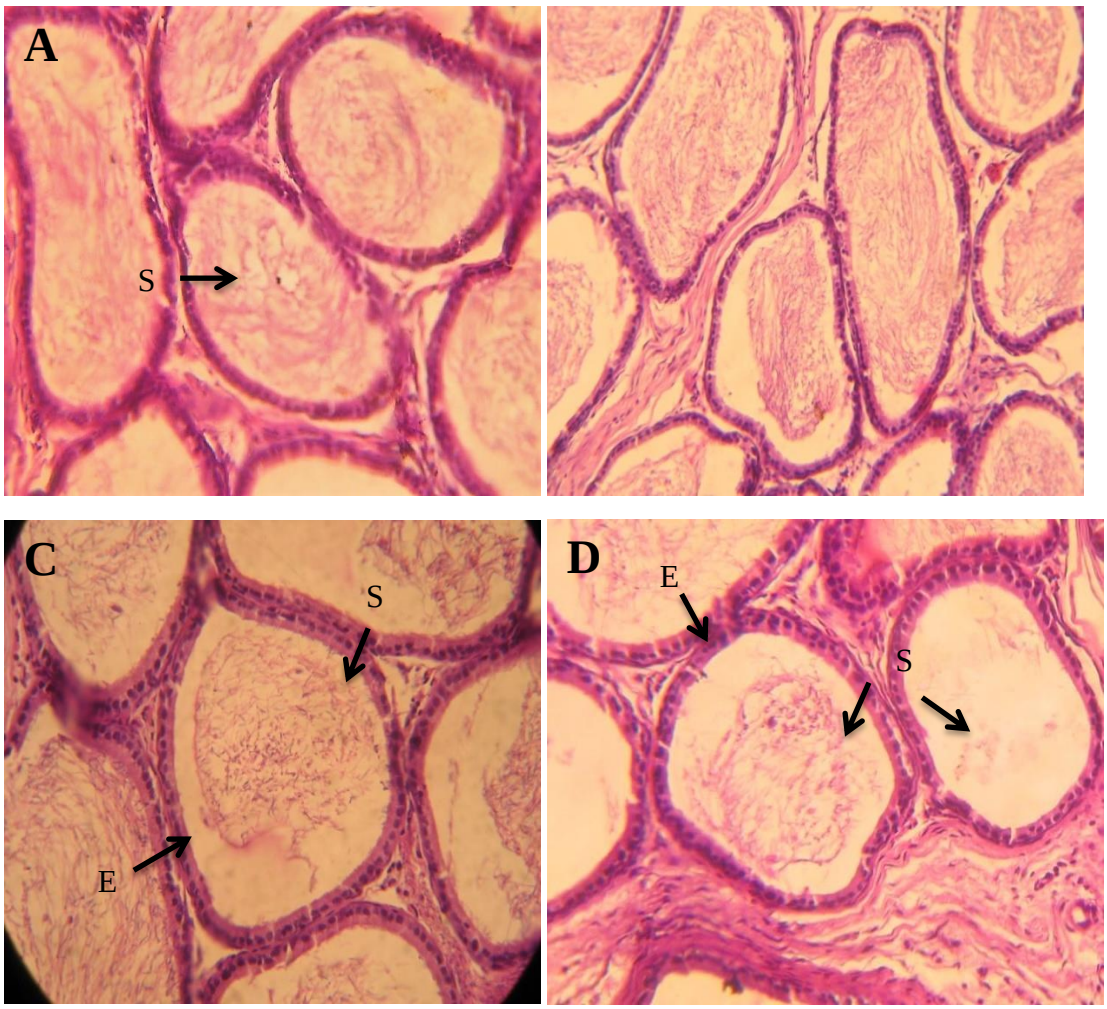


Figure 83: Photomicrograph of caput epididymis of adult male rats treated with methanolic extract of *A. integrifolia* (A) Control group; showing normal morphology of caput epididymis, thin pseudostratified epithelium lined with stereocilia and lumen filled with spermatozoa, (B) 50mg/kg group; showing compact tubules, normal epithelium and sperm filled lumen, (C) 100mg/kg group; showing less number of spermatozoa in lumen, (D) 150mg/kg group; showing normal tubular arrangement, thick epithelium and very little concentration of spermatozoa. Epithelium (E), Spermatozoa (S). Magnification 40X.

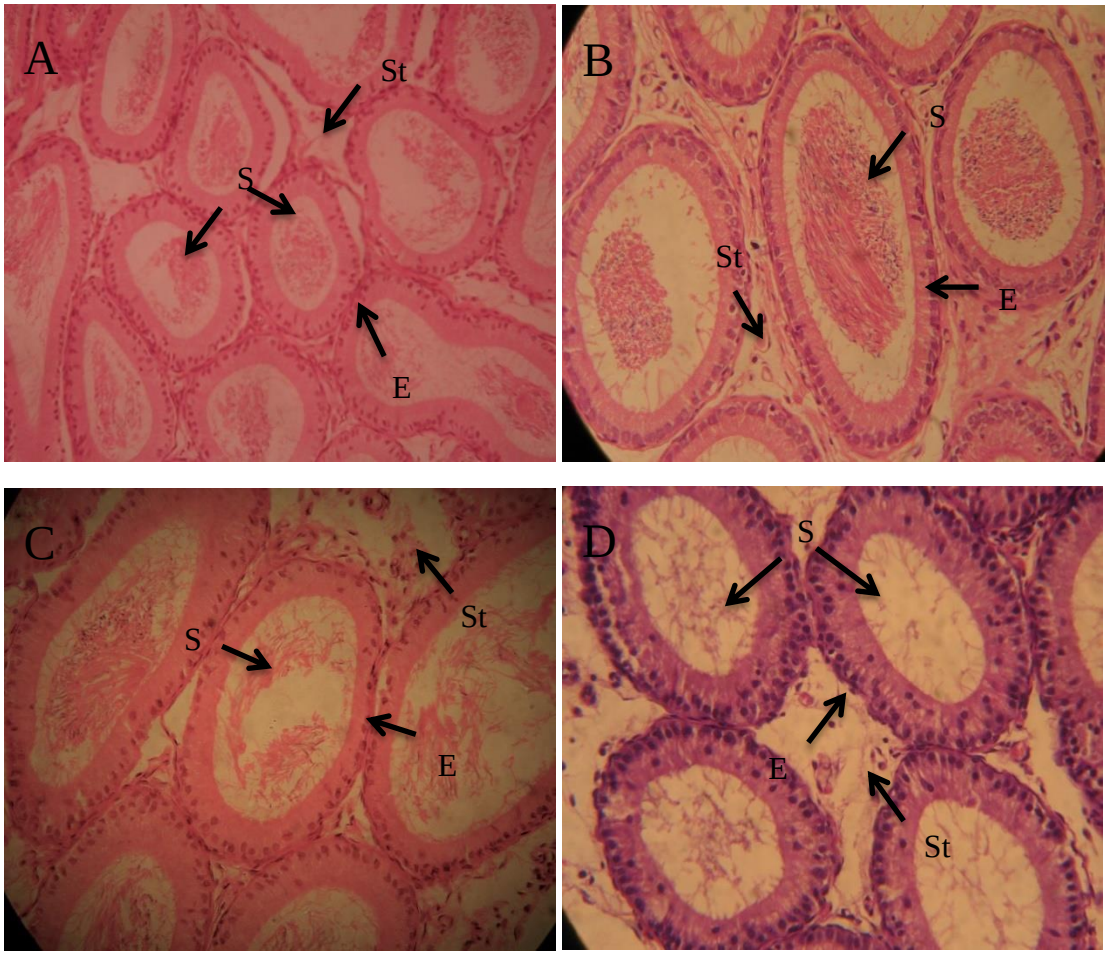


Figure 84: Photomicrograph of cauda epididymis of adult male rats treated with methanolic extract of *A. integrifolia* from: (A) Control; showing normal morphology of cauda epididymis, with thick epithelium, lumen filled with sperm, (B) 50mg/kg group; showing compactly arranged tubules thick epithelium and sperm filled lumen, (C) 100mg/kg group; showing normal tubules surrounded by stroma, lumen with reduced number of spermatozoa, (D) 150mg/kg group; showing thick epithelium with less lumen sperm concentration. Spermatozoa (S), Epithelium (E), Stroma (St). Magnification 40X.

Effect of extract on histomorphometeric changes in rat testis and epididymis

Testis

Parameters studied in testicular histology are given in Table 32. Change in diameter of seminiferous tubules was observed among all the experimental groups when compared to control, however, the change was not noteworthy. A little increase in luminal diameter of seminiferous tubules was also found in all three doses (50 mg/kg, 100 mg/kg and 150 mg/kg) treated groups as compared to control. But significant change ($p<0.05$) was noticed in 100 mg/kg and 150 mg/kg groups. The length of epithelial height was decreased with increase in dose concentration in contrast with control. Epithelial height reduced from 84.70±1.90 μm in control group to 78.64±1.67 μm in 100 mg/kg treated group and 76.78 ±1.54 μm in 150 mg/kg treated group (Figure 85). In addition, area of seminiferous tubule was decreased while area of interstitium was increased significantly ($p<0.01$) in 100 mg/kg and 150 mg/kg groups as compared to control (Table 32, Figure 86).

Table 32: Effect of *A. integrifolia* leaf extract on mean ± SEM of seminiferous tubule diameter (µm), tubular lumen diameter (µm), epithelial height (µm), area of seminiferous tubule (%) and interstitial space (%) of testis in control and treated groups.

Groups	Seminiferous tubule diameter (µm)	Lumen diameter (µm)	Epithelial height (µm)	Area of seminiferous tubule (%)	Interstitial space (%)
Control	232.06±8.29	32.04±1.06	84.70±1.90	87.24±0.56	12.76±0.56
50mg/kg	224.77±11.32	36.92±1.45	81.21±1.86	86.16±0.67	13.84±0.67
100mg/kg	221.03±7.87	38.52±1.37*	78.64±1.67*	84.00±0.53**	16.00±0.53**
150mg/kg	217.03±9.64	39.76±1.61*	76.78 ±1.54**	83.92±0.58**	16.08±0.58**

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

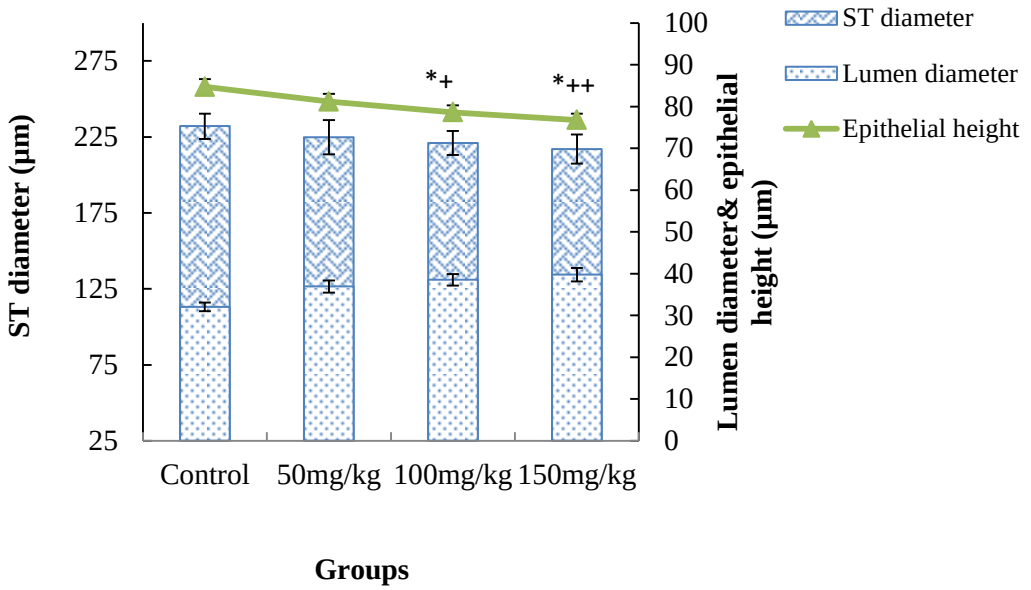


Figure 85: Mean ± SEM of seminiferous tubule diameter (µm), tubular lumen diameter (µm) and epithelial height (µm) in testes of control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

*= indicates significant difference of lumen diameter at probability of $p<0.05$ as compared to control.

+,++= indicates significant difference of epithelial height at probability of $p<0.05$, $p<0.01$ compared to control.

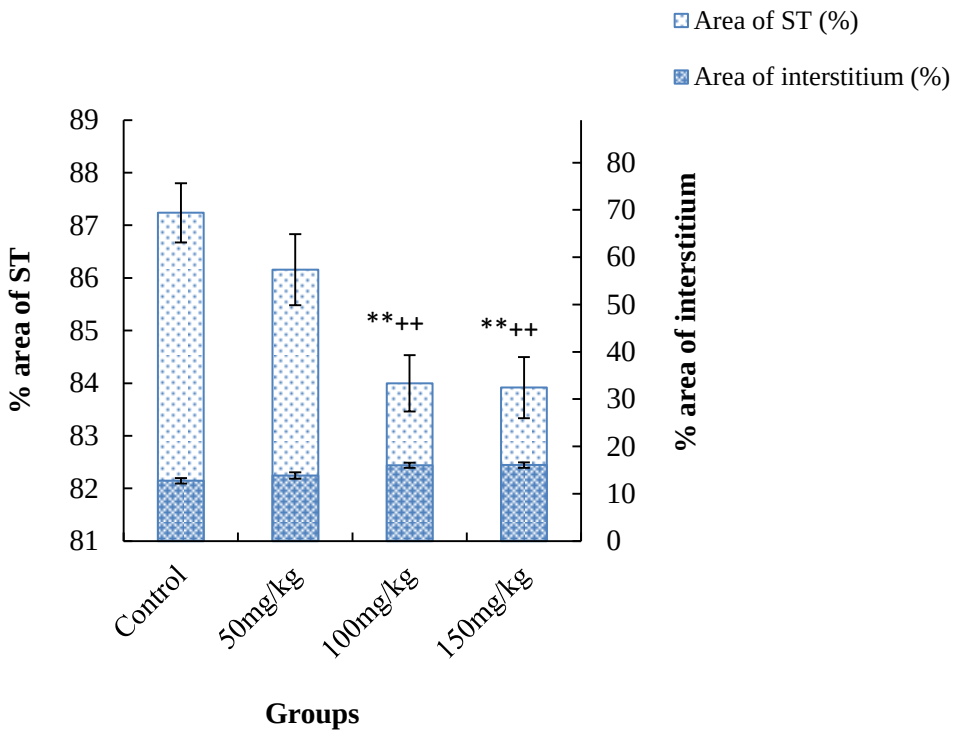


Figure 86: Mean ± SEM % area of seminiferous tubule and % area of interstitium in testes of control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

**= indicates significant difference of % area of ST at probability of $p<0.01$ as compared to control.

++= indicates significant difference of % area of interstitium at probability of $p<0.05$ compared to control.

Epididymis

Tubular diameter, lumen diameter and epithelial height of caput and caput are presented in table 33. Ductular diameter of caput epididymis was greatly reduced in all the dosage received groups. However, 100 mg/kg ($p<0.001$) and 150 mg/kg ($p<0.05$) treated rats showed most obvious decline in diameter. Furthermore, significant decrease ($p<0.001$) in lumen diameter of caput epididymis was only seen in 100 mg/kg treated group. Other groups did not show much remarkable change. Epithelial height for caput epididymis was decreased from control values of 31.94±2.06 µm to 21.87±2.52 µm and 21.45±1.61 µm respectively, in groups treated with 100 mg/kg and 150 mg/kg of plant extract (Figure 87).

The cauda epididymis did not show any notable difference in diameter of tubules and lumen of all the dose treated groups when compared with control one. While significant ($p<0.01$) decline in epithelial height was seen in 150 mg/kg dose treatment group as compare to control one. Other groups did not show marked differences (Table 33, Figure 88).

Table 33: Effect of *A. integrifolia* leaf extract on mean ± SEM of ductular diameter (µm), lumen diameter (µm) and epithelial height (µm) of caput and cauda epididymis in control and treated groups.

Groups	Ductular diameter (µm)		Lumen diameter (µm)		Epithelial height (µm)	
	Caput	Cauda	Caput	Cauda	Caput	Cauda
Control	363.18 ±9.72	447.36±17.90	274.05±14.77	412.13±17.72	31.94±2.06	38.52±2.38
50mg/kg	357.40±13.20	438.65±17.91	287.70±11.27	409.37±12.60	27.04±1.08	39.61±2.56
100mg/kg	273.63±23.32***	445.16±14.37	191.20±17.25***	394.61±12.78	21.87±2.52**	32.06±2.23
150mg/kg	271.57±10.35*	427.94±16.35	235.70±24.93	402.34±17.49	21.45±1.61*	27.21±2.46**

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test)

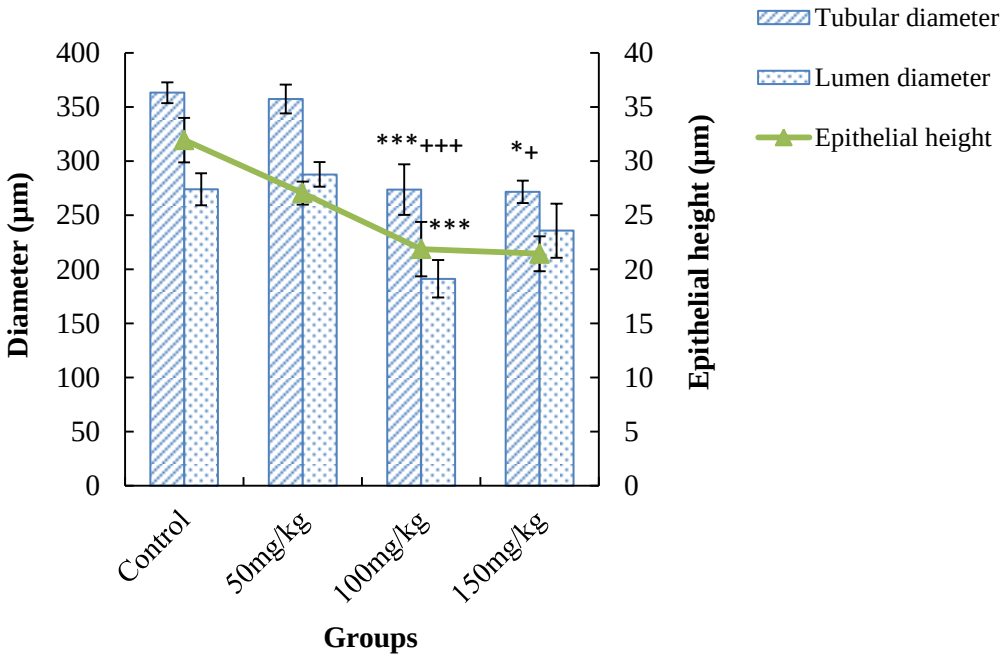


Figure 87: Mean ± SEM of ductular diameter (µm), lumen diameter (µm) and epithelial height (µm) in caput of control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

*,***= indicates significant difference of diameter at probability of p<0.01 as compared to control.

+,+++= indicates significant difference of epithelial height at probability of p<0.05 compared to control.

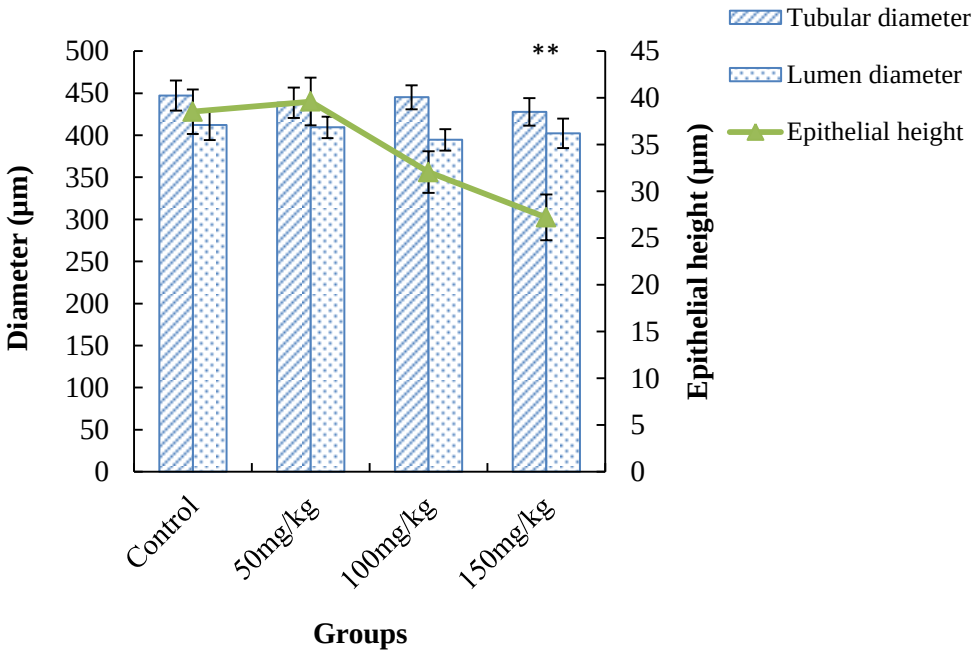


Figure 88: Mean ± SEM of ductular diameter (µm), lumen diameter (µm) and epithelial height (µm) in cauda of control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

**= indicates significant difference of epithelial height at probability of $p<0.01$ compared to control.

Effect of extract on plasma hormonal profile of rat

A significant decrease in plasma concentrations of testosterone ($p<0.001$) was obvious in all extract treated groups as compared with control group. Similarly, plasma LH concentration was lowered from 1.04±0.10 to 0.58±0.06 and 0.68±0.06 in 50 mg/kg ($p<0.01$) and 100 mg/kg ($p<0.05$) treated group respectively. Significant reduction ($p<0.001$) in FSH concentration was obvious in 100 mg/kg and 150 mg/kg dose treated groups when compared to control (Table 34, Figure 89).

Table 34: Effect of *A. integrifolia* leaf extract on mean ± SEM plasma Testosterone (ng/ml), LH (IU) and FSH (IU) concentration in control and treated groups.

Groups	Plasma Testosterone concentration (ng/ml)	Plasma LH concentration (IU)	Plasma FSH concentration (IU)
Control	2.90±0.30	1.04±0.10	1.18±0.16
50mg/kg	1.25±0.18***	0.58±0.06**	1.04±0.06
100mg/kg	1.03±0.01***	0.68±0.06*	0.37±0.06***
150mg/kg	1.01±0.03***	0.80±0.06	0.41±0.05***

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test)

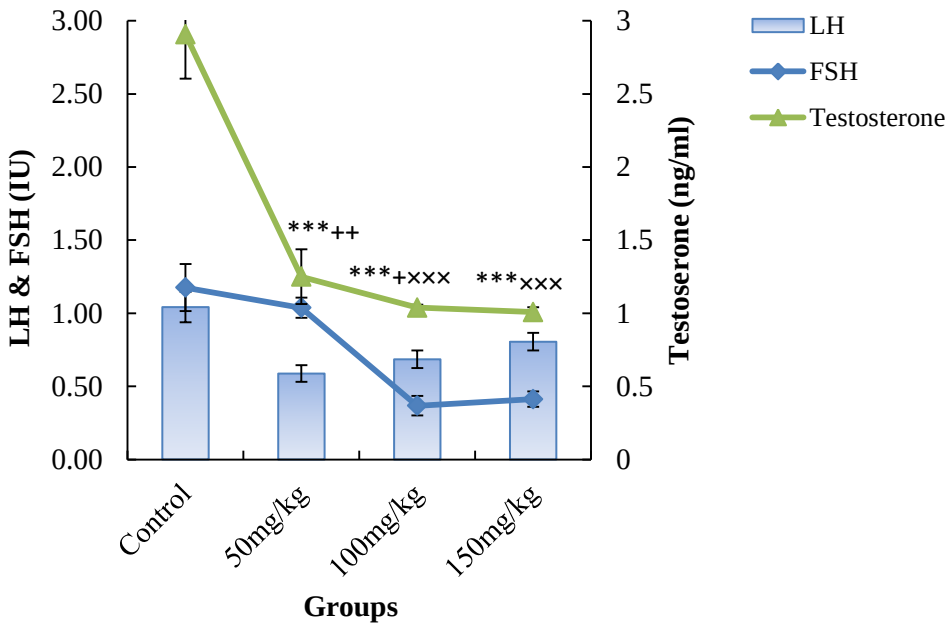


Figure 89: Mean ± SEM of plasma Testosterone, LH and FSH concentration in control and treated rats receiving methanolic leaf extract of *A. integrifolia* for 28 days.

***= indicates significant difference of Testosterone at probability of $p<0.001$ as compared to control.

+,++= indicates significant difference of LH at probability of $p<0.05$, $p<0.01$ compared to control.

×××= indicates significant difference of FSH at probability of $p<0.001$ compared to control.

DISCUSSION

For centuries, although use of traditional medicine has been practiced in most parts of the world but still it hasn't gain much prestige in western methods of treatment. Plant sources have been used in the isolation and development of incredible amount of modern drugs (Mustapha, 2013). A rising trend in investigation of medicinal plants and their phytochemicals properties has been seen during recent time; therefore, several traditional therapies have been introduced and practiced for various purposes even in western world. Among Asian countries, however, restricted traditional therapies have been implicated into conventional drugs. The field of andrology, itself, has thrown very little light on use of traditional medicines (Henkel *et al.*, 2012). This led us to study the pharmacological effect of methanolic leaf extract of *A. integrifolia* on the male reproductive system.

The results of present study revealed that gain in body weight as well as in reproductive organ weight of rats treated with methanolic leaf extract of *A. integrifolia* was significant. This reduction is mostly related to depletion of spermatogenic elements of deficiency of epididymal sperms (Singh and Gupta, 2016) and decreased production of semen (Malashetty and Patil, 2006). Similarly, sperm motility, viability and epididymal sperm count was significantly reduced after administration of methanolic leaf extract of *A. integrifolia* showing direct effect of plant extract on epididymal sperms. These results are in accordance to previous findings in which tulsi treatment (2 g/day) brings about a reduction in sperm count (Khanna *et al*., 1986) and reproductive hormones in male albino rabbits (Sethi *et al*., 2010). Khanna *et al*, have also reported significant decrease in sperm count and motility after long-term feeding of O. *Sanctum* leaves to male albino rats. Some plants are reported to cause drastic alterations in epididymal sperm quality, affecting sperm mobility and reducing sperm count (Khanna *et al*., 1986). The accessory reproductive organs of male are responsible for sperm motility, maturation and semen characteristics (Ahmed *et al*., 2002; Sandhyakumary *et al*., 2003; Gupta and Sharma, 2006; Azamthulla *et al*., 2015). The sperm attains motility and fertilizing capacity during its passage in the epididymis (Hamilton, 1975). So reduction in sperm motility in our study might be due to decreased reproductive organ weight, reduced androgen production and increased levels of reactive oxygen species.

Variations in energy metabolism of sperms may contribute to reduced sperm motility. The axoneme machinery of sperm needs a constant supply of energy in the form of ATP to maintain sperm motility. The motility of spermatozoa is initiated by phosphorylation of a contractile protein dynein causing flagellar movement. Following phosphorylation, the dynein ATPase becomes activated. The ATP hydrolysis releases energy which helps microtubules to slide past one another. Interference with enzymatic reactions involved in uncoupling of oxidative phosphorylation may be a reason for reduced sperm motility by *A.integrifolia* extract. Reduced motility can lead to inability of spermatozoa to travel through the Fallopian tubes and fertilize the egg, thus inducing sterility (Sharma and Jocob, 2001).

Sperm output can better be determined by DSP than epididymal sperm count. Findings of current study showed that treatment with 150mg/kg dose of *A.integrifolia* extract caused reduction in DSP after 28 days of treatment. It might suppress the hormonal levels and thus sperm production in testis. Previously, Jahan *et al*., have reported a decrease in daily sperm after exposure of rats with 20 and 60 mg/kg of ethanolic seed extract of *A. precatorius* (Jahan *et al*., 2009a).

In present study, in vivo exposure of plant extract caused dose dependent increase in oxidative stress within testicular tissue. This oxidative stress is contributed by increased production of free radical species i.e. TBARS and ROS. ROS are molecules having free unpaired electrons and oxygen, and after metabolism, these compounds generate free oxygen ions. The reactive oxygen species are synthesized in mitochondrion which serves as a primary site for oxidative phosphorylation. These oxidative species significantly affect cell signaling and homeostasis. The formation of ROS initiates the activation of a self-defense mechanism of antioxidant enzymes in response, within the cells (Jahan *et al*., 2009a). The antioxidants are enzymes that are capable of neutralizing free radicals and act at different stages/levels. Rise in antioxidant enzymes concentrations results in lessening of ROS. However, in case of decreased antioxidant enzymes levels, the cells undergo oxidative stress and fail to detoxify ROS. In this study, increase in levels of ROS and in lipid peroxidation like TBARS was detected in dose dependent manner. Some of the preventive antioxidants that attempt to halt the formation of ROS discussed here. These include superoxide dismutase, catalase and peroxidase. Reduction in these levels results in cell damage and death. Findings of current study reported reduction in antioxidant levels only in high dose treated group. This persistent reduction in antioxidant activity and increased

production of ROS caused degenerative changes in testicular tissue and disrupted spermatogenesis. These findings are similar to earlier studies (Ghosh *et al*., 2015).

The results of testicular tissue histology showed more pronounced changes in experimental groups. Reduction in tubular diameter of seminiferous tubule and epithelial height was seen when rats were treated with higher dose of methanolic extract of *A.integrifolia*. A significant increase in tubular lumen diameter was observed affecting normal process of spermatogenesis. These results were according to earlier reports by (Kusemiju *et al*., 2010; Obianime *et al*., 2010; Jain *et al*., 2012; Lotfi *et al*., 2013) in their respective studies.

Similarly, histomorphological changes of caput and cauda involves reduction in tubular diameter and epithelial height along with reduced number of luminal sperm concentration in high extract treated rats as compare with control. Similar results were also previously documented by Jahan *et al*. (2009) where exposure of mice exposed to 40 and 60 mg/kg/day extract of *Abrus precatorius* adversely affected tubular epithelium, reduction in sperm concentration and intraepithelial vacuoles formation (Jahan *et al*., 2009a)

Sertoli cells embrace and assist germ cells throughout process of spermatogenesis and are one of the chief components of seminiferous tubules. Total number of Sertoli cells is also significantly related to total sperm production. They provide an organized microenvironment for differentiating germ cells from spermatogonia to mature sperm, through modulation of hormonal stimuli function which monitors and regulate the process of spermatogenesis (Brinster, 2007). So appropriate number of Sertoli cells must be present to perform normal process of spermatogenesis.

Testosterone plays a crucial role in maturation, spermatogenesis and the maintenance of accessory sex organs and Testes. The plasma of animals treated with *A.integrifolia* extract exhibited reduction in the plasma concentrations of testosterone and assayed gonadotropins (LH and FSH) compared with control. Different scientists have reported similar findings in their respective studies (Sharma and Jocob, 2001; Daniyal and Akram, 2015; Singh and Singh, 2016). This indicated interference in the feedback mechanism among hypothalamus, pituitary, and testes. These hormones are synthesized and secreted under the influence of Gonadotropin-releasing hormone (GnRH) from the pituitary gland under the control of the hypothalamus. Treatment with *A.integrifolia* possibly decreased the levels of FSH and LH by inhibiting GnRH.

FSH and LH are the key enzymes, which trigger the Testosterone biosynthesis from the seminiferous tubules. Low levels of these hormones reduced testosterone secretion from the testes by suppressing testicular steroidogenesis and spermatogenesis since the pituitary-testicular axis is a central regulatory unit for the normal functioning of the testes and the production of spermatozoa (Kusemiju *et al*., 2010).

Conclusion

In the present study, dose-dependent treatment of *A. integrifolia* caused marked alterations in the male reproductive organs, which were reflected by the reduced concentrations of Testosterone, FSH, LH, sperm motility, viability and sperm density in reproductive organs. These effects altogether along with oxidative stress induced by ROS lead to the suppression of spermatogenesis and finally induced infertility in male rats. On the basis of these effects, we can conclude that *A. integrifolia* has a potential to develop an acceptable, easily available, non-toxic, safe and reversible herbal male contraceptive drug. However, further pharmacological and molecular studies are needed to explore the exact underlying mechanism of action.

CHAPTER 5:

Effect of methanolic leaf extract of *Rumex hastatus* D. Don on reproductive functions of adult male rats: A histological and biochemical study.

ABSTRACT

The current experiment was conducted to assess antifertility potential of methanolic extract of *Rumex hastatus* leaf on adult male rats. For this, rats were given different doses of extract (0, 50, 100 and 150 mg/kg) via oral gavage for 28 consecutive days. Results of current study showed no remarkable difference in body weight gain and testicular weight in dose treated groups when compared to control. A dose dependent decrease in sperm motility, viability and caput sperm number was noted while DSP, corpus and cauda sperm number remained unchanged. Similarly, extract treatment did not affect the concentration of CAT, SOD and POD in testicular tissue; however, levels of ROS and TBARS were elevated only in high dose regimen. Histomorphometric study of testis revealed arrangement of seminiferous tubules, in all treated groups, similar to control but lumen was wider with reduced number of sperms. No significant change was reported in seminiferous tubule diameter or lumen diameter while epithelial height was decreased in high dose treated groups. Likewise, treatment with plant extract (50 mg/kg and 100 mg/kg) showed normally arranged epididymis with slightly narrow lumen and relatively reduced number of sperms. However, 150 mg/kg dose treatment caused reduction in epithelial height and luminal diameter while sperm concentration was also significantly lowered when compared to control group. A significant reduction in concentrations of plasma Testosterone, FSH and LH was also recorded only in high extract treated animals when comparison was made with control. Percentage fertility and number of pups born per female paired with treated male rats were reduced non-significantly in high dose treated groups, after 60 days of treatment withdrawn. It is concluded that the methanolic leaf extract of *R. hastatus* affect the reproduction to some extant but it is not effective/potent to suppress fertility and accordingly used or considered as antifertility agent.

INTRODUCTION

Since ancient periods, medicinal plants have been consumed for a variety of therapeutic purposes. (Rao, 1996). Though, systematized research in this domain was started back in 1956 (Farnsworth *et al*., 1985) and later on such studies were gaining popularity and recognition due to declining plant population and loss of traditional knowledge. A large number of medicinal plants have been reported to be used in Ayurveda and Unani medicines for the treatment of variety of diseases and disorders (Kritikar and Basu, 1981; Singh *et al*., 1992). Even today this domain holds much more buried treasure as almost 80% of the human population in developing countries is dependent on herbal resources for healthcare. So it is utmost need of time to explore new ways of use of medicinal plants and their bioactive components to overcome the prevailing general health related issues and especially for fertility regulation.

Rumex is one of the most important genera of family "Polygonaceae" also known as "buckwheat" family. It is common perennial herb grow almost everywhere. *Rumex* plants have been used traditionally as a green salad, bactericidal, appetite stimulant, astringent, anti-inflammatory and anti-tumor (Xu *et al*., 2004). Both the leaves and tender stems *R. dentatus* are traditionally consumed in Egypt and other parts of the world (Tukan *et al*., 1998). The leaves of a Korean salad plant i-e *R. acetosa* are used as food antioxidant (Chon *et al*., 2008) . Additionally, *R. crispus* was reported to be used as an herbal hepato protection during antibiotic therapy (dose of 100-300 mg per day stimulates bile production and release) and a remedy of acute hepatitis, among many traditional folk medicines (Lee *et al*., 2007) . On the other hand, Rumex steudelii has been reported to possess antifertility properties by inhibiting ovarian folliculogenesis in rats (Solomon *et al*., 2010).

Rumex hastatus D. Don, commonly recognized as "Taroky", is a perennial flowering plant widely distributed in northern areas of Pakistan, Nepal, Afghanistan, and southwest China. It grows on plains as well as on slops at elevation ranging from 1000 to 2600m. Medicinal importance of whole plant has been described previously. It can be used as laxative or to cure various diseases including rheumatism (Shinwari and Gilani, 2003) and sexually transmitted diseases like AIDS (Vermani and Garg, 2002). Some previous studies substantiated that aerial parts of *R. hastatus* are abundant with tannins and phenols thus offer themselves as rich source of antioxidants (Sahreen *et al*., 2011). Another study have described the presence of

seven different types of phenols in the roots of *R. hastatus* (Zhang *et al*., 2009). Additionally, different medicinal plants and their active components are known to have antioxidant potential as they ameliorate the liver toxicity and reproductive impairments caused by oxidative stress in animal models (Tombolini and Cingolani, 1996; Premila and Wilfred, 2000). Sahreen *et al*, (2013) have studied that exposure to *R. hastatus* fractions enhanced the antioxidant activities in animal body thus combating oxidative damage caused by increased production of reactive oxygen species (Sahreen *et al*., 2013). It is obvious from aforementioned literature that *R. hastatus* has therapeutic potentials and few studies have documented their protective effect on reproduction. But no data is available to study its detailed sub- chronic exposure effects on sperm parameters or HPA axis. So this study was designed to investigate if methanolic leaf extract of *R. hastatus* cause any change/alteration in HPA axis or disturb hormonal profile.

Indigenous use for infertility:

Juice is made with water and taken before intercourse.

Taxonomy

The taxonomy of the plant according to National Plant Data Center, NRCS, USDA is as follows

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Caryophyllidae

Order: Polygonales

Family: Polygonaceae

Genus: *Rumex* L.

Specie: *Rumex hastatus* D. Don

Figure 90. Plant *Rumex hastatus* D. Don in its natural habitat.

Aims and Objectives

R. hastatus is traditionally used as an oral contraceptive but still no scientific experimentation is performed .The current study is planned to check the ability of *R. hastatus* in the induction of oxidative stress in testicular tissues and reproductive impairments, if any, in male rats.

MATERIALS AND METHODS

Plant collection and extract preparation

Rumex hastatus D. Don is locally known as "Taroky". It can widely procured from agricultural fields of Murree, Swat, Shangla, Kaghan, Naran, Dir and some areas of province Punjab, Pakistan. Leaf sample of *R. hastatus* were collected from fields of district 'Dir' and its vicinity located at altitude of 1420 m. Plant was identified by Dr. Mushtaq Ahmad at Plant Systematics and Biodiversity Lab (QAU) and the sample was placed in herbarium with accession number #26452.

Methanolic extract of *R.hastatus* leaves was prepared by method reported previously (Gulfraz *et al*., 2007). The detailed procedure is explained in chapter 1.

Animals

Adult male Sprague Dawley rats weighing (80-90 days old) were obtained from rodent and primate facility of Animal Sciences Department, Quaid-i-Azam University Islamabad. Animals were placed in aerated and ventilated room, provided with standard laboratory conditions of 14 hrs light/10 hrs dark cycle and temperature maintained at 25±1 C^{o}. Standard laboratory feed and water ad labitum was available for animals. All the animal handling and protocols were approved by ethical committee of Animal Sciences department.

Experimental Design

Current study was carried out to check the effect of methanolic leaf extract of *R. hastatus* on reproductive functions of adult male rats. *In vivo* experimentation followed by acute toxicity study was used for the evaluation of antifertility efficacy of plant extract

Acute oral toxicity

Acute oral toxicity study of methalonic leaf extract of *R. hastatus* was performed by using method described in chapter 3.

In vivo experiment

Animals were randomly divided into four groups each having ten rats (n=10 rats/group). Different concentrations of plant extract (0, 50, 100 and 150 mg/kg/day) were administered to rats via oral gavage for twenty eight consecutive days. Doses

were selected according to OECD guideline # 408 while the higher doses were selected according to previous related studies (Gebrie *et al*., 2005; Solomon *et al.*, 2010).

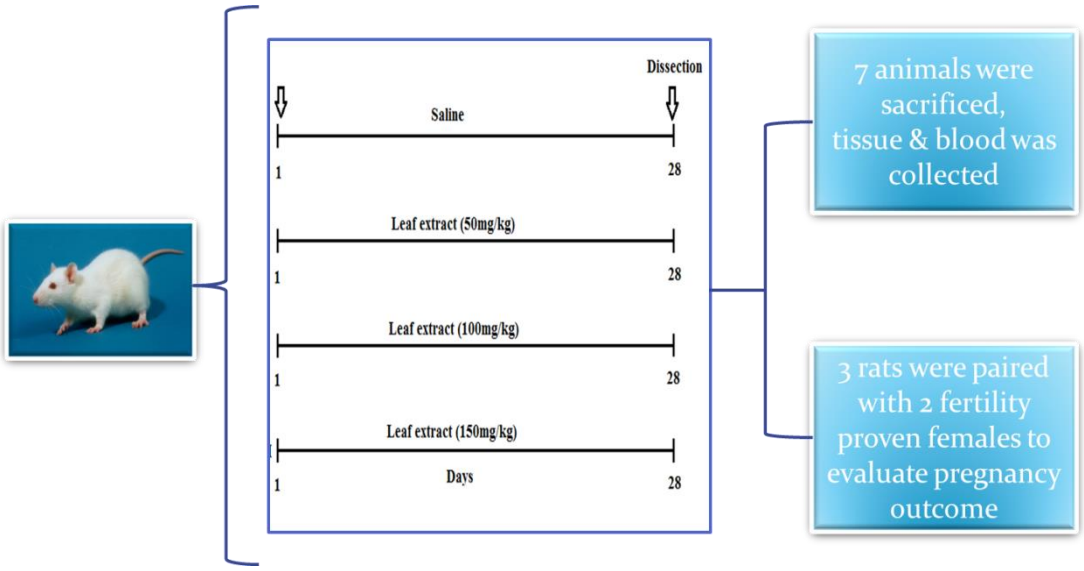


Figure 91: Schematic representation of experimental design of *in vivo* exposure to *R. hastatus* methanolic leaf extract.

After the completion of experiment, seven animals per group were weighed and killed by decapitation. Immediately after dissection, trunk blood was collected in heparinized syringes and subjected to centrifugation at 3000 rpm for 15 minutes. Plasma was separated and kept at -20 ºC until hormonal analysis. Reproductive organs were removed, weighed and washed in ice cold saline. Right testicular and epididymal tissue were fixed in 10% formalin for histological analysis. Left testis was stored in freezer for daily sperm production and biochemical investigation while, left epididymis was used for further assessment of sperm parameters.

Fertility test:

Fertility test was done according to method described in chapter 3.

Assessment of Sperm Motility and Viability

For the assessment of sperm motility and viability, method described Halvaei *et al.* 2012 was used. Detailed procedure is described in chapter 3 (Halvaei *et al*., 2012).

Epididymal Sperm count and DSP

Epididymal sperm count was determined by using procedure given by Thanga *et al.* 2012 (Thanga *et al*., 2012). For the assessment of DSP, frozen testicular tissues were thawed at room temperature, followed by homogenization in 3ml of 0.9% sodium chloride containing 0.5% triton X-100 (Robb *et al*., 1978). Detailed methodology of epididymal sperm count and DSP is given in chapter 3.

Biochemical Analysis

To check antioxidant status in testis, the stored samples were thawed and homogenized in 3 ml of phosphate buffer saline (pH 7.4). The homogenate was processed through centrifugation at 12000 rpm for 30 minutes at 4 °C. Supernatant was collected for determination of antioxidant status of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), thiobarbituricacid reactive species (TBARs) and reactive oxygen species (ROS) by using previously explained procedure given in chapter 2.

Histological analysis

Histology of testis and epididymis tissues was done according to method explained in chapter 3.

Hormonal Analysis

Plasma Testosterone, LH and FSH concentrations were assessed quantitatively by using enzyme immunoassay (EIA) kits as described in chapter 3.

Statistical analysis

All observed data was subjected to one way analysis of variance (ANOVA) followed by Dunnet test for comparison of various groups with control group using Graph pad Prism 5 software. All the results are shown in Mean ± SEM. Significance level was set at $p< 0.05$.

RESULTS

Acute toxicity

Results of acute oral toxicity of methanolic leaf extract of *R. hastatus* in rats after dose exposure (5 - 2000 mg/kg body weight) is given in table 35. There were no obvious symptoms of mortality or morbidity at all the tested doses.

Fertility Test:

Effect of *R. hastatus* leaf extract on the percentage fertility and mean number of pups born per female after 60 days of treatment withdrawal is given in table 36, figure 92. Vaginal smear of untreated female rats mated with treated male rats exhibited number of sperms comparable with healthy animal. Percentage fertility and number of pups born per female were reduced non-significantly in high dose treated groups. 50 mg/kg methanolic extract treatment did not reduce the fertility rate as it was 100%. However, in 100 mg/kg and 150 mg/kg treated group, 83% and 67% females conceived respectively.

Table 35: Results of acute toxicity study in rats after single oral dose of methanolic leaf extract of *R. hastatus*.

Dose (mg/kg body weight)	Number of animals	Short-term result (24 h)	Long-term results (14 days)
0	3	Survival	Survival
5	3	Survival	Survival
50	3	Survival	Survival
300	3	Survival	Survival
2000	3	Survival	Survival

Table 36: Effect of *R. hastatus* leaf extract on the fertility and the Mean ± SEM number of pups born per female after 60 days of treatment withdrawal.

Groups	**Percent fertility**	**Number of pups born/female**
Control	100	7.33 ± 0.71
50 mg/kg	100	7.16 ± 1.08
100 mg/kg	83.33	6.66 ± 1.38
150 mg/kg	66.66	5.16 ± 1.68

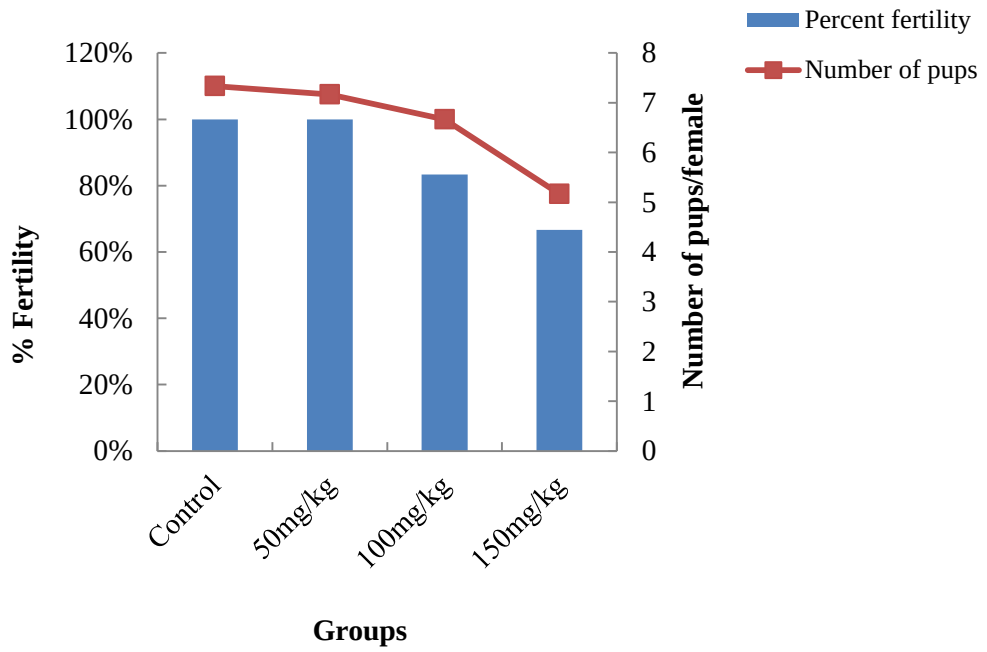


Figure 92: Effect of *R. hastatus* leaf extract on % fertility and the number of pups born per female after 60 days of treatment withdrawal.

Body weight and reproductive organ weights

There was no remarkable difference in body weight gain seen among animals of all treated groups when compared to control group (table 37, figure 93). A significant decrease ($p<0.05$) in right testicular weight was observed in 100 mg/kg treated group as compared to control group. However, non significant decrease in testicular weight was noted among all other treated groups when comparison was made with control animals as shown in table 37, figure 94.

A significant reduction ($p<0.05$) in left and right epididymal weight was seen in animals of 100 mg/kg and 150 mg/kg groups when compared with control group. Likewise, seminal vesicle weight was decreased considerably in 100 mg/kg ($p<0.05$) and 150 mg/kg ($p<0.001$) dose treated rats. While no significant change was seen in mean prostate weight of all extract treated groups as compared to control group (Table 37).

Table 37: Effect of *R. hastatus* leaf extract on mean ± SEM body weight (g) and testicular weight (g) of control and treated groups.

Groups	Body weight gain (g)	Testicular weights (g)		Epididymus weight (g)		Seminal vesicle weight (g)	Prostate weight (g)
		Right	Left	Left	Right		
Control	55 ± 5.82	1.29 ± 0.03	1.31 ± 0.05	0.57 ± 0.03	0.59 ± 0.03	1.50 ± 0.19	0.79 ± 0.10
50mg/kg	56.6 ± 5.44	1.22 ± 0.02	1.17 ± 0.03	0.53 ± 0.06	0.50 ± 0.05	1.23 ± 0.08	0.70 ± 0.07
100mg/kg	55.4 ± 10.4	1.14 ± 0.03*	1.16 ± 0.03	0.41 ± 0.03	0.38 ± 0.04*	1.02 ± 0.10*	0.74 ± 0.02
150mg/kg	63.2 ± 7.62	1.20 ± 0.05	1.21 ± 0.05	0.37 ± 0.03*	0.38 ± 0.05*	0.51 ± 0.01***	0.55 ± 0.11

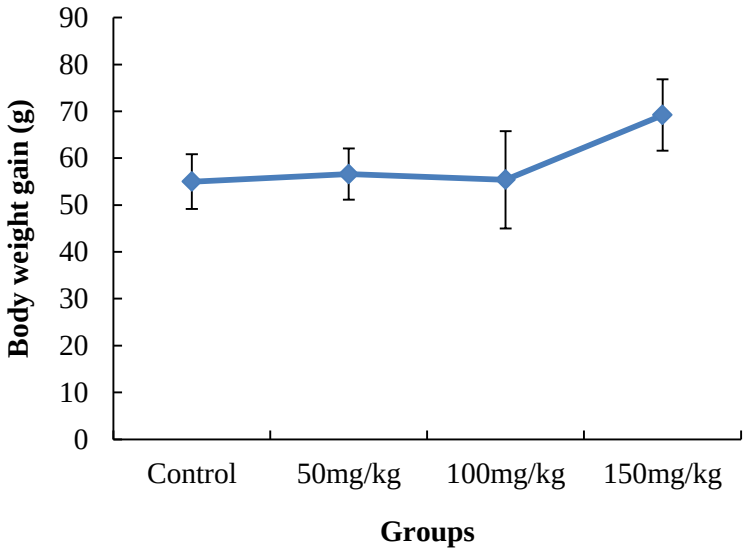


Figure 93: Mean ± SEM body weight gain (g) of control and treated groups of rats, receiving methanolic leaf extract of *R. hastatus* for 28 days.

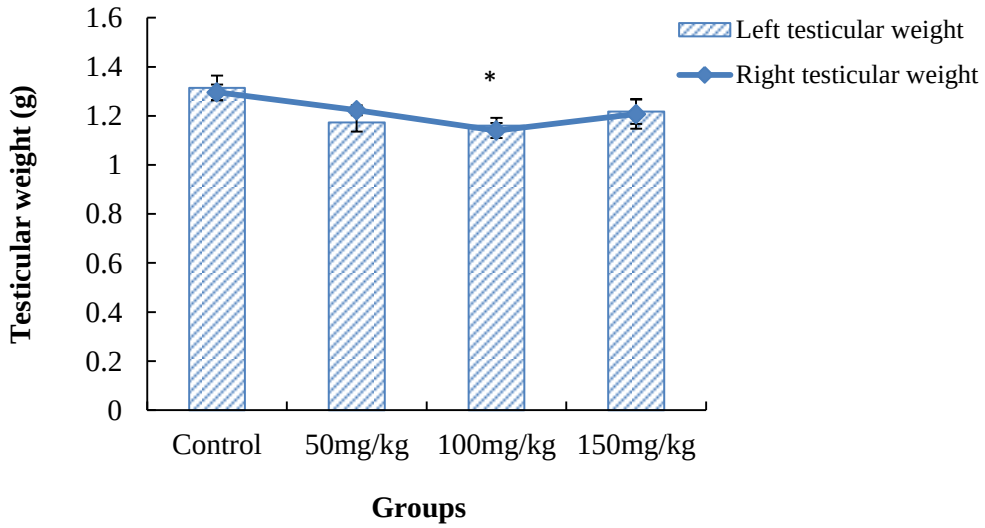


Figure 94: Mean ± SEM of Testicular weight gain (g) of control and treated groups of rats, receiving methanolic leaf extract of *R. hastatus* for 28 days.

*= indicates significant difference of Right testicular weight at probability of $p<0.05$ compared to control.

Sperm Parameters, DSP and epididymal sperm count

Mean ± SEM of sperm motility (%), viability (%) and DSP of control and treated groups are presented in Table 38. A significant reduction ($p<0.001$) in % sperm motility and viability rate was seen in all extract treated groups in dose dependent manner when compared to control group (Figure 95). A non-significant decrease in daily sperm production was observed when 50 mg/kg and 100 mg/kg dose treated groups were compared to control group. However, 150 mg/kg dose treatment caused significant reduction ($p<0.05$) in DSP as compared to control (Figure 97).

Mean ± SEM of epididymal sperm count in control and treated groups are shown in table 38, figure 96. Caput sperm number was significantly decreased ($p<0.05$) when 50 mg/kg group was compared to control group, similarly, significant reduction ($p<0.001$) was also observed in 100 mg/kg and 150 mg/kg dose treatment groups. No significant change in corpus sperm number was noticed in all treatment groups as compared to control. Cauda sperm number was reduced in dose dependent manner but significant reduction ($p<0.05$) was only observed in 100 mg/kg extract treated rats.

Table 38: Effect of *R. hastatus* leaf extract on mean ± SEM of % sperm motility, viability rate, DSP and epididymal sperm count among control and treated groups.

Groups	% Motility rate	% Viability rate	DSP × 10^5 /testis	Caput sperm count	Corpus sperm count	Cauda sperm count
Control	73.2±2.67	78.6±3.31	5.35±0.23	1585.00±31.22	1298.75±65.68	1943.75±41.30
50mg/kg	55.8±1.8**	56.00±2.1***	4.56±0.29	1343.75±73.15*	1218.75±37.32	1693.75±82.52
100mg/kg	47.00±4.43***	50.2±3.17***	4.24±0.40	1250.00±20.41***	1181.25±50.38	1600.00±75.69*
150mg/kg	43.2±2.22***	43.00±2.00***	4.09±0.29*	1175.00±48.94***	1118.75±90.92	1762.50±75.34

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

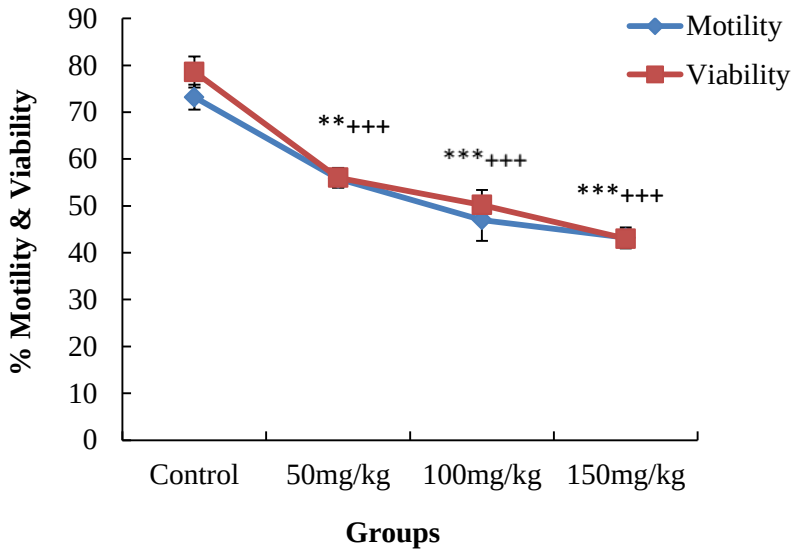


Figure 95: Mean ± SEM of % sperm motility & viability rate in control and treated rats receiving methanolic leaf extract of *R. hastatus* for 28 days.

***= indicates significant difference of % motility at probability of p<0.001 as compared to control.

+++= indicates significant difference of % viability at probability of p<0.001 compared to control.

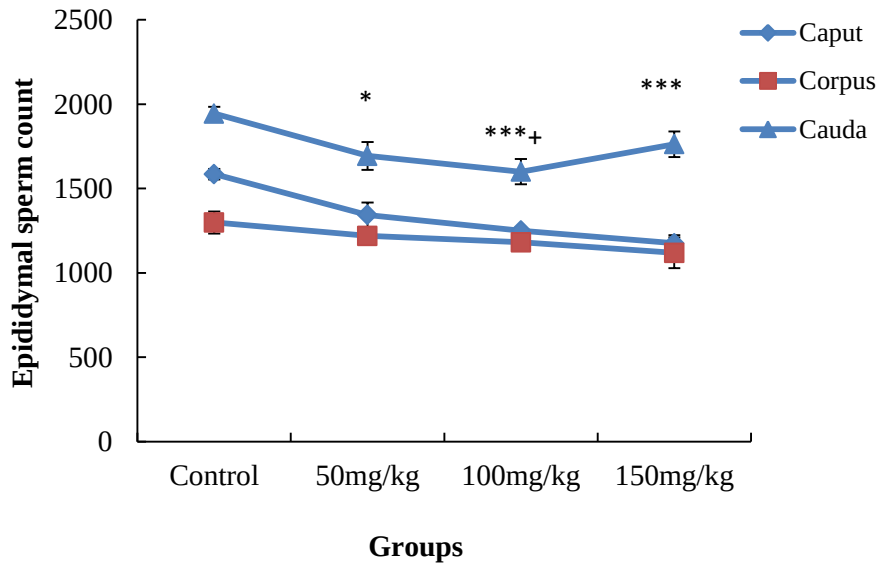


Figure 96: Mean ± SEM of epididymal sperm count in control and treated rats receiving methanolic leaf extract of *R. hastatus* for 28 days.

*,***= indicates significant difference of caput sperm number at probability of $p<0.05$ and $p<0.001$ as compared to control.

+= indicates significant difference of cauda sperm number at probability of $p<0.001$ compared to control.

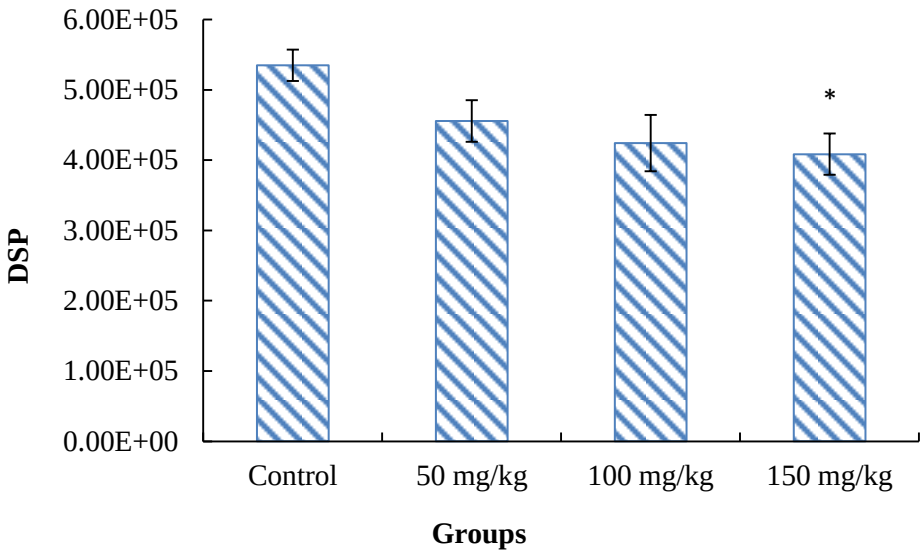


Figure 97: Mean ± SEM of DSP in control and treated rats receiving methanolic leaf extract of *R. hastatus* for 28 days.

*= indicates significant difference at probability of $p<0.05$ as compared to control.

Antioxidant Enzymes Status

Mean ± SEM specific activity of antioxidant enzymes including CAT, SOD, POD, ROS and TBARS in the testis of control and treated groups of adult male rats after 28 days of treatment is sown in table 39.

Exposure to methanolic extract of *R. hastatus* resulted in dose dependent decrease in catalase activity (U/mg), superoxide dismutase (U/mg protein) and peroxidase activity (nmole) within testis of all the treated groups when compared to control group (Figure 98 & 99). However, this decrease was not significant as shown in table 39. A non-significant increase in ROS and TBARS was reported in 50 mg/kg and 100 mg/kg treated groups as compared to control group. While, 150 mg/kg dose treated rats showed significant increase in ROS ($p<0.05$) and TBARS ($p<0.01$) values when compared with control group rats (Table 39, Figure 100).

Table 39: Effect of *R. hastatus* leaf extract on mean ± SEM antioxidant activity of testicular tissues of adult male rats in control and treated groups.

Groups	CAT (U/mg)	SOD (U/mg)	POD (nmole)	ROS (µmol/min)	TBARS (nM/mg)
Control	29.27 ± 1.95	45.00 ± 5.11	13.89 ± 1.38	0.82±0.15	0.36 ± 0.06
50mg/kg	27.74 ± 1.52	41.19 ± 2.16	12.46 ± 1.12	0.97±0.06	0.87 ± 0.33
100mg/kg	25.97 ± 2.12	39.40 ± 2.80	11.00 ± 1.25	0.89±0.05	1.01 ± 0.25
150mg/kg	25.57 ± 3.44	33.38 ± 2.77	9.73 ± 1.37	1.35±0.21*	2.25 ± 0.28***

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

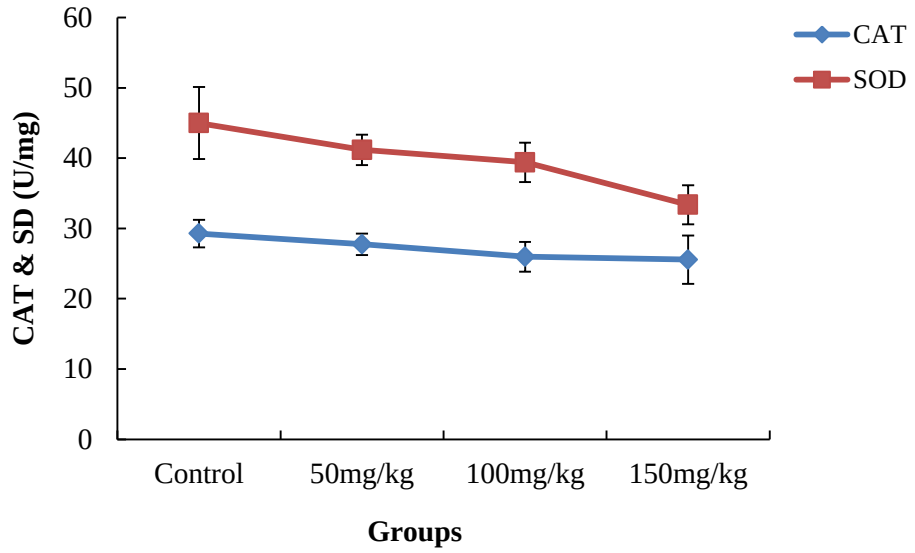


Figure 98: Specific activity of CAT and SOD in testis of control and treated rats receiving methanolic leaf extract of *R. hastatus* for 28 days.

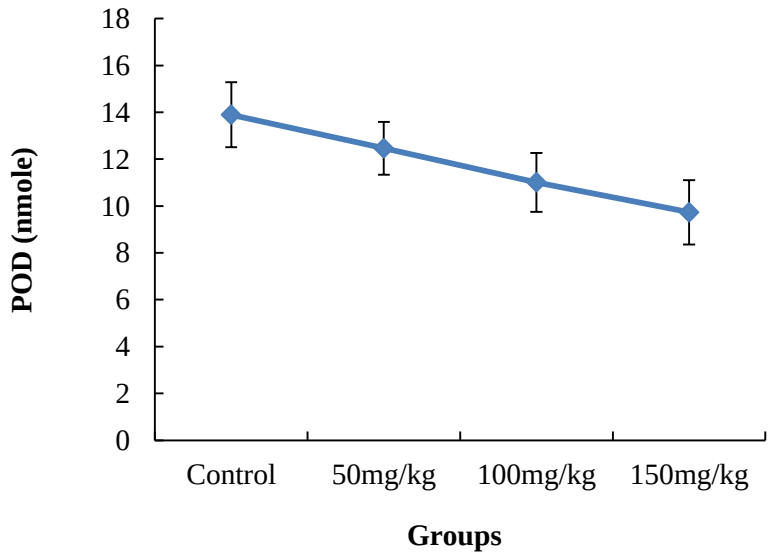


Figure 99: Specific activity POD of in testis of control and treated rats receiving methanolic leaf extract of *R. hastatus* for 28 days.

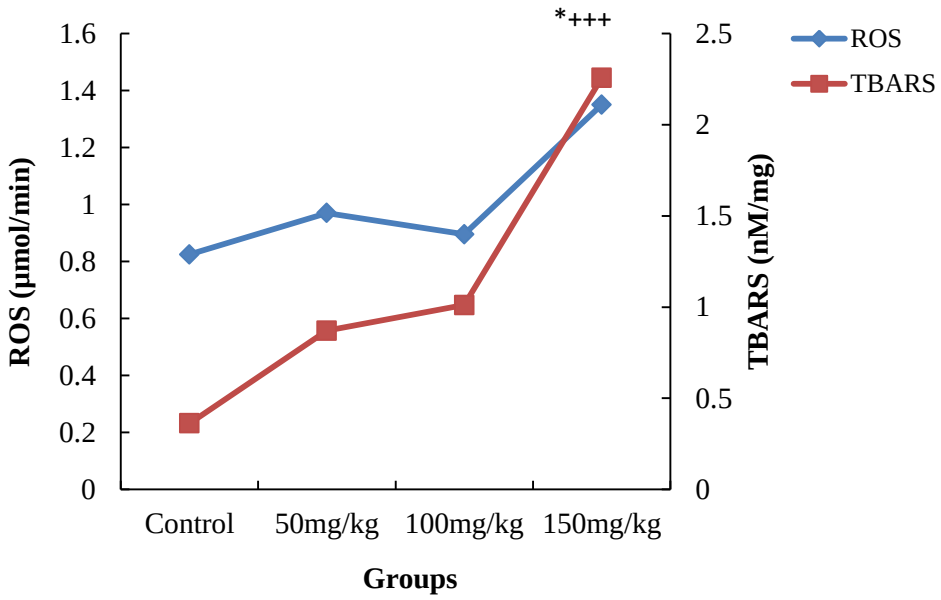


Figure 100: Specific activity of ROS & TBARS in testis of control and treated rats receiving methanolic leaf extract of *R. hastatus* for 28 days.

*= indicates significant difference of ROS at probability of $p<0.05$ as compared to control.

+++= indicates significant difference of TBARS at probability of $p<0.001$ compared to control.

Histological Analysis

Morphological Changes in testis

Histological examination of testis of control group showed intact seminiferous tubules covered by thick layer of tunica albuginea with normal spermatogenesis. A thick stratified germinal epithelium with lumen filled with mature spermatozoa was observed. Arrangement of seminiferous tubules in all treated groups was similar to control but lumen was wider with reduced number of sperms. With comparison to control group, exposure to high doses (100 mg/kg and 150 mg/kg) resulted in decreased tubular diameter and disrupted germinal epithelium (Figure 101).

Morphometric results

Various parameters of testicular morphometry are given in table 40. A non-significant difference in tubular and lumeninal diameter was observed in all dose treated groups when compared to control group. However, this reduction in tubular diameter was significant ($p<0.05$) in 150 mg/kg treated animals. In case of epithelial height, significant change ($p<0.001$) was reported in 100 mg/kg and 150 mg/kg dose treated groups than control (Figure 102). Percentage area of seminiferous tubule and interstitium remained unchanged in all treated groups compared to control group (Figure 103).

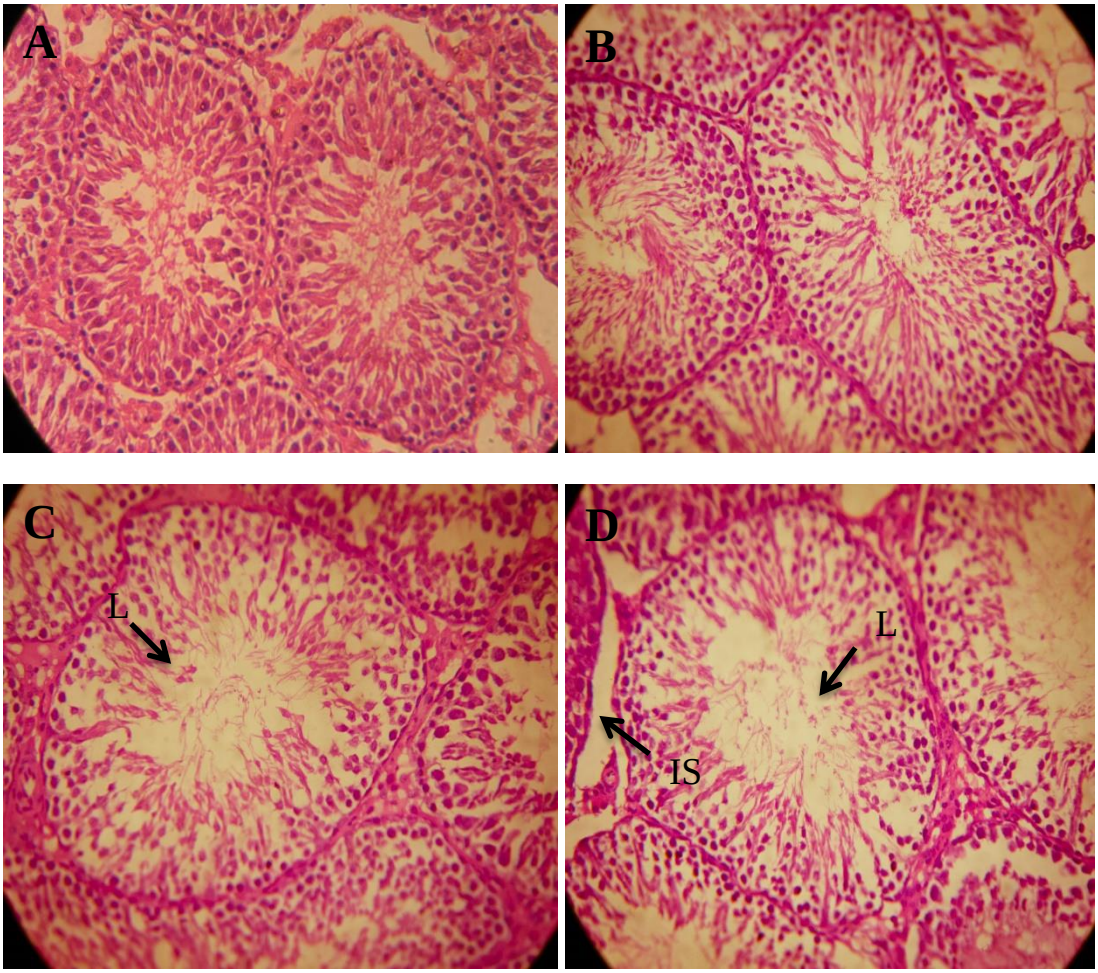


Figure 101: Photomicrograph of seminiferous tubules of adult male rats receiving doses of *R. hastatus*. Regularly arranged tubules, lumen filled with spermatids with normal germ cells shown in (A) Control; represents normal process of spermatogenesis, lumen filled with mature spermatozoa, (B) 50mg/kg groups; showing normal morphology like control group, (C) 100mg/kg group; showing increased lumen (L) diameter filled with immature spermatozoa and amplified epithelial height, (D) 150mg/kg group; thin epithelium, lumen is empty, large interstitial space (IS) are present. Magnification 40X.

Table 40: Effect of *R. hastatus* leaf extract on mean ± SEM of seminiferous tubule diameter (µm), tubular lumen diameter (µm), epithelial height (µm), area of seminiferous tubule (%) and interstitial space (%) of testis in control and treated groups.

Groups	Seminiferous tubule diameter (µm)	Tubular lumen diameter (µm)	Epithelial height (µm)	Area of seminiferous tubule (%)	Interstitial space (%)
Control	250.88 ± 10.68	85.24 ± 3.36	82.06 ± 1.98	82.81±0.61	17.19±0.61
50mg/kg	244.34 ± 11.17	90.57 ± 6.72	77.34 ± 2.16	83.02±0.55	16.98±0.55
100mg/kg	226.97 ± 7.45	94.79 ± 4.70	67.02 ± 2.87***	81.85±0.80	18.15±0.80
150mg/kg	213.10 ± 10.08*	96.41 ± 4.56	64.53 ± 2.01***	80.63±0.59	19.37±0.59

Values are expressed as mean ± SEM

*,**,*** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

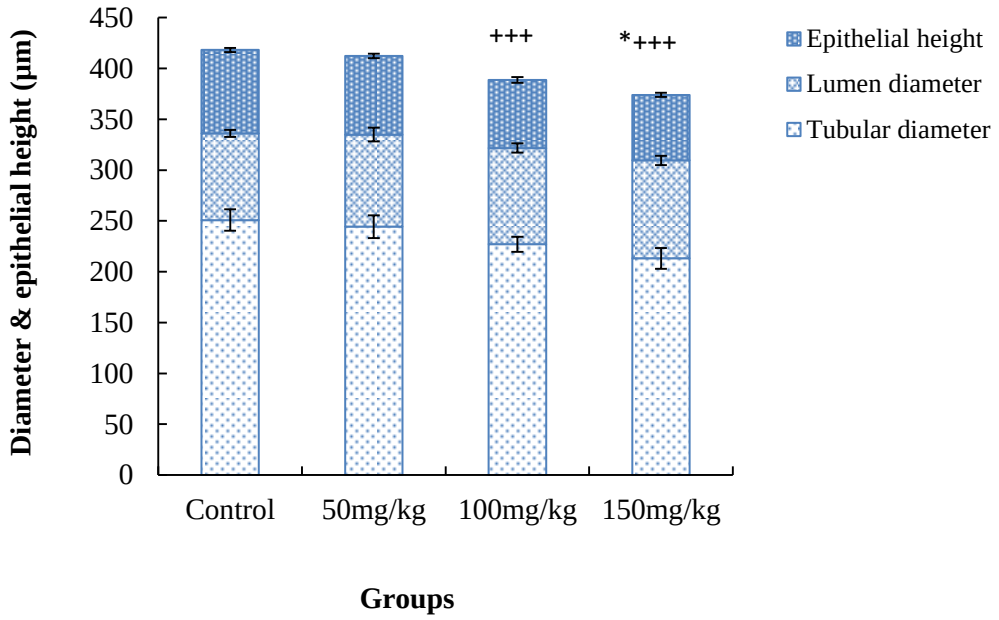


Figure 102: Mean ± SEM of seminiferous tubule diameter (µm), tubular lumen diameter (µm) and epithelial height (µm) in testes of control and treated rats receiving methanolic leaf extract of *R. hastatus* for 28 days.

*= indicates significant difference of tubular diamter at probability of $p<0.05$ as compared to control.

+++= indicates significant difference of epithelial height at probability of $p<0.001$ compared to control.

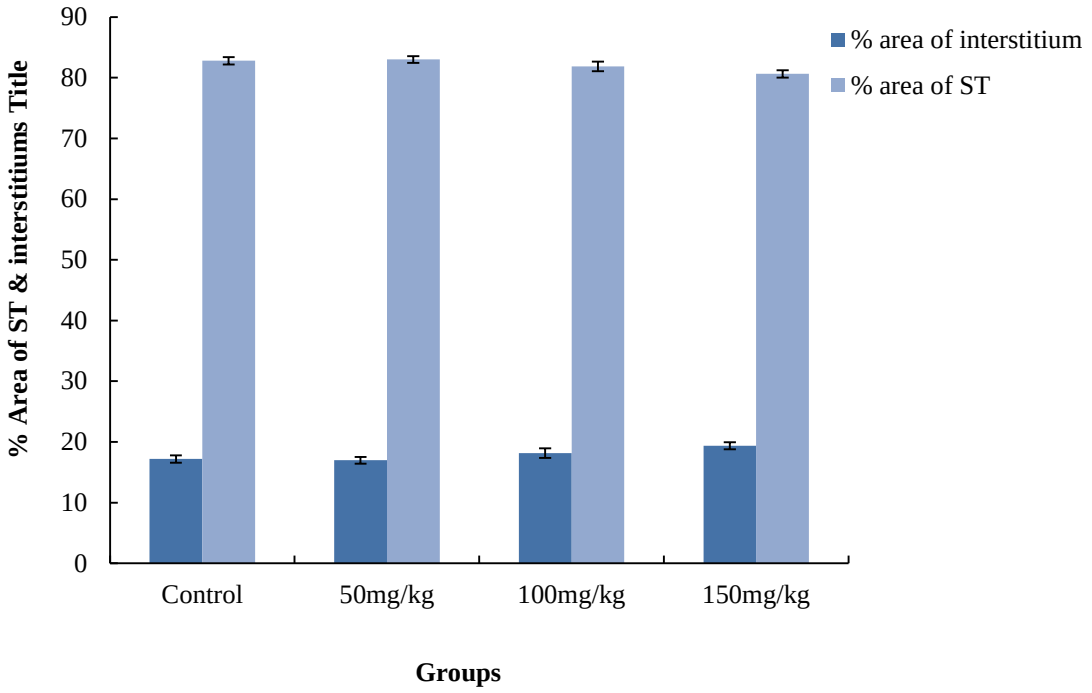


Figure 103: Mean ± SEM % area of seminiferous tubule and % area of interstitium in testes of control and treated rats receiving methanolic leaf extract of *R. hastatus* for 28 days.

Epididymis:

Table 41 shows histomorphometric results for caput and cauda epididymis.

Epididymis

Caput and cauda epididymis of control group showed larger diameter, wider lumen and thin pseudostratified epithelium lined with stereocilia. The epididymis was compactly arranged and lumen was filled with large number of spermatozoa. Treatment with plant extract (50 mg/kg and 100 mg/kg) showed normally arranged epididymis with slightly narrow lumen and relatively reduced number of sperms. However, 150 mg/kg dose treatment caused reduction in epithelial height and luminal diameter while sperm concentration was also significantly lowered when compared to control group (Figure 104 & 105).

Tubular diameter, lumen diameter and epithelial height of caput and cauda epididymis are given in table 41. No significant change in tubular and lumen diameter of epididymis was seen in all dose treated groups when compared to control. However, lumen diameter of caput was increased significantly ($p<0.05$) in 150 mg/kg extract treated group. The epithelial cell height was found to be reduced in dose dependent manner in all the extract treated groups. While significant change ($p<0.01$) in both caput and cauda was noted in 150 mg/kg extract treated rats (Figure 106 & 107).

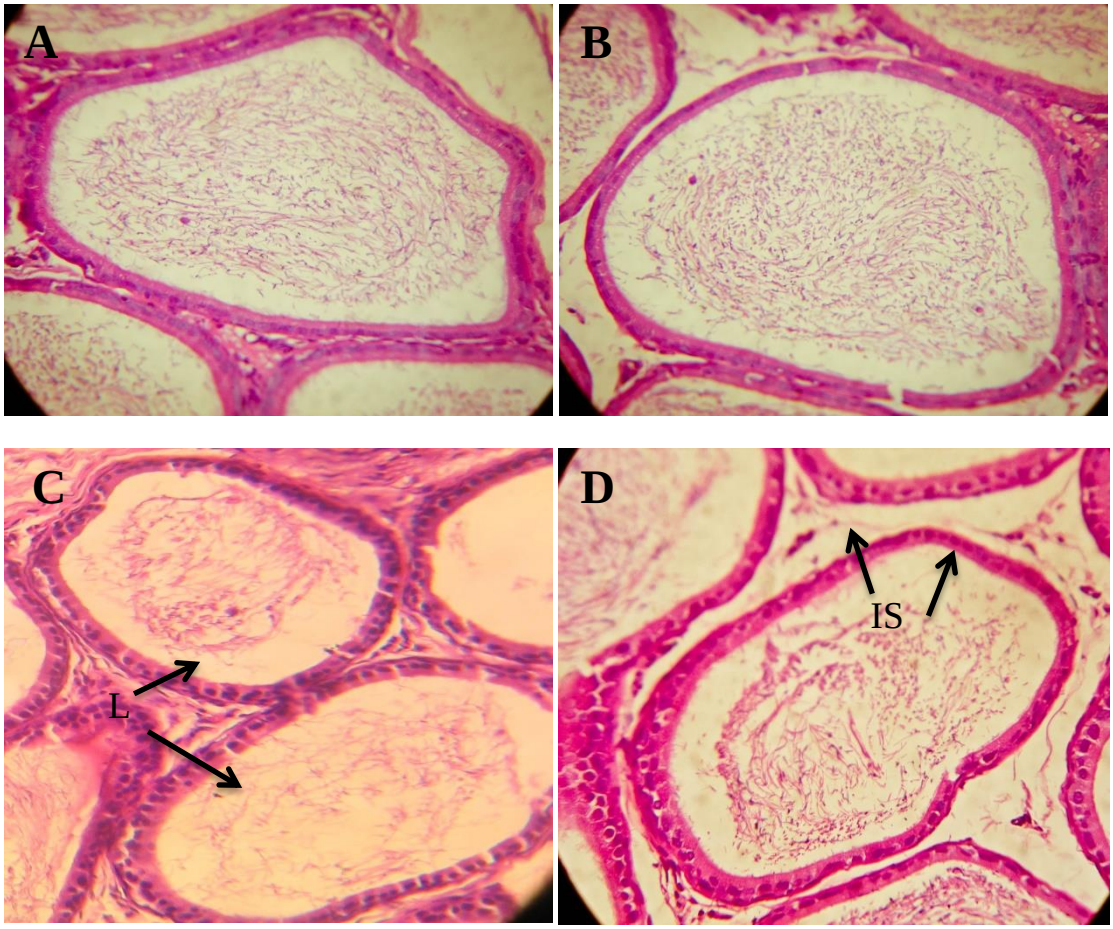


Figure 104: Photomicrograph of caput epididymis of adult male rat receiving doses of *R. hastatus* from: (A) Control group; with normal morphology of caput epididymal cells having thin pseudostratified epithelium lined with stereocilia. Lumen is heavily filled with spermatozoa, (B) 50mg/kg group; normal morphology of caput epididymal cells like control, (C) 100mg/kg group; showing reduction in number of spermatozoa, low number of spermatozoa in lumen (L) (D) 150mg/kg group; showing increase in pseudostratified epithelium and and large interstitial space (IS) compared to other two groups group. Magnification 40X.

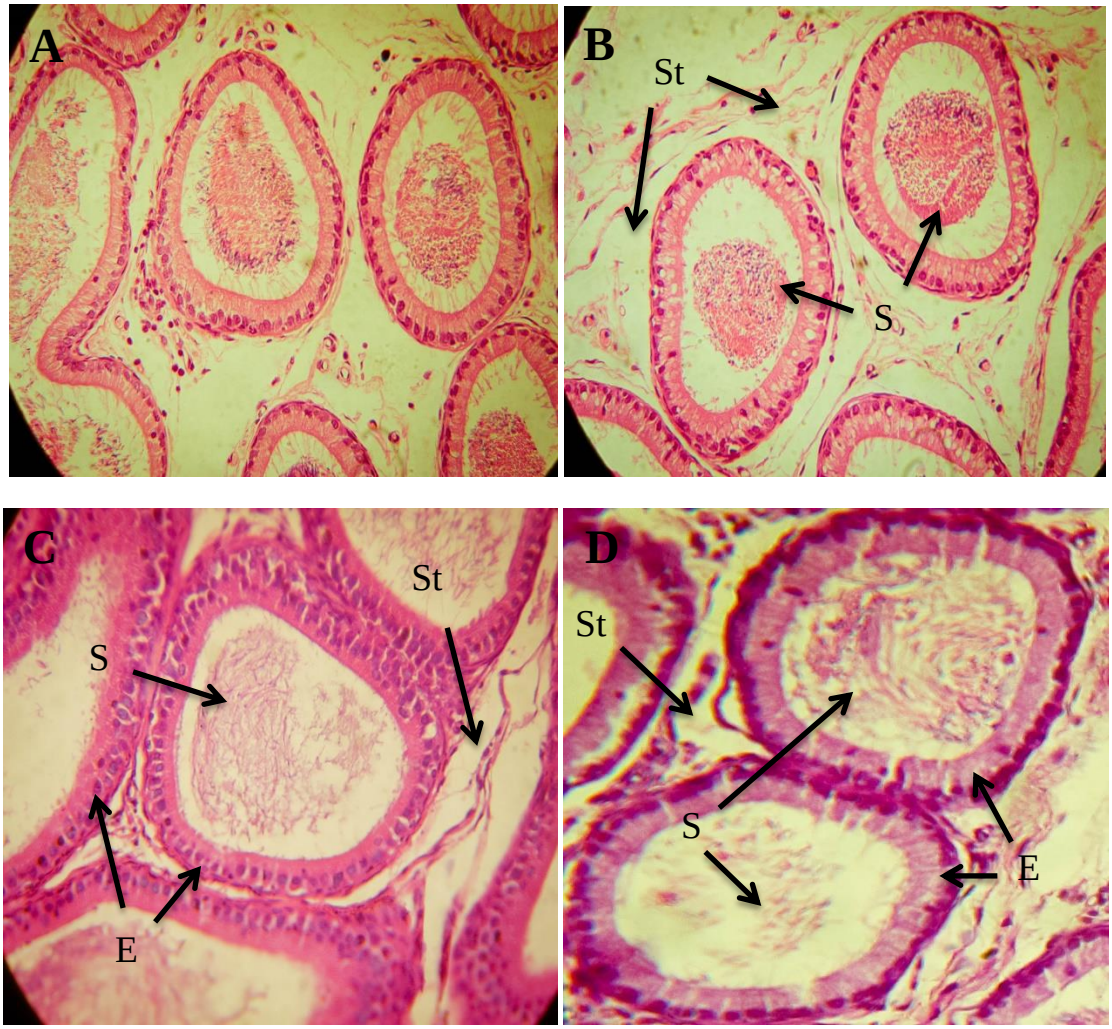


Figure 105: Photomicrograph of cross section of cauda epididymis of adult male rats receiving doses of *R. hastatus* showing (A) Control; exhibiting normal morphology of cauda epididymis; thick epithelium (E) and lumen filled with mature sperms, (B) 50mg/kg group; showing normal morphology with thick epithelium (E) while lumen filled with sperms (S), (C) 100mg/kg group; showing tubules surrounded by stroma (St) lumen has very little number of spermatozoa (S), (D) 150mg/kg group; showing further maximum increase in epithelial height and a little sperm concentration in lumen (S). Magnification 40X.

Table 41: Effect of *R. hastatus* leaf extract on mean ± SEM of ductular diameter (µm), luminal diameter (µm) and epithelial cell height (µm) of epididymis in control and treated groups.

Groups	Ductular diameter (µm)		Lumen diameter (µm)		Epithelial height (µm)	
	Caput	Cauda	Caput	Cauda	Caput	Cauda
Control	269.42±15.48	298.31±12.71	220.44±28.13	251.79±11.15	32.58±2.64	37.28±1.95
50mg/kg	261.93±19.40	282.89±9.19	225.60±17.48	244.59±9.65	28.50±1.60	34.54±1.74
100mg/kg	260.35±14.15	272.84±12.67	232.92±21.56	242.64±12.32	27.60±1.24	34.42±1.30
150mg/kg	252.08±15.22	289.70±18.68	239.30±14.78*	246.64±15.29	23.76±1.32**	26.85±2.38**

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

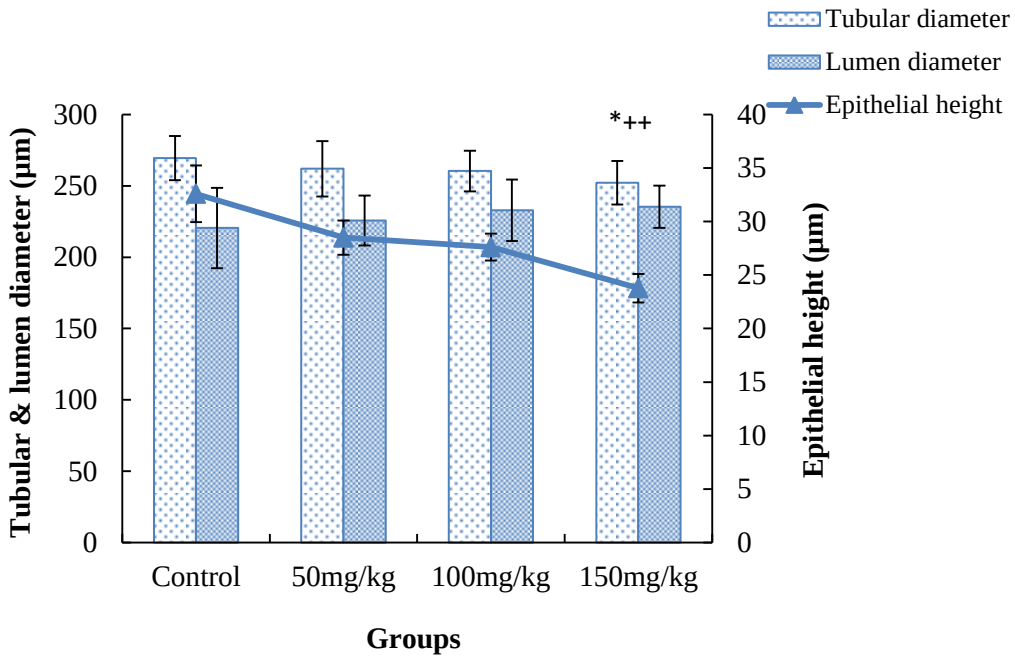


Figure 106: Mean ± SEM of ductular diameter (µm), lumen diameter (µm) and epithelial height (µm) in caput of control and treated rats receiving methanolic leaf extract of *R. hastatus* for 28 days.

*= indicates significant difference of lumen diameter at probability of $p<0.05$ as compared to control.

++= indicates significant difference of epithelial height at probability of $p<0.01$ compared to control.

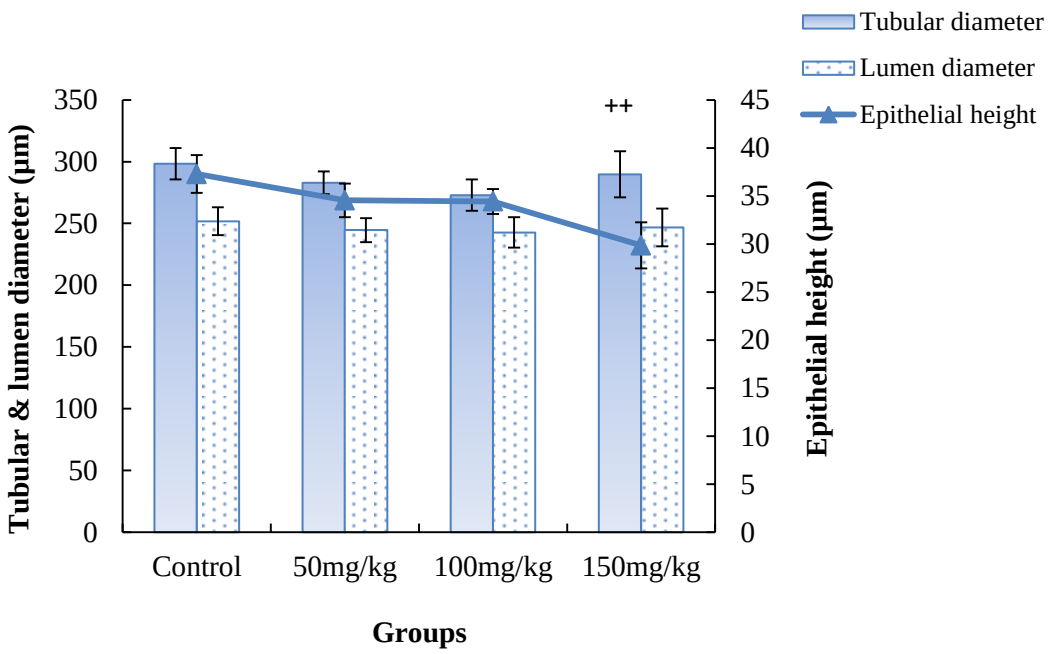


Figure 107: Mean ± SEM of ductular diameter (µm), lumen diameter (µm) and epithelial height (µm) in cauda of control and treated rats receiving methanolic leaf extract of *R. hastatus* for 28 days.

++= indicates significant difference of epithelial height at probability of $p<0.01$ compared to control.

Hormonal Analysis

Hormonal concentration of plasma Testosterone (ng/ml), LH (IU) and FSH (IU) in adult male rats following 28 days of treatment has been given in table 42. A significant reduction ($p<0.001$) in plasma testosterone level was seen in all treated groups as compared to control group. Similarly, plasma LH concentrations (IU) were decreased in dose dependent manner; however, this reduction was significant in 100 mg/kg ($p<0.01$) and 150 mg/kg ($p<0.001$) dose treated groups when comparison was made with control group. Plasma FSH levels were also reduced significantly ($p<0.05$) in 150 mg/kg group as compared to control, while change in other groups is non-significant (Figure 108 & 109).

Table 42: Effect of *R. hastatus* leaf extract on mean ± SEM plasma Testosterone (ng/ml), LH (IU) and FSH (IU) concentrations in control and treated groups.

Groups	Plasma Testosterone concentration (ng/ml)	Plasma LH concentration (IU)	Plasma FSH concentration (IU)
Control	2.83±0.17	1.26±0.08	2.01±0.39
50mg/kg	1.02±0.12***	0.93±0.07	1.07±0.12
100mg/kg	0.88±0.11***	0.61±0.15**	1.29±0.16
150mg/kg	0.78±0.09***	0.49±0.06***	0.83±0.19*

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

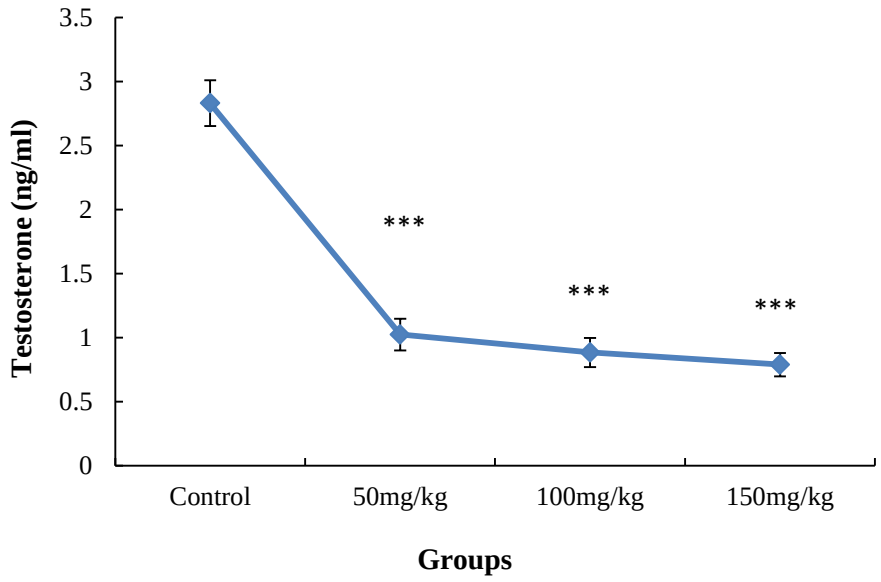


Figure 108: Mean ± SEM of plasma Testosterone concentration in control and treated rats receiving methanolic leaf extract of *R. hastatus* for 28 days.

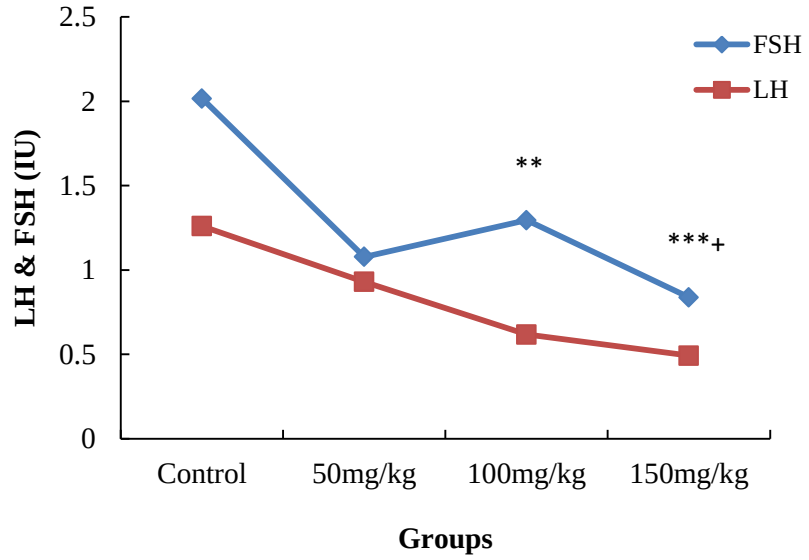


Figure 109: Mean ± SEM of plasma Luteinizing hormone and Follicle stimulating hormone concentration in control and treated rats receiving methanolic leaf extract of *R. hastatus* for 28 days.

,*= indicates significant difference of LH at probability of $p<0.01$, $p<0.001$ as compared to control.

+= indicates significant difference of FSH at probability of $p<0.05$ compared to control.

DISCUSSION

In current study, effect of mathanolic leaf extract of *R. hastatus* on fertility of adult male rats was evaluated. Different doses (50 mg/kg, 100 mg/kg and 150 mg/kg) were administered for 28 consecutive days; testicular and epididymal tissues were analyzed for sperm motility and other sperm parameters, biochemical, hormonal and histopathological changes. Percentage fertility and pregnancy outcome were also considered after withdrawal of treatment to check reversibility of toxicity if any.

Results of fertility test showed that vaginal smear of untreated female rats mated with treated male rats exhibited number of sperms comparable with healthy animal. No significant effect was observed on percentage fertility and number of pups born per female in any dose treated groups as compared to control.

In our study, body weight gain and weight of accessory sex organ was remained unchanged among animals of all treated groups; however, testis and epididymis weight was reduced in high dose treated group. This reduction in reproductive organ weight might be due to decreased sperm concentration in epididymis or reduced levels of seminal fluid (Malashetty and Patil, 2006; Singh and Gupta, 2016). Sperm motility and viability were decreased in all extract treated groups in dose dependent manner. However, reduction in epididymal sperm count and DSP was observed only in high dose treatment group. Similar results were obtained in previous study in which oral administration of 100 mg/rat/day dose of *Tinospora cordifolia (Willd.)* stem extract caused 100% fertility inhibition by reducing sperm motility and sperm density (Gupta and Sharma, 2006). It is well known that sperm attains motility, maturation and fertilizing capacity during its passage through epididymis (Hamilton, 1975). So reduction in sperm motility and sperm count in our study might be the reason of decreased reproductive organ weight and reduced androgen production.

Sperm motility is important for fertilization. Several plant based contraceptives target sperm motility and sperm number to induce male sterility (Sharma and Jocob, 2001; Thanga *et al*., 2012). As it is vital for sperm to pass through epididymis for germ cell maturation, but due to reduced motility, immature spermatids are released in lumen as confirmed by current histological findings.

Present study revealed that antioxidant enzyme status was remained unchanged after exposure of rats with *R. hastatus* leaf extract. No significant reduction in antioxidant enzymes level was noticed. However, concentration of oxidative stress markers (ROS and TBARS) was significantly increased in high dose treatment group.

The results of testicular histology exhibited no change in lumen diameter and tubular diameter, while, reduction in epithelial height was observed in high dose treated animals. Similarly, histological analysis of caput and cauda epididymis showed no significant change in their morphology except reduction in epithelial height. Histomorphmetric analysis also showed no change in tubular and lumen diameter of epididymis. However, decrease in epithelial cell height of both caput and cauda was noticed in high dose treated animals. The degenerative changes of seminiferous epithelium are associated with alterations in steroidogenesis or disruption of the hypothalamus-pituitary-gonadal axis. Previously, a study has reported that methanolic extract of *Rumex steudelii,* related specie of our selected plant, caused atrophic changes in the uterus and disrupted ovarian folliculogenesis in female albino rats (Solomon *et al*., 2010). Another study reported similar results in which oral dose of 140 mg/kg *Mucuna urens* extract caused degenerative changes in testicular tissues of Male Guinea-pigs (Udoh and Kehinde, 1999).

Results of hormonal analysis showed reduction in hormonal concentrations after exposure to plant extract. A significant reduction in plasma testosterone level was seen in all treated groups. Similarly, plasma LH and FSH concentrations were also decreased in dose dependent manner. Androgens are necessary for germ cell survival, maturation and successful spermatogenesis to occur. Their reduced levels as observed in our study are associated with spermatogenic arrest. Sertoli cells synthesize 17- β – estradiol which bind to estrogen receptor in the Leydig cells and inhibit testosterone production through negative feedback mechanism (Sandhyakumary *et al*., 2003). This might be the possible cause of decreased levels of testosterone in current study. Many scientists have reported antiandrogenic effects of different plants previously in their respective studies (Sandhyakumary *et al*., 2003; Malashetty and Patil, 2006). Treatment with *R. hastatus* possibly decreased the levels of FSH and LH by inhibiting GnRH. FSH and LH are the key enzymes, which trigger the Testosterone biosynthesis from the seminiferous tubules. Their low levels inhibit testosterone production by

suppressing steroidogenesis and spermatogenesis by regulating hypothalamus-pituitary-testicular axis.

Conclusion

The results of present experiment revealed that although methanolic leaf extract of *R. hastatus* is capable of inducing reproductive toxicity to some extant but it does not have potential to suppress fertility. Hence, this plant extract cannot be used as antifertility agent.

CHAPTER 6:

Assessment of Reproductive Toxicity induced by methanolic leaf extract of *Hedera nepalensis* K. Koch: A search for herbal contraceptive.

ABSTRACT

This study was designed to assess antifertility efficacy of methanolic extract of *H. nepalensis* leaf on male rats. For this, various selected extract doses (0, 50, 100 and 150 mg/kg) were given to rats via oral gavage for 28 consecutive days. A marked gain in body weight was reported in medium dose treated group as compared to control. While dose dependent reduction in testicular weight and accessory organs weight was seen in all extract treated groups. Results of sperm parameters exhibited notable reduction in sperm motility and viability in all extract treated animals while DSP was reduced only in high dose regimens. A marked reduction in concentration of CAT, SOD and POD was noted in 100 mg/kg and 150 mg/kg dose treated rats. Increase in levels of ROS was prominent only in 150 mg/kg dose treated group while concentration of TBARS was quite evident only in all dose regimens. Histopathological observations revealed normal tubular arrangement with reduced epithelial height and wider lumen having less number of spermatozoa in 100 mg/kg and 150 mg/kg dose treated groups as compared to control group. Additionally, significant decrease in spermatogonial populations and number of mature spermatids was also noted. Epididymal histology showed normally arranged epididymis with slightly narrow lumen and relatively reduced number of sperms in plant extract treated (100 mg/kg and 150 mg/kg) groups as compared to control group. A significant decline in plasma Testosterone concentrations was seen in all extract treated groups, while plasma LH and FSH concentrations were decreased significantly in 100 mg/kg and 150 mg/kg dose treated groups when comparison was made with control group. Fertility test of present study also found reduced pregnancy outcome in the females paired with treated male rats as compared to those mated with control animals after 60 days of treatment withdrawn. Litter size was significantly reduced in 150 mg/kg dose treatment group, but no signs of morbidity or mortality were observed in resultant pups. In conclusion, it is suggested that the methanolic leaf extract of *H. nepalensis* has the potential to suppress male fertility that can be restored after treatment withdrawal.

INTRODUCTION

The eventual fate of life on the planet is under the pressure of the population explosion, which is a noteworthy reason for pollution and neediness, particularly in the developing and under developed countries (Thanga *et al*., 2012). Fertility regulation including contraceptive methods and infertility management has become the major concern to improve reproductive health (Gupta and Sharma, 2006). Despite the fact that advances have been made for the development of safe, improved and effective contraceptive agent for female, progress on male is still lagged far behind. Currently available methods for male contraception results either unintended pregnancies or lead to complete sterility (El-Kashoury *et al*., 2009). Hence, development of new, safe, reversible and biologically active contraceptives for male is necessary and can provide remarkable public and social health benefits (Dehghan *et al*., 2006).

Traditional herbal medicines have long been practiced worldwide to prevent different diseases for centuries. Many plants having antifertility agents have also been reported with antispermatogenic, semen coagulant and spermicidal activities (Gupta and Tandon, 2004; Sharma *et al*., 2013). In the recent past, research has been done to explore the biologically active components of medicinal plants that interfere with spermatogenic progression or natural process of reproduction (Jensen, 2002; Aladakatti *et al*., 2010).

Hedera, commonly called as "ivy" is a genus of flowering plants belonging to the family Araliaceae which have about 70 genera and 700 species. It is widely distributed throughout Europe Asia and North Africa. This genus is highly valuable economically despite of its uncertain taxonomy and phylogenetic relationships (Ackerfield and Wen, 2002).

Hedera nepalensis, locally known as "Kurie" is a flowering plant traditionally used for the treatment of many diseases including diabetes (Frohne and Pfander, 2004), fever, pulmonary infections, inflammatory bronchial disease and rheumatism (Shah, 2006). Its leaves and berries have cathartic and diaphoretic properties and widely used to cure indolent ulcers and abscesses. It is also known to have hypoglycemic and anticancer properties (Hamayun *et al*., 2006; Shah, 2006). *H. nepalensis* is locally used as a food for animals and inhabitants, while, commercially used in fibers, dyes,

cosmetics, agricultural and food industries (Ahmad *et al*., 2012). This plant has also been evaluated for different biological activities including phytotoxic, antitumor, cytotoxic and antifungal activities (Inayatullah *et al*., 2007; Meng and Wang, 2010). Previously, inflorescence of *H. nepalensis* has been reported to halt male reproduction by immobilization of human spermatozoa (Pant *et al*., 1988). But no clear picture about its effect on reproduction is available.

Indigenous use for antifertility: Flowers are dried, ground and taken in small quantity with water early in the morning.

Taxonomy

The taxonomy of the plant according to National Plant Data Center, NRCS, USDA is as follows

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Apiales

Family: Araliaceae

Genus: *Hedera* L.

Specie: *Hedera nepalensis* K.Koch

Figure 110. Plant *Hedera nepalensis* K.Koch in its natural habitat.

So, based on traditional knowledge and available literature, current study was designed to investigate the effect of methanolic extract of *H. nepalensis* on male reproduction. Histological and biochemical approaches were utilized and fertility test was performed to assess the reversibility of fertility suppression following the treatment withdrawal.

MATERIALS AND METHODS

Plant collection and extract preparation

Hedera nepalensis K.Koch is a wide spread herb located in Murree, AJK, Kaghan, Naran, Swat, Galyat and in some areas of Punjab, Pakistan. It is commonly known as "Kurie". Plant sample for this study was collected from fields of Galyat located at height of 2500 m. It was recognized by Dr. Mushtaq Ahmad at Plant Systematics and Biodiversity Lab (QAU) and specimen was deposited in herbarium with accession number #130046.

Methanolic leaf extract of *H. nepalensis* was prepared according to method described in chapter 2.

Animals

Adult male Sprague Dawley rats (150±10g), gained from rodent facility of Animal Sciences Department, Quaid-i-Azam University Islamabad, were housed in stainless steel cages under standard laboratory conditions. They were given with standard laboratory pelleted food and water was available *ad labitum*. All the animal handling and protocols were agreed by ethical committee of Animal Sciences department.

Experimental Design

Acute toxicity and *in vivo* sub chronic study was used for the evaluation of antifertility efficacy of *H. nepalensis* in male rats.

Acute oral toxicity

Acute oral toxicity study of methalonic leaf extract of *H. nepalensis* was performed according to method described in chapter 3.

In vivo experiment

Rats (80-90 days old) were divided into four study groups each having ten rats (n=10 rats/group). Four different doses (0, 50, 100 and 150 mg/kg/day) of plant extract were administered orally for twenty eight consecutive days. Stock solution of plant extract was prepared in methanol and further diluted with normal saline. Final methanol concentration was 0.5-1% in saline. Different doses used in this study were selected according to OECD guideline # 408 and duration of experiment was selected according to OECD guideline # 407 (one dose level daily during 28 days).

After the completion of experiment, seven animals from each group were killed by diethyl ether anesthesia; trunk blood was collected and plasma was separated for hormonal analysis. Reproductive organs were dissected out, weighed and processed for sperm parameters, biochemical and histological analysis.

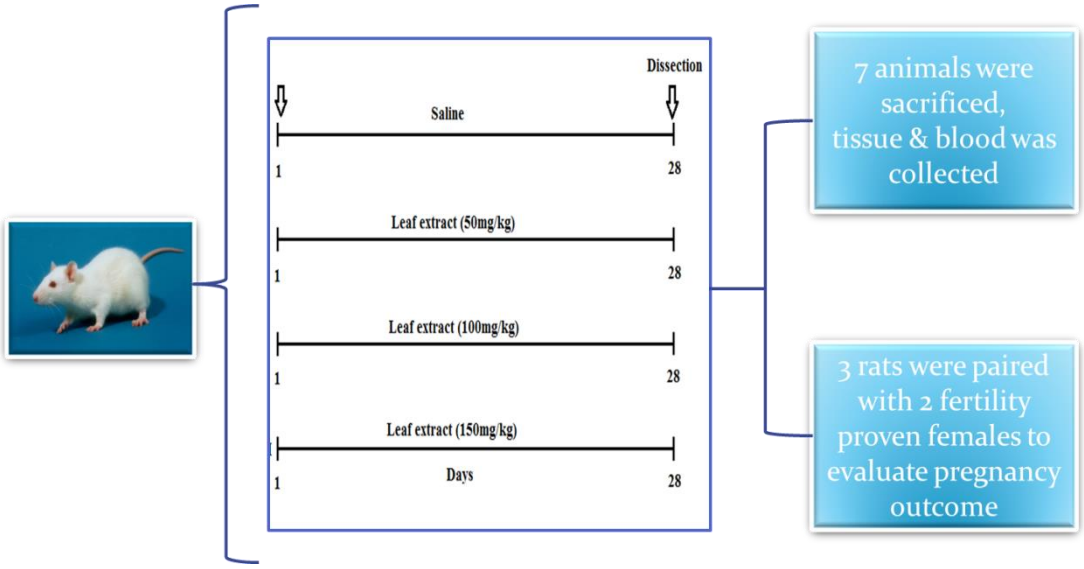


Figure 111: Schematic representation of experimental design of *in vivo* exposure to *H. nepalensis* methanolic leaf extract.

Fertility test:

Fertility test was done according to method described in previous chapter.

Assessment of Sperm parameters

For the assessment of sperm motility, viability and sperm count freshly dissected cauda epididymis was used according to method explained in chapter 3; while testicular homogenate was used for DSP as described in chapter 3.

Biochemical Analysis

Antioxidant enzymes (SOD, POD, CAT), TBARS and ROS were estimated in testicular tissues as given in chapter 2.

Histological analysis

Tissue histology was done according to method given in chapter 3.

Hormonal Analysis

Plasma Testosterone, LH and FSH concentrations from were assessed quantitatively by using enzyme linked immune sorbent assay (ELISA) kits. Detailed procedure is described in chapter 2 & 3.

Statistical analysis

Data is shown Mean ± SEM. Results were analyzed by one way analysis of variance (ANOVA) followed by Dunnet test using Graph pad Prism 5 software. Significance level was set at $p<0.05$.

RESULTS

Acute toxicity

Results of acute toxicity of methanolic leaf extract of *H. nepalensis* in rats are presented in table 43. Death of a single animal from high dose was reported within 24 hours of single dose of 2000 mg/kg extract while 2 animals were noted to be dead after 14 days. While, no obvious change in animal's behavior was seen except increase in water intake during the first 24 hours in high dose treated groups.

Table: 43. Results of acute toxicity study in rats after single oral dose of methanolic leaf extract of *H. nepalensis.*

Dose (mg/kg body weight)	Number of animals	Short-term result (24 h)	Long-term results (14 days)
0	3	Survival	Survival
5	3	Survival	Survival
50	3	Survival	Survival
300	3	Survival	Survival
2000	3	Mortality (1 animal)	Mortality (2 animals)

Fertility Test:

Effect of *H. nepalensis* leaf extract on the fertility and mean number of pups born per female after 60 days of treatment withdrawal is given in table 44, figure 112. A dose dependent decrease in percentage fertility and number of pups born per female were observed in all dose regimens when compared to control group. Low dose treatment (50 mg/kg) showed 83% fertility rate, while, percent fertility was observed 50% in 100 mg/kg treated group and only 16% in 150 mg/kg leaf extract treated group. The no of pups born per female were significantly reduced ($p<0.05$) in high dose treatment but no signs of morbidity or mortality were observed in resultant pups.

Table 44: Effect of *H. nepalensis* leaf extract on the fertility and the Mean ± SEM number of pups born per female after 60 days of treatment withdrawal.

Groups	Percent fertility	Number of pups born/female
Control	100	6.16 ± 0.83
50 mg/kg	83.33	5.66 ± 1.28
100 mg/kg	50	2.66 ± 1.28
150 mg/kg	16.66	1.50 ± 1.5*

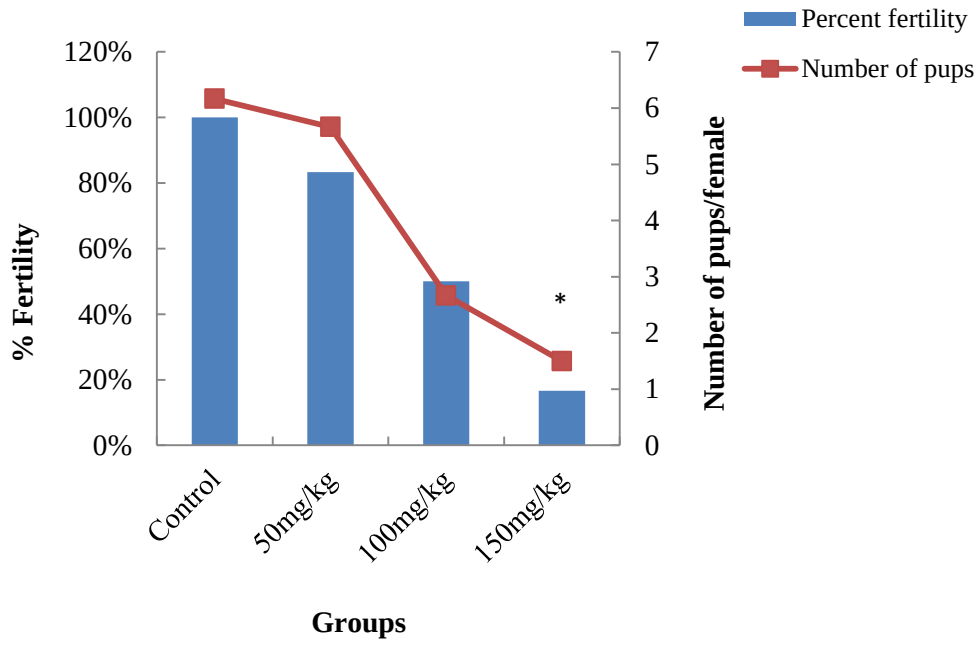


Figure 112: Effect of *H. nepalensis* leaf extract on % fertility and the number of pups born per female after 60 days of treatment withdrawal.

*= indicates significant difference of number of pups born/female at probability of $p<0.05$ compared to control.

Effect of extract on body weight gain and reproductive organ weight

There was no obvious change in body weight gain noted in all treated groups when compared with control. But this change was significant ($p<0.05$) in 100 mg/kg extract treated rats (Table 45, Figure 113). A significant decrease in both right and left testicular weight was seen in 50 mg/kg ($p<0.001$) and 100 mg/kg ($p<0.05$ and $p<0.001$ respectively) treated group as compared to control group. However, little change was observed in testicular weight of 150 mg/kg dose treated rats when comparison was made with control group (Table 45, Figure 114).

Similarly, significant decrease in left and right epididymal weight was seen in animals of 50 mg/kg ($p<0.001$) and 100 mg/kg ($p<0.05$) groups as compared to control group. In addition, weight of seminal vesicle was reduced significantly ($p<0.001$) in all dose treated groups. A significant reduction ($p<0.05$) in mean prostate weight was seen among 100 mg/kg while non-significant change in 50 mg/kg and 150 mg/kg extract treated rats was observed when compared to control group (Table 45).

Table 45: Effect of *H. nepalensis* leaf extract on mean ± SEM body weight (g) and reproductive organ weights (g) of control and treated groups.

Parameters	Groups			
	Control	**50mg/kg**	**100mg/kg**	**150mg/kg**
Body weight gain (g)	49.2 ± 4.91	38.6 ± 8.48	24.4 ± 2.60*	53.3 ± 5.47
Right testicular weight (g)	1.25 ± 0.05	0.94 ± 0.04***	1.03 ± 0.02*	1.20 ± 0.06
Left testicular weight (g)	1.23 ± 0.03	0.90 ± 0.02***	1.02 ± 0.03***	1.29 ± 0.03
Right epididymis weight (g)	0.55±0.02	0.35±0.02***	0.42±0.02*	0.59±0.04
Left epididymis weight (g)	0.56±0.01	0.38±0.01***	0.44±0.03*	0.56±0.03
Seminal vesicle weight (g)	1.48±0.17	0.70±0.05***	0.67±0.07***	0.75±0.08***
Prostate weight (g)	0.73±0.08	0.52±0.03	0.51±0.03*	0.69±0.05

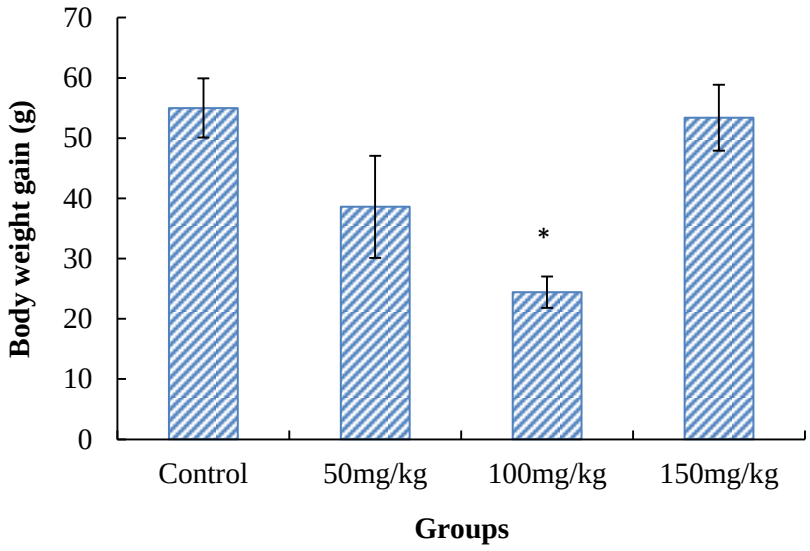


Figure 113: Mean ± SEM body weight gain (g) of control and treated groups of rats, receiving methanolic leaf extract of *H. nepalensis* for 28 days.

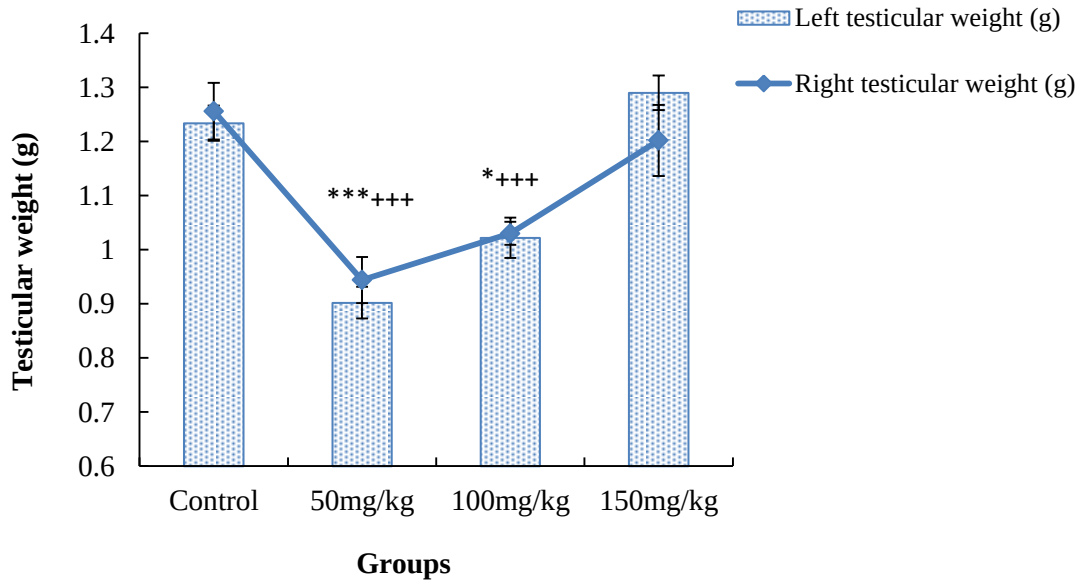


Figure 114: Mean ± SEM of Testicular weight gain (g) of control and treated groups of rats, receiving methanolic leaf extract of *H. nepalensis* for 28 days.

*,***= indicates significant difference of Right testicular weight at probability of $p<0.001$ as compared to control.

+++= indicates significant difference of Left testicular weight at probability of $p<0.001$ compared to control.

Sperm parameters and DSP

A significant reduction ($p<0.001$) in % sperm motility and viability rate was seen in all extract treated groups when compared to control group as shown in table 46, figure 115. Similarly, daily sperm production was significantly reduced ($p<0.05$) in 150 mg/kg treated group when compared to control group. However, no significant change in DSP was seen among low dose treated rats as compared to control group (Table 46, Figure 117).

Mean ± SEM of epididymal sperm count in control and treated groups are shown in table 46, figure 116. No significant difference was seen in epididymal sperm count of 50 mg/kg group as compared to control group. However, significant reduction in sperm number of caput, cauda ($p<0.01$) and corpus ($p<0.05$) was observed in 100 mg/kg dose treatment group. Similarly, 150 mg/kg treated rats exhibited significant decrease in caput ($p<0.05$), corpus and cauda ($p<0.001$) sperm count when compared with control group animals.

Table 46: Effect of *H. nepalensis* leaf extract on mean ± SEM sperm parameters of control and treated groups.

Parameters	Groups			
	Control	**50mg/kg**	**100mg/kg**	**150mg/kg**
Motility rate (%)	70.4±1.21	49.8±2.11***	40.2±3.20***	32.4±2.29***
Viability rate (%)	72.6±2.11	40.00±5.14***	42.2±3.08***	30.8±4.65***
DSP × 10^5 /testis	5.01±1.83	3.93±0.35	4.09±0.29	3.46±0.47*
Caput sperm count	1548.75±20.08	1465±31.65	1381.25±32.20**	1396.25±31.78*
Corpus sperm count	1398.75±10.73	1280±44.88	1266.25±21.76*	1177.5±24.32***
Cauda sperm count	1893.75±14.10	1768.75±19.14	1697.5±44.33**	1645±36.68***

Values are expressed as mean ± SEM

*, **, *** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

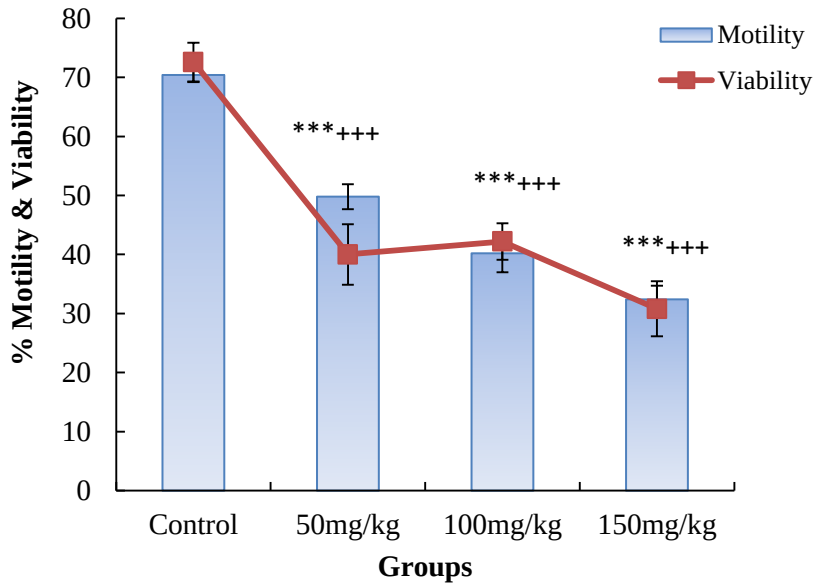


Figure 115: Mean ± SEM of % sperm motility & viability rate in control and treated rats receiving methanolic leaf extract of *H. nepalensis* for 28 days.

***= indicates significant difference of % motility at probability of p<0.001 as compared to control.

+++= indicates significant difference of % viability at probability of p<0.001 compared to control.

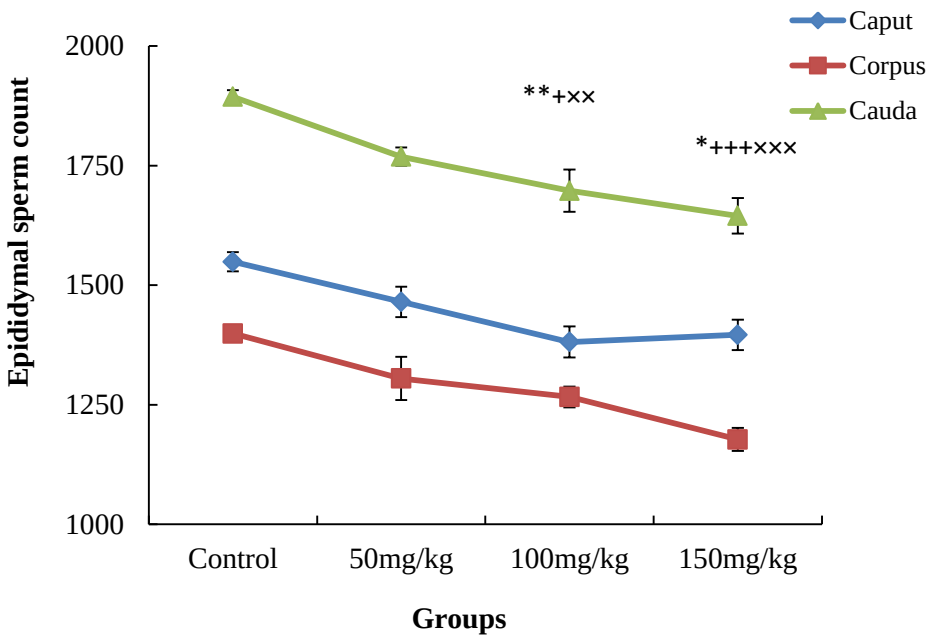


Figure 116: Mean ± SEM of epididymal sperm count in control and treated rats receiving methanolic leaf extract of *H. nepalensis* for 28 days.

*,**= indicates significant difference of caput sperm number at probability of $p<0.05$, $p<0.01$ as compared to control.

+,+++= indicates significant difference of carpus sperm number at probability of $p<0.05$, $p<0.001$ compared to control.

××,×××= indicates significant difference of cauda sperm number at probability of $p<0.01$, $p<0.001$ compared to control.

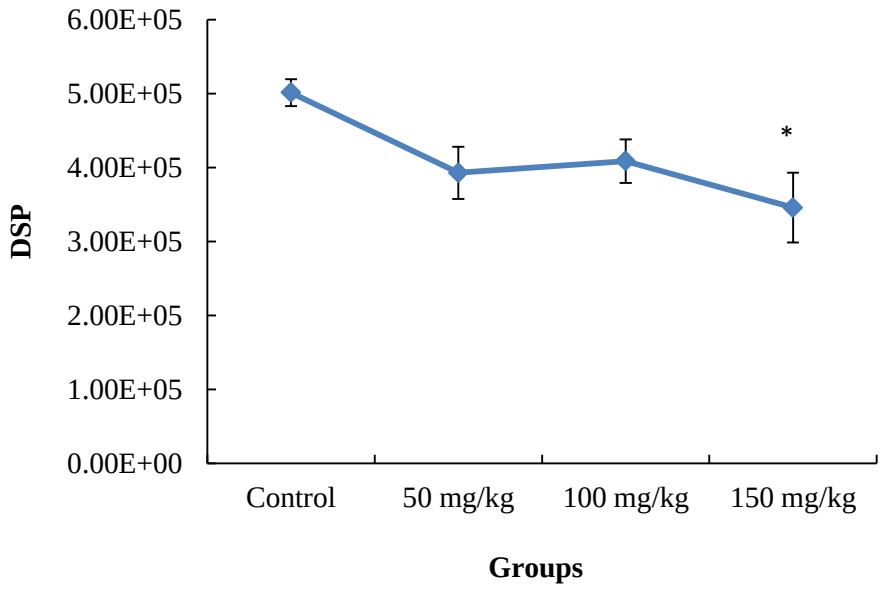


Figure 117: Mean ± SEM of DSP in control and treated rats receiving methanolic leaf extract of *H. nepalensis* for 28 days.

*= indicates significant difference at probability of $p<0.05$ as compared to control.

Biochemical analysis

Antioxidant enzymes activity including CAT, SOD, POD, ROS and TBARS in the testis of control and treated groups of adult male rats after 28 days of treatment are presented in table 47.

A dose dependent decrease in catalase activity (U/mg) was observed in all treated groups, however, this reduction was significant ($p<0.01$) in 100 mg/kg and 150 mg/kg extract treated groups as compared to control group. Similarly, superoxide dismutase (U/mg protein) concentration was reduced significantly ($p<0.001$) in all extract treated groups dose dependently when comparison was made with control (Figure 118). A significant reduction in peroxidase (nmole) level was reported from 13.38 ± 1.22 in control to 7.21 ± 0.97 in 100 mg/kg ($p<0.01$) and 9.18 ± 0.80 in 150 mg/kg ($p<0.05$) dose treated groups (Table 47, Figure 119).

Plant extract treatment caused increased production of oxidative stress markers (ROS and TBARS) within testis of adult male rats as shown in table 47. A non significant increase in ROS was seen in 50 mg/kg and 100 mg/kg treated groups while significant increase ($p<0.05$) in 150 mg/kg dose treated group was noted as compared to control group. Similarly, TBARS level was increased significantly ($p<0.001$) in all extract treated groups when compared with control group (Table 47, Figure 120).

Table 47: Effect of *H. nepalensis* leaf extract on mean ± SEM antioxidant activity of testicular tissues of control and treated groups.

Groups	CAT (U/mg)	SOD (U/mg)	POD (nmole)	ROS (µmol/min)	TBARS (nM/mg)
Control	18.06 ± 1.23	49.41 ± 4.45	13.38 ± 1.22	0.99±0.05	1.23 ± 0.10
50mg/kg	14.92 ± 1.18	30.96 ± 2.92**	12.79 ± 0.78	1.12±0.16	2.48 ± 0.25**
100mg/kg	10.02 ± 1.74**	27.69 ± 2.79***	7.21 ± 0.97**	1.24±0.15	3.08 ± 0.19***
150mg/kg	10.21 ± 1.51**	21.39 ± 1.44***	9.18 ± 0.80*	1.61±0.20*	2.98 ± 0.17***

Values are expressed as mean ± SEM

*,**,*** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

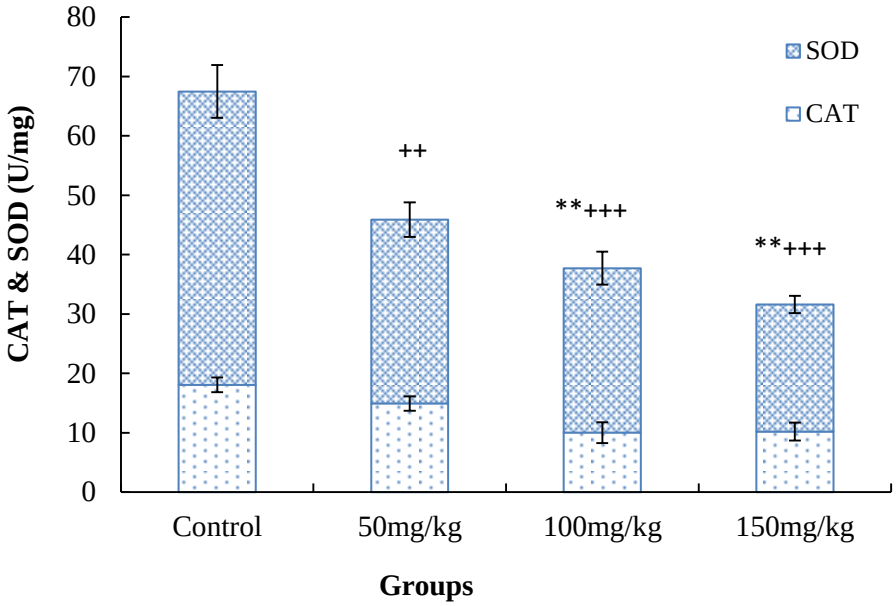


Figure 118: Specific activity of CAT and SOD in testis of control and treated rats receiving methanolic leaf extract of *H. nepalensis* for 28 days.

**= indicates significant difference of CAT at probability of $p<0.01$ compared to control.

++,+++= indicates significant difference of SOD at probability of $p<0.01$, $p<0.001$ compared to control.

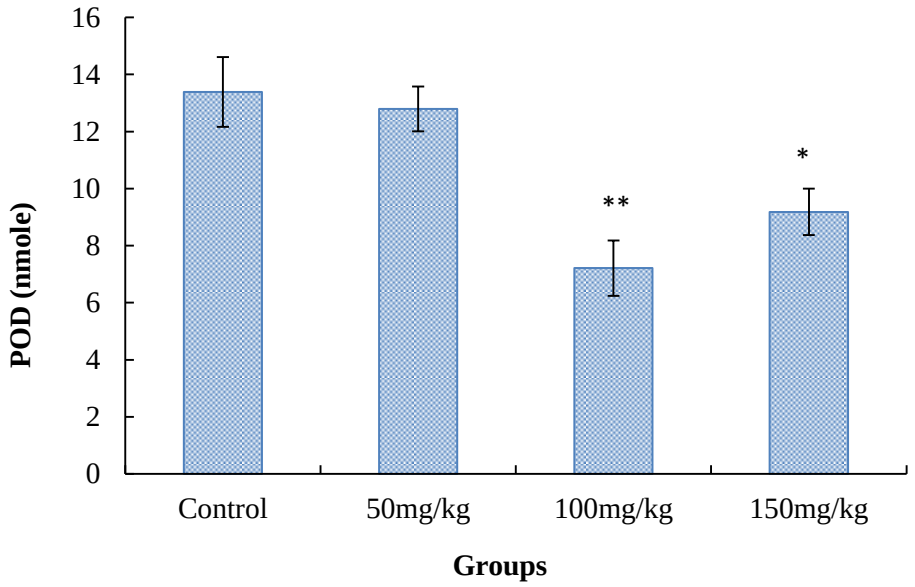


Figure 119: Specific activity POD of in testis of control and treated rats receiving methanolic leaf extract of *H. nepalensis* for 28 days.

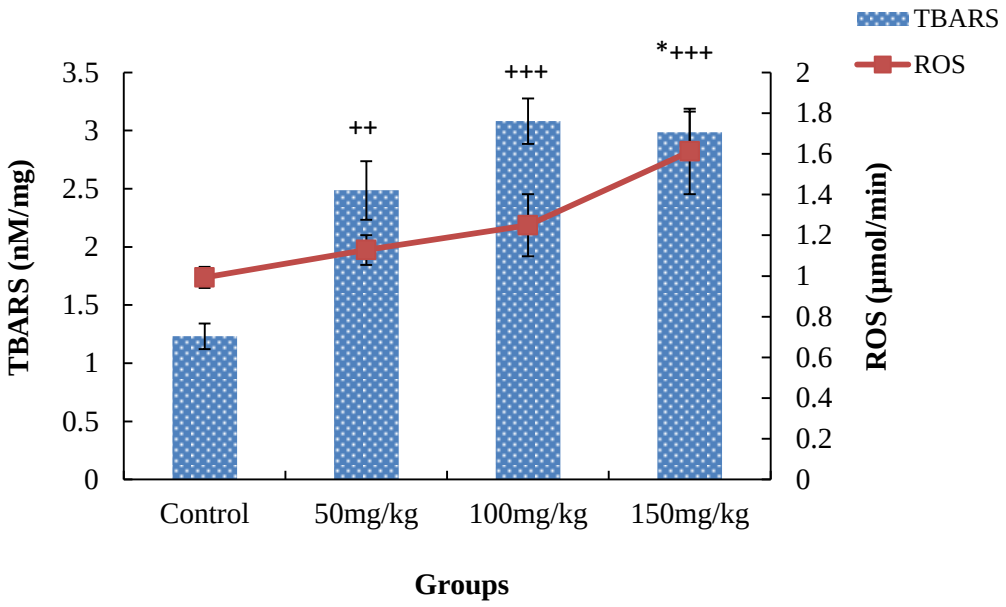


Figure 120: Specific activity of ROS & TBARS in testis of control and treated rats receiving methanolic leaf extract of *H. nepalensis* for 28 days.

*= indicates significant difference of ROS at probability of $p<0.05$ compared to control.

++,+++= indicates significant difference of TBARS at probability of $p<0.01$ and $p<0.001$ compared to control.

Histological Analysis

Morphological Changes in testis

Closely arranged seminiferous tubules along with thick stratified epithelium and narrow lumen filled with mature spermatids was present in testicular section of control group Arrangement of seminiferous tubules in 50 mg/kg treated group was similar to control group with thick epithelium and lumen concentrated with sperms. On the other hand, high dose treated groups with 100 mg/kg and 150 mg/kg groups showed normal tubular arrangement with wider lumen having less number of spermatozoa and germinal epithelium as compared to control group (Figure 121).

Morphometric results

Morphometric parameters of testicular tissue are given in table 48. A non significant decrease in seminiferous tubule diameter was observed in all extract treated groups when compared to control. While, tubular lumen diameter was significantly increased in 100 mg/kg ($p<0.05$) and 150 mg/kg ($p<0.01$) dose treated groups, however, no change was seen in 50 mg/kg treated group. In case of epithelial height, significant change was reported in 100 mg/kg ($p<0.01$) and 150 mg/kg ($p<0.001$) dose treated groups than control (Figure 122). Similarly, area of seminiferous tubules and interstitial space (%) were disturbed significantly in all dose treated groups i.e. 50 mg/kg ($p<0.05$), 100 mg/kg ($p<0.01$) and 150 mg/kg ($p<0.001$) (Figure 123).

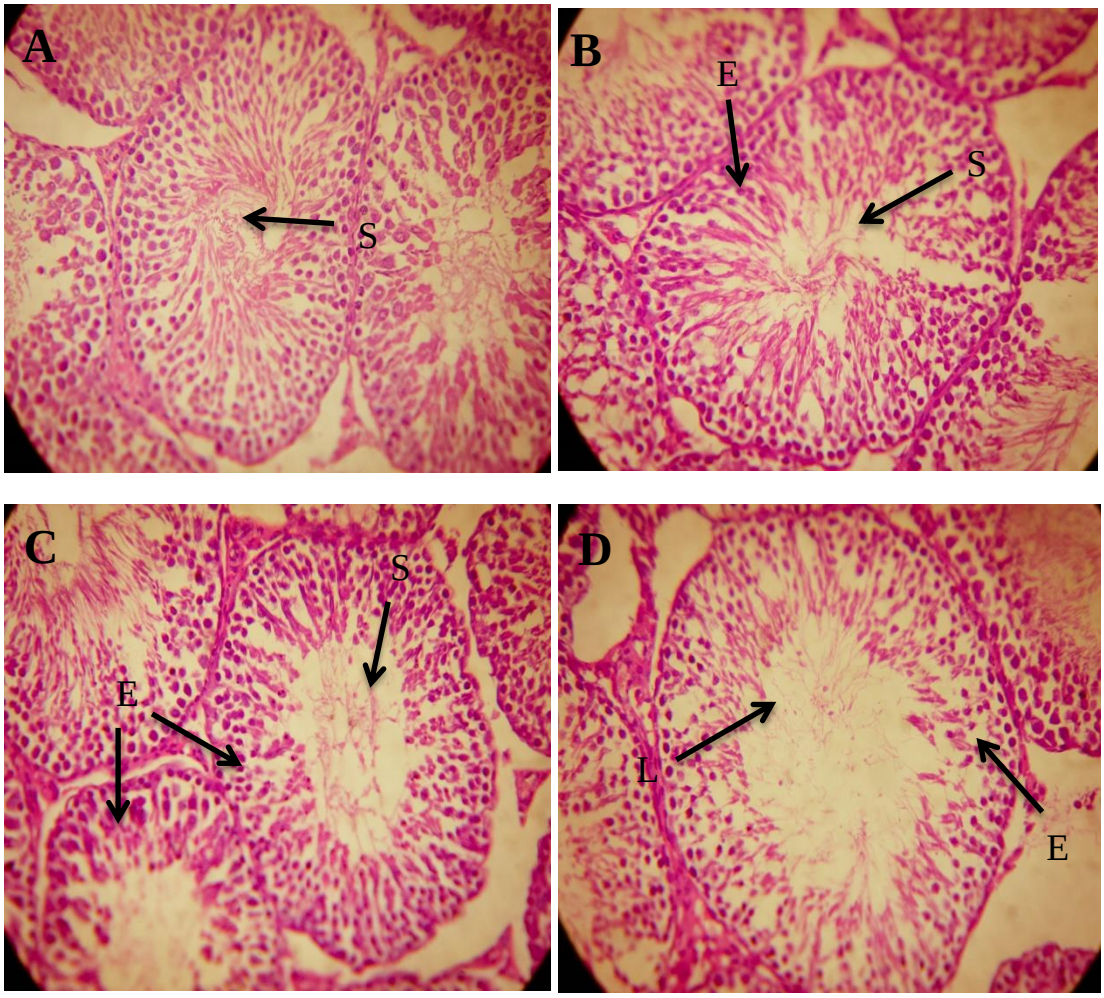


Figure 121: Photomicrograph of seminiferous tubules of adult male rats treated with methanolic leaf extract of *H. nepalensis*. Regularly arranged tubules, lumen filled with spermatids with normal germ cells shown in (A) Control; represents normal process of spermatogenesis, lumen filled with mature spermatozoa, (B) 50mg/kg groups; showing tubules with immature spermatids (S) released in lumen, (C) 100mg/kg group; showing increased lumen diameter (L) and amplified epithelial height (E), tubules with interstitial spaces (IS) are present, (D) 150mg/kg group; thin epithelium (E), lumen is empty (L), large interstitial space (IS) are present. Magnification 40X.

Table 48: Effect of *H. nepalensis* leaf extract on mean ± SEM of seminiferous tubule diameter (µm), tubular lumen diameter (µm), epithelial height (µm), area of seminiferous tubule (%) and interstitial space (%) of testis in control and treated groups.

Groups	Seminiferous tubule diameter (µm)	Tubular lumen diameter (µm)	Epithelial height (µm)	Area of seminiferous tubule (%)	Interstitial space (%)
Control	230.73 ± 5.71	189.02 ± 3.95	77.53 ± 1.92	85.52±0.62	14.48 ±0.62
50mg/kg	227.97 ± 4.46	180.89 ± 3.39	73.87 ± 2.19	83.44±0.30*	16.56 ±0.30*
100mg/kg	219.12 ± 3.18	177.66 ± 2.82*	67.28 ± 2.39**	82.28±0.65**	17.72 ±0.65**
150mg/kg	222.69 ± 4.33	171.72 ± 2.93**	59.39 ± 1.92***	79.34±0.57***	20.66 ±0.57***

Values are expressed as mean ± SEM

*,**,*** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

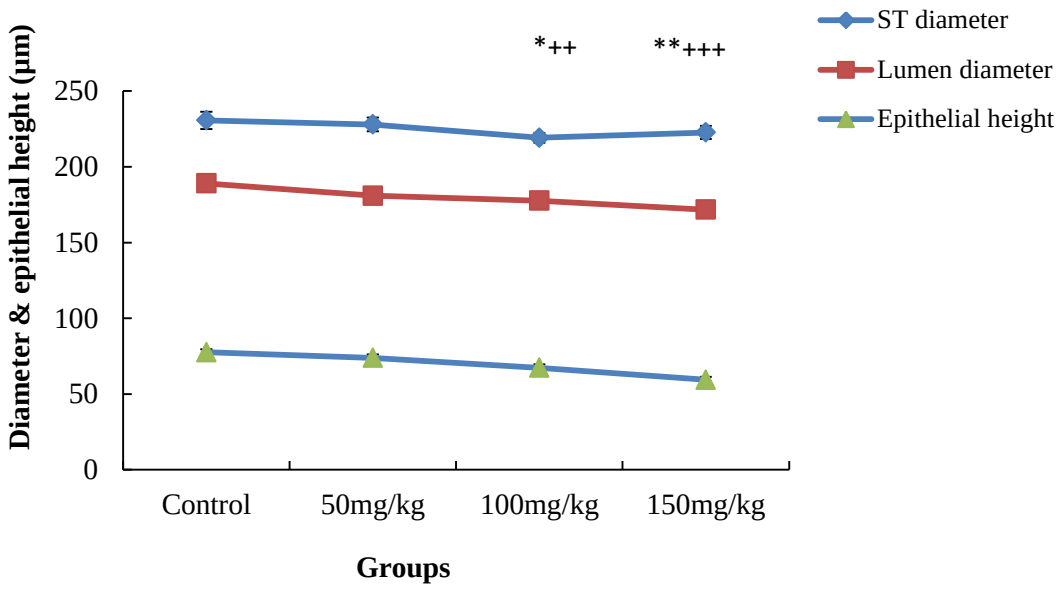


Figure 122: Mean ± SEM of seminiferous tubule diameter (µm), tubular lumen diameter (µm) and epithelial height (µm) in testes of control and treated rats receiving methanolic leaf extract of *H. nepalensis* for 28 days.

*,**= indicates significant difference of lumen diameter at probability of $p<0.05$ and $p<0.01$ compared to control.

++,+++= indicates significant difference of epithelial height at probability of $p<0.01$ and $p<0.001$ compared to control.

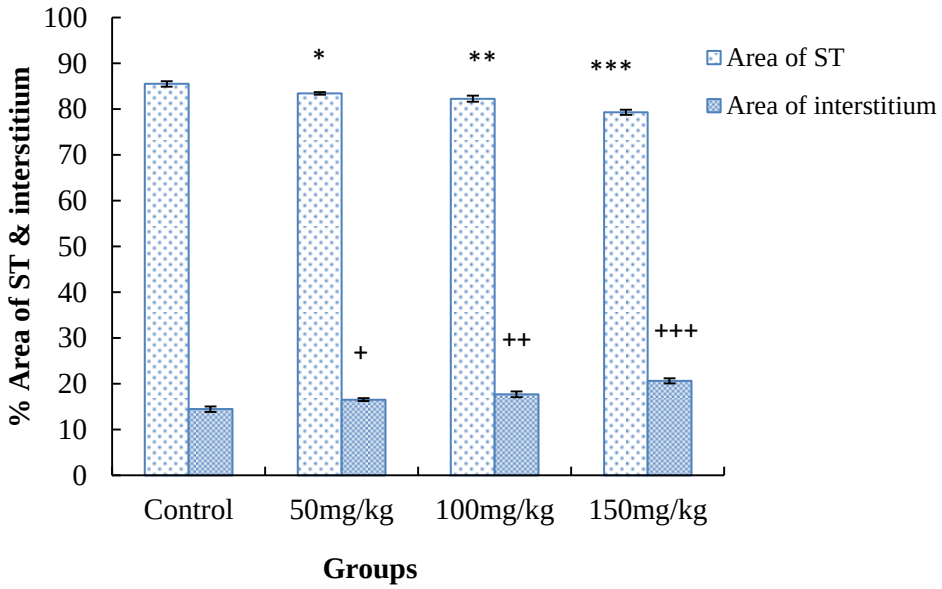


Figure 123: Mean ± SEM % area of seminiferous tubule and % area of interstitium in testes of control and treated rats receiving methanolic leaf extract of *H. nepalensis* for 28 days.

*,**,***= indicates significant difference of % area of ST at probability of $p<0.05$, $p<0.01$ and $p<0.001$ compared to control.

++,+++= indicates significant difference of % area of interstitium at probability of $p<0.05$, $p<0.01$ and $p<0.001$compared to control.

Epididymis:

Histological analysis of caput and cauda epididymis of control group showed normal tubular arrangement, larger diameter and wider lumen with thin pseudostratified epithelium. The epididymis was closely arranged and lumen was filled with large number of spermatozoa. Low dose treated group (50 mg/kg) did not affect tubular arrangement and sperm concentration in lumen was similar to control. Treatment with plant extract (100 mg/kg and 150 mg/kg) showed normally arranged epididymis with slightly narrow lumen and relatively reduced number of sperms as compared to control group (Figure 124 & 125).

Tubular diameter, lumen diameter and epithelial height of caput and cauda epididymis are given in table 49. Non significant difference in tubular diameter of epididymis was seen in all dose treated groups when compared to control. However, tubular diameter of caput epididymis was decreased significantly ($p<0.001$) in 150 mg/kg extract treated group. Similarly, lumen diameter of caput was significantly reduced ($p<0.01$) in all treated groups while no change was seen in lumen diameter of cauda epididymis when comparison was made with control group (Table 49, Figure 126). .

Epithelial height of caput epididymis was reduced significantly ($p<0.01$) only in 150 mg/kg treated group, while other extract treated groups showed non significant change as compared to control group. The epithelial cell height of cauda epididymis was found to be reduced in dose dependent manner in all the extract treated groups with significant reduction ($p<0.001$) in 100 mg/kg and 150 mg/kg extract treated groups compared to control group (Table 49, Figure 127).

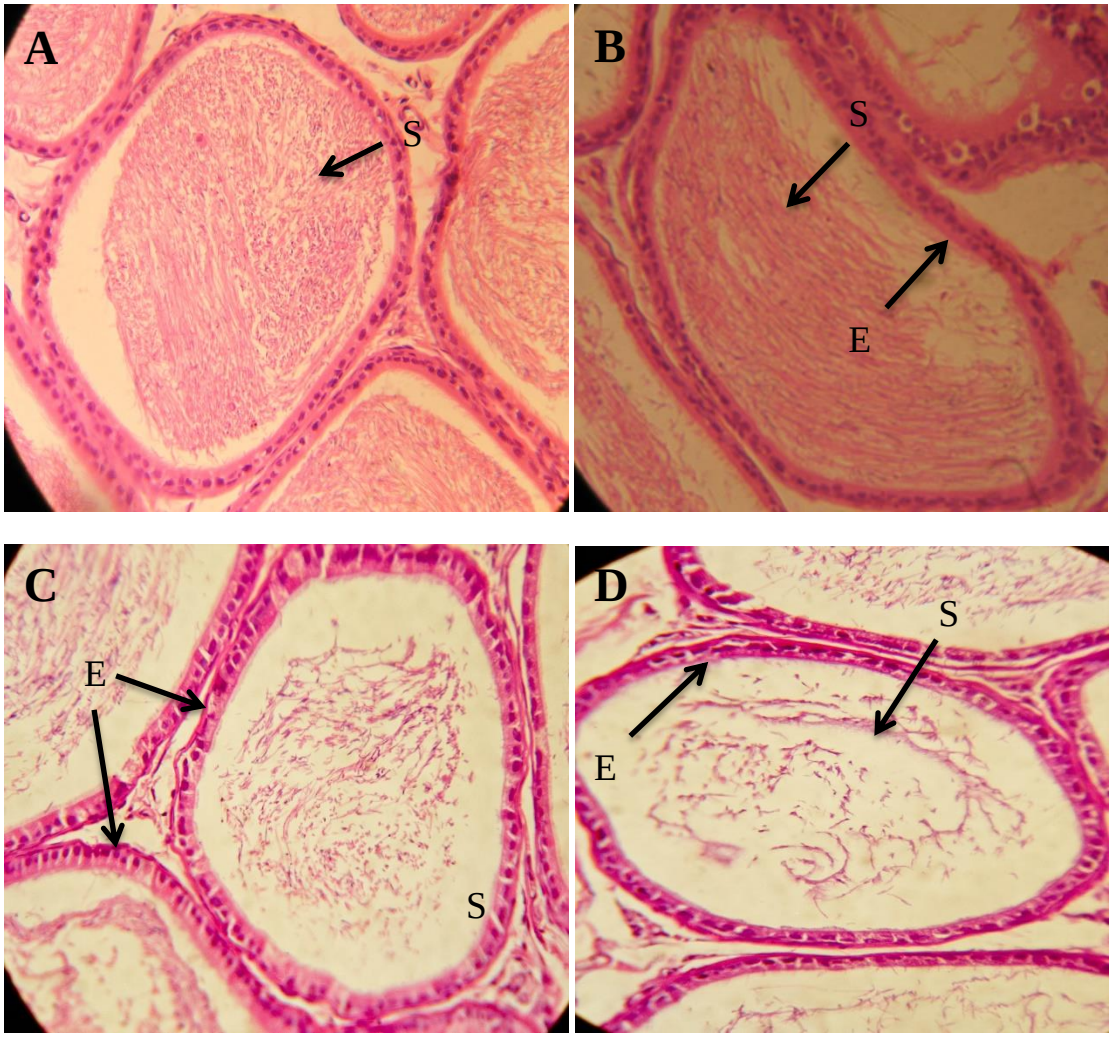


Figure 124: Photomicrograph of caput epididymis of adult male rats treated with methanolic leaf extract of *H. nepalensis* from: (A) Control group; with normal morphology of caput having thin pseudostratified epithelium lined with stereocilia. Lumen is heavily filled with spermatozoa (S), (B) 50mg/kg group; reduced pseudostratified epithelium with lumen filled with spermatozoa (S), (C) 100mg/kg group; showing reduction in number of spermatozoa, low number of spermatozoa in lumen and quite thick epithelium (E), (D) 150mg/kg group; showing sloughing of epithelium and very little concentration of spermatozoa in lumen. Magnification 40X.

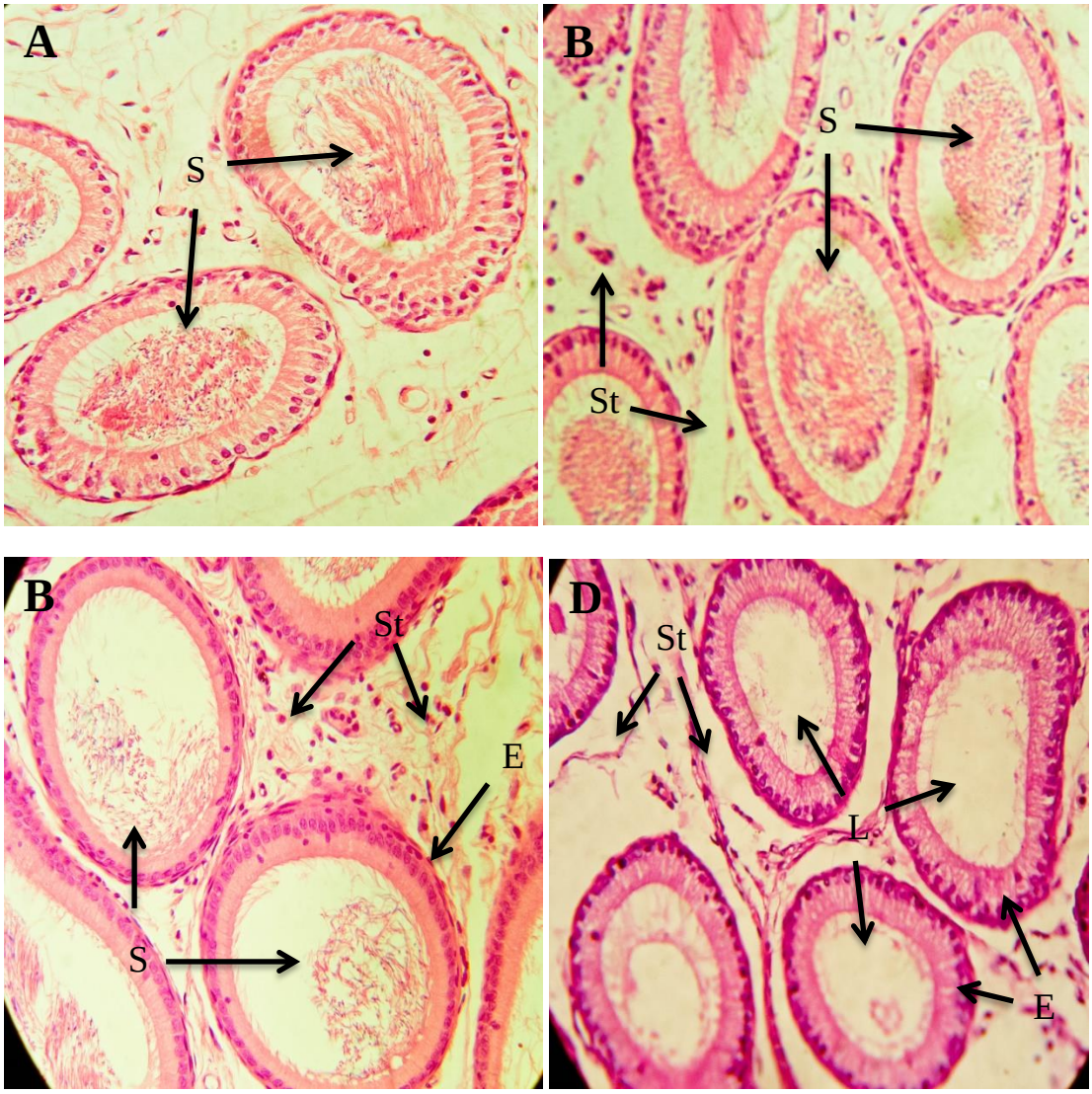


Figure 125: Photomicrograph of cauda epididymis of adult male rats receiving doses of *H. nepalensis* showing (A) Control; exhibiting normal morphology of cauda epididymis; thick epithelium (E), lumen filled with mature sperms (S), (B) 50mg/kg group; showing changes in structure of tubule with thick epithelium (E) while lumen filled with sperms (S), (C) 100mg/kg group; showing irregular arrangement of tubules surrounded by stroma (St), lumen has very little number of spermatozoa (S), (D) 150mg/kg group; showing further maximum increase in epithelial height (E) and a little sperm concentration in lumen (L). Magnification 40X.

Table 49: Effect of *H. nepalensis* leaf extract on mean ± SEM of ductular diameter (µm), luminal diameter (µm) and epithelial cell height (µm) of epididymis in control and treated groups.

Groups	Ductular diameter (µm)		Lumen diameter (µm)		Epithelial height (µm)	
	Caput	Cauda	Caput	Cauda	Caput	Cauda
Control	265.09±4.26	312.64±5.18	246.75±3.45	267.15±6.55	28.52±0.89	34.06±1.60
50mg/kg	257.60±5.88	308.03±4.74	227.02±5.48**	268.96±6.39	25.80±0.95	32.80±0.96
100mg/kg	249.95±4.67	299.24±3.41	227.57±5.16**	262.78±6.82	26.22±0.84	22.03±0.89***
150mg/kg	226.83±4.01***	304.21±3.99	198.39±3.99***	256.55±5.91	24.09±0.87**	24.57±0.85***

Values are expressed as mean ± SEM

*,**,*** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

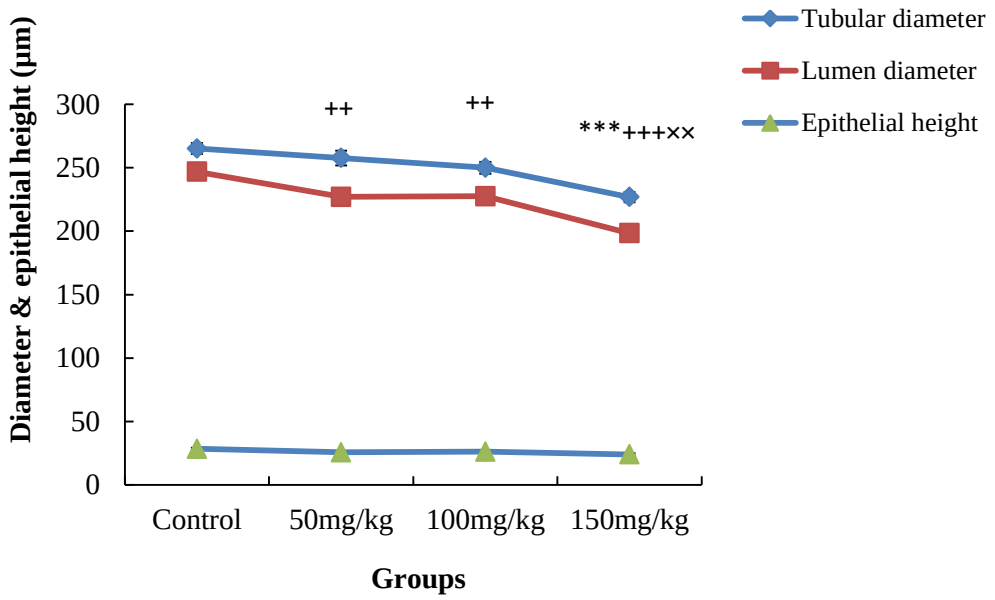


Figure 126: Mean ± SEM of ductular diameter (µm), lumen diameter (µm) and epithelial height (µm) in caput of control and treated rats receiving methanolic leaf extract of *H. nepalensis* for 28 days.

**= indicates significant difference of ductular diameter at probability of p<0.01 compared to control.

++,+++= indicates significant difference of lumen diameter at probability of p<0.01 and p<0.001compared to control.

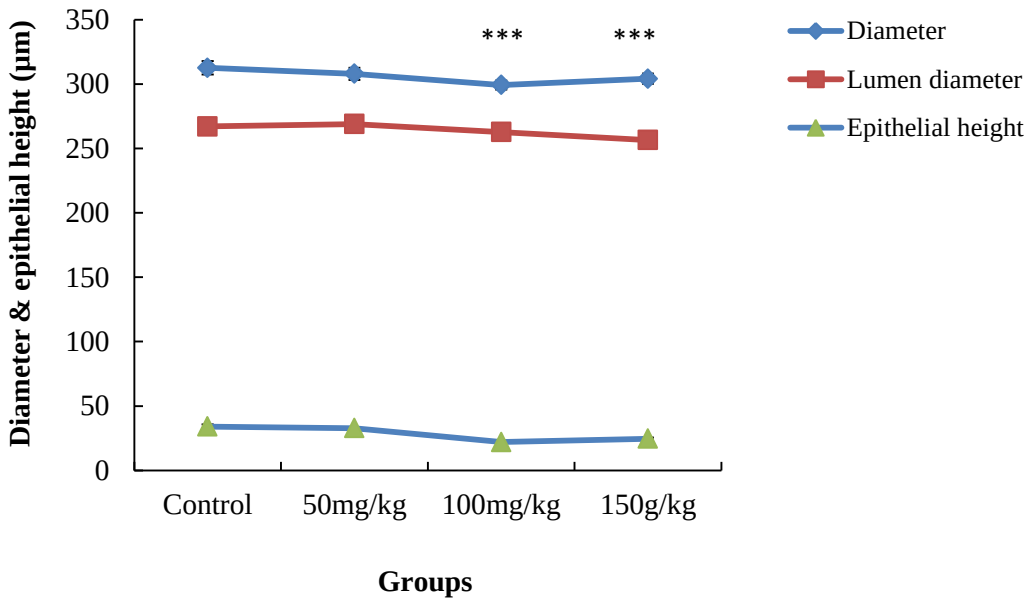


Figure 127: Mean ± SEM of ductular diameter (µm), lumen diameter (µm) and epithelial height (µm) in cauda of control and treated rats receiving methanolic leaf extract of *H. nepalensis* for 28 days.

***= indicates significant difference of epithelial height at probability of $p<0.001$ compared to control.

Hormonal Analysis

Hormonal concentration of plasma Testosterone (ng/ml), LH (IU) and FSH (IU) in adult male rats following 28 days of treatment has been given in Table 50. A significant decline ($p<0.001$) in plasma testosterone level was seen in all treated groups as compared to control group. Likewise, plasma LH and FSH concentrations were decreased in dose dependent manner; however, this reduction was significant ($p<0.001$) in 100 mg/kg and 150 mg/kg dose treated groups when comparison was made with control group (Figure 128 & 129).

Table 50: Effect of *H. nepalensis* leaf extract on mean ± SEM plasma Testosterone (ng/ml), LH (IU) and FSH (IU) concentrations in control and treated groups.

Groups	Plasma Testosterone concentration (ng/ml)	Plasma LH concentration (IU)	Plasma FSH concentration (IU)
Control	3.08±0.12	2.21±0.14	1.03±0.08
50mg/kg	2.13±0.07***	1.88±0.24	0.91±0.08
100mg/kg	1.16±0.03***	1.11±0.06***	0.44±0.07***
150mg/kg	1.06±0.05***	1.09±0.04***	0.51±0.05**

Values are expressed as mean ± SEM

*,**,*** indicates significant difference at probability $p<0.05$, $p<0.01$ and $p<0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

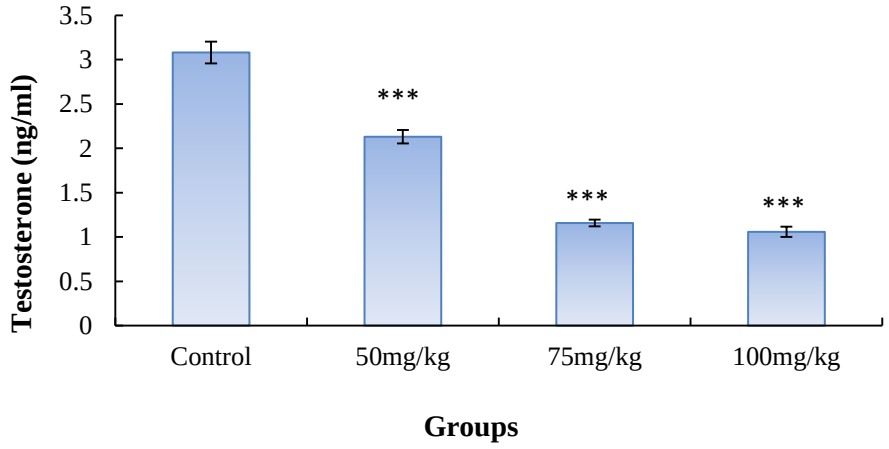


Figure 128: Mean ± SEM of plasma Testosterone concentration in control and treated rats receiving methanolic leaf extract of *H. nepalensis* for 28 days.

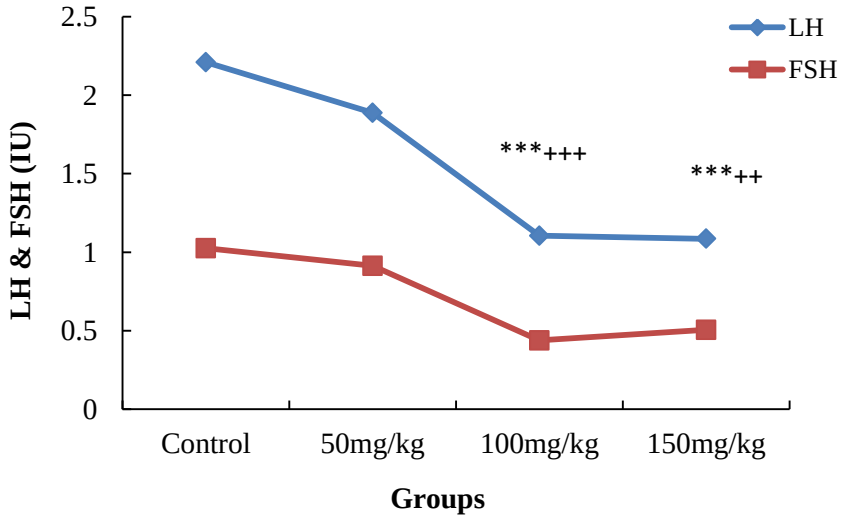


Figure 129: Mean ± SEM of plasma LF and FSH concentration in control and treated rats receiving methanolic leaf extract of *H. nepalensis* for 28 days.

***= indicates significant difference of LH at probability of p<0.001 compared to control.

++,+++= indicates significant difference of FSH at probability of p<0.01 and p<0.001compared to control.

DISCUSSION

Currently, regulation of population growth is one of the major problems in developing countries. Plants have been used globally as a safe and natural source of medicine among varied cultures and civilizations since ancient times. The knowledge about plants and their medicinal properties was delivered orally through generations but written records were prepared as time passed with growth of civilizations (Kritikar and Basu, 1981). Many plants are known to have contraceptive potential; exerting their action in animals either by impairing normal process of spermatogenesis or steroidogenesis in (Dixit *et al*., 1978; Udoh and Kehinde, 1999). *Hedra nepalensis* commonly known as "Kurie" has been traditionally used for the treatment of numerous diseases including diabetes, fever, pulmonary infections, ulcers, abscesses and rheumatism (Frohne and Pfander, 2004; Shah, 2006). Previous studies have confirmed its hypoglycemic, antiproliferative, cytotoxic and antifungal properties (Hamayun *et al*., 2006; Shah, 2006). A former study reported that *H. nepalensis* exerts antifertility effects in humans by immobilization of spermatozoa (Pant *et al*., 1988) but no study has been conducted to find out underlying mechanism. Therefore, current study was designed to explore antifertility actions of methanolic leaf extract of *H. nepalensis* on reproductive system of male rat.

The acute toxicity studies showed no mortality in rats at any given dose of methanolic leaf extract of *H. nepalensis* highlighting that it is not toxic enough to induce mortality even at 2000 mg/kg of plant extract. Reduced fertility rate and pregnancy outcome was observed among females mated with extract treated male rats after withdrawal of treatment. This might be attributed to poor quality of sperms and reduced sperm motility. Sperm motility is crucial for normal fertilization and one of the most important parameters for the assessment of the fertilizing ability of ejaculated sperm. Results of present study showed significant reduction in percent sperm motility and viability among all dose treated groups. Similarly, daily sperm production and epididymal sperm count was also reduced when animals were treated with high dose of extract showing its toxic effects on sperm parameters. Similar results have also been reported previously by various researchers (Ahmed *et al*., 2002; Cárdenas-Valencia *et al*., 2008; Saba *et al*., 2009; Shweta *et al*., 2011). As circulating levels of testosterone are necessary for maintenance of normal structure and function of accessory sex organs, so, reduced daily sperm production and epididymal sperm

concentration are might be due to low testosterone concentration in blood (Sethi *et al*., 2010).

A dose dependent decrease in antioxidant levels (CAT, SOD, POD) was seen within testicular tissues of rats exposed to methanolic leaf extract of *H. nepalensis* while production of oxidative stress markers (ROS and TBARS) was increased, respectively. Our findings are supported by previous study in which administration of *Cannabis* extracts caused oxidative stress in testis by reducing antioxidant levels associated with increased lipid peroxidation. This prolonged oxidative stress did damage to basement membrane, sloughing of germinal epithelium and shrinkage of seminiferous tubules thus leading to complete spermatogenic arrest (Alagbonsi and Olayaki, 2017).

Antiandrogenic potential of plant extract was supported by disrupted arrangement of seminiferous epithelium thus decreasing tubular diameter and affecting normal process of spermatogenesis. Previously, Udoh & Kehinde reported similar findings in which oral administration of crude ripe seeds of papaya caused germ cell apoptosis and degeneration of germinal epithelium (Udoh and Kehinde, 1999). In another study, exposure of rats to different concentrations of crude extract of garlic (*Allium sativum*) caused reduction in testosterone levels leading to germ cell death. It was observed that expression of caspase inhibitors i.e. Baculoviral IAP repeat-containing protein 3 (BIRC3) and Baculoviral IAP repeat-containing protein 2 (BIRC2) and activation of caspase-3 increases in germ cell apoptosis (Hammami *et al*., 2009).

Synthesis of testicular androgens along with highly regulated feed-back mechanism involving HPG axis is vital for maintenance of spermatogenesis. Deficiency of testosterone leads to decline in germ cell population thus affecting spermatogenesis (Sofikitis *et al*., 2008). GnRH secreted from hypothalamus induces production of LH and FSH in pituitary that in turn, regulates the synthesis of androgen in testis by negative feedback mechanism (Dufau *et al*., 1984).

Testosterone play crucial role in sustaining the body shape, and increasing muscle mass and strength. Thus, reduced levels of testosterone adversely affect androgen-dependent functions including spermatogenesis (Yakubu *et al*., 2007). In present study, hormonal concentration of plasma Testosterone, Luteinizing hormone and Follicle stimulating hormone was reduced when rats were treated with higher doses of

plant extract. Similar results have been reported formerly, showing reduced testosterone concentration and Leydig cell degeneration after exposure to different plant extracts (Gupta and Kachhawa, 2007; Yakubu *et al*., 2007; Ogbuewu *et al*., 2011). In present study, reduced concentrations of testosterone and gonadotropins might induce histological alterations in testis and epididymis indicating *H. nepalensis* possesses anti-androgenic and anti-spermatogenic properties.

Conclusion

From these results, it can be speculated that methanolic leaf extract of *H. neaplensis* have the potential to suppress fertility either by disturbing sperm parameters or interfering with HPG axis. Reduced production of gonadotropins and testosterone along with increased oxidative stress in testis caused histological alterations ultimately leading to spermatogenic arrest.

GENERAL DISCUSSION

Growing population is a worldwide problem particularly in developing and under developed countries. Ever increasing population throughout the world, however, has inevitable effects on life supporting resources on the earth and development (Gupta and Kachhawa, 2007) such as health, education, employment, housing and sanitation. According to a survey in 2005, world population is estimated to be 6.5 billion and has been expected to become 9 billion in 2050 (Gupta and Sharma, 2006a; b). Latest official current world population estimate is 6,790,062,216 (Azamthulla *et al*., 2014; 2015). Now there is a need of time to control the population status to get rid of resource availability issues. Currently, prevalence of contraceptive methods among human population is 8.1 percent (Thakur *et al*., 2010a; b).

Almost 90% women use contraceptives from the overall population (Joshi *et al*., 2011). Regardless of the advancement for development of reversible and more effective contraceptive methods for female, males are lagging far behind in this regard (Gupta and Sharma, 2006a; Dehghan et al., 2009). Presently attention is diverted towards male methods of contraception. According to the World Health Organization (WHO), basic health related facilities of about 80 % of population, are fulfilled by using plant resources (Okigbo *et al*., 2008) and about 70% of the available drugs are derived from natural and botanicals products (Newman and Cragg, 2016). Therefore, plants grabbed the attention of many scientists as a key source of naturally occurring fertility regulating agents due to their minimum side effects (Umadevi *et al*., 2013). Most of the contraceptive techniques used in family planning program are for female as male have less options for reversible and effective contraceptives. Hence there is utmost need to develop plant based safe, effective and reversible male contraceptives for the equitability of male and female in birth control program thus sorting the issue of population growth.

Ethno-botanical Aspects:

In current study, attempt was made to explore and screen antifertility activity of four different medicinal plants from Pakistan; *C. ambrosioides, A. integrifolia, R. hastatus* and *H. nepalensis*, based on available literature and traditional claims for their use in folklore medicines for the treatment of several diseases and as antifertility agents.

Chenopodium is a genus of numerous perennial or annual herbaceous flowering plants species which are cosmopolitan in distribution (Gelin *et al*., 2003). *C. ambrosioides* is a perennial, aromatic herbaceous shrub of family Chenopodiaceae that is extensively cultivated in Eastern and Central USA, Europe, Maryland, Mexico and Canada. *C. ambrosioides* is plentiful in flavonoids and terpenoids compounds, it is pharmacologically used as cancer chemopreventive agent (Kiuchi *et al*., 2002; Liu, 2004), and for the treatment of several metabolic disorders (De Pascual *et al*., 1980). It was previously shown that aqueous extract of *C. ambrosioides* negatively influence the reproduction in Drosophila melanogaster (Wohlenberg and Lopes-da-Silva, 2009). So it can be hypothesized that *C. ambosioides* can be used to suppress fertility.

Ajuga L. is a genus of annual and perennial herbaceous flowering plants belonging to mint family Lamiaceae. Members of this family are being used in traditional medicine and in insect control (Kew *et al*., 2010). *A. integrifolia* is low growing, erect and perennial herb, harvested for local medicinal use and potential source of essential oil. It is found in Northeast and east tropical Africa, Indian Subcontinent, Afghanistan, China, Indonesia, Philippines and New Guinea. In Pakistan, it is located in Murree, Swat, Shangla, Dir and many regions of Punjab province. Traditionally, decoction of the fresh leaves are drunk by *Ethiopian* people to treat diarrhea and malaria while, paste made of leaves is applied on the affected part for treating wound (Gedif and Hahn, 2003). In Pakistan, this herb is traditionally used among Pakhtun population of Shangrilla, Dir and its locality for contraceptive purposes in men. However, there is no scientific evidence to back up these claims and so its antifertility potential is not scientifically proven.

Rumex is one of the most important genera of family "Polygonaceae" also known as "buckwheat" family. It is common perennial herb grow almost everywhere. *R. hastatus* D. Don is commonly known as "khatimal." It is distributed in northern Pakistan, northeast Afghanistan, and southwest China, growing between 700 and 2500m, and sometimes grows as a pure population. It is reported that the whole plant possess medicinal importance. It is laxative, tonic, alterative and is used in rheumatism (Shinwari and Gilani, 2003) and sexually transmitted diseases including AIDS (Vermani and Garg, 2002). *R. hastatus* is traditionally used as an oral contraceptive but still no scientific experimentation is performed.

Hedera, commonly called as "ivy" is a genus of flowering plants belonging to the family Araliaceae which have about 70 genera and 700 species. It is widely distributed throughout Europe Asia and North Africa. *H. nepalensis* commonly known as "Kurie" has been traditionally used for the treatment of numerous diseases including diabetes, fever, pulmonary infections, ulcers, abscesses and rheumatism (Frohne and Pfander, 2004; Shah, 2006). Previous studies have confirmed its hypoglycemic, antiproliferative, cytotoxic and antifungal properties (Hamayun *et al*., 2006; Shah, 2006). A former study reported that *H. nepalensis* exerts antifertility effects in humans by immobilization of spermatozoa (Pant *et al*., 1988). But no clear picture about its effect on reproduction is available.

Characterization and Biological activities:

In current study, methanolic extracts of four selected plants were evaluated for total phenolic contents, total flavonoid contents, antioxidant potential and protein kinase inhibition activity. Experimental findings of phytochemical analysis revealed highest amount of TFC, TPC and total antioxidant capacity were quantified by methanolic leaf extract of *R. hastatus.* While, highest 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity was shown by methanolic leaf extract of *A. integrifolia* with 17 µg/mL IC_{50} value. In our study, methanolic extract of *H. nepalensis* showed high protein kinase inhibition against Streptomyces growth with 27.6 mm zone of inhibition/cytotoxicity at highest concentration, followed by *A. integrifolia*, *R. hastatus* and *C. ambrosioides* indicating their cytotoxic potential. Cytotoxicity of selected plant extracts was determined by using Brine shrimps lethality assay (Chapter 1). Experimental findings showed maximum percent mortality at 1000 µg/ml with methanolic leaf extract of *R. hastatus*< *A. integrifolia*< *C. ambrosioides* < *H. nepalensis*. Previously, Ahmed *et al*., 2002 tested various fractions of *H. nepalensis* by BSLA and reported that crude methanolic extract showed 50% cytotoxicity at 1000µg/ml (Ahmed *et al*., 2002). The significant mortality of shrimp's larvae after exposure to tested plant extracts revealed that these plants may have cytotoxic components that should be isolated and investigated to support their traditional use. To evaluate the phytotoxic potential of methanolic leaf extract of selected plants, *Lemna minor* bioassay was performed. Relatively moderate activity was observed by *C. ambrosioides* (45%), *A. integrifolia* (38%) and *R. hastatus* (49%) at highest concentration (1000 µg/ml) while, *H. nepalensis* showed highest % inhibition (51%)

towards *Lamna minor* at 1000µg/ml. Previously, *Lemna minor* bioassay was used to evaluate phytotoxic potential of different plant extracts including *R. nepalensis, R. australe, R. dentatus, P. persicaria, R. hastatus* and *P. plebejum* (Hussain *et al*., 2010). In current study, inhibitory activity of methanolic leaf extract of four different plant extracts on total ROS generation was investigated. As summarized in chapter 1, % inhibition values of methanolic leaf extract of *A. integrifolia, R. hastatus, H. nepalensis and C. ambrosioides* on total ROS generation were 24.2%, 14.5%, -8.4% and -15.4% respectively. None of the plant showed significant activity towards ROS generation. These results are in contrast to previous studies (Toiu *et al*., 2016; Jafri *et al*., 2017).

In present study, phytochemical components of methanolic leaf extract of four selected plants were analyzed by GC-MS (chapter 2). Some identified compounds present in methanolic leaf extract of *C. ambrosioides* include saturated fatty acids, lipids, amino acids and phytol. Most of them are known to have anticancer, antioxidant, antimicrobial and anti-inflammatory activities (Screening of nutritional, phytochemical, antioxidant and antibacterial activity of *C. album* (Pandey and Gupta, 2014). *A. integrifolia* revealed eleven phytoconstituents when analyzed by GC-MS. Most of the components are used in food as flavoring agent (Pohanish, 2017). In methanolic leaf extract of *R. hastatus*, identified compounds include carboxylic acids, straight-chain saturated and unsaturated fatty acids with antifungal and antibacterial properties but their excessive amount is associated with neurological disorders (El-Masri, 2005; NYEEM *et al*., 2006). GC-MS analysis of methanolic leaf extract of *H. nepalensis* identified only three compounds including Hexanal, Glycidol and 1-Methyldecylamine.

Reviewing the available literature, no clear data was found related to qualitative and quantitative analysis of methanolic leaf extracts of these plants. Additionally, phytochemical constituents were identified by GC-MS analysis. Results obtained from the study support their use in traditional medicine as well as provide basis and direction for the further pharmacological investigations.

***In vitro* and *In vivo* experimental approach using rat model:**

In present set of experiments, *in vitro* and *in vivo* approaches were used to evaluate contraceptive/antifertility efficacy of four selected plants by using adult male Sprague

Dawley rats. For this, different parameters were studied including antioxidant enzymes concentration, reproductive hormones production, sperm parameters and testicular and epididymis histology. Fertility test was also performed to check reversibility of treatment.

In current study, oxidative stress and antioxidant enzymes levels in testis were determined through in vitro and in vivo exposure of different plant extracts. In the in vitro study, increased oxidative stress was observed in highest dose regimen (1000 µg/mL) of methanolic leaf extract of *C. ambrosioides* and *A. integrifolia.* While, in vitro exposure to *R. hastatus* and *H. nepalensis* caused slight increase in ROS production at 1000 µg/mL dose without affecting antioxidant enzymes status. In the in vivo study, oral administration of adult male rats with different concentrations of plants extracts for 28 days shifted normal oxidative/antioxidant status of body and induced production of ROS. This increase in levels of reactive oxygen species and lipid peroxidation like TBARS was prominent in higher doses of *C. ambrosioides* and *H. nepalensis* than *A. integrifolia* and *R. hastatus* extract exposure. The antioxidant enzyme activity was measured for two important enzymes, CAT and SOD. POD is another enzyme involved in cell defense mechanism which converts hydrogen peroxide into water through oxidation-reduction reaction and prevents cell damage and death. Reduction in antioxidant levels was observed within testicular tissues of rats in high doses of *C. ambrosioides, A. integrifolia* and *H. nepalensis,* while antioxidant enzyme status was remained unchanged after exposure of rats with *R. hastatus* leaf extract (Dufau *et al*., 1984). In the *in vitro* study, decline in testicular testosterone level was noticed after two hours incubation with all the doses of *C. ambrosioides* and *A. integrifolia* leaf extract, however, *R. hastatus* and *H. nepalensis* showed reduction only at higher doses regimens (100 and 1000 µg/mL). In current *in vivo* study, hormonal concentration of plasma testosterone and FSH was reduced when rats were treated with *C. ambrosioides* leaf extract while level of LH remained unchanged. In addition, *A. integrifolia* and *R. hastatus* extract exposure caused reduction in testosterone and gonadotropins concentrations but this decline was more prominent after exposure to *H. nepalensis* plant extract. Similar results have been reported previously, showing reduced testosterone concentration and Leydig cell degeneration after exposure to different plant extracts (Gupta and Kachhawa, 2007; Yakubu *et al*., 2007; Ogbuewu *et al*., 2011). The male hypothalamic-pituitary-gonadal

(HPG) axis which controls the production of male gametes is regulated through a negative feedback mechanism. A recently discovered mechanism of interest in the hypothalamic regulation of reproduction indicates that the plant compounds might act as phytoestrogens. They might suppress the expression of Kiss 1 gene in arcuate nucleus, thereby inhibiting the production of kisspeptin, a ligand for the G-protein-coupled receptor 54 (GPR54), which participates in regulating the secretion of GnRH and integration of the negative feedback effect of T on the hypothalamus (Manetti and Honig, 2010). In present study, reduced concentrations of testosterone and gonadotropins might induce histological alterations in testis and epididymis exhibiting anti-androgenic and anti-spermatogenic properties.

In present study, alteration is sperm parameters and sperm number in caput, corpus and cauda region of epididymis was seen after *in vivo* exposure of all the tested plant extracts. But, marked decline in epididymal sperm count was observed in dose dependent manner when adult male rats were exposed to *H. nepalensis* for 28 days. Similarly, DSP was reduced after treatment with *C. ambrosioides* and *A. integrifolia* extract, however, *R. hastatus* and *H. nepalensis* exposure showed slight reduction in DSP.

Histopathological studies of testes showed methanolic extract of *A. integrifolia* leaf and *H. nepalensis* caused more pronounced increase in lumen diameter as well as sloughing off of germinal epithelium affecting normal process of spermatogenesis. Seminiferous tubules were visible with empty lumen, degenerated epithelial layer and increased interstitial space in high dose group. Histomorphology of caput and cauda exhibited reduction in tubular diameter, epithelial height and less number of luminal sperms after exposure with high extract dose. However, the results of testicular histology of *C. ambrosioides* and *R. hastatus* exhibited slight change in lumen diameter and reduction in epithelial height only in high dose treated animals. Similarly, histological analysis of caput and cauda epididymis showed no change in their morphology except reduction in epithelial height. Androgens are necessary for germ cell survival, maturation and successful spermatogenesis to occur. Their reduced levels as observed in our study are associated with spermatogenic arrest. Previously, Udoh & Kehinde reported similar findings in which oral administration of crude ripe seeds of papaya caused germ cell apoptosis and degeneration of germinal epithelium (Udoh and Kehinde, 1999). In another study, exposure of rats to different

concentrations of crude extract of garlic (*Allium sativum*) caused reduction in testosterone levels leading to germ cell death. It was observed that expression of caspase inhibitors i.e. Baculoviral IAP repeat-containing protein 3 (BIRC3) and Baculoviral IAP repeat-containing protein 2 (BIRC2) and activation of caspase-3 increases in germ cell apoptosis (Hammami *et al*., 2009).

Complete spermatogenic arrest is not necessary for male contraception; fertility can be eliminated by altering structure or function of spermatozoa (Dwivedi *et al*., 1990). Many studies have related the plant based male sterility with decreased sperm number and sperm motility (Watcho *et al*., 2001; Thanga *et al*., 2012). This reduction might be due to capability of plant extract to cross blood testes barrier (BTB) and hinder with normal process of spermatogenesis in seminiferous tubules either by effecting sperm proteins and sertoli cells, altering epididymal function and effecting motility of mature spermatozoa, or altering androgen synthesis and feedback regulation of HPG axis shifting spermatogenesis (Naik *et al*., 2016).

Fertility test was performed to check the reversibility of male fertility after 60 days of treatment withdrawal. Results of current study revealed that withdrawal of selected plant extracts treatment after 28 days for a period of eight weeks induced partial recovery of fertility as it is revealed from the attained pregnancy of untreated female rats when mated with treated males and their litter size. Effect of *C. ambrosioides* leaf extract on the percent fertility showed that 83%, 66% and 50% females conceived in 50 mg/kg, 100 mg/kg and 150 mg/kg leaf extract treated groups respectively. While in case of *A. integrifolia*, percent fertility 66%, 50% and 33% was noted in 50 mg/kg, 100 mg/kg and 150 mg/kg extract treated groups respectively. Percentage fertility and number of pups born per female mated with methanolic extract of *R. hastatus* exhibited that fertility rate was 100% after in 50 mg/kg. However, in 100 mg/kg and 150 mg/kg treated group, 83% and 67% females conceived respectively. Methanolic leaf extract of *H. nepalensis* revealed that low dose treatment (50 mg/kg) showed 83% fertility rate, while, percent fertility was observed 50% in 100 mg/kg treated group and only 16% in 150 mg/kg group after 60 days of treatment withdrawal. When percent fertility for each selected plant at similar dose was compared among themselves, *H. nepalensis* treated group showed maximum fertility suppression for long period of time as less number of females conceived after 60 days of treatment withdrawal; followed by *A. integrifolia*>*C. ambrosioides*>*R. hastatus.* From these

observations, it can be acclaimed that methanolic leaf extract of *R. hastatus* might interfere with spermatogenesis but it is not potent to suppress fertility. However, methanolic leaf extract of *H. nepalensis, A. integrifolia and C. ambrosioides* are capable for reversible fertility suppression. Further studies are needed to know whether longer recovery period would lead to complete recovery of fertility or not.

General Conclusion

The findings of current study demonstrate that methanolic leaf extract of all the four selected plants are rich with phenolic and flavonoid contents and possess remarkable antioxidant activities. However, methanolic leaf extract of *R. hastatus* possess high flavonoids and phenolic contents with highest antioxidant potential followed by *A. integrifolia*, *C. ambosioides* and *H. nepalensis*. Moreover, methanolic extract of *H. nepalensis* is quite effective against brine shrimp and *Lemna minor* revealing its cytotoxic potential. These reported plant extracts possess variety of components which could be used as novel contraceptive drug discovery. The results of *in vitro* and *in vivo* sets of experiments revealed that methanolic leaf extract of all the plants have certain effects on fertility and reproduction.

- In the *in vitro* study, *C. ambrosioides* and *A. integrifolia* exposure showed increased oxidative stress while in the *in vivo* study, prominent increase in ROS and lipid peroxidation was exhibited by *C. ambrosioides* and *H. nepalensis.* However, antioxidant enzyme status was remained unchanged after exposure of rats with *R. hastatus* leaf extract.
- *In vitro* study revealed loss of cell viability associated with induction of sperm DNA damage after incubation with selected plant extracts.
- Reduction in testicular testosterone and gonadotropins was more prominent by *H. nepalensis* plant extract exposure, suggesting that it can suppress fertility by interfering HPG axis.
- Alteration in sperm number and sperm production was obvious after *in vivo* exposure of all plant extracts at higher doses with prominent change with *H. nepalensis* and *C. ambrosioides* administration.
- *A.integrifolia* and *H. nepalensis* exposure caused spermatogenic arrest by affecting testicular architecture and interfering germ cell maturation. Reduced concentrations of testosterone and gonadotropins might induce histological alterations in testis and epididymis exhibiting anti-androgenic and anti-spermatogenic properties of these plants.
- Percent fertility and number of pups/female were reduced after treatment withdrawal in order of *H. nepalensis*>*A. integrifolia*>*C. ambrosioides*>*R. hastatus* extract treatments.

From these points, it is obvious that although methanolic leaf extract of *R. hastatus* affect reproduction to some extant but it is not capable of suppressing fertility in male rats. On the other hand, *H. nepalensis* have the potential to suppress fertility either by disturbing sperm parameters or interfering with HPG axis. *C. ambrosioides* and *A. integrifolia* caused partial male sterility by disturbing spermatogenic cycle, inducing oxidative stress and hormonal imbalance. Although, fertility was compromised, but fertility suppression was reversible after cessation of treatment and no fetal mortality or morbidity was evident by fertility test.

Traditional medicines practiced for fertility regulation are very important in various tribal/rural cultures worldwide. In summary, results of current study are in agreement with ethno-medicinal/traditional use of *H. nepalensis*, *A. integrifolia* and *C. ambrosioides* as antifertility agents. These plants certainly possess antifertility potential without adverse toxicity. Current interest in traditional medicine has led to study these plants for contraception purpose. Novel information gathered from the current data is important in preserving folk indigenous knowledge as well as in the discovery of novel potential compounds with promising antifertility potential. Overall, the investigation of pharmacological potential of selected medicinal plants to be used as male contraceptive justify the knowledge coming from pharmacology and its roots in ancient herbal medicine, in addition to incredible possibility in the development of natural contraceptive drug. Therefore, result of present study could be used to provide new insight about use of traditional plants in male fertility regulation.

FUTURE PERSPECTIVES

In current study, antifertility potential of plants has been investigated comprehensively using rat as experimental model. However, further studies are required as several gaps remained unfilled and questions remained unanswered that need to be investigated.

- Several bioactive compounds should be isolated and purified from different parts of these plants and their activity can be evaluated to find most active compound leading to development of synthetic approach for active analogue.
- Metabolic pathways used by plants and their secondary metabolites for fertility suppression need to be elucidated.
- Molecular approach should be used to study exact underlying mechanism of action of these plants at cellular level and the target site of action.
- Pharmacokinetic and pharmacodynamic properties of active components should be studied.
- Effect of ecological and genetic variations on the activity of these plant species as well as on the quality of their secondary metabolites should be studied.
- Another aspect of this study to be focused on is to find out effect of other plant parts and their active constituents as we use only aerial parts of the plants.
- Human metabolism greatly influences the effect of natural products as they pass through digestive system. This results in modifications of natural product through metabolic pathways. This aspect should not be ignored to fully understand the mechanism of action.

SUMMARY

Chenopodium ambosioides

Methanolic Extract Preparation

In Vitro

- Increased oxidative stress
- Reduced TT
- Increased DNA damage

In Vivo

- Interfere with Sperm Parameters
- Increased oxidative stress
- Alter testicular and epididymis histology
- Reduced reproductive hormonal levels
- Reduced fertility rate

Male Infertility

Figure 130: Graphical representation of effects of *C. ambrosioides* on reproductive functions of adult male rats.

SUMMARY

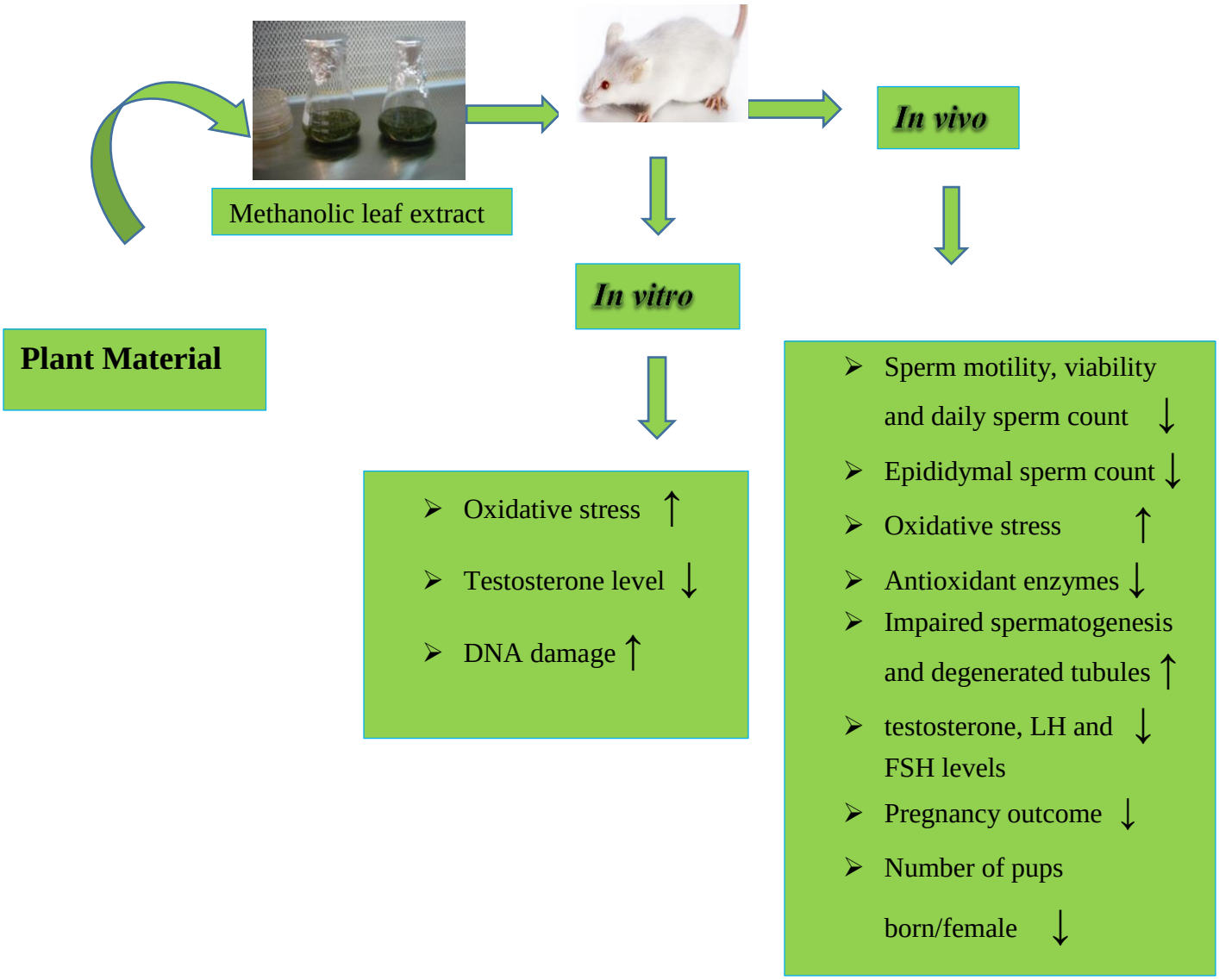
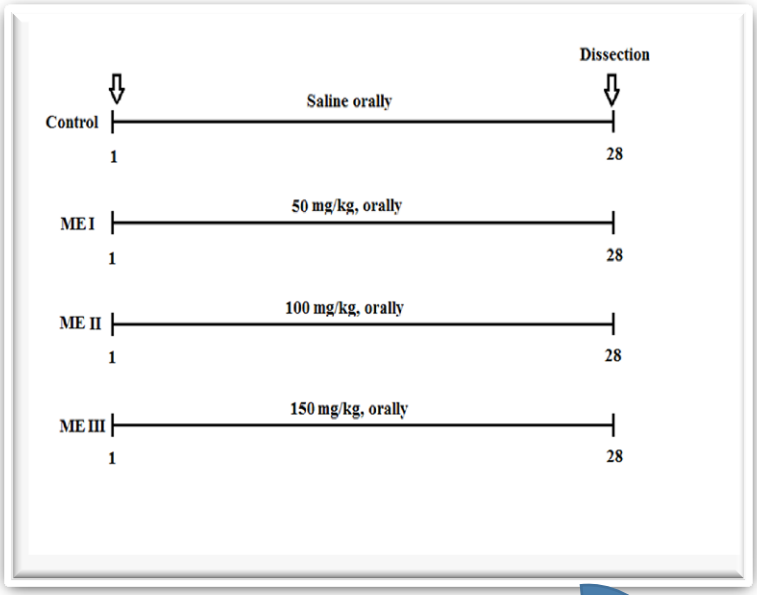


Figure 131: Graphical representation of effects of *A. integrifolia* and *H. nepalensiss* on reproductive functions of adult male rats.

SUMMARY

Methanolic leaf extract of *R. hastatu*

Adult male rats



Testis Sperm

2 hours Incubation

- Affect Sperm motility and viability
- Did not affect DNA integrity
- Slight increase in ROS generation
- Intact testicular and epididymis little effect on morphology.
- Alter hormonal level to some extant
- No effect of fertility rat

Fertility not affected

Figure 132: Graphical representation of effects of *R. hastatus* on reproductive functions of adult male rats.

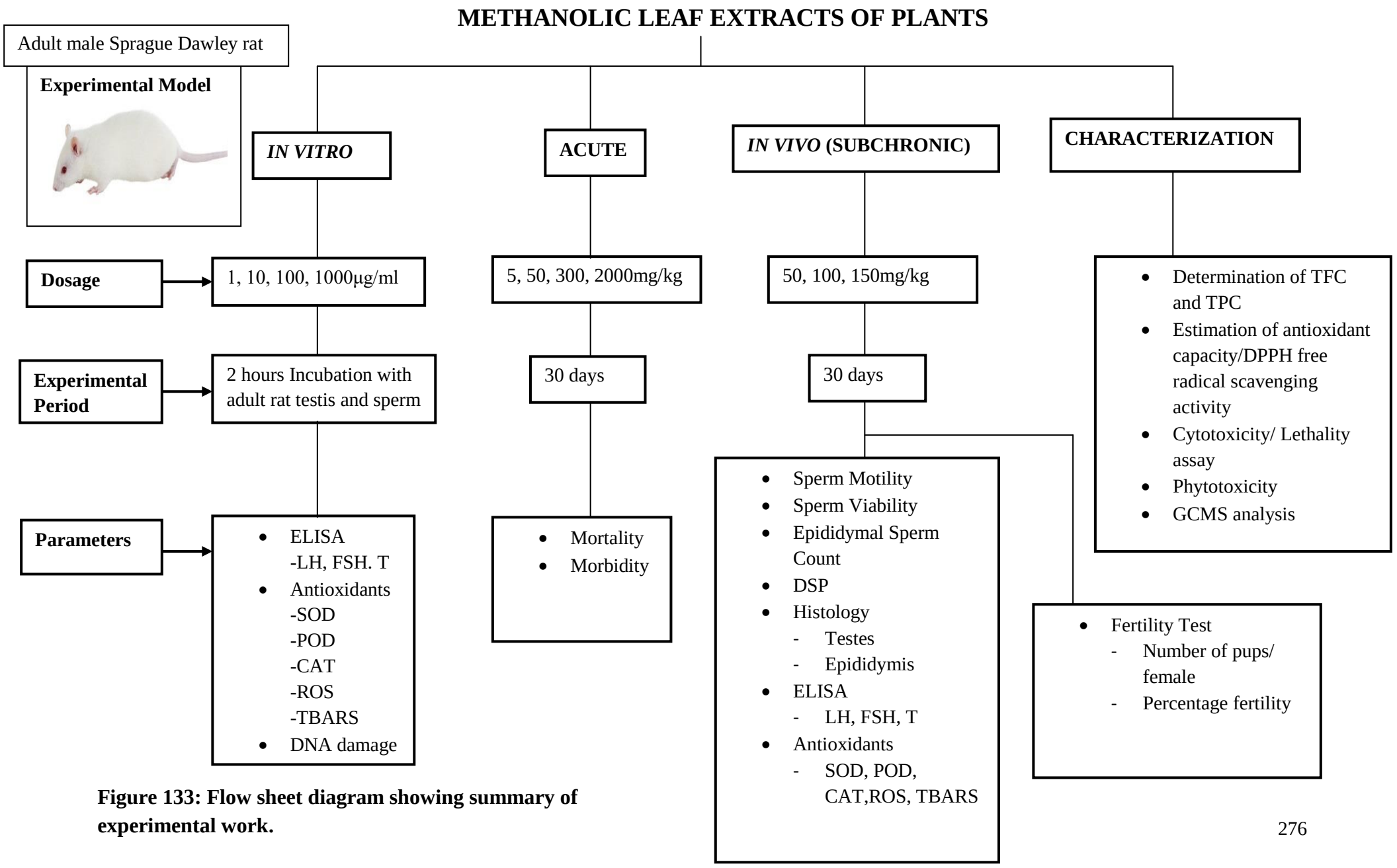


Figure 133: Flow sheet diagram showing summary of experimental work.

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