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Nigella Sativa Supplementation Effects on Growth, Organ Development, and Reproduction in Sprague-Dawley Rats

by

Usochukwu Chimaobim

A Thesis

Submitted to the Graduate Faculty of

St. Cloud State University

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

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Thesis Committee:
Oladele Gazal, Chairperson
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Abstract

Nigella sativa (NS) is a small herbaceous plant belonging to the Ranunculaceae family and is cultivated across several regions in Asia, Southern Europe, and the Middle East. Several studies have investigated the effects of alcoholic NS extracts on reproduction, but few have investigated the effects of the dietary supplementation of NS seeds at up to 15% inclusion on pregnancy and pregnancy outcomes. Further, few studies have investigated the effect of acute or chronic aqueous NS extracts administration on hematology, hormone secretion, and the histology of major organs regulating metabolism and reproduction in the male rat. Thus, our studies were intended to bridge those gaps in knowledge.

In our first study designed to investigate the effects of the dietary supplementation of NS seeds on pregnancy and pregnancy outcome, 32 mature female Sprague-Dawley rats weighing 250-350 g and between seven and ten weeks of age were utilized. Rats were assigned to one of four groups: Control group (0% Nigella sativa seed inclusion; n=8), Low group (5% Nigella sativa seed inclusion; n=8), Medium group (10% Nigella sativa seed inclusion; n=8), and High group (15% Nigella sativa seed inclusion; n=8). Rats were mated and allowed to carry pups in-utero to term. Our results indicated no observable injury in conceived pups in all experimental groups. Lowest incidence of pregnancy was seen in the 10% supplementation group. Mean pup weights in all NS-supplemented groups remained significant higher up to seven days post-partum when compared to the control group. Dietary supplementation of NS seeds up to 15% had no deleterious effect on pregnancy and pregnancy outcomes.

In the second study, we utilized 30 mature male Sprague-Dawley rats weighing 250-350 g, and between seven and ten weeks of age. Each rat was weighed and randomly assigned to receive either Saline, i.p. (Control group, n = 10) or 25mg/Kg BW NS aqueous extract, i.p. (Low dose group, n = 10), or 50mg/Kg BW NS aqueous extract, i.p. (High dose group, n = 15) in a total volume of 200 µl for either 15 days (acute phase; n=15) or for 30 days (chronic phase; n=15). At the end of each phase, five rats from each experimental group were euthanized and hormonal analyses and hematological, liver and kidney enzymes and function tests were determined. Further, histological analyses of key organs of metabolism and reproduction were performed.

Administration of NS aqueous extract had no effect on liver enzyme levels and weights of the liver, kidney, testes, prostate gland and the epididymis. However, the chronic administration of NS extract increased blood urea nitrogen, reduced testicular and epididymal weights and caused testicular scarring and severe abdominal adhesion. NS aqueous extract administration, either in the acute or chronic phases, had no effect on luteinizing hormone, follicle-stimulating hormone and testosterone hormone levels. However, whereas phase significantly (P=0.03)increased mean LH levels, irrespective of treatment, it decreased mean plasma FSH levels (P=0.001) and this decrease was also not treatment-dependent.

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Taken together, these results indicate that NS supplementation had no deleterious effect on pregnancy and parturition in the female rat and increased pup weight, possibly as a result of its galactogogue effect. Further, acute NS extract administration had no effect in the male rat but chronic administration although without an effect on liver and kidney histology but induced structural changes in the testes and the epididymides. The NS-induced adhesion observed in the present study could be due to thymoquinone, an active ingredient in NS.

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May the Almighty bless you all.

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Chapter I: Introduction

For as long as man has existed, plants have been used as food, cosmetics, fuel and more importantly, medicine (Jamshidi-Kia, Lorigooini, & Amini-Khoei, 2017). Although orthodox medicine constitutes a large percentage of prescribed medicine, the use of plants as medicine dates to ancient European, Asian and African civilizations (Petrovska, 2012). Interestingly, modern day drugs such as codeine, morphine, atropine, and quinine are all plant-derived (Rates, 2001). Plants have been employed in the treatment of a myriad of ailments but not limited to viral, bacterial, and fungal infections, neurological and endocrine disorders related to infertility (Upadhyay & Singh, 2015) Due to a myriad of environmental, metabolic and endocrine factors, infertility in human mammals emanates (Anwar & Anwar, 2016). Physiological and endocrine differences in the human population also complicate treatment of infertility-related cases.

Reproduction in mammals is regulated by the hypothalamic-pituitary-gonadal axis (Goodman, 2009). The hypothalamus produces gonadotrophin releasing hormone (GnRH) a decapeptide which stimulates the production of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior region of the pituitary gland. LH stimulates the release of testosterone in male mammals and estrogen in female mammals, while FSH stimulates the production of androgen binding protein in male mammals and gametogenesis in both males and females, (Silverthorn & Johnson, 2010). Reactive oxygen species (ROS), noise, and the unregulated consumption of herbal remedies has been known to initiate and expedite damage to cells contained in the hypothalamus, pituitary gland, and gonads of mammals (Drougard, Fournel, Valet, & Knauf, 2015). Predominantly consumed in the Middle East, *Nigella sativa* is a plant whose seeds have been employed in the treatment of variety a variety of ailments including

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infertility. However, the mechanisms of how *Nigella sativa* affects reproduction have not been studied exhaustively.

Nigella sativa (NS) is a commonly consumed herb indigenously grown in parts of Africa, Asia and the Middle East. Seeds from *Nigella sativa*, commonly referred to as black cumin or black seeds have been consumed for a myriad of ailments including but not limited to bacterial, fungal and viral infections, neurological and endocrinological disorders (Randhawa & Alenazi, 2016).

There have been contradictory reports regarding the effect of NS on reproduction. While several studies have indicated stimulatory effects of NS on reproduction hormones (Parandin, Yousofvand, & Ghorbani, 2012), other results have suggested inhibitory effects on the estrous cycle and pregnancy (Keshri, Singh, Lakshmi, & Kamboj, 1995; Yadav & Agarwal, 2011). Such discrepancies necessitated the development of a systematic study to properly evaluate the effect of administering aqueous extracts *Nigella sativa* seeds on the synthesis and secretion of reproduction hormones, the regulation of the estrous cycle and pregnancy outcomes in a mammalian model.

The safe consumption of herbs such as *Nigella sativa* becomes a matter of concern specifically under special physiological conditions like pregnancy. The presence of intrinsic compounds such as flavonoids, alkaloids and their derivatives have been reported to disrupt pregnancy when consumed acutely or chronically at several graded doses (Galluzzo & Marino, 2006). In parts of Asia and Africa, where access to orthodox medicine is limited, individuals turn to extracts of medicinal plants when plagued by illness. Depending on the severity of illness, individuals may consume these extracts acutely or chronically at either low or high doses.

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Several studies have evaluated the effect of the acute or chronic administration of alcoholic extracts of *Nigella sativa* seeds (Al-Sa'aidi, Al-Khuzai, & Al-Zobaydi, 2009; Kolahdooz, Nasri, Modarres, Kianbakht, & Huseini, 2014; Yadav & Agarwal, 2011); however, very few studies have compared the effects of administering the aqueous extracts of the NS seeds, acutely versus chronically, in other to properly elucidate the short and long-term effects of their administration.

In this study we used the in vivo rat model to determine the effects of supplementing *Nigella sativa* seeds on the estrous cycle, pregnancy, pregnancy outcomes. Further we evaluated the effects of administering aqueous extracts of these seeds acutely and chronically on reproductive hormones, serum levels of liver and kidney enzymes, several hematological parameters and organ development in male Sprague-Dawley rats.

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Chapter II: Literature Review

Neural Regulation of Reproduction in Mammals

In mammals, reproductive ability spurns from synchronized communication between neural populations present in the hypothalamus and pituitary gland, and receptors in the gonads. Puberty initiates maturation and activation of gonadotrophin releasing hormone (GnRH) neurons leading to the pulsatile release of GnRH in the arcuate nucleus. An important key neuro-peptide kisspeptin, encoded by the *Kiss 1* gene and synthesized by neural populations domiciled in both the arcuate (ARC) and anteroventral periventricular (AVPV) areas in the hypothalamus, stimulates GnRH secretion. These strategic locations of kisspeptin neurons suggest possible modulation of pulsatile and surge secretion of GnRH as nuclei in the ARC and AVPV are involved in pulsatile and surge secretions, respectively. Nuclei in the ARC and AVPV have been shown to react differently to estradiol-17β released from the ovaries. While kisspeptin expression in the ARC is modulated by negative feedback inhibition of Estradiol-17β, Estradiol-17β facilitates the positive feedback of kisspeptin in AVPV. Pre-puberty, homeobox transcription factors such as ventral anterior homeobox 1 (*Vax1*) also affect maturation of GnRH neurons and pituitary gonadotrophs synthesizing luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH GnRH-dependent secretions facilitate gonadal maturation and ultimately gonadal hormone secretion. FSH in females stimulates ovarian follicular development and androgen binding protein in Sertoli cells in males. Androgen binding protein plays a pivotal role in spermatocyte maturation in males. LH stimulates testosterone synthesis in males and estrogen production in females. Pre-ovulation, as LH producing gonadotroph cells become hyper-sensitized to tonic pulsatile secretions of GnRH from the hypothalamus, this leads to key surges

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of LH principally involved in modulating inflammatory pathways in mature graafian follicles geared towards ovulation.

Pregnancy Endocrinology in Mammals

Pregnancy in mammals is an evasive process which takes place after fertilization of ovum or ova at the ampullary-isthmic junction in the uterus. Preceding pregnancy, the recruitment and development of ovarian follicles rely heavily on the synchronized coordinated release of hormones along hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamus synthesizes and secretes GnRH a decapeptide hormone. GnRH travels through the hypothalamic-hypophyseal portal vessel binds to receptors in the anterior pituitary and modulates the synthesis and secretion of gonadotrophins, LH and FSH. LH and FSH are heterodimeric glycoproteins consisting of a common α subgroup and different β subgroups. Distinct populations of LH and FSH gonadotrophic cells exhibit peculiar differences regarding GnRH-dependent secretion, with slow GnRH pulses favoring FSH secretion and fast GnRH pulses favoring LH secretion (Wildt et al., 1981)

At birth in a human mammalian female, up to 400,000 primordial follicles exist in each ovary. Following initial menses in female mammals (menarche) once every menstrual cycle an ovum is ovulated from a mature graafian follicle into the oviduct. Preceding ovulation, several primordial follicles selected by recruitment develop into singled-layered primary follicles which develop into multilayered secondary follicles. Endocrine control on follicular selection begins after the onset of FSH receptor expression in secondary follicles. Through a coordinated two-cell cross talk system between outer theca and inner granulosa cells in secondary follicles, estrogens necessary for follicular growth are produced. This two-way cross talk system initiated by LH

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stimulated theca cells produces androgens which get converted to estrogens in granulosa cells by a cytochrome P450 superfamily enzyme called aromatase. During this process, granulosa cells are stimulated by FSH. When follicular cohort estrogen synthesis peaks, negative feedback inhibition of FSH due to high ovarian estrogen levels occurs. Resulting ovarian FSH level decline leads to the emergence of one dominant periovulatory Graafian follicle in monovulatory mammals. Cell mediated apoptosis (atresia) occurs in remaining follicles due to lack of FSH. This developmental phase in the menstrual/ovarian cycle preceding ovulation is referred to as the follicular phase.

During the follicular phase, increased estrogen production by follicular cohort activates *kiss1* neurons in the AVPV, overriding repression by ovarian steroids and increasing GnRH pulse frequency and amplitude. This in turn markedly stimulates LH synthesis leading to a pre-ovulatory LH surge. The resulting LH surge has been shown to activate several important signaling pathways and factors involved in initiating follicular wall thinning and proteolysis prior to ovulation. Follicular rupture and ovulation leave remnant theca and granulosa cells remodeled into aggregation of luteal cells called the corpus luteum. Essential follicular remodeling involving key matrix metalloproteinases and rapid angiogenesis are required for neo-vascularization involved in corpus luteum formation. The corpus luteum is a transient endocrine organ responsible for the synthesis of vital steroidal hormones such as estrogen and progesterone necessary for the overall sustenance of pregnancy. Post fertilization, syncytiotrophoblasts from implanting conceptus synthesize human chorionic gonadotrophin hormone (hCG) implicated in the inhibition of pro-luteolytic factors such prostaglandin F2 α ($PGF_{2\alpha}$) thus leaving the corpus luteum intact. Luteolytic regression of the corpus luteum into scar tissue (corpus albicans)

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occurs in the absence of fertilization. Post fertilization, rapid division of fertilized ovum develops into a 16-celled morula which further develops into a blastocyte or pre-embryo. The formed pre-embryo implants itself in the endometrium of the uterus which serves as a point of nourishment to the conceptus.

Post-implantation, the conceptus initially relies on steroidal estrogen and progesterone hormones produced by the corpus luteum for development and sustained implantation. In the human mammal, the corpus luteum disappears by the 13th week of pregnancy. At this point, a developing fetal endocrine organ called the placenta takes over the production of key pro-gestion hormones. Additionally, the placenta also acts as a conduit between maternal and embryo/fetal vascular circulatory units.

During mammalian pregnancy, estrogen modulates events leading to the initiation of labor, biosynthesis of progesterone, transfer mechanisms modulating maturation of fetal hypothalamic-pituitary-adrenocortical axis involved in fetal organogenesis and fetus well-being in utero. This protective action of estrogen was displayed when administered estrogen in ovariectomized pregnant rats reduced incidence of injured fetus in utero (Tamada & Ichikawa, 1980).

Progesterone is a steroidal hormone involved in a vast array of pro-gestation events including but not limited to preparation of the endometrium for implantation, maintenance and facilitation of conceptus implantation, suppression of pro-luteolytic factors such PGF2α, suppression of endometrial contractility via the pathways involved in upregulation of calcitonin a peptide hormone involved in calcium homeostasis. Pro-gestation cytokines such as interferon tau ($IFN\text{-}_{T}$) are involved in the upregulation of progesterone receptors in the endometrium and the

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direct inhibition of $PGF_{2\alpha}$ a pro-luteolytic factor that facilitates luteolysis. Hence critical evaluations of maternal dietary habits during gestation remain paramount as foods containing phytochemicals functioning as progesterone, estrogen or pro-pregnancy cytokine antagonists could interfere with the sustenance of pregnancy in mammals.

Relevance of Plants on Infertility, Pregnancy, and Organ Development

Consumed chiefly for energy, certain plants contain compounds implicated in the treatment of fertility related disorders. These compounds influence reproduction directly or indirectly at different levels of the HPG axis. The presence of xenoestrogen compounds in plants could pose as endocrine disruptors impinging upon the occurrence and maintenance of pregnancy at several crucial stages such ovulation, implantation, gestation and parturition (Galluzzo & Marino, 2006). Certain plant-derived flavonoids have also been reported to act as estrogen or progesterone antagonists affecting the bioavailability of these hormones needed for the maintenance of pregnancy and optimal fetal development. Flavonoids were initially identified as endocrine disruptors when sheep known to exclusively consume red clover were discovered to suffer from reproductive dysfunction (Birt, Hendrich, & Wang, 2001).

Estrogen has protective properties on vital organs such as the brain, heart, small intestine and liver (Yu & Chaudry, 2009). With cardiac functional recovery increasing in ovariectomized human females administered estrogen after traumatic hemorrhage (Jarrar, 2000) and beneficial effects on organ development in men also treated with estrogen, a strong case for the therapeutic pro-organ property of estrogen is plausibly argued for. The consumption of plants possessing anti estrogenic properties could alter organ development in not only women but also in men who also produce minute amounts of estrogen (Galluzzo & Marino, 2006).

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Organ development and maintenance in utero, prepuberty, in-puberty, and post puberty in humans is greatly associated with the constitution of consumed diet. Organ development is strongly linked to the presence of essential elements, vitamins and compounds contained in plants and derived processed products in diet. In third world countries, the consumption of water and alcohol-derived herbal concoctions remain prevalent due to inaccessibility to modern orthodox medicine caused by an array of financial and infrastructural factors. Due to inadequate regulatory machinery in such countries, the unbridled consumption of these herbal concoctions leads to incidences of hepatorenal diseases highly prevalent in the male populace. Studies surveying these incidences in Africa have reported nephrotoxicity in a few herbal remedies and plants resulting majorly in tubular degeneration (Liwa & Jaka, 2016). Cape aloe extensively used in Southern Africa (*Aloe capensis*) has been indicated to induce parenchymatous nephritis, primarily due to the presence of Aloins and Aloinosides (Luyckx et al., 2002). Also, concoctions made from violet tree (*Securidacea longepedunculata*), also indigenous to Southern Africa, induced renal cortical necrosis and intestinal nephritis in individuals who consumed them, due to the presence of securinine, saponins and methylsalicylate (Dapar, Aguiyi, Wannang, Gyang, & Tanko, 2007).

Extensive systematic evaluations need to be carried out on popularly consumed plants to ensure their safety and endocrine stability before being consumed pre-conception and post-conception.

Nigella Sativa: Botany, Chemistry, and Distribution

Nigella sativa (NS) is a small herbaceous plant belonging to the Ranunculaceae family. It is cultivated across several regions in Asia, Southern Europe, and the Middle East (Paarakh,

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2010). Ground *Nigella sativa* seeds are used as cooking seasoning and prophylactic or curative concoctions claimed to cure most aliments. The *Nigella sativa* plant grows up to 45cm in height, with the leaves being between 2.5 cm to 5.0 cm long. The flowers are pale blue, and they occur solitary on long peduncles. The seeds are small and trigonus in shape; measuring about 2.5mm- x 1.5mm. They are black on the outside and white on the inside. The seeds are bitter to taste and smell slightly aromatic. Studies evaluating the phytochemical components of *Nigella sativa* seeds have reported the presence of flavonoids, terpenes, and several fatty acids. Major active constituents cited by literature include, thymoquinone (30%-48%), p cymene (7%-15%), carvacrol (6%-12%) 4-terpineol (2%-7%), t – anethole (1%-4%) and the sesquiterpene longifolene (1%-8%) (Paarakh, 2010).

Physiological Properties of *Nigella Sativa*

The intrinsic properties of *Nigella sativa (*NS) are consequences of potent phenol derivatives and other compounds (Randhawa & Alenazi, 2016). Flavonoids, a group of polyphenolic compounds found in *Nigella sativa* have been reported to possess estrogen mimicking properties which invariably make them xeno-estrogens. Estradiol-17β, the most active form of estrogen has been has shown to protect pre-menopausal women from cardiovascular related diseases (Baker et al., 2003) and various debilitating neurological issues (Beheshti, Khazaei, & Hosseini, 2016). Post-menopausal symptoms such as depression and anxiety can be attributed to depleted levels of estradiol-17β, with several studies also elucidating estrogen mimicking activities displayed by flavonoids (Randhawa & Alenazi, 2016; Wharton, Gleason, Sandra, Carlsson, & Asthana, 2012).

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NS extract administration has caused improvements in short term recall in adolescents and decreased symptoms of neurological disorders such as Parkinson's and Alzheimer's diseases by chelation of transition metals necessary in amyloid beta protein aggregation thus inhibiting amyloid beta protein plaque formation necessary for pathophysiology.

Interestingly, NS extracts have also been reported to possess anticancer properties as demonstrated in research done with cancer cell lines (Shafi, Munshi, Hasan, Alshatwi, Jyothy & Lei, 2009; Thabrew, Mitry, Morsy, & Hughes, 2005). Methanolic NS extracts have demonstrated inhibitory capabilities against gram-positive and gram-negative bacteria including *Staphylococcus aureus*, *Bacillus subtilis, Salmonella typhi, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa* (Islam, Ahmad, & Salman, 2013). NS extracts have also been shown to possess biocidal effects against all stages of *Schistosoma mansoni* (in vitro) and inhibitory effects on the egg-laying ability of the adult female worms (Mohamed, Metwally, & Mahmoud, 2005).

Diabetes presents as a complex pathology induced either by the inadequacy of β islet cells of the pancreas in the production of insulin, a peptide hormone involved in facilitating glucose uptake in cells, or the down regulation of insulin receptors. Research exploring anti diabetic properties of NS extracts have demonstrated not only their modulation of glucose and cholesterol capabilities (Sultan et al., 2014), but also their stimulatory effects on insulin synthesis (Salama, 2011).

Nigella Sativa and Reproduction

Nigella sativa (NS) extracts have been implicated in the treatment of fertility-related disorders either induced in suitable study models or ailing individuals. NS extract administration

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has increased levels of reproductive parameters such as sperm count, motility and semen volume in humans and Sprague-Dawley rats (Cho Ping, Hashim, & Hasan Adli, 2014; Kolahdooz et al., 2014;) and has also reduced excitation time and increased libido in Sprague Dawley rats.

NS extract administration could stimulate cellular anti-apoptotic pathways by upregulating anti apoptosis genes such B-cell lymphoma 2 (BCL-2) genes in Leydig and Sertoli cells preventing death and facilitating cellular repair and proliferation as observed when NS extract administration led to higher populations of Leydig cells, Sertoli cells, primary spermatocytes and secondary spermatocytes (Al-Sa'aidi et al., 2009).

Normal duration in phases of the estrus cycle in polyestrous mammals serve as indicators of optimal reproductive competence. In rats, the estrous cycle typically lasts four to five days and it is divided into four different phases. The proestrus and estrus phases last 12-14 hours and 25-72 hours each while the metestrus and diestrus phases last six to eight hours and 55-57 hours respectively (Westwood, 2008). The proestrus and estrus phases correlate to the follicular and peri-ovulatory phases while the metestrus and estrus phases correlate to luteal phases in higher mammals. Durational changes in these phases could be indicative of anti-reproductive activity. Administration of aqueous extracts of NS (200mg/ kg BW) to non-pregnant rats decreased durations of proestrus and metestrus phases and increased durations of the estrus and diestrus phases of the cycle (Yadav & Agarwal, 2011). This suggests that NS extracts may have anti-reproductive properties.

There have been several conflicting reports as to the effect of NS on pregnancy. Although the administration of Phytovagex, a pessary formulation of NS, demonstrated no deleterious effects on pregnancy outcomes (Salarinia, Rakhshandeh, Oliaee, Gul Ghasemi, &

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Ghorbani, 2016) however NS extracts administered at 2g/kg BW in female rats resulted in pregnancy inhibition (Keshri, et al., 1995).

Contradiction in research raises several questions as to the actual effect of NS extract administration on reproduction. The dosage, solvent utilized for extraction and duration of extract administration might play key roles in elucidating effects of NS on reproduction. Several studies on reproduction have been carried using several alcoholic extracts of NS but very few have been done using aqueous extracts.

In countries were NS seeds are consumed, these seeds are usually ground up and used as food spices. NS seeds contain several phytochemicals that could either have pro-reproductive or anti-reproductive effect upon consumption due to the inhibition or down regulation of estrogen or progesterone. It therefore becomes paramount to develop research models that would greatly mimic consumption in humans to investigate the safely of consumption during pregnancy. Thus far, studies conducted have investigated the effects of NS extracts and NS pessary formulations on pregnancy (Keshri et al., 1995; Salarinia et al., 2016), but few studies have investigated the effect of the dietary supplementation of NS at levels up to 15% on pregnancy and pregnancy outcomes. The highest NS seed dietary supplementation thus far is at 13% in pregnant Barki Ewes (El Hawy et al., 2018). It is important to know what level of NS supplementation presents possible pathophysiology in pregnant female mammals.

Thymoquinone: Reported Properties

Thymoquinone is a monoterpene molecule and has been reported to be the major active compound in *Nigella* sativa (Al-Saleh, Billego, & Inam, 2006). Chemically, thymoquinone is known as 2-methyl-5-isoproply-1,4-benzoquinone (Goyal et al., 2017). Known in its role as a

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potent antioxidant, thymoquinone has been reported to have reticent effects on COX2 expression and prostaglandin production in allergic airway inflammation in mice (El Mezayen et al., 2006) Thymoquinone also has cardioprotective (Ojha et al., 2015), gastro-protective (Lebda, Ahmed, Abd El Samad, & Shawky, 2011), neuroprotective (Ahmad et al., 2013), antimicrobial (Umar et al., 2016), anti-arthritic (Taka et al., 2015) hepatoprotective (Suddek, 2014), respiratory organ protective (Suddek, Ashry, & Gameil, 2013) and nephroprotective properties (Evirgen et al., 2011). Immune stress has been long implicated in various pathologies of reproduction (Peter, Bosu, & DeDecker, 1989). Interestingly, thymoquinone has been observed to stimulate production of mitogen activated protein kinases (MAPKs) and extracellular related signal kinases (ERKs) in chondrocytes found in rabbits (Yu & Kim., 2015). This is pivotal since MAPK/ERK pathways have been shown to play essential roles in the expression of LHβ and FSHβ genes (Kanasaki, Purwana, Oride, Mijiddorj, & Miyazaki, 2012). This raises the possibility for thymoquinone to stimulate LHβ and FSHβ genes expression in the pituitary gland. Furthermore, thymoquinone administration has led to increased Leydig cell populations, testosterone levels, LH and FSH expression in rats (Aithal, Haseena, Das, & Saheb, 2016; Widjan & Khafaje, 2015).

Phytochemical Analysis of *Nigella Sativa* Extracts

Table 1 shows the phytochemical make up of several NS extracts. Ishtiaq, Ashraf, Hayat, and Asrar (2013) showed that aqueous NS extracts contained higher amounts of flavonoids and coumarins. In addition to thymoquinone, medicinal properties of aqueous NS could also be attributed to coumarins. Coumarins consists of large group of phenolic substances found in plants. Coumarins have been shown to possess a myriad of curative pharmacological properties

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not limited to antioxidant, neuroprotective, antibacterial, antifungal, anti-cancer and antihypertensive properties (Venugopala, Rashmi, & Odhav, 2013). However, many studies have reported the teratogenic effect of coumarins on pregnancy (Van Driel et al., 2002) raising questions as to what levels of NS dietary supplementation become physiologically harmful.

Table 1

Phytochemical Analysis of Nigella Sativa Extracts

EXTRACTS	Methanol	Ethanol	Chloroform	DEE	Acetone	Aqueous	Butanol	Hexane
Steroids	+	+	++	++	+++	-	-	+
Tannins	++	++	-	-	-	++	+	-
Terpenoids	-	-	+++	-	-	-	+++	-
Flavonoids	++	++	+	-	-	+++	-	-
Anthocyanins	-	-	-	-	-	-	-	-
Leucoanthocyanins	-	-	-	-	-	-	-	-
Coumarins	+++	+	+	+	+	+++	-	+
Cardiac Glycosides	++	+	+	+	++	-	+	+
Saponins	++	-	-	-	-	-	+	-
Diterpenes	++	-	-	-	-	+	+	-

+=weakly present, *++= Moderately present* *+++= Strongly present* *-=Not present*

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Chapter III: Hypothesis

We hypothesize:

- **H_0**: supplementation of NS seeds up to 15% in rat chow will have no effect on feed intake, body weight and pregnancy outcomes in female Sprague-Dawley rats.
- **H_1**: supplementation of NS seeds up to 15% in rat chow will have an effect on feed intake, body weight and pregnancy outcomes in female Sprague-Dawley rats.
- **H_0**: intraperitoneal administration of aqueous NS seeds will have no deleterious effect on organ development, circulating plasma levels of gonadotrophins, luteinizing hormone (LH), and follicle stimulating hormone (FSH), and Testosterone in male Sprague-Dawley rats.
- **H_1** intraperitoneal administration of aqueous NS seeds will have an effect on organ development, circulating plasma levels of gonadotrophins, luteinizing hormone (LH), and follicle stimulating hormone (FSH), and Testosterone in male Sprague-Dawley rats.

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Chapter IV: Materials and Methods

Approval

The research protocols in this study were approved by the Institutional Animal Care and Usage Committee (IACUC) of St. Cloud State University, St. Cloud, Minnesota, USA. All studies were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (National Library of Medicine, 2011). All experiments were carried out in the vivarium at the integrated science and engineering laboratory facility (ISELF) building at St. Cloud State University, St. Cloud, Minnesota.

Plant Acquisition and Preparation

Nigella sativa seeds were purchased from a local Somali store in St. Cloud Minnesota, U.S.A. Seeds were washed before being blended to fine powder using a single-speed food blender (Waring Products, Torrington, CT, USA).

Experiment 1: The Effect of Nigella Sativa Seeds in Rat Feed on Pregnancy in Female Sprague-Dawley Rats

The objective of this study was to determine the effect of graded doses of *Nigella sativa* seed in rat chow on the incidence of pregnancy, parturition, and litter characteristics in female Sprague-Dawley rats.

Experimental Diet

Normal rat chow (NRC) pellets were ground with a ceramic pestle and mortar after which they were electrically blended to fine powder using a single-speed food blender (Waring Products, Torrington, CT, USA). *Nigella sativa* seeds (NSS) were ground to fine powder similarly. To produce 5% or 10% or 15% *Nigella sativa* seed pellets (NSSP),1g of NSS was mixed with 19g of NRC, 2g NSS with 18g NRC and 3g NSS with 17g of NRC, respectively.

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NRC and NSSP were mixed thoroughly with hand mixer (Proctor Silex 5 speed mixer, Hamilton Beach, NC, U.S.A). Resulting mixtures were made into slurries and filled into hand-held cookie presser (Wilton Comfort Grip Cookie Press, Ware Deals Inc). Obtained slurry pieces were left to dry at the surgical suite in the vivarium at ISELF. Dried pellets were stored in zip lock bags in -80°F freezer in WSB 235, St Cloud State University until used in the experiment.

Experimental Animals

Thirty-two mature female Sprague-Dawley rats weighing 250-350 g and between seven and ten weeks of age were used for this study. This study took place in the vivarium of ISELF. Rats were housed in individual plastic cages for ten days prior to the experiment for acclimation. The rats were maintained under a lighting regime of 12 hours under light and 12 hours in the dark at 25°C. During the acclimation period, rat feed and water were provided ad libitum.

After the acclimation period, each rat was weighed and randomly assigned to one of four groups namely, Control group (0% Nigella sativa seed inclusion; n=8), Low group (5% Nigella sativa seed inclusion; n=8), Medium group (10% Nigella sativa seed inclusion n=8); or High group (15% Nigella sativa seed inclusion; n-=8). All rats were weighed at the start of the study and every two days thereafter. Female rats were fed their daily allowance for a 30-day period and daily feed intakes and refusals were determined. Thereafter, male rats were paired with female rats (1:1), to enable coitus. Pairing was allowed for a five-day period after which male rats were removed. Determination of the rats that were pregnant and those that underwent parturition was done. Female rats were continuously fed until parturition. At parturition, litter size was determined, and each pup was weighed. Postpartum rats were maintained on normal rat chow (NRC). Pup body weights were determined at Days 3 and 7 postpartum.

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Statistical Approach

The effect of NS supplementation on body weights, feed intake, feed refusal, percentage pregnancy, percentage parturition, litter size, birth weights, Day 3 post parturition weights, and Day 7 post parturition weights was determined. All data were expressed as mean ± standard error of the mean (SEM). The effect of NS supplementation dose on all data was determined using the analysis of variance technique (ANOVA) on JMP analytical software (Cary NC, U.S.A). A probability of $P < 0.05$ was set as the level of significance. Tukey post hoc test was used to separate the means where significant differences existed.

Experiment 2: The Effects of Acute and Chronic Treatment of Aqueous Extracts of Nigella Sativa on Metabolic and Growth Indices, Reproductive Hormones, and Fertility Indices in Male Sprague-Dawley Rats

The objective of this study was to determine the effect of the intraperitoneal administration of aqueous extracts of NS seeds on the serum levels of reproduction hormones: LH, FSH, and testosterone, hematological parameters, and organ development in male Sprague-Dawley rats. Organs of emphasis included the kidney, liver, prostate gland, seminal vesicles, epididymides and testes.

Aqueous Extraction of NS Seeds

NS seeds were mixed with deionized water (1 g/100 mL). The solution was filtered with a vacuum through Whatman qualitative Filter Paper Grade 1 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The filtrate was then dehydrated in an oven at 37ºC for 24 h. The NS aqueous extract (NSE) was stored in an air -tight container at 4°C.

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Experimental Animals

Thirty mature male Sprague-Dawley rats weighing 250-350 g and between seven and ten weeks of age were used for this study. Rats were housed in individual plastic cages for ten days prior to the experiment for acclimation. This study took place in the vivarium of the ISELF building at St Cloud State University Mn, U.S.A. Rats were maintained under a lighting regime of 12 hours under light and 12 hours in the dark at 25°C. During the acclimation period rat feed and water was provided ad libitum.

After the acclimation period, each rat was weighed and randomly assigned to one of three groups, Control (Saline, i.p., n = 10), Low dose (25mg/kg BW of NS extract; n = 10), or High dose (50mg/kg BW of NS extract; n = 10). The study was conducted in two phases namely the Acute Phase (15 days) and the Chronic Phase (30 days). During the duration of study, rats were weighed weekly and daily administered intraperitoneal (i.p.) injections of the vehicle (Saline) or NS extract in a final volume of 200 µl.

Euthanization

At the end of each phase, five rats were randomly selected from each study group, administered an overdose of anesthetic tribromoethanol at 300mg/BW (University of Arizona, Guidelines for Anesthetics and Analgesics Use in the Laboratory) and euthanized by decapitation following approved IACUC guidelines.

Plasma Collection

Whole trunk blood was collected into EDTA tubes upon decapitation. Blood samples were centrifuged at 3000 rpm for 10 minutes and plasma was harvested and stored at -80ºC for subsequent radioimmunoassay of LH, FSH, and testosterone.

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Hematological Analyses

Whole blood samples from each rat were collected into EDTA tubes at euthanization. Hematological analyses were done at Idexx Laboratories Inc Bloomington, Minnesota, U.S.A.

Harvest of Organs

At euthanization, kidneys, livers, prostate glands, epididymis and testes from each rat were harvested, weighed, sectioned and placed in plastic histological cassettes and stored in plastic containers containing formalin until further histological processing.

Histology

For histology, previously explained protocols as described by Thomas et al., 2017 were followed. Cut pieces of kidney, liver, lungs, prostate gland, seminal vesicle, epididymis, and testes tissue from each male rat were packed into histological cassettes and fixed for a month. Prior to analyses collected tissue samples were dehydrated through a series of ethanol and xylene baths with a Leica automated tissue processor 1050 (Leica, Wetzlar, Germany). Dehydrated samples were embedded in paraffin using a Thermo Scientific Microm EC 350–1 embedding station (Waltham, MA). Sectioning was carried at 0.5µm thickness using Reichert-Jung cassette microtome (Leica, Wetzlar, Germany). Staining was carried out using hematoxylin and eosin counter staining techniques as described by Carson (1997). Slides were checked for normalcy and morphological changes.

Radioimmunoassay of LH

LH concentrations were determined utilizing the techniques described by Bernard, Valet, Beland, and Lambert (1983) LH was iodinated using ^{125}Iodine (Perkin Elmer Laboratories, Waltham, MA, USA) by the Chloramine-T method. The LH stock (NIDDK rLH-RP-3) used for

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making standards (0.8, 1.6, 3.1, 6.2, 12.5, 25 and 50 ng/mL) and in iodination was obtained from Dr A.F Parlow (National Hormone and Peptide Program, Los Angeles Biomedical Research Institute, Harbor-UCLA Medical center, California, U.S.A).The first antiserum (Anti-rat LH, AFP240580) was also obtained from Dr A.F Parlow (National Hormone and Peptide Program, Los Angeles Biomedical Research Institute, Harbor-UCLA Medical center, California, U.S.A) and used at a 1:40,000 dilution. The second antibody utilized was goat anti-rabbit immunoglobulin G (GARGG, Equitech-Bio, Inc, Kerrville, TX, USA) at a dilution of 1:1 with 0.01 M phosphate buffered saline (PBS) and EDTA. Briefly on day 1, standards (200 µL), plasma samples (100 µL) in duplicates, first antibody (100 µL) and tracer (125I-LH; 20,000 cpm/100 µL) were pipetted into 12x75 mm borosilicate glass tubes (VWR International, Radnor, PA, USA). The tubes were then vortexed briefly before being stored at 4°C for 48 h. On day 3, the second antibody GARGG (200 µL) was pipetted into the tubes along with 6% polyethylene glycol (500 µL). Afterwards, the tubes were vortexed briefly and centrifuged at 3,000 rpm at 4°C for 15 minutes using the Sorvall RT7 plus centrifuge (Thermo Scientific, Waltham, MA, USA). Supernatant were discarded into a waste receptacle. The tubes were then loaded and counted in a Packard Cobra II gamma counter (Perkin Elmer, Waltham, MA, USA).

Radioimmunoassay of FSH

FSH concentration was determined utilizing techniques described by Odell, Parlow, Cargille, and Ross (1968). FSH was iodinated using ^{125}Iodine (Perkin Elmer Laboratories, Waltham, MA, USA) by the chloramine-T method. The FSH stock (USDA-oFSH-SIAFP-RP2; AFP-4117A) used for making standards (0.125, 0.25, 0.5, 1.5, 2.5, 5.0, 10.0, 20.0 and 50 ng/mL) was obtained from Dr A.F Parlow (National Hormone and Peptide Program, Los

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Angeles Biomedical Research Institute, Harbor-UCLA Medical center, California, U.S.A). The first antiserum (NIDDK-anti-oFSH, AFP-C5288113) utilized was obtained in a lyophilized state from Dr A.F Parlow and used at a dilution of 1:15000. The second antibody used was GARGG (Equitech-Bio, Inc, Kerrville, TX, USA) at a dilution of 1:1 with 0.01 M PBS and EDTA. The radioimmunoassay (RIA) for FSH is a five-day assay. On Day 1, standards (200 µL), plasma samples (150 µL) in duplicates and first antibody (200 µL) were pipetted into 12x75 mm borosilicate glass tubes (VWR International, Radnor, PA, USA). The tubes were then vortexed briefly before being stored at 4°C for 24 hours. On Day 2, the tracer (^{125}I-oFSH; 20,000 cpm/100 µL) was pipetted into all tubes, vortexed briefly, and then stored at 4°C for 24 hours. On Day 3, 200 µL SARGG (GARGG diluted with 0.01M PBS EDTA at 1:1) was pipetted into all tubes except total count (TC) tubes containing only iodinated oFSH. After the addition of SARGG, tubes were vortexed briefly and placed in a refrigerator for 48 hours. On Day 5, ice cold 0.01 M PBS working solution (3ml) was added to all tubes except TC tubes. All tubes except TC tubes were vortexed briefly and immediately centrifuged for 60 minutes at 3600 rpm at 4^0C. When centrifugation was complete, supernatants from tubes were decanted into a waste receptacle. After which, tubes rims were gently blotted against absorbent paper towels. Decanted tubes along with TC tubes were then loaded into the gamma counter for counting.

Radioimmunoassay of Testosterone

Plasma testosterone concentrations were assayed utilizing a one-day, double-antibody testosterone radioimmunoassay kit (MP Biomedicals, Solon, OH 44139, USA). 50µl of standards (0.1ng/ml, 0.25ng/ml,0.5ng/ml,1.0ng/ml,2.5ng/ml,5.0ng/ml,10.0ng/ml) or plasma samples were pipetted into 12x75 mm borosilicate glass tubes (VWR International, Radnor, PA, USA) and

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incubated with 500µl Testosterone ^{125}Iodine tracer. 500µl of first anti-body (anti-testosterone) was added to all tubes apart from total count (TC) and nonspecific binding (NSB) tubes. Tubes were then vortexed and allowed to incubate for 120 minutes. After incubation, 100µl of second antibody was added to all tubes except TC and NSB tubes, vortexed and allowed to incubate again at room temperature for 60 minutes. Subsequently, tubes were centrifuged at 2500rpm for 15 minutes. Supernatant from centrifuged tubes were decanted after which tube rims were blotted against absorbent paper towels. TC tubes and precipitate pellets in assay tube were counted in gamma counter.

Statistical Analyses

Data were obtained in kidney weights, liver weights, prostate gland weights, epididymis weights, testes weights and liver enzyme concentration in whole blood. All data were expressed as mean ± standard error of the mean (SEM). The effect of the intra peritoneal administration of aqueous extracts of NS seeds on all data was determined using the analysis of variance technique (ANOVA) on SAS analytical software (Cary NC, U.S.A). A probability of $P < 0.05$ was set as the level of significance. Tukey's post hoc test was used to separate the means where significant differences existed.

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Chapter V: Results

Experiment 1: Effect of the Dietary Supplementation of NS on Feed Intake and Body Weight in Female Sprague-Dawley Rats

The effect of the dietary supplementation of NS on feed intake and body weight are shown in Figures 1 and 2, respectively. Pre-coitus, dietary supplementation of NS did not significantly affect daily feed intake at any of NS supplementation levels (0%=16.2, 5%=15.8g, 10%=15.9g,15%=15.9). Similarly, dietary supplementation of NS did not significantly affect mean body weight (control:208.1g, 5% :205.1g, 10%:205.0g, 15%:209.8g). Although non-significant, highest body weight (209.8g) was recorded in the 15% treatment group.

Post coitus, feed intake was statistically similar among all groups 0% (19.45g), 5% (19.81) and 15% (19.50g) except in the 10% supplementation group (18.75g) which was significantly lower (p=0.0008). Post-coitus, rats fed rat chow supplemented at 5% had the highest feed intake (19.81g), while rats fed rat chow substituted at 10% had the lowest feed intake (18.76g) across all treatment groups. Body weights remained similar amongst treatment groups while the highest body weight was recorded in the 15% supplementation group (270.2g). Overall, mean feed intake during pregnancy (19.4g) was significantly higher (P <0.0001) than mean feed intake during the pre-mating period (15.9g) indicating a possible effect of pregnancy on feed intake. Similarly, overall body weight during pregnancy (261.9g) period was also significantly higher than overall mean pre-mating bodyweight (207.2g) indicating also, a possible effect of pregnancy on weight gain.

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Effect of the Dietary Supplementation of NS Seeds on Pregnancy Outcomes in Female Sprague-Dawley Rats

The effects of the dietary supplementation of NS seeds on pregnancy outcomes in female Sprague-Dawley rats are shown in Table 1. Dietary supplementation of NS seeds in feed did not significantly (P>0.05) affect conception rates and litter sizes across all NS supplemented groups when compared with the control. Furthermore, supplementation of NS seeds significantly increased (P<0.0001) birth weights and post-partum pup weights. However, this increase was not dose-dependent. Post parturition the highest incidence of pup mortality (7%) was seen in the 15% NS inclusion group.

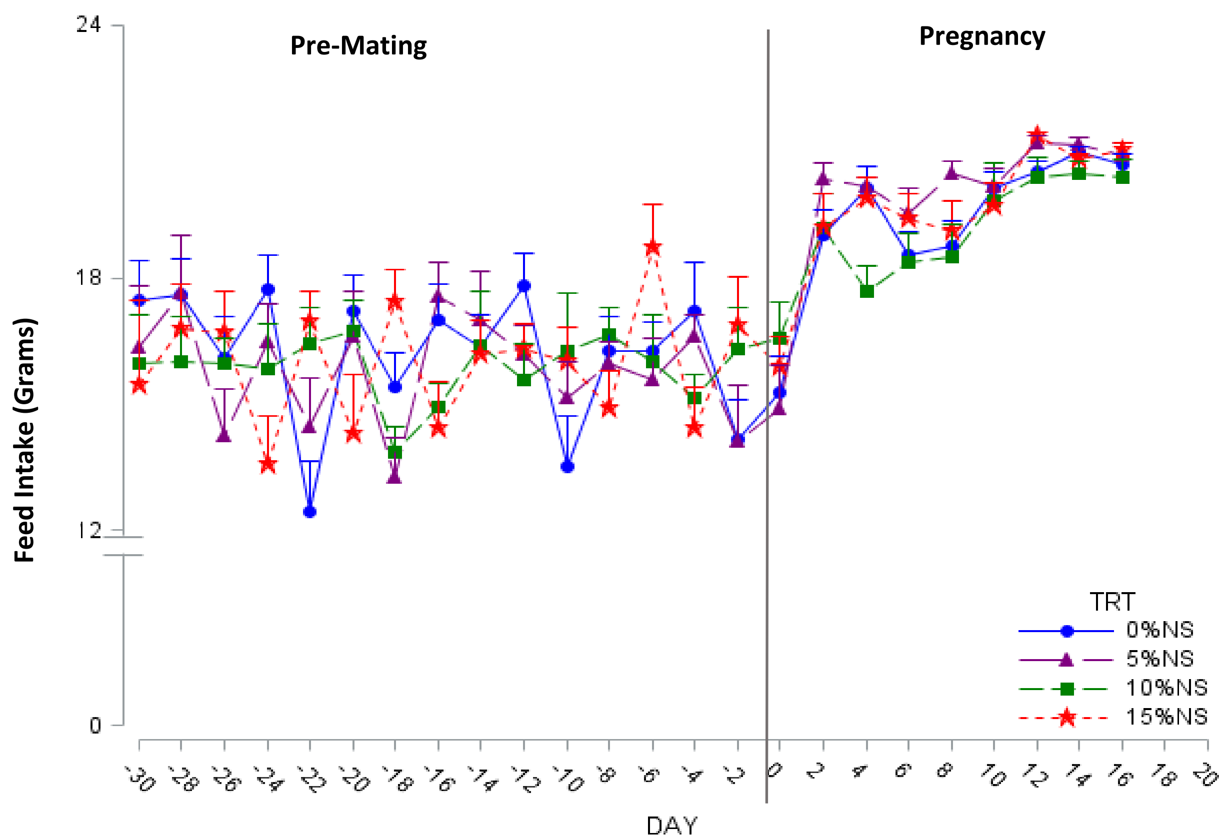

Figure. 1. Effects of NS Supplementation On Feed-Intake in Female Sprague-Dawley Rats Pre and Post Pregnancy. Days with negative symbol indicate pre-mating period while days with positive symbol indicate post-mating period.

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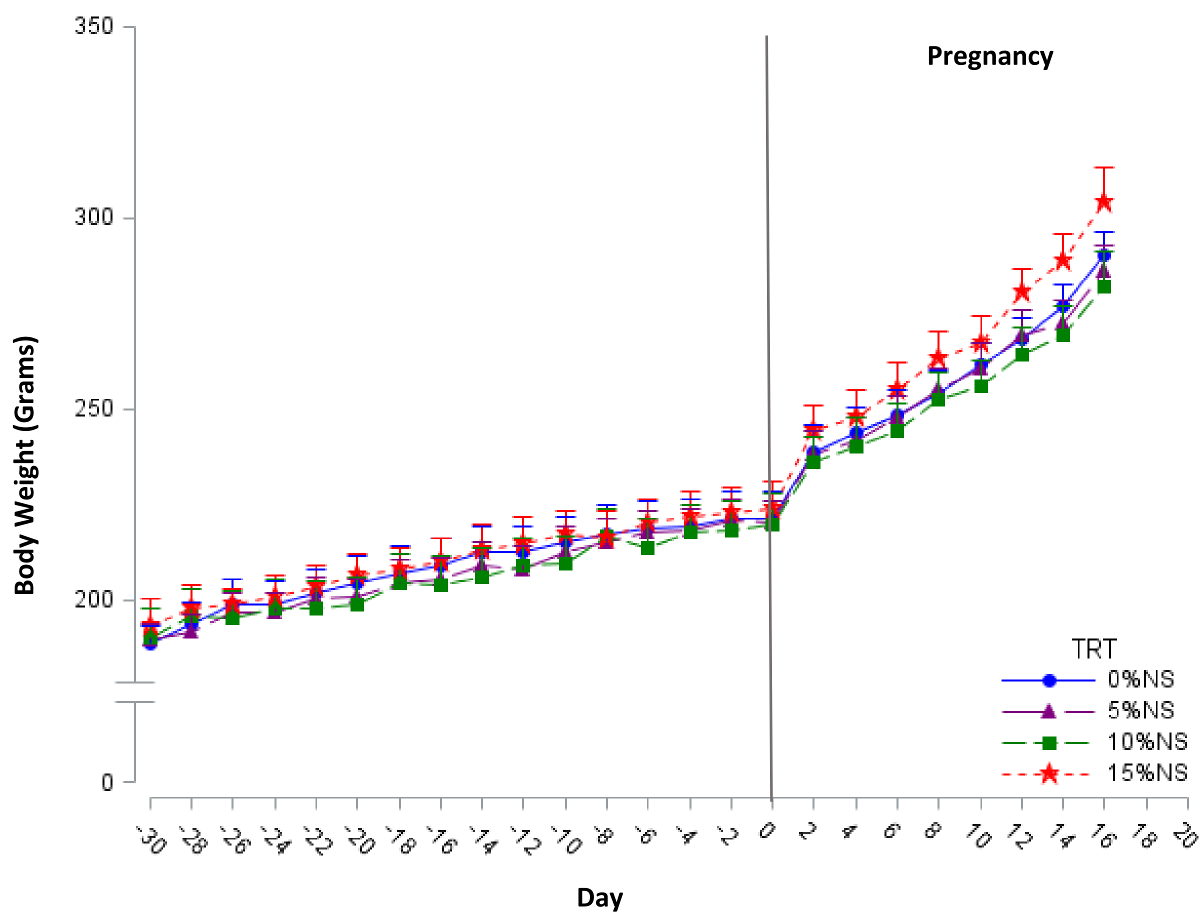

Figure 2. Effects of NS Supplementation on Body Weight in Female Sprague-Dawley Rats, Pre and Post Pregnancy. Days with negative symbol indicate pre-mating period while days with positive symbol indicate post-mating period.

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Table 2

Effect of the Dietary Supplementation of Nigella Sativa on Pregnancy Outcomes in Female Sprague-Dawley Rats.

	Treatment Groups			
Parameters	0%NS	5%NS	10%NS	15%NS
Rats Bred	8	8	8	8
Conception %	88	100	75	100
Litter Size	13±1	12±3	11±1	11±3
Pup birth weight, g	5.8±0.11^{a}	6.5±0.11^{b}	6.9± 0.06^{b}	6.6±0.09^{b}
Day 3 Post Parturition Weight, g	7.7±0.19^{a}	8.8±0.19^{b}	9.0±0.23^{b}	8.2±0.19^{b}
Day 7 Post Parturition Weight, g	11.5±0.19^{a}	13.1±0.31^{b}	13.7±0.20^{b}	12.2 ± 0.40^{b}
Percent Pup Mortality	1	2	0	7
Runt	0	1	0	0

Results are mean ± S.E.M
^{a,b} Means within a row with different superscripts are statistically different, $P<0.05$

Experiment 2: Acute and Chronic Effect of the Intraperitoneal NSS Extract Administration on Visceral Organ Weights

There were no treatment effects ($p>0.05$) of administering NS extracts on the weights of the liver, kidney, prostate gland, epididymis and testes as shown in Tables 4, 5, 6, 7, and 8, respectively. However, chronic NS aqueous extract administration significantly reduced testicular ($p=0.0005$) and epididymis weight. ($p=0.0039$)

Acute and Chronic Effect of Intraperitoneal NS Administration on Key Hematology Parameters

There were no treatment effects of NS administration on creatinine, blood urea nitrogen (BUN) levels, white blood cell counts, red blood cell counts, hemoglobin, and mean corpuscular

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hemoglobin concentration levels during the duration of the study as shown in Figures 8, 9, 10, 11, 12, and 13 respectively. However, chronic administration of NS extracts significantly increased blood urea nitrogen levels (p=0.03) while reducing (p=0.0002) mean corpuscular hemoglobin concentration levels.

Acute and Chronic Effect of Intraperitoneal NS Administration on Visceral Organ Histology

The administration of our NS extract led to no visible morphological changes in the histology of the kidney and liver. Healthy, normal looking spermatogonia, spermatocyte, and spermatozoa populations were observed in the control obtained after the acute and chronic phases of the second study. However, formation of fibrous tissue and depletion in the levels of spermatogonia, spermatocyte and spermatozoa populations were noticed in all Treatment Groups C, D, E, F as shown in Figure 3. Microscopic analyses of epididymides tissue slides of controls from the acute and phases of the study revealed intact epithelia and normal optimal spermatozoa populations. However, disintegration of epididymides epithelia and overall depletion in spermatozoa populations were noticed in the both the acute & chronic low and high dose groups. Analyses of prostate gland tissue slides processed from rats in acute and chronic control groups showed no evidence of pathology, while a general trend of histological damage was noticed in sample prostate gland slides processed from all NS treated groups.

Acute and Chronic Effect of Intraperitoneal NS Administration on Liver Enzymes

Wide variations were observed in the levels of the liver enzymes namely aspartate dehydrogenase, alanine aminotransferase and alkaline phosphatase levels (Tables 8, 9, and 10)

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Acute and Chronic Effect of Intraperitoneal NS Administration on Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), and Testosterone in Male Sprague-Dawley Rats

There was no treatment effect of NS administration on FSH or LH levels during of the study as shown in Table 11 and 12. However, chronic NS administration reduced mean FSH levels ($p=0.0014$) and increased mean LH levels ($p=0.03$) in study rats. There was no treatment or duration effect of NS administration on testosterone concentration as shown in Table 13.

Adhesion Effect of intraperitoneal NS administration

Chronic administration of NS caused severe adhesion in our study rats as seen in Figure 14.

Table 3

Effect of the Acute and Chronic Administration of NS Extracts on Liver Weight (g/100gBW)

	Treatment Duration	
Treatment Group	15 days (Acute)	30 days (Chronic)
Saline (Control)	4.3 ± 0.50	4.5 ± 0.20
25mg/kg BW NS (Low dose)	3.8 ± 0.40	4.0 ± 0.00
50mg/kg BW NS (High dose)	4.8 ± 0.08	3.9 ± 0.00

Results are the mean ± S.E.M.
Statistical significance was set at $p<0.05$.
Treatment with NS extracts caused no statistical significance within group
No treatment effect of NS administration was observed, $p=0.6172$
No period effect of NS administration was observed, $p=0.6552$

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Table 4

Effect of the Acute and Chronic Administration of NS Extracts on Kidney Weight (g/100gBW)

	Treatment Duration	
Treatment Group	15 days (Acute)	30 days (Chronic)
Saline (Control)	0.9 ± 0.09	0.8 ± 0.07
25mg/kg BW NS (Low dose)	0.8 ± 0.02	0.8 ± 0.04
50mg/kg BW NS (High dose)	0.8 ± 0.06	1.0 ± 0.01

Results are the mean ± S.E.M.
Statistical significance was set at $p<0.05$.
Treatment with NS extracts caused no statistical significance within groups
No treatment effect of NS administration was observed, $p=0.4723$
No ped effect of NS administration was observed, $p=0.6467$

Table 5

Effect of the Acute and Chronic Administration of NS Extracts on Prostate Gland Weight (g/100gBA)

	Treatment Duration	
Treatment Group	15 days (Acute)	30 days (Chronic)
Saline (Control)	0.2 ± 0.03	0.2 ± 0.20
25mg/kg BW NS (Low dose)	0.2 ± 0.03	0.1 ± 0.00
50mg/kg BW NS (High dose)	0.1 ± 0.08	ND

Results are the mean ± S.E.M.
Statistical significance was set at $p<0.05$.
Treatment with NS extracts caused no statistical significance within groups
ND= Not determinable due to adhesion shown in Figure 8
No treatment effect of NS administration was observed, $p=0.2027$
No period effect of NS administration was observed, $p=0.8685$

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Table 6

Effect of the Acute and Chronic Administration of NS Extracts on Epididymis Weight (g/100gBW)

	Treatment Duration	
Treatment Group	15 days (Acute)	30 days (Chronic)
Saline (Control)	0.5 ± 0.04	0.4 ± 0.02
25mg/kg BW NS (Low dose)	0.5 ± 0.02	0.4 ± 0.01
50mg/kg BW NS (High dose)	0.4 ± 0.03	0.3 ± 0.03

Results are the mean ± S.E.M.
Statistical significance was set at $p<0.05$.
Treatment with NS extracts caused no statistical significance within groups.
Chronic administration of NS aqueous extract significantly reduced epididymis weight, $P=0.0039$
No treatment effect of NS administration observed, $p=0.6172$
Period effect was not due to NS administration, $p=0.6485$

Table 7

Effect of the Acute and Chronic Administration of NS Extracts on Testes Weight (g/100gBW)

	Treatment Duration	
Treatment group	15 days (Acute)	30 days (Chronic)
Saline (Control)	1.2 ± 0.07	1.0 ± 0.04
25mg/kg BW NS (Low dose)	1.2 ± 0.09	0.7 ± 0.21
50mg/kg BW NS (High dose)	1.3 ± 0.13	0.9 ± 0.03

Results are the mean ± S.E.M.
Statistical significance was set at $p<0.05$.
Treatment with NS extracts caused no statistical significance within groups.
Chronic administration of aqueous NS extract significantly reduced testes weight $p=0.0005$
No treatment effect of NS administration was observed, $p=0.4336$
Period effect was not significant, $p=0.2662$

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Acute and Chronic Effect of the Intraperitoneal NS Administration on Testes Histology

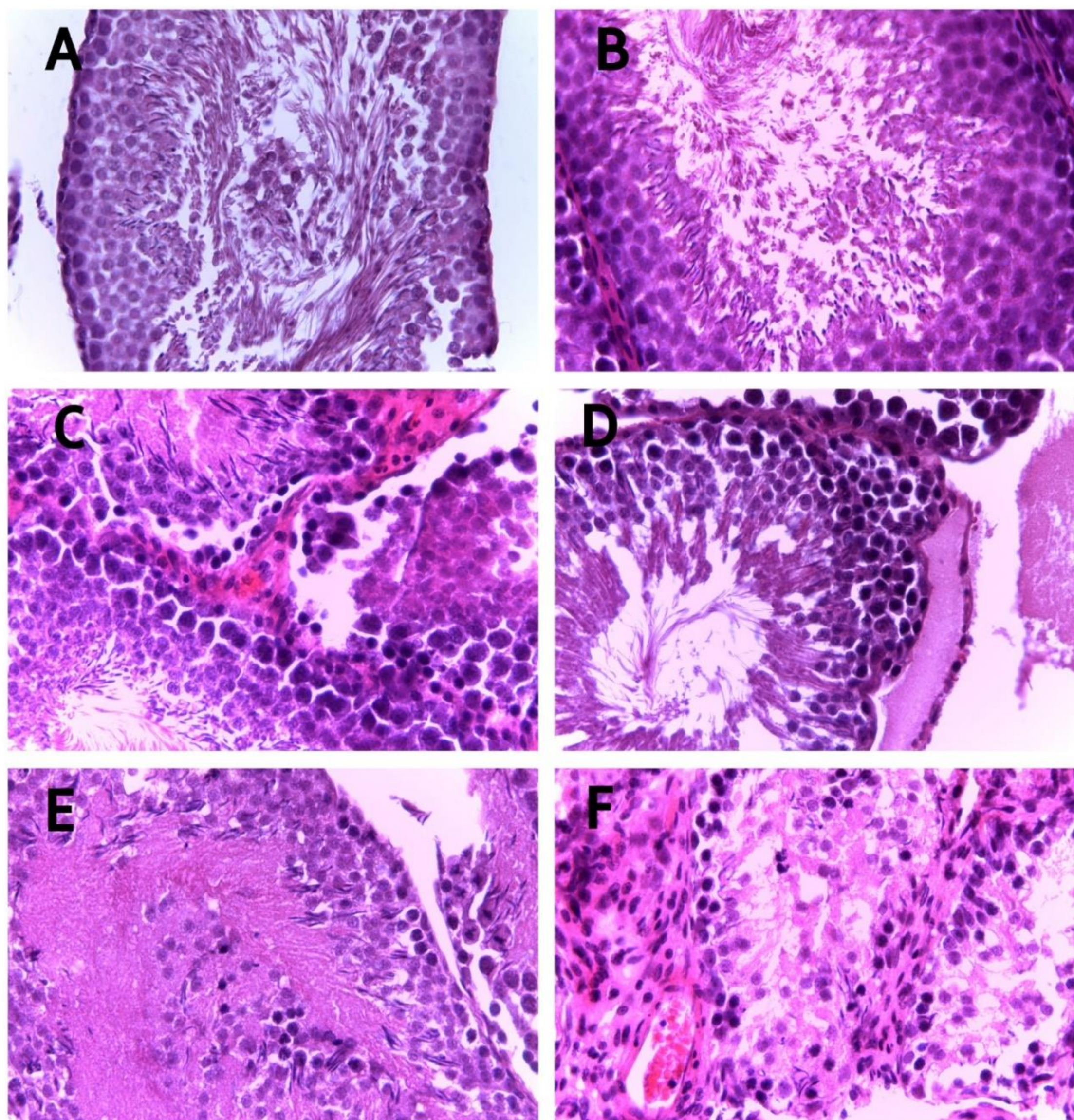

Figure 3. Testes of Male Sprague-Dawley Rats. Rats were intraperitoneally administered:
A. 0.9% Saline Acutely
B. 0.9% Saline Chronically
C. 25mg/kg BW NSE acutely
D. 25mg/kgBW NSE chronically
E. 50mg/kg BW acutely
F. 50mg/kg BW chronically

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Acute and Chronic Effect of the Intraperitoneal NS Administration on Epididymis Histology

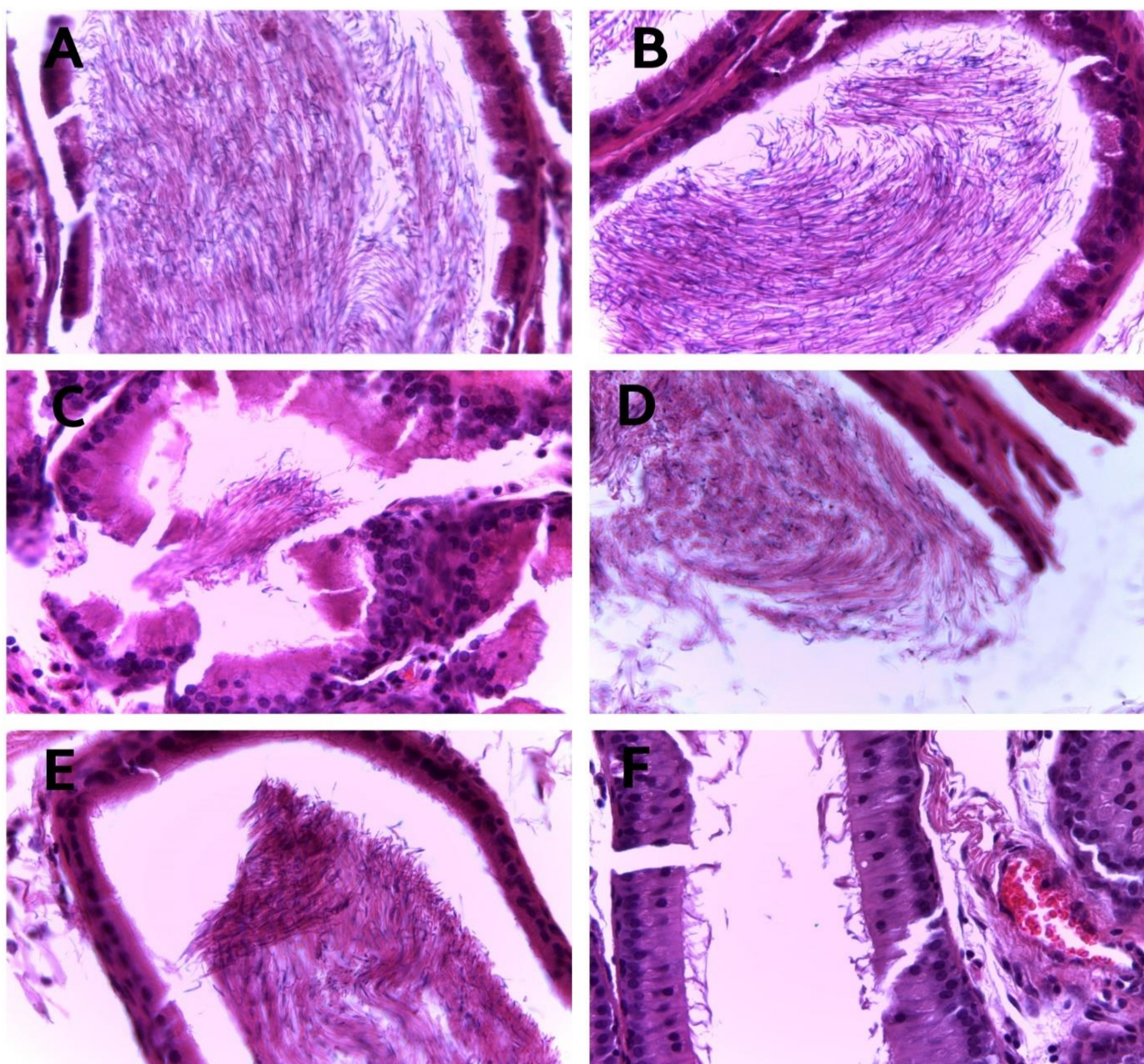

Figure 4. Epididymis of Male Sprague-Dawley Rats. Rats were intraperitoneally administered:

A. 0.9% saline acutely
B. 0.9% saline chronically
C. 25mg/kg BW NSE acutely
D. 25mg/kg BW NSE chronically
E. 50mg/kg BW acutely
F. 50mg/kg BW chronically

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Acute and Chronic Effect of the Intraperitoneal NS Administration on Prostate Gland Histology

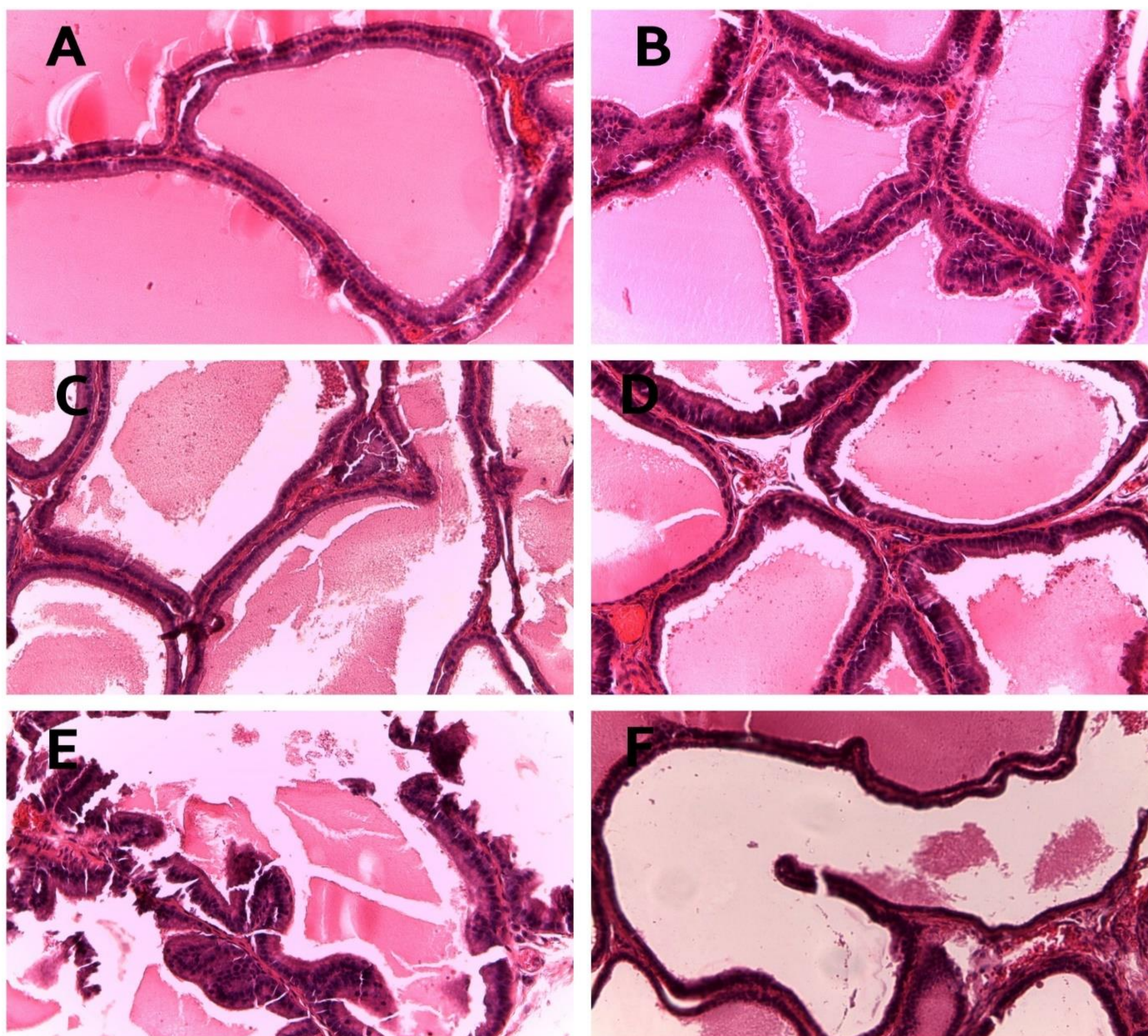

Figure 5: Prostate of Male Sprague-Dawley Rats. Rats were intraperitoneally administered:

A. 0.9% saline acutely
B. 0.9% saline chronically
C. 25mg/kg BW NSE acutely
D. 25mg/kg BW NSE chronically
E. 50mg/kg BW acutely
F. 50mg/kg BW chronically

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Acute and Chronic Effect of the Intraperitoneal NS Administration on Kidney Glomeruli Histology

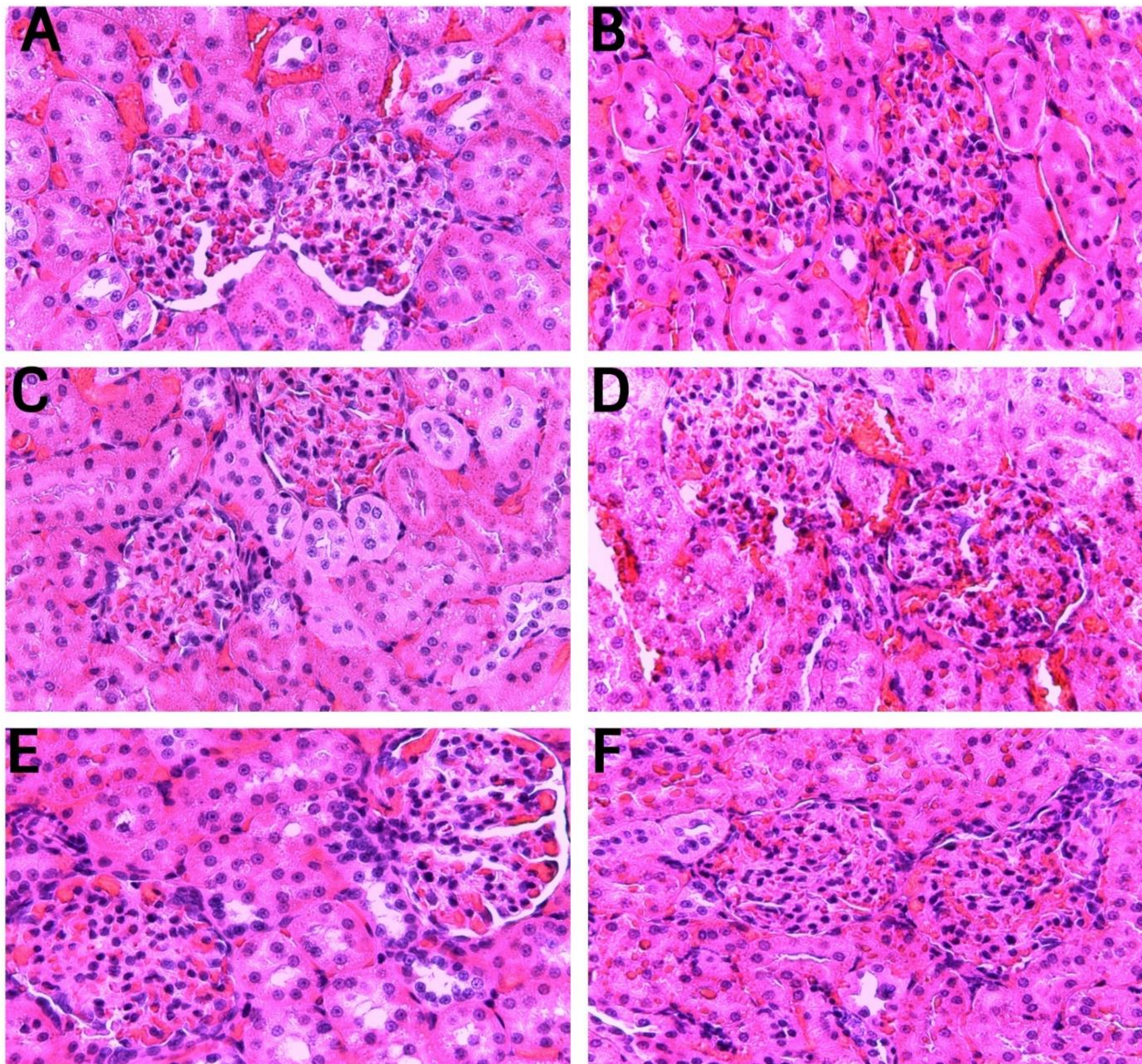

Figure 6. Glomeruli of Male Sprague-Dawley Rats. Rats were intraperitoneally administered:
A. 0.9% saline acutely
B. 0.9% saline chronically
C. 25mg/kg BW NSE acutely
D. 25mg/kg BW NSE chronically
E. 50mg/kg BW acutely
F. 50mg/kg BW chronically

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Acute and Chronic Effect of the Intraperitoneal NS Administration on Liver Histology

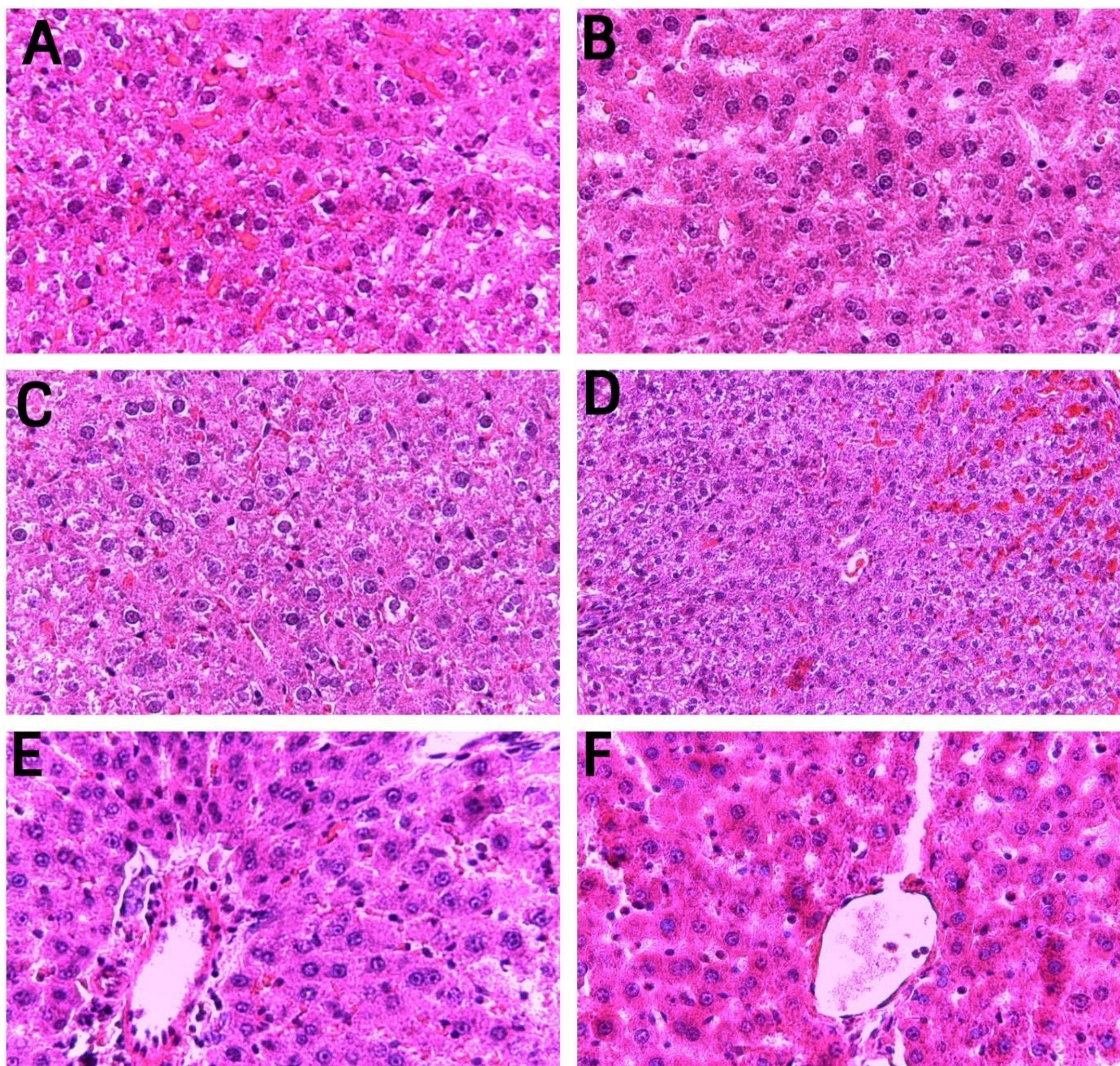

Figure 7. Liver of Male Sprague-Dawley Rats. Rats were intraperitoneally administered:
A. 0.9% saline acutely
B. 0.9% saline chronically
C. 25mg/kg BW NSE acutely
D. 25mg/kg BW NSE chronically
E. 50mg/kg BW acutely
F. 50mg/kg BW chronically

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Table 8

Effect of the Acute and Chronic Administration of NS Extracts on Aspartate Dehydrogenase Level (U/L)

	Treatment Duration	
Treatment Group	15 days (Acute)	30 days (Chronic)
Saline (Control)	585.7 ± 233.66	648.5 ± 277.50
25mg/kg BW NS (Low dose)	875.0 ± 146.00	410.0 ± 152.00
50mg/kg BW NS (High dose)	524.0 ± 262.87	1628.5 ± 1345.50

Results are the mean ± S.E.M.
Statistical significance was set at $p<0.05$
Treatment with NS extracts caused no significant effect

Table 9

Effect of the Acute and Chronic Administration of NS Extracts on Plasma Alanine Transaminase Levels (U/L)

	Treatment Duration	
Treatment Group	15 days (Acute)	30 days (Chronic)
Saline (Control)	125.3 ± 33.27	136.0 ± 31.00
25mg/kg BW NS (Low dose)	298 ± 99.00	93.0 ± 40.00
50mg/kg BW NS (High dose)	169.0 ± 105.53	216 ± 154.00

Results are the mean ± S.E.M
Statistical significance was set at $p<0.05$.
Treatment with NS extracts caused no significant effect

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Table 10

Effect of the Acute and Chronic Administration of NS Extracts on Plasma Alkaline Phosphatase Levels (U/L

	Treatment Duration	
Treatment Group	15 days	30 days
Saline	0.0 ± 0.00	2.5 ± 1.50
25mg/kg BW NS	0.0 ± 0.00	2.5 ± 2.50
50mg/kg BW NS	0.0 ± 0.00	25.5 ± 21.50

Results are the mean ± S.E.M.
Statistical significance was set at $p<0.05$.
Treatment with NS extracts caused no significant effect

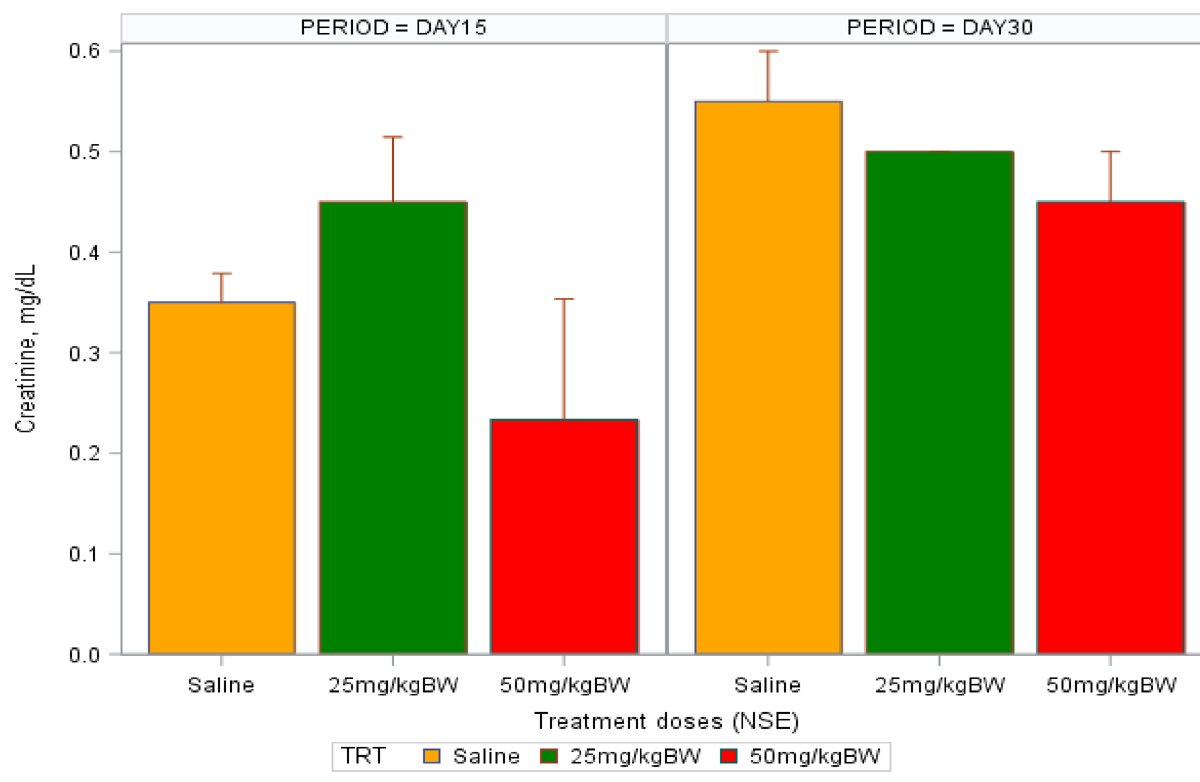

Figure 8. Comparison of Creatinine Concentrations Between Acute 15 Day and Chronic 30 Treatment Periods. Orange bar represents saline control, green bar represents low dose of NS extract, red bar represents high dose of NS extract.

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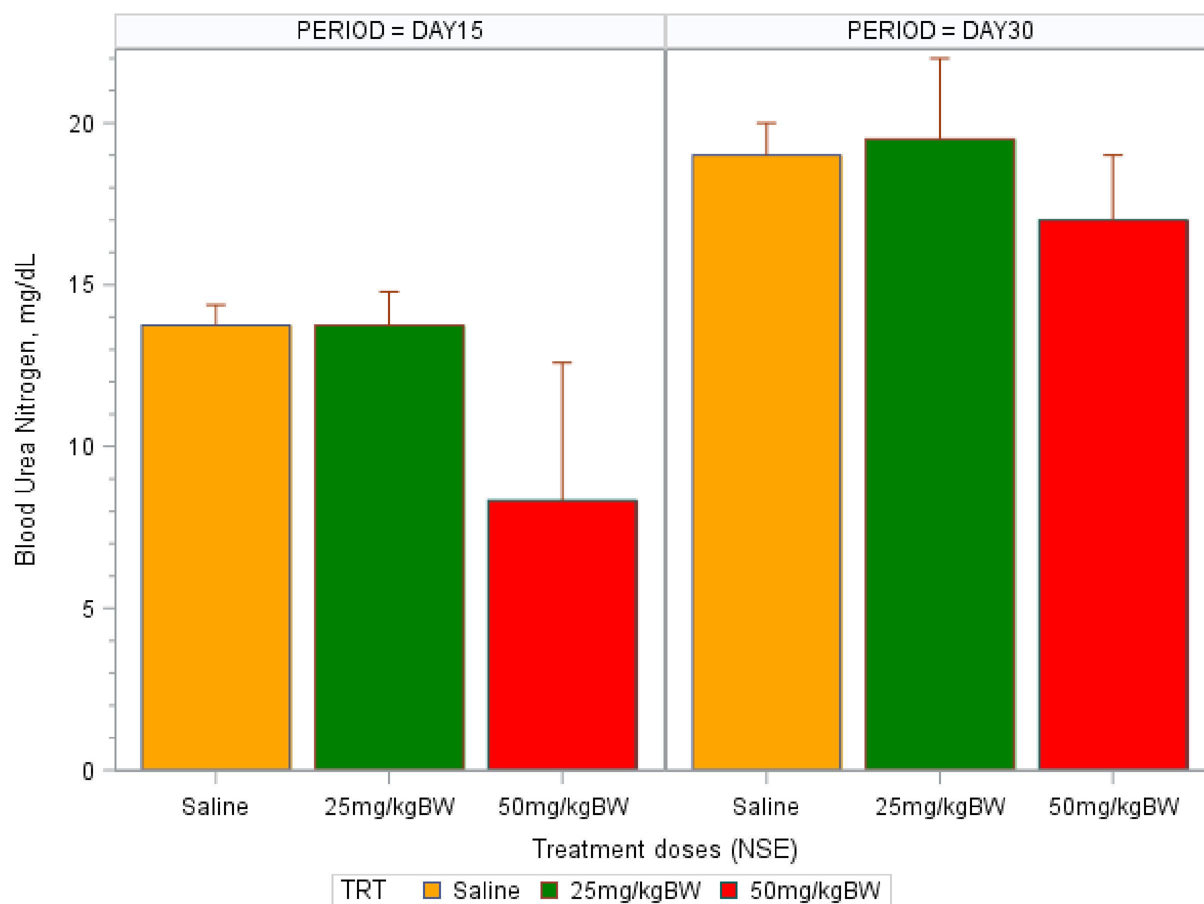

Figure 9. Comparison of Blood Urea Nitrogen Concentrations Between Acute 15 Day and Chronic 30 Treatment Periods. Orange bar represents saline control, green bar represents low dose of NS extract, red bar represents high dose of NS extract.

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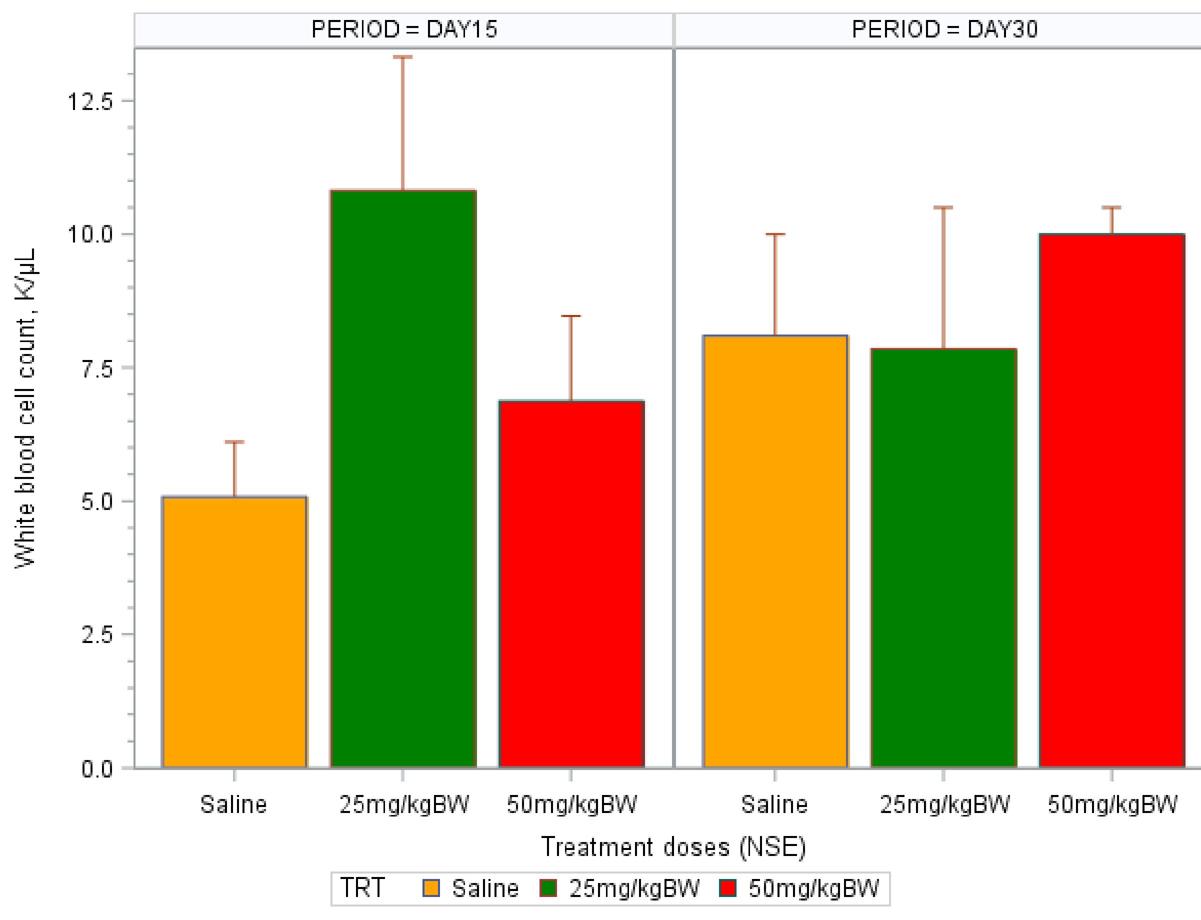

Figure 10. Comparison of White Blood Cell Count Between Acute 15 Day and Chronic 30 Treatment Periods. Orange bar represents saline control, green bar represents low dose of NS extract, red bar represents high dose of NS extract.

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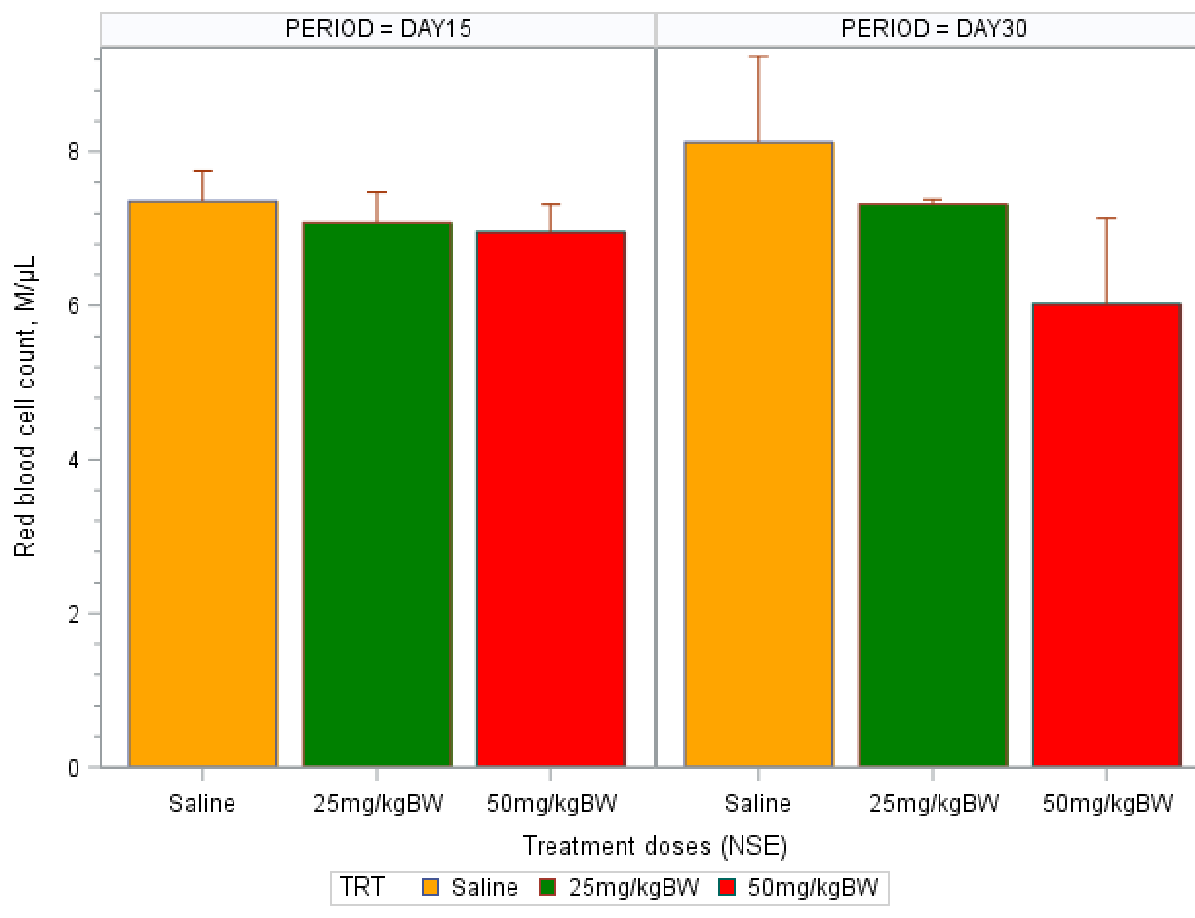

Figure 11. Comparison of Red Blood Cell Count Between Acute 15 Day and Chronic 30 Treatment Periods. Orange bar represents saline control, green bar represents low dose of NS extract, red bar represents high dose of NS extract.

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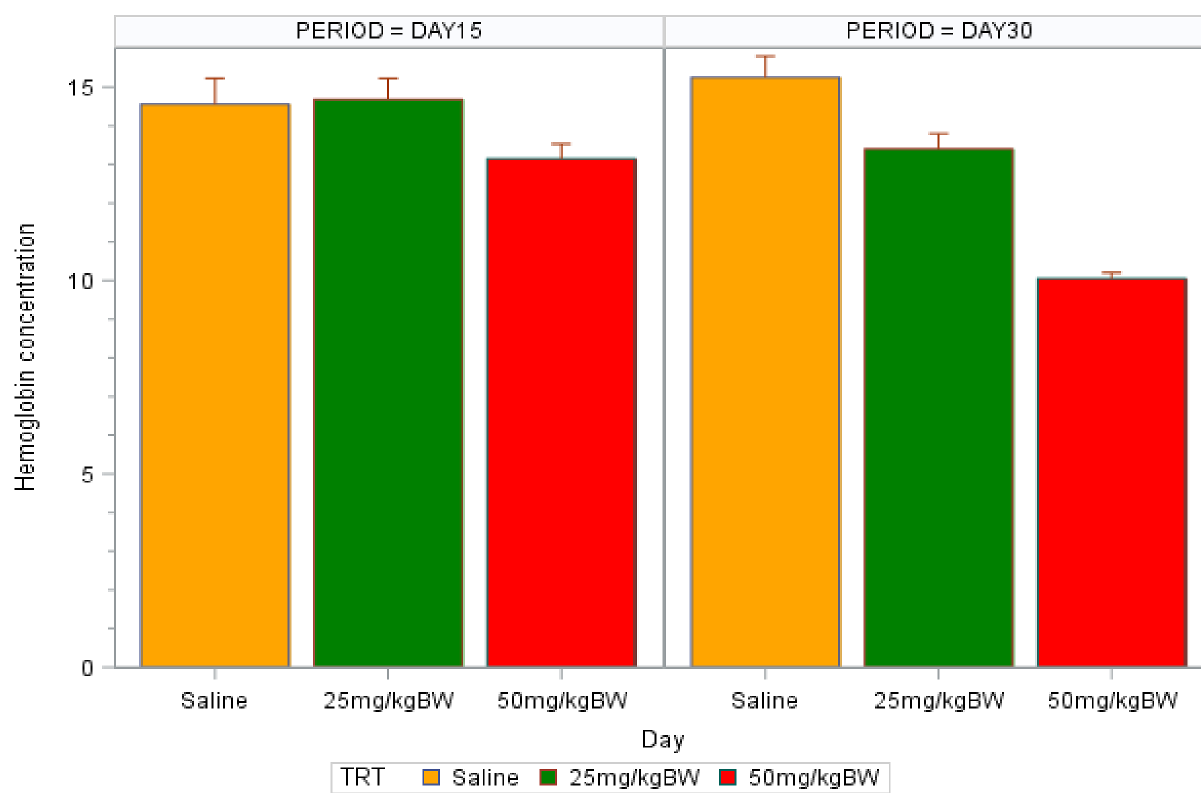

Figure 12. Comparison of Hemoglobin Concentration Between Acute 15 Day and Chronic 30 Treatment Periods. Orange bar represents saline control, green bar represents low dose of NS extract, red bar represents high dose of NS extract.

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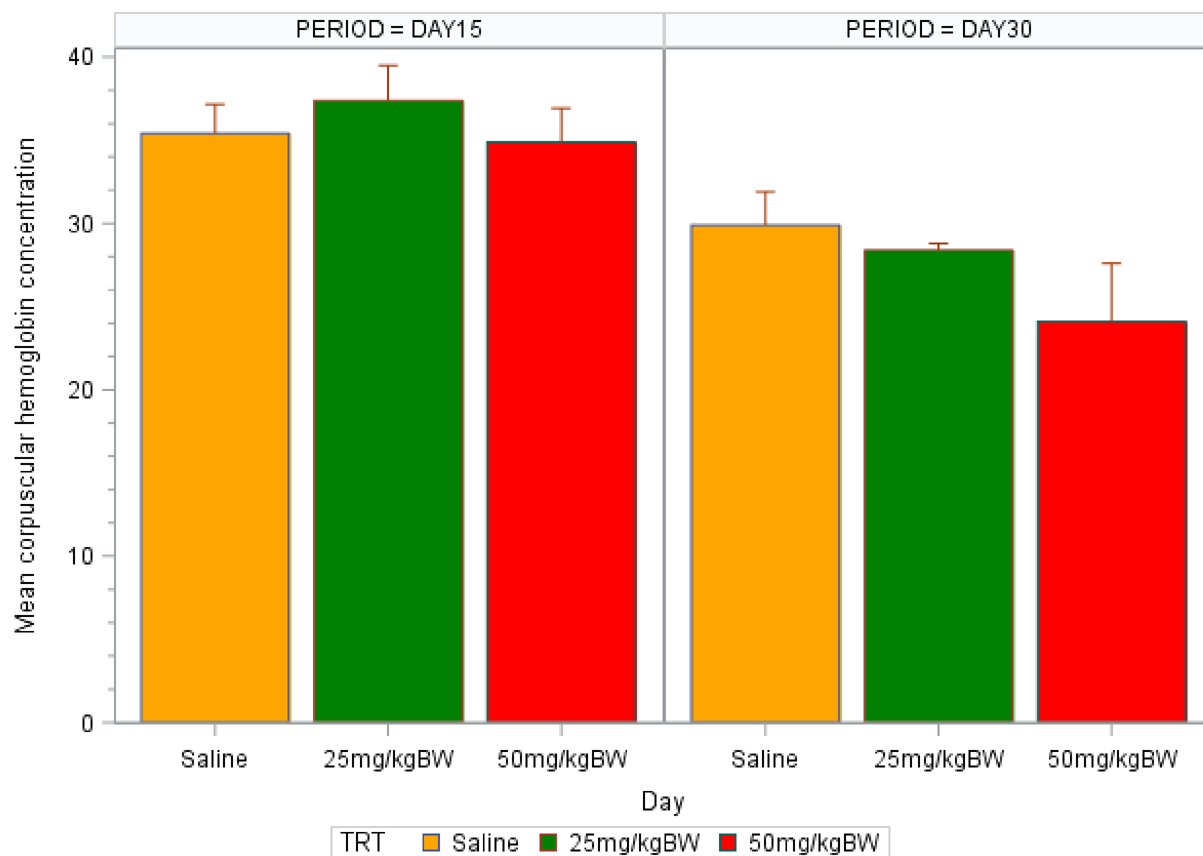

Figure 13. Comparison of Mean Hemoglobin Corpuscular Concentration Between Acute 15 Day and Chronic 30 Treatment Periods. Orange bar represents saline control, green bar represents low dose of NS extract, red bar represents high dose of NS extract.

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Table 11

Effect of the Acute and Chronic Administration of NS Extracts on Plasma Luteinizing Hormone Levels (ng/ml)

	Treatment Duration	
Treatment Group	15 days (Acute)	30 days (Chronic)
Saline (Control)	1.4 ± 0.29^{a}	1.9 ± 0.11^{b}
25mg/kg BW NS (Low dose)	1.7 ± 0.05^{a}	2.1 ± 0.33^{b}
50mg/kg BW NS (High dose)	1.3 ± 0.25^{a}	1.9 ± 0.21^{b}

Results are the mean ± S.E.M.
[a,b]Means within a row with different superscripts are statistically different
Treatment with NS extracts caused no statistical significance effect on mean plasma LH levels. Mean LH levels were significantly (P=0.03) greater at the end of the Chronic phase compared to the acute phase, irrespective of the treatment.

Table 12

Effect of the Acute and Chronic Administration of NS Extracts on Plasma Follicle Stimulating Hormone Levels (ng/ml)

	Treatment Duration	
Treatment Group	15 days (Acute)	30 days (Chronic)
Saline (Control)	0.3 ± 0.04^{a}	0.1 ± 0.01^{b}
25mg/kg BW NS (Low dose)	0.2 ± 0.02^{a}	0.1 ± 0.00^{b}
50mg/kg BW NS (High dose)	0.2 ± 0.02^{a}	0.1 ± 0.00^{b}

Results are the mean ± S.E.M.
[a,b]Means within a row with different superscripts are statistically different
Treatment with NS extracts caused no statistical significance effect on mean plasma FSH levels. Mean FSH levels were significantly (P=0.0014) lower at the end of the Chronic phase compared to the acute phase, irrespective of the treatment.

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Table 13

Effect of the Acute and Chronic Administration of NS Extracts on Plasma Testosterone Hormone Levels (ng/ml)

	Treatment Duration	
Treatment Group	15 days (Acute)	30 days (Chronic)
Saline (Control)	1.2 ± 0.85	1.5 ± 0.14
25mg/kg BW NS (Low dose)	1.3 ± 0.00	0.3 ± 0.22
50mg/kg BW NS (High dose)	0.7 ± 0.41	0.02 ± 0.01

Results are the mean ± S.E.M.
Statistical significance was set at $p<0.05$.

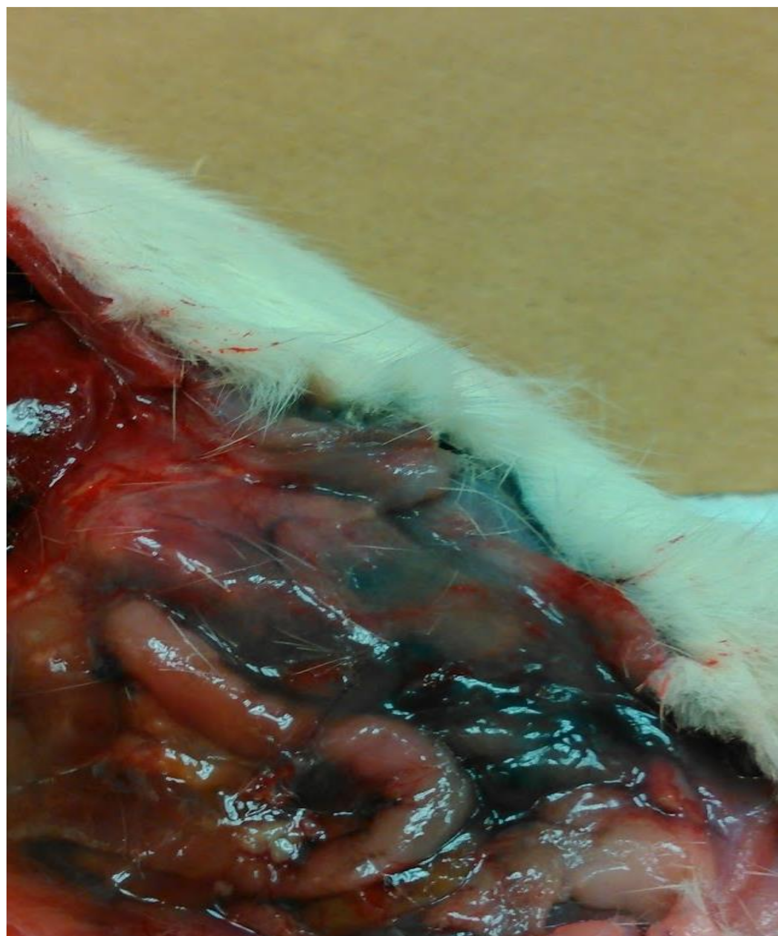

Figure 14. Image Shows Severe Adhesion Noticed During Study. Study was initially intended to last 90 days but was discontinued due to adhesion.

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Chapter VI: Discussion

Pregnancy presents a fragile physiological condition webbed in an intricate interplay of hormonal events. Dietary accommodations during pregnancy are important as essential nutrients for successful gestation are required. Herbs contain a myriad of active constituents such as phytosterols which support or have untoward effects on pregnancy. Our first experiment was carried out to ascertain the effects of the dietary supplementation of NS extracts on metabolic parameters such as feed intake, body weight and pregnancy outcomes in Sprague-Dawley rats. Prior to mating and conception, our results indicated that NS supplementation of had no significant effect on feed-intake and body weight in our experimental rats. In contrast to our findings, Al-Sa'aidi et al. (2008) reported that alcoholic extracts of NS enhanced body weight in male rats. Further, *Nigella sativa* has also been reported to cause significant changes in body weights of Barki lambs and calves (Mahmoud & Bendary, 2014) and broilers (Nasir & Grashorn, 2010). Further, NS supplementation in feed was reported to increase weight gain (El-Ghammry, EL-Mallah, & EL-Yammy, 2002) and increase feed conversion ratio (Al-Betawi & El-Ghousein, 2008) in broilers. NS supplementation in Demeshgi goats also stimulated daily weight gain (Abd El-Rahman, Abedo, Salman, Mohamed, & Shoukry, 2011). It seems that the duration of NS administration, the type of extract used, and the experimental animal model may also determine the body weight response to NS.

Interestingly however, post-conception body weight and feed intake increased in female rats across all groups, this could have been attributed to increased consumption of feed arising from additional units of energy needed due to pregnancy (Butte, Wong, Treuth, Ellis, & O-Brian Smith, 2004). Post-parturition, body weights of pups from all NS-treated groups were

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significantly higher than those obtained in the control. This has been attributed to the reported galactogogue attribute of *Nigella sativa* (Hosseinzadeh, Mohsen, Mojdeh, & Taghiabadi, 2013). *Nigella sativa* has been reported to contain t-Anethole, a phytosterol dopamine inhibitor (Hosseinzadeh et al., 2013). Although precise mechanisms as to how t-Anethole stimulates milk production have not been elucidated, it has been suggested that binding of t-Anethole directly causes the upregulation of the prolactin gene enabling enhanced milk production, or indirectly upregulating the prolactin gene via protein kinase A and phospholipase C in anterior pituitary lactotrophic cells (Tabares, Bedoya Jaramillo, & Ruiz-Cortés, 2014). Proliferation in cell survival, and the upregulation of prolactin receptors in myoepithelial cells found in the mammary gland could have also stimulated milk synthesis leading to increased weights of treated pups (Tabares et al., 2014).

A peculiar observation noticed during experiment 1 was the high mortality of pups in the 15% supplementation group. This could have been due to chance or several plausible mechanisms. Instinctively, parturient carnivore or omnivore dams exhibit a tendency to ingest their newly delivered pups, only a constellation of stimuli such as warmth and vocalizations at certain frequencies (22-23kHz) emanating from viable newborns inhibit ingestion by mothers (Kristal, 2009). Weak or moribund newborns cannot produce these stimuli and are therefore eaten (Noirot, 1972; Peters & Kristal, 1983). Caging of rodents during experiments is also a great source of stress. The induction of stress via direct or indirect channels could have led to mal-development of fetuses in-utero which could have in turn resulted in weak or still born neonates (Bale, 2005; Malmkvist & Damm, 2007) ultimately leading to their consumption. The non-observation of cannibalistic behavior in other dams could be due to individual differences in the

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levels of maternal activity, usually associated with different levels of dopamine in the nucleus accumbens, rostral to the pre-optic area in the hypothalamus (Champagne et al., 2004). Increased *Nigella sativa* supplementation at 15% could have reduced dopamine availability needed for the expression of maternal behavior in females leading to the high incidence of pup mortality. This inhibitory effect of *Nigella sativa* on dopamine could have also stimulated prolactin secretion, as dopamine synthesized in the hypothalamus inhibits prolactin production by pituitary lactotroph cells (Fitzgerald & Dinan, 2008) removing the break on prolactin secretion. This increased prolactin could have stimulated higher milk production in NS supplemented group implicating possible galactogogue properties as reported by (Agrawala, Achar, & Tamankar, 1971). Phytochemical analysis of aqueous NS extracts has shown that they contain a class of phenolic coumarin compounds (Ishtiaq et al., 2013). Coumarins have been reported to possess teratogenic properties (Van Driel et al., 2002). Hence, high amounts of coumarins contained in 15% NS supplemented feed in our first study could have led to fetal injury in-utero, prompting consumption post-parturition by dams. *Nigella sativa* seeds have been reported to stimulate estrogen production (Parhizkar, Latiff, & Parsa, 2016). Supplementation of NS at 15% might have induced down-regulation of estrogen receptors likely caused by high plasma estrogen levels as a result of high supplementation rate. Reduction in the possible NS-mediated production of GABA via estrogen stimulatory pathways could have also resulted in higher levels of anxiety in the 15% supplementation group due to a break on the GABAergic inhibition of corticotrophin releasing neurons in the parvocellular area of the paraventricular nucleus in the hypothalamus (Herman et al., 2003; Larsen et al., 2003; Ulrich & Herman, 2009),

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leading to an unbridled production of cortisol, ultimately resulting in higher stress levels in the 15% supplemented dam.

The emergence of herbal remedies as prophylactics and curatives has seen a widespread proliferation in first and third world countries in the last decade (Calapai 2008). In countries where herbal extract remedies are consumed by males to stimulate and enhance sexual prowess, mal development and malfunction to major organs remains a potential eventuality. Our second study was carried out to not only examine the effects of NS on the plasma levels of reproductive hormones LH, FSH and testosterone, but to also ascertain if administration altered the structure and function of major organs. Results obtained from our study indicated that neither acute nor chronic administration of NS extracts had significant effects on the weights of the kidney, liver, prostate gland, epididymis and testes. The kidney is a central detoxification organ perfused with as much as 1.2 liters of blood per minute (Tortora & Derrickson, 2006). High levels of urea and creatinine serve as signals indicative of kidney distress (Salazar, 2014). Our results showed similar values for creatinine and blood urea nitrogen levels between NS treatment and Control groups indicating non-toxic effects of administering NS extracts acutely or chronically, agreeing with studies undertaken by El-Kholy, Hassan, Nour, Abe Elmageed, and Matrougui (2009) and Al-Ameen et al. (2011). In contrast to our results, administration of NS extracts significantly reduced plasma levels of creatinine and blood urea nitrogen in male Sprague-Dawley rats (Dollah, Parhizkar, Latiff, & Bin Hassan, 2013), which could have resulted from the interaction of several active compounds: beta pinene, thymoquinone, oleic acid and thymol contained in seeds utilized (Saleh, El-Darra, Raafat, & Ghazzawi, 2018). For our second study, however, the

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aqueous extract of NS was used, which has been reported to contain thymoquinone as the main active constituent (Saleh et al., 2018).

Translocation of liver enzymes such as Aspartate amino transferase (ASP), Alanine amino transferase (ALT) and Alkaline phosphatase (ALP) from the cytosol to plasma in blood is clinically indicative of liver injury. While ASP is found in a myriad of organs such as the brain, muscle, kidney, heart and liver, ALT and ALP are found predominantly in the liver and more accurate markers of liver function. Our results indicate that short-term and long-term administration of NS extracts for up to a month resulted in normal levels of ASP, ALT, and ALP in treatment groups. This was surprising as severe adhesion was noticed in NS treated rats. This normalcy in both kidney and liver function could have resulted from the hepato-renal protective feature of the key major compound found in Nigella sativa which is thymoquinone (Dollah et al., 2013).

This could be due to stimulatory capacity of thymoquinone on increasing levels of potent antioxidants such as Glutathione (Sankaranarayanan & Pari, 2011). High levels of glutathione could have subdued production of harmful reactive oxygen species (ROS) thereby leading to overall protection of kidney and liver. After more than 15 days, swelling and rigidity of the lower abdomen was noticed in NS treated rats. Treated rats were also observed to be lethargic with a few abruptly dying during the administration of NS extracts. Autopsies carried out revealed adhesion of visceral organs, abscesses, leakage of fecal matter, blood clots and general inflammation. In a study carried out by Abukhader (2012) adhesion due to intraperitoneal administration of NS extracts was attributed to possible increased uptake of glucose by peripheral tissues induced by thymoquinone leading ultimately to lipolysis. Several studies have

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reported a relationship between hyperlipidemia and acute pancreatitis (Gianfrate & Ferraris, 1998; Gumaste, 1996). In our study, administration of utilized extracts could have punctured ceca of experimental rats, spilling micro-organism rich fecal matter, inducing pancreatitis and eventual peritonitis. This hypothesis raises another question. Why was this adhesion not seen in the control groups? The viscous nature of NS extracts could have made administration more forceful leading to puncture of ceca. Peritonitis could have led to the production of inflammatory cytokines TNF-α, IL-6 and IL-1β (Haupt et al., 1998; Margetts et al., 2002) which in turn could have facilitated adhesion and production of pro-adhesion molecules.

Intraperitoneal NS extract administration caused no significant change in red blood cell (RBC) counts intra-group, after either the acute or chronic phases of Experiment 2. Although not statistically significant, a step wise decline in RBC counts were noticed between the control and treatment groups as shown in Figure 8. Decreased levels of hemoglobin and mean hemoglobin corpuscular volume counts were recorded after the chronic phase of the study. Already reported has been the relationship between pro inflammatory tumor necrosis factor α (TNFα) and hemoglobin concentration. In 1987, Blick, Sherwin, Rosenblum, and Gutterman reported a decrease in the level of hemoglobin in cancer patients when they were treated with TNFα. The underlying mechanism for this decline could have resulted from a possible inhibition of key erythroid transcription factors GATA-1 and NF-E2 as reported by Liu, Hou, and Shen in 2003 and Morceau et al. in 1996. Down regulation of transcription factor GATA-1, has been proposed to bring about apoptosis of erythroblasts facilitated by downregulation of anti-apoptosis gene Bcl-X_L, since its expression is also regulated by GATA-1.

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Although several studies (Aithal, Haseena, Das, & Saheb, 2016; Marbat, Ali, & Hadi, 2013) have indicated a stimulatory effect of NS on plasma testosterone levels, we recorded a reduction in testosterone levels from the chronic administration of NS extracts. Stress induced production of cortisol could have inhibited testosterone production (Fenske, 1996) through reduction in pulse amplitude of GnRH and LH (Debus et al., 2002). However, our results revealed that administration of NS extracts had a non-stimulatory effect of LH and FSH levels in contrast to several studies (Al-Sa'aidi et al.,2009; Marbat et al., 2013). This could have resulted from the production of gonadotrophin inhibitory cytokines (Dondi, Limonta, Montagnani Marelli, & Piva, 1998; Watanobe & Hayakawa, 2003) spurred from peritonitis in our NS treated rats.

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Chapter VII: Conclusion.

NS seeds were observed to have no untoward or deleterious effect pre and post parturition in female Sprague-Dawley rats. However, delivered pups from dams in NS substituted groups were significantly larger than pups delivered by dams in the control group, pointing thereby to the galactagogue and possible stimulatory effect of NS seeds on prolactin. Also, aqueous extracts of NS seed had no significant effect on plasma liver enzyme and reproductive hormone (LH, FSH, testosterone) levels.

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