College of Health Sciences

School of Medicine

Department of Anatomy

Ph.D. dissertation

Evaluation of Acute and Subacute Toxicity, Teratogenicity, Reproductive Toxicity and Extended First Generation Developmental Toxicity of Ethanol Leaf Extract of *Urtica simensis* Hochst. Ex A. Rich in Wistar Albino Rats

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A dissertation submitted to the Department of Anatomy, College of Health Sciences, Addis Ababa University for partial fulfillment of the requirements for the degree of Doctor of Philosophy in Anatomy

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ABBREVIATIONS AND ACRONYMS

AGD	Anogenital Distance
ALP	Alkaline phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of variance
AST	Aspartate Aminotransferase
CRL	Crown rump length
EPHI	Ethiopian Public health institute
GD	Gestational day
IRB	Institutional review board
KOH	Potassium hydroxide
LD	Lethal dose
LOAEL	Lowest observable adverse effect level
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
NOAEL	No observable adverse effect level
OECD	Organization for Economic Cooperation and Development
PND	Postnatal day
RBC	Red blood cell
SDM	Standard deviation of mean
SPSS	Statistical package for social science
TM	Traditional medicine
WBC	White blood cell
WHO	World Health Organization

ABSTRACT

Introduction: *Urtica simensis* is a native nutraceutical herb in Ethiopia. It has been used to treat various ailments such as malaria, hypertension, diabetes, gonorrhea, gastritis, body swelling, and wound infections. However, the safety of its repeated oral intake has not been evaluated yet. Therefore, this *in-Vivo* experimental study was conducted to evaluate the acute and subacute toxicity, teratogenicity, reproductive toxicity, and extended first-generation developmental toxicity in Wistar albino rats. **Methods:** For the acute and subacute toxicity study, sixty rats (30 males and 30 females) were randomly assigned to six groups. The median lethal dose (LD_{50}) was determined in the acute toxicity study. Groups' I to III received 250, 500 and 1000 mg/kg of *U. simensis* leaf extract daily for 4 weeks, while Group IV received distilled water. The satellite 1000 mg/kg and satellite control groups were monitored for an additional 2 weeks. After 4 weeks, rats were euthanized for organ weight, blood chemistry and histopathological evaluations. For teratogenic studies of the leaf extract and essential oil, fifty pregnant rats were randomly assigned to five groups of ten gravid rats for each embryo and fetal experiments. Groups I to III were given the leaf extract and the essential oil at doses of 250, 500 and 1000 mg/kg daily from the 6^{th} to the 12^{th} days of gestation, respectively. Groups IV and V were pair fed and ad libitum controls. The developing embryos and fetuses were retrieved on the 12^{th} and 20^{th} days of gestation, respectively. Embryos were evaluated for growth and developmental delays. Fetuses were assessed for growth retardation, external and visceral anomalies. The skeletal ossifications and histopathology of fetal placentae were also evaluated. For the reproductive toxicity study, 20 rats per sex and group were randomly assigned into four groups. Groups' I to III were treated with 250, 500 and 1000 mg/kg leaf extract daily for 10 weeks, respectively: two premating, two mating, three pregnancy, and three lactational weeks. The effects of leaf extract on the daily food intake, body weight changes,

and histology of primary reproductive organs, liver and kidneys were assessed and evaluated. Biochemical tests including thyroid function were measured and analyzed. Reproductive indices and pregnancy outcomes were also assessed and evaluated. The pups were assessed for gross anomalies at birth. The weights of pups were measured on the postnatal days zero, four, seven, fourteen and twenty one. In addition, the pups' anogenital distances were measured on postnatal day zero. For the extended first generation developmental toxicity studies, the pups were randomly assigned into three cohorts at weaning: F1 cohort-1 for developmental reproductive toxicity, F1 cohort-2 for developmental neurotoxicity and F1 cohort-3 for developmental immunotoxicity. All pups assigned to the three cohorts were orally treated on a daily basis with similar doses of leaf extract as the parental rats. F1 cohort-1 rats, 20 rats per sex and group, were administered up to postnatal day 90. The tests conducted on the parental rats were repeated on F1 cohort-1 pups. F1 cohort-2 rats, 10 rats per sex and group, were treated until postnatal day 70, and the effects of the extract on the body weight and histopathology of brain and spinal cord were evaluated. F1 cohort-3 rats, 10 rats per sex and group, received the leaf extract daily until postnatal day 60, and the effects of the extract on body weight and histopathology of the spleen, thymus, and mandibular lymph nodes were evaluated. **Results:** The LD_{50} exceeded 5 g/kg animal body weight. No adverse effects were observed at 250 mg/kg. However, 1000 mg/kg subacute dose caused significant weight gain in both male and female rats. Doses of 500 and 1000 mg/kg significantly elevated ALT in both sexes of rats. Male rats given 1000 mg/kg showed significantly increased serum creatinine, while females exhibited reduced hemoglobin. Some female rats treated with 1000 mg/kg had liver parenchymal necrosis, kidney glomerular distortion and spleen white pulp depletion. In the embryonic experiment, somite numbers and morphological scores were significantly decreased in gravid rats given 1000 mg/kg of leaf extract. Embryonic developments

of the caudal neural tube (CNT), otic system, olfactory system and limb buds were significantly delayed in gravid rats given 1000 mg/kg of extract. The 500 mg/kg dose also caused significant developmental delays in the CNT and olfactory system. In the fetal experiment, fetal resorption was significantly increased; whereas crown rump length and fetal weight were significantly decreased in pregnant rats given 1000 mg/kg of leaf extract. Oral doses of 250 and 500 mg/kg of essential oil did not reveal adverse observed effects in all embryos and fetuses. However, somite numbers and morphological scores were significantly decreased in pregnant rats treated with 1000 mg/kg of essential oil. Embryonic developments of the caudal neural tube and forebrain were significantly delayed in pregnant dams administered 1000 mg/kg of essential oil. The crown rump length and fetal weight were significantly decreased in gravid rats given 1000 mg/kg of essential oil. The gravid rats received 1000 mg/kg of essential oil also revealed a significant increase in fetal resorption. In the reproductive toxicity study, female parental rats treated with 500 and 1000 mg/kg of leaf extract showed a significant pre-gestational weight gains. However, during gestation, the 1000 mg/kg treated female parental rats exhibited significantly lower weight gains. Lactational weight changes remained similar across all groups. Similarly, male parental rats treated with 1000 mg/kg of leaf extract revealed a significant weight gains. On the other hand, female parental rats treated with 1000 mg/kg of leaf extract had significantly lengthened estrous cycles. Both parental rats treated with *U. simensis* leaf extract did not have any adverse effects on mating or fertility indexes. However, the rats treated with 500 and 1000 mg/kg doses showed significant prolonged mating latency. Female parental rats administered the leaf extract at 500 and 1000 mg/kg doses revealed a significant decrease in litter size. Both parental male and female rats treated with 500 and 1000 mg/kg of leaf extract exhibited liver parenchymal necrosis. The biochemical analysis of rats treated with highest dose of leaf extract revealed a significant rise in liver enzyme markers. In

the first offspring (F1) cohort-1 study, male rats received the 1000 mg/kg dose showed significant weight gains. Female cohort-1 rats treated with 500 and 1000 mg/kg doses also revealed a significant weight gains. In addition, F1 cohort-1 rats treated with 1000 mg/kg leaf extract showed significant prolongation of the estrous cycle and vaginal cornification. Both male and female offspring exhibited statistically significant elevations in liver enzyme tests at the highest dose, supporting findings in the parental rats. Furthermore, F1 cohort-1 rats that received 500 and 1000 mg/kg of extract also exhibited liver parenchymal necrosis, similar to the observations made in their parental rats. The F1 cohort-2 rats treated with *U. simensis* leaf extract did not show any signs of behavioral aberrations. The histopathological assessment of F1 cohort-2 rats treated with leaf extract did not reveal any structural changes of tissue sections taken from cerebral cortex, cerebellum, spinal cord, sympathetic ganglia and peripheral nerve fibers. The F1 cohort-3 rats treated with *U. simensis* leaf extract did not have any signs and symptoms of toxicity during the daily cage side clinical observations. The histological examination of key immunological organs (spleen, thymus, and mandibular lymphoid) of F1 cohort-3 rats treated with the leaf extract revealed no histological abnormalities. **Conclusions:** In this study, the no observable adverse effect level (NOAEL) for *U. simensis* leaf extract was 250 mg/kg, while the lowest observable adverse effect level (LOAEL) was 500 mg/kg. The adverse effects observed at the LOAEL were elevated liver enzymes with parenchymal necrosis, embryo-fetal developmental delays, and prolonging of some reproductive functions particularly lengthening of estrous cycle and mating latency. These findings suggest that the repeated oral intakes of high-dose *U. simensis* derivatives for purported nutraceutical benefits are not without risks and should be approached with caution, particularly during pregnancy and reproductive ages.

Keywords: *Urtica simensis*, Teratogenicity, Developmental Toxicity, Reproductive Toxicity

1. INTRODUCTION

1.1 Background

Toxicology is a scientific discipline that overlaps with biology, chemistry, pharmacology and medicine [1]. It studies the adverse effects of biological agents or chemical substances on living organisms as well as identifying and treating exposures to toxins and toxicants [2]. Most toxicologists prefer to use the term toxic instead of poison. The dose, frequency, route and timing of exposure determine whether the substance is considered toxic or safe [3]. Toxicity refers to potential adverse impacts of chemicals on humans, animals or environment following single or repeated exposure [4].

The purpose of toxicity testing is to generate reliable data that support the implementation of appropriate safety measures and ensure the protection of public health against harmful effects of chemicals and drugs [5, 6]. The adverse effects of biological agents or synthetic chemicals are assessed through organ toxicity and toxicity endpoints [7]. Toxicological studies constitute an essential part of the process of transforming natural remedies into a pharmacological products [8].

The global surge in herbal medicinal product usage either as primary therapy or as complementary and alternative medicine, has raised concerns regarding their safety and efficacy profiles [9]. Obtaining reliable estimates of health risks associated with herbal products remains challenging due to the widespread misconception that "natural" equates to "safe." This often leads users to overlook potential adverse effects compounded by poor communication between patients and healthcare providers regarding herbal use, the prevalence of substandard products, and the circulation of counterfeit goods [10].

Pharmacovigilance is therefore essential for generating reliable information on the safety of herbal medicines, as practiced in Europe and the United States [11]. The safety of these medicines has been a growing concern for regulatory agencies, as several serious side effects such as liver injury, kidney dysfunction, and allergic responses have been reported [12, 13]. Acknowledging the increasing global importance of herbal therapies, the World Health Organization (WHO) has developed guidelines to facilitate the monitoring of herbal safety within existing pharmaceutical surveillance frameworks [14]. The classification of adverse reactions is widely established in conventional medicine and is also applicable to herbal therapies [15].

Traditionally the safety of herbs was primarily based on empirical knowledge and was effective in identifying acute toxic effects, with symptoms appearing within hours or days after consumption [11]. However, this traditional approach is inadequate for detecting herbs that cause cumulative, long-lasting or delayed toxicities. Aristolochic acid nephropathy serves as notable example of chronic toxicity as its effects are cumulative and renal symptoms may not manifest until up to two years after discontinuation of the herbal preparation [16].

Although traditional medicine (TM) has been used by communities for generations and is widely regarded as beneficial, its mechanisms of action remain incompletely understood within the framework of modern scientific investigation [17]. Concerns have been raised about the safety and efficacy of commonly used herbs due to potential side effects and interactions with conventional medications [18]. Studies have shown that numerous plants can be highly toxic when used or in traditional medicine [19, 20]. Moreover, the WHO has stated that the inappropriate use of herbal medicines or traditional practices can lead to harmful outcomes, emphasizing the need for further research to ensure safe and effective therapeutic systems [21].

The growing global interest in the use of herbal medicines underscores the importance of understanding their potential adverse effects [22, 23]. Some herbs employed in the pharmaceutical industries have been found to contain toxic substances [24]. For instance, patients who consumed *Hagenia abyssinica* have reportedly experienced vision impairment and behavioral changes [25]. Additionally, the use of certain herbal preparations for weight control has been associated with neuropathy and coma [24].

Teratogenic studies form an important component of developmental and reproductive toxicology (DART) focusing on identifying the teratogens [26, 27]. These studies are critical for pharmaceutical development, chemical safety assessment and environmental toxicology [26]. A teratogen is any substance that can alter capable of disrupting normal embryonic or fetal development, leading to congenital anomalies or deformities [28]. Beyond the exchange of oxygen and nutrients, the uteroplacental circulation can also transfer teratogenic substances to the developing embryo or fetus. Therefore, conducting teratogenic evaluations of herbal products is crucial prior to their widespread use [29].

Teratogenicity studies provide the strongest evidences of adverse embryonic or fetal outcomes that may be detectable at birth [30]. The primary objective of such studies is to determine the potential of a test substance to induce embryo-fetal abnormalities, fetal or embryonic death, reduced fetal weight, length, or other adverse effects on maternal health [31]. Several reports have demonstrated the potential teratogenic of certain herbs [32]. For instance, administration of *Ruta graveolens* extracts during the first week of the embryonic development has been shown to disrupt preimplantation processes [33], while an alcoholic extract of *Coleus barbatus* was found to delay embryonic development [34].

Reproductive toxicity studies investigate the adverse effects of test substances on sexual function and fertility in both males and females [35]. Such effects may manifest during sexual development, sexual behavior and execution, germ cell synthesis, fertilization, implantation, and pregnancy outcomes [36]. Additionally, reproductive toxicants can impair nesting and lactation behaviors [37, 38]. They may also affect reproductive function such as the ovarian cycle, ovulation, and the survival rate of offsprings during lactation [39-41].

Traditional medicines and therapeutic practices are gaining increasing popularity worldwide [42]. In developing countries, indigenous medicinal herbs are often preferred due to their accessibility, affordability, and cultural acceptance [43]. Herbal therapy occupies a prominent place in Ethiopian traditional medicine [44]. Ethiopia hosts a rich diversity of plant species that are widely utilized for medicinal purposes [45]. These plants are typically collected from their natural habitats by skilled traditional practitioners [46].

Urtica simensis Hochst. ex A. Rich belongs to the genus *Urtica* within the *Urticaceae* family [47]. It is an Ethiopian nettle species commonly known as 'samma' in Amharic [48], 'Amei'e' in Tigrigna [49], Sanamik in Halaba [50] and 'Dobii' or 'Gurgubbee' in Oromifaa [51]. The leaves of *U. simensis* have been traditionally used to manage various ailments including gastritis [52], stomach ulcer [53], intestinal parasites [54], sexually transmitted diseases [48], heart failure [55], bleeding wound [56], diabetes [57], hemorrhoids [58], nyctalopia [59] and gonorrhea [60]. In Ethiopia, pregnant women inhale fresh *U. simensis* steam vapor to fumigate their body [48, 61], prepared it as a tea by boiling the powdered or dried plant material [62], consumed it as juice after roasting and grinding [63], or eaten it as stew [61]. When fresh herb is boiled, the essential oil present in the plant material is released and inhaled [64].

1.2 Statement of the problem

More than 85% of the world's population relies on medicinal herbs for the prevention and treatment of diseases [65]. In Ethiopia , 80% of the population used TM to maintain primary health care [44, 66]. Herbal medicines are essential for maintaining the health and well-being of the population; however, the main challenges that hinder their effective use are lack of quality control and safety measures [67].

The use of plant medicine among pregnant mothers has substantially increased throughout the world, especially in the sub-Saharan Africa [68]. Utilization of herbal remedies lacking clear pharmacological activities may pose risks to pregnant women [69]. In Africa, the average use of herbal medicines among pregnant women ranges from 32% to 45% [70]. In Ethiopia, 47.77% of mothers having prenatal care used herbal medication during pregnancy [71], ranging from 10.9% [72] to 73.1% [73]. The adverse effects of traditional herbal medicines ranged from allergic reactions to organ toxicity [74, 75] and their nutraceutical use currently come into question due to toxic effects [18].

Reported studies have shown that numerous plants are exceedingly harmful when they are utilized as nourishment or herbal medicine [19]. Their toxicities spanned from cellular and biochemical components of blood to histological changes of organs [76]. Some herbs can lead to infertility, hormonal disruption, or testicular and ovarian toxicity [77, 78]. They can result in reduced sperm count and motility, damage to ovarian follicles, and hormonal imbalances. Other herbs contain compounds that could lead to teratogenicity, embryo lethality, and developmental delays [32, 79]. Blindness and neuronal dysfunction have been reported in traditional use of *Hagenia abyssinica* [25]. Oral ingestion of *Aristolochia fangchi* also caused kidney disease and urothelial cancer [80].

A recent study on herbal medicine use during pregnancy reported that topical application of almond oil was significantly associated with preterm birth, oral consumption of raspberry leaf with caesarean delivery, and excessive intake of licorice with early preterm birth [81]. The leaves of *U. simensis* have been reported to possess antidiabetic [82], antiulcer [83], wound-healing [84], antimicrobial [85], cardioprotective [86], and antioxidant [87] activities. Moreover, the essential oil isolated from the aerial parts of *U. simensis* exhibited antiproliferative activity against ovarian and leukemia cancer cell lines [88].

Despite the reported nutraceutical potential of *U. simensis*, the safety profile of this herb or its derivative has not yet been established. The effects of repeated oral administration of *U. simensis* leaf extract on vital organs, histopathology, hematological, and biochemical parameters have not been evaluated. Furthermore, the potential impact of repeated exposure to the plant's leaf extract on embryo-fetal development, reproductive performance, mating behavior, birth outcomes, and offspring survival remains unexplored. Therefore, the present study was conducted to assess the acute and subacute toxicity, teratogenicity, reproductive toxicity, and extended first generation developmental toxicity of ethanol leaf extract of *U. simensis* in rats.

1.3 Significances of the study

Animal-based toxicological studies are essential for assessing and classifying chemicals, pharmaceuticals, and other compounds according to their potential impact on human health [89]. Nutraceutical herbs must undergo toxicological evaluation to verify their safety prior to human consumption. Such investigations are essential for determining the potential toxicity of herbal extracts and identifying adverse effects, including hepatotoxicity, nephrotoxicity and allergic reactions. They also help establish safe dosage ranges and prevent overdose risks. Furthermore,

toxicological assessments are crucial for regulatory approval, consumer confidence, and dispelling misconceptions.

Accordingly, the findings of this study provide valuable insights into the effects of single and repeated doses of *U. simensis* leaf extract on hematological parameters and major visceral organs. In addition, the study examines the impact of the plant's leaf extract on the embryo-fetal development during the prenatal period. Furthermore, it provides insights into the influence of *U. simensis* leaves on sexual function, mating behavior, birth outcomes, and early and late life stages of first-generation offspring. Moreover, the study establishes the no-observed-adverse- effect level (NOAEL) of the plant material. Ultimately, the findings serve as valuable reference data for regulatory guidance and as baseline information for future studies, including potential drug development from this nutraceutical herb.

2. LITERATURE REVIEW

2.1 The genius *Urtica*

Urtica simensis belongs to the genus *Urtica* within the family *Urticaceae* [90]. The *Urticaceae* family, commonly known as stinging nettle, comprises 54 genera and over 2000 plant species [91]. The genus name *Urtica* is derived from Latin word *urere*, meaning “to burn”. Species of this genus are primarily distributed across tropical and subtropical regions of the world. Approximately 30 to 45 species of *Urtica* are found in the cosmopolitan temperate regions [50, 92]. In these areas, they typically grow as perennial plants across Asia, America, and Europe, favoring deforested and fertile soils [93].

The most well-known species include *U. dioica* L. (English : stinging nettle) and *U. urens* L. (small nettle), which are native to the Europe, Africa, Asia, and North America [94]. *Urtica* species are herbaceous perennials that can reach heights of up to two meters. They have serrated, oppositely arranged leaves along the stem, and both the leaves and stems are covered with fine hairs, some of which deliver a characteristic sting that define the genus [95]. However, the European species of *U. galeopsifolia* lacks the stinging hairs [96]. These plants spread via distinctive yellow underground roots and produce small, greenish-white flowers with four petals, clustered in dense, elongated inflorescences near the stem tips.

The common name 'nettle' derives from the Anglo-Saxon word *noedl*, meaning 'needle,' while the Latin genus name, *Urtica*, translates as 'to burn,' aptly describing the plant's notorious stinging ability. This stinging effect is caused by nearly invisible hollow hairs, known as trichomes that coat the leaves and stems. When brushed against the skin, these needle-like structures release irritants, causing a characteristic burning sensation and temporary rash. Upon contact with the skin, the fragile tips of the trichomes snap off, exposing sharp, hypodermic-like tubes [97]. These microscopic needles

penetrate the epidermis, injecting a potent cocktail of irritants, including formic acid (also found in the ant and bee venom), histamine, acetylcholine, and serotonin. This toxic mixture triggers an immediate inflammatory response, producing intense itching and burning pain that can persist for 12 hours or more. This sophisticated defense mechanism evolved primarily as protection against herbivorous insects. Upon contact with skin, the fragile tips of the trichomes snap off, exposing sharp, hypodermic-like tubes [97]. Most *Urtica* species grow in nitrogen-rich soil and are commonly found in areas with high concentrations of inorganic nitrates and heavy metals [98].

The current species, *U. simensis* Hochst. Ex. A rich., is endemic to Ethiopia and is locally known as 'samma' in Amharic [48], 'Amei'e' in Tigrigna [49], Sanamik in Halaba [50] and 'Dobii' or 'Gurgubbee' in Oromifaa [51]. This *Urtica* species has genetic diversity, with a Shannon information index of 0.6197 [99]. The stinging hairs on the stems and the undersides of the leaves of *U. simensis* are prominent [100]. It is widely distributed across the Ethiopian highlands including Gondar [99], Showa [101], Gojam [102], Wollo [103] and southern Ethiopia, at elevations ranging from 1500 to 3500 meters above the sea level [104]. *Urtica simensis* is a dioecious, erect, perennial herb that grows up to 1 m tall [105] (Fig.1).

Fig. 1: A photograph of *Urtica simensis* Hochst.ex.A.Rich taken around Debre Markos town, 2022

2.2 Nutritional importance of *Urtica simensis*

Cultural norms and values show a strong correlation with the use of *U. simensis*, indicating that the stigma surrounding the plant is associated with its perception as a food for impoverished individuals or as an emergency food during famine [106]. Nevertheless, *U. simensis* also plays an important role addressing food insecurity and enhancing societal well-being in Ethiopia. The traditional preparation of *U. simensis* as a meal involves the following steps [106]:

1. Collecting young shoots from the field while wearing thick clothing to protect against stings;
2. Using a traditional grass sieve to mash the leaves;
3. Boiling the leaves in clay pots with plenty of water;
4. Removing the leaves from the liquid after 5–10 minutes of boiling and discarding the water;

5. Recooking the partially boiled leaves for about 15 minutes after mixing them with prepared barley flour (hulled, lightly roasted, and ground barley grains);
6. Refrigerating the mixture overnight; and
7. Finally, serving it the next day with *injera* (a traditional fermented flatbread made from teff and/or sorghum or maize).

The crude fat, crude fiber and total ash contents of fresh *U. simensis* leaves decrease after boiling, while their moisture and crude protein contents increase [107]. Similarly, the levels of minerals such as iron, zinc and calcium are reduced following boiling. Although fresh *U. simensis* leaves are rich in vitamin C, this nutrient is also diminished by heat treatment. The concentrations of tannins and oxalates in raw leaves decline after boiling as well [108].

Bread is a widely consumed food, and incorporating *U. simensis* leaf flour into bread formulations helps combat malnutrition by improving protein and mineral contents [109, 110]. The addition of the *U. simensis* leaf flour to wheat bread at 5%, 10%, and 15% significantly enhances the protein, crude fiber and ash, fat, mineral, vitamin C and beta-carotene levels. Increasing the proportion of *U. simensis* leaf flour further elevates these nutritional parameters in the bread. Substituting up to 15% *U. simensis* leaf flour in bread formulation yields products with higher minerals with higher mineral, vitamin C, and β-carotene contents [109]. However, increasing the proportion of *U. simensis* leaf flour leads to a decline in sensory attributes such as color, taste, and texture. A 5% substitution level produces bread with desirable sensory qualities [109].

Nutritious noodles can be prepared by blending wheat flour with dried *U. simensis* leaf flour [111]. Traditional noodles made from wheat semolina or regular wheat flour are rich in carbohydrates, but much of the fiber, vitamins, and minerals are lost during flour refinement [112, 113]. Noodles

enriched with up to 15% *U. simensis* leaf flour (by weight) show significantly higher protein, ash, fiber, calcium, iron and zinc contents compared to conventional wheat noodles.

Conversely, incorporating *U. simensis* leaf flour reduces the fat, carbohydrate, and total energy content of the noodles [114]. Sensory accessibility was rated above average for all sensory attributes [114]. The moisture content of noodles plays a key role in determining microbial growth and shelf-life [115], and it was found to depend on the proportion of *U. simensis* flour incorporated. This is because *U. simensis* leaf flour has a higher moisture content than wheat flour [114].

The nutritional composition and sensory acceptability of flat bread enriched with *U. simensis* leaves showed that the addition of nettle flour improved the proximate composition (crude protein, crude fat, crude fiber, ash and carbohydrate) as well as the mineral content (calcium, zinc, and iron) of the flat bread [116]. However, sensory acceptability declined with increasing levels of the *U. simensis* leaf flour.

2.3 Medicinal values of *Urtica simensis*

In Ethiopia, the different parts of *U. simensis* have long been used as traditional medicine to treat a range of ailments. The plants' roots, fresh leaves, and young twigs are employed in managing conditions such as gonorrhea, gastritis, body swelling, rhinitis and heart failure. It also plays an important role in controlling bacterial and fungal infections [50, 117, 118]. Traditionally, *U. simensis* leaves have been used in the treatment of diabetes mellitus [82].

For gastritis, *U. simensis* leaves are prepared in two different ways [117]. In one method, the leaves are roasted and crushed, and the extracted juice is taken orally. In another, the leaves are boiled in water and consumed with *injera*, a traditional flatbread made from *Eragrostis teff* flour. To treat gonorrhea, powdered *U. simensis* leaves and roots were mixed with water, and the filtrate is

administered orally [119]. For the treatment of body swelling, the leaves of the *U. simensis* are either heated and applied directly to the affected area, or crushed and mixed with butter to form an ointment, which is then applied topically to the affected site [119].

The leaves of *U. simensis* are traditionally roasted and ground into small pieces, and the resulting juice is taken orally; alternatively, the fresh leaves may be cooked and eaten with *injera* [120]. The plant is also used to treat acute abdominal pain (by taking the juice orally) and heart failure (by inhaling vapor from the fresh leaves through the nose and fumigating the body) [117].

In addition, the root of the *U. simensis* is traditionally used for the treatment of plasmodial malaria infection. The root is crushed, shade-dried, mixed with tap water, and one glass of the concentrate is taken orally, followed by the consumption of milk [48, 121].

Urtica simensis has been attributed with multiple medicinal properties including diuretic, anti-hypertensive, hemostatic, anti-asthenic, anti-anemic, antispasmodic, and anti-rheumatic effects, as well as being used as a remedy for headaches and chills [122, 123]. It is also traditionally employed in the management of spleen, renal, and dermal disorders [123, 124].

A study reported in 2009 demonstrated the antidiabetic activity of the hydroalcoholic and aqueous extracts, along with various solvent fractions of *U. simensis*, in streptozotocin-induced diabetic mice [82]. Oral administration of 300 mg/kg of the methanol and aqueous fractions reduced blood glucose levels by 17.9% and 29.9%, respectively. The aqueous fraction of *U. simensis* exhibited both antidiabetic antihypertensive effects in a dose-dependent manner.

In a study using acetic acid-induced ulcer models in rats [83], the hydromethanolic crude leaf extract of *U. simensis* significantly reduced ulcer severity compared to the negative control. The extract also exhibited dose dependent anti-secretory effects in the pylorus ligation-induced ulcer

model, with the highest gastroprotective activity (67.68%) was observed at a dose of 400 mg/kg/day of the 80% methanolic crude extract. Furthermore, administration of 100, 200 and 400 mg/kg/day doses resulted in ulcer healing rates of 33.54%, 58.33%, and 67.07%, respectively, demonstrating a marked improvement over the control groups.

Another *in-vivo* study investigating the effects of crude and solvent fractions of *U. simensis* leaves on cyclophosphamide-induced myocardial injury in rats revealed that the extract prevented the deleterious effects of cyclophosphamide on body weight, heart weight to body weight ratio, cardiac biomarkers (including troponin I, alanine aminotransaminase [ALT], aspartate aminotransferase [AST], as well as lipid profiles [triglycerides and total cholesterol]) [125]. In this study, *U. simensis* leaf extract possessed free radical scavenging activity, with IC_{50} values of 63.27 μg/mL for the crude extract, 136.38 μg/mL for the aqueous fraction, and 258.70 μg/mL for the hexane fraction.

Another study reported that leaves of *U. simensis* exhibited substantial antioxidant activity ranging between 2.28 and 2.42 mg ascorbic acid equivalent/g of dried leaves [87]. The essential oi extracted from the aerial parts of *U. simensis* exhibited anti-proliferative activity against ovarian and leukemia cancer cell lines [126].

2.4 Phytochemicals of *Urtica simensis*

A qualitative phytochemical screening of hydromethanolic crude extract of *U. simensis* revealed the presence of anthraquinones, terpenoids, saponins, tannins, flavonoids, alkaloids, and phenolic compounds [83]. In spite of their medicinal importance, the tannins are anti-nutrients that precipitate proteins, decrease mineral ion and vitamin utilization, and prevent the activities of digestive enzymes [127].

The essential oil extracted from the aerial parts of *U. simensis* contained forty-seven identified compounds [126]. Its major constituents included aromatic hydrocarbons (such as *p*-xylene, *o*-cymene, and *p*-cymene), monoterpenes, and organosulfur compounds, including 3,5-dimethyl-1,2,4-trithiolane, 2,4,6-trimethyl-1,3,5-trithiane, and 3,6-dimethyl-1,2,4,5-tetrathiane.

A study conducted by Bayba et al. [128] on the chemical compositions of the *U. simensis* leaves showed the presence of ash (17.2-24.3%), crude fat (3.19-3.50%), crude protein (3.42-6.38%), crude fiber (9.37-14.0%), and carbohydrate (56.7-63.7%) contents. The total polyphenols, flavonoids, and the antioxidant activities were also determined and their values ranged from 2.18 to 4.84 mg gallic acid, 1.35 to 4.46 mg catechin, and 1.58 to 3.36 mg ascorbic acid, respectively, equivalents per gram of dry sample. The study also identified a total of 16 fatty acids with linoleic acid, palmitic acid, and linolenic acid are the major fatty acids, with average compositions of 36.6%, 20.7%, and 15.5% of total fatty acids, respectively. The lipid fractions of the *U. simensis* leaves have been found to be rich in essential fatty acids (α-linolenic acid and linoleic acid) which are vital for the human nutrition.

Another study conducted on the leaves of *U. simensis* in Ethiopia reported total phenolic contents ranging from 15.75 to 22.67 mg gallic acid equivalent (GAE) per gram of dried leaves. The total tannin content ranged from 0.496 and 1.54 mg GAE per gram, while the total flavonoid expressed as catechin equivalent (CE), varied between 6.89 and 9.03 mg per gram of dried leaves [87]. A study conducted by Keflie T [129] demonstrated that the *U. simensis* leaves are a rich source of tocols, highlighting their potential as a natural source of vitamin E. The total tocol content varied with the drying method: sun-dried leaves contained 14.1 ± 1.1 mg/100 g, shade-dried leaves 13.8 ± 1.1 mg/100 g, and lyophilized (freeze dried leaves 16.9 ± 1.2 mg/100 g, with freeze-drying producing the highest concentrations.

2.5 Safety profiles of *Urtica simensis* leaves

An acute oral toxicity study on female albino rats revealed that no signs of toxicity in behavioral, autonomic, neurologic, or physical parameters [83]. The study reported that the median lethal dose (LD_{50}) of the *U. simensis* leaf extract was greater than 2000 mg/kg animal body weight. Similarly, another acute toxicity study conducted by Tesfaye et al [125] showed that the 70% ethanolic extract and solvent fractions of the *U. simensis* leaves administered at a dose of 2000 mg/kg body weight, did not produce any behavioral changes, including alterations in hair texture, pupil size, or feeding behavior. No animal deaths were observed at this limit dose, and the LD_{50} was likewise determined to be greater than 2000 mg/kg body weight. Furthermore, histological examinations, revealed normal cardiocyte structures with no pathological abnormalities in any of the treated groups that received the crude leaf extract or aqueous fraction [125].

3. OBJECTIVE

3.1 General objective

- To evaluate the acute and subacute toxicity, teratogenicity, reproductive toxicity and extended first generation (F1) developmental toxicity of the ethanol leaf extract of *Urtica simensis* in Wistar albino rats

3.2 Specific objectives

- To evaluate acute and subacute toxicity of leaf extract of *U. simensis* in Wistar albino rats
- To assess the teratogenic potential of the leaf extract of *U. simensis* in Wistar albino rats
- To assess the teratogenic potential of the essential oil of *U. simensis* in Wistar albino rats.
- To evaluate the reproductive toxicity of the leaf extract of *U. simensis* in Wistar albino rats
- To evaluate developmental reproductive toxicity of leaf extract of *U. simensis* in F1 rats
- To investigate the developmental neurotoxicity of leaf extract of *U. simensis* in F1 rats
- To evaluate the developmental immunotoxicity of the leaf extract of *U. simensis* in F1 rats

4. MATERIALS AND METHODS

4.1 Study setting

This study was conducted at the laboratories of the Traditional and Modern Medicine Research Center, the Ethiopian Public Health Institute, and the Department of Pathology at the College of Health Sciences, Addis Ababa University. This *in-Vivo* experimental study consisted of five components: (1) the acute oral toxicity testing of *U. simensis* ethanol leaf extract, (2) subacute toxicity study of the *U. simensis* leaf extract, (3) teratogenicity evaluation of the ethanol leaf extract of *U. simensis*, (4) teratogenicity study of the *U. simensis* essential oil, and (5) the reproductive toxicity with extended one generation developmental toxicity study of the ethanol leaf extract in Wistar albino rats.

4.2 Study period and design

This study was conducted from September 2022 to December 2024, following OECD guidelines 425, 407, 414, and 443 for its design.

4.3 Plant collection and authentication

Fresh *U. simensis* were collected around Debremarkos town, located at GPS coordinates 10°20′ North, 37°43′ East, approximately 310 km Northwest of Addis Ababa, the capital city of Ethiopia. To ensure proper hygiene, sanitized protective gloves were used to detach leaves from the stem starting at the bottoms toward the tips based on their stalk positions. The identification and authentication of the plant material were confirmed at the National Herbarium of Addis Ababa University (AAU) where voucher specimen (collection number: bw-001) was deposited for future reference.

4.4 Crude extraction

The collected fresh *U. simensis* leaves were washed with distilled water, air-dried and manually crushed into pieces before being coarsely pulverized with an electric grinder. The coarsely milled leaves were weighed using an electronic balance (Mettlertoledo, Switzerland). These roughly ground leaves were mixed with 70% ethanol in powder to solvent ratio of 1 to 10 (w/v) and constantly oscillated for 24 hours using an orbital shaker (Bibby scientific limited stone, UK). The Whatman No. 1 filter paper (Maid stone, UK) was applied to filter the agitated mixture. After filtration, the residue underwent double maceration process over two days with fresh ethanol. The obtained filtrate was placed in the Rota vapor (Büchi R-200, Switzerland) at controlled temperature not exceeding 40 °C. The residual aqueous solution was desiccated in 40 °C water bath for fortnight. The desiccated extract was stored in the refrigerator until it was used [130].

4.5 Proximate analysis

The moisture, ash, crude protein, crude fat, crude fiber and carbohydrate contents of *U. simensis* leaves were analyzed using the Association of Official Analytical Chemists (AOAC) standard procedures [131]. The air dried leaves of *U. simensis* (10 g) were placed in 100 °C oven to determine the moisture content. The weight of the dish before drying (W1) and after drying (W2) was weighed and their difference (W2 minus W1) was determined. The moisture content was calculated by subtracting the weight difference of dish from weight of the sample (10 g), divided by sample weight and multiplied by 100. The same amount of *U. simensis* leaf was heated in muffle furnace at 600 °C to determine total ash content. Then, the ash content was determined by subtracting weight of the crucible from weight of the crucible with ash, divided by 10 g and multiplied by 100. The crude fat content of 10 g of air-dried leaves of *U. simensis* was also determined using a Soxhlet apparatus with hexane extraction. It was calculated by subtracting

weight of the flask from weight of flask with fat, dividing by 10 g and multiplying by 100. The total crude fiber content of 10 g sample was also determined by successively boiling in 0.313 M H_2SO_4 acid and 0.313 M KOH base solutions, rinsed in boiled water and acetone, dried, weighed and incinerated in the furnace at 550 °C. The crude fiber content was determined by subtracting weight of the crucible with ash from weight of crucible with fiber, dividing by 10 g and multiplying by 100. The total crude protein of the sample was estimated using the Kjeldahl digestion and distillation method with conversion factor of 6.25 based on the total nitrogen content and multiplied by dilution factor [132]. Finally, the subtraction method was used to determine total carbohydrate content. It was computed by subtracting total dry weight of the sample from crude protein, crude fiber, total ash and crude fat [133].

4.6 Phytochemical screening

The qualitative phytochemical screening tests for the 70% ethanol extract of *U. simensis* leaves were carried out to detect the presence of phenols, flavonoids, tannins, terpenoids, saponins, steroids, alkaloids and anthraquinones according to the methods described by Khan et al. [134].

4.7 Experimental animals

Healthy male and female (nulliparous, non-pregnant) Wistar albino rats, aged ten to twelve weeks, were procured from the animal breeding unit of the Ethiopian Public Health Institute. The rats were adapted to the laboratory environment for one week before the experiment. The conventional pellet diet and clean water were given to the animals, and they were kept at room temperature of 22 ± 3 °C, relative humidity of 50 to 60% and 12 hours light-dark cycle until end of the experiment.

4.8 Acute oral test

The acute oral test was carried out in line with the OECD guideline test number 425 [135]. Five female rats were used as test group while other five female rats were taken as control group. The

rats were fasted overnight from food but not water prior to administration of the *U. simensis* leaf extract. The doses were estimated using the fasting body weight of rats. The test group rats were given a single oral dose of 5000 mg/kg of *U. simensis* leaf extract whereas control group rats received only the vehicle, distilled water. Cage-side close monitoring was carried out for the first four hours, every hour for the next twenty four hours and every day for the next 14 days.

4.9 Subacute toxicity study

Sixty albino rats (5 males and 5 females per group) were used based on the OECD guideline test number 407 [136]. The rats were randomly divided into six groups of ten animals. Groups I to III were administered *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg animal body weight, respectively while group IV received only distilled water. The calculated doses were administered through oral gavage every morning for 28 days. The remaining two satellite groups (SG), satellite control which was given distilled water, and satellite 1000 mg/kg were administered the plant leaf extract for four weeks and remained untreated for two more weeks for additional observations. The experimental doses were adjusted based on the acute toxicity study, taking the highest dose as one-fifth of the median lethal dose, which was 1000 mg/kg, and descending dose levels with two folds (500 mg/kg as middle dose and 250 mg/kg as low dose) to evaluate the cumulative effects. The animals were inspected and examined for any behavioral and physical signs of toxicity. Furthermore, the daily diet intake and body weight changes were measured on day one of treatment, weekly thereafter and at the end of treatment period.

4.9.1 Hematology and biochemical analysis

As described by Jegnie et al [137] and Abebe et al [138], five to six ml of fasting blood was taken through cardiac puncture while the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (150 mg/kg). The blood was taken through two sets of test tubes. The first

set of test tubes with anticoagulant (ethylene diamine tetra acetic acid) were used for hematological analysis, and the second set of test tubes with no anticoagulant were used for biochemical analysis. The hematological test tubes were processed for white blood cell (WBC) count, differentials, platelet (PLT) and red blood cell (RBC) counts. The mean corpuscular volume (MCV), hemoglobin (HG), hematocrit (HCT), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) values were also analyzed. Serum was obtained from the biochemical test tubes using micropipette after electrical centrifuge for 10 minutes. The automated clinical chemistry analyzer (Huma Star80, Germany) recorded each biochemical tests related to the liver and renal functions such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total cholesterol, urea, creatinine, low density lipoprotein (LDL), high density lipoprotein (HDL), sodium ion (Na^+), potassium ion (K^+) and chlorine ion (Cl^-).

4.9.2 Organ weights

After phlebotomy of blood, the rats were given sodium pentobarbital (150 mg/kg) intraperitoneal injection for laparotomy [139]. The key visceral organs such as brain, heart, lungs, stomach, pancreas, kidneys, adrenal glands, spleen and liver were surgically removed. Furthermore, male testicles and female ovaries were promptly removed. These major visceral organs were weighted and examined for any gross morphological changes.

4.9.3 Histopathology

Tissue samples were taken for histopathological evaluations in the possibly toxic substance target organs, specifically the liver, kidneys and spleen [140, 141]. The samples were fixed overnight with 10% formalin. The Bancroft's practice and theory of histology procedures were used for tissue processing and staining [142]. In a nutshell, an increasing series of alcohol concentrations (40, 50,

70, 80, 90, and 100) were applied to dehydrate tissues. Then, these dehydrated tissues were cleared with xylene and impregnated with paraffin wax. The five µm slice was produced using a Leica microtome (Leica RM 2125 RT, Germany) to stain the tissues. Slides with ribbons were heated in the oven (40 to 45 °C) for 30 minutes to remove extra wax and tissue clinging. The hematoxylin and eosin were used to stain the sliced tissues [142, 143]. A senior pathologist examined the slides through binocular light microscope for any treatment related changes. The histological differences between experimental and control groups were evaluated. Photomicrograph was taken with an automated camera (Zeiss Primo Star, Germany) at 100× total magnifications. The severity of hepatic injury was assessed based on (1) sinusoid dilatation, (2) inflammatory infiltration, (3) sinusoidal congestion, and (4) hydropic degeneration (e.g. cytoplasmic vacuolization or hepatocyte swelling) [144, 145]. Similarly, the renal injury was assessed based on (1) tubular injury, (2) glomerular injury, (3) Tubulointerstitial fibrosis, and (4) vascular injury [146, 147]. The splenic injury was also assessed through five criteria: (1) red pulp congestion, (2) hemorrhage, (3) inflammatory infiltration, (4) white pulp atrophy or depletion, and (5) necrosis or apoptosis [147, 148]. Each attribute of the histopathological lesions was adapted and modified from published works and scored as 0 (normal), 1 (mild), 2 (moderate), and 3 (severe).

4.10 Teratogenic study of ethanol leaf extract of *U. simensis*

4.10.1 Experimental animals

Wistar albino rats, consisting of 25 males and 50 nulliparous females, aged ten to twelve weeks, were chosen for each experiment. After one week of acclimatization, rats were mated overnight by pairing one male rat with two female rats. The next morning, female rats were examined for copulatory plugs, and vaginal smears were taken for microscopic examination of spermatozoa.

The first-day observation of spermatozoa in the vaginal swab was taken as day one of pregnancy [149].

4.10.2 Experimental designs

The experimental doses were determined with two to five fold intervals based on the LD_{50} dose. The highest dose was set as 1000 mg/kg which was one fifth of the LD_{50} dose. The middle dose was 500 mg/kg, half of highest dose and lowest dose was 250 mg/kg, one fourth of highest dose. Then after, five groups of twenty gravid rats were randomly assigned using OECD guidelines for the prenatal developmental toxicity studies [150, 151]. Groups I to III were treatment groups whereas group IV and group V were pair fed and ad libitum control groups, respectively. The treatment groups were administered *U. simensis* ethanol leaf extract daily doses of 250, 500, and 1000 mg/kg animal body weight. The pair fed group received only distilled water at 1 ml/100 g animal body weight. The ad libitum control was given food and water freely. The extract was administered through gavage from 6^{th} to 12^{th} days of gestation as it was a critical period of embryogenesis and organogenesis in the rats [152]. The daily dietary intake of each group was measured. The weight of each rat was also taken at first confirmation of pregnancy, day 6, day 12 and at day 20 of gestations [153].

4.10.3 Embryonic experiment

On the 12^{th} days of gestation at noon, gravid rats received an intraperitoneal injection of sodium pentobarbital at a dosage of 150 mg/kg to induce stupor [139]. Afterward, the rats were positioned supine on the operating table. Then, an abdominal vertical incision was done to reveal abdominal cavities. The pins were used to fix skin flaps and abdominal muscles on both sides. The uterine horns were retrieved and placed in Hank's solution. An incision was made through anti-mesometrial boundary of the uterine horns to reveal the developing embryos [154, 155]. The

embryonic membranes were meticulously separated to expose the visceral yolk sac and the circulation was observed through dissecting microscope. The embryonic development of each system was assessed using the Brown and Fabro criteria [156] later adopted for *in-Vivo* studies by Seyoum and Persaud [157]. The crown rump length and somites of each embryo were also measured and counted.

4.10.4 Fetal experiment

On the 20th day of gestation at noon, pregnant rats were given pentobarbital 150 mg/kg through intraperitoneal injection to induce sedation [139]. The techniques used to recover the fetuses were similar to the embryonic experiment. The uterine horns were fully exposed and examined. The yellowish nodules of metrial glands along mesometrial border of uterine horns were assessed to count the implantation sites. The resorptions were identified by metrial glands that were not held by alive or deceased fetuses. Gently pressing on the fetus revealed whether it was alive or dead.

4.10.4.1 Fetal external examination

The fetuses were retrieved and their placentae were detached. The crown rump length was measured from top of the head to the buttocks. The fetal and placental weights were recorded. Each fetus was carefully examined for the craniofacial development anomalies (exencephaly, anencephaly, microphthalmia and anophthalmia), limb development abnormalities (syndactyly, adactyly and polydactyly), vertebral column malformations (neural tube defect, kyphosis and scoliosis), tail development disorders (lost tail) and external genitalia abnormalities. After external examination, about two to three fetuses from each dam were immersed in 95% ethanol for one week. These alcohol preserved fetuses were eviscerated through laparotomy and subjected to skeletal staining using the revised techniques of Rigueur and Lyons [158]. The remaining fetuses were fixed in Bouin's solution for visceral examination [159].

4.10.4.2 Fetal visceral examination

The Bouin's solution fixed fetuses were serially sectioned for visceral examination through the dissecting microscope and the revised Wilson technique (24). The coronal section of the head and transverse section of the rest of the body were performed to examine for readily apparent visceral abnormalities in the head, neck, thorax and abdominopelvic regions.

4.10.4.3 Fetal skeletal examination

A skeletal scoring chart developed by Nash and Persaud [160] was used to evaluate the skeletal prenatal developmental process. The alizarin stained skeletons were examined through the dissecting microscope. Evaluation and quantification of ossification centers were conducted. The degree of ossification of the sternebrae, metacarpal, metatarsal and sacrococcygeal bones were taken as a key markers of skeletal development [161]. Furthermore, ossifications of the skull, hyoid, sternum, ribs, vertebrae and limb bones were also scrutinized.

4.10.4.4 Examination of fetal placenta

Each fetal placenta was assessed for any gross morphological abnormalities. Placentae of two to three fetuses per dam and group were taken for histopathological evaluations. About 4 mm size sections were sampled from each placenta; and 10% formalin was used for fixation. After fixation, the tissues were dehydrated by an ascending series of alcohol (40%, 50%, 70%, 80%, 90%, and 100%). Then it was cleared by xylene (I, II, and III, 1:30 hour in each phase) and impregnated with two phase paraffin wax, 1:30 hour in each. Each sample tissue was placed in an embedding cassette, and filled with melted paraffin wax. A five μm section was made for every block and the ribbon was placed on frosted slide. Then it was kept in a hot oven (40-45°C) for 20-30 minutes. For staining, the tissue was dewaxed with three-phase xylenes for five minutes. Descending series of alcohol (absolute alcohol I, absolute alcohol II, 90% alcohol, 80% alcohol, 70% alcohol) were

used for rehydration. Running tap water was used for washing for two minutes; and stained with Harris hematoxylin for 5-10 minutes, and immersed in acid alcohol for 2-3 seconds. The tissue again was counterstained by eosin Y for 1-2 minutes. The stained slides were dehydrated by ascending series of alcohol (80%, 95%, and absolute alcohol I and II) for two minutes in each and cleared with xylene I, II, and III, two minutes in each. The cleared slides were mounted with DPX; and covered with a cover-slip [162]. Finally, the structural integrity of the placentae were investigated using a binocular light microscope. The decidual zone, labyrinthine zone, giant cells, and trophoblasts of the placenta were evaluated; and important findings were photographed with an automated inbuilt digital microscope camera (Leica EC4, Germany).

4.11 Teratogenic study of *U. simensis* essential oil

4.11.1 Essential oil extraction

Fresh *U. simensis* aerial part (2 kg) were chopped into pieces and hydro-distilled for 3 hours using clevenger apparatus. The resultant condensate was isolated with 100 ml of hexane three times using a separatory funnel. To remove moisture, 15 g of anhydrous sodium sulfate was added to the hexane extract. Then, hexane was removed under vacuum at a temperature range of 30 to 35°C, resulting in a pale yellow essential oil that had an unpleasant odor. The extracted oil was kept in tightly sealed jar at -20 °C until it was used [130].

4.11.2 GC-MS analysis

The GC-MS analysis was conducted using an Agilent Technologies 7890B gas chromatography (GC) and an Agilent Technologies 5977A Network mass spectrometry (MS). The GC used an Agilent Technologies HP-5MS non-polar column, measuring 30 m in length, 250 μm in internal diameter, and 0.25 μm in film thickness. A 1 μL sample was injected using a Gerstel cooled injection system, starting at −20 °C. The injector temperature was ramped to 320 °C during a 1:20

split injection. The GC separation procedure was carried out using hydrogen as carrier gas at a flow rate of 1.15 mL/min. Prior to injection, samples were diluted 1:1000 in methanol. The oven temperature program began at 40 °C (held for 3 minutes), then increased to 150 °C at 6 °C/min, followed by a ramp to 320 °C at 10 °C/min, and finally held at 320 °C for 3 minutes. Mass spectra were recorded using electron impact ionization at 70 eV in the range of 50 up to 550 amu.

4.11.3 Experimental animals

Healthy male and female (nulliparous, non-pregnant) Wistar albino rats, weighing 220-240 g and aged 10-12 weeks, were recruited from the animal breeding unit of Ethiopian Public Health Institute (EPHI). The animals had not previously undergone any experimental procedures. Rats of the same sex were housed in standard stainless steel cages, maintained under a 12-hour light and dark cycle, at room temperature of 23 ± 3°C and relative humidity of 50 ± 10%. The rats were provided a conventional laboratory diet and had free access to drinking water.

4.11.4 Acute oral test

Acute oral test was conducted on five female rats in accordance with OECD guideline 425 [135]. Initially, a single female rat was given 5000 mg/kg of essential oil through oral gavage. The remaining four female rats were administered 5000 mg/kg of essential oil 48 hours later since there were no fatalities in the first rat. As a control group, another five female rats were given the vehicle (distilled water with 2% Tween 80). After oral administration, clinical observation was conducted every 30 minutes for the first 4 hours and then daily for the next 14 days. Toxicity signs such as mortality, coma, convulsion, feces consistency, hair, itching, respiration, salivation and other behavioral patterns were assessed.

4.11.5 Mating and administration

After one week of acclimatization, rats were mated overnight by placing male rats in a cage with virgin female rats in 1:1 ratios. Female rats were checked next morning for copulatory plug and vaginal smears were taken for microscopic examination of spermatozoa. The first day detection of spermatozoa in the vaginal swab was taken as day zero of pregnancy [149]. Thereafter, pregnant rats were randomly assigned to five groups, with each group consisting of twenty gravid rats, in accordance with OECD guidelines [150, 151]. Groups I to III were administered 250, 500 and 1000 mg/kg of essential oil, respectively, based on the acute oral test. Groups IV and V were pair-fed and ad libitum control, respectively. The administration was given through oral gavage from 6^{th} to 12^{th} days of gestation since it was a critical period of organogenesis in rats [163]. The pair-fed control rats received only the vehicle (distilled water with 2% Tween 80) at 1 ml/100 g body weight. The ad libitum control rats were fed freely and kept untouched during the experiment.

4.11.6 Clinical observations

The clinical observation of pregnant rats was made daily at the same time for all signs of overt toxicity including mortality, morbidity and pertinent behavioral changes.

4.11.7 Food intake and weight changes

The daily food intake of pregnant dams were measured every morning until end of the experiment. Each pregnant dam's weight was taken at 1^{st} confirmation of pregnancy, 6^{th}, 12^{th} and 20^{th} days of gestation [153].

4.11.8 Embryonic experiment

On the 12^{th} day of gestation, at noon, fifty pregnant rats (ten per group) were anesthetized using an intraperitoneal injection of sodium pentobarbital (150 mg/kg) [139]. Subsequently, they were placed supine on an operating table. The extremities were stretched out and set in place, and a

vertical abdominal skin incision was made to reveal the abdominal cavity. Pins were applied to hold skin flaps and abdominal muscles on both sides. The uterine horns were removed and placed in Hank's solution. In order to expose the developing embryos, uterine horns were incised along the anti-mesometrial boundary [154, 155]. The membranes of the embryos were dissected with fine forceps to show the underlying visceral yolk sac. The development and circulation of yolk were looked through dissecting microscope. The progression of each developmental system was assessed using the Brown and Fabro criteria, which contained 16 noticed embryonic developmental endpoints [156, 157]. There were up to six developmental stages, and a score ranging from 0 to 5 was assigned for each developmental endpoint. The CRL of each embryo was measured, and the morphological score was also computed.

4.11.9 Fetal experiment

Similar to the evaluation of embryos, fifty near-term pregnant rats (ten per group) on the 20^{th} day of gestation were dissected through vertical abdominal incision [139]. The uterine horns were fully exposed and examined. The metrial glands, which are yellowish nodules along the mesometrial border of uterine horns, were counted to assess implantation sites. The number of prior resorptions was reflected by metrial nodules that were not occupied by living or recently deceased fetuses. Gently pressing on the fetus revealed whether it was alive or dead. The uterine horns have been cut along the anti-mesometrial border to reveal fetuses, fetal membranes, and placentas. The fetuses were explanted, and the placenta was removed. The CRL of each fetus was measured from the top of the head to the buttocks. Each fetus's anogenital distance (AGD) was measured from the cranial edge of the anus to the caudal border of the genital tubercle. Each fetus's placental weight was also measured.

4.11.9.1 Fetal external and visceral examinations

Each fetus underwent examination for craniofacial developmental anomalies, limb developmental abnormalities, vertebral column malformations, tail development disorders and abnormalities of the external genitalia. After external examination, some fetuses from each pregnant rat were preserved for skeletal staining. The remaining fetuses were fixed in Bouin's solution for visceral examination [159]. These Bouin's solution-fixed fetuses were serially sectioned using a dissecting microscope and a modified Wilson technique [164]. The craniocaudal section was done through the jaw and posteriorly above the ear and the presence of palatine cleft was assessed. Coronal section of the head and transverse section of the rest of the body were also done to assess any readily apparent abnormalities in the brain, neck, thorax and abdominopelvic regions.

4.11.9.2 Fetal skeletal staining

Two to three fetuses from each gravid rat were placed in 95% ethanol for one week. These alcohol-preserved fetuses were eviscerated through midline incision and underwent skeletal staining using revised techniques of Rigueur and Lyons [158]. Following alcohol preservation, the fetuses underwent a clearing process using 1% potassium hydroxide (KOH) solution until the bones became transparent, which typically took two days. Thereafter, the fetuses were transferred to fresh 1% KOH solution and stained with a few drops (0.4 ml) of alizarin red. The staining process was allowed to proceed overnight, and any excess staining was addressed by placing the specimens in a Mall's solution (79% distilled water, 20% glycerin, and 1% KOH). The specimens were placed in a gradual transition through increasing concentrations of glycerin (20%, 40%, 60%, and 80%) over the course of approximately one week for each concentration and stored in 100% glycerin. A small thymol crystal was added as a precautionary measure to prevent fungal growth and contamination. Finally, the skeletal scoring chart developed by Nash and Persaud was used to

evaluate skeletal ossification [160]. The sternum, metacarpal, metatarsal and sacrococcygeal bones degree of ossification were taken as the key markers of skeletal development in rats [161]. The ossifications of the skull, hyoid, ribs, vertebrae and limb bones were also examined.

4.11.9.3 Histopathology of fetal placenta

The placentas were examined for morphological abnormalities, with 2 to 3 samples per dam collected for histopathology. Tissues were fixed in 10% formalin, dehydrated through graded alcohols (40–100%), cleared in xylene, and embedded in paraffin. Sections (5 µm) were cut using a microtome, mounted on slides, and dried at 40–45°C. For staining, slides were dewaxed in xylene, rehydrated through descending alcohols, and rinsed in water. Tissues were stained with hematoxylin (5–10 min), differentiated in acid alcohol, counterstained with eosin (1–2 min), and dehydrated again. Finally, slides were cleared in xylene and mounted with DPX (Dibutylphthalate Polystyrene Xylene). A pathologist analyzed placental structure using a Leica EC4 microscope (Germany) at 100× total magnification, with digital images captured for documentation [162].

4.12 Reproductive and first generation developmental toxicity study

4.12.1 General description

The extended first generation toxicity study was conducted in accordance with OECD guideline test number 443 [165]. This long-term study aimed to assess the toxicity of ethanol leaf extract from *U. simensis* on the structure and function of the reproductive systems of parental (P) rats and their first-generation offspring (F1). The toxic effects of the *U. simensis* leaf extract were assessed for histopathology of liver, kidneys, adrenal gland, thyroid and parathyroid glands. Following prenatal and postnatal exposure to the leaf extract of parental rats, F1 rats were observed for possible developmental reproductive toxicity, developmental neurotoxicity, and developmental immunotoxicity effects.

4.12.2 Experimental animals

Young, healthy male and nulliparous, non-pregnant female Wistar albino rats aged between 10 and 12 weeks were obtained from the animal breeding unit of EPHI and housed for the experiment in the animal care room. The process of animal handling and care was similar to the teratogenic studies.

4.12.3 Grouping and dosing of parental rats

After a one-week acclimatization period, 20 parental rats per sex and group were randomly assigned to four groups. Groups' I to III received the *U. simensis* ethanol leaf extract through gavage at doses of 250, 500, and 1000 mg/kg, respectively, until necropsy, while Group IV (control group) received only the vehicle (distilled water). The extract was administered daily for ten successive weeks. Among this, two weeks were used for the premating period. The premating period was adjusted to cover three to four estrous cycles for female rats and epididymal sperm maturation for male rats [149]. After two weeks of treatment, rats were mated in a one to one male to female ratios. The female rats were kept with unrelated males of proven fertility for two weeks. Every morning, the female rats were inspected for the presence of vaginal plug. The vaginal smear test was conducted to assure the presence of spermatozoa with in the vaginal fluid. This was considered as day zero of pregnancy. The treatment was continued for the next two mating weeks, in which the female rats were housed with a male rat until pregnancy was confirmed. The female rats continued to receive the treatment during pregnancy and lactation until the pups started weaning. The male rats were also treated for the same duration as the female rats. Both the male and female rats were housed separately except for mating. In the near term, each pregnant rat was housed separately in a maternity cage.

4.12.4 Clinical observations

Cage-side clinical observations were made every morning throughout the treatment period. Any signs of toxicity changes in the skin, motor and sensory function, unusual respiratory pattern, and self-mutilation were assessed and recorded before and after dosing the extract. All rats were also scrutinized daily for severe toxicity, morbidity, and mortality.

4.12.5 Food intake and body weight changes

On the first day of dosing, all parental rats were weighed. The male rats were weighed weekly, with weight gain calculated accordingly. The female rats were weighed weekly before pregnancy, then on gestational days (GD) 0, 7, 14, 20 and lactational days (LD) 1, 4, 7, 14 and 21. The pre-gestational, gestational and lactational weight gains were computed and compared across groups. In addition, the daily food intake was measured every morning throughout the treatment period.

4.12.6 Reproductive toxicity study in parental rats

4.12.6.1 Reproductive indices in parental rats

The reproductive endpoint indices were evaluated for both parental male and female rats. The dates of pairing, insemination, and parturition were recorded. The pre-coital interval and the length of pregnancy were calculated. The number of parental male rats capable of insemination and the number of pregnant parental female rats were also recorded. The mating index was calculated by dividing the number of rats with evidence of mating by the number of paired rats and multiplying by hundred. The male rats' fertility index was calculated as the number of males siring a litter divided by the number of pairs times hundred. The female rats' fertility index was also calculated as the number of females with evidence of pregnancy divided by the number of pairs times hundred. In addition, the gestational indices were calculated by dividing the number of females delivering a viable litter by the number of females with evidence of pregnancy and multiplying by

hundred. The number of live births, stillbirths, and the sex of the pups were recorded. Additionally, the gestational survival index, the number of postnatal deaths of litters on postnatal days (PNDs) 1, 4, 7, 14, and 21, as well as the sex ratio, were calculated.

4.12.6.2 Estrous cycle assessment

The *U. simensis* leaf extract related effect on the estrous cycle was assessed through vaginal smear test. The parental female rats were held in a restrainer and the vaginal fluid was collected by a plastic pipette filled with 10 µl of normal saline (0.9% Na Cl). The tip of the plastic pipette was gently inserted into the rat's vagina. The aspirated vaginal fluids were fairly distributed over the labeled glass slides. The slides were kept at room temperature until dried. In order to stain, few droplets of crystal violet was added on the aspirated fluids and kept for one minute. The crystal violet was washed with distilled water, and glycerol was added to increase the optical property. Finally, it was covered by cover-slip and examined using a binocular light microscope [166]. The microscopic examination was done to identify nucleated epithelial cells, cornified cells and leukocytes. The phases of estrous cycles (proestrus, estrus, metestrus, and diestrus) were identified. The mean length of estrous cycles were computed and compared between the treatment and control groups [167, 168].

4.12.7 Necropsy of parental rats

4.12.7.1 Gross examination

All paternal rats were euthanized with an intraperitoneal injection of 150 mg/kg sodium pentobarbital. The anterior abdominal incision was done to reveal the visceral organs. Gross examinations of different internal organs were carried out for any observed abnormalities or pathological changes.

4.12.7.2 Organ weights of parental rats

Following necropsy, the weights of key reproductive organs of parental rats such as the ovaries, fallopian tubes, uterus with cervix, testes, epididymis, prostate, and seminal glands were measured.

4.12.7.3 Histopathology of parental rats

The ovarian and testicular tissue samples were taken from all groups, fixed in 10% formalin, and stained with hematoxylin and eosin (H and E). A comprehensive microscopic view with histopathological analysis was conducted. Photomicrographs were obtained using an integrated digital microscope camera (Leica EC4, Germany) at 4×, 10×, and 40× objective lens magnifications, corresponding to total magnifications of 40×, 100×, and 400×, respectively.

4.12.7.4 Sperm analysis

Both the testis and epididymis were retrieved and dissected. The tail of epididymis was excised with surgical blade to disperse the spermatozoa in a glass containing diluted fluid (sodium bicarbonate (5 g) in normal saline, and 1 ml 40% formalin). The suspension was diluted and mixed [169]. The spermatozoa was counted and evaluated for the morphological abnormalities. A single drop of sperm suspension was placed into the Neubauer hemocytometer chamber and was settled in a humid place for 10 minutes. The number of spermatozoa in five squares of the hemocytometer was counted though a microscope. The number of sperm cells were calculated as the number of spermatozoa times dilution factor multiplied by the depth factor and divided by the number of areas counted [170]. The percentage of abnormal sperm cells were also calculated [171]. The slides were prepared as a fixed wet preparation [172] and classified as either normal or abnormal [40].

4.12.8 Systemic toxicity of the parental rats

4.12.8.1 Biochemical analysis

The biochemical analysis of the liver and kidney functions in both parental rats was conducted. About five to six ml of blood was taken through cardiac puncture. The withdrawn blood was placed in a test tube for an hour and centrifuged for ten minutes to isolate the serum. The serum was collected through micropipette and kept in the refrigerator with a vial. Then, it was analyzed by an automated clinical chemistry analyzer, and the alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea, creatinine, total protein, albumin, glucose, and total cholesterol were analyzed.

4.12.8.2 Histopathology of liver and kidney

The parental rats were given sodium pentobarbital intraperitoneal injection and underwent laparotomy. Gross examination was conducted for any abnormalities of the liver and kidney. Tissue samples were taken from the liver and kidney for treatment related histological changes. The samples were fixed in 10% formalin and stained with H and E.

4.12.9 First generation developmental toxicity study

The first generation (F1) rats were assessed for the developmental reproductive toxicity, neurological and immune toxicities of *U. simensis* leaf ethanol extract. The F1 rats were grouped and followed into the three cohorts: F1 chort-1, F1 cohort-2 and F1 cohort-3. The number and sex of pups, stillbirths, live births and any deformity were evaluated at the post-natal day zero (PND0). Every week, the in life pups were weighed separately on PNDs 0, 4, 7, 14, and 21 and at necropsy. The distance between the anus and genital tubercle of each pup was measured at PND zero. In male pups, the presence of nipples or areolae was also assessed on PND twelve.

4.12.9.1 Selection of pups for cohort study

The pups were housed with their dams until weaning (PND 21). All F1 offsprings were identified at PND zero. The litter of origin, sex, and their parental doses were recorded. The developing pups were separated from their dams and grouped for post-weaning studies. At least one pup per sex per litter was selected from each dam for post-weaning cohort studies: cohort 1-reproductive toxicity, cohort 2-neurotoxicity, and cohort 3-immunotoxicity. Based on the OECD guideline 443, twenty male and twenty female pups per dose level were randomly assigned for cohort 1, but ten male and ten female pups were assigned for cohort 2 and cohort 3 studies. The selected pups in the three cohort studies were used to assess the effects of *U. simensis* leaf extract on the development of reproductive, neurological, and immune systems.

4.12.9.2 F1 reproductive toxicity study / F1 cohort-1 study

The extended effects of *U. simensis* leaf extract were assessed in the reproductive system, liver, kidney, thyroid, parathyroid and adrenal glands of F1 rats starting from the PND 21. The pups received the treatment indirectly from their dams during pregnancy and lactation. At PND 21, direct oral administration was started. Groups' I to III received daily oral doses of 250, 500 and 1000 mg/kg of *U. simensis* leaf extract. However, the control group received the vehicle, distilled water. Every week, the weights of F1 cohort 1 pups were measured from PND 21 to necropsy (PND 90). The weight gain was computed for all groups. The daily food intake was also recorded.

4.12.9.3 Preputial separation and vaginal opening

Each day, all F1 male pups were assessed for balanopreputial separation starting from PND thirty five. The full balanopreputial separation was considered when the prepuce was completely retract from the glans penis. The female pups were also inspected for the presence of vaginal opening starting from PND twenty eight. The vagina was considered open when the membranous sheath

covering the vaginal orifice was completely break. The day of preputial separation or vaginal opening with respective weight was measured and recorded. The sexual maturity of F1 cohort 1 rats was compared with the physical development by determining age and body weight at balanopreputial separation or vaginal opening for males and females, respectively. Any abnormalities of genital organs like persistent vaginal thread, hypospadias or cleft penis was assessed and recorded [173]. In the F1 cohort 1 rats, estrous cycle assessment was done for four weeks. The length of estrous cycle was compared between the treatment and control groups. Gross examination, weight measurement and histopathologic assessment of F1 cohort 1 rats were carried out for the reproductive organs, liver, kidneys, thyroid, parathyroid and adrenal glands. Biochemical and thyroid hormone tests were also analyzed.

4.12.9.4 Assessment of endocrine toxicity

The potential endocrine toxicity of *U. simensis* leaf extract was assessed through thyroid function tests. Histopathological examinations of thyroid, parathyroid and adrenal glands were conducted. Any toxicity related microscopic changes of aforementioned endocrine glands were evaluated.

4.12.9.5 F1 developmental neurotoxicity study /F1 cohort -2 study

The postnatal developmental neurotoxicity of *U. simensis* leaf extract was assessed in the cohort-2 study. On the PND 21, F1 cohort-2 rats were isolated from their dams; and assigned into four groups. Groups' I to III were treated with daily oral doses of 250, 500 and 1000 mg/kg of *U. simensis* leaf extract, respectively until necropsy (PND 70); while the group IV pups received distilled water. The daily food intake and body weight changes were also recorded as similar to cohort-1 rats. On the PND 70, cohort-2 rats were anesthetized by intraperitoneal injection of sodium pentobarbital, the rat's scalp were removed by scissor and the skull was bilaterally separated through the sagittal suture. The brain was carefully detached from the meninges and

cranial cavity. Then, it was inspected for any signs of gross morphological changes. The weight of the brain was also recorded with digital balance. Ten percent formalin was injected through trans cardiac perfusion for each rats [174]. The skull was opened and the brain was removed from the cranial cavity. The lamina of each vertebra was broken craniocaudally to reveal the spinal cord. Then, brain and spinal cord were immersed in 10% formalin for 24 hours. The cerebrum, cerebellum and spinal cord was sectioned for tissue processing. Tissue samples were taken from the cervical, thoracic and lumbar regions of the spinal cord. Microscopic examinations were done for the presence of any neuropathological changes to the sampled tissues.

4.12.9.6 F1 developmental immunotoxicity study / F1 cohort -3 study

The developmental immunotoxicity study was conducted to assess the effects of *U. simensis* leaf extract on the immune system. The gross morphology, organ weight and histopathology of key lymphoid organs were assessed for each cohort-3 rats for possible developmental immunotoxicity. On the PND 21, ten female pups and ten male pups were randomly selected from each dam to assess the potential immunotoxicity nature of *U. simensis* leaf extract. Groups' I to III were given daily oral doses of 250, 500 and 1000 mg/kg of leaf extract while the control group, group IV, received distilled water. The body weight changes and daily food intakes were recorded. Organ weights of the spleen, thymus and mandibular lymph nodes were measured immediately after necropsy (PND 60). Tissue samples were gathered from the spleen, thymus and submandibular lymph nodes for histopathological examinations.

4.13 Data processing and analysis

Data were entered using EPI data version 3.1; and exported to stata software version 14 for analysis. The one way analysis of variance (ANOVA) was done for the comparison of treatment and control groups related to the dietary intakes, weight changes, embryo-fetal outcomes,

reproductive indices and histopathological scores. The post hoc tests (Tukey and Games-Howell) were conducted to identify the statistical significance relied with in the groups. The Games-Howell test was applied if the assumption of homogeneity was violated. The Dunnett's test was also applied to assess the difference between the control group and treatment groups. The Shapiro-Wilk test of normality was conducted to assess the distribution of data scores. Homogeneity of variance was assessed using the Levene's test before the ANOVA. The chi-square test was used to analyze data related to eternal or visceral development abnormalities. The data were expressed using the means, standard deviations and percentages. The p-value less than 0.05 was taken as statistically significant.

4.14 Ethical considerations

Ethical approval (protocol number: 045/22/Anatomy) was obtained from the Department of Anatomy graduate committee and the Institutional Review Board (IRB) of the College of Health Sciences, Addis Ababa University in compliance with the OECD guidelines for the care and use of the experimental animals [149, 175, 176]. The Wistar albino rats were kept at EPHI's biomedical research laboratory in accordance with the highest standards for animal care. The rats were not subjected to any unnecessary painful or terrifying situations. Every precaution was taken to protect them from pathogens. To avoid pain and suffering, the rats were sedated with sodium pentobarbital before being sacrificed. The unused pups and sacrificed parental rats were disposed of humanely manner by the laboratory standards of EPHI.

5. RESULTS

5.1 Proximate compositions of *U. simensis* leaf extract

The proximate analysis of *U. simensis* leaf revealed 7.4% moisture, 28% total ash, 31% crude protein, 3.2% crude fat, 7% crude fiber and 30.8% carbohydrate.

5.2 Phytochemicals of *U. simensis* leaf extract

Tannins and phenols were found in the ethanol, hexane and chloroform extracts of the *U. simensis* leaf, but they were absent in the aqueous extract. Moderate amounts of steroids were present in all solvent extracts. The terpenoids were only present in the ethanol extract of *U. simensis* leaf. The high amounts of alkaloids and phenols were detected in the ethanol extract, which was relatively rich in phytochemicals. However, anthraquinones and glycosides were not found in all extracts of the *U. simensis* leaves (Table 1).

Table 1: Phytochemical screening of *U. simensis* leaf with different solvents

Phytochemicals	Aqueous Extract	Ethanol Extract	Chloroform Extract	Hexane extract
Phenols	-	+++	+	++
Flavonoids	-	++	-	+
Tannins	-	+	+	+
Terpenoids	-	+	-	-
Saponins	+	++	-	+
Glycosides	-	-	-	-
Steroids	++	++	++	++
Alkaloids	-	+++	-	+
Anthraquinones	-	-	-	-

(-) = not detected, (+) = small amount, (++) = moderate amount, (+++) = high amount

5.3 Acute effects of *U. simensis* leaf extract

The oral administration of 5000 mg/kg extract from the *U. simensis* leaves caused toxicity signs in the two female rats. One rat showed loss of appetite, piloerection, dizziness, and lethargy, while another rat showed repetitive circling and diarrhea. These symptoms gradually faded and vanished in the first 24 hours of observation. However, no other indicators of toxicity or deaths were observed during the 14 days follow up period. This suggested that the median lethal dose (LD_{50}) of the *U. simensis* leaf extract was greater than 5000 mg/kg of animal body weight.

5.4 Subacute effects of *U. simensis* leaf extract

5.4.1 Food intake and body weight effects

The 28-day oral administration of *U. simensis* leaf extract did not have significant effect on the daily food intake of either male or female rats. However, the female rats revealed lower daily food intake compared to the male rats. The four week oral doses of *U. simensis* leaf extract revealed relatively higher mean weight gain as compared to the control group. There were significant body weight variations within groups in both male and female rats. The mean weight gains of rats received 1000 mg/kg of *U. simensis* leaf extract in both sexes were significantly increased as compared to the respective control group. However, the satellite-1000 mg/kg treated group showed a non-significant increase in weight gain compared to the corresponding satellite-control group (Table 2).

Table 2: Effects of 28 days oral dose of *U. simensis* leaf extract on food intake and body weight of rats (N = 30 female rats, N = 30 male rats)

		G1-250 mg/kg	G2-500 mg/kg	G3-1000 mg/kg	Control	SG-1000 mg/kg	SG-control
Daily food intake	F	100.24±1.54	99.05±0.96	98.04±0.71	101.11±0.59	103.09±0.25	103.56±0.12
	M	102.19±0.72	101.86±0.45	101.08±0.39	104.86±0.39	104.59±1.16	104.38±1.07
Initial weight	F	216±2.45	214±4.15	217±2.35	221±2.29	219±3.25	217±1.78
	M	218±2.50	220±1.29	224±5.56	223±1.65	222±2.45	223±3.36
Final weight	F	263±3.74	261±5.50	265±3.61	267±3.39	287±3.19	285±1.55
	M	286±3.71	289±2.77	294±6.60	291±2.79	296±2.51	294±1.49
Weight gain	F	46.61 ± 1.29	46.85±1.35	48.11 ± 1.26*	46.17±1.10	73.89±3.21	72.45±2.27
	M	68.12 ± 1.21	68.92±1.48	70.08 ± 1.05*	68.04±1.14	78.92±2.37	76.98±3.09

SG = satellite group, values are expressed as mean ± standard deviation, *statistically significant ($P < 0.05$, one-way ANOVA) as compared to respective control group, F = female rats, M = male rats

5.4.2 Organ weight effects

The gross examination of visceral organs during laparotomy revealed no changes in texture or color except for fibrotic changes of the liver in some female rats. The average weights of the liver, kidneys, and spleen in female rats treated with 1000 mg/kg subacute dose of *U. simensis* leaf extract significantly increased as compared to those in the control group female rats. However, there were no statistically significant weight differences in the adrenals, stomach, pancreas, heart, lungs, brain, and ovaries between the treatment and control group female rats. Male rats given a 1000 mg/kg subacute dose also revealed significantly higher mean weights of kidneys and liver than the respective control groups. However, the two weeks post treatment follow up did not reveal any significant organ weight changes between the two satellite groups, SG-1000 mg/kg and SG-control (Table 3).

Table 3: Effects of 28 days oral doses of *U. simensis* leaf extract on organ weights of rats (N = 30 female rats, N = 30 male rats)

Organs		G1-250 mg/kg	G2-500 mg/kg	G3-1000 mg/kg	Control	SG-1000 mg/kg	SG-control
Liver	F	5.88 ± 1.20	5.95 ± 1.31	6.72 ± 0.51*	5.92 ± 1.08	6.06 ± 0.52	5.98±0.50
	M	7.89 ± 0.44	7.97 ± 0.35	9.24 ± 0.26*	7.81 ± 0.52	8.12 ± 0.16	8.04±0.14
Kidneys	F	1.23 ± 0.18	1.38 ± 0.10	1.58 ± 0.14*	1.22 ± 0.04	1.24 ± 0.12	1.24±0.10
	M	1.42 ± 0.23	1.53 ± 0.21	1.71 ± 0.13*	1.40 ± 0.34	1.51 ± 0.33	1.49±0.28
Adrenals	F	0.033±0.001	0.029±0.002	0.028±0.001	0.031±0.001	0.034±0.001	0.033±0.02
	M	0.034±0.002	0.031±0.001	0.030±0.002	0.036±0.001	0.040±0.001	0.039±0.02
Stomach	F	2.18±0.21	2.23±0.16	2.19±0.22	2.20±0.24	2.25±0.15	2.24±0.17
	M	2.26±0.51	2.24±0.62	2.20±0.39	2.27±0.45	2.30±0.32	2.31±0.08
Pancreas	F	0.25±0.01	0.24±0.02	0.22±0.01	0.26±0.01	0.25±0.02	0.26±0.01
	M	0.27±0.01	0.25±0.03	2.26±0.02	0.28±0.01	0.29±0.01	0.28±0.02
Spleen	F	0.67 ± 0.05	0.69 ± 0.02	0.87 ± 0.05*	0.63 ± 0.04	0.70±0.03	0.68±0.03
	M	0.68 ± 0.02	0.72 ± 0.03	0.74 ± 0.01	0.68 ± 0.01	0.75±0.02	0.73±0.01
Heart	F	1.35±0.23	1.33±0.14	1.33±0.15	1.34±0.19	1.36±0.21	1.37±0.05
	M	1.37±0.16	1.36±0.21	1.35±0.19	1.36±0.11	1.37±0.13	1.37±0.16
Lungs	F	1.96±0.22	1.94±0.16	1.95±0.13	1.98±0.17	1.97±0.20	1.98±0.01
	M	2.11±0.12	2.10±0.08	2.13±0.07	2.14±0.09	2.19±0.06	2.18±0.04
Brain	F	1.54±0.12	1.51±0.01	1.49±0.02	1.50±0.02	1.54±0.01	1.53±0.03
	M	1.55±0.13	1.53±0.02	1.50±0.03	1.55±0.14	1.61±0.13	1.59±0.11
Ovaries	F	0.17±0.01	0.16±0.01	0.15±0.02	0.16±0.02	0.17±0.01	0.16±0.03
Testes	M	2.74±1.01	2.71±1.01	2.70±1.02	2.69±1.02	2.72±0.09	271±0.10

SG = satellite group, values are expressed as mean ± standard deviation, *statistically significant ($P < 0.05$, one-way ANOVA) as compared to respective control group

5.4.3 Hematological effects

The rats treated with *U. simensis* leaf extract revealed a dose-dependent increase in WBC, MCV, MCH, and MCHC. However, rats treated with the plant leaf extract showed a dose-dependent decrease in RBC, PLT, HG, and HCT values. The female rats that received a 1000 mg/kg dose of the leaf extract exhibited a significant increase in WBC count, along with a notable decrease in HG levels when compared to the control female rats. Similarly, male rats treated with a 1000 mg/kg leaf extract exhibited a significant increase in WBC count as compared to the respective control male rats. Rats treated with 1000 mg/kg of the extract for four weeks, followed by a two week treatment free period, showed no significant differences in hematological parameters when compared to the satellite control group (Table 4).

Table 4: Effects of 28 days oral dose of *U. simensis* leaf extract on hematological parameters of rats (N = 30 female rats, N = 30 male rats)

Parameters		G1-250mg/kg	G2-500 mg/kg	G3-1000mg/kg	Control	SG-1000mg/kg	SG- control
WBC($\times 10^3/\mu L$)	F	7.19± 0.07	8.01± 0.33	8.23± 0.15*	7.06±0.09	7.42±0.14	7.40±0.12
	M	9.36 ±2.64	9.89 ±0.51	10.04 ± 0.35*	8.42 ±1.13	8.62±0.54	8.59±0.38
RBC($\times 10^6/\mu L$)	F	6.85± 1.41	6.25± 1.22	6.05±1.12	6.90± 1.16	6.95±1.29	7.01±1.05
	M	7.55 ± 2.11	7.45 ± 1.06	7.25 ± 1.26	7.61 ± 1.32	7.89±1.55	7.90±1.24
PLT ($\times 10^3/\mu L$)	F	997.45±166.27	978.00±152.87	952.00±149.77	1001.80±163.37	999.74±170.18	1001.63±167.22
	M	1038.00±142.1	1011.43±138.32	1007.00±129.17	1041.60±140.37	1053.26±132.20	1055.08±128.45
HG (g/dl)	F	14.42 ± 0.36	14.13 ± 0.11	14.02 ± 0.12*	15.00 ± 0.08	14.92±0.07	15.13±0.01
	M	16.72± 0.41	16.09 ± 0.20	16.00 ± 0.10	16.82 ± 0.50	16.70±0.44	16.77±0.93
HCT (%)	F	45.58 ± 0.39	45.41 ± 0.32	45.28 ± 0.53	45.77 ± 0.33	46.01±0.13	46.09±0.02
	M	46.28 ± 0.19	46.08 ± 0.69	46.03 ± 0.65	46.91 ± 0.74	47.22±0.05	47.15±0.11
MCV (pg)	F	54.17 ± 1.02	54.39 ± 1.70	54.57 ± 1.20	54.12 ± 1.05	54.09±1.12	54.25±0.73
	M	55.67 ± 0.42	55.71 ± 1.31	55.87 ± 0.89	55.67 ± 0.60	56.18±0.24	55.92±0.44
MCH (pg)	F	17.67 ± 0.81	17.70 ± 0.90	18.09 ± 1.07	17.33 ± 0.06	17.83±0.69	17.59±0.37
	M	18.22 ± 1.07	18.36 ± 1.11	19.08 ± 1.20	18.03 ± 0.04	18.41±1.02	18.33±0.90
MCHC (g/dl)	F	30.89 ± 1.07	30.92 ± 1.88	31.17 ± 1.23	30.55 ± 1.10	31.23±0.65	30.98±0.77
	M	31.07 ± 1.17	31.66 ± 1.31	32.04 ± 1.24	31.10 ± 0.69	31.87±1.14	31.36±0.49

Table 4: Effects of 28 days oral dose of *U. simensis* leaf extract on hematological parameters of rats (continued)

Neutrophil (%)	F	29.64±1.27	27.22±1.56	26.18±1.22	26.95±1.42	27.26±1.31	26.75±1.23
	M	30.53±1.10	30.72±1.19	30.92±1.34	30.85±1.49	30.89±1.55	30.57±1.45
Lymphocyte (%)	F	62.48±1.32	63.59±2.45	64.33±2.44	65.39±2.21	65.48±2.19	65.51±2.02
	M	63.08±2.89	63.26±0.27	65.31±0.38	66.88±2.67	67.09±2.35	67.14±2.11
Monocyte (%)	F	3.67±0.45	3.53±0.38	3.71±0.12	3.44±0.29	3.50±0.18	3.29±0.66
	M	3.34±0.12	3.36±0.11	3.35±0.13	3.33±0.06	3.30±0.15	3.41±0.08
Eosinophils (%)	F	2.21±0.35	2.34±0.22	2.48±0.19	2.42±0.17	2.45±0.13	2.40±0.15
	M	3.65±0.12	3.66±0.14	3.44±0.13	3.94±0.19	3.86±0.09	3.78±0.16
Basophils (%)	F	1.25±0.02	1.20±0.01	1.19±0.10	1.26±0.01	1.27±0.03	1.25±0.04
	M	1.34±0.11	1.35±0.02	1.39±0.12	1.30±0.12	1.37±0.14	1.35±0.15

SG = satellite group, values are expressed as mean ± standard deviation, *statistical significant ($p<0.05$, one-way ANOVA) difference as compared to respective control group, HG = hemoglobin, HCT = hematocrit, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MCV = mean corpuscular volume, PLT = platelets, RBC = red blood cells, WBC = white blood cell

5.4.5 Biochemical effects

Rats treated with 500 and 1000 mg/kg *U. simensis* leaf extract revealed significantly elevated serum ALT compared to the respective control group. In addition, rats that received the leaf extract showed dose-dependent increases in urea and creatinine levels. However, only the serum creatinine levels of male rats treated with a 1000 mg/kg were significantly elevated compared to those of the respective control male rats. Furthermore, rats treated the leaf extract, serum levels of LDL declined while protein, glucose and HDL increased, but not statistically significant as compared to the respective control group. However, no significant differences were observed in the biochemical test results between the two satellite groups, SG-1000 mg/kg and SG-control (Table 5).

5.4.6 Histopathological effects

The mean histopathological scores of the liver, kidney, and spleen in the female rats treated with 1000 mg/kg *U. simensis* leaf extract were 1.20±0.74, 0.40±0.40, and 0.60±0.60, respectively; however, these scores were not statistically significant as compared to the corresponding controls (Table 6). The 4-week oral dose of *U. simensis* leaf extract did not cause any histopathological changes of the liver, kidney, and spleen in the male rats (Fig. 2). In contrast, some female rats treated with 1000 mg/kg leaf extract showed liver parenchymal necrosis, kidney glomerular distortion, and spleen white pulp depletion (Fig. 3). However, no notable histopathological changes were observed in the 1000 mg/kg treated satellite groups.

Table 5: Effects of 28 days oral dose of *U. simensis* leaf extract on biochemical parameters of rats (N = 30 female rats, N = 30 male rats)

Parameters		GI-250mg/kg	GII-500mg/kg	GIII-1000mg/kg	Control	SG-1000mg/kg	SG-control
ALT(U/L)	F	45.15±2.36	48.93 ±3.14*	49.21 ±2.44*	45.02±3.17	46.29 ± 2.41	46.07±2.29
	M	46.23±3.09	51.60±4.29*	54.33 ±5.01*	45.96±3.86	49.02 ± 1.78	48.63±1.57
AST(U/L)	F	200.53 ± 6.01	201.82 ± 1.18	201.04 ± 1.68	199.87±5.55	200.26 ± 3.11	200.45±2.89
	M	210.49±13.55	211.22±15.07	211.71±20.08	209.63±11.4	210.82 ± 14.31	209.94±15.06
ALP(U/L)	F	92.21 ± 5.25	92.78± 7.44	93.08± 4.54	92.91 ± 8.12	92.51 ± 6.14	92.48±7.53
	M	153.04±12.33	153.61±19.02	154.11 ± 9.84	154.27±10.1	153.29 ± 11.35	154.08±10.21
Urea (mg/dL)	F	40.24± 1.10	40.61± 1.05	41.04± 0.2	40.43± 0.18	40.39 ± 0.07	40.45±0.03
	M	41.19 ± 3.03	41.46± 3.91	42.06± 2.15	41.08± 3.14	41.74 ± 2.03	41.69±1.92
Creatinine(mg/dL)	F	0.34± 0.01	0.35± 0.03	0.38± 0.01	0.33± 0.04	0.34 ± 0.06	0.33±0.09
	M	0.39 ± 0.01	0.40± 0.07	0.41±0.05*	0.38± 0.02	0.40 ± 0.01	0.39±0.01
Albumin(g/dL)	F	3.93± 0.03	4.09± 0.02	4.16± 0.01	3.99± 0.04	4.11± 0.03	4.05±0.03
	M	4.29± 0.02	4.33± 0.01	4.51± 0.04	4.22± 0.03	4.37 ± 0.05	4.33±0.06
Protein(g/dL)	F	6.03± 0.04	6.13± 0.01	6.30± 0.06	6.10± 0.04	6.24 ± 0.02	6.21±0.03
	M	6.17± 0.08	6.41± 0.05	6.53± 0.06	6.29± 0.07	6.38 ± 0.09	6.35±0.11
Cholesterol(mg/dL)	F	40.45 ± 1.63	40.73 ± 1.08	41.04 ± 1.03	40.21 ± 1.10	40.56 ± 1.15	40.49±1.18
	M	42.46± 1.32	42.06 ± 0.59	42.67 ± 0.81	41.39 ± 0.70	42.16 ± 0.57	41.95±0.14
Glucose (mg/dL)	F	109.72 ± 5.23	110.02 ± 4.59	110.77 ± 2.77	109.95±6.02	110.49 ± 2.25	109.57±3.07
	M	120.63 ± 4.24	121.53 ± 4.12	122.08 ± 3.65	120.84±5.01	121.05 ± 1.89	120.86±2.14
LDL (mg/dl)	F	5.59±0.31	5.71±0.51	5.84±0.62	5.44±0.29	5.47 ± 0.31	5.41±0.29
	M	5.55±0.33	5.49±0.29	5.36±0.20	5.77±0.17	5.70 ± 0.01	5.69±0.12

Table 5: Effects of 28 days oral dose of *U. simensis* leaf extract on biochemical parameters of rats (continued)

HDL (mg/dl)	F	45.74±2.11	44.90±2.64	45.85±2.20	43.99±2.19	43.92 ± 1.68	43.82±1.57
	M	20.44±0.36	20.59±0.24	20.97±0.22	19.82±0.16	20.65 ± 0.31	20.38±0.55
Na+ (mmol/l)	F	141.58±1.92	142.39±1.55	142.81±1.47	141.33±1.28	142.19 ± 1.22	141.65±1.27
	M	144.11±0.22	145.09±0.31	145.11±0.40	143.60±0.18	144.08 ± 0.51	143.95±0.32
K+ (mmol/l)	F	4.49±0.14	4.66±0.16	4.90±0.13	4.42±0.15	4.72 ± 0.12	4.68±0.14
	M	5.49±0.02	5.36±0.03	5.35±0.01	5.44±0.02	5.40 ± 0.01	5.42±0.01
Cl− (mmol/l)	F	104.10±1.22	103.64±1.09	103.88±1.16	104.55±1.20	104.23 ± 1.29	104.36±1.04
	M	101.12±0.34	100.89±0.42	100.95±0.29	101.22±0.47	100.65 ± 0.51	101.41±0.08

SG = satellite group, values are expressed as mean ± standard deviation, *statistical significant ($p<0.05$, one-way ANOVA) difference as compared to respective control group, ALT = alanine aminotransferase; AST = aspartate aminotransferase, ALP = alkaline phosphatase, LDL = low-density lipoprotein, HDL = high-density lipoprotein, ALP = alkaline phosphatase, Na^+ = sodium, K^+ = potassium; Cl^- = chlorine, F = female rats, M = male rats

Table 6: Histopathological scores of liver, kidney and spleen in rats treated with *U. simensis* ethanol leaf extract

	G1-250mg/kg		G2-500mg/kg		G3-1000mg/kg		Control		SG-1000mg/kg		SG-control	
	M	F	M	F	M	F	M	F	M	F	M	F
Liver	0	0	0	0	0	1.20±0.74 (2)	0	0	0	0	0	0
Kidney	0	0	0	0	0	0.40±0.40 (1)	0	0	0	0	0	0
Spleen	0	0	0	0	0	0.60±0.60 (1)	0	0	0	0	0	0

Lesion severity was scored on a scale of 0 to 3: 0 = normal, 1 = mild/minimal, 2 = moderate, 3 = severe. Mean ± standard deviation, Kruskal–Wallis test was used for comparison within and between groups. Numbers in parentheses denote the count of female rats with histopathological lesions (M = male rats, F = female rats

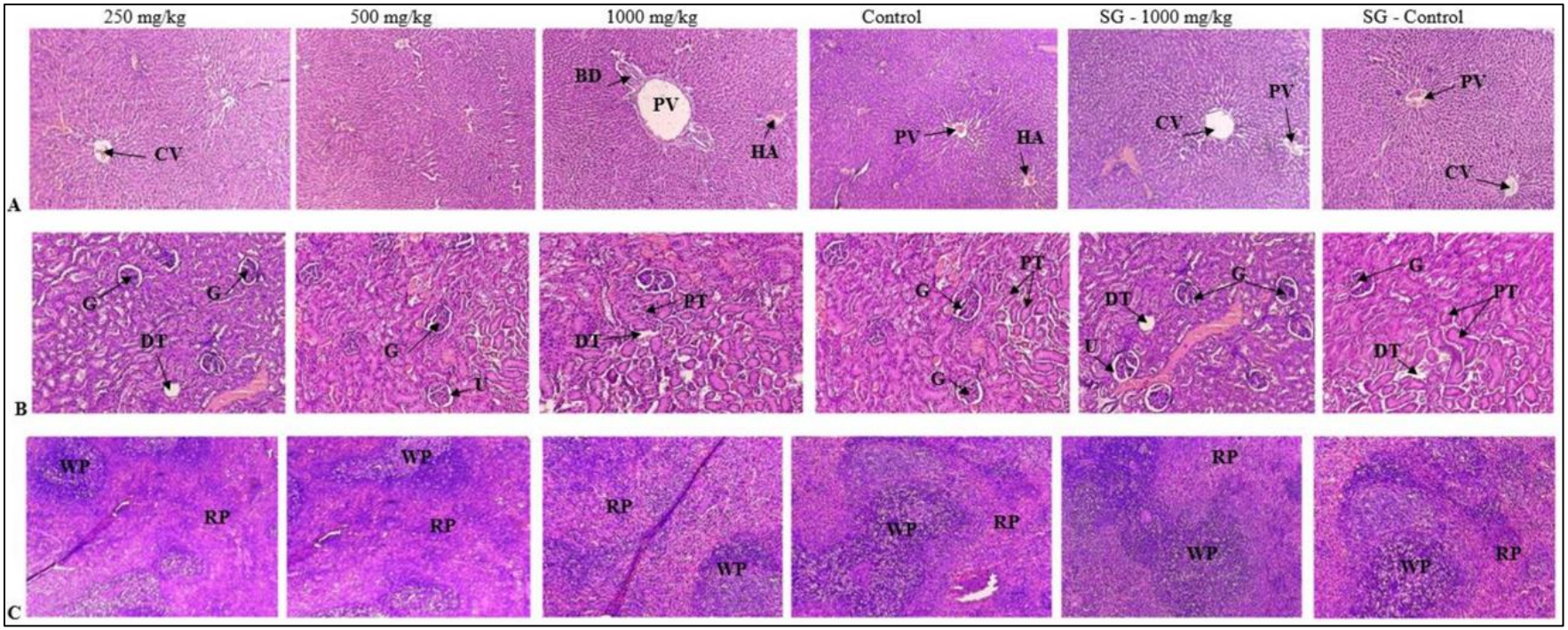

Figure 2: Photomicrographs of male rats' liver (A), kidney (B) and spleen (C) (total magnification: x100 H&E): showing effects of oral doses of *U. simensis* leaf extract on histopathology. Treatment groups which received 250 mg/kg, 500 mg/kg, and 1000 mg/kg of *U. simensis* leaf extract revealed normal structures of liver (hepatocytes, portal vein, hepatic artery, bile duct, and central vein), kidney (proximal tubules, distal tubules, and glomerulus), and spleen (red pulp and white pulp): SG = satellite group, CV = central vein, PV = portal vein, HA = hepatic artery, BD = bile duct, G = glomerulus, DT = distal tubule, PT = proximal tubule

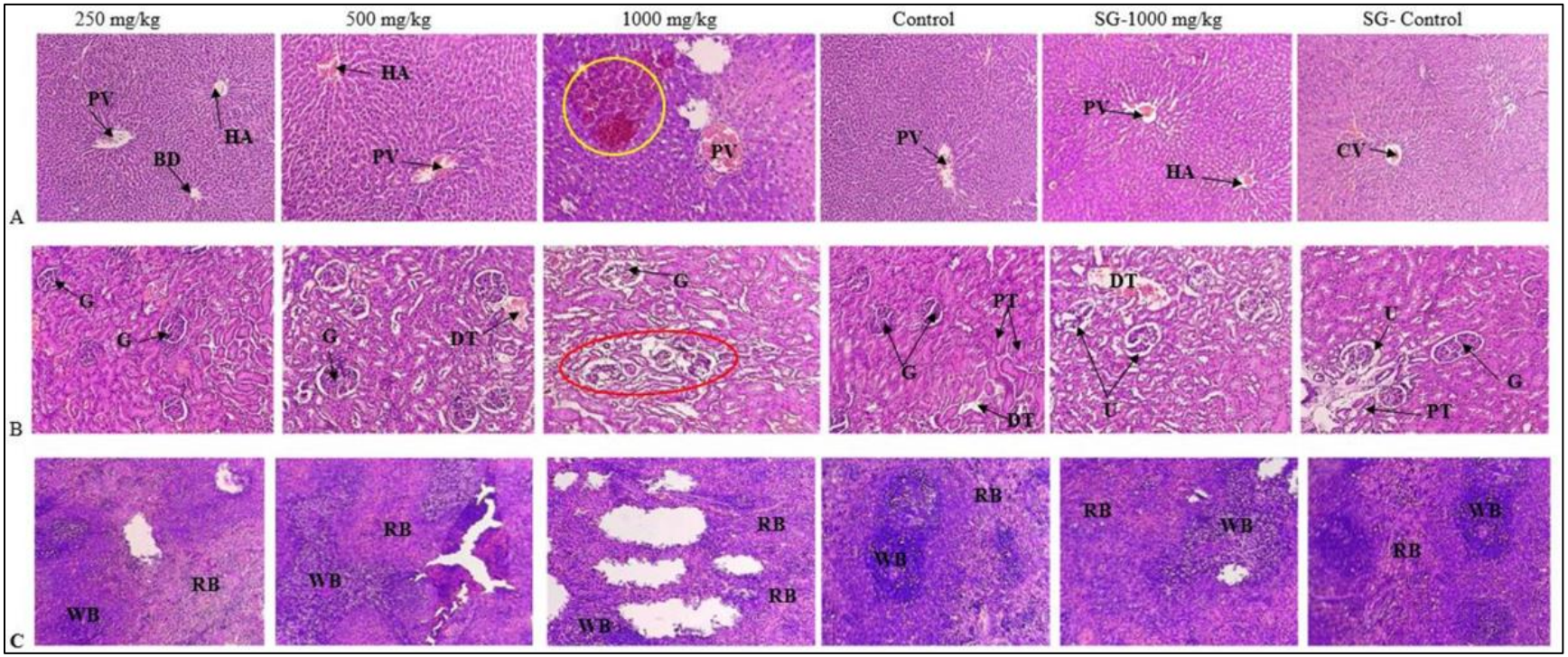

Figure 3: Photomicrographs of female rats' liver (A), kidney (B) and spleen (C) (total magnification: x100 H&E): showing effects of oral doses of *U. simensis* leaf extract on histopathology. Treatment groups which received 250 mg/kg, 500 mg/kg and 1000 mg/kg of *U. simensis* leaf extract showed liver parenchymal necrosis (yellow circle), kidney glomerular distortion (red circle) and spleen white pulp depletion: SG = satellite group, CV = central vein, PV = portal vein, HA = hepatic artery, BD = bile duct, PT = proximal tubule, G = glomerulus, DT = distal tubule, WP = white pulp, RP = red pulp

5.5 Teratogenicity of *U. simensis* leaf extract

5.5.1 Food and body weight gain effects

There was no significant difference in the daily dietary intakes of pregnant dams treated with *U. simensis* leaf extract as compared to pair fed and ad libitum control groups. In addition, the pregnant rats treated with the *U. simensis* leaf extract revealed a dose-dependent decrease in body weight gain compared to the two control groups. However, this difference was not statistically significant (Table 7).

Table 7: Food intake and weight gain of pregnant rats treated with *U. simensis* leaf extract

Groups	Food intake /day(g)		Weight gain /dam(g)	
	Day-12	Day-20	Day-12	Day-20
Group I (250 mg/kg)	200.19 ± 1.02	201.09 ± 0.61	13.75 ± 0.23	18.21 ± 0.51
Group II (500 mg/kg)	199.48 ± 1.23	200.26 ± 1.05	13.12 ± 0.39	17.99 ± 0.46
Group III (1000mg/kg)	197.82 ± 1.31	199.29 ± 0.71	12.64 ± 0.18	16.86 ± 0.32
Group IV (pair fed)	200.57 ± 1.04	201.52 ± 0.22	14.09 ± 0.43	18.40 ± 0.22
Group V(ad libitum)	200.73 ± 1.25	201.80 ± 0.31	14.11 ± 0.27	18.61 ± 0.19

Results are written as mean and standard deviation

5.5.2 Embryo outcomes

5.5.2.1 Embryonic growth effects

The embryonic growth indices were decreased in gravid rats given *U. simensis* leaf extract compared to the pair fed and ad libitum control groups. The gravid rats given 1000 mg/kg leaf extract had a significantly lower somite number per litter compared to pair fed and ad libitum control groups. In addition, the pregnant dams administered 1000 mg/kg leaf extract showed a significant reduction in morphological score per litter in comparison to control groups (Table 8).

Table 8: Embryonic growth indices of rat embryos treated with *U. simensis* leaf extract

Groups (doses)	Embryonic growth indices		
	Crown rump length/litter (cm)	Somite number/litter	Morphological score/litter
G –I (250 mg/kg)	3.90 ± 0.15	31.63 ± 0.22	43.98 ± 1.06
G-II (500 mg/kg)	3.88 ± 0.11	30.45 ± 0.52	43.07 ± 0.64
G-III (1000 mg/kg)	3.82 ± 0.14	29.03 ± 0.27*	42.58 ± 1.05*
G-IV (pair fed)	3.99 ± 0.18	31.86 ± 0.19	44.25 ± 0.77
G-V(ad libitum)	4.01 ± 0.02	32.04 ± 0.02	45.08 ± 0.05

*Significant difference ($P < 0.05$) as compared to control groups (pair fed and ad libitum)

5.5.2.2 Embryo developmental effects

The embryonic developments of the circulatory system, neurological system, musculoskeletal and craniofacial regions were assessed using the morphological endpoints (Fig. 4). The pregnant rats given 1000 mg/kg of *U. simensis* leaf extract revealed a significant embryonic developmental delays of the caudal neural tube, otic system and olfactory system compared to the pair fed and ad libitum control groups. In addition, the pregnant dams received the repeated dose of 500 mg/kg of *U. simensis* leaf extract showed significantly low developmental scores of the caudal neural tube and the olfactory system as compared to the corresponding control groups. Furthermore, the embryo developmental scores of the forelimb and hindlimb buds significantly decreased in those gravid dams treated with 1000 mg/kg of *U. simensis* leaf extract as compared to pair-fed and ad libitum control groups (Table 9).

Table 9: *In-Vivo* embryonic developments of rats treated with *U. simensis* leaf extract

Morphological end points	GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-pair fed	GV-ad libitum
Number of embryos	110	107	106	110	109
Yolk sac circulation	3.14 ± 0.09	3.12 ± 0.07	3.08 ± 0.05	3.22 ± 0.02	3.17 ± 0.09
Flexion	2.15 ± 0.22	2.16 ± 0.09	2.14 ± 0.09	2.24 ± 0.01	2.24 ± 0.19
Heart	2.87 ± 0.52	2.87 ± 0.50	2.89 ± 0.51	2.85 ± 0.52	2.87 ± 0.52
Caudal neural tube	3.77 ± 0.02	3.51 ± 0.02*	3.49 ± 0.01*	3.82 ± 0.02	3.88 ± 0.01
Hind brain	2.71 ± 0.32	2.70 ± 0.30	2.69 ± 0.33	2.71 ± 0.34	2.71 ± 0.32
Mid brain	2.73 ± 0.31	2.73 ± 0.31	2.70 ± 0.29	2.75 ± 0.32	2.75 ± 0.31
Fore brain	2.98 ± 0.26	2.91 ± 0.22	2.82 ± 0.20	2.99 ± 0.27	2.99 ± 0.30
Otic system	2.54 ± 0.08	2.47 ± 0.05	2.11 ± 0.05*	2.67 ± 0.26	2.67 ± 0.28
Optic system	2.49 ± 0.16	2.49 ± 0.13	2.40 ± 0.16	2.52 ± 0.18	2.52 ± 0.20
Olfactory system	0.87 ± 0.52	0.58 ± 0.30*	0.53 ± 0.22*	0.88 ± 0.53	0.89 ± 0.52
Branchial bars	2.91 ± 0.28	2.90 ± 0.27	2.89 ± 0.28	2.92 ± 0.29	2.92 ± 0.28
Maxillary process	1.37 ± 0.64	1.36 ± 0.61	1.32 ± 0.65	1.37 ± 0.63	1.38 ± 0.64
Mandibular process	0.9 ± 0.07	0.89 ± 0.04	0.82 ± 0.07	0.9 ± 0.10	0.9 ± 0.12
Fore limb	1.88 ± 0.67	1.65 ± 0.37	1.07 ± 0.39*	1.90 ± 0.61	1.91 ± 0.57
Hind limb	1.79 ± 0.45	1.68 ± 0.05	1.09 ± 0.85*	1.82 ± 0.75	1.89 ± 0.73

*significant difference ($p<0.05$) as compared to control groups (pair fed and ad libitum)

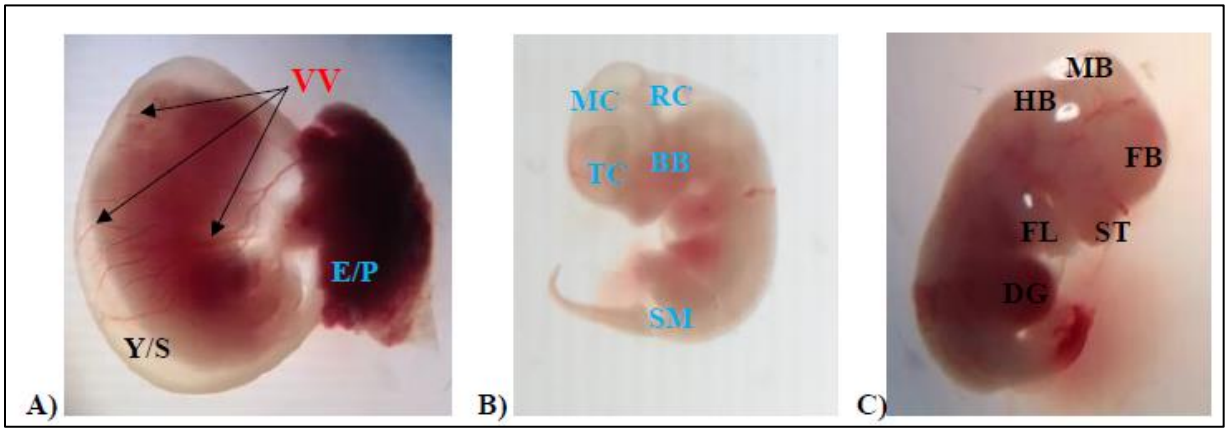

Figure 4: Examinations of 12 days old rat embryos through the dissecting microscope: **A)** 500 mg/kg of *U. simensis* leaf extract: Vitelline Vasculature (**VV**), Intact Yolk sac (**Y/S**) and Embryonic Placenta (E/P); **B)** 1000 mg/kg of *U. simensis* leaf extract: Telencephalon (TC), Mesencephalon (MC), Rhombencephalon (RC), Brachial bars (BB) and Somites (SM); **C)** ad libitum control: Fore brain (**FB**), Mid brain (**MB**), Hind brain (**HB**), Stomodeum (**ST**), Fore limb bud (**FL**), and Developing gut (**DG**)

5.5.3 Fetal outcomes

The pregnant rats treated with the *U. simensis* leaf extract had a dose-dependent increase in the fetal resorption as compared to the pair fed and ad libitum control groups. Those gravid rats received 1000 mg/kg of *U. simensis* leaf extract revealed a significant increase in fetal resorption as compared to the two control groups, pair fed and ad libitum. However, there was no significant difference in the implantation sites per dam between the treatment and control groups. No dead fetus was retrieved in all experimental groups (Table 10 and Fig. 5).

Table 10: Fetal outcomes of pregnant rats treated with *U. simensis* leaf extract

Group (G)	Implantation site /dam	Resorption site/dam	Live fetuses/dam	Dead fetuses/dam
G –I (250 mg/kg)	10.28 ± 0.07	0.25 ± 0.21	10.15 ± 0.25	0.00 ± 0.00
G-II (500 mg/kg)	10.30 ± 0.09	0.32 ± 0.22	10.13 ± 0.13	0.00 ± 0.00

G-III (1000 mg/kg)	10.16 ± 0.05	0.54 ± 0.14*	10.11 ± 0.14	0.00 ± 0.00
G-IV (pair-fed)	11.08 ± 0.14	0.20 ± 0.02	11.07 ± 0.12	0.00 ± 0.00
G-V(ad libitum)	11.07 ± 0.21	0.19 ± 0.01	11.06 ± 0.13	0.00 ± 0.00

*significant difference ($p<0.05$) as compared to control groups (pair fed and ad libitum)

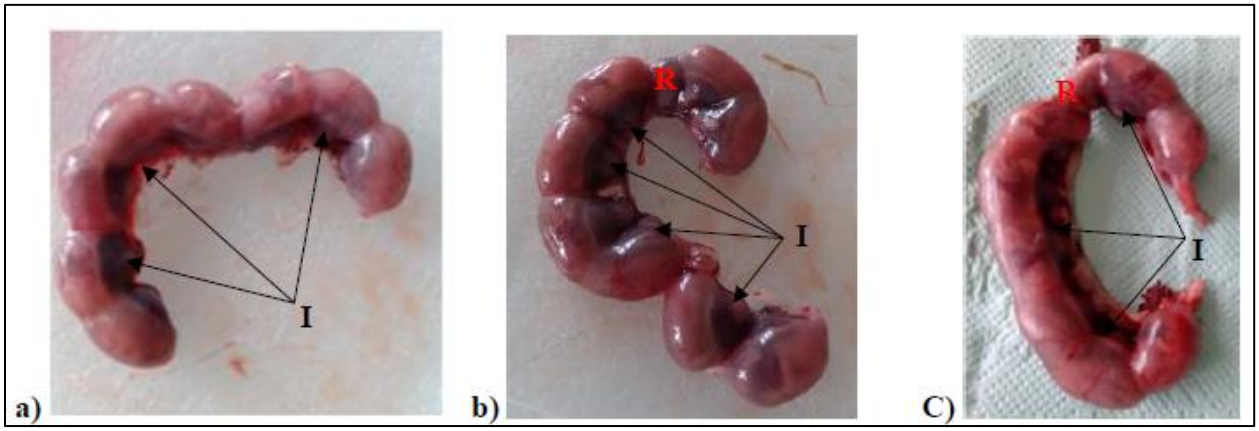

Figure 5: Implantation (**I**) and resorption (R): a) ad libitum control, b) 500 mg/kg, and c) 1000 mg/kg of *U. simensis* leaf extract treatment groups

5.5.3.1 Fetal growth effects

The pregnant rats exposed to the *U. simensis* leaf extract showed a dose-dependent reduction in the fetal growth indices compared to the pair fed and ad libitum pregnant rats. The gravid rats given 1000 mg/kg of *U. simensis* leaf extract revealed a significantly reduced fetal weight as compared to the pair fed and ad libitum control groups. In addition, pregnant dams treated with 1000 mg/kg of *U. simensis* leaf extract showed a significant decrease in the fetal crown rump length as compared to pair fed and ad libitum control groups. The mean placental weight in those pregnant rats received 1000 mg/kg of *U. simensis* leaf extract was low as compared to the pair fed and ad libitum controls, but not statistically significant (Table 11).

Table 11: Fetal growth and placental weight of pregnant rats treated with *U simensis* leaf extract

Groups	Litter weight/fetus (g)	CRL/fetus (cm)	Placental weight/fetus (g)
G –I (250mg/kg)	3.25 ± 0.21	4.19 ± 0.15	0.52 ± 0.11

G-II (500mg/kg)	3.22 ± 0.13	4.11 ± 0.01	0.53 ± 0.02
G-III (1000mg/kg)	3.06 ± 0.11*	4.07 ± 0.11*	0.51 ± 0.18
G-IV (pair-fed)	3.40 ± 0.04	4.49 ± 0.03	0.58 ± 0.12
G-V(ad libitum)	3.39 ± 0.12	4.51 ± 0.01	0.57 ± 0.15

*significant difference ($p<0.05$) as compared to pair fed and ad libitum control, CRL = crown rump length

5.8.2.1 Fetal external and visceral anomalies

The explanted fetuses were examined for external anomalies from cranial to caudal as part of treatment related developmental anomalies such as limb deformities, vanishing tails, craniofacial malformations, vertebral column discrepancies and apparent genital defects (Fig. 6). However, there was no visible external anomalies in all experimental groups (Table 12). In addition, the Bouin's solution fixed fetuses were serially sectioned at the head, neck, chest, abdomen and abdominopelvic regions for visceral examinations (Fig.7). These sequential sections were carefully inspected through the dissecting microscope for any visceral anomalies. The presence of cleft palate, hydrocephalus, eye-related anomalies, and abnormalities in thyroid gland and trachea were assessed in the head and neck regions. Furthermore, diaphragmatic hernia, agenesis of abdominal viscera and external genitalia were also assessed. Yet, no visible visceral abnormalities were observed through the dissecting microscope (Table 13).

Table 12: External anomalies of 20 days old rat fetuses exposed to *U. simensis* leaf extract

Groups	External anomalies							
	AC	EC	SB	KY	SC	LD	MT	AEG
Group 1 (250 mg/kg)	0	0	0	0	0	0	0	0
Group II (500 mg/kg)	0	0	0	0	0	0	0	0
Group III (1000 mg/kg)	0	0	0	0	0	0	0	0
Group IV (pair fed)	0	0	0	0	0	0	0	0

Group V (ad libitum)	0	0	0	0	0	0	0	0

AC: anencephaly, AEG: external genitalia agenesis, EC: exencephaly, KY: kyphosis, LD: limb defect, MT: missed tail, SB: spina bifida, SC: scoliosis

Table 13: Visceral anomalies of 20 days old rat fetuses exposed to *U. simensis* leaf extract

Groups	Visceral anomalies										
	HC	MO	AO	CP	NSD	REAA	VSD	DH	RA	HU	CT
Group 1 (250 mg/kg)	0	0	0	0	0	0	0	0	0	0	0
Group II (500 mg/kg)	0	0	0	0	0	0	0	0	0	0	0
Group III (1000 mg/kg)	0	0	0	0	0	0	0	0	0	0	0
Group IV (pair fed)	0	0	0	0	0	0	0	0	0	0	0
Group V (ad libitum)	0	0	0	0	0	0	0	0	0	0	0

AO: anophthalmia, CP: cleft palate, CT: Cryptorchid testes, DH: diaphragmatic hernia, HC: hydrocephalus, HU: Hydroureters, MO: microphthalmia, NSD: nasal septal defect, RA: renal agenesis, REAA: retroesophageal aortic arch, VSD: ventricular septal defects

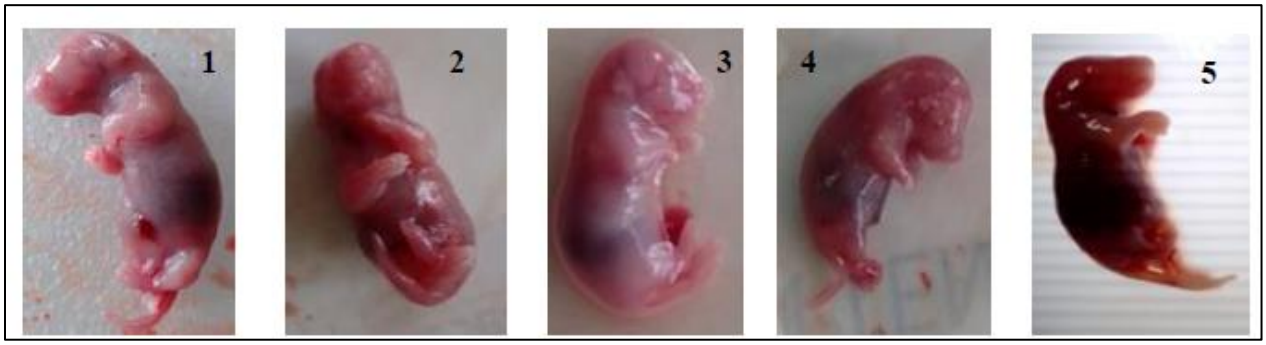

Figure 6: External examination of 20 days old rat fetuses exposed to *U. simensis* leaf extract: ad libitum control group (**1**), pair-fed control group (**2**), 250 mg/kg (**3**), 500 mg/kg (**4**) and 1000 mg/kg (**5**) of leaf extract treated groups

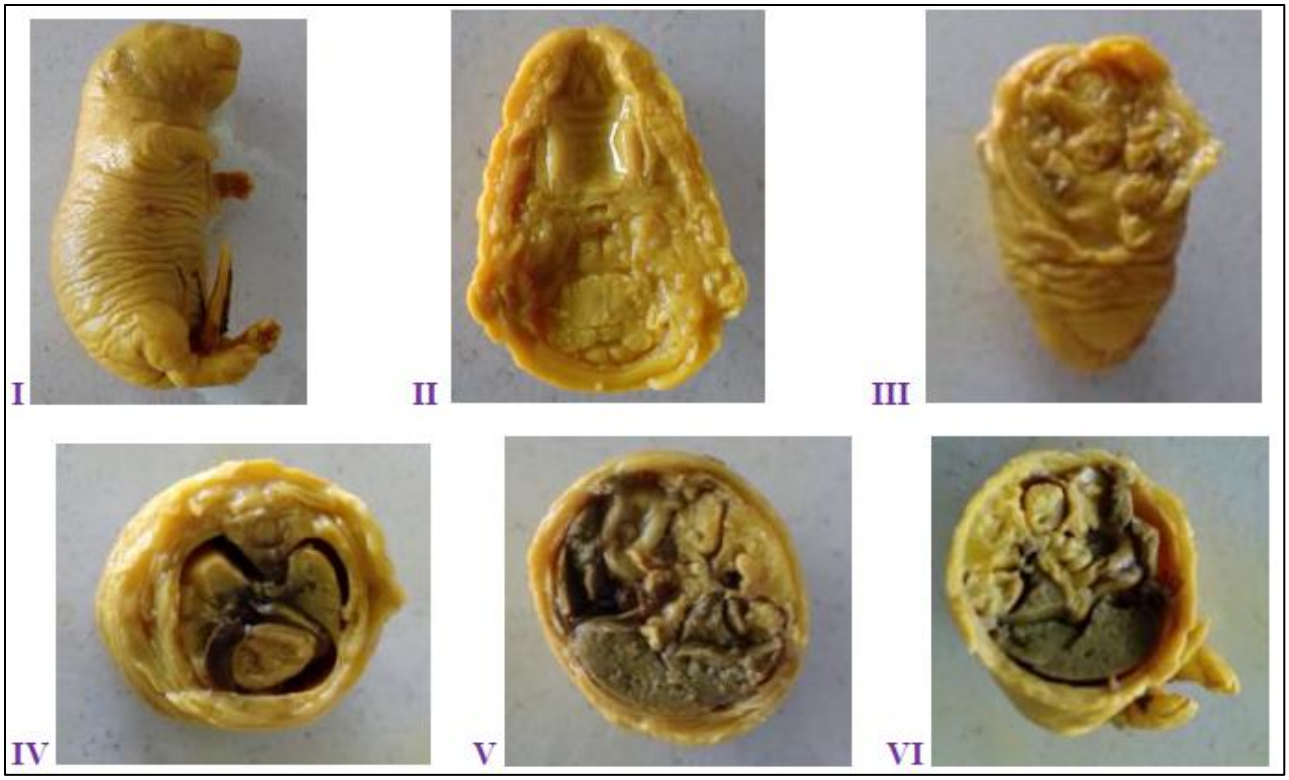

Figure 7: Visceral examination of Bouin's solution fixed 20 days old rat fetus exposed to 1000 mg/kg of *U. simensis* leaf extract: (**I**) un-sectioned fetus, (**II**) coronal section of normal palate and brain tissue, (**III**) transverse section with normal viscera of neck, (**IV**) transverse section made through thorax with intact diaphragm, (**V**) transverse section made through the abdomen showed normal visceral organs and (**VI**) transverse section through the abdominopelvic region with normal visceral organs

5.5.3.3 Fetal skeletal ossification delays

This *in-Vivo* experimental study assessed the developments of axial and appendicular skeletons, but neither treatment nor control groups showed any notable skeletal deformities (Fig. 8). However, the *U. simensis* leaf extract treated group revealed relatively increased ossification delays in the sternum and caudal vertebrae as compared to the control groups, but not statistically significant (Table 14). In addition, the *U. simensis* leaf extract treated groups revealed dose dependent increase in ossification delays of the extremity bones as compared to the pair fed and

ad libitum control groups (Table 15). However, these developmental delays were not statistically significant as compared to either of the two control groups.

Table 14: Axial bones ossification delays of 20 days old rat fetuses exposed to *U. simensis* leaf extract

Groups	Ossification delay (%)						
	HD	SM[a]	RB[b]	CV[c]	TV[b]	LV[c]	SCV[d]
Group 1 (250 mg/kg)	0	19.5	0	0	0	0	9.4
Group II (500 mg/kg)	0	20	0	0	0	0	10.7
Group III (1000 mg/kg)	0	22	0	0	0	0	13
Group IV (pair fed)	0	17	0	0	0	0	9
Group V (ad libitum)	0	16.45	0	0	0	0	9

CV: cervical vertebrae, HD: hyoid bone, LV: lumbar vertebrae, RB: ribs, SCV: sacrocaudal vertebrae, SM: sternum, TV: thoracic vertebrae, a = no ossification signs on the hyoid bone and less than 4 ossification centers on the sternum, b = no ossification signs on the ribs and less than 13 ossification centers on the thoracic vertebrae, c = less than 7 ossification centers in cervical vertebrae and less than 5 ossification centers in lumbar vertebrae, d = less than 4 ossification centers in sacrocaudal vertebrae

Table 15: Extremity bone ossification delays of 20 days old rat fetuses exposed to *U. simensis* leaf extract

Groups	Ossification delay (%)			
	Metacarpals[a]	Metatarsals[a]	FL phalanges[b]	HL phalanges[b]
Group 1-250 mg/kg	6.7	6.5	5.8	6
Group II -500 mg/kg	9	7	8	6.4
Group III -1000 mg/kg	10	8	8.5	7
Group IV -pair fed	7	6	6.8	5
Group V -ad libitum	6.9	5.9	6.5	5.3

FL = forelimb, HL = hindlimb, a = less than 3 metacarpus and less than 3 metatarsus, b = no proximal phalanges of forelimb and no proximal phalanges of hindlimb

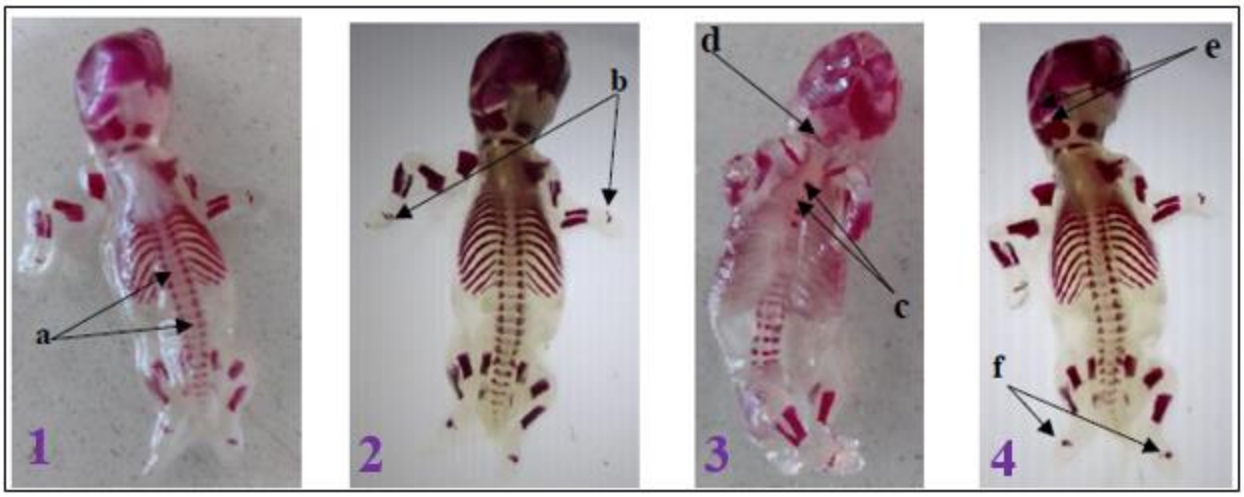

Figure 8: Photomicrographs of alizarin red stained 20-day old rat fetuses exposed to *U. simensis* leaf extract showing skeletal ossifications: 250 mg/kg **(1),** Vertebrae (a), 500 mg/kg **(2)**, Metacarpals (b), 1000 mg/k **(3),** Sternum (c), Hyoid (d), ad libitum control **(4),** Parietal and Occipital (e) and Metatarsals (f)

5.5.3.4 Effects of *U. simensis* leaf extract on histology of the placenta

A microscopic analysis of the placenta was performed to evaluate any histological irregularities, such as necrosis and hemorrhage (Table 16). However, no histological abnormalities were observed in any of the experimental groups (Fig. 9).

Table 16: Histopathological scoring of fetal placenta exposed to *U. simensis* leaf extract

Groups	Necrosis	Hemorrhage	Intervillous thrombosis	Calcification	Vascular dilatation
Group I - 250 mg/kg	0	0	0	0	0
Group II - 500 mg/kg	0	0	0	0	0
Group III - 1000 mg/kg	0	0	0	0	0
Group IV - Pair fed	0	0	0	0	0
Group V - ad libitum	0	0	0	0	0

Zero (0) values indicate no pathological lesion for each fetal placenta

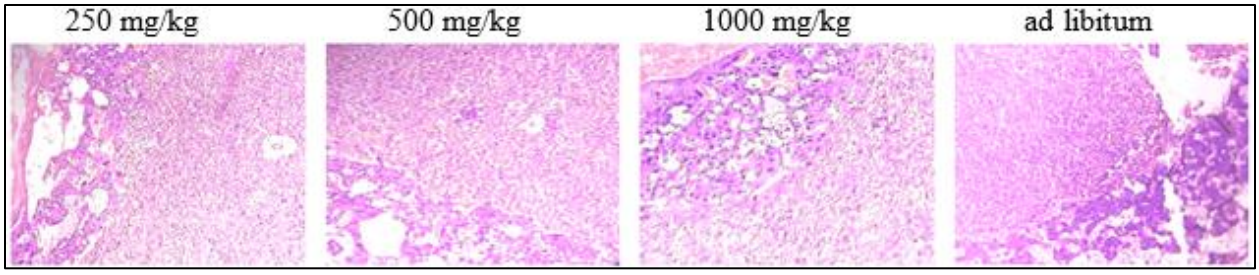

Figure 9: Photomicrographs of fetal placentae (H and E stain, 100x total magnification): Microscopic examinations of fetal placentae exposed to *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg alongside the ad libitum control. All groups exhibit normal placenta histological architecture

5.6 Teratogenicity of *U. simensis* essential oil

5.6.1 Chemical compositions

The GC-MS analysis of *U. simensis* essential oil revealed 43 compounds, accounting for 67.69% of the total composition. The most abundant constituents were 3, 5-dimethyl-1, 2, 4-trithiolane (12.5%), followed by cis-3-hexen-1-ol (5.62%), linalool (5.41%), citronellol (5.10%), methyl chavicol (4.50%), and cis-3-hexenyl acetate (4.20%) (Table 17).

Table 17: chemical compositions of *U. simensis* essential oil identified through GC-MS analysis

Formula	Name	Area (%)	RI	Formula	Name	Area (%)	RI
$C_8H_{16}O$	1-Octen-3-Ol	0.23	970	$C_8H_{14}O$	6-Methyl-5-hepten-2-one	3.25	984
$C_{10}H_{22}$	n-Decane	0.29	989	$C_8H_{14}O_2$	cis-3-Hexenyl acetate	4.20	1008
$C_9H_{12}O_2$	4-Ketoisophorone	0.33	1137	$C_{10}H_{16}O$	Camphor	0.16	1138
$C_{11}H_{10}$	1-Methyl-naphthalene	1.21	1300	$C_{11}H_{10}$	2-Methyl-naphthalene	0.50	1317
$C_9H_{20}O$	n-Nonanol	0.68	1159	$C_{10}H_{18}O$	α-Terpineol	0.53	1180
$C_{10}H_{12}O$	Methyl chavicol	4.50	1209	$C_{10}H_{16}O$	trans-4-caranone	0.44	1175
$C_7H_{12}O$	cis-4-Heptenal	0.75	897	$C_7H_{14}O$	Heptanal	0.20	905
$C_{12}H_{24}O_2$	Dodecanoic acid	0.26	1554	$C_{15}H_{24}O$	Caryophyllene oxide	4.32	1576
$C_{10}H_{18}O$	1,8-Cineole	0.28	1020	$C_7H_{14}O$	cis-4-Hepten-1-ol	0.26	960
$C_{15}H_{26}O$	Bulnesol	0.67	1675	$C_{15}H_{26}O$	4-*epi*-*cis*-Dihydroagarofuran	0.15	1504
$C_{13}H_{20}O$	β-Ionone	1.22	1493	$C_6H_{14}O$	1-Hexanol	0.18	874
$C_6H_{12}O$	cis-3-Hexen-1-ol	5.62	860	$C_{10}H_{18}O$	Linalool	5.41	1090
$C_{10}H_{16}O$	Fenchone	1.56	1079	$C_4H_8S_3$	3,5-Dimethyl-1,2,4-trithiolane	12.35	1140
$C_{10}H_{14}$	1,3,8-p-Menthatriene	0.52	1120	$C_{10}H_{18}O$	Geraniol	1.33	1259
$C_{10}H_{20}O$	Citronellol	5.10	1219	$C_6H_{10}O$	trans-2-hexenal	0.20	850
$C_4H_8S_4$	3,6-dimethyl-1,2,4,5-tetrathiane	0.25	1398	$C_8H_{18}O$	n-Octanol	0.24	1066
$C_9H_{10}O_2$	p-Vinyl guaiacol	3.28	1312	$C_6H_{12}S_3$	2,4,6-Trimethyl-1,3,5-trithiane	1.35	1302
$C_{10}H_{14}O$	Pinocarvone	0.23	1667	$C_{10}H_{18}O$	Borneol	0.15	1161
$C_{10}H_{12}O$	p-Ethylacetophenone	0.62	1275	$C_{12}H_{18}O_2$	Thymohydroquinone dimethyl ether	0.38	1449
$C_{10}H_{12}O_2$	Eugenol	2.82	1360	$C_{11}H_{18}O_2$	Neryl formate	0.24	1285
$C_7H_{16}O$	n-Heptanol	0.24	950				
C_8H_8	Styrene	0.71	879				
$C_{12}H_{12}$	1,3-Dimethyl-naphthalene	0.48	1422				
Total identified = 67.69%, Total unidentified = 32.31%							

RI - retention indices

5.6.2 Acute effects of *U. simensis* essential oil

No mortality was observed in any of the animals treated with a single oral dose of 5000 mg/kg of essential oil. The daily clinical observations did not show any signs of toxicity. In addition, there were no significant differences in daily dietary intake or weight gain between essential oil-treated rats and the vehicle control rats. The median lethal dose (LD_{50}) of the essential oil exceeded 5 g/kg animal body weight.

5.6.3 Clinical observation of pregnant dams

The daily cage-side clinical observations showed no instances of abortion or maternal death in any of the experimental groups. In addition, all pregnant dams showed no signs of overt toxicity or morbidity.

5.6.4 Effects of *U. simensis* essential oil on dietary intake and body weight

The pregnant dams received the essential oil demonstrated a dose-dependent decrease in daily dietary intake compared to the control groups, but it was not statistically significant. However, pregnant rats treated with 1000 mg/kg of essential oil experienced significantly lower weight gain compared to the pair-fed and ad libitum control groups (Table 18).

Table 18: Food intake and weight gain of pregnant dams treated with *U. simensis* essential oil

Groups	Food intake (g/day)		Water intake (ml/day)		Weight gain /dam(g)	
	Day-12	Day-20	Day-12	Day-20	Day-12	Day-20
Group I - 250 mg/kg	199.74±0.88	200.97± 0.48	26.67±1.16	27.33±1.56	14.63±0.64	18.20±2.64
Group II - 500 mg/kg	199.48±1.05	200.72±0.48	26.42±1.31	27.08±1.44	13.36±0.51	17.90±2.53
Group III-1000mg/kg	199.23±0.95	200.71 ± 0.90	26.42±1.50	26.92±1.44	10.60±0.55*	14.50±2.42*
Group IV – vehicle	200.20±1.25	200.97± 0.93	26.75±1.05	27.42±1.44	14.15±2.25	18.40±2.32
Group V- ad libitum	199.91 ±1.01	201.10 ± 0.82	26.92±1.17	27.75±1.37	14.20±1.05	18.60±2.00

Data were written as mean ± SD (standard deviation),*significant differences as compared to pair fed and ad libitum control group

5.6.5 Effects of *U. simensis* essential oil on embryonic growth and development

No statistically significant differences were observed in the crown-rump length (CRL) of embryos between treatment and control groups. However, litters from dams treated with 1000 mg/kg of the essential oil exhibited a significantly lower mean somite number and morphological score compared to both pair-fed and ad libitum controls (Table 19).

Table 19: Embryonic growth indices of rat embryos exposed to *U. simensis* essential oil

Groups	Number of embryos	CRL/litter (cm)	Somite number/litter	Morphological score/litter
Group I-250 mg/kg	91	3.80±0.01	29.13±1.33	40.79±1.08
Group II- 500mg/kg	89	3.79±0.02	29.12±1.31	40.76±1.12
Group III-1000mg/kg	90	3.79±0.02	27.91±1.04*	39.60±1.03*
Group IV-pair fed	90	3.80±0.02	29.19±1.36	40.87±1.04
Group V-ad libitum	91	3.80±0.02	29.18±1.31	40.90±1.07

Data were written as mean ± SD (standard deviation), CRL = crown rump length, *significant difference as compared to pair fed and ad-libitum controls

The *in-Vivo* embryonic developments were evaluated through morphological endpoints in the circulatory, neurological, musculoskeletal and craniofacial systems (Fig.10). The embryo developmental scores exhibited a dose-dependent decrease for each system (Table 20 and Table 21). Notably, gravid dams administered 1000 mg/kg of the essential oil showed significantly reduced developmental scores in the caudal neural tube and forebrain compared to both pair-fed and ad libitum control groups.

Table 20: Developmental scores of the nervous system of rat embryos exposed to *U. simensis* essential oil

Groups	Caudal neural tube	Hind brain	Mid brain	Fore brain	Otic system	Optic system	Olfactory system
Group I - 250 mg/kg	3.08±0.67	2.82±0.70	2.56±0.77	3.01±0.68	2.99±0.64	2.49±0.67	2.88±0.71
Group II - 500 mg/kg	3.03±0.69	2.76±0.69	2.48±0.74	2.90± 0.67	2.93±0.60	2.44±0.62	2.82±0.68
Group III - 1000 mg/kg	2.83±0.71*	2.72±0.70	2.44±0.72	2.81±0.69*	2.84±0.65	2.41±0.63	2.77±0.70
Group IV - pair fed	3.14±0.68	2.97±0.73	2.53±0.72	3.11±0.68	3.07±0.60	2.59±0.70	2.96±0.73
Group V - ad libitum	3.12± 0.70	2.96±0.74	2.68±0.83	3.14±0.67	3.09±0.63	2.64±0.74	3.00±0.76

Data were written as mean ± SD (standard deviation), *significant difference as compared to pair fed and ad-libitum control

Table 21: Developmental scores of cardiovascular and musculoskeletal of rat embryos exposed to *U. simensis* essential oil

Groups	Yolk sac circulation	Heart	Flexion	Branchial bars	Maxillary process	Mandibular process	Fore limb	Hindlimb
Group I-250 mg/kg	3.05±0.71	2.84±0.64	2.40±0.50	2.98±0.61	1.58±0.49	0.84±0.37	2.16±0.76	1.59±0.49
Group II-500 mg/kg	3.01±0.75	2.78±0.65	2.35±0.48	2.90±0.64	1.51±0.50	0.78±0.42	2.11±0.74	1.53±0.50
Group III - 1000 mg/kg	2.99±0.71	2.76±0.64	2.33±0.52	2.88±0.63	1.49±0.50	0.73±0.44	2.10±0.73	1.52±0.50
Group IV - pair fed	3.10±0.74	2.88±0.67	2.44±0.50	3.04±0.65	1.59±0.49	0.84±0.36	2.24±0.74	1.64±0.51
Group V - ad libitum	3.14±0.80	2.90±0.66	2.46±0.50	3.07±0.64	1.62±0.48	0.85±0.36	2.27±0.73	1.68±0.55

Data were written as mean ± SD (standard deviation)

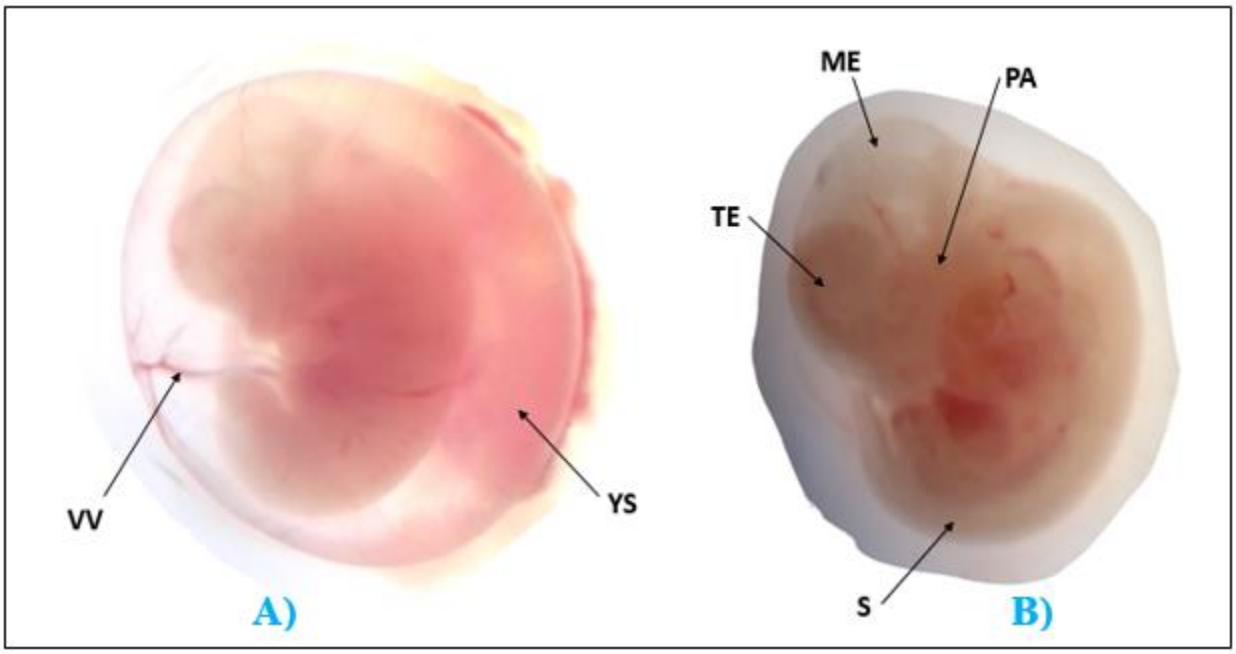

Figure 10: *In-Vivo* developments of 12-day old rat embryos exposed to *U. simensis* essential oil: **A)** 1000 mg/kg, **VV**-Vitelline Blood Vessels, **YS**-Yolk Sac, **B)** ad libitum control, **TE**-Telencephalon, **ME**-Mesencephalon, **PA**-Pharyngeal Arch, **S**-Somites

5.6.6 Effects of *U. simensis* essential oil on fetal outcomes

No dead fetuses were found in any of the experimental groups. The mean values of the implantation and resorption sites per litter were computed and analyzed for both the treatment and control groups. The pregnant dams treated with 1000 mg/kg of essential oil showed a statistically significant increase in resorption per litter compared to both the pair fed and ad libitum control groups. However, there was no statistically significant difference in the implantation sites per litter between the gravid rats treated with the essential oil and the control groups (Table 22 and Fig. 11).

Table 22: Fetal outcomes of gravid rats treated with *U. simensis* essential oil

Groups	Live fetuses	Dead fetuses	Implantation sites/litter	Resorption sites/litter	Male fetuses /dam	Female fetuses/dam
Group I-250 mg/kg	92	0	9.20±0.78	0.40±0.52	4.30±1.45	4.80±1.23
Group II-500 mg/kg	90	0	9.00±0.83	0.50±0.71	4.20±0.79	4.80±0.78
Group III-1000 mg/kg	91	0	9.10±0.87	1.30±1.25*	4.10±1.37	5.00±1.05
Group IV-Pair fed	92	0	9.20±0.1.14	0.30±0.48	4.40±1.71	4.80±1.68
Group V-ad libitum	93	0	9.30±1.34	0.20±0.42	4.40±1.58	4.90±1.91

Data were written as mean ± SD (standard deviation), *significant difference as compared to pair fed and ad-libitum controls

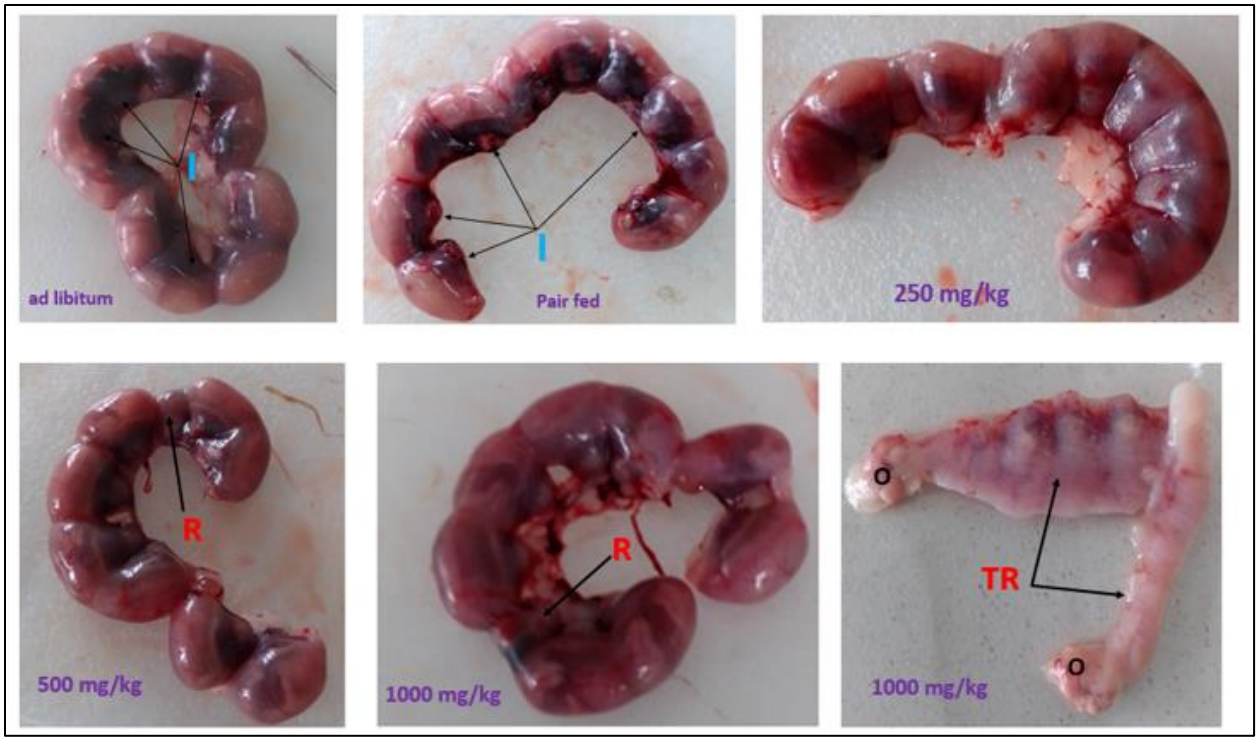

Figure 11: Implantations and resorptions of 20-day old rat fetuses exposed to *U. simensis* essential oil: **I**-implantation, **R**-resorption, **TR**-total resorption only in one gravid rat, **O**-ovary

5.6.7 Effects on fetal growth and androgen dependent endpoints

The mean fetal weight in gravid rats treated with 1000 mg/kg of essential oil was 3.47±0.18 g, significantly lower than that of the pair fed (3.55±0.17 g) and ad libitum (3.56±0.18 g) control groups. The mean CRL was also significantly lower in the high-dose treated group (4.54±0.18 cm) as compared to the pair fed (4.62±0.15 cm) and ad libitum (4.63±0.17 cm) controls. However, there was no statistically significant difference in the mean weight of fetal placentas between the treatment and control groups. The anogenital distance, presence of multiple nipples/areola and external genital abnormalities were used to assess the essential oil's androgen related effects. However, there were no visible abnormalities in the nipples, areola, and external genitalia. There were also no significant differences in AGD between the treatment groups exposed to essential oil and the control groups in both male and female fetuses (Table 23).

Table 23: Fetal growth, anogenital distance and placental weight of rat fetuses treated with *U.simensis* essential oil

Groups	Fetal weight (g)	CRL (cm)	Female AGD (mm)	Male AGD (mm)	Placental weight (g)
Group I - 250 mg/kg	3.53±0.17	4.58±0.16	4.44±0.78	6.02±0.62	0.54±0.06
Group II - 500 mg/kg	3.52±0.16	4.56±0.16	4.42±0.71	5.95±0.66	0.53±0.06
Group III - 1000 mg/kg	3.47±0.18*	4.54±0.18*	4.40±0.75	5.73±0.63	0.52±0.06
Group IV - pair fed	3.55±0.17	4.62±0.15	4.48±0.82	5.93±0.72	0.54±0.06
Group V - ad libitum	3.56±0.18	4.63±0.17	4.49±0.80	6.05±0.60	0.55±0.07

Data were written as mean ± SD (standard deviation), *significant difference compared to pair fed and ad libitum controls, CRL = crown rump length, AGD = anogenital distance

5.6.8 Effects on fetal external and visceral structural formations

The explanted fetuses were assessed from the head to tail for the treatment related developmental defects (Fig 12). Limb deformities, tail abnormalities, craniofacial malformations, vertebral column discrepancies, and obvious genital defects were examined, but no discrete external anomaly was observed.

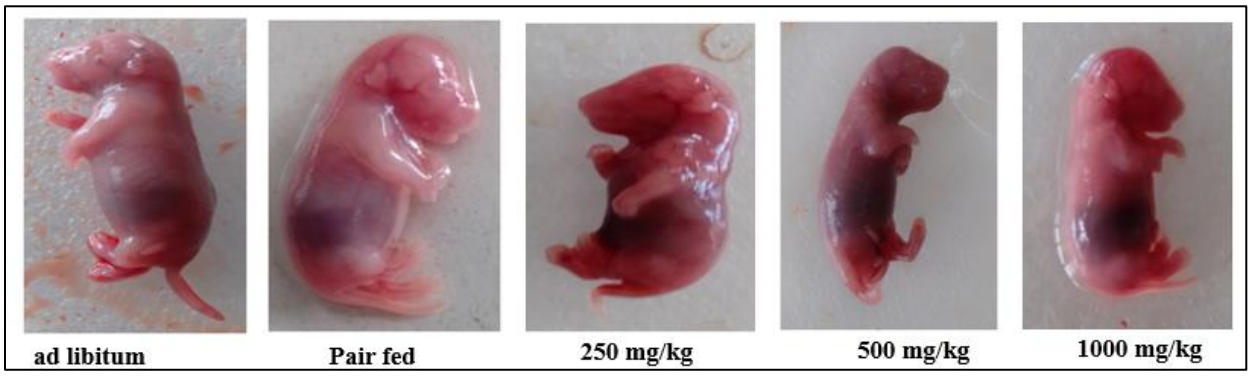

Figure 12: External examination of 20-day old rat fetuses exposed to *U. simensis* essential oil

In addition to the external examinations, the fetuses fixed in the Bouin's solution were serially sectioned and examined for visceral anomalies (Fig 13). The head, neck, chest, abdomen and abdominopelvic regions were thoroughly inspected for any anomalies using a dissecting microscope. Conditions such as cleft palate, hydrocephalus, eye-related anomalies, and abnormalities in the neck, trachea, and cardiac septum were assessed in the head and neck regions. Furthermore, the diaphragmatic hernia, agenesis of abdominal viscera, and external genitalia were also examined. However, no visible visceral abnormalities were observed through the dissecting microscope.

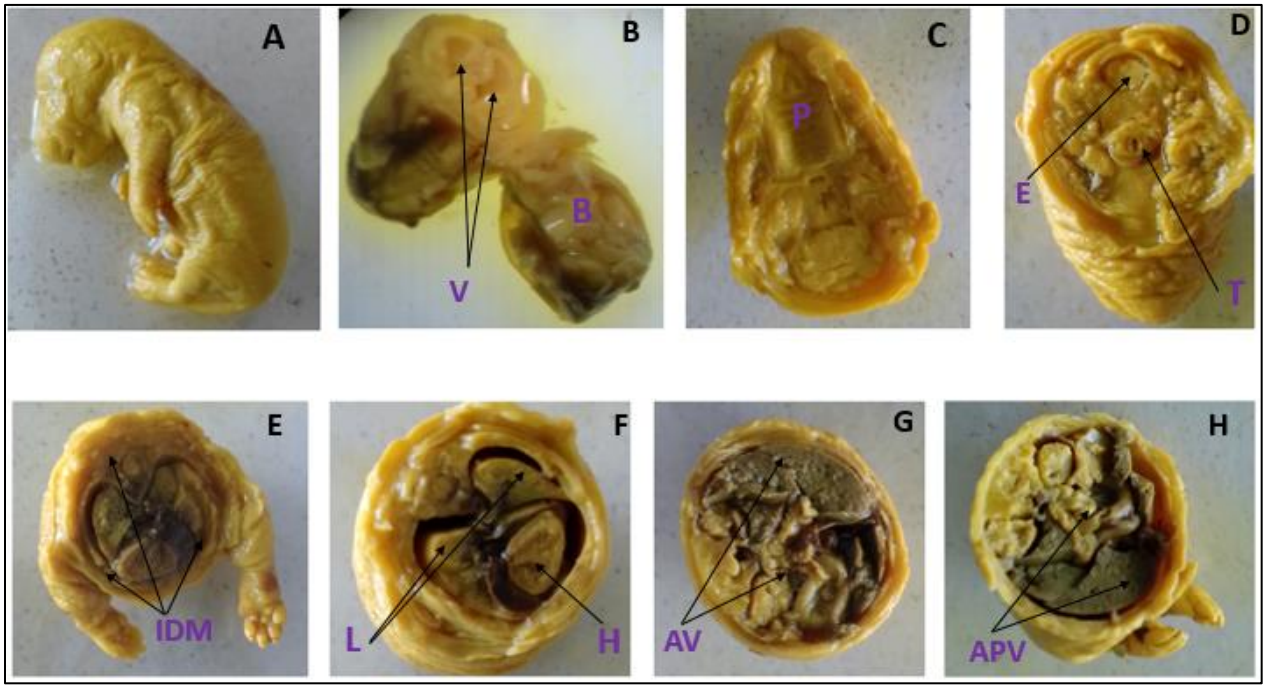

Figure 13: Visceral examinations of Bouin's solution fixed 20-day old rat fetuses exposed to 1000 mg/kg of *U. simensis* essential oil:(**A**) un-sectioned fetus, (**B**) coronal section of head, **V**-ventricles, **B**- brain tissue, (**C**) section made through jaw and above the ear, **P**-palate, (**D**) transverse section of neck, **E**-esophagus, **T**-trachea, (**E**) transverse section of thorax, **IDM**-intact diaphragm, (**F**) mediastinal viscera, **L**-lung, **H**-heart, (**G**) horizontal section of abdomen, **AV**-abdominal viscera-liver and intestine, (**H**) horizontal section of abdominopelvic region, **APV**-abdominopelvic viscera

5.6.9 Effects of *U. simensis* essential oil on fetal skeletal ossifications

As shown in Table 24, the mean ossification centers in the axial and extremity bones of 20 days old rat fetuses treated with the *U. simensis* essential oil were counted and analyzed. However, there was no significant difference in the skeletal ossifications between the essential oil-treated and control animals, whether pair fed or ad libitum controls (Fig 14).

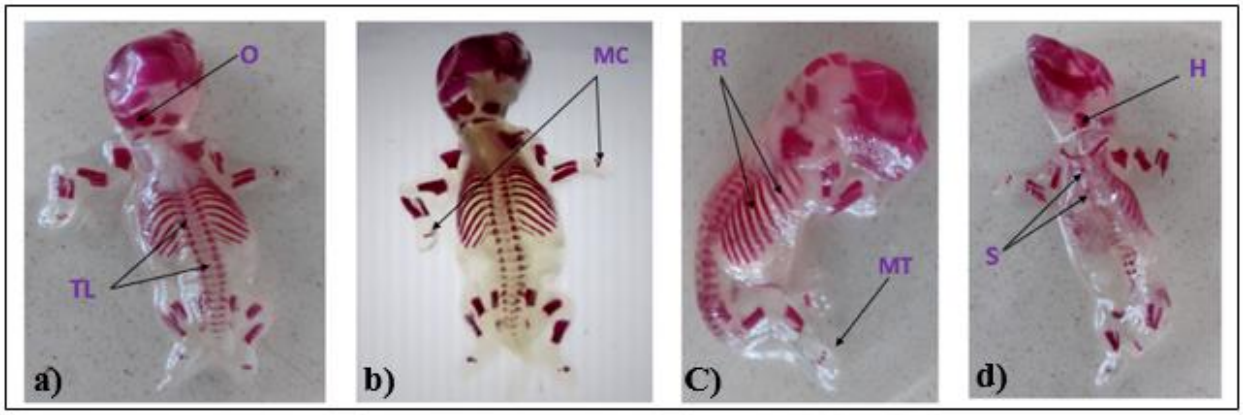

Figure 14: Photomicrographs of alizarin red stained 20-day old rat fetuses exposed to *U. simensis* essential oil showing skeletal ossifications: **a)** ad libitum control, **TL**-Thoracic and Lumbar vertebrae, **O**-Occipital bone, **b)** 250 mg/kg, **MC**-Metacarpals, **c)** 500 mg/kg, **R**- Ribs, **MT**-Metatarsals, **d)** 1000 mg/kg, **H**-Hyoid, **S**-Sternum

Table 24: Axial and extremity bone ossification centers of rat fetuses treated with *U. simensis* essential oil

Groups	number of ossification centers per group								
	Sternum	Ribs	Thoracic	Lumbar	Caudal	FL phalanges	HL phalanges	Metacarpals	Metatarsals
Group I-250 mg/kg	4.00±0.71	24±0.0	12±0.0	5±0.0	3.92±0.57	3.72±0.45	3.64±0.49	4.12±0.44	3.84±0.47
GroupII-500 mg/kg	3.96±0.74	24±0.0	12±0.0	5±0.0	3.88±0.53	3.68±0.47	3.56±0.50	4.08±0.40	3.80±0.41
GroupIII-1000 mg/kg	3.84±0.62	24±0.0	12±0.0	5±0.0	3.80±0.64	3.60±0.50	3.52±0.51	4.04±0.54	3.76±0.44
Group IV - pair fed	4.08±0.64	24±0.0	12±0.0	5±0.0	4.04±0.54	3.72±0.46	3.60±0.50	4.16±0.55	3.84±0.37
Group V - ad libitum	4.20±0.65	24±0.0	12±0.0	5±0.0	4.12±0.52	3.76±0.43	3.68±0.48	4.20±0.64	3.88±0.33

Results are written as mean ± SD (standard deviation), FL = Fore limb, HL = Hind limb

5.6.10 Effects of *U. simensis* essential oil on histology of the placenta

A microscopic analysis of the placenta was performed to evaluate any histological irregularities, such as necrosis and hemorrhage (Table 25). However, no histological abnormalities were observed in any of the experimental groups (Fig 15).

Table 25: Histopathological scoring of fetal placenta exposed to *U. simensis* essential oil

Groups	Necrosis	Hemorrhage	Intervillous thrombosis	Calcification	Vascular dilatation
Group I - 250 mg/kg	0	0	0	0	0
Group II - 500 mg/kg	0	0	0	0	0
Group III - 1000 mg/kg	0	0	0	0	0
Group IV - Pair fed	0	0	0	0	0
Group V - ad libitum	0	0	0	0	0

Zero (0) values indicate no pathological lesion for each fetal placenta

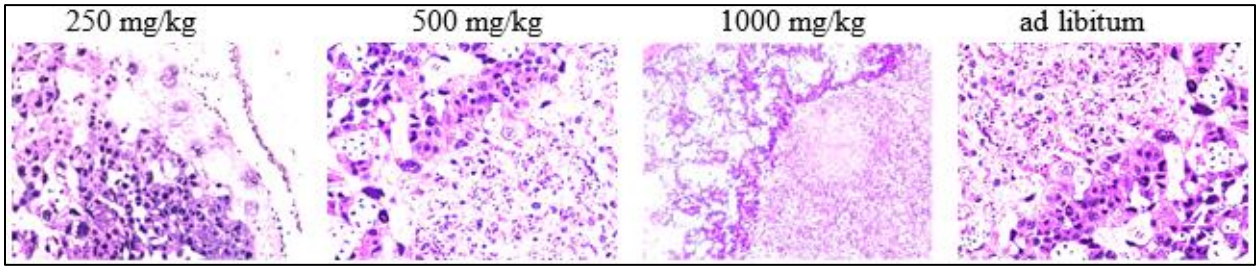

Figure 15: Photomicrographs of fetal placentas (H and E stain, 100x total magnification): Microscopic examinations of fetal placentas exposed to *U. simensis* essential oil at doses of 250, 500 and 1000 mg/kg alongside the ad libitum control. All groups exhibit normal placenta histological architecture

5.7 Reproductive toxicity of *U. simensis* leaf extract

5.7.1 Clinical observations

Daily cage-side clinical observations were conducted throughout the treatment period. The observations showed no changes in the skin, fur or mucous membranes of the oral and nasal cavities. In addition, no abnormalities in the respiratory patterns, locomotor activity, self-mutilation or other signs of toxicity were observed, and no mortality occurred.

5.7.2 Effects on food intake and body weight changes

The daily food intake and body weight changes of female parental rats treated with *U. simensis* leaf extract in the pre-gestational, gestational and lactational periods are presented in Table 26. All experimental groups of animals (250, 500 and 1000 mg/kg groups) showed comparable daily food intake to that of the control group. The pre-gestational weight gain was significantly higher in the 500 and 1000 mg/kg treatment groups compared to the controls. However, the 1000 mg/kg treated group during gestation exhibited significantly lower weight gain compared to the corresponding controls. Lactational weight changes remained similar across all groups.

Table 26: Food intake and body weight changes of female parental rats treated with *U. simensis* leaf extract

Groups	Food intake (g/day)	Pre-gestational weight gain (g)	Gestational weight gain (g)	Lactational weight gain (g)
G1-250 mg/kg	415.30±3.45	13.10±2.95	56.65±4.61	9.30±2.35
GII-500 mg/kg	413.28±3.27	13.80±1.82*	54.65±5.05	9.23±2.62
GIII-1000 mg/kg	414.36±2.42	14.30±1.63*	52.45±6.98*	8.95±2.49
GIV-control	415.99±2.60	12..90±1.29	57.40±2.47	9.58±2.16

Results were written as mean and standard deviation, *statistically significant as compared to control groups

The effects of *U. simensis* leaf extract on the daily food intake and body weight changes in male parental rats were assessed in comparison to the control group (Table 27). All the treated groups showed higher weight gain than the control group. The 1000 mg/kg treated group had the highest weight gain (55.20 ± 2.16), which was statistically significant compared to the control group and the 250 mg/kg treated group. The 500 mg/kg treated group also showed notable weight gains (54.85 ± 8.25), but not statistically significance as compared to the control group.

Table 27: Food intake and body weight changes of male parental rats treated with *U. simensis* leaf extract

Groups	Food intake (g/day)	Initial weight	Final weight	Weight gain
G1-250 mg/kg	415.17±7.56	243.30 ±4.54	290.65±4.41	47.35± 4.19
G II-500 mg/kg	414.83±4.57	240.60±5.08	295.45±5.63	54.85±8.25
GIII-1000 mg/kg	414.51±5.10	242.95±4.43	298.15±3.67	55.20±2.16$^{*!}$
GIV-control	415.29±6.56	243.85±4.62	291.00±4.55	42.95±1.98

Results were written as mean and standard deviation, *statistically significant as compared to control groups, $^{!}$statistical significant as compared to the 250 mg/kg treated groups

5.7.3 Effects of *U. simensis* leaf extract on the estrous cycles

The effects of *U. simensis* leaf extract on estrous cycle duration in the female parental rats were assessed across different dose groups (250, 500, and 1000 mg/kg) in comparison to the control group (Table 28). The 1000 mg/kg treated group showed a significantly longer estrous cycle (4.80 ± 0.61 days) as compared to both the controls (4.30 ± 0.85 days) and the 250 mg/kg treated group (4.35 ± 0.50 days). The 250 mg/kg and 500 mg/kg groups had estrous cycles similar to the control group, suggesting no significant effect at lower doses.

Table 28: Mean estrous cycles of female parental rats treated with *U. simensis* leaf extract

Groups	mean estrous cycle (days/dam)
G1-250 mg/kg	4.35±0.50
G II-500 mg/kg	4.40±0.72
GIII-1000 mg/kg	4.80±0.61*!
GIV-control	4.30±0.85

Results were written as mean and standard deviation, *statistically significant as compared to control groups, !statistical significant as compared to the 250 mg/kg treated groups

5.7.4 Effects of *U. simensis* leaf extract on the reproductive indices

Table 29 shows the reproductive indices in parental rats treated with *U. simensis* leaf extract. There were no detrimental effects on the mating or fertility indices. All the tested and control groups demonstrated full success in the mating index (males and females), fertility index (males and females), and pregnancy success (females produced viable litters in all groups). There was a significant increase in the time taken to mate (pre-coital interval) at the higher doses; 500 mg/kg (5.30 ± 0.92) and 1000 mg/kg (6.50 ± 1.98) showed prolonged mating latency compared to both the control (4.60 ± 1.14) and 250 mg/kg treated group (4.10 ± 1.16). However, no significant differences were observed in the gestational index, which remained at hundred percent across all groups, nor in the duration of pregnancy, which was approximately 21 days for all groups.

Table 29: Reproductive indices of parental rats treated with *U. simensis* leaf extract

Groups	GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
Paired	20	20	20	20
Mated	20	20	20	20
Males siring a litter	20	20	20	20
Females being pregnant	20	20	20	20
Females delivered viable litter	20	20	20	20
Males mating index	100	100	100	100
Females mating index	100	100	100	100

Male fertility index	100	100	100	100
Female fertility index	100	1000	100	100
Pre-coital interval	4.10±1.16	5.30±0.92*!	6.50±1.98*!	4.60±1.14
Gestational index	100	100	100	100
Duration of pregnancy	21.50±0.68	21.20±0.41	21.60±0.68	21.70±0.80

Results were written as mean and standard deviation, *statistically significant as compared to control groups, !statistical significant as compared to the 250 mg/kg treated groups

5.7.5 Effects on birth outcomes

The birth outcomes in the gravid rats treated with *U. simensis* leaf extract were presented in Table 30. The litter size (total pups per dam) significantly reduced in the 500 mg/kg (10.30 ± 0.92) and 1000 mg/kg (10.10 ± 1.16) treated groups compared to the control (11.30 ± 1.12) groups. The 250 mg/kg group (10.90 ± 0.85) treated showed no significant differences, suggesting a dose-dependent decrease in litter size at higher doses. The statistical analysis showed no significant effect on the number of male or female pups per litter across the groups. The sex ratio (M/F) remained unchanged, indicating that there was no selective impact on the survival of male or female offspring. The zero dead pups in all experimental groups, confirming the no adverse effects of the *U. simensis* leaf extract on the neonatal survival.

Table 30: Birth outcomes of female parental rats treated with *U. simensis* leaf extract

	GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
Total pups /dam	10.90±0.85	10.30±0.92*	10.10±1.16*!	11.30±1.12
Male pups/dam	4.80±1.19	4.90±1.07	4.10±0.85	5.40±0.68
Female pups /dam	6.10±0.71	5.40±1.23	6.00±1.02	5.90±1.16
Sex ratio (M/F)	0.81±0.27	1.00±0.51	0.71±0.24	0.95±0.23
Dead pups/dam	0	0	0	0

Results were written as mean and standard deviation, *statistically significant as compared to control groups, !statistical significant as compared to the 250 mg/kg treated groups

5.7.6 Effects on the lactational weight of pups

Table 31 shows the postnatal weights of pups exposed to the *U. simensis* leaf extract. At the PND zero, the male pups in the 1000 mg/kg treated group had significantly lower weight (4.30 ± 0.65) compared to the control (5.10 ± 0.71) group. However, there was no significant difference in the 250 and 500 mg/kg treated groups as compared the control group. In addition, there were no significant differences in the birth weight of female pups in the extract-treated and control groups at the PND zero. The male pups from the 1000 mg/kg treated group had gained significantly more weight (9.00 ± 0.72) than the corresponding control group (7.55 ± 0.68) during early postnatal growth (PND 4). Female pups showed no significant difference. There were no significant variations in the male or female pup weights after PND 4 in the later growth (PND 7, 14, 21). Female pups in high-dose treated groups (500 and 1000 mg/kg) had a non-significant trend towards lower weight at PND 7 and 21, but this was not statistically significant.

Table 31: Postnatal weights of pups exposed to *U. simensis* leaf extract

	Sex of pups	GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
PND 0	Male	4.65±0.55	4.40±0.50	4.30±0.65*	5.10±0.71
	Female	4.60±0.75	4.55±0.82	4.45±1.09	4.75±0.85
PND 4	Male	7.44±0.91	8.15±0.74	9.00±0.72*	7.55±0.68
	Female	7.35±0.93	7.30±1.03	7.30±1.17	7.60±1.23
PND 7	Male	10.00±1.14	10.05±0.75	10.15±0.74	10.10±0.71
	Female	9.20±0.69	8.75±0.78	8.50±0.68	9.35±0.58
PND 14	Male	16.15±1.98	17.45±1.31	17.70±1.17	17.10±1.48
	Female	15.45±1.63	15.30±1.75	15.25±1.51	15.70±1.38
PND 21	Male	28.25±3.19	29.20±1.47	29.30±1.89	29.15±1.53
	Female	26.25±1.77	25.75±1.68	25.65±1.87	27.30±2.00

Results were written as mean and standard deviation, *statistically significant as compared to control groups

5.7.9 Effects on the androgen dependent endpoints

Table 32 presents the anogenital distances (AGD) of male and female rat pups exposed to the varying doses of *U. simensis* leaf extract, measured on PND 0. Exposure to 250 mg/kg dose resulted in the minimal changes as compared to the control groups. At 500 mg/kg dose, AGD decreased to 4.70 ± 0.73 mm in the male pups and 3.20 ± 0.61 mm in the female pups. The 1000 mg/kg dose induced a statistically significant reduction in both sexes, with males at 4.35 ± 0.81 mm and females at 3.10 ± 0.64 mm.

Table 32: Anogenital distances of pups exposed to the *U. simensis* leaf extract

	Sex of pups	Anogenital distance (mm) /pup
GI-250 mg/kg	Male	5.30±0.47
	Female	3.35±0.67
GII-500 mg/kg	Male	4.70±0.73
	Female	3.20±0.61
GIII-1000 mg/kg	Male	4.35±0.81*
	Female	3.10±0.64*
GIV- control	Male	5.40±0.50
	Female	3.45±0.51

Results were written as mean and standard deviation, *statistically significant as compared to control groups

5.7.8 Effects on the sperm count and morphology

The sperm morphologies of male rats treated with the *U. simensis* leaf extract have been examined (Table 33). There was no significant difference in total sperm count across the groups (205-207 × 10^6/mL). The extract also has no effect on the sperm production at all dose levels (250-1000 mg/kg). The 1000 mg/kg treated group had a slight but statistically significant increase in the abnormal sperm morphology compared to the control group.

Table 33: Sperm counts of male parental rats treated with *U. simensis* leaf extract

	GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
Total counts (10^6/ml	206.60±11.23	205.60±12.14	205.00±11.64	207.20±11.11
Abnormal sperms (%)	9.30±2.34	9.45±2.85	10.20±2.62*	9.10±1.99

Results were written as mean and standard deviation, *statistically significant as compared to control groups

5.7.9 Effects on the reproductive organ weights

Table 34 shows the reproductive organ weights of both male and female parental rats treated with *U. simensis* leaf extract. The female reproductive organs did not show any significant weight changes across all treatment doses as compared to the corresponding control groups. The weights of fallopian tube and uterus revealed slight reductions at the 500 and 1000 mg/kg treatment doses, but no statistical significance compared to the control groups. Similarity, the weights of male reproductive organs, which include the testes, epididymis, seminal vesicles, and prostate, showed no significant differences in all doses when compared to the control group.

Table 34: Reproductive organ weights of both parental rats treated with *U. simensis* leaf extract

	GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
Female parental rats				
Ovary (g)	0.28±0.01	0.28±0.02	0.27±0.03	0.31±0.01
Fallopian tube (g)	0.39±0.063	0.38±0.05	0.36±0.05	0.42±0.08
Uterus with cervix (g)	0.31±0.01	0.30±0.01	0.30±0.01	0.32±0.02
Male parental rats				
Testes (g)	2.73±0.24	2.72±0.20	2.71±0.17	2.72±0.25
Epididymis (g)	1.79±0.03	1.78±0.05	1.78±0.04	1.79±0.05
Seminal vesicles (g)	1.45±0.07	1.43±0.07	1.42±0.06	1.44±0.06

Prostate gland (g)	0.38±0.02	0.38±0.01	0.37±0.02	0.39±0.03

Results were written as mean and standard deviation

The male parental rats treated with the *U. simensis* leaf extract (250, 500 and 1000 mg/kg) revealed the normal microscopic testicular structures (Fig.16). This suggests that the leaf extract, at the administered doses, did not have any adverse effects on the histological architecture of the testes. The seminiferous tubules are tightly packed with clear intact basement membranes. These tubules contain cells at the various stages of spermatogenesis, but it was difficult to distinguish each cell types clearly at this level of magnifications. The interstitial cells are hormone producing cells located in the spaces between the seminiferous tubules. The Sertoli cells are supportive nurse cells inside the seminiferous tubules.

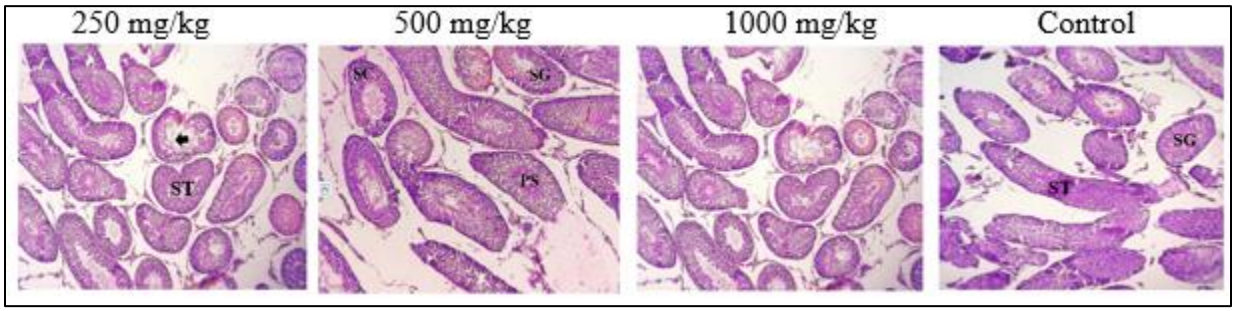

Figure 16: Microscopic examination of testicular tissue sections taken from male parental rats (H and E stain, 100x total magnification) treated with 250, 500 and 1000 mg/kg *U. simensis* leaf extract and the control group showing normal structures, developing spermatocytes (arrow head), seminiferous tubules (**ST**), spermatogonia (**SG**), and primary spermatocytes (**PS**)

Similarly, the female parental rats treated with 250, 500 and 1000 mg/kg doses of *U. simensis* leaf extract showed normal ovarian histological architectures as that of the control groups (Fig. 17). The leaf extract did not disrupt the normal histology of the ovary. It did not deplete the pool of primordial follicles. In addition, there was no evidence of follicular atresia, inflammation or cystic formations.

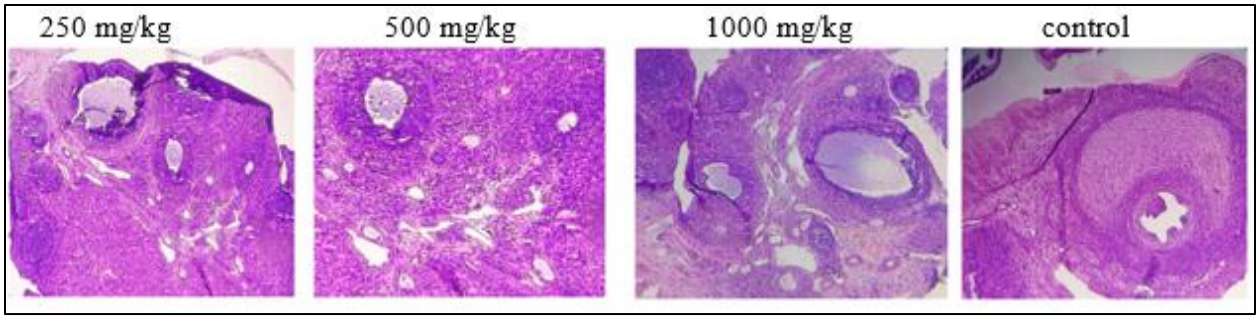

Figure 17: Microscopic examination of ovarian tissue sections taken from female parental rats (H and E stain, 100x total magnification) treated with 250, 500 and 1000 mg/kg of *U. simensis* leaf extract and the control group showing normal ovarian histological structures

5.7.10 Hepato-renal effects of *U. simensis* leaf extract

Table 35 displays the liver and kidney weights of the parental rats treated with *U. simensis* leaf extract. The weights of the liver in the male rats treated with 250, 500 and 1000 mg/kg of *U. simensis* leaf extract had no significant differences as compared to the control group. A slight rise of liver weight was observed at 1000 mg/kg treated male rats; however, it was not statistically significant. Similarly, the female parental rats treated with *U. simensis* leaf extract did not show any significant differences of liver weights as compared to that of the control female parental rats. Furthermore, there were no significant differences in the kidney weights of both parental rats treated with *U. simensis* leaf extract at all dose levels.

Table 35: Liver and kidney weights in parental rats treated with *U. simensis* leaf extract

		GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
Liver	Male	10.20±0.76	10.40±0.88	10.75±1.06	10.00±0.91
	Female	8.80±0.76	8.95±0.75	9.20±0.95	8.65±0.67
Kidneys	Male	1.38±0.05	1.39±0.05	1.41±0.06	1.37±0.05
	Female	1.27±0.06	1.27±0.05	1.28±0.05	1.26±0.05

Results were written as mean and standard deviation

The biochemical profiles of both male and female parental rats treated with *U. simensis* leaf extract (250,500 and 1000 mg/kg) are presented in Table 36. The functional biomarkers of the liver, kidney and metabolism were analyzed. The liver functional enzymes (ALT, AST and ALP) were significantly increased in both male and female parental rats treated with 1000 mg/kg dose as compared to the control groups. However, there were no significant changes in the kidney function markers (urea and creatinine) in both parental rats treated with the plant leaf extract at all doses. At the 1000 mg/kg of *U. simensis* leaf extract, the female parental rats had significantly higher total protein levels than the corresponding control groups.

Table 36: Biochemical profiles of parental rats treated with *U. simensis* leaf extract

		GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
ALT (U/L)	M	47.60±5.13	48.25±5.32	52.30±6.82$^{*!}$	46.85±5.08
	F	60.55±3.05	61.05±3.63	63.70±5.23*	60.20±3.23
AST(U/L)	M	130.80±5.92	132.00±6.75	136.70±9.11$^{*!}$	130.05±5.78
	F	168.80±6.10	169.45±5.76	173.10±6.64*	167.80±5.80
ALP (U/L)	M	73.20±2.62	74.20±3.54	76.20±4.14$^{*!}$	72.35±2.34
	F	117.60±1.90	117.95±2.21	119.30±3.46*	116.90±1.77
Urea (mg/dL)	M	53.20±2.62	53.55±2.66	54.40±3.53	52.20±2.41
	F	64.40±3.70	65.40±4.38	65.90±6.03	63.95±3.66
Creatinine (mg/dL)	M	0.44±0.02	0.45±0.02	0.45±0.03	0.43±0.02
	F	0.41±0.02	0.42±0.03	0.43±0.04	0.40±0.02
Albumin (g/dL)	M	4.13±0.29	4.14±0.30	4.15±0.31	4.12±0.30
	F	3.90±0.25	3.93±0.24	3.96±0.26	3.88±0.25
Total protein (g/dL)	M	6.00±0.64	6.15±0.81	6.40±1.04	5.85±0.67
	F	5.55±0.88	5.85±0.93	6.10±0.78*	5.25±0.78

Glucose (mg/dL)	M	112.20±5.63	113.20±5.50	115.20±4.93	111.05±5.15
	F	109.60±4.61	110.95±3.91	111.35±5.21	108.60±4.63
Total cholesterol (mg/dL)	M	43.80±1.19	44.00±1.16	44.20±1.47	43.50±1.43
	F	58.40±4.56	59.40±4.59	60.05±5.06	57.15±4.59

Results were written as mean and standard deviation, *statistically significant as compared to control groups, !statistical significant as compared to the 250 mg/kg treated groups

The ethanol leaf extract of *U. simensis* has a dose-dependent effects on the liver histology in male parental rats. At the 250 and 500 mg/kg doses, the *U. simensis* leaf extract appears safe, causing no observable damage to the liver tissues. However, at the 1000 mg/kg dose, two male parental rats showed liver parenchymal necrosis (Fig.18).

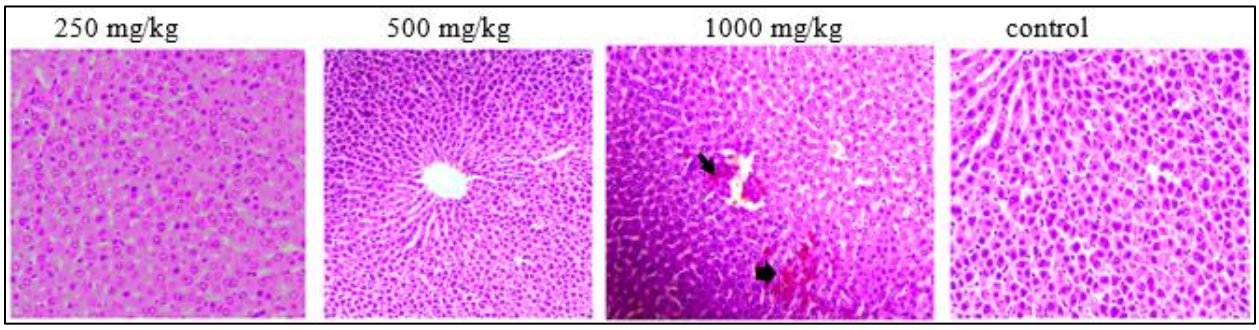

Figure 18: Microscopic examination of liver tissue sections taken from male parental rats treated with *U. simensis* leaf extract (H&E stain, 100x total magnification). The male rats treated with 250 and 500 mg/kg exhibit normal hepatic histological architecture as the control group. However, two male rats treated with 1000 mg/kg revealed liver parenchymal necrosis (arrow heads)

Figure 19 shows the histopathological effects of the *U. simensis* leaf extract on the liver of female parental rats. The hepatic tissue architecture remained normal and comparable to the control group in the female parental rats treated with 250 mg/kg of *U. simensis* leaf extract. However, evidence of liver parenchymal necrosis was observed in one female parental rat treated with 500 mg/kg of

U. simensis leaf extract and five female parental rats treated with 1000 mg/kg of *U. simensis* leaf extract.

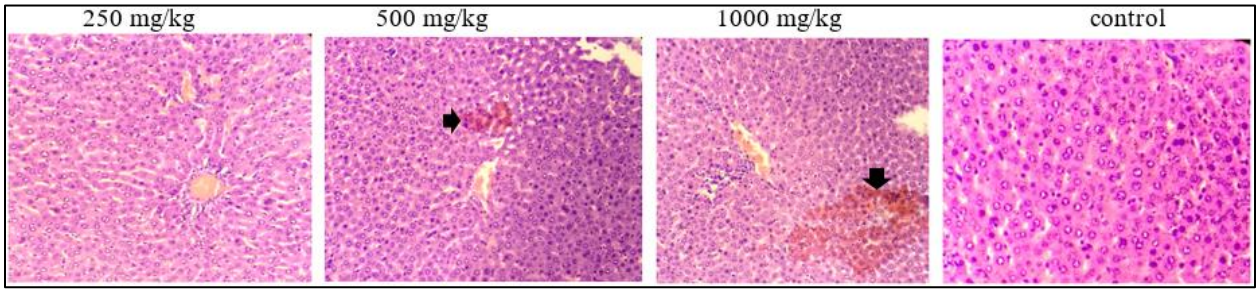

Figure 19: Microscopic examination of liver tissue sections taken from female parental rats treated with *U. simensis* leaf extract (H&E stain, 100x total magnification). The female rats treated with 250 mg/kg exhibit normal hepatic histological architecture similar to that of the control group. However, one female rat that received 500 mg/kg and five female rats treated with 1000 mg/kg dose showed evidence of liver parenchymal necrosis (arrowheads).

The microscopic examinations of kidney tissue sections taken from parental male rats treated with *U. simensis* leaf extract revealed normal renal histological architecture in all treatment and control groups (Fig. 20). The glomeruli, renal tubules and interstitial tissues in the parental male rats treated with 250, 500 and 1000 mg/kg were intact and comparable to those observed in the vehicle control group. These indicate that the *U. simensis* leaf extract did not induce any detectable renal histological aberrations at all administered doses.

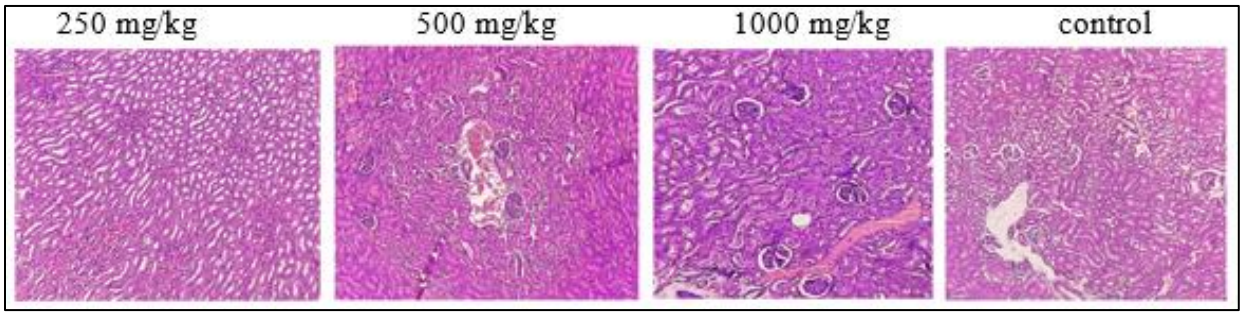

Figure 20: Microscopic examination of renal tissue sections taken from male parental rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal renal histological architecture

The histological assessment of renal tissue sections taken from the female parental rats treated with *U. simensis* leaf extract demonstrated normal renal architecture across all dose levels (Fig.21). The microscopic examination revealed intact glomerular structure, normal renal tubules, and unaffected interstitial tissue, with no observable differences between the *U. simensis* leaf extract treated groups and the vehicle control groups. The histological analysis showed absence of treatment related nephrotoxicity at the administered doses in all female parental rats.

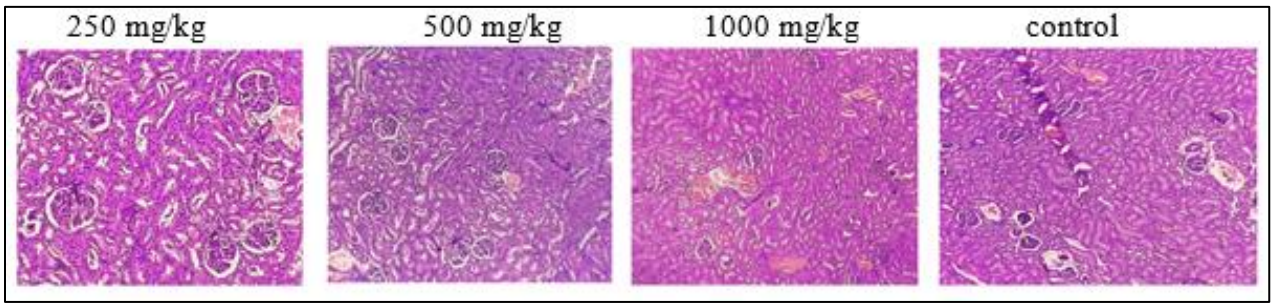

Figure 21: Microscopic examination of renal tissue sections taken from female parental rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal renal architecture

5.8 Extended first generation (F1) developmental toxicity of *U. simensis* leaf extract

5.8.1 Developmental reproductive toxicity /F1 cohort-1 rats

5.8.1.1 Food intake and body weight effects

Table 37 reveals the dose dependent and sex-specific effects of the *U. simensis* leaf extract on the daily food intake and body weight gains of the first generation (F1) offspring cohort-1 rats. While the food intake exhibited a non-significant decrease in both male and female cohort-1 rats across all the treatment doses (250,500, 1000 mg/kg) in comparison to the control group. Despite the reduced food intake, F1 cohort-1 male offsprings had significant weight gain at the highest dose (1000 mg/kg) compared to the corresponding control groups, whereas the female cohort-1

offspring showed a significant weight gain at both 500 and 1000 mg/kg doses of *U. simensis* leaf extract.

Table 37: Food intake and bod weight gains of F1 cohort-1 rats treated with *U. simensis* leaf extract

		GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
Food intake (g)	M	405.31±2.89	404.42±2.62	401.43±2.63	407.85±2.70
	F	390.30±3.44	388.13±3.27	385.86±2.71	392.99±2.60
Weight gain (g)	M	122.40±1.90	123.50±3.5	125.40±5.98*	120.80±1.82
	F	115.00±3.49	116.40±3.53*	117.50±5.83*	111.75±3.95

Results were written as mean and standard deviation, *statistically significant as compared to control groups

5.8.1.2 Effects of *U. simensis* leaf extract on puberty of F 1 cohort-1 rats

The sub chronic gavage administration of *U. simensis* leaf extract did not have a significant adverse effects on the timing of sexual maturation endpoints in the F1 cohort-1 rats. The puberty endpoints were assessed through the mean days of preputial separation in the males and vaginal opening in the females, as shown in Table 38. Both parameters showed nearly identical developmental timelines across all treatment groups (250, 500, 1000 mg/kg) compared to the control groups, with preputial separation occurring at approximately 42 days and vaginal opening around 32 days of age in all groups. The observed minimal variations, ranging from 42.00 to 42.85 days for the preputial separations and 31.95 to 32.75 days for the vaginal opening, were not statistically significant. This indicates that the *U. simensis* leaf extract did not have any adverse effects on the accelerating or delaying the onset of puberty in either sex at any of the tested doses.

Table 38: Preputial separation and vaginal opening of F1 cohort-1 rats treated with *U. simensis* leaf extract

	GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
Preputial separation/day	42.20±1.76	42.75±1.91	42.85±2.13	42.00±1.86
Vaginal opening/day	32.20±1.76	32.60±1.87	32.75±2.02	31.95±1.93

Results were written as mean and standard deviation

5.8.1.3 Effects on the estrous cycles of F1 cohort-1 rats

The effects of the *U. simensis* leaf extract on the estrous cycles of F1 female cohort-1 rats were presented as shown in Table 39. While the low and middle doses (250, 500 mg/kg) exhibited minimal impact, the highest dose (1000 mg/kg) resulted in a statistically significant prolongation of the estrous cycle (4.60 ± 0.59 days) compared to the control group (4.25 ± 0.55 days). There was also an increased duration of vaginal cornification (2.55 ± 0.51 days) in comparison to the control group (2.25 ± 0.55 days).

Table 39: Estrous cycles of F1 cohort-1 rats treated with *U. simensis* leaf extract

	GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
Estrous cycle /day	4.40±0.50	4.45±0.51	4.60±0.59*	4.25±0.55
Vaginal cornification/day	2.40±0.50	2.50±0.51	2.55±0.51*	2.25±0.55

Results were written as mean and standard deviation, *statistically significant as compared to control groups

5.8.1.4 Reproductive organ weight effects of F1 cohort-1 rats

The *U. simensis* leaf extract administration to F1 generation cohort-1 rats had no significant effect on the reproductive organ weights across all tested doses (250, 500, 1000 mg/kg) (Table 40). In the female offspring, there was a consistent but non-significant slight reduction of weights in all examined reproductive organs (ovaries, fallopian tubes, and uterus with cervix) compared to

control groups, with the most substantial decrease seen in the ovarian weight at the highest dose (0.36 ± 0.03 g versus control 0.40 ± 0.01 g). The male reproductive organs (testes, epididymis, seminal vesicles, and prostate gland) also showed a non-significant decreases across all treatment groups as compared to the control group.

Table 40: Reproductive organ weights of F1 cohort-1 rats treated with *U. simensis* leaf extract

	GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
F1 cohort-1 female rats				
Ovary (g)	0.38±0.01	0.37±0.02	0.36±0.03	0.40±0.01
Fallopian tube (g)	0.48±0.06	0.47±0.05	0.46±0.05	0.51±0.08
Uterus with cervix (g)	0.40±0.01	0.39±0.01	0.39±0.01	0.41±0.02
F1 cohort-1 male rats				
Testes (g)	2.83±0.24	2.82±0.20	2.81±0.17	2.82±0.25
Epididymis (g)	1.89±0.03	1.88±0.05	1.88±0.04	1.89±0.05
Seminal vesicles (g)	1.55±0.07	1.53±0.07	1.53±0.06	1.55±0.06
Prostate gland (g)	0.48±0.02	0.48±0.01	0.47±0.02	0.49±0.03

Results were written as mean and standard deviation

The microscopic evaluation of ovarian tissue sections taken from the female F1 cohort-1 rats treated with varying doses of *U. simensis* leaf extract revealed no adverse effects on ovarian histoarchitecture. As presented in Figure 22, all experimental groups, including those administered the extract at doses of 250, 500 and 1000 mg/kg, exhibited normal ovarian morphology comparable to the corresponding vehicle control group. All stages of the follicular development such as the primordial, primary, secondary and tertiary follicles were clearly identifiable and appeared normal across all treatment groups received the *U. simensis* leaf extract. The follicles were characterized by the intact oocytes, granulosa cells and theca cells. The stromal tissue surrounding the follicles showed a typical dense, cellular composition without any signs of edema, hemorrhage, or

inflammatory cell infiltration. The absence of pathological alterations such as follicular atresia, cyst formation, necrosis or disruption of the germinal epithelium in the extract-treated groups indicates that the gavage administration of *U. simensis* leaf extract, even at the highest dose of 1000 mg/kg, did not induce any overt histopathological damage to the ovarian tissue.

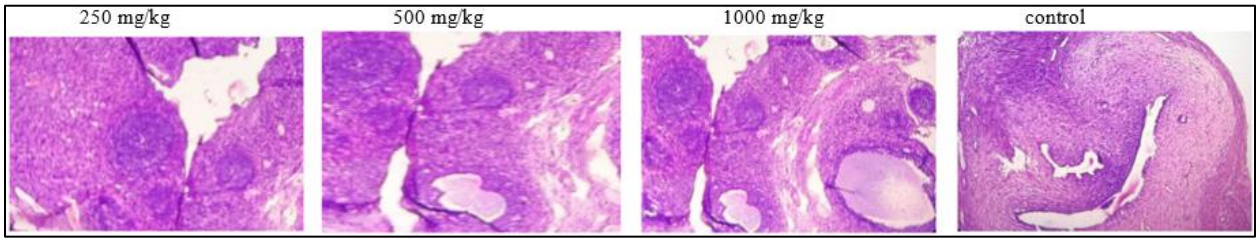

Figure 22: Microscopic examination of ovarian tissue sections taken from female cohort-1 rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500, and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal ovarian histological architecture.

As shown in Figure 23, the microscopic examination of fallopian tube tissue sections taken from all female cohort-1 experimental groups revealed the normal histological architecture, with no observable differences between the extract-treated groups (250, 500 and 1000 mg/kg) and the vehicle control group. The mucosal folds maintained a branching pattern, and the epithelial lining was intact with no evidence of erosion or atrophy. Furthermore, the muscularis layer showed no signs of hypertrophy, degeneration, or abnormal fibrosis. There was no luminal dilation, inflammatory cell infiltration, fibrosis, or edema in any of the treatment groups received the *U. simensis* leaf extract.

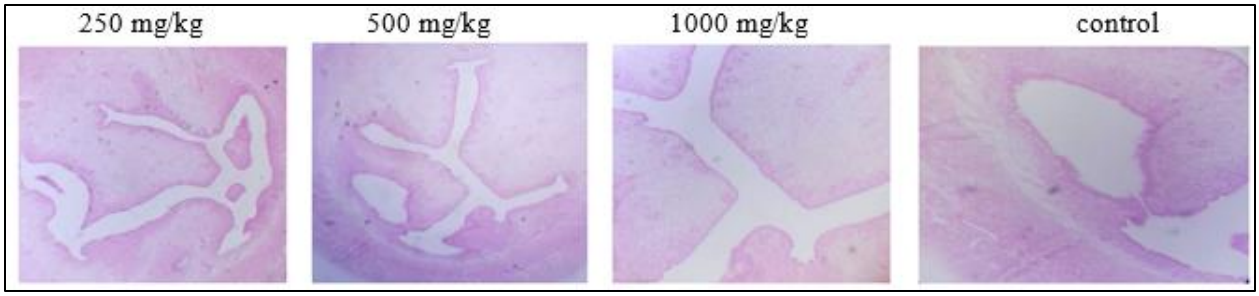

Figure 23: Microscopic examination of fallopian tissue sections taken from female cohort-1 rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500, and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal fallopian tube histological architecture.

The microscopic evaluation of the uterine tissue sections taken from all female cohort-1 rats demonstrated that the sub chronic treatment with *U. simensis* leaf extract did not induce any adverse histopathological changes. As presented in Figure 24, all experimental groups, including those administered the leaf extract at doses of 250, 500 and 1000 mg/kg, exhibited a normal uterine histological architecture as the corresponding vehicle control group. The endometrium showed a typical columnar epithelial lining with intact, regularly arranged uterine glands. The endometrium appeared normal without signs of edema, leukocyte infiltration, or fibrosis. The overall histological architecture of the uterus, including the luminal shape, was normal across all treatment groups. The absence of pathological changes such as glandular dilation, epithelial hyperplasia, pyknotic nuclei, necrosis or inflammatory lesions, indicated that the oral administration of *U. simensis* leaf extract at the tested doses did not adversely affect the histological integrity of the uterine tissues.

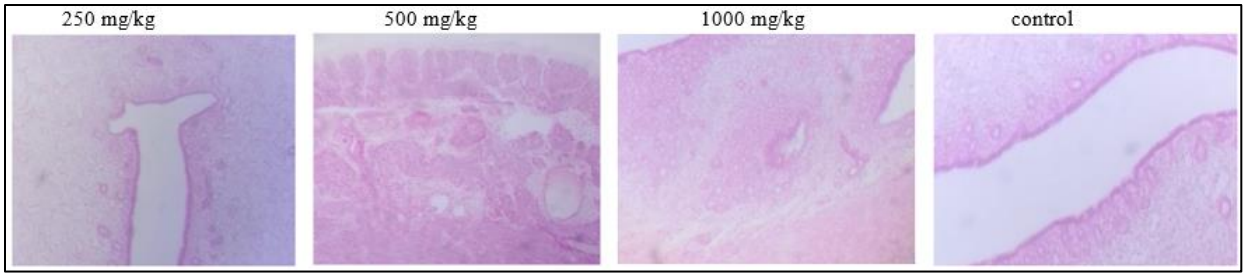

Figure 24: Microscopic examination of uterine tissue sections taken from female cohort-1 rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500, and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal uterine histological architecture.

The microscopic evaluation of testicular tissue sections taken from male cohort-1 rats revealed no treatment-related abnormalities in the spermatogenesis or testicular structure. As illustrated in Figure 25, the histological architecture of the testes remained normal and well-preserved in all groups administered the *U. simensis* leaf extract (250, 500, and 1000 mg/kg) and was comparable to the vehicle control group. The seminiferous tubules in all tissue sections appeared intact with a regular and oval shape. All stages of the spermatogenic cell series including spermatogonia, primary and secondary spermatocytes and spermatids were present and arranged in orderly layers. The mature and elongated spermatids were observed in the lumina of the testicular tubules. The sertoli cells were clearly visible and exhibited normal morphology. The interstitial spaces between the tubules displayed normal leydig cells, blood vessels and connective tissue with no signs of congestion, hemorrhage or inflammatory cell infiltration.

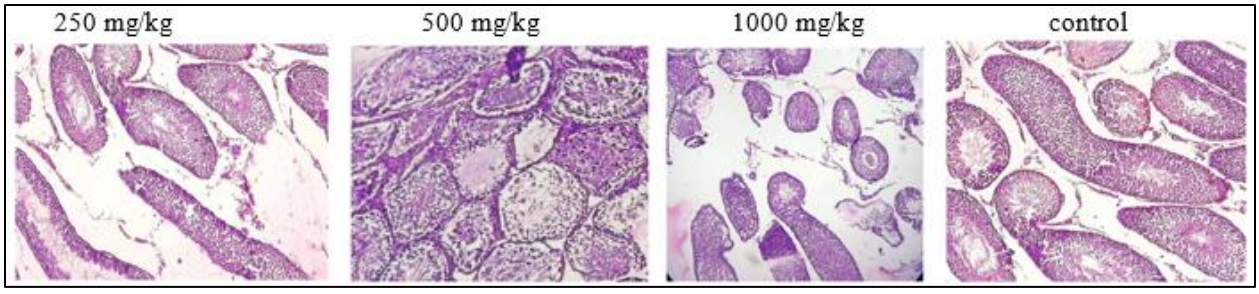

Figure 25: Microscopic examination of testicular tissue sections taken from male cohort-1 rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500, and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal testicular histological architecture.

Microscopic analysis of the epididymal tissue sections taken from male cohort-1 rats demonstrated that the gavage treatment with *U. simensis* leaf extract did not induce any structural abnormalities of epidydimal histology. As presented in Figure 26, the histological architecture of the epididymis in all extract-treated groups (250, 500, and 1000 mg/kg) was normal and comparable to the vehicle control group. The sampled tissue sections displayed the characteristic features of the epididymis, comprising a network of ducts lined with a pseudostratified columnar epithelium. The principal cells with their stereocilia were clearly identifiable and appeared intact. The lumina of the ducts were patent and contained abundant spermatozoa, indicating normal sperm maturation and storage. The surrounding stromal and smooth muscle layers maintained their typical organization and thickness, with no evidence of fibrosis, inflammation, or edema.

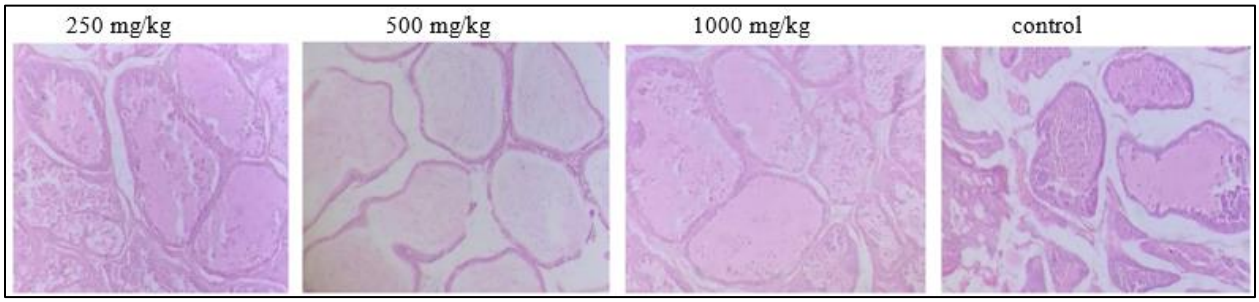

Figure 26: Microscopic examination of epididymis tissue sections taken from male cohort-1 rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500,

and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal epidydimal histological architecture.

Microscopic evaluation of the seminal vesicle tissue sections taken from male cohort-1 rats indicated the no adverse effects following treatment with *U. simensis* leaf extract. As shown in Figure 27, the histological architecture of the seminal vesicles in all experimental groups, including those administered doses of 250, 500 and 1000 mg/kg, was normal as the vehicle control group. The sampled tissue sections exhibited the characteristic folded mucosa lined by a pseudostratified columnar epithelium, which appeared intact and well-organized. The underlying lamina propria and the surrounding thick layers of smooth muscle maintained their typical structure and thickness. The glandular lumina contained the characteristic eosinophilic secretory material, and its density and distribution appeared consistent across all groups. The absence of pathological changes, such as epithelial atrophy or hyperplasia, inflammatory cell infiltration, fibrosis, vascular congestion or any disruption of the normal glandular architecture, demonstrates that the oral administration of *U. simensis* leaf extract across the specified dose ranges did not detrimentally affect the histology of the seminal vesicles.

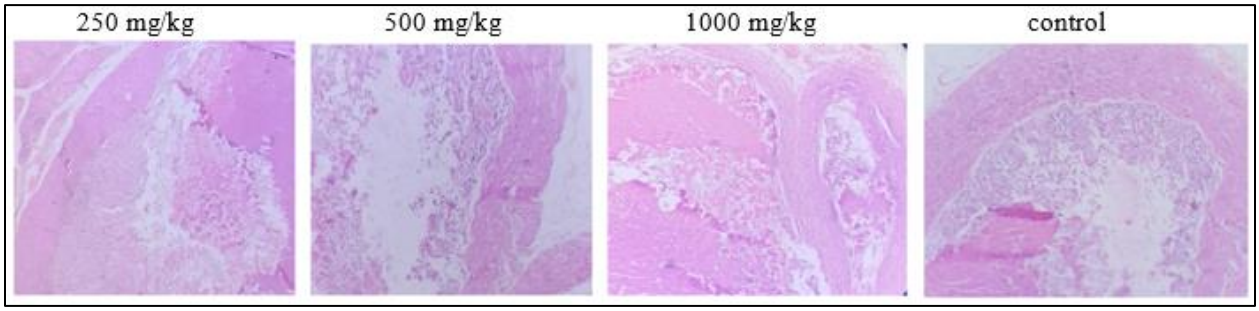

Figure 27: Microscopic examination of seminal vesicle tissue sections taken from male cohort-1 rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500, and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal seminal vesicle histological architecture.

Microscopic examination of the prostate tissue sections taken from male cohort-1 rats demonstrated that treatment with *U. simensis* leaf extract did not induce any histopathological alterations. As presented in Figure 28, the histology of the prostate gland in all extract-treated groups (250, 500, and 1000 mg/kg) was normal and comparable to the vehicle control group. The absence of pathological features, such as epithelial hyperplasia, inflammatory cell infiltration, stromal fibrosis, atrophy of the glandular units, or intra-luminal cellular debris, indicates that the sub chronic gavage administration of *U. simensis* leaf extract at the tested doses did not adversely affect the histological integrity of the prostate gland.

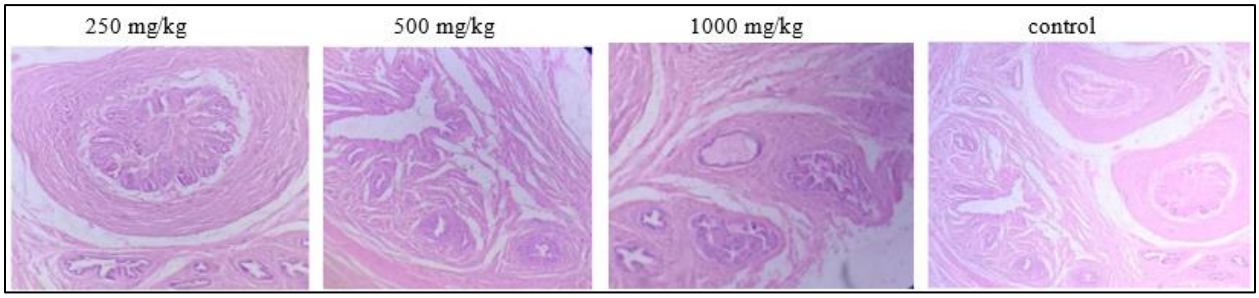

Figure 28: Microscopic examination of prostate tissue sections taken from male cohort-1 rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500, and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal prostate histological architecture.

5.8.1.6 Hepato-renal and endocrine toxicity in the F1 cohort-1 rats

Table 41 shows the liver, renal, thyroid, and adrenal gland organ weight changes in the F1 generation cohort-1 rats treated with *U. simensis* leaf extract. The female cohort-1 rats treated with the extract showed a substantial increase in the liver weight at the highest dose compared to the corresponding controls (1000 mg/kg: 11.30 ± 0.23 g versus control 10.15 ± 0.67 g). The endocrine organs, the thyroid and adrenal glands, did not exhibit any significant weight differences in either male or female cohort-1 rats treated with the *U. simensis* leaf extract.

Table 41: Hepato-renal, thyroid and adrenal glands of F1 cohort-1 rats treated with *U. simensis* leaf extract

Organs	GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
F1 chort-1 female rats				
Liver	10.30±0.76	10.45±0.75	11.30±0.23$^{*!}$	10.15±0.67
Kidney	1.46±0.06	1.47±0.05	1.48±0.05	1.458±0.05
Thyroid	0.035±0.003	0.033±0.003	0.034±0.003	0.035±0.003
Adrenal	0.036±0.001	0.035±0.001	0.035±0.002	0.036±0.001
F1 chort-1 male rats				
Liver	11.70±0.76	11.90±0.88	12.25±0.06	11.50±0.91
Kidney	1.58±0.05	1.59±0.05	1.61±0.06	1.57±0.05
Thyroid	0.03±0.001	0.029±0.001	0.029±.001	0.031±0.002
Adrenal	0.04±0.003	0.039±0.003	0.038±0.004	0.041±0.003

Results were written as mean and standard deviation, *statistically significant as compared to control groups, $^{!}$statistically significant compared to 250 mg/kg treated group

The biochemical profile findings in Table 42 showed that the *U. simensis* leaf extract has a dose-dependent effects in the F1 generation cohort-1 rats, with the most consistent changes observed in the liver enzyme function markers. Both male and female cohort-1 offspring treated with the leaf extract showed a statistically significant increases in the liver enzymes (ALT, AST, and ALP) at the highest dose (1000 mg/kg), as compared to the corresponding controls. The female cohort-1 rats treated with the leaf extract showed a more pronounced biochemical alterations, including a significant increase in the total protein (6.10 ± 0.78 g/dL versus control 5.25 ± 0.78 g/dL) at 1000 mg/kg dose, but males only showed a non-significant rise. Notably, the kidney function markers (urea and creatinine) remained unchanged across all groups. The *U. simensis* leaf extract had no significant effect on the metabolic parameters (glucose and cholesterol) or thyroid hormones (T3, T4, and TSH) in both male and female cohort-1 progenies.

Table 42: Biochemical profiles of F1 chort-1 rats treated with *U. simensis* leaf extract

Parameters		GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
ALT (U/L)	M	50.60±5.13	51.25±5.32	55.30±6.82*	49.85±5.08
	F	63.55±3.05	64.05±3.63	66.70±5.23*	63.20±3.23
AST(U/L)	M	133.80±5.92	135.00±6.75	139.70±9.11*	133.05±5.78
	F	171.80±6.10	172.45±5.76	176.10±6.64*	170.80±5.80
ALP (U/L)	M	76.20±2.62	77.20±3.54	79.20±4.14*	75.35±2.34
	F	120.60±1.90	120.95±2.21	122.30±3.46*	119.90±1.77
Urea (mg/dL)	M	55.20±2.62	55.55±2.66	56.40±3.53	54.20±2.41
	F	66.40±3.70	67.40±4.38	67.90±6.03	65.95±3.66
Creatinine (mg/dL)	M	0.45±0.02	0.46±0.02	0.46±0.02	0.44±0.02
	F	0.42±0.02	0.43±0.03	0.44±0.04	0.41±0.02
Albumin (g/dL)	M	4.18±0.29	4.19±0.30	4.20±0.31	4.17±0.30
	F	3.95±0.25	3.98±0.24	4.01±0.26	3.93±0.25
Total protein (g/dL)	M	7.00±0.65	7.15±0.80	7.40±1.03	6.79±0.61
	F	6.52±0.85	6.78±0.91	7.12±0.80*	6.24±0.77
Glucose (mg/dL)	M	115.20±5.62	114.20±5.49	113.03±4.94	117.05±4.93
	F	110.60±4.61	109.95±3.91	108.35±5.21	110.89±4.63
Total cholesterol (mg/dL)	M	45.80±1.20	46.00±1.17	46.20±1.47	45.50±1.42
	F	60.36±4.54	61.40±4.55	62.05±5.06	59.15±4.59
Triiodothyronine (T3) (ng/dl)	M	68.90±8.41	68.15±8.63	67.15±7.68	69.35±8.56
	F	78.80±3.96	78.10±3.83	77.65±4.28	78.80±3.96
Thyroxine (T4) (μg/dL)	M	2.64±0.37	2.55±0.38	2.53±0.40	2.73±0.31
	F	4.420±0.33	4.35±0.32	4.37±0.35	4.47±0.34
Thyroid stimulating hormone (TSH) (ng/ml)	M	2.41±0.35	2.36±0.32	2.33±0.34	2.46±0.34
	F	1.96±0.32	1.92±0.32	1.90±0.30	1.98±0.34

Results were written as mean and standard deviation, *statistically significant as compared to control groups

The histopathological effects of the *U. simensis* leaf extract on the liver of F1 cohort-1 male rats are presented in Figure 29. The hepatic histological architecture in the vehicle control group and the low-dose (250 mg/kg) treatment group was normal, exhibiting normal features of hepatocyte cords radiating from the central vein, intact sinusoidal spaces, and no evidence of pathological

alteration. In contrast, treatment with higher doses of *U. simensis* leaf extract induced dose-related hepatocellular damage. Two F1 cohort-1 male rats treated with 500 mg/kg extract showed focal evidence of liver parenchymal necrosis. This effect was more pronounced in the high-dose treated group (1000 mg/kg), where three F1 cohort-1 male rats exhibited clear and more extensive signs of hepatic necrosis, as indicated by the arrowheads in the Figure 29.

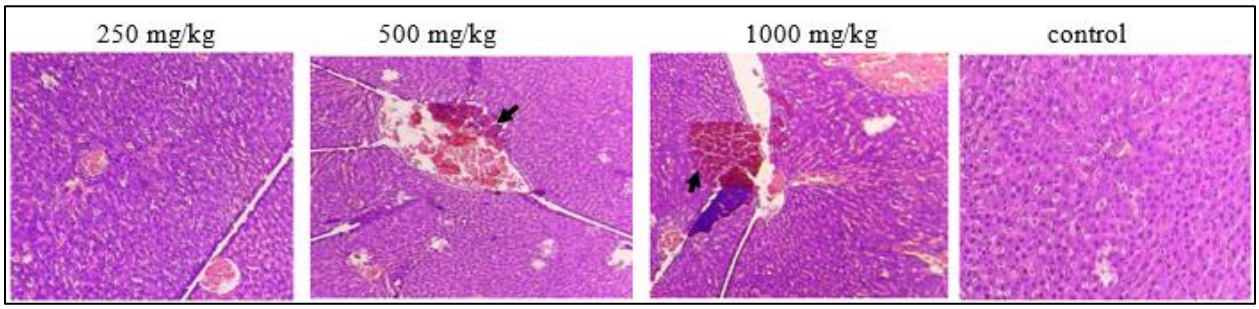

Figure 29: Microscopic examination of liver tissue sections taken from F1 cohort-1 male rats (H&E stain, 100x total magnification) treated with *U. simensis* at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. The male rats treated with 250 mg/kg exhibit normal hepatic histological architecture similar to that of the control group. However, two male rats that received 500 mg/kg and three male rats that received 1000 mg/kg showed evidence of liver parenchymal necrosis (arrowheads).

The hepatic effects of the *U. simensis* leaf extract on the F1 cohort-1 female rats are shown in Figure 30. The microscopic examination revealed that the hepatic histoarchitecture in the female cohort-1 rats treated with the low dose (250 mg/kg) extract was comparable to that of the vehicle control group, presenting normal lobular structure and hepatocyte morphology. However, the sub chronic oral administration of higher doses (500, 1000 mg/kg) of *U. simensis* leaf extract resulted in a substantial hepatic injury. The evidences of hepatic parenchymal necrosis were observed in five female F1 cohort-1 rats treated with 500 mg/kg and 1000 mg/kg of *U. simensis* leaf extract, as indicated by the arrowheads in Figure 30. The necrotic changes were characterized by clusters

of hepatocytes with eosinophilic cytoplasm and loss of cellular nuclei. Notably, the incidence and severity of necrosis appeared similar between the 500 mg/kg and 1000 mg/kg dose groups.

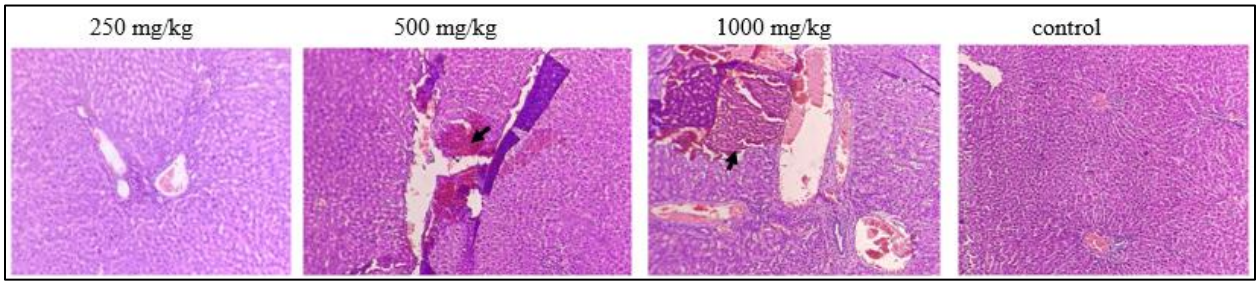

Figure 30: Microscopic examination of liver tissue sections taken from F1 cohort-1 female rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. The female rats treated with 250 mg/kg exhibit normal hepatic histological architecture similar to that of the control group. However, five female rats received 500 and 1000 mg/kg showed evidence of liver parenchymal necrosis (arrowheads).

The effects of *U. simensis* leaf extract on the renal histology of F1 cohort-1 male rats are presented in Figure 31. The microscopic evaluations revealed the normal renal histological architecture across all experimental groups. The kidneys of F1 cohort-1 rats treated with all doses of the *U. simensis* leaf extract (250, 500, and 1000 mg/kg) were histologically comparable to those of the vehicle control group. All sampled tissue sections showed preserved glomerular structure, intact tubules, and no evidence of histopathology such as tubular degeneration, necrosis, inflammation, or casts. This histological findings indicates that treatment with *U. simensis* leaf extract at the doses administered did not induce any detectable pathological integrity in the renal histology of F1 cohort-1 male rats.

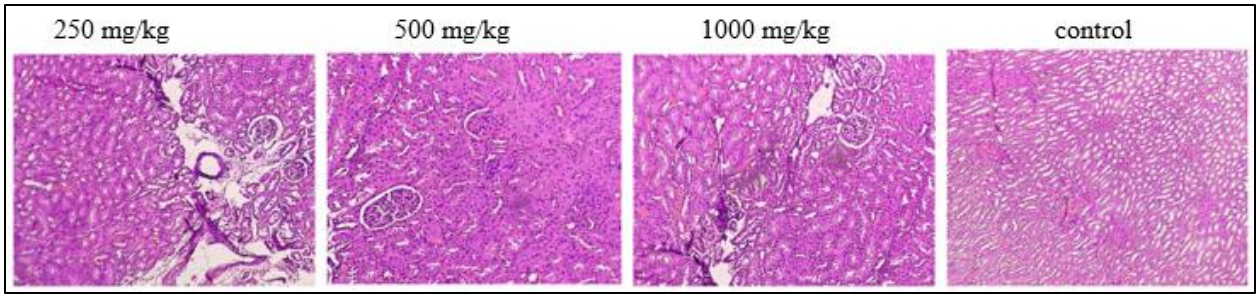

Figure 31: Microscopic examination of kidney tissue sections taken from F1 cohort-1 male rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal renal histological architecture

The renal histoarchitecture of F1 cohort-1 female rats following treatment with *U. simensis* leaf extract is shown in Figure 32. The microscopic examination revealed no treatment related pathological findings in any of the dose groups. All renal tissue sections from F1 cohort-1 female rats administered 250, 500, and 1000 mg/kg of the leaf extract were histologically normal as those of the vehicle control group. The renal parenchyma in all F1 cohort-1 female rats displayed normal glomeruli, intact tubules (proximal and distal convoluted tubules, and collecting ducts), and the absence of degenerative changes, necrosis, inflammatory cell infiltrates, or proteinaceous casts. These indicate that the *U. simensis* leaf extract, at the doses tested, did not induce any observable histopathological alterations in the renal histology of F1 cohort-1 female rats.

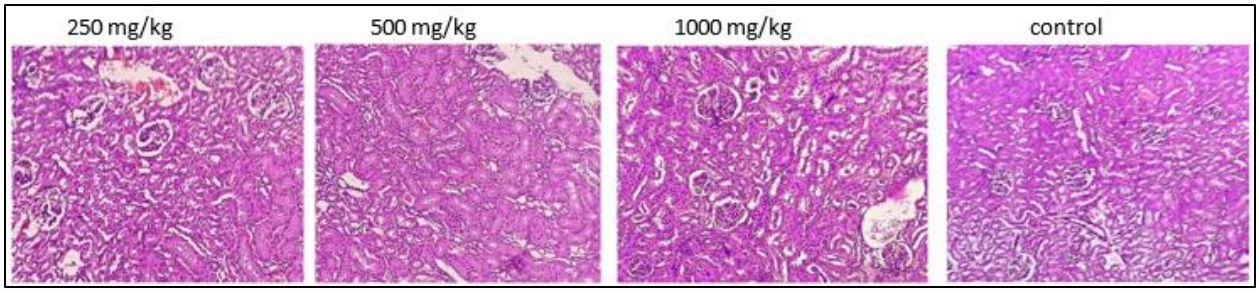

Figure 32: Microscopic examination of renal tissue sections taken from F1 cohort-1 female rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500

and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal renal histological architecture.

The histological integrity of the thyroid gland in F1 cohort-1 male rats following treatment with *U. simensis* leaf extract is presented in Figure 33. The microscopic evaluation revealed normal thyroid follicular architecture across all treatment groups. The thyroid tissue sections taken from the F1 chort-1 rats treated with 250, 500, and 1000 mg/kg of the *U. simensis* leaf extract were comparable to those from the vehicle control group. All sampled thyroid tissue sections exhibited follicles of varying sizes lined by a single layer of cuboidal follicular epithelium and filled with homogenous and eosinophilic colloids. There was no evidence of treatment-related pathological alterations, including follicular hypertrophy or hyperplasia, necrosis, inflammatory infiltrates, or colloid depletions.

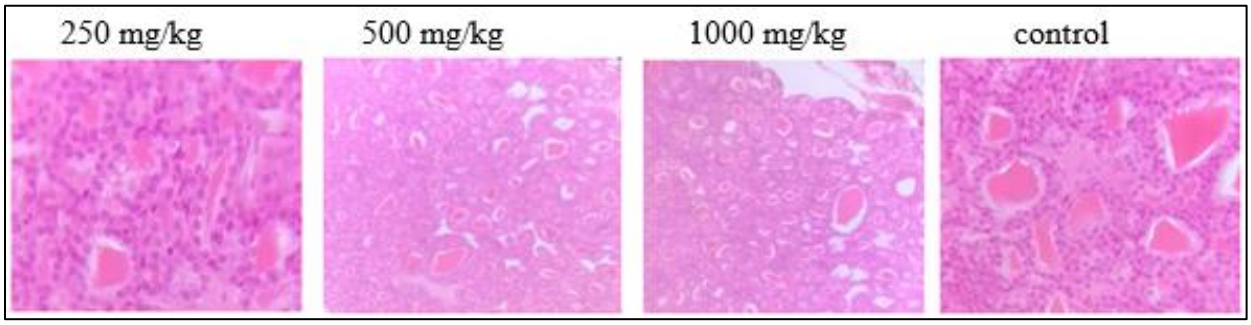

Figure 33: Microscopic examination of thyroid tissue sections taken from F1 cohort-1 male rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal thyroid histological architecture.

The microscopic examination of thyroid tissue sections taken from F1 cohort-1 female rats revealed no morphological alterations following treatment with *U. simensis* leaf extract (Figure 34). The thyroid microscopic architecture in all extract treated groups (250, 500, and 1000 mg/kg) was histologically normal as that of the vehicle control group. The glands displayed typical

follicular structure, characterized by follicles of varying sizes lined by a single layer of cuboidal follicular epithelial cells and filled with uniformly stained colloids. There were no observed treatment related findings, such as follicular cell hypertrophy, hyperplasia, necrosis, inflammatory cell infiltrates, or abnormalities in colloid density.

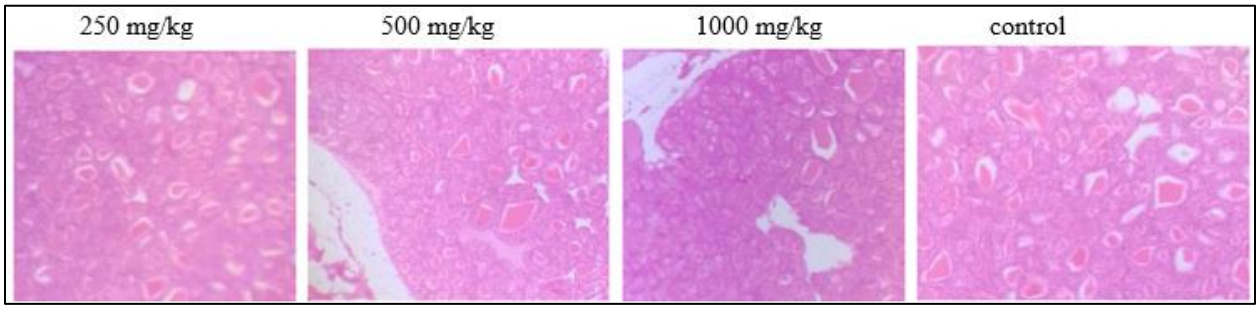

Figure 34: Microscopic examination of thyroid tissue sections taken from F1 cohort-1 female rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal thyroid histological architecture.

The microscopic evaluations of the parathyroid gland tissue sections taken from F1 cohort-1 male rats demonstrated no adverse effects after sub chronic treatment with the *U. simensis* leaf extract (Figure 35). The histological architecture of the parathyroid tissue was normal in all rats across every dose treated groups, including the vehicle control and those administered 250, 500, and 1000 mg/kg of *U. simensis* leaf extract. The glands exhibited the normal morphological parenchyma, composed of tightly packed, uniform chief cells with round, densely stained nuclei and pale cytoplasm. There was no evidence of pathological alterations such as cellular vacuolization, hypertrophy, hyperplasia, necrosis, inflammatory infiltrates, or fibrosis. These findings indicate that treatment with the *U. simensis* leaf extract, at the doses tested, did not induce any observable histopathological changes in the parathyroid glands of F1 cohort-1 male rats.

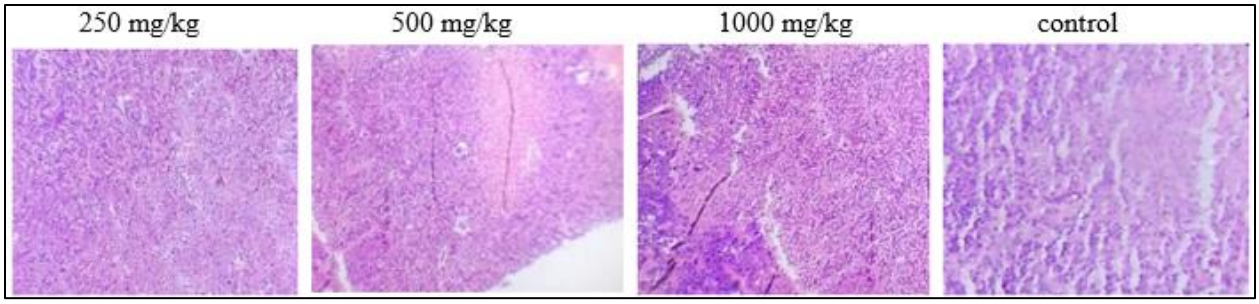

Figure 35: Microscopic examination of parathyroid tissue sections taken from F1 cohort-1 male rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal parathyroid tissue architecture.

The microscopic examinations of the parathyroid tissue sections taken from F1 cohort-1 female rats revealed no morphological alterations following sub chronic treatment with *U. simensis* leaf extract (Figure 36). The parathyroid microscopic architecture in all treatment groups (250, 500, and 1000 mg/kg) was histologically normal and comparable to the vehicle control group. The glands displayed characteristic normal parenchymal structure consisting of tightly arranged chief cells with typical round, hyperchromatic nuclei and pale eosinophilic cytoplasm. No treatment related histological changes were observed, including absence of cellular vacuolization, hypertrophy, hyperplasia, inflammatory infiltrates, necrosis, or stromal fibrosis. These results indicate that the oral administration of the *U. simensis* leaf extract at the tested dose levels did not produce any detectable histopathological alterations in the parathyroid glands of F1 cohort-1 female rats.

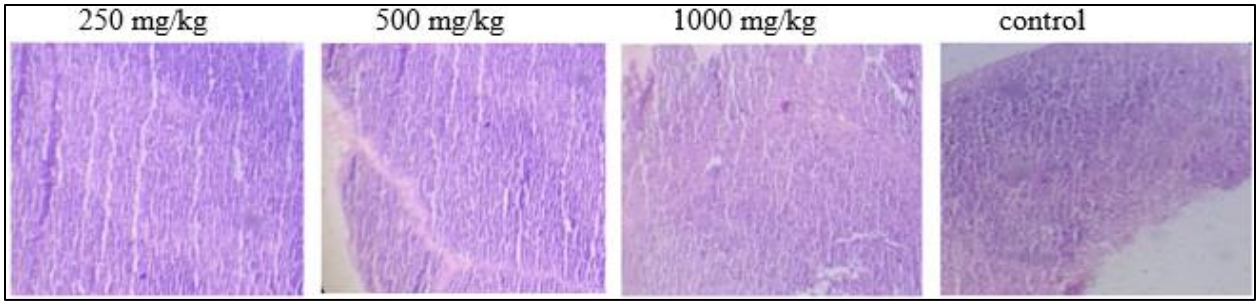

Figure 36: Microscopic examination of parathyroid tissue sections taken from F1 cohort-1 female rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal parathyroid tissue architecture.

The adrenal tissue sections taken from F1 cohort-1 male rats treated with *U. simensis* leaf extract at doses of 250, 500, and 1000 mg/kg was histologically comparable to that of the vehicle control group (Fig.37). All zones of the adrenal cortex (zona glomerulosa, zona fasciculata, and zona reticularis) exhibited normal cellular organization, thickness, and histological appearance. The medulla likewise appeared normal, with typical chromaffin cells and no evidence of pathological changes. There were no treatment related findings such as hemorrhage, necrosis, vacuolization, inflammatory infiltrates, or cellular degeneration in any of the dose groups. These results indicate that administration of *U. simensis* leaf extract at the tested dose levels did not produce any detectable histopathological alterations in the adrenal glands of cohort-1 male rats.

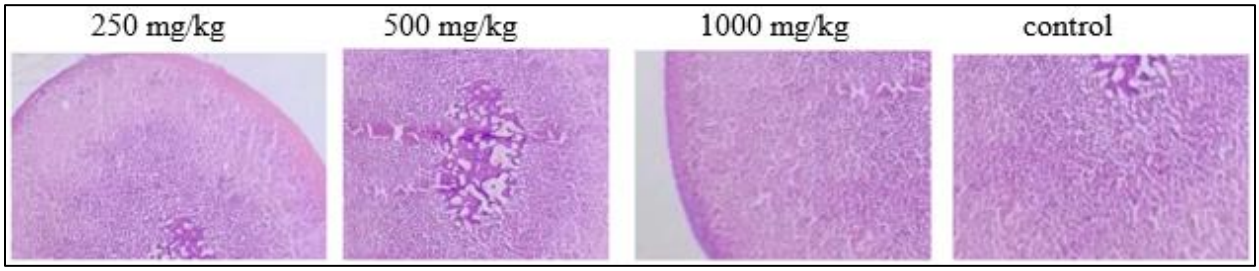

Figure 37: Microscopic examination of adrenal tissue sections taken from F1 cohort-1 male rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500

and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal adrenal histological architecture

Microscopic examination of the adrenal gland tissue sections taken from the F1 cohort-1 female rats revealed no morphological alterations following treatment with *U. simensis* leaf extract (Figure 38). The adrenal histological architecture in all treatment groups (250, 500, and 1000 mg/kg) was normal and comparable to the vehicle control group. The histological morphology displayed the characteristic of cortical zonation into distinct zona glomerulosa, zona fasciculata, and zona reticularis, all with typical histological features. The adrenal medulla exhibited normal morphology with appropriately arranged chromaffin cells. No treatment related pathological changes were observed, including absence of hemorrhage, necrosis, inflammatory infiltrates, cellular vacuolization, or disruption of the normal microscopic architectural organization. These indicate that the gavage administration of *U. simensis* leaf extract at the tested dose levels did not produce any detectable histopathological alterations in the adrenal glands of cohort-1 female rats.

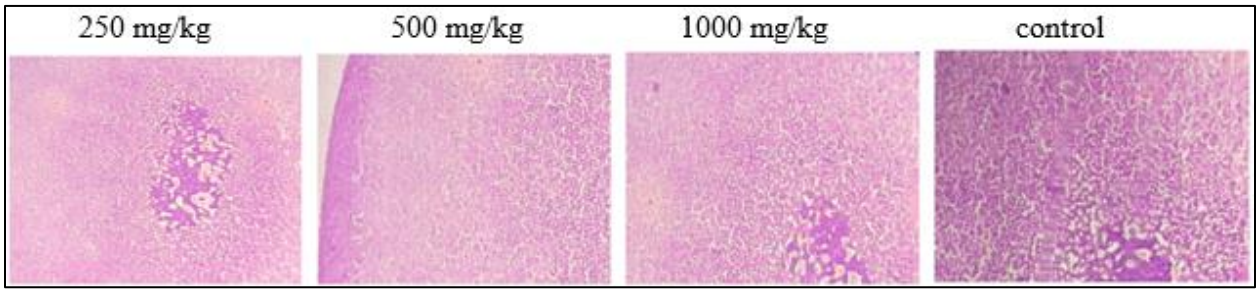

Figure 38: Microscopic examination of adrenal tissue sections taken from F1 cohort-1 female rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal adrenal tissue architecture

5.8.2 Developmental neurotoxicity / F1 cohort-2 rats

5.8.2.1 Food intake and body weight effects

The food intakes and weight gains of F1 cohort-2 rats treated with *U. simensis* leaf extract at various doses were compared to the control group (Table 43). The cohort-2 male rats showed no statistically significant differences in the daily food consumptions between the extract-treated groups and the control group, though slight reductions were observed at higher doses. Similarly, the cohort-2 female rats exhibited no significant changes in the daily food intakes across all tested doses. The male rats treated with the highest dose (1000 mg/kg) of *U. simensis* leaf extract resulted in a significant increase in weight gains (122.40 ± 5.98 g) as compared to the control group (118.80 ± 1.82 g). The low and middle doses (250 and 500 mg/kg) of extract had no significant effect in weight gain differences. The female rats treated with 500 and 1000 mg/kg doses showed a significant weight gains (113.40 ± 3.53 g and 114.50 ± 5.83 g, respectively) relative to the control group (108.75 ± 3.95 g).

Table 43: Food intakes and weight gains of F1 cohort-2 rats treated with *U. simensis* leaf extract

		GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
Food intake (g)	M	402.31±2.89	396.42±2.62	396.42±2.62	407.85±2.66
	F	387.30±3.44	385.12±3.27	382.85±2.71	389.98±2.60
Weight gain (g)	M	119.40±1.90	120.50±3.51	122.40±5.98*	118.80±1.82
	F	112.00±3.49	113.40±3.53*	114.50±5.83*	108.75±3.95

Results were written as mean and standard deviation, *statistically significant as compared to control groups

5.8.2.2 Brain and spinal cord effects

The effects of the *U. simensis* leaf extract on the weights of the brain and spinal cord in the F1 cohort-2 rats treated at three dose levels (250, 500, and 1000 mg/kg) were assessed and compared

to the corresponding control group that received only the vehicle (Table 44). Both male and female cohort-2 progeny rats treated with the *U. simensis* leaf extract showed no statistically significant changes in the brain and spinal cord mean weights as compared to the respective control groups.

Table 44: Brain and spinal cord weights of F1 cohort-2 rats treated with *U. simensis* leaf extract

		GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
Brain (g)	M	1.94±0.15	1.91±0.14	1.90±0.12	1.96±0.16
	F	1.82±0.13	1.80±0.15	1.77±0.14	1.84±0.14
Spinal cord (g)	M	0.41±0.03	0.40±0.03	0.40±0.03	0.42±0.03
	F	0.35±0.04	0.34±0.04	0.34±0.03	0.35±0.04

Results were written as mean and standard deviation

The microscopic examinations of the cerebral tissue sections taken from F1 cohort-2 male rats revealed no evidences of treatment related histopathological alterations. As shown in Figure 39, the cerebral histological architectures in all groups, including the vehicle control and those treated with the *U. simensis* leaf extract at doses of 250, 500, and 1000 mg/kg, was normal and well-preserved. The cerebral cortex in all treated animals exhibited normal histoarchitecture. The neurons appeared morphologically intact with large, vesicular nuclei and prominent nucleoli. No signs of neuronal degeneration, necrosis, pyknosis, or eosinophilia were observed. The neuropil was uniform and displayed a normal density and distribution of glial cells. Furthermore, there were no observable indications of edema, inflammatory cell infiltration, hemorrhage, or vascular abnormalities in any of the treatment groups. The absence of any pathological findings across all dose levels indicates that the oral administrations of *U. simensis* leaf extract at doses up to 1000 mg/kg did not induce any adverse histopathological changes in the cerebral tissue of the F1 male offsprings.

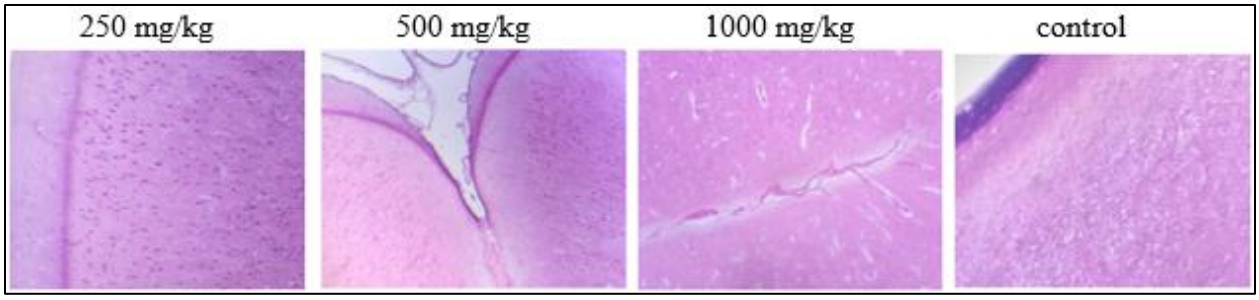

Figure 39: Microscopic examination of cerebral tissue sections taken from F1 cohort-2 male rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal cerebral histology

The microscopic evaluations of the cerebral tissue sections taken from F1 cohort-2 female rats demonstrated no adverse effects following treatment with *U. simensis* leaf extract. As presented in Figure 40, the histological architecture of the cerebrum was normal and comparable across all experimental groups, including the vehicle control and those administered the leaf extract at doses of 250, 500, and 1000 mg/kg. All sampled tissue sections exhibited intact meninges and a well-organized cerebral cortex with clearly distinguishable layers. The neurons revealed standard morphological characteristics, including prominent nucleoli, with no evidence of degeneration, necrosis, or shrinkage. The neuropil appeared uniform with an expected distribution of glial cells. Furthermore, there were no observable pathological alterations such as vacuolation, edema, perivascular cuffing, inflammatory cell infiltrates, or hemorrhage in any of the treatment groups. These findings indicate that the sub-chronic administration of *U. simensis* leaf extract across the tested dose ranges did not induce any neurohistopathological alterations in the F1 offspring female rats.

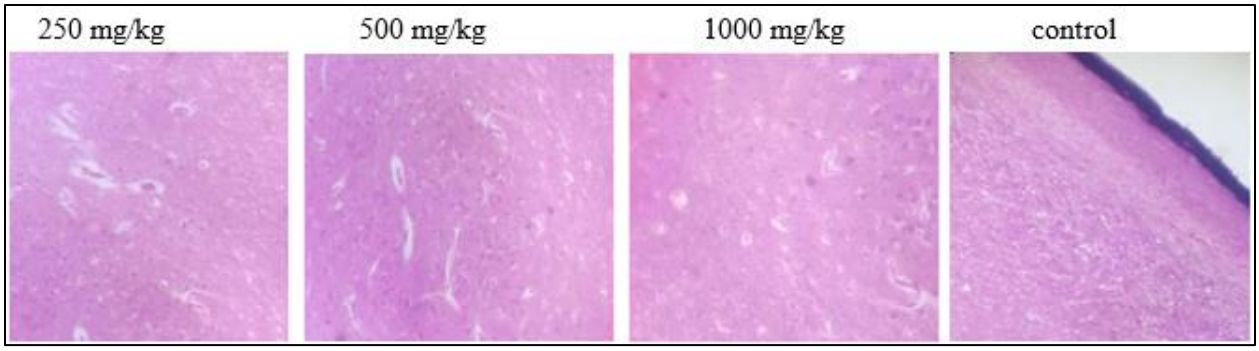

Figure 40: Microscopic examination of cerebral tissue sections taken from F1 cohort-2 female rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal cerebral histology

Histopathological assessment of the cerebellar tissue taken from F1 cohort-2 male and female rats revealed no abnormalities associated with the administration of *U. simensis* leaf extract. As illustrated in Figure 41 and figure 42, the histoarchitecture of the cerebellum was well-preserved and normal for all experimental groups, including the vehicle control and those receiving doses of 250, 500, and 1000 mg/kg of leaf extract. In all examined tissue sections, the characteristic trilaminar organization of the cerebellar cortex, consisting of the molecular layer, Purkinje cell layer, and granular layer, was clearly visible and structurally intact. The Purkinje cells appeared normal, exhibiting their typical large, flask-shaped with prominent nuclei, and were arranged in a single, continuous layer without signs of displacement, degeneration, or loss. The molecular layer showed a normal, sparse cellularity, and the granular layer displayed a high density of uniformly small, deeply stained granule cell nuclei. Furthermore, the underlying white matter tracts appeared normal with no evidence of edema or gliosis. The absence of any pathological findings indicates that treatment with *U. simensis* leaf extract at the doses tested did not induce any adverse histopathological effects on the cerebellar tissue of both male and female F1 offspring rats.

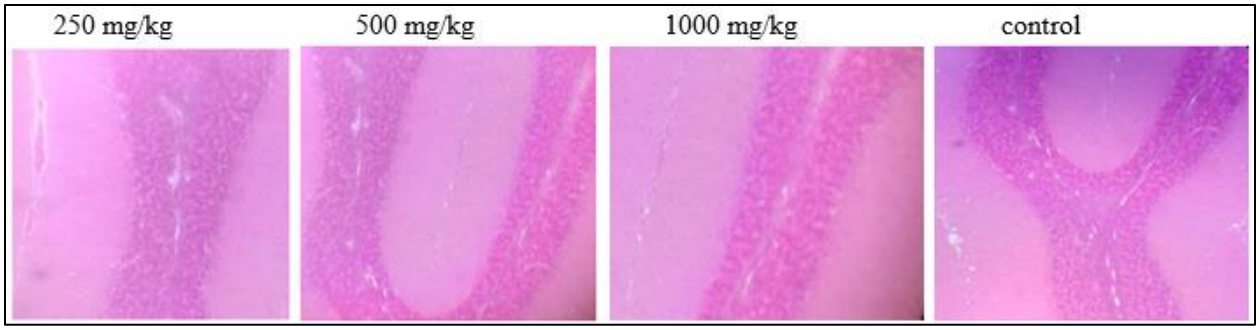

Figure 41: Microscopic examination of cerebellar tissue sections taken from F1 cohort-2 male rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal cerebellar histology

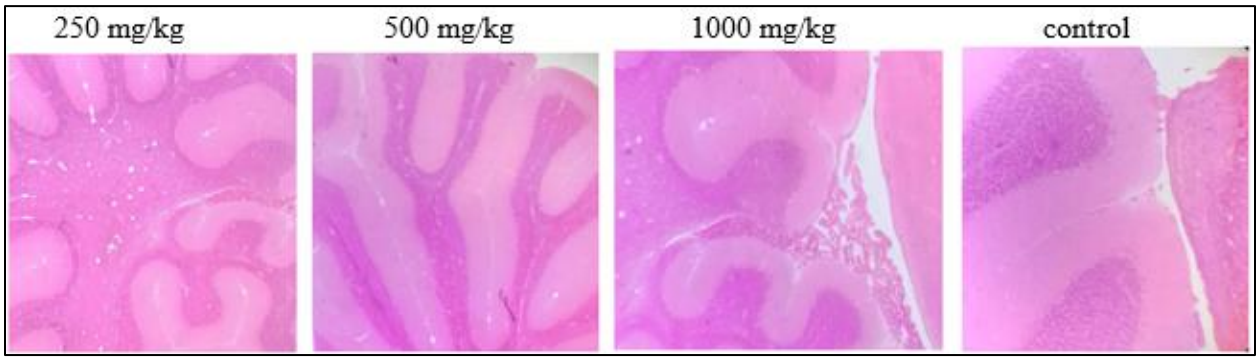

Figure 42: Microscopic examination of cerebellar tissue sections taken from F1 cohort-2 female rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal cerebellar histology

The histopathological assessment of the spinal cord tissue sections taken from both male and female F1 cohort-2 rats revealed no abnormalities associated with the oral administrations of *U. simensis* leaf extract. As illustrated in Figure 43 and Figure 44, the histoarchitecture of the spinal cord was well-preserved and normal for all treatment groups in both sexes, including the vehicle control and those receiving doses of 250, 500, and 1000 mg/kg of leaf extract. In all examined tissue sections, the distinct histological organization of the spinal cord, featuring the central canal, surrounding grey matter (with prominent anterior and posterior horns), and the peripheral white

matter tracts, was clearly defined and structurally intact. No signs of neuronal degeneration or necrosis were observed. The white matter tracts exhibited normal architecture with no evidences of edema, vacuolation or gliosis. Furthermore, there were no indications of inflammatory cell infiltrates or vascular abnormalities in any of the treatment groups. The absence of any histopathological findings across both sexes and all dose levels indicate that treatment with *U. simensis* leaf extract at the doses tested did not induce any adverse histopathological effects on the spinal cord histology of the F1 offspring rats.

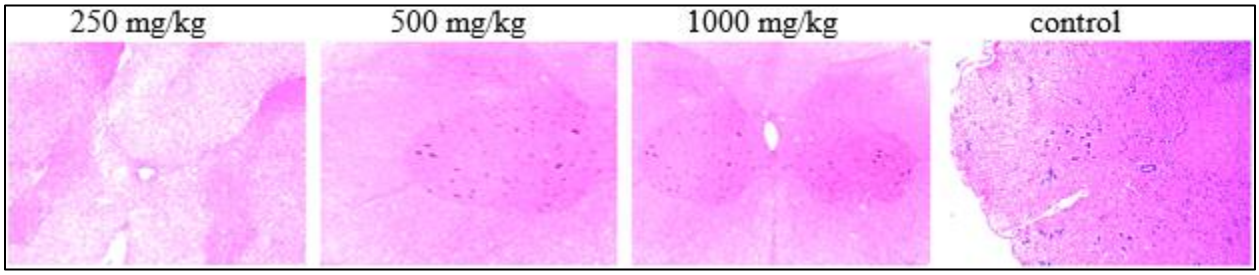

Figure 43: Microscopic examination of spinal cord tissue sections taken from F1 cohort-2 male rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal spinal cord histology.

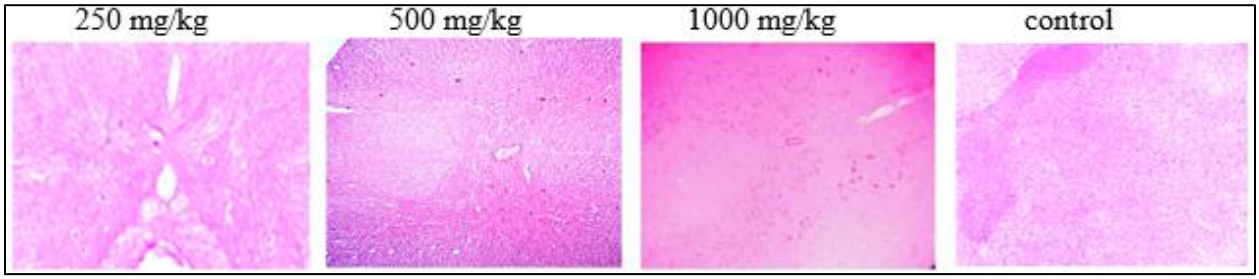

Figure 44: Microscopic examination of spinal cord tissue sections taken from F1 cohort-2 female rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal spinal cord histology

The microscopic examination of sympathetic ganglia tissue sections taken from both male and female rats in the F1 cohort-2 study revealed no treatment related histopathological alterations. As shown in Figure 45 and Figure 46, the tissue architectures in all groups, including the vehicle control and those treated with *U. simensis* leaf extract at doses of 250, 500, and 1000 mg/kg, was observed as normal and comparable. The ganglia exhibited the normal histological organizations with no evidences of the neuronal degeneration, necrosis or satellitosis. The ganglion cells displayed a normal morphological appearance, including a large, vesicular nucleus with prominent nucleolus. The surrounding capsule cells and supporting stromal elements also appeared normal. No signs of inflammation, hemorrhage, vacuolation, or other degenerative changes were observed in any of the treatment groups at any of the tested doses. These findings indicate that the gavage administration of *U. simensis* leaf extract at doses up to 1000 mg/kg did not induce any adverse histopathological effects on the sympathetic ganglia in the F1 offsprings.

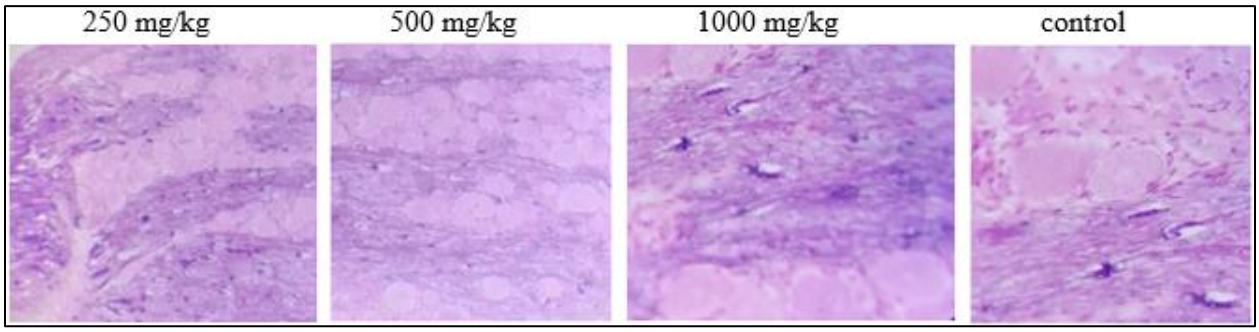

Figure 45: Microscopic examination of sympathetic ganglia tissue sections taken from F1 cohort-2 male rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal sympathetic ganglia tissue architecture

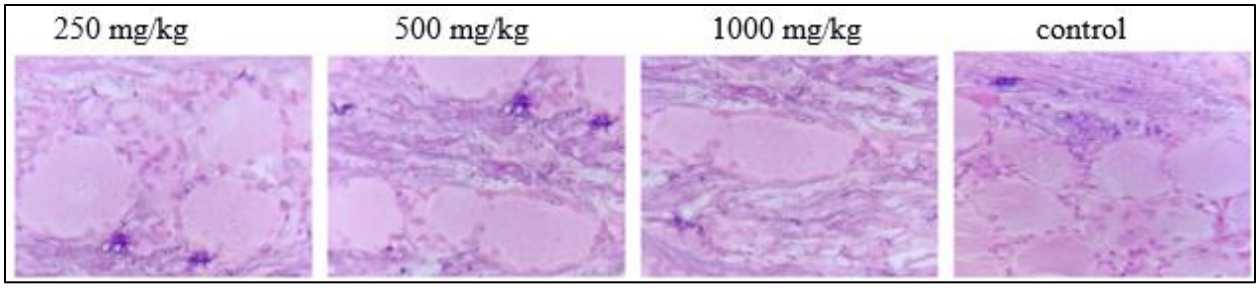

Figure 46: Microscopic examination of sympathetic ganglia tissue sections taken from F1 cohort-2 female rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal sympathetic ganglia tissue architecture

The histological examination of peripheral nerve fibers taken from F1 cohort-2 male and female rats demonstrated no morphological alterations attributable to the treatment with *U. simensis* leaf extract. As shown in Figure 47 and Figure 48, the nerve fibers from all experimental groups; including the vehicle control and the groups administered oral doses of 250, 500, and 1000 mg/kg, exhibited normal histoarchitecture. The nerve bundles displayed intact and well organized neuronal fibers. The axons appeared uniform and continuous with no evidences of axonal swellings. The surrounding connective tissue layers were intact and exhibited a typical and non-inflammatory appearances. No histopathological findings such as inflammatory cell infiltrates, edema, and hemorrhage or fiber necrosis were observed in any of the treatment groups. These indicate that sub chronic oral administrations of *U. simensis* leaf extract across the tested dose ranges did not induce any adverse toxicological effects on the peripheral nerve fibers in the F1 offsprings.

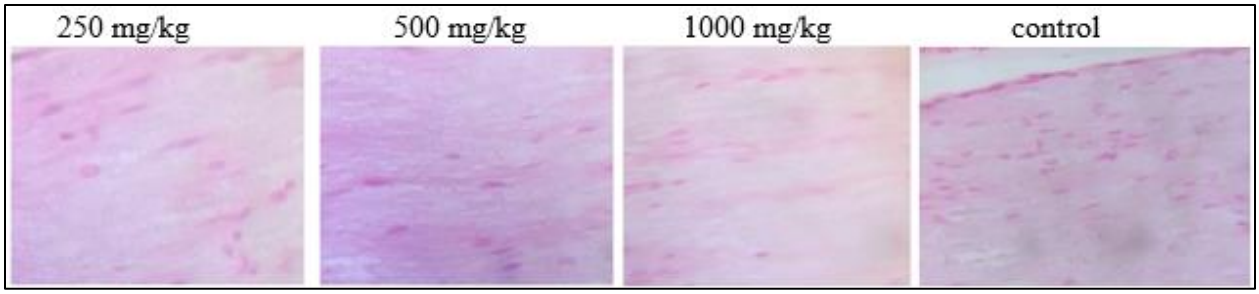

Figure 47: Microscopic examination of nerve fiber tissue sections taken from F1 cohort-2 male rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal nerve fiber histology

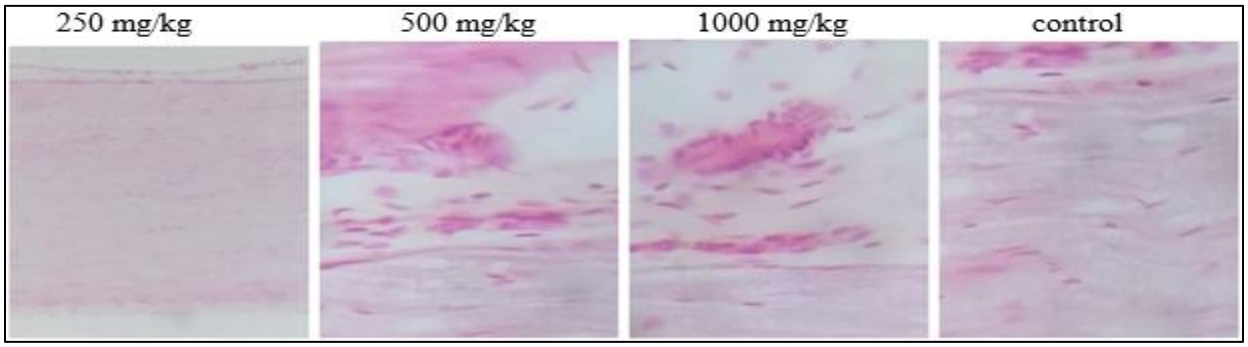

Figure 48: Microscopic examination of nerve fiber tissue sections taken from F1 cohort-2 female rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal nerve fiber histology

5.8.3 Developmental immunotoxicity effects /F1 cohort-3 rats

5.8.3.1 Food intake and body weight effects

The effects of *U. simensis* leaf extract on the daily food intake and body weight gains in the F1 cohort-3 rats were examined at three dose levels (250, 500, and 1000 mg/kg) and compared to a control group (Table 45). Both male and female cohort-3 rats had slightly lower food intake in the treatment groups compared to the respective control group, but the differences were not statistically significant. There was a dose-dependent increase in weight gains in both male and

female rats received the leaf extract as compared to the control group, but no statistically significant.

Table 45: Food intake and body weight gains of F1 cohort-3 rats treated with *U. simensis* leaf extract

		GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
Food intake (g)	M	397.31±2.89	391.42±2.62	391.30±2.72	400.85±5.87
	F	382.30±3.44	380.12±3.27	377.85±2.71	384.98±2.60
Weight gain (g)	M	114.40±1.90	115.50±3.51	117.40±5.98	113.80±1.82
	F	107.00±3.49	108.40±3.53	109.50±5.83	103.75±3.95

Results were written as mean and standard deviation

5.8.3.2 Lymphoid organ effects

The effects of *U. simensis* leaf extract on the weights of key lymphoid organs (spleen, thymus and mandibular lymph nodes) in the F1 cohort-3 rats across three dose levels (250, 500, and 1000 mg/kg) were evaluated and compared to a control group that received the vehicle (Table 46). Both male and female progenies received the *U. simensis* leaf extract showed non-significant decreases in the spleen weight as compared to the corresponding control group. The weights of thymus and mandibular lymph nodes in both sexes of progenies treated with *U. simensis* leaf extract were not significantly different from those of the corresponding controls.

Table 46: Weights of key lymphoid organs of F1 cohort-3 rats treated with *U. simensis* leaf extract

		GI-250 mg/kg	GII-500 mg/kg	GIII-1000 mg/kg	GIV-control
Spleen (g)	M	0.724±0.081	0.708±0.083	0.704±0.082	0.733±0.082
	F	0.624±0.063	0.613±0.076	0.610±0.069	0.631±0.062
Thymus (g)	M	0.322±0.068	0.316±0.075	0.311±0.070	0.327±0.064

	F	0.270±.029	0.264±0.033	0.260±0.034	0.276±0.024
Mandibular	M	0.0332±0.004	0.0326±0.005	0.0327±0.004	0.0341±0.005
Lymph node (g)	F	0.0258±0.002	0.0251±0.002	0.0250±0.002	0.0263±0.002

Results were written as mean and standard deviation

The microscopic evaluation of spleen tissue sections taken from F1 cohort-3 male and female rats revealed no treatment related abnormalities. As illustrated in Figure 49 and Figure 50, the spleen histological architecture in all groups, including the vehicle control and those treated with *U. simensis* leaf extract at doses of 250, 500, and 1000 mg/kg, was histologically normal and comparable to the controls. Both the white pulp, consisting of periarteriolar lymphoid sheaths and lymphoid follicles, and the red pulp, composed of splenic cords and sinusoids, appeared normal morphology and exhibited typical cellular organizations. No evidences of pathological lesions, such as lymphoid depletion or hyperplasia, fibrosis, inflammatory cell infiltrates, congestion, or pigment deposition, was observed in any of the animals across the treatment groups. These findings demonstrate that the oral administration of *U. simensis* leaf extract at doses up to 1000 mg/kg did not induce any adverse histopathological effects on the spleen in the F1 offsprings.

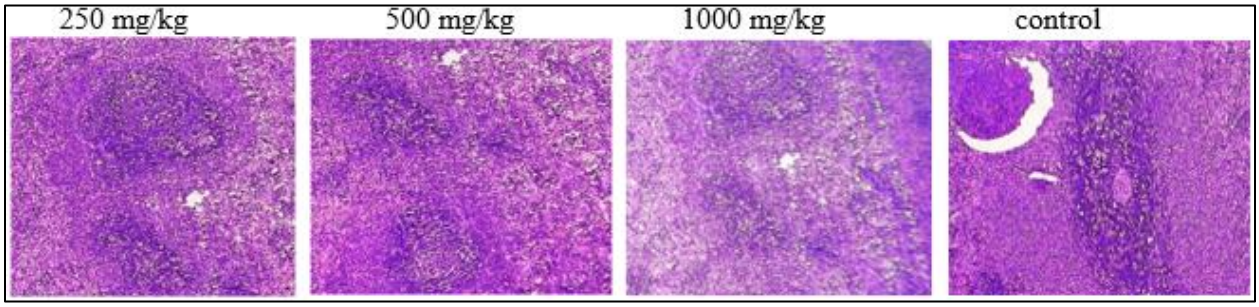

Figure 49: Microscopic examination of spleen tissue sections taken from F1 cohort-3 male rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal spleen histological architecture

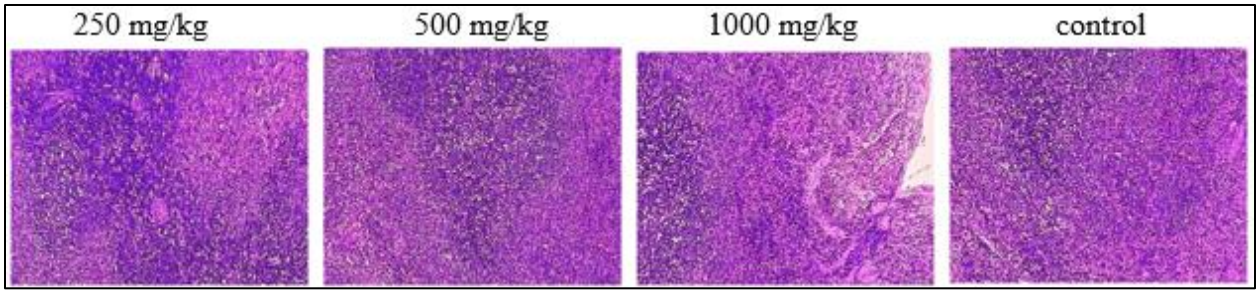

Figure 50: Microscopic examination of spleen tissue sections taken from F1 cohort-3 female rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal spleen histological architecture

The histological examinations of the thymus tissue sections taken from F1 cohort-1 male and female rats demonstrated normal histological architectures across all treatment groups. As presented in Figure 51 and Figure 52, the thymus histological morphology in the F1 rats treated with *U. simensis* leaf extract at doses of 250, 500, and 1000 mg/kg was comparable to that of the vehicle control group. The sample thymus tissue sections from all F1 offspring rats exhibited a normal corticomedullary demarcation, with a dense cortex populated by lymphocytes and a less dense medulla. The medulla contained clearly distinguishable Hassall's corpuscles, which appeared normal in morphology and distribution. There was no microscopic evidences of treatment related pathological changes, such as lymphocyte depletion, atrophy, fibrosis, cystic degeneration, or inflammatory cell infiltrations. These indicate that the sub chronic oral administration of *U. simensis* leaf extract at the tested doses did not induce any adverse histopathological alterations in the histology of thymus in the F1 offsprings, suggesting absence of tissue injury to this lymphoid organ.

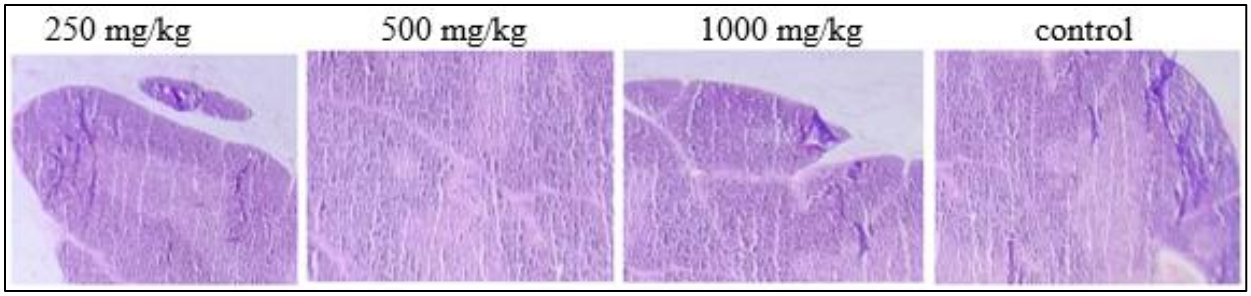

Figure 51: Microscopic examination of thymus tissue sections taken from F1 cohort-1 male rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal thymus tissue architecture

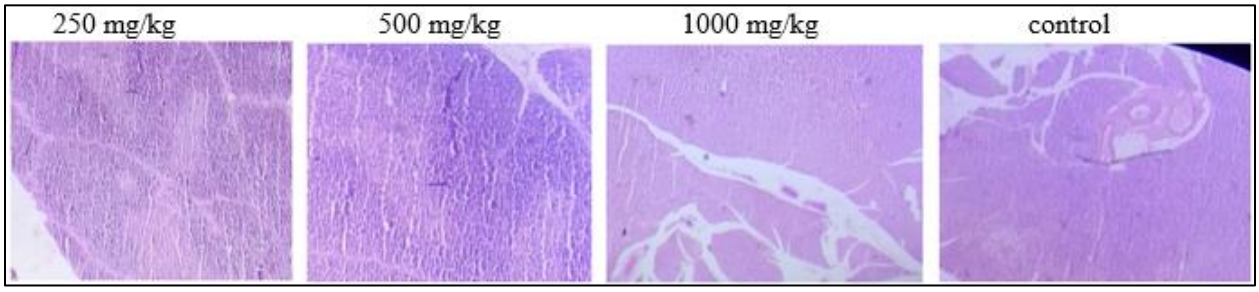

Figure 52: Microscopic examination of thymus tissue sections taken from F1 cohort-3 female rats (H&E stain, 100x total magnification) treated with *U. simensis* at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal thymus tissue architecture

The microscopic examinations of the mandibular lymphoid tissue sections taken from F1 cohort-3 male and female rats revealed no morphological alterations following treatment with *U. simensis* leaf extract. As shown in Figure 53 and Figure 54, the histological architecture of the lymphoid tissue in all treatment groups, including the vehicle control and groups administered doses of 250, 500, and 1000 mg/kg, was normal and comparable. The lymph nodes exhibited a well-defined cortex, paracortex, and medulla. The cortical regions showed normal lymphoid follicles with distinct germinal centers, indicating active immune functions. The paracortical (T-cell dependent) and medullary regions, including the medullary sinuses and cords, also appeared intact and exhibited typical cellularity. No histopathological findings, such as lymphoid depletion

or hyperplasia, sinusoidal distension, hemorrhage, granuloma formation, or inflammatory cell infiltrates, were observed in any of the treatment groups. These demonstrate that the sub chronic gavage administration of *U. simensis* leaf extract at doses up to 1000 mg/kg did not induce any adverse effects on the architecture or cellular integrity of the mandibular lymphoid tissue in the F1 offsprings.

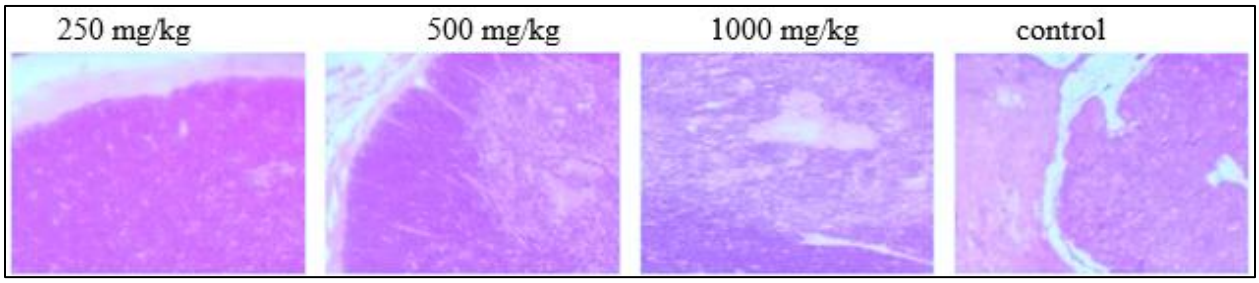

Figure 53: Microscopic examination of mandibular lymphoid tissue section taken from F1 cohort-3 male rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit normal mandibular lymphoid tissue architecture

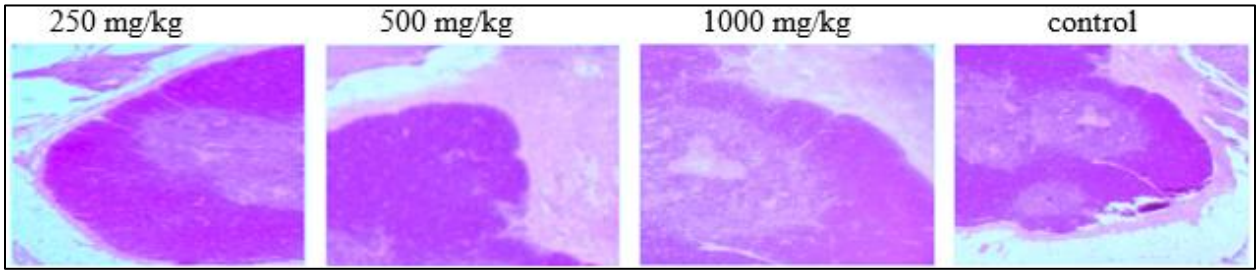

Figure 54: Microscopic examination of mandibular lymphoid tissue sections taken from F1 cohort-3 female rats (H&E stain, 100x total magnification) treated with *U. simensis* leaf extract at doses of 250, 500 and 1000 mg/kg, alongside a vehicle control group. All groups exhibit mandibular lymphoid tissue architecture

6. DISCUSSIONS

Single and repeated dose toxicity studies are fundamental components of toxicology assessments providing essential information about the harmful effects of chemicals, drugs or other substances following exposure [136, 177]. These studies are critical for evaluating the safety profiles of a substance, identifying its potential hazards and establishing safe exposure levels for both humans and the environment [178]. Acute toxicity tests are short-term assessments conducted to evaluate the immediate effects of a substance following a single exposure [137, 179].

Subacute toxicity tests, on the other hand, are designed to evaluate the effects of repeated exposure, dose response relationships, and potential target organ damage that may not be evident in the acute studies. In subacute studies, the substance is typically administered to the animals daily for a period of 4 weeks [179, 180]. These fundamental experimental toxicology studies have been conducted to identify the potential hazardous effects of the herbal therapies, primarily in the liver and kidney of rodents [181]. Among the various toxic effects reported, liver injury is the most well-documented organ damage associated with botanical remedies [130, 182]. Owing to its high metabolic activity, the liver is particularly susceptible to substance-induced toxicity [183]. The kidneys are another major target of toxic substances [138]; they are especially vulnerable to blood-borne toxins due to their high blood flow, role in urine concentration, and biochemical responsiveness to foreign substances [184].

In addition to the repeated-dose studies, developmental and reproductive toxicity assessments have been carried out to evaluate the effects of test substances on reproductive performance, including gonadal function, mating behavior, conception, embryo-fetal development, and parturition [185]. Extended-generation reproductive toxicity studies were also carried out to assess the effects of the test chemicals on specific life stages not covered by other types of toxicity studies, as well as to

evaluate potential outcomes resulting from prenatal and postnatal chemical exposures [35]. The present study aimed to evaluate the acute and subacute toxicity, embryo-fetal developmental toxicity, reproductive toxicity and extended first generation developmental toxicities of the ethanol leaf extract of *U. simensis* in Wistar albino rats.

6.1 Acute and subacute toxicity studies

This study was conducted to evaluate the acute and subacute toxicity of *U. simensis* leaf extract in the albino rats. The no observed adverse effect level (NOAEL) of the extract in rats was determined to be 250 mg/kg body weight. Despite this, *U. simensis* leaves are widely used in traditional medicine at unspecified doses for the treatment of diabetes [57], wounds [186], gastritis [187], heart disease [188], and sexually transmitted diseases [48]. Four weeks of oral administration of the extract at doses of 500 mg/kg and 1000 mg/kg resulted in alterations in body weight, organ weights, hematological and biochemical parameters, as well as histopathological changes in some female rats.

Rats treated with *U. simensis* leaf extract exhibited a dose dependent increase in body weight compared to the respective control groups. This effect might be due to the nutritional composition of *U. simensis* leaf as reported by Diddana et al. [116]. However, the current findings contradict earlier reports in which experimental animals administered *U. simensis* leaf extract experienced weight loss [82, 86]. These discrepancies may be attributed to variations in extraction methods, administered doses, and duration of exposure.

In the present study, the weights of the kidneys, liver and spleen were significantly increased in rats treated with 1000 mg/kg of *U. simensis* leaf extract as compared to the corresponding control group. This effect might be associated with the action of the extract itself, as similar organ weight

increases were reported by Nouioura et al. [189]. Raina et al. [190] reported that organ weights can serve as important indicators of animal's health and well-being.

In the present study, rats treated with the *U. simensis* leaf extract exhibited a dose-dependent increase in the white blood cell (WBC) count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Conversely, the red blood cell (RBC) count, platelet count, hemoglobin (Hb), and hematocrit (HCT) values decreased in a dose-dependent manner compared to the respective control groups. These hematological alterations suggest that blood parameters are highly sensitive indicators of exposure to toxic substances [191]. This may be attributed to physiological adjustments to the animals' bodies. Blood samples often provide valuable insights into the body responses to adverse conditions such as exposure to harmful substances [192].

The adverse effects of herbal medicines can be assessed through changes in hematological and biochemical markers [193]. The significant increase in WBC count observed in this study might reflect an immune response to the *U. simensis* leaf extract, serving as a defense mechanism against toxic insults [194]. Conversely, the reduction in RBC count and increase in MCH could suggest that the extract induced macrocytic anemia, a condition in which erythropoiesis is impaired and the newly formed RBCs are larger than normal [195]. The significant decrease in Hb levels observed in female rats treated with 1000 mg/kg of *U. simensis* leaf extract could be attributed to the reduction in RBC count. This decline in Hb levels may also be associated with hemodilution or interference of the extract with iron absorption; although further investigations are needed to confirm these possibilities.

In the present study, rats treated with 500 and 1000 mg/kg of *U. simensis* leaf extract showed significantly elevated serum ALT levels compared to their respective control groups. The mean

ALT values for rats treated with highest dose exceeded the normal range reported for Wistar albino rats (females: 46 U/L; males: 33 U/L) [196]. Alterations in liver function test parameters are well-documented indicators of hepatotoxicity [183]. Specifically, elevated serum levels of AST, ALT and ALP are markers of hepatic injury [197, 198], with ALT considered the most specific indicator as it is mostly localized in the liver cytosol [199]. The alkaloids detected in the *U. simensis* leaf extract may be responsible for the elevated liver functions, as previous studies have reported that some plant alkaloids possess toxic properties [200, 201].

In the present study, serum creatinine in male rats treated with 1000 mg/kg of *U. simensis* leaf extract was significantly higher than those in the respective control group. The best indicator of renal function is serum creatinine [202]. High serum creatinine level indicates nephron dysfunction [202, 203]. This might be due to the phytochemicals present in the plant, particularly tannins which decrease the efficiency of glomerular filtration but control serum creatinine concentration [204, 205]. The non-significant increase in serum cholesterol, LDL, and HDL observed in rats treated with 1000 mg/kg of *U. simensis* leaf extract may suggest a potential adverse effect of the extract on lipid metabolism.

Furthermore, some female rats treated with 1000 mg/kg of *U. simensis* leaf extract showed liver parenchymal necrosis, kidney glomerular distortion, and spleen white pulp depletion. This might be due to the possible adverse effects of the phytochemicals present in the extract, such as alkaloids and tannins. On the other hand, the observed hepatic parenchymal necrosis might be attributed to the xenobiotic metabolism resulting from repeated administration of the leaf extract [206]. The parenchymal necrosis of the liver was also evidenced by elevated liver function tests. Serum ALT levels were elevated in rats of both sexes; however, the histopathological alterations were observed only in some female rats.

The sex-specific effect may be attributed to the role of estrogen as a potent inducer of coagulation factor synthesis in the hepatocytes [207, 208], potentially contributing to the liver parenchymal necrosis observed in female rats treated with *U. simensis* leaf extract. The renal glomerular distortion observed in some female rats may be attributed to urinary space dilatation associated with the hydronephrosis, while the depletion of white pulp in the spleen could indicate a potential immunotoxicity effect of the leaf extract. However, the female rats in the satellite group treated with 1000 mg/kg of the *U. simensis* leaf extract did not show any notable histopathological changes, which might be due to recovery or healing following withdrawal of the treatment. To clarify the discrepancies between the physiological indicators and histological findings, a chronic administration of the *U. simensis* leaf extract is recommended.

6.2 Teratogenicity of *U. simensis* leaf extract

In this study, the embryonic and fetal toxic effects of ethanol extract of *U. simensis* leaves were evaluated in the pregnant Wistar albino rats. The extract of *U. simensis* leaves was administered between 6 and 12 days of pregnancy, a critical period for growth and development in rats [150]. Crown rump length, somites and morphological scores are key indicators of embryonic growth [209]. Morphological scores which have predictable relationship with embryonic age were used to estimate embryonic development [156].

No deaths or noticeable behavioral changes were observed in either the treatment or control groups throughout the study period. Pregnant rats administered 1000 mg/kg of *U.simensis* leaf extract showed reduced dietary intake and weight gain compared to the pair-fed and ad libitum control groups, but the differences were not statistically significant. This finding was consistent with previous reports on the cardioprotective and antidiabetic properties of the *U. simensis* leaf extract, which showed experimental animals receiving the extract lost weight [82, 86]. The substantial

reduction in weight gain observed in the present study could be attributed to the presence of polyphenols in the *U. simensis* leaves, which have been reported to induce weight loss effects [210]. Toxicity or adverse reactions to treatment would be other possible explanations for decreased weight gain in the pregnant dams [211, 212].

Pregnant rats treated with 1000 mg/kg of *U. simensis* leaf extract exhibited significant decreases in somite numbers and morphological scores compared to pair-fed and ad libitum control groups. Because a crude extract was used, it was not possible to know which compound was responsible for the reduction in embryonic growth parameters. However, the effect might be attributed to the presence of naphthalene, eugenol or organosulfur compounds in *U. simensis* leaves, as reported in a previous study [126]. These compounds have been reported to interfere with the cell cycle and promote apoptosis [213-215]. The dose dependent decrease in the embryonic crown rump length may be associated with the presence of alkaloids or compounds such as linalool in the *U. simensis* leaves which have been reported to halt growth and development [126, 216, 217].

In the present study, significant embryonic developmental delays of the caudal neural tube, otic system, olfactory system, forelimb and hindlimb were shown in pregnant rats administered 1000 mg/kg of *U. simensis* leaf extract. Significant developmental lags in the caudal neural tube and olfactory system were also observed in pregnant rats treated with 500 mg/kg of *U. simensis* leaf extract. These embryonic developmental delays could be attributed to the presence of tannins and alkaloids in the extract, which are known to inhibit growth and development by interfering with cholinergic neurotransmission [218, 219].

In the fetal experiment of the current study, external morphological evaluations of fetuses revealed no discernible treatment-related anatomical deformities in the head, neck, thoracic, or abdominopelvic visceral organs. However, the crown-rump length, and also fetal and placental

weights were reduced in a dose-dependent manner in pregnant rats administered *U. simensis* leaf extract as compared to the pair-fed and ad libitum control groups. The reduction of these fetal biometrics may be associated with the presence of some phytochemicals of *U. simensis* leaves, especially 1,8-cineole and eugenol which are known to induce apoptotic cell death [88, 220]. It may also be attributed to other components of *U. simensis* leaves, such as phenolic substances that can reach the placenta and impede the placental steroidogenesis [221]. Furthermore, the significant increases of fetal resorption in pregnant rats treated with a dose of 1000 mg/kg *U. simensis* leaf extract may be attributed to the phytochemicals present in the leaves [126]. The aromatic hydrocarbons present in *U. simensis* leaves [88] may also have a role in the resorption due to their intermediary effects on fertility [222].

In the present study, the ossifications of the sternum, ribs, vertebrae, hyoid, metacarpal, metatarsal, forelimb, and hindlimb bones did not differ significantly between the treatment and control groups. While the extract-treated groups exhibited fewer ossification centers compared to the control groups, the difference was not statistically significant. These findings suggest that the *U. simensis* leaf extract exerts minimal or no effect on skeletal ossification. The current study provides scientific evidence on the prenatal developmental toxicity of *U. simensis* leaf extract in rat embryos and rat fetuses. However, it was not without some limitations. The first limitation was the extract was administered only from the sixth to twelfth day of pregnancy. The second was the study used the minimum acceptable numbers of experimental animals due to limited resources to handle the animals.

6.3 Teratogenicity of *U. simensis* essential oil

The pharmaceutical sector is strongly looking for the natural remedies with biological properties [223]. Essential oils are volatile and fragrant extracts derived from different parts of aromatic

plants such as flowers, leaves, stems, bark or roots. These oils are renowned for their distinct fragrances and have been used for centuries across various cultures for their therapeutic, cosmetic and culinary purposes [224-226]. The major components of *U. simensis* essential oil are aromatic hydrocarbons, organosulfur compounds and monoterpenoids [88]. Among these, the aromatic hydrocarbons are the principal group of substances responsible for oil's distinct aroma. The organosulfur compounds and monoterpenoids also contribute to the overall composition and medicinal properties of the essential oil [226, 227]. Although the oil has been reported to have therapeutic and nutritional values [88], its safety following prenatal exposure has not yet been studied.

This study aimed at evaluating the prenatal developmental toxicity of *U. simensis* essential oil in rat embryos and rat fetuses. Pregnant dams were administered *U. simensis* essential oil by oral gavage at doses of 250, 500, and 1000 mg/kg from the sixth to twelfth day of gestation. The findings of this study showed that oral administration of 250 and 500 mg/kg of the oil did not produce any observable adverse effects in either rat embryos or rat fetuses. However, the gravid rats treated with 1000 mg/kg of the oil showed significant developmental delays and growth retardations in both rat embryos and rat fetuses.

The mean somite and morphological scores of twelve-day-old rat embryos exposed to the 1000 mg/kg of the oil were significantly lower than those of the pair fed and ad libitum control groups. The mean crown-rump length of the developing embryos treated with the oil was lower than the control groups, but it was not statistically significant. The significant decrease in the somite and morphological scores may be attributed to the presence of naphthalene, eugenol and organosulfur compounds found in the essential oil [88], which interfere with the cell cycle [213-215]. The dose-dependent reductions in the embryonic growth indices may also be attributed to the presence of

certain alkaloids in the emulsified hydrosol of the oil as well as other acyclic monoterpenoids, especially linalool, known to inhibit growth and development [126, 216, 217]. In this study, the in vivo embryonic development of the caudal neural tube and forebrain in the 12-day-old rat embryos exposed to 1000 mg/kg of the oil was significantly delayed compared to the pair-fed and ad libitum control groups. This delay could be attributed to the presence of tannins and alkaloids in trace amounts in the aromatic water of the oil, which might have halted growth and development as evidenced by reported studies [218, 219].

External and visceral morphological assessment of 20-day-old rat fetuses revealed no noticeable treatment related anomalies in the cranial, nasal, oral, trunk and visceral organs. However, fetuses exposed to 1000 mg/kg of *U. simensis* essential oil exhibited significant reduction in the crown rump length and fetal weight compared to both the pair-fed and ad libitum control groups. In addition, the mean fetal resorption significantly increased in gravid rats treated with 1000 mg/kg of the oil compared to control groups. Reductions in crown rump length and fetal weight might be associated with certain constituents of the oil, particularly, 1,8-cineole and eugenol, which have been shown to impede cell proliferation, as evidenced by their anti-proliferative effects of cancer cell lines [88, 220]. Some studies also reported that exposure to aromatic hydrocarbons results in developmental toxicity [222, 228]. The increased fetal resorption in 1000 mg/kg treated dams could be attributed to the apoptosis role played by *U. simensis* essential oil [126] and the role of polycyclic aromatic hydrocarbon constituents of the oil, such as naphthalene, as an intermediary effect of infertility [222]. The anogenital distance in twenty days old male and female fetuses showed no significant differences between pregnant rats exposed to *U. simensis* essential oil and the control groups. Furthermore, no abnormalities were observed in the nipples, areola or external

genitalia. This finding suggests that *U. simensis* essential oil might not produce any androgen-related observable adverse effects.

In this study, the ossification (foci or centers) of the sternum, ribs, vertebrae, hyoid, metacarpal, metatarsal, forelimb, and hindlimb bones did not show any significant differences between gravid rats treated with *U. simensis* essential oil and those in the pair fed and ad libitum control groups. However, the treatment groups had lower number of ossification centers compared to the control groups. This difference was not statistically significant, indicating that *U. simensis* essential oil had no observable adverse effects on the skeletal ossification. Similarly, oral administration of the oil did not affect histopathology of the placenta. This could indicate that the essential oil either does not alter placental morphology or possesses placental regenerative effect, since the treatment was limited to days 6 to 12 of pregnancy. The current study provides scientific evidence on the toxic effects of the *U. simensis* essential oil in rat embryos and fetuses. However, the study did not address the postnatal effects of the essential oil. Therefore, further studies are recommended to investigate the postnatal effects of the *U. simensis* essential oil to gain better understanding of its toxicological properties.

6.4 Reproductive toxicity of *U. simensis* leaf extract

In this study, a reproductive toxicity evaluation was conducted to assess the potential effects of the ethanol leaf extract of *U. simensis* on reproductive indices, as well as on biochemical changes and histopathological alterations in the liver and kidney of both male and female parental rats. Female parental rats that received 500 and 1000 mg/kg of *U. simensis* leaf extract showed a significant pre-gestational weight gain compared to the control groups. However, during gestation, the 1000 mg/kg treated female parental rats exhibited significantly lower weight gain as compared to the corresponding controls. Lactational weight changes remained similar across all groups. Similarly,

the male parental rats that received 1000 mg/kg of *U. simensis* leaf extract showed a significant weight gain compared to the corresponding controls. The observed biphasic weight modulation in female rats was characterized by an increased pre-gestational weight gain at doses of 500 and 1000 mg/kg of the leaf extract, however, the 1000 mg/kg dose also resulted in reduced gestational weight gain. This finding is consistent with the established knowledge regarding the interactions of phytochemicals with metabolic and the reproductive physiology [229].

The significant weight gain at higher doses (500 and 1000 mg/kg), despite unchanged food consumption, is consistent with previous studies reporting that improved nutrient partitioning increased the lean mass without hyperplasia [230]. The pre-gestational weight gain may be attributed to bioactive constituents, specifically flavonoids, which enhance protein synthesis and improve insulin sensitivity for glucose uptake [231-233]. The gestational weight reduction seen in the 1000 mg/kg treated-group contrasts with the pre-gestational effects. However, it aligned with reports on *Urtica dioica*, which has been shown to enhance metabolic process, such as insulin sensitivity and lipid profile regulation, factors commonly linked to weight management [234]. This finding was supported by reports that ursolic acid analogs, widely distributed in medicinal plants, possess uterine relaxant properties that may reduce placental blood flow, thereby limiting gestational weight [235].

The normalization of weight changes during lactation across all doses indicates an adaptive metabolic resetting. This observation aligns with prolactin-mediated metabolic flexibility, which appears to override the earlier phytochemical effects [236]. In addition, it might also be due to lactation and rearing effects [237]. Although pre-gestational anabolic effects could benefit underweight females, gestational hazards should be carefully considered. The 500 mg/kg dose of *U. simensis* leaf extract may offer the optimal balance possibly offering benefits without

gestational trade-offs. Male parental rats treated with the *U. simensis* leaf extract showed higher weight gains than the control group. The extract, particularly at a dose of 1000 mg/kg, significantly increased the weight gains compared to both the control group and those receiving lower doses. This suggested the potential anabolic or growth promoting effects of the extract at higher dose [238].

On the other hand, female parental rats treated with 1000 mg/kg of the extract showed significantly lengthened estrous cycle. The lower doses (250 and 500 mg/kg) had no notable effects on the estrous cycles. The significant prolongation of the estrous cycle at a dose of 1000 mg/kg, indicates a potential hormonal or reproductive modulating effect of the extract at high doses. This may be attributed to the phytochemical constituents present in *U. simensis* leaf extract. Previous studies have demonstrated that secondary metabolites such as alkaloids, tannins and flavonoids possess antigonadotrophic effects and suppress ovulation [239, 240].

In the current study, both male and female parental rats treated with *U. simensis* leaf extract did not produce any adverse effects on mating or fertility indices, and female rats in all groups produced viable litters. This indicates that the extract did not affect fundamental reproductive functions at the studied doses. However, parental rats treated with 500 and 1000 mg/kg of the extract showed significant prolonged mating latencies compared to their respective control groups. This may indicate a mild sedative or libido reducing effects of the extract at the higher doses.

The observed significant delay in mating (the pre-coital interval) at the higher doses is possibly look into whether this delay is associated with hormonal modulation, stress responses, or behavioral changes. While female parental rats receiving higher doses (500 and 1000 mg/kg) of *U. simensis* leaf extract showed a significant decrease in litter size, those treated with the lowest dose (250 mg/kg) exhibited no significant effects, suggesting the presence of a threshold for the

extract's reproductive impacts. This could be associated with a mild antifertility effect of the extract at the higher doses (e.g. reduced ovulation or implantation) without affecting pup viability. Although the extract may modulate fertility at higher doses, it did not cause fetal mortality or sex ratio biases.

The reduced litter size could stem from hormonal changes, ovarian effects or embryo-implantation interferences. Several researchers have indicated that some alkaloids could traverse the placental membrane and influence the embryonic development [241]. At birth (PND 0), the male offsprings from the 1000 mg/kg treatment group showed significantly reduced body weight compared to the controls, while female pups remained unaffected. This sex specific reduction in the birth weight suggests the potential gender dependent fetal growth modulation under high-dose exposures. These male pups demonstrated a rapid compensatory growth response, exhibiting significantly increased weight gain by the PND 4 compared to controls.

No persistent effects on the body weight were observed in either sex of the pups beyond PND 7, indicating that the high-dose exposure induced transient effects rather than permanent growth alterations. The male pups appeared to exhibit effective metabolic adaptation that normalized their growth trajectories. While the female pups showed greater resilience to the extract's growth modifying effects. These findings highlight a sexually dimorphic response patterns, where male pups experienced both initial growth restriction and subsequent compensatory response, while the female pups maintained stable growth throughout all developmental stages. While the transient nature of these effects suggests that *U. simensis* leaf extract might temporarily influence the fetal growth pathways at high doses, it did not cause long-lasting impairment of postnatal development.

Sperm parameters of parental male rats treated with *U. simensis* leaf extract showed no significant differences across all groups. The extract did not affect the sperm production at all tested doses

(250, 500, and 1000 mg/kg). This might indicate that the *U. simensis* leaf extract had no disruptions of spermatogenesis. However, the 1000 mg/kg treated group showed a small but statistically significant increase in the abnormal sperm morphology compared to the corresponding control group. On the other hand, the lower doses (250 and 500 mg/kg) had no effect, suggesting a threshold dose for the sperm development and maturation effects. The significant increase in the abnormal sperm morphology were observed at 1000 mg/kg represents a biologically modest change, with only about a 1% absolute increase compared to the control group. The potential causes might be oxidative stress, hormonal modulation, or mild toxicity associated with high doses.

U. simensis leaf extract did not cause any measurable changes in the reproductive organ weights in either male or female parental rats at doses up to 1000 mg/kg. The weights of female reproductive organs showed slight numerical decreases, but these were not statistically significant. Combined with the functional data (longer estrous cycle, fewer litters) findings, this suggests possible hormonal modulation without structural damages. The male reproductive organs remained entirely unaffected, further supporting the safety of the extract with regard to testicular and accessory gland functions.

Histopathological evaluation is a critical component of toxicological investigations, that look into as it helps to identify lesions and reveal underlying toxic processes [242, 243]. However, in the current study, parental rats treated with the *U. simensis* leaf extract showed normal histology of the primary reproductive organs, the testis and ovaries. This implied that oral administration of the extract did not affect the histological architecture of the ovaries and testes. Both male and female parental rats treated with the extract exhibited no significant difference in the mean weights of the liver and kidneys compared to the corresponding control groups. Organ weight alteration is a good

indicator of toxicity, disease progression and drug efficacies [211]. The slight increase in mean liver weight observed in the 1000 mg/kg treated group of both male and female parental rats may indicate mild metabolic adaptive hypertrophy.

However, histopathological examination revealed liver parenchymal necrosis in rats treated with 500 and 1000 mg/kg of the extract. All male and female parental rats treated with the extract did not show any renal histological aberration. Biochemical analysis of parental rats treated with the highest dose of *U. simensis* leaf extract showed a significant increase in liver enzyme markers (ALT, AST, and ALP) compared to the respective control groups. The significant increase in liver enzyme levels corroborates the parenchymal necrosis observed under microscopic examination.

The concurrent increase in all the three enzyme markers (ALT, AST, and ALP) indicates a mixed patter of liver injury, involving both hepatocellular damage (primary damage to liver hepatocytes) and cholestatic injury (impairment of bile flow). ALT is the most sensitive liver enzyme that detects liver cell injury and bile duct obstruction. Liver injury or bone diseases could all cause a rise in serum ALP levels [197, 244]. Elevated ALT, AST, and ALP serum levels indicated the subclinical hepatotoxicity of the *U. simensis* leaf extract. Liver parenchymal necrosis could result from certain alkaloids or toxins that might directly disrupt the integrity of hepatocyte membranes or the essential cellular processes [245].

In the current study, both male and female parental rats treated with the extract showed normal serum urea and creatinine values, which aligned with the unchanged kidney weights confirming the absence of renal toxicity. Creatinine values reflect glomerular filtration rate, but serum urea levels reflect renal excretion capacity [246]. This was congruent with the histological findings of the kidney, which revealed no morphological alterations in any of the extract treated groups.

Parental rats treated with *U. simensis* leaf extract did not show any significant differences in protein, glucose, and lipid profiles. However, only female parental rats treated with the highest dose of the extract exhibited significant increase in protein metabolism. The pronounced elevation in total protein metabolism in the female parental rats was possibly due to hormonal or metabolic differences. The non-significant reductions in blood glucose levels observed in parental rats treated with the highest dose of the extract suggest a potential antidiabetic effect of *U. simensis* leaves [82].

6.5 Developmental reproductive toxicity in F1 cohort-1 rats

The F1 cohort-1 rats were exposed to *U. simensis* leaf extract indirectly during intrauterine development and lactation. This was followed by daily oral administration from weaning until PND 90. This pattern was intended to assess the prolonged effects of *U. simensis* leaf extract on the postnatal development, particularly on the reproductive, hepato-renal, and endocrine systems.

In this study, both male and female F1 rats treated with the extract exhibited a non-significant reduction in daily food intake. However, male offspring rats that received 1000 mg/kg of the extract showed a significant weight gain compared to the control group that received only the vehicle. The female offspring rats treated with 500 and 1000 mg/kg doses also showed a significant weight gain compared to the corresponding controls. This effect might be due the nutritional values of the *U. simensis* leaves. The weight gain observed in F1 cohort-1 rats was similar to those of the parental rats in that treatment with high doses of the extract increased weight gain. Such weight increase at high doses, despite reduced food intake, may be associated with modulation of gut microbiota or alteration energy metabolism by the plant extract. It may also be attributed to the anabolic effect of the extract, such as enhancement of protein synthesis, which contribute to the observed weight gain.

The timing of preputial separation in males and vaginal opening in females was assessed in F1 cohort-1 rats. Cornification of the epithelium between the glans penis and the prepuce initiates the separation of the prepuce [247]. When the epithelial cornification reached the ventral end of the glans penis, preputial separation was considered to be complete. It was an androgen dependent process that served as an external marker of puberty [247, 248]. Female rats' puberty onset has been identified as the age of vaginal opening and the first appearance of cornified epithelial cells in the vagina [249]. These are triggered by increment of estradiol level and usually coincide with the very first wave of the developing ovarian follicles. In the present study, the F1 offspring rats treated with *U. simensis* leaf extract did not show any significant effects on the timing of sexual maturation markers as measured by the preputial separation in male rats and vaginal opening in female rats. At the tested doses, the extract did not speed up nor slow down the beginning of puberty in either male or female rats. These may indicate that the extract does not have significant endocrine disrupting actions in these specific areas of reproductive developmental endpoints within the study dose ranges.

In the current study, the F1 cohort-1 female rats treated with 1000 mg/kg of the extract showed a significant prolongation of the estrous cycle and increased vaginal cornification. These effects were consistent with those seen in the parental female rats, demonstrating estrogenic or hormonal modulating activity at high doses in F1 female generations. This suggests that exposure to a repeated high dose of the extract subtly changes hormonal regulation of the estrous cycle, possibly through effects on the hypothalamic-pituitary-ovarian axis. Prolonged cornification time indicates persistent estrogenic stimulation resulting from the treatment. However, there were no aberrant histological abnormalities observed on the ovarian tissues. The functional disturbance without structural changes may be attributed to the phytochemical constituents of *U. simensis* leaves, such

as flavonoids, which could prolong the estrous cycle by disrupting the hypothalamic-pituitary-ovarian axis and interfering with the ovarian follicular development [250].

In the female progenies, there was a persistent but non-significant lower weights in all examined reproductive organs, such as the ovaries, fallopian tubes, and uterus with cervix, compared to control groups, with the most pronounced decrease in the ovarian weight of animals treated with the highest dose. In male offsprings, the reproductive organs, such as the testes, epididymis, seminal vesicles and prostate glands, did not reveal any significant organ weight changes across all treatment groups and control groups. The non-significant decrease in the ovarian weight in the F1 female rats was potentially related to the observed changes in the estrous cycles. The retention of normal organ weight in the male progenies was consistent with prior results of unaffected sperm counts and fertility indices.

Histological evaluation of the ovaries and testes in the F1 cohort-1 rats exhibited normal histological morphology in both the treatment and control groups. The normal morphological architecture of the ovaries implies that the ovarian cycle variations were caused by functional rather than structural changes. Results from this cohort study revealed a significant increase in liver weight in rats treated with the highest dose *U. simensis* leaf extract. The mean liver weight in F1 male rats showed only a non-significant increase compared to the respective control groups. Kidney weights remained unaffected in both male and female F1 rats across all treatment groups. Endocrine organs (thyroid and adrenal glands) maintained normal weights in all F1 cohort-1 rats. The increase in liver weight observed in the F1 female progeny treated with 1000 mg/kg dose was consistent with the findings from the parental female rats, which showed elevated enzyme levels, suggesting a sex-dependent metabolic response to high-dose exposure to the extract.

Biochemical profile data in F1 cohort-1 rats showed dose-dependent effects of the *U. simensis* leaf extract, with the most consistent changes observed in the liver function markers. Both male and female offsprings exhibited statistically significant elevations in liver enzymes (ALT, AST, and ALP) at the highest dose, consistent with the findings observed in the parental rats. In addition, the F1 cohort-1 rats treated with 500 and 1000 mg/kg of the extract also exhibited liver parenchymal necrosis, similar to those observed in the parental rats. This indicates a clear, dose-dependent hepatotoxic effects of the extract with heritable impacts observed across generations, affecting both the parental and F1 offspring rats. ALT, AST, and ALP are normally contained within the liver cells, hepatocytes and their significant elevation in the blood stream is a classic clinical indicator of liver cell damage and rupture [251].

Microscopic observations of liver parenchymal necrosis corroborated the biochemical data findings, indicating liver cell death. The most straight forward explanation is that the extract contains dose-dependent hepatotoxic compounds that are bioactivated in the liver cells into damaging agents. This finding is supported by a previous study which showing that some plant toxins are not inherently toxic but become harmful after metabolic activation in the body [252]. They are converted by hepatic cytochrome P450 enzyme into highly reactive intermediates [253]. These reactive metabolites bind covalently to cellular proteins and DNA; and disrupts critical cellular functions, leading to cell death, necrosis. The liver cells might use up their reserves of key antioxidants in an attempt to neutralize these reactive metabolites [254]. Once the key antioxidants are depleted, the cells are vulnerable to oxidative stresses. The reactive metabolites or other compounds in the extract could generate reactive oxygen species, which attack and damaged lipids in cell membranes, ultimately triggering necrotic cell death [255].

The F1 offsprings were exposed to the extract during the critical developmental windows, and their developing livers, experienced similar direct damage or necrosis from the extract, explaining the consistent findings. The female progenies showed more pronounced biochemical alterations, including a significant increase in the total protein levels at a dose of 1000 mg/kg, while the male progenies failed to show a significant difference compared to the corresponding controls. Notably, the kidney function parameters, urea and creatinine, remained unchanged across all treatment groups, demonstrating that *U. simensis* leaf extract is free from renal toxicity in the first progeny rats as evidenced by unaltered renal weight changes in all treatment groups.

The metabolic indices such as glucose, cholesterol, and thyroid function hormones (T3, T4, and TSH) did not change significantly, showing that glycemic control, lipid metabolism, and thyroid axis functions are all normal. The liver enzyme elevations observed in the 1000 mg/kg treatment group were consistent with the increased liver weight in the female progeny rats, implying a modest, dose dependent hepatotoxicity that was likely reversible as no corresponding changes were noted in synthetic functions such as albumin. The sex-specific responses observed in the female rats, notably in total protein levels, might be attributed to hormonal influences on hepatic protein metabolism [256, 257].

6.6 Developmental neurotoxicity in F1 cohort-2 rats

Due to its developmental state, the immature nervous system exhibits heightened susceptibility to neuro toxicants as compared to the adult neuronal system [258]. Perinatal exposure to a substance can induce permanent neurological damage at the lowest dose that produces no significant adverse effects in adults [259]. Several medicinal herbs have been reported to exhibit neurotoxic effects [260-263]. This study aimed to evaluate the neurotoxic effect of *U. simensis* leaf extract on the

postnatal development of F1 cohort-2 progeny rats. The assessment was conducted following prenatal and lactational exposures, as well as oral dosing after weaning.

F1 offspring rats treated with the extract did not show any neurobehavioral aberrations. F1 male progeny rats treated with 1000 mg/kg and female progeny rats treated with 500 and 1000 mg/kg of the extract showed statistically significant weigh gains compared to the respective control groups. However, all F1 cohort-2 offspring rats treated with the extract did not show any significant differences in the mean weights of brain and the spinal cord compared to the respective control groups.

The subtle trends and sex specific variations require interpretation in the context of existing literature on plant derived neuroactive substances. The mean brain weights of both male and female F1 ohort-2 progeny rats were comparable to their respective controls, with only minor, non-significant reductions observed at higher doses in the male offspring. Similar findings were reported for *Moringa oleifera*, where even high doses did not affect the brain weight despite systemic growth effects [264]. This might be due to the blood brain barrier which limits phytochemical penetration, protecting the brain and spinal cord from direct weight altering effects. The female progeny rats consistently showed lower absolute brain weights as compared to the male progeny rats, which was consistent with the established sexual dimorphism in the rodent brain morphology [265].

Histopathological assessment of F1 cohort-2 rats treated with *U. simensis* leaf extract did not show any structural changes of tissue sections taken from the cerebral cortex, cerebellum, spinal cord, sympathetic ganglia and peripheral nerve fibers. The absence of histopathological changes in the sampled neural tissues, despite the observed weight gains, provided substantial insights into the mechanism of action and safety profile of the extract. The lack of structural changes observed in

the brain, spinal cord, or nerve tissue samples following prolonged administration was a strong evidence for the safety of *U. simensis* extract. This suggests that the active constituents of *U. simensis* may not readily cross the blood brain barrier. This phenomenon is a common characteristic of many phytochemicals [266].

Molecular sizes, charges, or lipid solubility of these substances may limit their ability to enter the central nervous system at concentrations high enough to cause damage. However, they could still interact freely with peripheral organs, including the gut, liver, muscles, and fat. The findings of this cohort study indicates that oral administration of *U. simensis* leaf extract may not have adverse effects on the developing neural system and behavior.

6.7 Developmental immunotoxicity in F1 cohort-3 rats

The early developing immune system is commonly characterized by low serum complement activity, decreased neutrophil reserve, low antibody production, and reduced lymphocyte activity [267-269]. Some nutraceutical herbs have been reported to have immunotoxic effect [270, 271]. The immunotoxicity of *U. simensis* leaf extract was evaluated in F1 cohort-3 progeny rats after prenatal and lactational exposure through their dams as well as post-weaning oral administration.

In this study, the F1 progeny rats treated with the extract showed no signs or symptoms of toxicity during daily cage-side clinical observations. In addition, F1 cohort-3 rats treated with the extract did not show any significant body weight changes compared to the corresponding control groups. This indicates that the extract exhibited a favorable safety profile in the F1 cohort-3 progeny rats under subchronic administration.

The absence of cage-side clinical signs, such as lethargy, piloerection, respiratory distress, abnormal gait, mortality, along with stabile of body weight, strongly indicates that the extract lacks

overt toxicity. The stable body weight might be due to the nutraceutical-like action of *U. simensis* leaves. Many plant extracts function more as supplements rather than as potent drugs. Their effects are subtle and supportive, including enhancing antioxidant status and providing mild anti-inflammatory actions without disrupting core homeostasis, consistent with the stable body weight observed with some plant extracts [272, 273].

Histological examination of key immunological organs such as spleen, thymus, and mandibular lymph nodes, taken from F1 cohort-3 rats treated with *U. simensis* leaf extract (250, 500, and 1000 mg/kg) revealed no abnormalities. Absence of histopathological changes following subchronic oral administration indicates a safe immunomodulatory effect of the extract. The extract did not induce immunosuppression, immune hyperactivity or autoimmune pathology at the tested doses. The absence of abnormalities suggests that the plant leaves do not exert direct cytotoxic effect on immune cells, do not cause persistent immunological activation and not act as a nonspecific mitogens, a common toxic effect associated with certain plant lectins [274, 275]. The preserved histological architecture of these lymphoid organs further indicates that the extract does not disrupt the normal turnover and proliferation of immune cells essential for maintaining their structures and functions.

7. CONCLUSIONS

The present study confirmed that the median lethal (LD_{50}) dose of *U. simensis* leaf extract exceeded 5 g/kg body weight. The no observed adverse effect level (NOAEL) of *U. simensis* leaf extract was 250 mg/kg; while the least observed adverse effect level (LOAEL) of *U. simensis* leaf extract was 500 mg/kg. The liver was the target organ of toxicity for *U. simensis* leaf extract, evidenced by elevated serum liver enzymes and histopathological lesions at doses of 500 mg/kg and above. The leaf extract and essential oil of *U. simensis* were developmentally unsafe at 1000 mg/kg doses, causing significant embryonic growth retardations, development delays, and increased fetal resorptions. The 500 mg/kg of leaf extract also induced developmental lags. The extract from *U. simensis* leaves prolonged mating latency and reduced litter size at 500 and 1000 mg/kg doses. It also lengthened the estrous cycles in females, indicating an adverse effect on the reproductive system. The toxicity of *U. simensis* leaf extract was evident in the F1 generations, with progeny showing patterns of weight gain, prolonged estrous cycles, and liver toxicity similar to their parents. At the tested doses, the extract from *U. simensis* showed no evidence of causing neurobehavioral changes, structural damage to the nervous tissues, or pathological alterations in the key immune organs. Considering the observed effects, the repeated oral intakes of high-dose *U. simensis* derivatives for purported nutraceutical benefits should be approached with caution, particularly during pregnancy and reproductive ages.

8. RECOMMENDATIONS

- Future work should prioritize the isolation and identification of specific phytochemical compounds responsible for the observed adverse effects.
- Subsequent toxicological studies should be done beyond the observational endpoints to investigate the underlying molecular pathways for explaining mechanistic basis of the adverse outcomes reported here. Investigations involving embryo-culture biotechnology and immunohistochemistry also should be considered.
- To enable robust assessment of neurotoxicity, future studies should employ a standardized and comprehensive battery of behavioral tests evaluating motor activity, sensory function, learning, and memory. Concurrent investigation into the functional aspects of the immune system is also recommended.

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