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PhD Dissertation

Extended One-generation Reproductive Toxicity and Teratogenicity of Ethanol Leaf Extract of *Syzygium guineense* Wall. in Rats.

By: Melese Shenkut Abebe

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Extended One-generation Reproductive Toxicity and Teratogenicity of Ethanol Leaf Extract of *Syzygium guineense* Wall. in Rats.

By: Melese Shenkut Abebe

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Advisor:

- **Dr. Girma Seyoum (Associate Professor, Department of Anatomy, AAU)**

Co-advisors:

- **Professor Kaleab Asres (Professor, Department of Pharmaceutical Chemistry and Pharmacognosy, AAU)**
- **Dr. Yonas Bekuretsion (Associate Professor, Department of Pathology, AAU)**
- **Mr. Samuel W/kidan (Researcher, Traditional and Modern Medicine Research Directorate, EPHI)**
- **Mr. Eyob Debebe (Researcher, Traditional and Modern Medicine Research Directorate, EPHI)**

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Abstract

Background: Various extracts of *Syzygium guineense* Wall. leaves showed scientifically corroborated effects against hypertension, diabetic mellitus, breast and colon cancers, pain, inflammation, free radicals, snake venom, parasites, different bacterial strains, and fungi. It is widely stated that herbal products are presumed to be safe. However, validating the efficacy and assessing the safety of herbal products is mandatory. During pregnancy, an embryo or fetus can be affected by exposure to a variety of chemicals. The potential teratogenicity and reproductive toxicity of *S. guineense* leaf extract has not been determined yet. Therefore, the aim of this thesis research was to investigate the teratogenicity, reproductive toxicity, developmental neurotoxicity, and developmental immunotoxicity as well as glandular and hepato-renal toxicity of the ethanol extract of *S. guineense* leaves in rats.

Methods: For the teratogenicity study, five groups of Wistar albino rats, each consisting of ten pregnant rats were used as experimental and control animals. Groups I-III rats were treated with 250, 500, and 1000 mg/kg body weight of 70% ethanol extract of *S. guineense* leaves, respectively. Groups four and five were control and *ad libitum* control, respectively. Rats were treated beginning from day 6 to day 12 of gestation. Embryos and fetuses were respectively retrieved on day 12 and day 20 of gestation. The embryos were assessed for developmental anomalies and growth retardation. The fetuses were examined for developmental delays, growth retardation, gross external malformations, as well as skeletal and visceral anomalies. Histopathological alterations of the placenta also were evaluated for any treatment-related anomalies.

For the extended one-generation reproductive toxicity study, the parental Wistar rats, 20/sex/group, were randomly assigned into four groups. Groups one, two, and three received 250, 500, and 1000 mg/kg body weight of 70% ethanol extract of *S. guineense* leaf for 10 weeks, respectively: two premating, two mating, three pregnancy, and three lactational weeks. In the parental rats, the effect of extract administration on the food intake, weight gain, weight and histology of reproductive organs, liver, kidneys, adrenal glands, and thyroid gland were evaluated. Moreover, serum level of thyroid hormones and biochemical tests were measured. Sperm analysis was carried out and the length of estrous cycle was measured. Reproductive indices (pre-coital interval, pregnancy duration, mating, fertility, and gestation indices) and pregnancy outcomes also were evaluated. Once the pregnant dams gave birth, the pups were assessed for gross anomalies at

birth. The weight of pups was measured on postnatal day zero, four, seven, fourteen, and twenty-one. In addition, pups anogenital distance was measured on postnatal day four. The presence of nipple retention was assessed on postnatal day twelve. Moreover, postnatal death of pups was reported on postnatal day 1, 4, 7, 14, and 21. At weaning (postnatal day 21), the pups were randomly assigned into three cohort groups: to assess reproductive toxicity (set-1), developmental neurotoxicity (set-2), and developmental immunotoxicity (set-3). All pups assigned into the three cohort groups were orally treated on a daily basis with similar doses used for the parental rats. Set-1 pups, 20/sex/group were treated up to postnatal day 70. To investigate the extended effect of the test plant on the first-generation rats, the tests conducted on the parental rats were repeated on the set-1 pups. In addition, weight at and the day of vaginal opening/preputial separation were respectively evaluated in female and male set-1 rats. Set-2 pups, 10/sex/group, were treated until postnatal day 70 and the effect of the test plant extract on the weight and histopathology of the brain and spinal cord was investigated. Set-three pups, 10/sex/group, received the treatment until postnatal day 60 and the toxic effect of the plant extract on the weight and histopathology of spleen, thymus, and lymph nodes was evaluated. Data were analyzed by one-way analysis of variance and chi-square test using SPSS version 24.

Results: The results of potential teratogenicity assessment indicated that administration of 70% ethanol extract of *S. guineense* leaf resulted in a significant reduction of food intake and weight gain during pregnancy in high dose treated group. It also reduced the crown-rump length, and average morphological score of 12 days old rat embryos. Moreover, the crown-rump length of 20 days old rat fetuses was diminished by the treatment of 1000 mg/kg body weight of *S. guineense*. However, any of the doses of this plant did not produce significant effect on the number of implantations, resorptions, stillbirths, and live births. The external morphological and visceral examinations of rat fetuses did not reveal any detectable structural malformations in the cranial, nasal, and oral cavities as well as visceral organs. The ossification centers of fetal skull, vertebrae, hyoid, forelimb, and hindlimb bones were not significantly varied across all groups. However, although not statistically significant, high dose treated rat fetuses had a reduced number of ossification centers in the sternum, caudal vertebrae, metatarsal, metacarpal, and phalanges. The weight of the fetuses and the placentae were decreased. Decidual cystic degeneration was the most prevalent histopathological changes of the placenta of rats treated with 1000 mg/kg body weight of the test plant extract.

In the extended one-generation study, the administration of *S. guineense* extract resulted in significantly reduced food intake and weight gain of parental rats. Administration of 1000 mg/kg body weight of the extract prolonged the duration of estrous cycle and pre-coital interval of female parental rats. The mean number of litters and live births were significantly reduced in the treated groups. Rats treated with higher doses of the plant extract also showed significantly increased serum ALT, AST, ALP, and urea levels. Moreover, the blood glucose level of rats treated with 1000 mg/kg body weight of the extract was significantly decreased compared to that in the control groups. The serum level of thyroid hormone (T4) was significantly reduced in the rats treated with 500 and 1000 mg/kg body weight of *S. guineense* extract. Treatment of the rats with the high dose (1000 mg/kg body weight) of the plant extract significantly reduced the relative weight of the uterus and ovaries. No significant effect was observed in the number and morphology of spermatozoa, duration of gestation as well as mating, fertility, and gestation indices. The pup's weight, presence of nipple retention on male pups, anogenital distance, and number of postnatal deaths during lactation period were not significantly varied between the treatment and control groups. Furthermore, the weight and histopathology of reproductive organs (weight except for uterus and ovaries), liver, kidneys, adrenal glands, and thyroid gland were not significantly affected by treatment with *S. guineense* extract.

Similar to the parental rats, the result biochemical tests measured in the first-generation set-1 rats indicated that serum levels of ALT, AST, and ALP were significantly increased while food intake, weight gain, and serum levels of glucose and thyroid hormone were significantly decreased. In addition, the relative weight of the seminal gland, uterus, and ovaries was reduced by treatment with *S. guineense* extract (1000 mg/kg body weight). The relative weight of the other reproductive organs, liver, kidneys, adrenal glands, and thyroid gland was not significantly affected. In the first-generation rats, treatment with 1000 mg/kg body weight of *S. guineense* extract prolonged the length of estrous cycle. The weight at and the day of vaginal opening/preputial separation were not significantly altered by treatment with the test plant. Similarly, neither the relative organ weight nor the histopathology of the brain, spinal cord, spleen, thymus, and lymph nodes was affected by treatment with *S. guineense* extract.

Conclusion: In conclusion, administration of 70% ethanol leaf extracts of *S. guineense* resulted in decreased food intake and weight gain of pregnant and nonpregnant rats in the high dose treatment

group that indicated its toxicity at a high dose. Treatment of rats with the high dose of *S. guineense* extract revealed growth and developmental delays as evidenced by reduced crown-rump length and average morphological score of 12 days old rat embryos and lower crown-rump length of 20 days old rat fetuses as well as the average number of total and live births. The plant extracts also affected the blood chemistry, the length of estrous cycle, and the weight of reproductive organs that showed its toxicity at a high dose. Therefore, consumption of the plant, especially at a high dose, may be teratogenic and toxic. Thus, regulation and monitoring of the use of *S. guineense* leaves should be considered. Moreover, liberal consumption of *S. guineense* leaves should be taken curiously and cautiously. Further investigation should be conducted by increasing the number of test animals, extending the duration of treatment period, and including additional tests/organs and other test animals.

Keywords: Developmental Immunotoxicity, Developmental Neurotoxicity, Rats, Reproductive Toxicity, *Syzygium guineense* leaf, Teratogenicity.

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Dedication

This PhD dissertation is dedicated to my beloved son Pawlos Melese, who has been the wellspring of my philosophy of love.

Declaration

This is my declaration regarding this dissertation titled "Extended one-generation reproductive toxicity and teratogenicity of ethanol leaf extract of *Syzygium guineense* Wall. in Rats". The work presented in this thesis has been conducted by me Melese Shenkut in the Department of Anatomy, College of Health Sciences, Addis Ababa University in collaboration with TMMRD of EPHI. Each and every concept taken from literatures has been acknowledged and cited in the reference section. No part of this dissertation has been presented for any certificate in any other University.

Melese Shenkut Abebe	________________	______________
Name	Signature	Date

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List of abbreviation/acronyms

µm	Micrometer
AGD	Anogenital distance
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	One-way analysis of variance
AST	Aspartate aminotransferase
Cm	Centimeter
CRL	Crown-rump length
DIT	Developmental immunotoxicity
DNT	Developmental neurotoxicity
EPHI	Ethiopian Public health institute
EtOH	Ethanol
F	Female
F1	First generation offspring rat
g	Gram
g/dL	Gram/deciliter
GD	Gestational day
H & E	Hematoxylin and eosin
H	Hour
IRB	Institutional review board
kg	Kilogram
KOH	Potassium hydroxide

LD	Lactational day
M	Male
mg	Milligram
ml	Milliliter
°C	Degree centigrade
OECD	Organization for Economic Cooperation and Development
P	Parental rats
PND	Postnatal day
PNDs	Postnatal days
SDM	Standard deviation of mean
SG	*Syzygium guineense*
SPSS	Statistical package for social science
T3	Triiodothyronine
T4	Tetraiodothyronine (thyroid hormone)
TM	Traditional medicine
TMs	Traditional medicines
U/L	Unit per litter
WHO	World Health Organization

1. Introduction

1.1 Background

1.1.1 Traditional Medicine

The application of traditional medicine combines knowledge, skill, and practices of different cultures. It is based on cultural theories, reliance, and life experiences. Since long time ago, traditional medicine has been used for the prevention, diagnosis, and treatment of different diseases. Safe and effective traditional medicine contributes a lot for all people to get access to health care. Thus, millions of the world's population rely on it [1].

According to the World Health Organization (WHO) report, even in many developed countries, where modern medicine is available, a great portion of the population uses traditional medicine (TM) remedies, especially natural products of plant origin. On the other hand, in developing countries including Africa and other parts of the world, 65-80% of the population depend largely on medicinal plants for basic healthcare [1, 2].

The estimation of WHO indicated that majority of the world's population use plant products for their primary health care [2]. Similarly, a large number of Ethiopian populations use traditional medicine for their health care [3]. Plenty of factors are mentioned as a rationale to use products of TMs. In the developed nation, disaffection by contemporary medicine shoves them to use traditional medicine. On the contrary, the exorbitant cost of modern drugs, spares health care facilities, medical supplies, and medical professionals are reasons to rely on traditional medicines in developing countries [4].

Worldwide, traditional medicines (TMs) have different codifications. Among the well-known TMs, Chinese herbal medicine, Indian Ayurvedic medicine, the Japanese TM, and African TM can be mentioned. The Ethiopian TM system is a subclass of African TM. Even if it is partly influenced by the traditional philosophy of Egyptians and Greece, the Ethiopian TM system maintains its own attributes [4].

Mental illness, chronic diseases, and disease preventions are some aspects of health in which TMs exhibited promising efficacy [5]. As an illustration; the yellow bitter sap from *Aloe ferox* is an effective laxative, the juice extracts of *Momordica charantia* is a common remedy for diabetes

mellitus. Chewing and swallowing of *Zingiber officinale* are used for treating tonsillitis. Crushed leaves of *Calpurnia aurea* is a common treatment for the snake bite [6, 7].

1.1.2 Traditional Medicine in Ethiopia

Ethiopians history of traditional medicine dates back to the 15th century AD, according to some historians [5]. In Ethiopia, the practice of TMs includes product preparations from herbs, animals, and mineral substances, as well as spiritual healing, traditional midwifery, hydrotherapy, massage, cupping, counter-irritation, surgery, and bone setting. Ethiopians kept their TM practices orally. However, records are also found in religious manuscripts [5].

As reported by Pankhurst [8], Ethiopians have a long history of practicing TMs. Usually, they treat common ailments with local plants. For example, they drink Kosso (aqueous infusion of *Hagenia abyssinica* female inflorescence) in order to expel tapeworm.

Like any Africans, Ethiopians are also forced to use traditional medicines due to a variety of reasons. Among these: cultural linkage and trust in TMs, as well as accessibility and relatively low cost of TMs can be stated. Moreover, high price of commercial drugs and their casual infirmity also enforce to use TMs as a source of therapy. Due to accessibility and cost-effectiveness, the rural community mostly utilizes TMs [4].

1.1.3 Safety Assessment of Herbal Products

Recently, due to the global upsurge in the use of herbal products, WHO strongly recommends their safety evaluation. According to the recommendation of WHO, assessing the safety of herbal products is a fundamental principle prior to the incorporation into the health care system. Safety evaluation is also a vital component of quality control [9]. When dealing with the safety of medicinal plants, tests that are difficult or even impossible to detect clinically should be encouraged. Suggested tests include immunotoxicity, genotoxicity, carcinogenicity, teratogenicity, and reproductive toxicity [10].

1.1.4 Assessment of Potential Teratogenicity

Teratogen is any agent that can change the normal process of development and result in congenital anomaly or malformation [11]. Congenital anomalies may end up with death, structural abnormalities, or functional deficit [11]. Aside from nutrient and oxygen supply, uteroplacental circulation can transfer teratogenic chemicals to the developing embryo/fetus. Thus, the time of pregnancy can be a challenge for embryo or fetus. Teratogenicity studies also called prenatal developmental toxicity or embryo-fetal developmental study focus on maternal and embryo/fetal-related toxicity succeeding exposure to chemicals during embryogenesis [12]. Herbal products are tested for the potential teratogenicity using a rodent model before use by humans. Therefore, it is important to conduct teratogenicity prior to the marketing of herbal products [12].

The chief source of evidence for the unpleasant outcome of treatment on embryonic or fetal development that can be realized at birth is teratogenicity studies. Therefore, the objective of such studies is to investigate the potential of a test substance to cause embryo/fetal anomalies, embryo-fetal death, fetal weight (length) reduction, or any other adverse effects on maternal health [13].

There have been many reports that indicate the potential teratogenicity of herbs. For instance, administration of the aqueous extract of *Ruta graveolens* during the preimplantation period affected preimplantation development, i.e. it diminished the newly dividing number of cells and delayed embryo transport in mice [14]. On the other hand, an alcoholic extract from *Coleus barbatus* delayed fetal development and implantation process [15]. Even if some pieces of evidence support the teratogenic potential of herbal products, detailed information is limited and contradictory [16].

1.1.5 Reproductive and Developmental Toxicity

The deleterious effect of the test substances on sexual function and fecundity in males and females is investigated by reproductive toxicity studies. These effects can be observed on the onset of sexual maturity, sexual behavior and performance, germ cell synthesis and transportation, pattern of the sexual cycle, fertilization, implantation, labor, and pregnancy outcome. In addition, nesting and lactation behavior can be affected by reproductive toxicants [17].

Different studies indicated the adverse effect of test substances on the reproductive system of laboratory animals and humans. As reported by Smith and Asch, Zenick [18] and Clegg [19], and

Liu *et al* [20], reproductive toxicants affected reproductive function, ovarian cycle, ovulation, and survival rate of litters during lactation.

During developmental toxicity investigation, anogenital distance (AGD) and nipple retention are the two sensitive endpoints indicating endocrine (androgen) disruption. AGD, a distance from the anus to the genital tubercle, is reliant on the prenatal exposure of androgen. The androgen hormone regulates the differentiation of the genital bud (tubercle) into male external genitalia. Usually, in rats, the length of AGD in male pups is double of the females [21, 22]. In both males and females, the mammary crest develops into the nipple. However, the male nipple becomes regress in early intrauterine life due to the effect of dihydrotestosterone, whereas the female nipples are prominent and visible by the naked eyes in PND 12 [23, 24]. Overall, AGD measurement and nipple retention are the two critical and sensitive endpoints to be measured while investigating sexual development.

In female rats, the onset of puberty is measured by the opening of vagina and the presence of cornified vaginal epithelium showing the commencement of ovulation. The preputial separation, testicular enlargement, and beginning of sperm production are indicators of puberty onset in male rats. Research findings indicated that the onset of puberty can be affected by exposure to chemicals [25].

In the intrauterine life, the developing fetus is not fully protected from toxicants. Studies reported that many environmental chemicals can pass the placental membrane, and as high as 200 foreign chemicals have been detected in the blood samples taken from the umbilical cord [26]. The immature blood-brain barrier of the fetus permits passage of hydrophilic toxic agents to greater extent than in the adult brain [27, 28]. Due to the undeveloped metabolic function of the liver and excretion capacity of the kidney, the level of toxicants in the fetal brain is much greater than the maternal brain [29]. In addition, human studies indicated that toxicants are transferred to the newborn through breast milk [26]. The consequences of developmental neurotoxicity can be either permanent brain damage or lifetime functional deficit like reduced intelligence, impaired sensory and motor function and disrupted behavior [30].

Several chemicals (nearly 200) are known as developmental neurotoxicants. Such findings are emanated from both human and animal studies [31]. Among the most studied developmental

neurotoxicants: methylmercury [32, 33], lead [34, 35], polybrominated diphenyl ethers [36], and organophosphates [37] can be mentioned.

The developing immune system is exquisitely sensitive for most environmental toxicants compared with the adult immune system [38]. The results of immunotoxic insults are dependent on the nature of toxic agents and the time of exposure [39]. In early development, decreased serum complement activity [40], low neutrophil reserves in the bone marrow [41], diminished ability of neutrophil in adherence and chemotaxis [42], low antibody production capacity [43], and reduced cytotoxic activity of lymphocyte [44] are observed. These factors may contribute to the lower immune response observed in early development.

1.2 Statement of the Problem

Even though plenty of research works are available on the constituents and biological activities of medicinal plants of Africa, the development of therapeutic agents from these medicinal plants has remained a somewhat neglected subject [45]. Several investigations have shown that chemicals obtained from plants usually affect the physiology and survival of almost all animals in one way or another. Such physiological effects could have positive or negative consequences on the health of the animals. It could be based on such a general assumption that human beings rely on plant materials as sources of medicines for varieties of ailments since ancient times. It took a long time, however, until investigators gave scientific testimonies for the effects and mode of action of most plant extracts [10].

Traditional use of herbal remedies, such as taenicides is widely reported to be toxic. As evidence, blindness and central nervous system dysfunction have repeatedly been found in the people who took an overdose of *Hagenia abyssinica* [46]. A report from Nortier and his colleagues [47] indicated that the consumption of Chinese-herb (*Aristolochia fangchi)* causes renal disease and urothelial cancer.

In Africa, up to 90% and in Ethiopia 80% of the population use TMs to maintain their health care needs. Around 50% of pharmaceutical products are made from compounds extracted from herbs or animals [5].

Research finding indicates that medicinal plants have potential toxicity on cellular and biochemical parameters of blood and histopathology of various internal organs. Most findings of toxicity due to the use of herbal products are associated with hepatotoxicity and nephrotoxicity. However, other toxic effects including, the nervous system, blood, cardiovascular and dermatologic effects, have also been reported in several studies. These results signify possible dangers for patients who may use plant therapy for a long duration [48].

It is widely stated that herbal products are presumed to be safe. However, experts uphold appropriate toxicological investigations to be performed on herbal products. This will ensure the safety of herbal preparations. Toxic signs of the test substance can be observed at some expected period after its administration. These types of toxic effects are similar to human and experimental animals. Due to this reason, toxicological studies are usually conducted on animal models [49].

The world health assembly held in 2009 recommends the governors to esteem, maintain, and extensively be in touch with the knowledge of TMs [50]. Also, nowadays, the demand for herbal remedies has increased. Therefore, validating the efficacy and assessing the safety of herbal therapies is mandatory [49]. In addition to the general toxicity assessment, WHO recommends more extended specific toxicity studies. This includes immunotoxicity, genotoxicity, carcinogenicity, teratogenicity, and reproductive toxicity [10].

Syzygium guineense leaf has broader scientific importance, especially, its significant *in vivo* and *in vitro* antihypertensive effect make hope for a future source of antihypertensive drug [51]. It is tested for acute and sub-acute toxicity and it looks safe, but some controversial findings are reported [52-54]. However, its teratogenic effect and reproductive toxic profile are still unknown. Therefore, the aim of this thesis research was to investigate the teratogenicity, reproductive toxicity, developmental immunotoxicity, and developmental neurotoxicity as well as glandular and hepato-renal toxicity of *S. guineense*. The findings of this investigation can be a stepping stone in formulating drugs containing *S. guineense* origin.

1.3 Significance of the Study

Many toxicological investigations are done on the vital excretory organs like the liver, kidney, and blood. These investigations indicate the general effect of the tested plant. However, teratogenicity of the plant and reproductive toxicity are among the most pompous investigations to be performed on herbal products. If a plant has toxic effects on reproduction, its consequence will be to the generation.

Teratogenicity and reproductive toxicity profile of *S. guineense* has not been determined yet. The current study has an output on the teratogenicity potential of *S. guineense* on the whole rat embryo/fetus and the placenta. The toxic effect of the plant extract on the developing nervous system, immune system, and reproductive system structure and functions also were investigated in the parent (P) and first-generation (F1) rats. Moreover, hepatic, renal, and glandular toxicity of this plant was also investigated. This study is going to address a known gap in knowledge as it is the first of its kind investigating the teratogenic and reproductive, neuro, and immune toxic effects as well as glandular and hepato-renal toxicity of *S. guineense* and hence, advances the knowledge in the field of toxicology. This holistic data regarding the safety of *S. guineense* could also be used as baseline data for further investigation.

2. Literature Review

2.1 The Genius *Syzygium*

The word '*Syzygium'* is obtained from the Greek term 'syzygos' which means 'paired', that refers to the paired leaves and twigs. The specific name '*guineense'* means 'of Guinea', indicating where the tree was first collected. *S. guineense* is commonly named as 'water pear', since its wood looks like a pear and the plant prefer stream banks to grow [55].

The genus *Syzygium* is under the family *Myrtaceae.* It is one of the largest genera of flowering plants. It consists of nearly 1200–1800 species. Generally, the genus is distributed throughout tropical and subtropical regions of Africa including Madagascar, Asia, Oceania, and the Pacific region [56]. Some of the pharmacologically active species of syzygium includes: S*yzygium cumini, Syzygium jambos*, *Syzygium anisatum, Syzygium aqueum, Syzygium guineense, Syzygium aromaticum,* and *Syzygium cordatum* [56, 57].

2.2 *Syzygium guineense* Wall.

Syzygium guineense Wall. (Figure 1) is widely distributed in Africa. Its distribution covers Nigeria, Senegal, Eritrea, Ethiopia, Somalia, Zaire, Rwanda, Zambia, Malawi, Zimbabwe, Namibia, Uganda, Swaziland, Cameroon, and South Africa. It is a medium to large evergreen water-loving dicotyledon tree [58].

Syzygium guineense has different local names: Dokma in Amharic, Baddeessaa in Afaan Oromoo, Duwancho in Sidamigna, and Liham in Tigrigna. It is also called Water berry, Water-pear, or Snake-bean tree in English language [59].

The tree can grow in an altitude of 2300-2700 meter, mean annual temperature: 10-30°C, mean annual rainfall: 1000-2300 mm, and prefers fresh, moist, and well-drained soils with a high water table [60].

Figure 1: Picture of *Syzygium guineense* tree

Syzygium guineense can grow up to 30 m high. The bark varies in subspecies, it can be greyish-white or silver mottled. The bark of young trees is smooth while this smooth surface turns rough, flaky, and creamy when it gets old. The bark gives rise to red watery sap if cut [61].

The leaves of *S. guineense* are narrow at both ends. They reach 5-17.5 and 1.3-7.5 cm in length and width, respectively. They are simple, found in opposite pairs, have an ovoid shape, and slippery edge. Flowers are aromatic and the vagile reaches up to 1.5 cm in diameter [55].

Fruits are ovoid or ellipsoid drupes, 0.5-3.5 cm × 0.6-2.5 cm, red to purplish-black, tipped by the persistent calyx, usually single-seeded. Seeds are 1.3-1.4 cm in diameter, lemony to brownish in color, and orbital in shape [60, 62].

2.2.1 Traditional Uses of *Syzygium guineense*

Traditionally, *S. guineense* is used as a febrifuge and anti-abortifacient medication. It is also used for treating menstrual cycle disorder, malnutrition, nasopharyngeal infections, pain, pulmonary disorders, constipation, diarrhea, dysentery, arthritis, rheumatism, venereal diseases, malaria, asthma, wound, cancer, infertility, sleep disorder, and anemia [63-65]. The stem bark of *S. guineense* is used to treat skin disease like vitiligo by the people of Low and Middle Guinea [63].

Syzygium guineense leaves have been used by various communities to manage different health problems. It is used for the treatment of malaria in Congo [66], against stomach discomfort and ringworm infection, and in the treatment of wound in Uganda [67], against bacterial infections in Guinea [68], and herpes zoster in Namibia [64]. In Nigeria, *S. guineense* leaves in combination with leaves of *Jatropha curcas* are used to treat diabetes mellitus [69]. The bark is reported to be used against chronic diarrhea [66] and chest pain [70] in Tanzania.

Beyond the above traditional uses, Malians have extensive utilization of this plant. They use it for treating, pain, wounds, stomachache, dermatosis, infertility, malaria, fever, pneumonia, asthma, and sleep problems [71].

Dried leaves and roots of *S. guineense* with the leaves of *Osyris quadripartita* are crushed, mixed, decocted, and drunk for the treatment of cancer around Bahir Dar city, North West Ethiopia [65].

A study conducted on the people of Nigeria showed that the leaves, bark, and root are ethnobotanically used in the management of diabetes mellitus by Hausa people in Kano of northern Nigeria [72]. The leaf and bark of *S. guineense* are effective against tuberculosis, chronic diarrhea, cough, dysentery, malaria, amenorrhea, wounds, ulcers, rheumatism, and infections [73].

2.2.2 Pharmacologic Significance of *Syzygium guineense*

The different parts of *S. guineense* (leaves, root, bark, stem, and twig) have shown proven efficacy against pathogens (bacteria and fungus), hypertension, diabetic mellitus, snake venom, pain, inflammation, cancer, and free radicals.

Both *in vivo* and *in vitro* studies of the hydroalcoholic extract of *S. guineense* leaves induced a dose-dependent fall in blood pressure by vasodilatation in the rat model [51]. The leaves of *S. guineense* obtained from Cameron contain tannins as an active component. These tannins directly destabilize Wnt ligands. Breast cancer and colon cancer are dependent on the Wnt ligands. Therefore, *S. guineense* acts as a cancer preventive plant [74]. On the other hand, the leaves twig and stem bark extracts show spasmolytic and antidiarrhea activities [73].

A methanol extract of *S. guineense* leaves exhibited inhibition of alpha-glucosidase, scavenge free radicals, and increase intrahepatic glucose uptake and storage, by this it showed antihyperglycemic action in diabetic rats [75].

The investigation of Tadesse and Wubneh [76] revealed that the methanolic leaf extract of *S. guineense* showed substantial parasite suppression at doses of 600 and 400 mg/kg in a 4-day suppressive test with a chemosuppressive value of 59.39 and 49.09% respectively and dose-dependent schizontocidal activity.

In the other research conducted to screen its antioxidant property, the hydroalcoholic extracts of leaves and bark of *S. guineense* exhibited significant free radical scavenging and antioxidant properties. The free radical protective effect was attributed to the constituents of phenolic compounds [77].

Investigation conducted by Tsakala and colleagues [78] in the Democratic Republic of Congo reported that the dried aqueous decoction of *S. guineense* showed significant effect against the strains of *Salmonella enterica, Shigella dysenteriae, Shigella flexneri, Escherichia coli*, and *Enterobacter aerogenes* strains. Moreover, secondary metabolites identified from *S.guineense* showed significant activity against different bacterial strains mainly *Escherichia coli*, *Bacillus subtilis*, and *Shigella sonnei* [79]

The chloroform extract of *S. guineense* stem bark possessed two bioactive 3-β-hydroxylupane-type isoprenoids: betulinic acid methylenediol ester and betulinic acid which show anti-tuberculosis activity [80]. Ethanolic extract of the leaves of *S. guineense* administered to rats and mice showed significant analgesic and anti-inflammatory activities [81]. In another study, the methanol extract of the leaves of *S. guineense* showed potent Naja katiensis snake venom neutralizing capacity [82].

The ethanolic extract of stem bark of *S. guineense* showed molluscicidal activity [83]. A methanol extract of *S. guineense* bark (collected from Tanzania) inhibited intrinsic contractions in isolated ileum tissue of rabbits. It also produced sustained hypotension in anesthetized rats. A dose of 5 μg of this plant lowered systolic, diastolic, and mean blood pressure by 16%, 22%, and 17%, respectively. Maximum effect was obtained at a dose of 40 μg: the systolic, diastolic, and mean blood pressures were lowered by 23%, 36%, and 28%, respectively. The greater fall in blood pressure was in diastolic rather than in systolic blood pressure [62].

2.2.3 Phytochemical Property of *Syzygium guineense*

The ethyl acetate and *n*-butanol extracts of the leaves of *S. guineense* yield 10 triterpenes in chromatographic isolation and purification. These are betulinic acid, oleanolic acid, a mixture of 2-hydroxyoleanolic acid, 2-hydroxyursolic acid, arjunolic acid, asiatic acid, a mixture of terminolic acid, 6-hydroxyasiatic acid, a mixture of arjunolic acid, 28-β-glucopyranosyl ester, and the asiatic acid 28-β-glucopyranosyl ester [79].

In another phytochemical screening, *S. guineense* leaf extract showed the presence of alkaloids, terpenoids, anthraquinones, flavonoids, tannins, saponins, glycosides, triterpenes, and phenols [76].

Polysaccharides from the leaves of *S. guineense* contain fractions rich in arabinogalactan structures. These arabinogalactan polysaccharides potently stimulate macrophages, dendritic cells, and B cells. Thus, maintaining the immunomodulatory function by increasing phagocytic activity, promoting reactive oxygen species, and nitric oxide production, as well as enhancing proinflammatory and anti-inflammatory cytokines production [84].

The results of the phytochemical composition of *S. guineense* hydroalcoholic extracts from the leaves, barks, and roots show that all samples contain sugars, proteins, lipids, polyphenols, alkaloids, saponins, steroids, cardiac glycosides, flavonoids, and tannins. Exceptionally the extract of the bark contains more coumarins [77].

Essential oil extracted from dried leaves of *S. guineense* from Benin were analyzed by gas phase chromatography coupled to mass spectrometry. The main constituents identified were: caryophyllene oxide (7%), δ-cadinene (7.5%), viridiflorol (7.5%), *epi*-α-cadinol (9.8%), α-cadinol (12.7%), *cis*-calamenen-10-ol (14%), citronellyl pentanoate (15.2%), β-caryophyllene (20.1%), and α-humulene (39.5%) [85].

2.2.4 Safety Profile of *Syzygium guineense* Leaves

Some researchers investigated the safety profile of *S. guineense*. However, these studies were conducted based on short term administration of *S. guineense* extract and mainly assessed the cellular and biochemical aspects of blood and histology of liver and kidneys.

A six-week study, conducted by Abba *et al* [52], investigated the effect of aqueous extract of *S. guineense* leaves on the mice liver. They found hemorrhagic necrosis and cytoplasmic vacuolations of hepatocytes and increased liver weight in mice treated with 600 mg/kg of the extract. Another study conducted on mice reported hemorrhagic centrilobular necrosis and fatty cytoplasmic vacuolation of liver hepatocytes of mice treated with 400 and 600 mg/kg of aqueous extract of *S. guineense* [53].

On the other hand, Loha and colleagues [54] investigated the safety of *S. guineense* following a four-week administration of the methanol extract at the doses of 500 and 1500 mg/kg of body weight to rats. These results indicated that there was no sign of toxicity observed on the hematological parameters and histopathology of the liver and kidney.

3. Objective

3.1 General Objective

- To investigate potential teratogenicity and extended one-generation reproductive toxicity of 70% ethanol leaf extracts of *S. guineense* in rats.

3.2 Specific Objectives

- To investigate the teratogenic effects of *S. guineense* on day 12 rat embryos.
- To investigate the teratogenic effects of *S. guineense* on day 20 rat fetuses.
- To investigate the effects of *S. guineense* on the histopathology of placenta of 20 days old rat fetuses.
- To evaluate the reproductive toxic effects of *S. guineense* on the parental and F1 rats.
- To evaluate the endocrine toxic effects of *S. guineense* on the parental and F1 rats.
- To evaluate the effects of *S. guineense* on biochemical parameters of parental and F1 rats.
- To evaluate the effects of *S. guineense* on the histology of liver and kidney on parental and F1 rats.
- To assess the potential developmental neurotoxic effects of *S. guineense* on the rat litters.
- To evaluate the potential developmental immunotoxic effects of *S. guineense* on the rat litters.

4. Materials and Methods

4.1 Setting of the Experiment

This study has two major experiments, the first one was assessment of potential teratogenicity of *S. guineense* leaf following administration of the extract during the period of organogenesis. The second experiment was the assessment of extended one-generation reproductive toxicity. The experiments were conducted in the laboratories of the Ethiopian Public Health Institute (EPHI), Departments of Anatomy and Pathology and School of Pharmacy, Addis Ababa University.

4.2 Plant Collection and Extraction

The plant was collected from the outskirt of Woliso town, Oromia regional state, 113 km West of Addis Ababa, the capital city of Ethiopia. Plant identification and authentication were done by the National Herbarium, Department of Plant Biology and Biodiversity Management, Addis Ababa University, Ethiopia, where a voucher specimen (MS 001) was deposited. The leaves of *S. guineense* were cleaned, shade dried, manually broken into pieces, and coarsely powdered by an electric mill. The powder was mixed with 70% ethanol in a 1:10 powder to solvent ratio and rotated frequently for 24 hours by an orbital shaker at 130 g. The mixture was filtered by Whatman paper (No 1, 18 cm diameter). Then the filtrate was concentrated with a rotatory evaporator (Büchi Rota Vapor R-205, Switzerland) at 40°C and the concentrate was further dried in a hot water bath at 45°C. The dried extract (solvents were completely removed) was packed in a sealed glass and kept in a refrigerator at -4°C until used for the experiment [86].

4.3 Teratogenicity Test

The potential teratogenicity of *S. guineense* was evaluated following administration of the test substance at the critical period of organ formation in rats (day 6-12 of pregnancy). This test includes two separate experiments: day-12 and day-20 experiments. The day-12 experiment was designed to investigate the teratogenic effect of the test substance immediately at the cessation of extract administration. By this, the effect of the plant extract on embryonic development was evaluated. On the other hand, in the day-20 experiment, pregnancy of the rats was left to continue until one day before birth (20th day of pregnancy), but the treatment was terminated on the 12th day

of pregnancy. This experiment was performed to evaluate the residual effect of the test plant on further development of the fetus.

4.3.1 Experimental Animals

Wistar albino rats which were healthy, nulliparous, 220-240 g weight, and 10-12-weeks of age were used as an experimental and control animal. The rats were obtained from EPHI animal breeding unit and housed for the experiment in the animal facility of the Traditional and Modern Medicine Research Directorate (TMMRD) of EPHI. They were acclimatized for one week prior to the commencement of the experiment. The rats were maintained in a stainless-steel cage at a room temperature (23± 3°C) with a relative humidity of 50 ± 10% under a controlled alternating 12-hour light-dark cycle. Throughout the experiment, rats were served with a conventional laboratory diet and an unlimited supply of drinking water.

4.3.2 Mating of Rats and Pregnancy Confirmation

For mating, each female rat was placed with one randomly selected unrelated male rat with proven fertility. After an overnight mating period, female rats were inspected for the presence of a copulatory plug. A vaginal smear test was performed to check for the presence of spermatozoa and confirm pregnancy (Figure 2). The day of microscopic sperm detection was considered as day one of pregnancy.

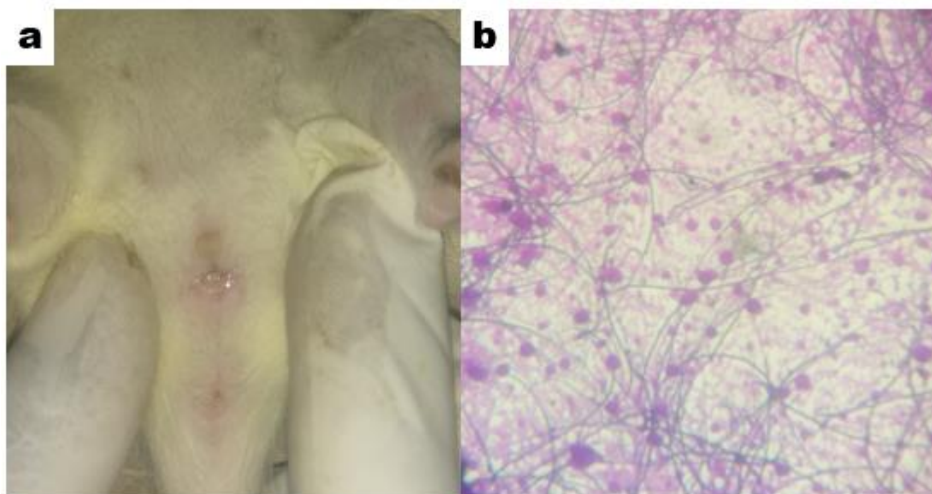


Figure 2: Pregnancy confirmation. (**a**) Vaginal sperm plug, (**b**) Light microscopy of vaginal smear showing the presence of spermatozoa; Crystal violet stain, 40x magnification.

4.3.3 Experimental Design

The teratogenicity test experiments were conducted in 12-day old rat embryos and 20-day old rat fetuses. For each experiment, 50 pregnant rats, with identification code marked on the tail, were randomly assigned into five groups, each consisted of ten rats, based on the recommendation of OECD guideline for developmental toxicity screening test [87] (Table 1). Pregnant rats were randomized using a computer-based random order generator. The three-treatment groups received the test substance at doses of 250, 500, and 1000 mg/kg body weight and the fourth one (a vehicle control group) received distilled water (1 ml/100 g body weight), this group was used to assure whether the outcomes were due to manipulation (stress) during intragastric administration or not. The control group was treated with distilled water since distilled water was used to dissolve the extract. The fifth group was *ad libitum* control (untouched control) and used as a comparison group. The doses of the plant extract were selected based on previous study reports [75]. The administration was done with an intragastric tube. Such root of administration was selected based on the traditional use of the plant. The treatment periods for day-12 and day-20 experiments were from day-6 to day-12 of gestation, every morning at 8:00 am. Pregnant rats were daily inspected for any treatment-related adverse effects. Outcomes measurement in both experiments was blindly done by the investigator unaware of the treated and control rats.

4.3.4 Day-12 Experiment

Day-12 experiment was aimed to detect the effect of the plant extract on the developing rat embryo at the critical time of organogenesis (day 6-12). Both the experimental and control rats were kept in a homogenous environment and served conventional laboratory food and water. Throughout the treatment period, the food intake of pregnant dams was measured daily. Additionally, pregnant rats were weighed at the confirmation of pregnancy, beginning of treatment (6th day of gestation), and at necropsy (12th day of gestation).

At the end of the treatment period, at 12:00 hours, the gravid rats were euthanized by intraperitoneal injection of pentobarbital (150 mg/kg body weight) [88]. The abdomen was opened by laparotomy and the uterine horns were removed. The number of implantation and resorption sites was counted. Then the uterine horns were placed in normal saline and incised along the antimesometrial border to reveal the embryos. With the help of a dissecting microscope and fine

forceps, the surrounding membranes of the embryo were removed to expose the visceral yolk sac. The development of allantois and yolk sac circulation was evaluated. In the yolk sac removed embryos, embryonic development was evaluated based on Brown and Fabro's [89] morphological scoring system of the rat embryo which was adopted for use in *in-vivo* study by Seyoum and Persaud [90]. The development of body systems (circulatory, nervous, and skeletal) and craniofacial development was assessed. In addition, crown-rump-length was measured and the number of somites was counted.

Table 1: Test animal grouping and dosing for day-12 and day-20 experiments

Treatment group	Number of rats	Treatment
Group I	10	SG extract 250 mg/kg body weight + food and water
Group II	10	SG extract 500 mg/kg body weight + food and water
Group III	10	SG extract 1000 mg/kg body weight + food and water
Group IV (Control)	10	DW (1 ml/100 g body weight) + food and water
Group V (*Ad-libitum* control)	10	Food and water

SG: *Syzygium guineense*, **DW**: distilled water

4.3.5 Day-20 Experiment

In the day-20 experiment, the feeding, rat's care, dosing, and duration of treatment were similar to the day 12-experiment. Food intake of pregnant dams was measured daily and pregnant rats were weighed at the confirmation of pregnancy, beginning of treatment (6th day of gestation), end of treatment (12th day of gestation), and at necropsy (20th day of gestation).

Rats were sacrificed on the 20th day of gestation following euthanasia by intraperitoneal injection of pentobarbital. The abdomen was opened by a longitudinal incision and the gravid uterus was kept intact and the following evaluations were done. The number of implantation sites (by counting the uterine glands which are yellowish nodules located along the mesometrial margin of the uterine horns) was counted. The numbers of fetal deaths and viable fetuses were counted by applying gentle pressure on the fetus. The number and degree of resorption (early or late) sites were counted by checking uterine nodules that were not occupied by living or recently dead fetuses. After the aforementioned examinations, the uterine horns were incised along the antimesometrial border.

Then the fetuses, fetal membranes, and the placenta were revealed. The placenta and fetal membranes were removed and measured separately. The sex of the fetuses was identified, the weight and crown-rump length (CRL) of each fetus were recorded. Subsequently, 2-3 fetuses from each rat were preserved in 95% ethanol for skeletal development examination. The rest fetuses were fixed in Bouin's solution (aqueous saturated solution of picric acid 75%, formalin 25%, and glacial acetic acid 5%) for gross external and visceral examination.

4.3.5.1 External Evaluation

Once each fetus was revealed and separated from the corresponding placenta, all fetuses were inspected head to tail for any gross developmental abnormalities. The examination includes: craniofacial developmental anomalies (exencephaly, anencephaly, microphthalmia, and anophthalmia), limb development abnormalities (syndactyly, adactyly, polydactyly), vertebral column malformations (neural tube defect, kyphosis, scoliosis), tail development disorder (missing tail), and external genitalia abnormalities.

4.3.5.2 Visceral Examination

Following an external examination of the fetuses at necropsy, additional soft tissue examination was conducted by serial sectioning. Serial sectioning was performed on the fetuses fixed in Bouin's solution for two weeks. The sectioning procedure was done by a surgical blade, based on the Modified Wilson technique [91]. Craniocaudally, sections were done at 1-2 mm intervals under a dissecting microscope (XTL3101, 6x magnification). The first section was made through the jaw and pass posteriorly above the ear. After removing the tongue, the palate was examined for the presence of any cleft. A coronal section on the head and a transverse section on the neck and parts below were done. The following organs were assessed for any visible anomalies: brain (hydrocephalus, dilation of ventricles, microphthalmia/anophthalmia), craniofacial region (nasal septum defect, cleft palate), thoracic region (lungs: lobar defect, heart: septal defect, retroesophageal aortic arch), abdominal region (liver, stomach, and gut anomalies), and pelvic region (kidneys: agenesis, ectopic, and hydronephrosis, gonads: testes, ovarian anomalies).

4.3.5.3 Skeletal Staining

Skeletal staining was done following a modified method of Rigueur and Lyons [92]. Two to three fetuses per litter were selected for skeletal examination. These fetuses were killed by an overdose of pentobarbital and eviscerated by a midline incision made through the anterior abdominal wall. Eviscerated fetuses were fixed in a bottle containing 95% ethanol (EtOH) for five days. To clear the soft tissues, specimens were placed in 1% potassium hydroxide (KOH) solution for 2-3 days, until the bones were clearly visible. To stain the bones, the specimens were transferred into a fresh KOH solution containing a few droplets (0.005%) of alizarin red S. The bones were stained within 24 hours. Over staining of the bones was corrected by Mall's solution (79% distilled water, 20% glycerin, and 1% KOH). After staining, the specimens were cleared by an increasing concentration of glycerin (20%, 40%, 60%, and 80%) for about one week in each concentration. Finally, they were kept in 100% glycerin until evaluation. Then, each specimen was examined under a dissecting microscope utilizing bright-field optics and white background.

4.3.5.4 Skeletal Evaluation

Toxic substances affecting fetal development may manifest themselves not only in terms of malformations but also in terms of retarded ossification [93]. The degree of ossification of the sternebrae, metacarpal, metatarsal, and sacrococcygeal bones has been reported to be the primary indices of skeletal development in rats [93]. The degree and number of ossification centers in each bone of the fetuses were examined under a dissecting microscope. The skull, hyoid, sternebrae, ribs, vertebrae, and limb bones were evaluated. Assessment of the skeletal development was performed by using a skeletal scoring chart designed by Nash and Persaud [94].

4.3.5.5 Examination of the Placenta

Each placenta was examined for any gross morphological abnormalities. Placentae of two-three fetuses/dam/group were selected for further histopathologic examination. A 3-4 mm size section was sampled from each placenta and immersed in 10% formalin for fixation. Following an overnight fixation, the tissues were dehydrated by an ascending series of alcohol (40%, 50%, 70%, 80%, 90%, 100). The tissues were then cleared by xylene (I, II, and III, 1:30 hour in each phase). After clearing, the tissues were impregnated with two-phase paraffin wax, 1:30 hour in each. Finally, 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 the frosted slide then kept in a hot oven (40-45°C) for 20-30 minutes [95].

Staining of the tissues was based on the following procedures: the slides were dewaxed with three-phase xylenes for five minutes in each, rehydrated with descending series of alcohol (absolute alcohol I, absolute alcohol II, 90% alcohol, 80% alcohol, 70% alcohol) for two minutes in each, washed with running tap water for two minutes. Then the slides were stained with Harris hematoxylin for 5-10 minutes, cleaned with running tap water for 10 minutes, immersed in acid alcohol for 2-3 seconds, and 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. Finally, the cleared slides were mounted with DPX and covered with a cover-slip [95].

In the stained slides, the structural integrity of the placentae was investigated using a binocular light microscope. The decidual zone, the labyrinthine zone, Giant cells, and trophoblasts of the placenta were investigated and important findings were photographed with an automated inbuilt digital microscope camera (Leica EC4, Germany) under 10x and 40x objective lens magnification, total magnification 100x and 400x respectively.

4.4 Extended One-generation Reproductive Toxicity Study

4.4.1 General Description

Extended one-generation reproductive toxicity study was conducted to investigate the toxic effect of the test plant on the structure and function of the reproductive system of P-rats and F1 offspring. Parallelly, the toxic effect of the plant extract on the adrenal glands, thyroid gland, liver, and kidneys was investigated. Following prenatal and postnatal exposure to the plant extract, F1 rats were observed for possible developmental neurotoxicity and immunotoxicity.

4.4.2 Animal Preparation

Young male and female Wistar albino rats, 220-260 g weight and age between 10-12 weeks were obtained from the animal breeding unit of EPHI and housed for the experiment in the animal facility of the TMMRD of EPHI. Female rats were nulliparous. The process of rat care and handling was as mentioned in the teratogenicity study.

4.4.3 Grouping and Dosing of Parental Animals

Following a week acclimatization period, male and female parental rats (P-rats), 20/sex/group, were randomly assigned into four groups. The three treatment groups (groups I-III) received 70% ethanol leaf extract of *S. guineense* and the fourth one, a control group, was treated with distilled water. The treatment groups I, II, and III were treated with 250, 500, and 1000 mg/kg body weight of *S. guineense* extract, respectively. The control group received 1 ml/100 g body weight of distilled water (Table 2). Rats were administered using an intragastric tube. The rats were treated with the plant extract continuously on a 7-days/week basis until necropsy. All rats were dosed by the same method.

Table 2: Test animal grouping and dosing for extended one-generation reproductive toxicity study

Group	Number of rats/sex/groups	Treatment
Group I	20	SG extract 250 mg/kg body weight + water + food
Group II	20	SG extract 500 mg/kg body weight + water + food
Group III	20	SG extract 1000 mg/kg body weight + water + food
Group IV (Control)	20	DW (1 ml/100 g body weight) + Water + food

SG: *Syzygium guineense*, **DW**: Distilled water

4.4.4 Treatment Schedule and Housing of Parental Rats

Generally, male and female P-rats were treated for ten consecutive weeks. Specifically, male and female P-rats were treated for two premating weeks period. The two-week premating period was expected to cover a 3-4 estrous cycle for female rats and epidydimal sperm maturation for male rats. This period is sufficient to detect the effect of the test substance on the length and normality of the estrus cycle and final sperm maturation process [96]. The treatment continues for the next two mating weeks, in which female rats were housed with a male rat until pregnancy was confirmed. Female rats were continued receiving the treatment during pregnancy and lactation (until litters started weaning), three weeks for each. Male rats were also treated for the same duration as female rats. For male rats, ten weeks cover full period of spermatogenesis. The treatment schedule is presented in Figure 3. Male and female rats were housed separately unless for mating. Near term, each pregnant rat was housed separately in a maternity cage.

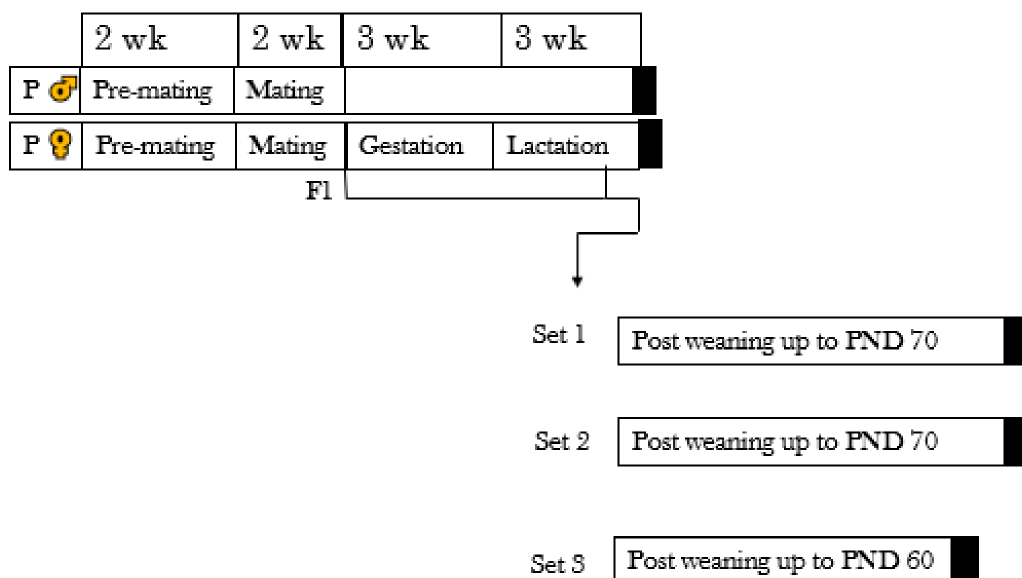


Figure 3: Schematic representation of the study design for extended one-generation reproductive toxicity study: dosing duration and grouping. The black box indicates time of necropsy.

4.4.5 Mating and Pregnancy Confirmation

Following two weeks of the premating treatment period, rats were mated in a 1:1 male to female ratio. Female rats were kept with unrelated males of proven fertility for two weeks until the pregnancy was confirmed. Within two weeks mating period, every morning, female rats were inspected for the presence of a vaginal plug. Then, a vaginal smear test was conducted to assure the presence of spermatozoa in the vaginal fluid. This day was considered as day one of pregnancy or gestational day (GD) one.

4.4.6 General Clinical Observations

Throughout the treatment period, a general clinical observation was made every morning. Any signs of toxicity: changes in the skin, motor and sensory function, unusual respiratory pattern, and self-mutilation were recorded before and after dosing period. All rats were also observed for severe toxicity, morbidity, and mortality daily.

4.4.7 Weight and Food Intake Measurement

All P-rats were weighed on the first day of dosing. Male rat's body weight was collected weekly throughout the study and finally at sacrifice, then the weight gain was calculated. In addition, P-

females were weighed weekly prior to pregnancy, on GD 0, 7, 14, and 20, and LD 1, 4, 7, 14, and 21. Pre-gestational, gestational, and lactational body weight gains were calculated and weight gain difference among groups was compared. Food intake was measured daily in the morning throughout the treatment period.

4.4.8 Assessment of Reproductive Toxicity

4.4.8.1 Reproductive Indices in Parental Rats

Different reproductive endpoint indices were calculated based on the assumption presented in Table 3. The date of pairing, insemination, and parturition was recorded. The pre-coital interval (pairing to insemination) and the length of pregnancy (insemination to parturition) were calculated. In addition, the number of males capable of insemination/fertilization and the number of pregnant females was also recorded.

The mating index was calculated by dividing the number of rats with evidence of mating by the number of paired and multiplied by hundred. Male fertility index was calculated as: (number of males siring a litter/ number of paired) x 100. Female fertility index was calculated as: (number of females with evidence of pregnancy/number of paired) x 100. In addition, gestation index was also calculated by dividing the number of females delivering a viable litter by the number of females with evidence of pregnancy and multiplied by hundred. The number of live births, stillbirth, and sex of the pups were recorded. Moreover, gestational survival index, number of postnatal deaths of litters on postnatal days (PNDs) 1, 4, 7, 14, 21 and sex ratio were calculated (Table 3). The day on which rats gave birth was considered as PND zero for pups and lactational day (LD) zero for dams.

Table 3: Calculation of reproductive indices

Reproductive indices	Description
Male mating index	(No. of males with evidence of mating/No. of paired) x 100
Female mating index	(No. of female with evidence of mating/No. of paired) x 100
Male fertility index	(No. of males siring a litter/No. of paired) x 100
Female fertility index	(No. of females with evidence of pregnancy/No. of paired) x 100
Gestation index	(No. of females delivering a viable litter/No. of female with evidence of pregnancy) x 100
Gestation survival index	Percentage of delivered pups alive at birth
Sex ratio on PND 0-Male: Female	Proportion of males to females at birth
Pre-coital interval	Days from pairing to insemination
Gestation length (days)	Days from insemination to parturition

4.4.8.2 Estrous Cycle Measurement

Test substance-related effects on estrous cycle were evaluated by conducting vaginal smear test daily. The estrous cycle was measured in the first two weeks of the premating period in P-rats. Ten rats per group were randomly selected for estrous cycle measurement. Rats were held in a restrainer and vaginal fluid was collected by a plastic pipette filled with 10 µl of normal saline (0.9% Na Cl). The tip of a plastic pipette was gently inserted into the rat's vagina. The aspirated vaginal fluid was fairly distributed over the labeled glass slides. The slides were kept at room temperature until they dry. For staining purposes, few droplets of crystal violet were added on it 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. Estrous cycle was always measured at 8:00-9:00 am [97].

Microscopic examination of the slides was conducted to identify the three types of cells: nucleated epithelial cells, cornified cells, and leukocytes. These cells were recognized using the following cellular characteristics. The round and nucleated ones were epithelial cells, the irregular ones without a nucleus were the cornified cells and the little round ones were the leukocytes. The proportion of these cells was used to determine the phases of estrous cycle. The four phases of

estrous cycle: proestrus, estrus, metestrus, and diestrus were identified. A proestrus phase was identified by the predominance of nucleated epithelial cells while an estrous phase primarily consisted of non-nucleated cornified cells. Smear with a nearly similar proportion of nucleated epithelial cells, cornified cells, and leukocytes were identified as metestrus phase of estrous cycle. Finally, smear in the diestrus phase was identified by the predominant leukocytes. The full duration of these phases was considered as the length of one estrous cycle [98, 99]. The difference in the length of estrous cycle was compared between the treatment and control groups.

4.4.8.3 Terminal Assessment

4.4.8.3.1 Macroscopic Examination

At the end of treatment schedule, all P-rats were killed with an intraperitoneal injection of pentobarbital. A median incision on the abdominal wall was performed to reveal visceral organs. The respective organs were macroscopically examined for any structural abnormalities or treatment-related pathological changes.

4.4.8.3.2 Organs Weight Measurement

The weight of the following organs was recorded immediately after necropsy. Paired organs were measured separately.

- Uterus (with oviducts and cervix)
- Ovaries
- Testes
- Epididymis
- Prostate
- Seminal gland

4.4.8.4 Histopathologic Investigation on Parental Rats

Histopathologic assessment of the organs listed above and the vagina was performed. Sample from each organ was fixed with 10% formalin and further processed and stained following the steps mention in placental histopathology (section 4.3.5.5). A detailed microscopic examination for any treatment-related changes was performed using a binocular light microscope. The histologic

appearance of organs from treatment groups was compared with the control group. After investigation, representative photomicrographs were captured with an automated inbuilt digital microscope camera (Leica EC4, Germany) under 4x, 10x, and 40x objective lens magnification, total magnification 40x, 100x, and 400x respectively.

4.4.8.5 Sperm Analysis

At termination, one epididymis was used for sperm evaluation. Testis and epididymis were removed and carefully dissected free of fat and blood vessels. The tail of the epididymis was excised with a surgical blade for sperm to disperse in a glass containing semen diluting fluid prepared by sodium bicarbonate (5 g) in 99 ml normal saline, and adding 1 ml formalin (40%) considering 20 times dilution factor. The suspension was carefully diluted and mixed [100]. The spermatozoa were counted and evaluated for morphological abnormalities.

4.4.8.5.1 Sperm Count

A single drop of sperm suspension was placed into the Neubauer hemocytometer chamber and keep settled in a humid place for 10 minutes. The number of spermatozoa in 5 squares of the hemocytometer was counted under a microscope. The total number of sperm cells was calculated as sperm count = number of spermatozoa x dilution factor x depth factor/number of areas counted [101].

4.4.8.5.2 Assessment of Sperm Morphology

For the assessment of sperm morphology, 200 sperm cells/rats were counted and the percentage of abnormal sperm cells was calculated [102]. Slides were prepared as a fixed wet preparation [103] and classified as either normal (both head and midpiece/tail appear normal) or abnormal (double head, absent head, misshaped heads, absent tail, or curved tail) [19].

4.4.9 Assessment of Hepatotoxicity and Renal Toxicity in Parental Rats

4.4.9.1 Clinical Chemistry Assessment in Parental Rats

Systemic toxic effects of the plant extract were determined on P-rats via clinical chemistry measurement and histopathologic investigation of the liver and kidneys. At termination, a fasted blood sample was taken from ten randomly-selected P male and female rats per group. For clinical

chemistry assessment, 5-6 ml of blood was collected via cardiac puncture. The blood was placed in a plane test tube (without anticoagulant) for an hour. To obtain serum, the blood was centrifuged for 10 minutes with an electrical centrifuge at 3500 rpm. Then serum was separately collected by micropipette and kept in a vial. Finally, the serum was immediately analyzed by an automated clinical chemistry analyzer and the following clinical chemistry investigations indicating renal and hepatic functions were recorded: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea, creatinine, total protein, albumin, glucose, and total cholesterol.

4.4.9.2 Histopathology of the Liver and Kidney

Once the blood was collected, the rats were dissected and macroscopic examination was carried out for any pathological alterations of the liver and kidney. Samples from the liver and kidney were obtained to assess treatment-related histopathologic changes. The samples were fixed in 10% formalin and further processed and stained by the steps mentioned for the reproductive organs.

4.4.10 Assessment of Endocrine Toxicity

The endocrine toxicity of the plant extract was assessed by measuring the thyroid hormone level and conducting the histopathologic investigation on the thyroid and adrenal glands. For the analysis of thyroid hormones (T4), the serum obtained for clinical chemistry was used.

The adrenal glands were weighed and fixed in 10% formalin for histopathologic investigation. The thyroid gland was also dissected with the trachea and fixed with formalin. The fixed glands were processed by similar steps mentioned for reproductive organs. Any toxicity-related microscopic changes were investigated and recorded.

4.4.11 Assessment of F1 Offspring

First-generation offspring were evaluated for the potential reproductive, systemic, glandular, neuro, and immune toxicities. They were grouped into three sets. On PND 0, the number and sex of pups, stillbirths, live births, and the presence of gross anomalies (externally visible abnormalities, including cleft palate, subcutaneous hemorrhages, and abnormal skin color or texture) were examined.

4.4.11.1 Weight Measurement of F1 Rats

Live pups were individually weighed on PNDs 0, 4, 7, 14, 21, weekly thereafter, and at necropsy.

4.4.11.2 Anogenital Distance Measurement and Presence of Nipples/Areolae

The anogenital distance (the distance between the anus and genital tubercle) of each pup was measured at PND 4. The presence of nipples/areolae in male pups was inspected on PND 12.

4.4.11.3 Selection of Pups for Post-Weaning Study

Litters were housed with their mothers until weaning (PND 21). All F1 offspring were identified when they are first examined on PND 0. The litter of origin, sex, and parental dose were recorded. Number of postnatal deaths was calculated on PND 0,4,7,14,21. On PND 21, pups were detached from their dam and grouped for post-weaning study. They were randomly assigned to one of the three cohort (set) group to examine the effects of the test substance upon specific aspects of development: Set-1, reproductive toxicity, Set-2, developmental neurotoxicity (DNT), Set-3, developmental immunotoxicity (DIT). A further illustration of the grouping is as follows and presented in Figures 3 and 4.

- **Set-1:** One male and one female from each litter (20/sex/group) were assigned to evaluate the effects of the test plant on the reproductive system as well as liver, kidney, thyroid, and adrenal glands on (PND 70).
- **Set-2:** One male and one female from each litter (10/sex/group) were assigned to evaluate DNT of *S. guineense* on PND 70.
- **Set-3:** One male and one female from each litter (10/sex/group) were assigned to evaluate DIT *S. guineense* on PND 60.

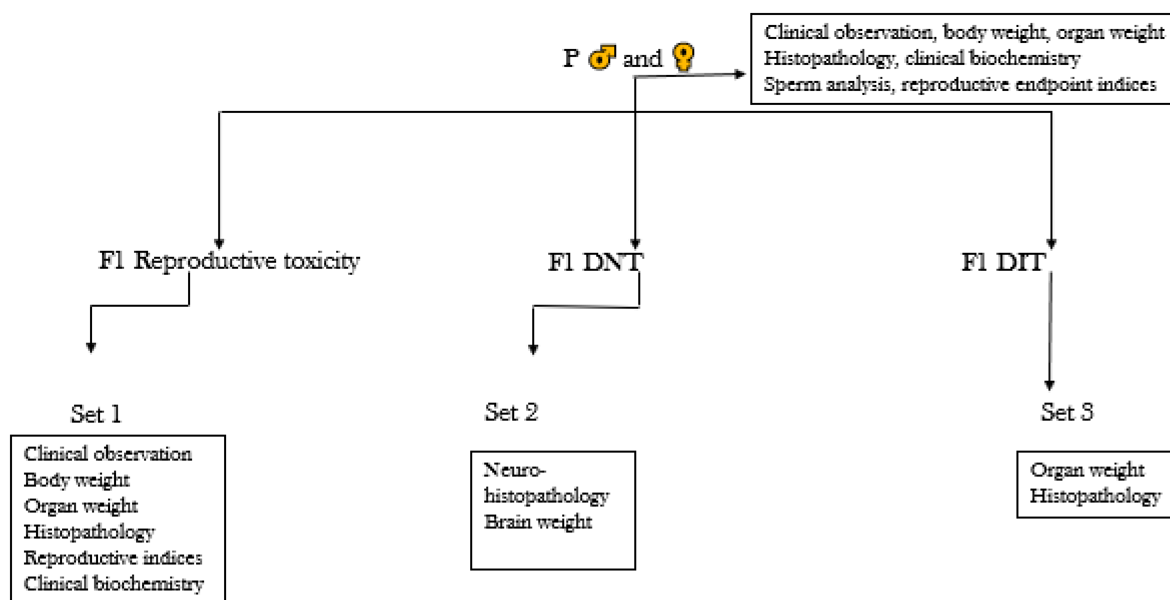


Figure 4: Schematic representation of the study design for the extended one-generation reproductive toxicity study: type of test in each group.

4.4.11.4 Reproductive Toxicity Assessment in F1 Set 1 Rats

At PND 21, 20 pups/sex/group were assigned to assess the extended effect of *S. guineense* on the reproductive system as well as liver, kidney, thyroid, and adrenal glands in F1 offspring. The pups received the treatment indirectly from their dams during pregnancy and lactation. From PND 21 onward, direct oral administration was initiated. Like that of the parents, treatment groups (I, II, and III) were treated with 250, 500, and 1000 mg/kg doses, respectively. While the control group was administered 1 ml/100 mg body weight of distilled water.

4.4.11.4.1 Weight and Food Intake Measurement

The weights of F1 set-1 pups were measured on weekly basis from PND 21 to necropsy (PND 70). The weight gain was calculated among the groups. Food intake was daily recorded to test the effect of the extract on the food intake in the pups.

4.4.11.4.2 Preputial Separation and Vaginal Opening

All F1 male pups were evaluated daily for balano-preputial separation starting from PND 35. Full balano-preputial separation was considered when the prepuce was completely retracted from the glans penis and folded beneath the glans [104]. On the other hand, female pups were also inspected for the presence of vaginal opening starting from PND 28. The vagina was considered open when the membranous sheath covering the vaginal orifice was completely broken and the lumen was patent [104]. The day of preputial separation or vaginal opening with respective weight was recorded. Sexual maturity of F1 rats was compared to physical development by determining age and body weight at balano-preputial separation or vaginal opening for males and females, respectively. Any abnormalities of genital organs (persistent vaginal thread, hypospadias, or cleft penis) were recorded.

4.4.11.4.3 Estrous Cycle Measurement (F1 Set-1 Rats)

In F1 set A rats, estrous cycle measurement was done for four weeks since the vaginal opening was confirmed. The date of first appeared cornified epithelium (time of ovulation) also was recorded following vaginal opening. Sample collection, staining procedure, staging of the phases of estrous cycle was as stated in the parental rats (section 4.4.8.2). The length of estrous cycle and day of first appeared of cornified epithelium was compared between the treatment and control groups.

4.4.11.4.4 Examination at Necropsy (F1 Set-1 Rats)

In F1 set-1 rats, gross examination, weight measurement, and histopathologic investigation in the reproductive organs, liver, kidneys, thyroid gland, and adrenal glands were carried out. In addition, clinical chemistry and thyroid hormone was analyzed by the same method done in the P-rats.

4.4.11.5 Assessment of Developmental Neurotoxicity (F1 Set-2 Rats)

Ten rats/sex/group (set-2 rats) was used to assess DNT of *S. guineense*. On PND 21 pups were separated from their dams and grouped into four groups. Groups I, II, and III were administered with 250, 500, and 1000 mg/kg of the *S. guineense* extract, respectively and group IV received distilled water (1 ml/100 g body weight). Rats were treated on daily basis until necropsy (PND 70). Food intake and weight were recorded as it was done for set-1 rats.

4.4.11.5.1 Brain Weight Measurement

At the end of the treatment period, rats were anesthetized by intraperitoneal injection of pentobarbital, the rat's scalp was removed by scissor and the skull was bilaterally separated following sagittal suture. The brain was carefully detached from the meninges and cranial cavity. Cuts were made uniformly from olfactory bulb cranially and medullary decussation caudally. The brain was inspected for any sign of gross morphological changes. Then the weight of each brain was recorded with digital balance.

4.4.11.5.2 Histopathology of Brain and Spinal cord

Before dissecting the brain and spinal cord, 10% formalin was injected into rats via trans-cardiac perfusion by the method described in Gage *et al* [105] study. After 10 minutes the skull was opened and the brain was meticulously removed from the cranial cavity. The lamina of each vertebra was broken craniocaudally to take the spinal cord out of the vertebral canal. The brain and spinal cord were immersed into 10% formalin for 24 hours. Representative samples from the main regions of the brain: cerebrum, cerebellum, brainstem, and diencephalon were sectioned for tissue processing. Regarding the spinal cord, samples were taken from the cervical, thoracic and lumbar regions. The rest tissue processing and staining protocol was done as mentioned earlier in P-rats (section 4.3.5.5).

The slides were examined with a binocular light microscope for the presence of any developmental neuropathological damage to the nervous system: cellular alterations (neuronal vacuolation, degeneration, necrosis) and tissue changes (gliosis, leukocytic infiltration, cystic formation). Changes in proliferation, migration, and differentiation, as evidenced by areas of excessive apoptosis or necrosis, clusters or dispersed populations of ectopic, disoriented or malformed neurons, and alterations in the relative size of various layers of cortical structures were investigated. Alterations in patterns of myelination, including an overall size reduction or altered staining of myelinated structures were taken into consideration.

4.4.11.6 Assessment of Developmental Immunotoxicity (F1 Set-3 rats)

Developmental immunotoxicity study was performed to determine the effect of the test substance on the immune system. It was assessed by measuring the weight and histopathologic evaluation of lymphoid organs. Set-3 rats were used to assess DIT. On PND 21, 10 pups/sex/group were

randomly selected to assess developmental immunotoxic nature of *S. guineense.* Groups I, II and III rats were treated with 250, 500, and 1000 mg/kg of the plant extract, while the control group received distilled water. As it was done for set-1 and 2 rats, weight and daily food intake were recorded.

4.4.11.6.1 Weight Measurement of Lymphoid Organs

At necropsy, weight of the spleen, thymus and lymph nodes were measured. Lymph nodes were obtained from submandibular, cervical and mesenteric regions.

4.4.11.6.2 Histopathology of Lymphoid Organs

Sample was collected from the spleen, thymus, and lymph nodes for histopathologic examinations. Tissue processing and staining protocols were similar to the other organs (see section 4.3.5.5).

4.5 Statistical Analysis

Data were entered and analyzed by a statistical package for social science (SPSS) version 24. The data regarding maternal food intake and weight gain, pregnancy outcomes, reproductive indices, embryonic development, fetal growth, relative organ weight, sperm count, and sperm morphology were analyzed using one-way analysis of variance (ANOVA). One-way ANOVA was used to determine whether the means of treatment and control groups were statistically different or not. To figure out exactly in which treatment group the difference lied, Post Hoc tests (Turkey and Games-Howell) were conducted. Games-Howell test was applied if the assumption of homogeneity was violated, otherwise Turkey tests was chosen. Moreover, the difference between the control group and each treatment group was evaluated by Dunnett's test. Dunnett's test was conducted to compare each treatment group with a single control group and found a statistical difference at least between the control and each treatment group. Shapiro-Wilk test of normality was conducted to check if the data scores were normally distributed or not. Data were subjected to Levene's test to meet the test of homogeneity of variance before conducting ANOVA. Data related to embryonic/fetal development abnormalities and placental abnormalities were analyzed using Chi-square test. The Chi-square test was carried out to compare the association between the frequency of anomalies and treatment with the test substance.

The experimental unit of analysis was different based on the type of experiment. Where appropriate, it could be an embryo, fetus, litter, or group. Clinical chemistry and hormone analysis were performed by sex considering the sexual differences in the value of clinical chemistry and hormones. Reproductive indices were calculated by a standard formula presented in Table 3. The data were expressed as mean ± standard deviation of mean (SDM) and percentages. P-value < 0.05 was considered statistically significant. The results were presented using tables and figures.

4.6 Ethical Approval

Prior to the experiment, letter of ethical approval was obtained from the Department of Anatomy graduate committee and institutional review board (IRB) of College of Health Sciences, Addis Ababa University with protocol number 012/19/ANAT and IRB form AAUMF03-008 in compliance with Organization for Economic Co-operation and Development (OECD) guidelines [87, 96, 106, 107] for the care and use of experimental animals. Rats used in this study were kept in the highest standards for the humane use of animals in the biomedical research laboratory of EPHI. They were not subjected to any unnecessary painful and terrifying situations. Administration of the test substance was carried out by experts and maximum effort was applied to prevent them from a pathogen. Before rats were sacrificed, to avoid pain and suffering, they were anesthetized with pentobarbital. Finally, unused pups and sacrificed parental rats were disposed of humanely by the laboratory standards of EPHI.

5. Results

5.1 Results of Teratogenicity Study

5.1.1 Day 12 Experiment

5.1.1.1 Maternal Food Intake and Body Weight Gain

The data regarding daily food intake, maternal body weight gain, and pregnancy outcomes were analyzed by one-way ANOVA, to check the presence of statistically significant difference in the mean between the treatment and control groups. Statistically significant reduction in food intake was observed in high dose (1000 mg/kg) treated group compared with the *ad libitum* control group (Table 4). Maternal body weight gain during gestational day 6-12 is presented in Table 4. Rats treated with 500 and 1000 mg/kg body weight of the plant extract had significantly reduced weight gain (13.7±4.5, and 10.5±8.7, respectively) compared to the untouched *ad libitum* control group (24.3±5.8) at p-value <0.05.

5.1.1.2 Pregnancy Outcomes

The number of implantation sites was counted per dam. The number of resorption sites was increased in higher doses (500 and 1000 mg/kg) treated groups. However, the difference was not statistically significant (Table 4).

Table 4: Food intake, maternal weight gain and pregnancy outcomes of rats treated with 70% ethanol extract of *Syzygium guineense* leaf:-day-12 experiment

	Group				
	250 mg/kg	500 mg/kg	1000 mg/kg	Control	*Ad libitum* control
Food intake in gram/day	193.4±7.8	189.2±10.8	181.4±9.6*	189.4±11.7	199.2±1.4
Maternal weight gain/dam (g)	16.2±5.7	13.7±4.5*	10.5±8.7*	14.8±1.9	24.3±5.8
Number of implantation/dams	12±0.6	12.2±0.8	12.8±0.8	11.3±1.4	11.7±1
Number of resorption /litters	0.5±0.55	0.83±0.98	0.83±0.98	0	0.17±0.41

Results are expressed as mean ± standard deviation of mean, *Significantly different from the *ad libitum* control group (p-value < 0.05), One-Way ANOVA.

5.1.1.3 Growth of the Embryo

The crown-rump length (CRL), number of somites and morphological scores were considered as embryonic growth indices [89, 108]. Generally, the CRL of the embryos was reduced dose-dependently in the treated groups. The CRL of rat embryos treated with 1000 mg/kg body weight of the test plant was significantly lower when compared to those in the control and *ad libitum* control groups. It was 5.1±0.4 in the *ad libitum* control and 4.3±0.4 in a high dose (1000 mg/kg) treated groups (Table 5).

The number of somites is an important predictor of embryonic development [89]. The mean number of somites was significantly lower (p-value<0.05) in the high dose treated group (1000 mg/kg) compared to the *ad libitum* control group. In spite of this, no significant difference was seen among the other groups (Table 5).

Morphological scores have a linear relationship with embryonic age and used to estimate the growth of the embryo [89]. The average morphological score was calculated based on Brown and Fabro [89] morphological scoring system of the rat embryo which was adopted for use in *in-vivo* study by Seyoum and Persaud [90] (Appendix 4). The mean morphological score was significantly lower in rats treated with 1000 mg/kg of the plant extract compared to the control and untouched *ad libitum* control groups. The mean score was 44.9±0.5 for rats treated with 1000 mg/kg body weight of *S. guineense* extract and 45.8±0.7 and 46±0.6 for the control and *ad libitum* control groups, respectively (Table 5).

Table 5: Embryonic growth following administration of 70% ethanol extract of *Syzygium guineense* leaf:-day-12 experiment

Group	Variables		
	CRL of the embryo/litter (mm)	Number of somites/litter	Morphological score/litter
Group I (250 mg/kg) n=115	4.7±0.4	28.4±1.3	45.6±0.3
Group II (500 mg/kg) n=113	4.6±0.4	29.1±0.8	45.5±0.3
Group III (1000 mg/kg) n=120	4.3±0.4**	27.7±0.8*	44.9±0.5**
Group IV (control) n=113	5.08±0.4	28.4±1.2	45.8±0.7
Group V (*ad libitum* control) n=115	5.1±0.4	29.8±0.9	46±0.6

Results are expressed as mean ± standard deviation of mean, *significant difference with the *ad libitum* control group, **significant difference with the control and *ad libitum* control groups (p-value <0.05), One-Way ANOVA; CRL: Crown-rump length, n: Number of embryos.

5.1.1.4 Embryonic Development

Delays in the development of various systems of the embryo are presented in Tables 6 to 8. Assessment of craniofacial development, embryonic circulatory, and musculoskeletal systems did not show significant difference in developmental retardation between treatment and control groups. However, embryos in the high dose group showed higher percentage of retarded development in olfactory system and somites score compared to the other groups (Tables 7 & 8). Figure 5 presents developmental status of the primordia of different systems of the embryo.

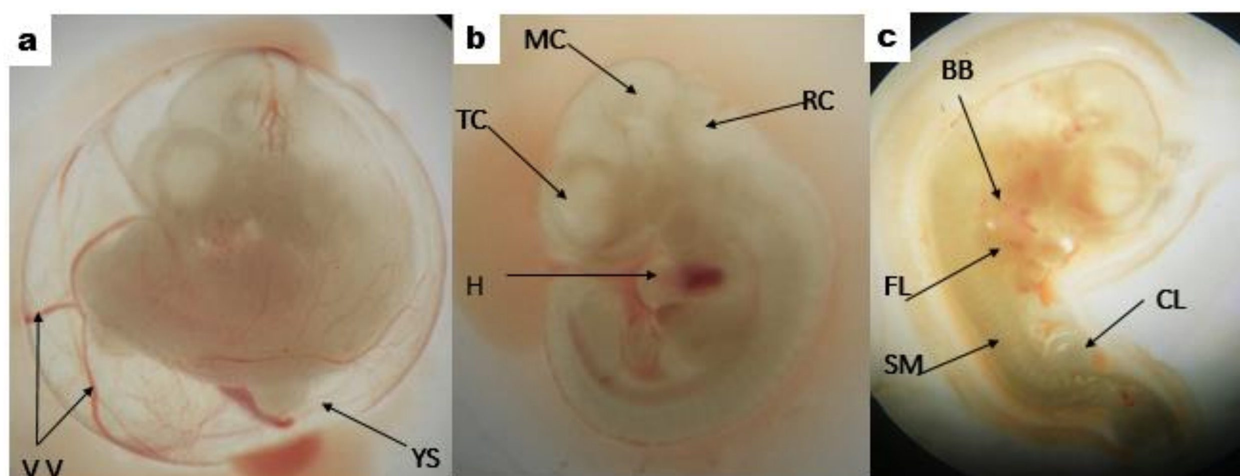


Figure 5: 12-Days old rat embryos treated with 1000 mg/kg of 70% ethanol extract of *Syzygium guineense* leaf showing the primordia of different organs. **a**: An embryo enclosed inside intact yolk sac (**YS**) with vitelline vasculatures (**VV**), **b**. Embryo with yolk sac and surrounding blood vessels removed showing heart (**H**), telencephalon (**TC**), mesencephalon (**MS**), rhombencephalon (**RC**), **c**: Showing branchial bars (**BB**), forelimb bud (**FL**), somites (**SM**), and caudal limb bud (**CL**).

Table 6: Embryonic circulatory system development following administration of 70% ethanol extract of *Syzygium guineense* leaf:-day-12 experiment

Group	Percent of retarded development		
	Yolk sac circulation	Allantois	Heart
Group I (250 mg/kg) n=115	0	0	0
Group II (500 mg/kg) n=113	0	0	2
Group III (1000 mg/kg) n=120	2	0	2
Group IV (control) n=113	0	0	0
Group V (*ad libitum* control) n=115	0	0	0

Results are expressed as percentages of retarded development (Chi-Square); n: Number of embryos.

Table 7: Embryonic nervous system and sense organs development following administration of 70% ethanol extract of *Syzygium guineense* leaf:-day-12 experiment

Group	Percent of retarded development						
	Caudal Neural tube	Hind-brain	Mid-brain	Fore-brain	Otic system	Olfactory system	Optic system
Group I (250 mg/kg) n=115	0	0	0	0	2	2	0
Group II (500 mg/kg) n=113	0	0	0	2	2	4	0
Group III (1000 mg/kg) n=120	0	0	0	2	4	8	0
Group IV (control) n=113	0	0	0	0	0	0	0
Group V (*ad libitum* control) n=115	0	0	0	0	0	0	0

Results are expressed as percentages of retarded development (Chi-Square); n: Number of embryos.

Table 8: Embryonic musculoskeletal system development following administration of 70% ethanol extract of *Syzygium guineense* leaf:-day-12 experiment

Group	Percent of retarded development						
	Flexion	Branchial Bars	Maxillary Process	Mandibular process	Fore-limb	Hind-limb	Somites score
Group I (250 mg/kg) n=115	0	0	0	2	0	2	4
Group II (500 mg/kg) n=113	0	0	2	4	0	4	4
Group III (1000 mg/kg) n=120	0	0	6	4	0	4	12
Group IV (control) n=113	0	0	0	0	0	2	2
Group V (*ad libitum* control) n=115	0	0	0	0	0	0	2

Results are expressed as percentages of retarded development (Chi-Square); n: Number of embryos.

5.1.2 Day-20 Experiment

5.1.2.1 Maternal Food Intake and Weight Gain

The levels of food intake are presented in Table 9. The high dose (1000 mg/kg) treated group consumed significantly low amount of food compared with the controls and low dose (250 mg/kg) treated groups. The *ad libitum* control group ate significantly high amount of food compared with all the other groups. The weight gain of pregnant rats was measured between pregnancy day 6-12 and 13-20. The *ad libitum* control group showed significantly increased weight gain in the first 6-12 days of pregnancy, compared with all the other groups. In addition, rats treated with high dose of *S. guineense* extract showed significant weight gain reduction during day 13-20 of pregnancy compared with the control and *ad libitum* control groups (Table 9).

Table 9: Maternal weight gain and food intake of pregnant rats treated with 70% ethanol extract of *Syzygium guineense* leaf:-day-20 experiment

Weigh gain and food intake	Group				
	Group I 250 mg/kg	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV Control	Group V *Ad-libitum* control
Food intake/day (g) n=10	199.2±4.5	191.4 ±8**	180±12.1*!	196.6±10	214±4.5*
Maternal weight gain/dam (g) Day 6-12	17.8±8.5	17.2±7.4	15.8±7.5	19±12.5	40.2±5.8*
Day 13-20	69.7±3.4	67.2±6.6	60.5±5.6***	72.2±5.6	72.8±9.6

The results are expressed as mean ± standard deviation of mean, * Significant difference with all the other groups, *! significant difference with Group I, IV and V, ** significant difference with group V, *** significant difference with group IV and V (for all p-value < 0.05), One-Way ANOVA.

5.1.2.2 Pregnancy Outcomes

In the current study, the pregnancy outcomes measured were the number of implantation sites, resorptions, live/dead fetuses, and sex of the fetus (Table 10). Treatment of pregnant rats with *S. guineense* extract did not significantly alter the average number of either implantation or resorption

sites (Figure 6). In addition, no significant change in the number of live or dead fetuses was observed between the treatment and the control groups (Figure 7).

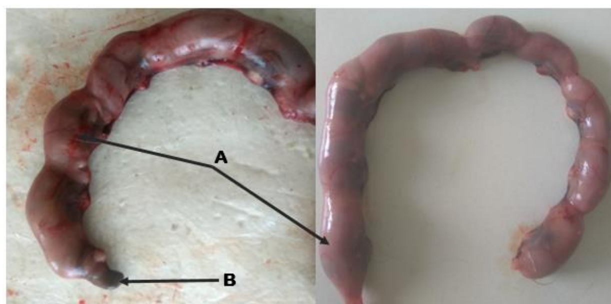


Figure 6: Gravid uterus of rat indicating implantation sites (A) and fetal resorption (B) following administration of 70% ethanol extract of *Syzygium guineense* leaf (1000 mg/kg).

Table 10: Pregnancy outcomes of rats treated with 70% ethanol extract of *Syzygium guineense* leaf:-day-20 experiment

Variables	Group				
	250 mg/kg n=10	500 mg/kg n=10	1000 mg/kg n=10	Control n=10	*Ad libitum* control n=10
Number of fetuses	93	91	88	95	115
Number of implantation sites/litter	9.8±2.1	10±2.5	10±1.4	10.5±0.5	11.7±1.2
Number of resorption sites/litter	0.5±0.8	0.9±0.9	1±0.9	1±1.6	0.2±0.4
Live fetuses/litter	9.3±2.7	9.1±3.5	8.8±1.6	9.5±1.9	11.5±1.4
Dead fetuses/litter	0	0	0.2±0.4	0	0
Number of male fetuses/dam	4.5±1.6	4.3±1.2	3.7±1.5	5±1.1	5.3±1.2
Number of female fetuses/dam	4.8±2.7	4.8±2.4	5.3±2.3	4.5±2.4	6.2±0.8

The results are expressed as mean ± standard deviation of mean, One-Way ANOVA; n: number of dams.

5.1.2.3 Fetal Growth

Regarding the fetal growth indices (CRL, fetal weight, and placental weight), a significant reduction of CRL was observed in fetuses treated with 1000 mg/kg body weight of the plant extract, compared with the control and *ad libitum* control groups. The CRL of fetuses in the high

dose and *ad libitum* control groups was 5±0.4 and 5.7±0.4, respectively. The weight of the fetuses and the placentae showed reduction in the treatment groups. However, this was not statistically significant, compared to the control or *ad libitum* control groups (Table 11).

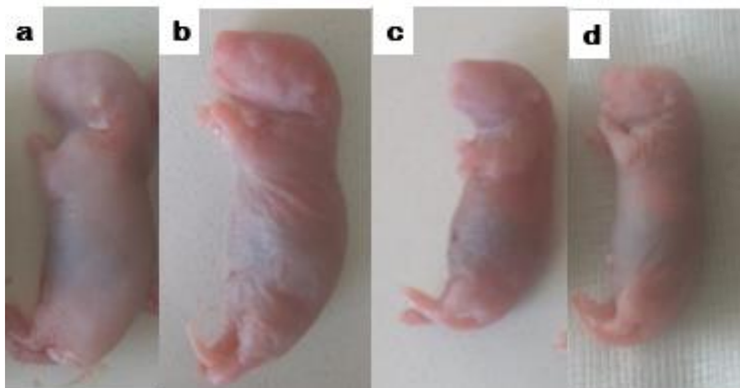


Figure 7: 20-Days old, live rat fetuses in group: **a** (control), **b** (250 mg/kg), **c** (500 mg/kg) and **d** (1000 mg/kg).

Table 11: Fetal growth indices of rat fetuses following administration of 70% ethanol extract of *Syzygium guineense* leaf:-day-20 experiment

Variables	Group				
	250 mg/kg n=10	500 mg/kg n=10	1000 mg/kg n=10	Control n=10	*Ad libitum* control n=10
CRL/fetus (cm)	5.3±0.2	5.2±0.4	5.0±0.4*	5.5±0.2	5.7±0.4
Fetal weight (g)	5.8±0.9	5.1±0.5	4.9±0.5	5.5±0.6	5.1±0.8
Placental weigh (g)	0.7±0.2	0.6±0.1	0.6±0.1	0.7±0.1	0.6±0.05

The results are expressed as mean ± standard deviation of mean. * significant difference with control and *ad libitum* control groups (p-value <0.05), One-Way ANOVA; CRL: Crown-rump length.

5.1.2.4 External and Visceral Morphological Anomalies

Explanted fetuses were freshly examined head to tail for the presence of external malformations. Craniofacial developmental anomalies, limb defect, vertebral column anomalies, missed tail, and

external genitalia related abnormalities were inspected. However, our investigation did not reveal any treatment related external malformations on the near-term rat fetuses (Table 12).

Fetuses fixed with Boiun's solution were serially sectioned for visceral soft tissue examination. Serial sectioning was made at the level of head, neck, chest, and abdomen. The sections were carefully examined for the existence of any visceral anomalies under a dissecting microscope. At the head region, cleft palate, hydrocephalus, and eye-related abnormalities were inspected. The thyroid, thymus, trachea, and cardiac septum associated abnormalities were also checked at the level of the neck and chest. The occurrence of diaphragmatic hernia, agenesis of abdominal viscera, and external genitalia were also investigated. Like the external morphological examination, no visible visceral abnormalities were detected (Table 13 and Figure 8).

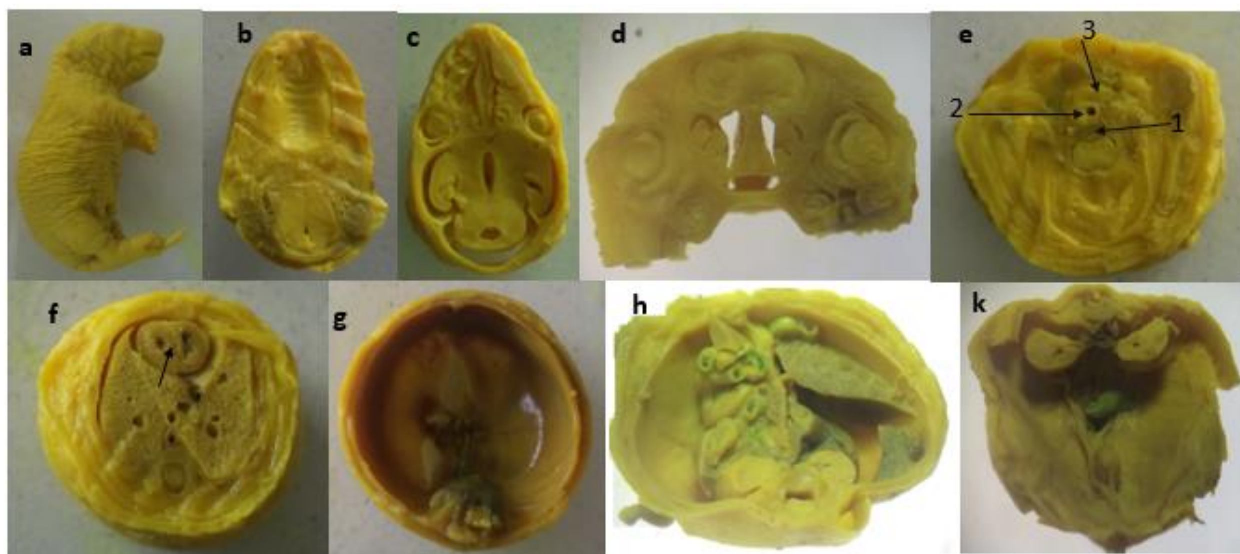


Figure 8: 20-Days old rat fetus fixed in Bouin's solution for visceral examination (treated with 1000 mg/kg of *Syzygium guineense* leaf extract). **a**: Un-sectioned fetus, **b**: Normal palate, **c & d**: Transverse section and a coronal section of the brain showing normal ventricle and eyeball respectively, **e**: A section made through the neck showing normal 1-esophagus, 2-trachea, and 3-thyroid, **f**: A section through the chest showing normal interventricular septum (arrow), **g**: Intact diaphragm, **h & k**: Sections made though the abdomen showing normal visceral organs including the kidney.

Table 12: External malformations of rat fetuses following administration of 70% ethanol extract of *Syzygium guineense* leaf:-day-20 experiment

Group	Fetus examined	Observed malformations (%)							
		EC	AE	SB	KY	SC	LD	MT	AEG
Group I (250 mg/kg)	93	0	0	0	0	0	0	0	0
Group II (500 mg/kg)	91	0	0	0	0	0	0	0	0
Group III (1000 mg/kg)	88	0	0	0	0	0	0	0	0
Group IV (control)	95	0	0	0	0	0	0	0	0
Group V (*Ad libitum* control)	100	0	0	0	0	0	0	0	0

Results are expressed as percentages of malformations (Chi-Square). **EC**: Exencephaly (extrusion of the brain from the cranium), **AE**: Anencephaly (absence of brain and calvaria), **SB**: Spina bifida (protrusion of the spinal cord via the defects in vertebral arch), **KY**: Kyphosis (increased outward curvature of the thoracic vertebrae), **SC**: Scoliosis (lateral bending of the vertebrae), **LD**: Limb defect (any abnormalities in the forelimb and hindlimb), **MT**: Missed tail (complete absence of tail), **AEG**: Agenesis of external genitalia (complete absence of external genitalia).

Table 13: Visceral malformations of rat fetuses following administration of 70% ethanol extract of *Syzygium guineense* leaf:-day-20 experiment

Group	Fetus examined	Observed malformations (%)										
		HC	MO	AO	CP	NSD	REAA	VSD	DH	RA	HU	CT
Group I (250 mg/kg)	50	0	0	0	0	0	0	0	0	0	0	0
Group II (500 mg/kg)	50	0	0	0	0	0	0	0	0	0	0	0
Group III (1000 mg/kg)	60	0	0	0	0	0	0	0	0	0	0	0
Group IV (control)	50	0	0	0	0	0	0	0	0	0	0	0
Group V (*Ad libitum* control)	50	0	0	0	0	0	0	0	0	0	0	0

Results are expressed as percentages of malformations (Chi-Square). **HC**: Hydrocephalus (enlarged ventricles of the brain), **MO**: Microphthalmia (small eyeball), **AO**: Anophthalmia (complete absence of eyeball), **CP**-Cleft palate (an opening in the roof of the mouth), **NSD**: Nasal

septal defect (the presence of a hole in the bony/cartilaginous wall of the nasal cavity), **REAA**: Retroesophageal aortic arch (aortic arch cross the midline posterior to the esophagus), **VSD**: Ventricular septal defects (presence of a communicating slit between the two ventricles of the heart), **DH**: Diaphragmatic hernia (the presence of abnormal opening in the diaphragm), **RA**-Renal agenesis (complete absence of one/both kidneys), **HU**: Hydroureters (dilation of the ureter), and **CT**: Cryptorchid testes (absence of testis in the scrotum).

5.1.2.5 Skeletal Malformations

The results of the skeletal evaluation are presented in Figure 9 and Tables 14 and 15. The observation conducted on the skull, thoracic vertebrae, sternum, hyoid, and metatarsals did not reveal statistically significant skeletal malformations between treatment and control groups. The ossification centers of caudal/coccygeal vertebrae, forelimb phalanges, and hindlimb phalanges indicated variation. Fetuses of middle and high dose group rats showed decreased number of ossifications in the sternum and caudal vertebrae. However, it was not statistically significant. A total of 56.7% of rat fetuses from mid and high dose groups had no proximal hindlimb phalanges. Nevertheless, this variation did not show statistical significance.

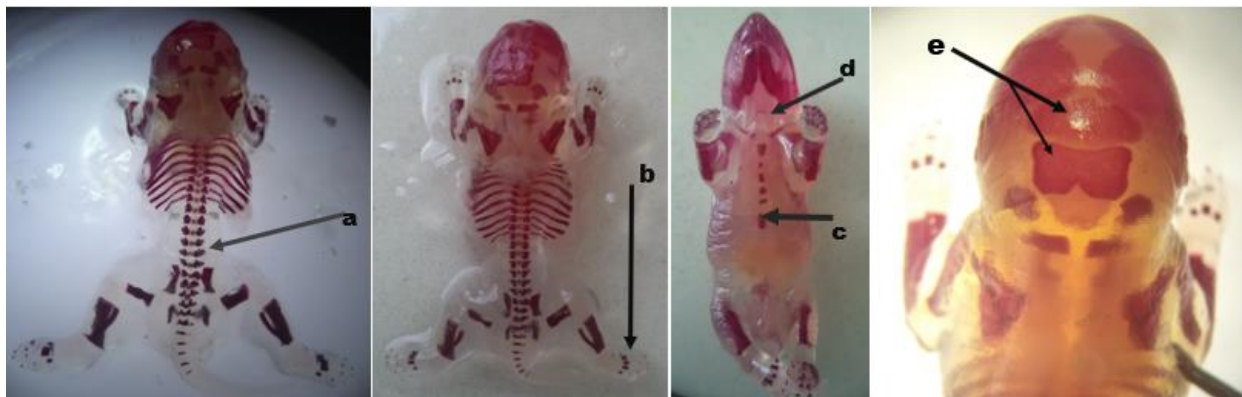


Figure 9: Alizarin red stained rat fetuses showing different ossification centers. **A**: Vertebrae, **b**: Metatarsal, **c**: Sternebra, **d**: Hyoid, **e**: Supraoccipital and Inter-parietal.

Table 14: Skeletal malformations of 20 days old rat fetuses following treatment with 70% ethanol extract of *Syzygium guineense* leaf

Group	Percent of skeletal malformations				
	Sternum *	Hyoid*	Ribs	Thoracic vertebrae**	Caudal vertebrae***
Group I (250 mg/kg) n=30	10	100	100	100	13.3
Group II (500 mg/kg) n=30	23.3	100	100	100	33.3
Group III (1000 mg/kg) n=30	26.7	100	100	100	30
Group IV (control) n=30	16.7	100	100	100	13.3
Group V (*Ad libitum* control) n=30	13.3	100	100	100	16.7

Results are expressed as percentages of skeletal malformations (Chi-Square). * Sternum with 4 ossification centers and hyoid bone showing signs of ossification, ** Thoracic centra with 13 ossification centers, and *** Caudal vertebrae with 4 ossification centers.

Table 15: Skeletal (limb bones) malformations of 20-days old rat fetuses following treatment with 70% ethanol extract of *Syzygium guineense* leaf

Group	Percent of skeletal malformations of limb bones			
	Metacarpus*+	Metatarsal*+	Forelimb *! phalanges	Hindlimb *! phalanges
Group I (250 mg/kg) n=30	0	0	10	43.3
Group II (500 mg/kg) n=30	13.3	0	16.7	56.7
Group III (1000 mg/kg) n=30	13.3	6.7	20	56.7
Group IV (control) n=30	10	3.3	13.3	20
Group V (*Ad libitum* control) n=30	6.7	0	10	33.3

Results are expressed as percentages of skeletal malformations (Chi-Square).

*+ presence of <=3 metacarpus and metatarsus and *! Absent proximal phalanges.

5.1.2.6 Placental Histopathology

Microscopic examination of the placenta showed some structural changes in the decidual and labyrinthine zones of the placenta (Figures 10 & 11). Decidual cystic degeneration, hemorrhage, and trophoblast proliferation were the histopathological changes observed. Although the frequency of decidual cystic degeneration was higher in rats treated with high a dose of the plant extract, none of the above changes were statistically significant (Table 16).

Table 16: Microscopic placental abnormalities in rats following administration with 70% ethanol extract of *Syzygium guineense* leaf

Group	Percent of placental abnormalities		
	Decidual cystic/ degeneration	Hemorrhage/ hematoma	Trophoblast proliferation
Group I (250 mg/kg)	0	0	0
Group II (500 mg/kg)	10	10	10
Group III (1000 mg/kg)	20	0	10
Group IV (control)	10	10	0
Group V (*Ad libitum* control)	0	0	0

Results are expressed as percentages of placental abnormalities, Chi-square.

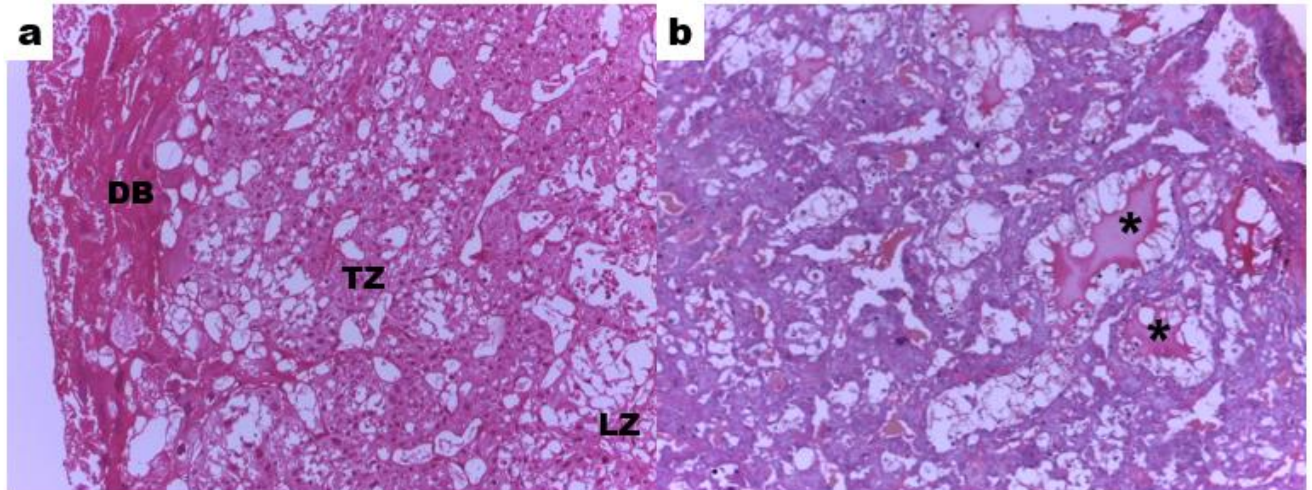


Figure 10: Photomicrograph of rat placenta, (**a**) Section taken from the control group showing normal architecture of the placenta, (**b**) Placenta of rat treated with 1000 mg/kg of 70% ethanol extract of *Syzygium guineense* leaf showing decidual cystic degeneration (*). **DB**: Decidua basalis, **TZ**: Trophoblastic zone, **LZ**: Labyrinthine zone; H and E stain, 100x total magnification.

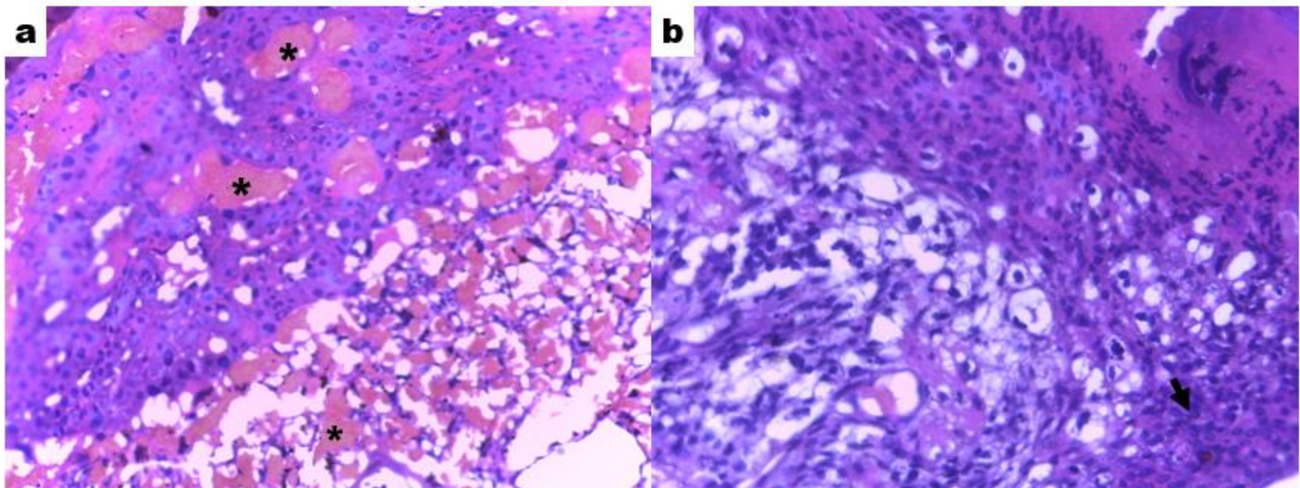


Figure 11: Photomicrograph of rat placenta, (**a**) Section taken from rat treated with 500 mg/kg of 70% ethanol extract of *Syzygium guineense* leaf showing hematoma in the different zones of the placenta (*), (**b**) Placenta of rat treated with 1000 mg/kg of 70% ethanol extract of *Syzygium guineense* leaf showing trophoblast proliferation (arrow head); H and E stain, 100x total magnification.

5.2 Results of the Extended One-generation Reproductive Toxicity Study

5.2.1 Cage Side Clinical Observation Results of the Parental Rats

A cage side clinical observation was conducted daily before and after dosing periods. The result of these records did not exhibit any changes in the skin, hair, and mucus membranes of the oral and nasal cavities. Moreover, no abnormal changes in respiratory pattern, motor activity, self-mutilation, any other sign of toxicity, or death were observed.

5.2.2 Food Consumption and Weight Gain of the Parental Rats (P Rats)

The results of weight gain and food intake during the treatment period are presented in Table 17. Regarding the food consumption of P male rats, a significant reduction in food intake was observed in the high dose (1000 mg/kg body weight) treated group compared to all the other groups. P male rats treated with 500 mg/kg body weight of the test plant also showed a significant reduction of food intake compared to that in the control group. In the P female rats, those treated with 500 mg/kg body weight (compared to the control group) and 1000 mg/kg body weight (compared to the control and low dose treated groups) of the test substance indicated a significant reduction in food consumption (Table 17).

A significant reduction in weight gain was recorded in male parental rats treated with 1000 mg/kg body weight of *S. guineense* extract compared to the control and low dose treated groups. Similarly, the pregestational and gestational weight gain of all treated female parental rats showed significant reduction compared to the control group. In addition, during lactation period, a significant (-27.3±7.1) weight reduction was observed in a high dose (1000 mg/kg) treated P female rats compared with all the other groups (Table 17).

Table 17: Weight gain and food intake of parental rats treated with 70% ethanol extract of *Syzygium guineense* leaf

		Group			
		Group I 250 mg/kg	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV Control
Male rats	Weight gain (g)	91.2±6.1	85±12.8	70±10*!	93.8±6.6
	Food intake (g) n=20	462.3±28.8	438.7±40**	370.7±33.5*	477.8±22.6
Female rats	Pregestational weight gain (g)	17.4±5.5	14±4.2	11.2±6.9	26±5*
	Gestational weight gain (g)	101.4±6.6	95.3±7.2	95.7±3.4	113.2±5.6*
	Lactational weight gain (g)	-7.9±9.1	-6±11	-27.3±7.1*	-1.8±7.5
	Food intake (g) n=20	321±16.6	301.3±27.9**	296.8±10.6*!	333.3±17.3

- Results are expressed as mean ± standard deviation of mean,
- * significant difference with all the other groups,
- ** significant difference with group IV,
- *! significant difference with group I and IV,
- For all p-value <0.05, One-Way ANOVA,
- NB: Pregestational weight gain (weight at pregnancy confirmation minus weight at the beginning of dosing), gestational weight gain (weight at gestational day 21 minus weight at gestational day zero), lactational weight gain (weight at lactational day 21 minus weight at lactational day zero).

5.2.3 Reproductive Toxicity Assessment Results

The potential reproductive toxic effect of 70% ethanol leaf extract of *S. guineense* was assessed by measuring the length of the estrous cycle, mating performance, pregnancy outcomes, postnatal survival of litters, and histopathology of reproductive organs.

5.2.3.1 Effects on the Estrous Cycle of Parental Rats

Changes in the length and normality of the estrous cycle is an index for the effect of the plant extract in the hypothalamic-pituitary–ovarian axis [109]. The length of the estrous cycle and its

four stages, namely: proestrus, estrus, metestrus, and diestrus were recorded based on the examination of a vaginal smear. The result displayed in Table 18 indicated that the length of the estrous cycle was significantly longer for the high dose group (5.8±1.4) compared to those in the control group (4.3±0.5), p-value <0.05.

Table 18: Length of estrous cycle of the parental rats treated with 70% ethanol extract of *Syzygium guineense* leaf

	Group			
	Group I 250 mg/kg	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV Control
Length of estrous cycle (days/dam)	5.1±1.2	4.5±0.9	5.8±1.4*	4.3±0.5

Results are expressed as mean ± standard deviation of mean. * Significantly different from control group (p-value <0.05), One-Way ANOVA, Games-Howell post Hoc test.

5.2.3.2 Effects on the Reproductive Indices

Twenty male and female rats were mated. All males in the four groups showed evidence of copulation. All female rats in the treatment and control groups, except two rats in the high dose treated group (1000 mg/kg) were successfully mated and were pregnant (Table 19).

Table 19: Reproductive data of parental rats treated with 70% ethanol extract of *Syzygium guineense* leaf

Reproductive data		Group			
		Group I 250 mg/kg	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV Control
No of paired	Male	20	20	20	20
	Female	20	20	20	20
No of mated	Male	20	20	20	20
	Female	20	20	20	20
No male siring a litter		20	20	18	20
No of pregnant females		20	20	18	20
No of females delivered viable litter		20	20	18	20

Results are expressed as the number of rats.

The results of reproductive indices presented in Table 20 were considered as indicators of the reproductive performance of male and female rats. Reproductive indices were calculated for 20 male and female parental rats based on the assumptions presented in Table 3. Administration of 70% ethanol leaf extracts of *S. guineense* for parental rats did not bring significant change on the mating index ((Number of rats with evidence of mating/ Number of paired) x 100), fertility index ((Number of rats siring a litter or with evidence of pregnancy/ Number of paired) x 100), and gestation index ((Number of females delivering a viable litter/ Number of females with evidence pregnancy) x 100). All female rats, except two (10%), in group three (1000 mg/kg) were pregnant (Table 20).

The pre-coital interval (the days required to be inseminated since pairing) was significantly longer (7±3.6) in rats administered with 1000 mg/kg body weight of the test substance, compared to the control group (2.8±1.2). However, the duration of pregnancy was not significantly altered by the treatment. The mean duration of pregnancy was 22.2±0.9, 21.5±1.2, 21.4±0.7, and 21.5±0.7 days for group I-IV, respectively (Table 20).

Table 20: Reproductive indices of parental rats treated with 70% ethanol extract of *Syzygium guineense* leaf

Reproductive indices		Group			
		Group I 250 mg/kg	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV Control
Mating Index (%)	Male	100	100	100	100
	Female	100	100	90	100
Fertility index (%)	Male	100	100	90	100
	Female	100	100	90	100
Gestation index (%)		100	100	90	100
Pre-coital interval (days/dam)		5.6±4.7	3.2±2.8	7±3.6*	2.8±1.2
Pregnancy duration (days/dam)		22.2±0.9	21.5±1.2	21.4±0.7	21.5±0.7

Results are expressed as mean ± standard deviation of mean and percentage. *Significantly different from control group (p-value <0.05), One-Way ANOVA.

5.2.3.3 Effects on Pregnancy Outcomes

Data related to the pregnancy outcomes are presented in Table 21 and Figure 12. The mean number of litters (total), male, and live births were significantly reduced in the treatment (low and middle doses) groups, compared to those in the control group. The number of stillbirth litters did not reveal a significant change between the treatment and control groups. Concerning the sex ratio, a higher number of male litters (1.2:1, male: female) were reported from the high dose treated group. On the contrary, the number of male litters was apparently lesser (0.58:1, male: female) in the low dose group, but not statistically significant.

Table 21: Birth outcomes of parental rats treated with 70% ethanol extract of *Syzygium guineense* leaf

Birth outcomes		Group			
		Group I 250 mg/kg (n=20)	Group II 500 mg/kg (n=20)	Group III 1000 mg/kg (n=18)	Group IV Control (n=20)
No of litter/dam	Male	3.2±1.3*	3.8±1.6*	4.7±1.7	6±1.8
	Female	5.4±1.9	4.2±2.1	4.0±1.7	4.6±1.6
	Total	8.6±1.9*	8.0±2.3*	8.7±1.9	10.6±0.8
	Live birth	8.2±1.9*	7.9±2.2*	8.3±2.2	10.2±0.8
	Stillbirth	0.4±0.8	0.1±0.3	0.4±0.7	0.4±0.7
Male: Female ratio		0.58:1	0.9:1	1.2:1	0.77:1

Results are expressed as %, mean ± standard deviation of mean, and ratio. *Significantly different from control group (p-value <0.05), One-Way ANOVA; n: means number of dams.

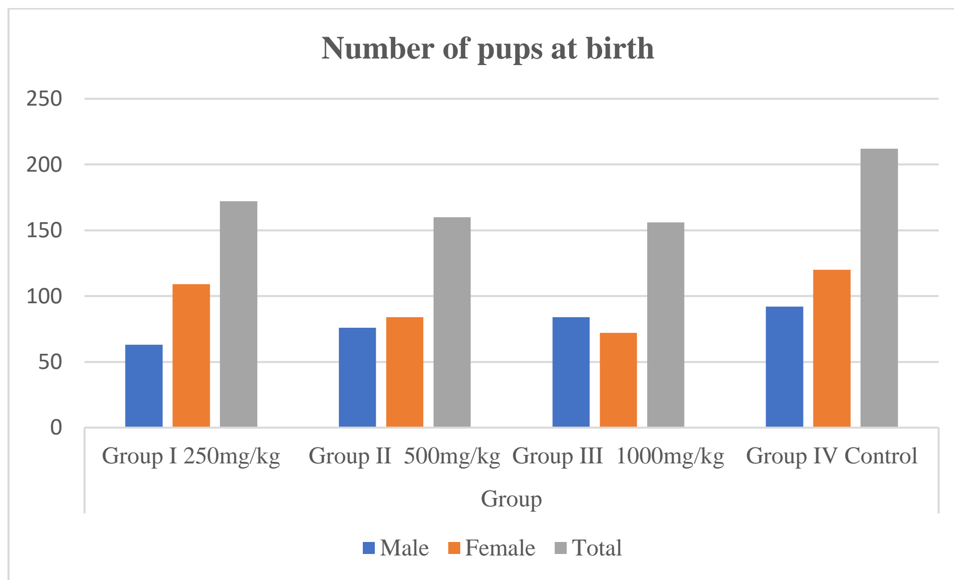


Figure 12: Number of pups at birth. The number of litters in group III were from 18 dams while the rests were from 20 dams. The colors indicate the number of males, females, and total pups.

5.2.3.4 Effects on the Postnatal Survival of Litters

Gestational survival index (viability at birth) was more than 95% in the treatment and control groups. Therefore, no significant variation was observed between treatment and control groups. The mean postnatal death of litters was calculated on postnatal days (PNDs) 1, 4, 7, 14, and 21. There was no pup death reported on PND 1 in any of the groups. From PNDs 4-21, the mean postnatal death of litters was not significantly varied between treatment and control groups (Table 22).

Table 22: Gestation survival index and postnatal death of litters treated with 70% ethanol extract of *Syzygium guineense* leaf

Reproductive indices		Group			
		Group I 250 mg/kg	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV Control
Gestation survival index %		95.8	98.8	96.2	96.5
No of postnatal death	PND 1	0.0	0.0	0.0	0.0
	PND 4	0.33±0.5	0.6±1.6	1.4±2.1	0.63±1.4
	PND 7	0.0	0.3±0.7	0.1±0.3	0.1±0.4
	PND 14	0.0	0.9±1.4	0.0	0.1±0.4
	PND 21	0.0	0.3±0.7	0.1±0.3	0.0

Results are expressed as mean ± standard deviation of mean and percentages, One-Way ANOVA and Chi square test.

5.2.3.5 Effects on the Lactational Weight of Pups

Before receiving direct treatment of the extract, pups got access to the extract during the intrauterine life and lactation period. Therefore, the effect of the extract on the growth of the pups was investigated by measuring the weight of pups. The weight of male and female pups was separately recorded on postnatal day zero, four, seven, fourteen, and twenty-one (Figures 13 & 14) and the results are presented in Table 23. The mean weight of pups from the high dose treated group, on PND 0 and PND 21 was 6.4±0.4 and 27.1±4.7 for males and 5.8±0.3 and 27.4±6.6 for females, respectively, while the record of control pups in the above days was 6.3±0.4 and 30.2±4.9

for males and 6±0.3 and 28.8±5 for females, respectively. However, statistically significant variation among groups was not seen.

5.2.3.6 Effects on the Androgen-Dependent Endpoints

In the current study, anogenital distance, presence of nipple/areola in male pups, and abnormalities of external genitalia were used as indicators of androgen-related effect of the plant extract.

Anogenital distance (AGD) is a valuable morphometric measurement that indicates androgen disturbance during development [110]. On PND 4, AGD was measured from the anus to external genitalia (Figure 15). Although no significant difference existed between the treatment and control groups, the longest AGD (6.11±0.41 mm) of male pups was recorded in the control pups. Also, female pups in the high dose group had longer AGD (3.08±0.3 mm). The results are presented in Table 23.

Table 23: Preweaning weight and anogenital distance of pups treated with 70% ethanol extract of *Syzygium guineense* leaf

Group	Sex	Pup's weight (g)					AGD on
		PND 0	PND 4	PND 7	PND 14	PND 21	PND 4 (mm)
One (250 mg/kg)	Male	6.3±0.5	8.8±1	11.6±1.4	21.4±2.3	30.7±4.9	6±0.41
	Female	6.2±0.4	8.6±1.1	11.2±1.7	20.1±2.5	29.1±4.1	3.06±0.17
Two (500 mg/kg)	Male	6.2±0.5	8.5±0.8	12.6±1.8	21.6±4.9	28±5.6	6.07±0.34
	Female	5.9±0.4	8.2±1.1	11.7±2.2	20.7±4.6	26.7±4.7	3.06±0.19
Three (1000 mg/kg)	Male	6.4±0.4	8.9±1	12.8±1.7	20.7±2.8	27.1±4.7	6.05±0.4
	Female	5.8±0.3	8.7±1	12.3±1.8	20.5±4.6	27.4±6.6	3.08±0.3
Four (control)	Male	6.3±0.4	8.3±0.9	11.5±1.1	20.8±1.3	30.2±4.9	6.11±0.41
	Female	6±0.3	7.7±0.8	10.7±1.2	19.5±1.3	28.8±5	3±0.53

Results are expressed as mean ± standard deviation of mean, One-Way ANOVA.

Figure 13: Developmental stages of rat pups treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf, **a**: on PND 0, **b**: on PND 4, **c**: on PND 7, **d**: on PND 14, and **e**: on PND 21.

Figure 14: Developmental stages of rat pups in the control group, **a**: on PND 0, **b**: on PND 4, **c**: on PND 7, **d**: on PND 14, and **e**: on PND 21.

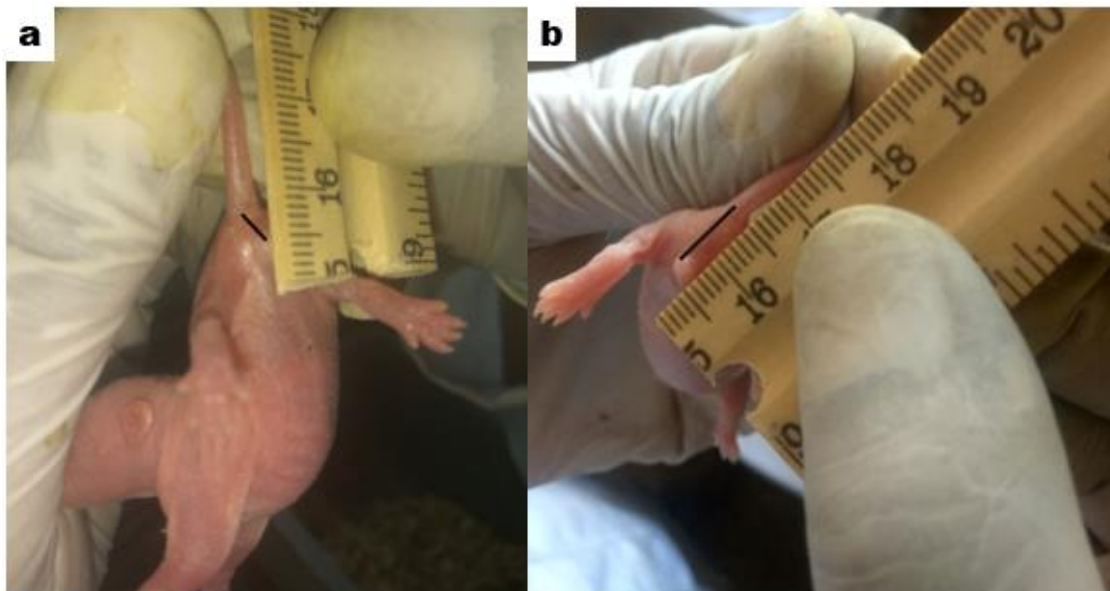


Figure 15: Anogenital distance measurement of rat pups on PND 4, **a**: Female and **b**: Male.

The presence of nipple retention on male pups was measured on PND 12. No nipple/areola was observed on male pups (Figure 16). In addition, no visible abnormalities external genitalia were observed in any of the treatment groups.

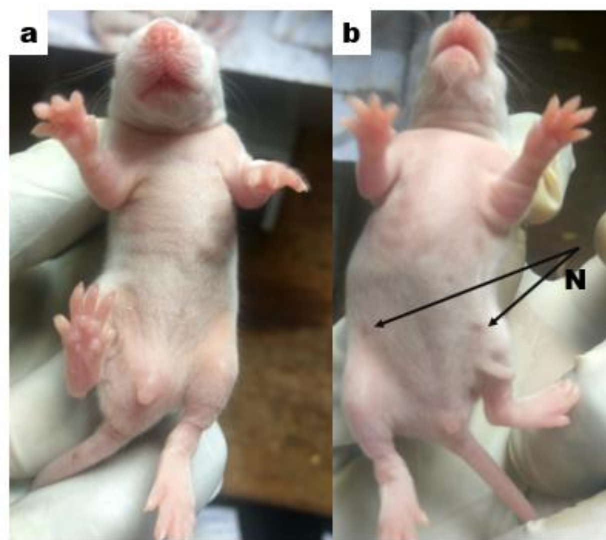


Figure 16: Absence (**a**) and presence (**b**) of nipple retention on rat pups treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf measured on PND 12, **a**: Male and **b**: Female pup; **NR**: Nipple retention.

5.2.3.7 Sperm Count and Morphology Assessment Results

As presented in Table 24, no significant variation in the mean number of total sperm count and abnormal morphology of spermatozoa was observed across all experimental parental rats.

Table 24: Sperm count and percentage of abnormal sperm cells of parental rats treated with 70% ethanol extract of *Syzygium guineense* leaf

Group	Sperm count (10^6/ml)	Abnormal sperm cells (%)
One (250 mg/kg)	206.72±16.41	8.64±3.08
Two (500 mg/kg)	204.52±13.31	9.12±2.32
Three (1000 mg/kg)	203.88±8.56	10.41±1.77
Four (control)	206.04±15.9	8.61±2.96

- Results are expressed as mean ± standard deviation of mean, One-Way ANOVA,
- Abnormal sperm (double head, absent head, misshaped heads, absent tail, curved tail).

5.2.3.8 Gross Examination and Weight of Reproductive Organs of Parental Rats

Each reproductive organ listed in Figure 17 was freshly examined for any gross abnormalities prior to microscopic examination. The result of macroscopic examination did not reveal any abnormalities in the treated or control groups.

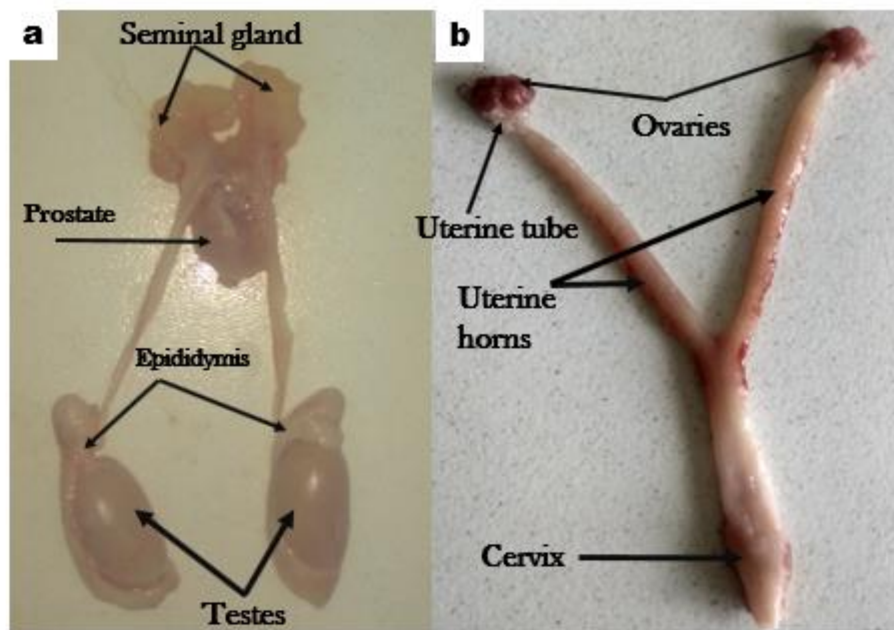


Figure 17: Male (**a**) and female (**b**) reproductive organs of rats treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf.

Research findings indicate that there is a strong correlation between organ weight and body weight [111]. Therefore, in the current study, relative organ weight was calculated by dividing absolute organ weight by rat weight at necropsy and multiplied by a hundred. The result of relative organs weight of male and female parental rats is presented in Table 25. Administration of ethanol leaf extract of *S. guineense* did not produce a significant change in the relative weight of male reproductive organs, namely: testis, epididymis, prostate, and seminal gland. However, in female reproductive organs (uterus and ovary), there was a significant reduction of relative organ weight of rats treated with 500 and 1000 mg/kg body weight of *S. guineense* extract compared to that in the control group.

Table 25: Relative organ weight of parental rats treated with 70% ethanol extract of *Syzygium guineense* leaf

Organ weight (g)		Group			
		Group I (250 mg/kg)	Group II (500 mg/kg)	Group III (1000 mg/kg)	Group IV (Control)
Testis		0.431±0.03	0.423±0.046	0.44±0.044	0.384±0.061
Epididymis		0.187±0.025	0.178±0.019	0.179±0.017	0.170±0.013
Seminal gland		0.327±0.182	0.282±0.022	0.318±0.039	0.36±0.077
Prostate gland		0.354±0.082	0.3±0.62	0.259±0.032	0.263±0.055
Uterus		0.173±0.083	0.145±0.035*	0.123±0.045*	0.257±0.103
Ovary		0.024±0.005	0.018±0.002*	0.018±0.003*	0.025±0.004
Male	Liver	3.74±1.3	2.73±0.093	2.78±0.17	3.3±0.79
	Kidney	0.349±0.056	0.321±0.02	0.319±0.027	0.331±0.019
Female	Liver	3.39±0.482	3.5±0.39	3.64±0.52	3.72±0.36
	Kidney	0.354±0.017	0.344±0.028	0.344±0.027	0.383±0.051

- Results are expressed as mean ± standard deviation of mean,
- *Significantly different from control group (p-value was <0.05), One-Way ANOVA.

5.2.3.9 Histopathological Findings in Reproductive Organs

Light microscopic examination of Hematoxylin and eosin (H & E) stained tissues of the testis, epididymis, prostate gland, and seminal glands were performed, and thus no morphological changes were observed in the treated and control rats. In the testis, the seminiferous tubules appeared to be normal in the outline and presentation of the cellular elements. Spermatogonia and Sertoli cells rested on the basal compartment of the seminiferous tubules. The primary spermatocytes were the largest cell in the spermatogenic lineage with rounded nuclei. The spermatids and the spermatozoa were placed towards the lumen of the seminiferous tubules (Figure 18).

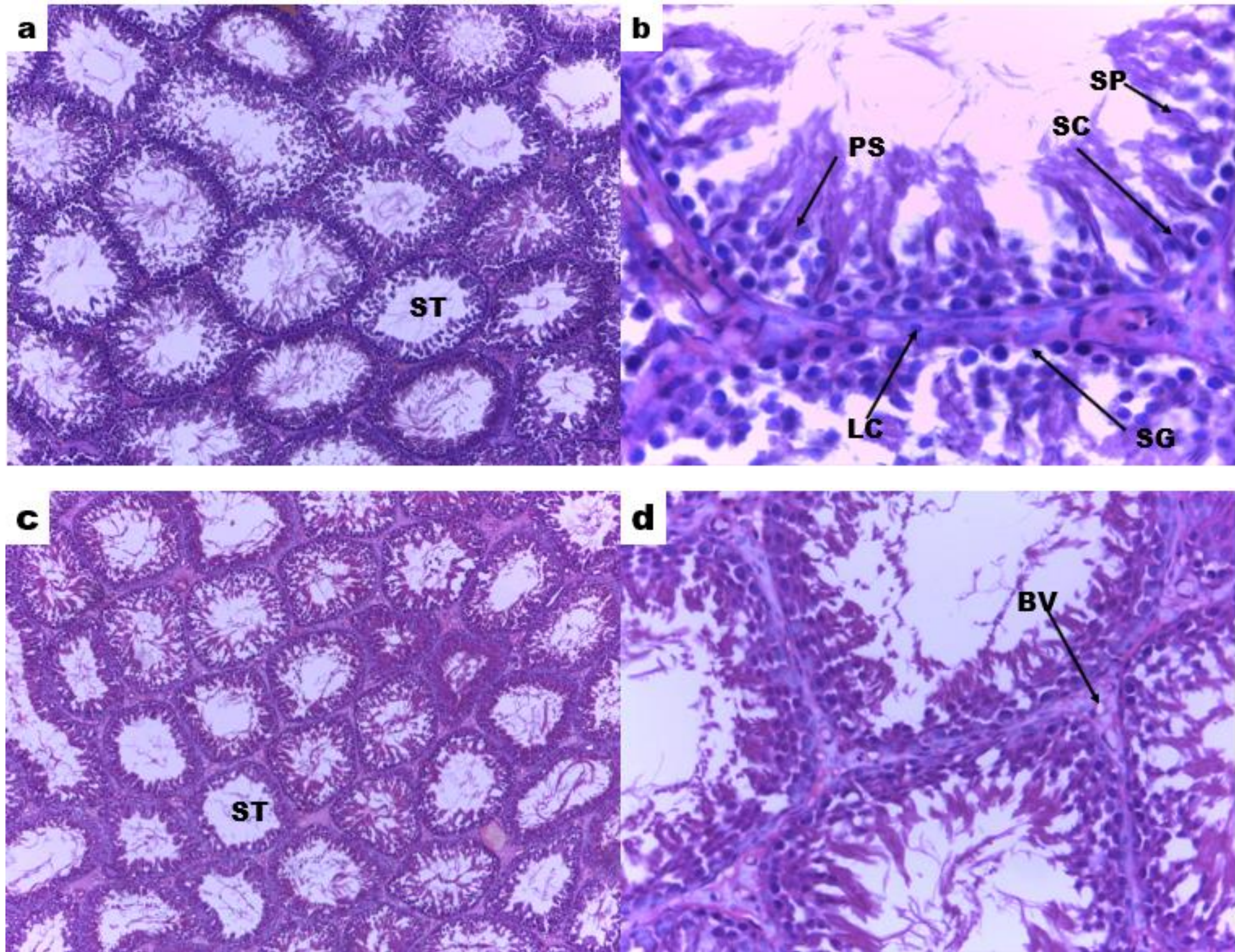


Figure 18: Photomicrograph of P-rat testis showing normal microscopic structures, (**a & b**) Sections taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf, (**c & d**) Sections taken from control group. **ST**: Seminiferous tubule, **SC**: Sertoli cell, **SG**: Spermatogonium, **PS**: Primary spermatocyte, **S**: Spermatid, **LC**: Interstitial cells of Leydig, and **BV**: Interstitial blood vessel; H and E stain, a & c 100x, b & d 400x total magnification.

The epididymis is an organ where post-testicular maturation and temporary storage of sperm occurs [103]. In the microscopic examination of the epididymis, normal epithelial lining and sperm cells in the lumen were observed (Figure 19).

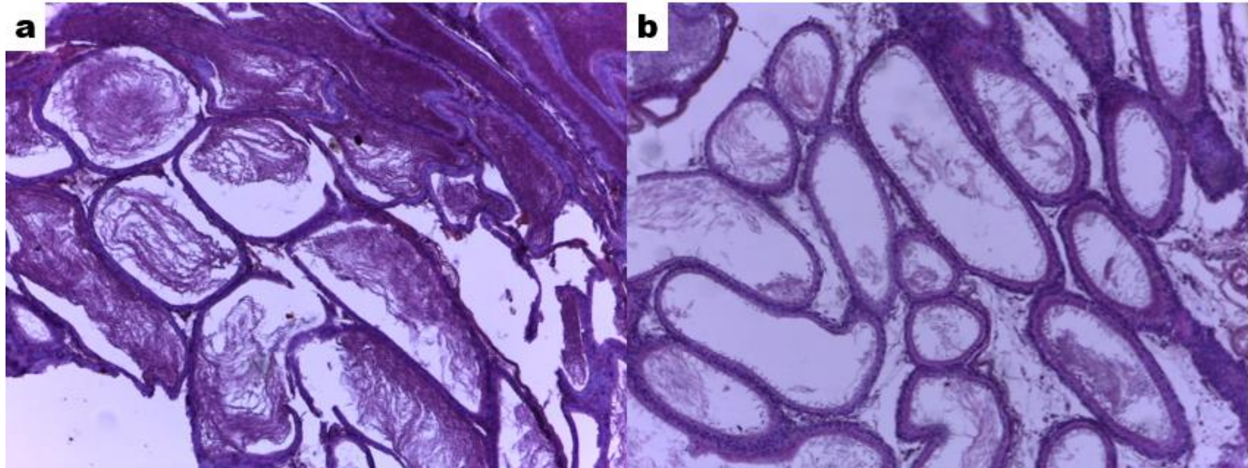


Figure 19: Photomicrograph of P-rat epididymis showing normal microscopic structures, (**a**) Section taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf, (**b**) Section taken from control group; H and E stain, 100x total magnification.

Examination of the prostate gland indicated that, the secretory portion, duct system, and fibromuscular stroma were not affected by the treatment of the plant extract. Similarly, there was no variation in seminal glands mucosa, which was highly folded, ducts and stromal components between the treated and control groups (Figures 20 & 21).

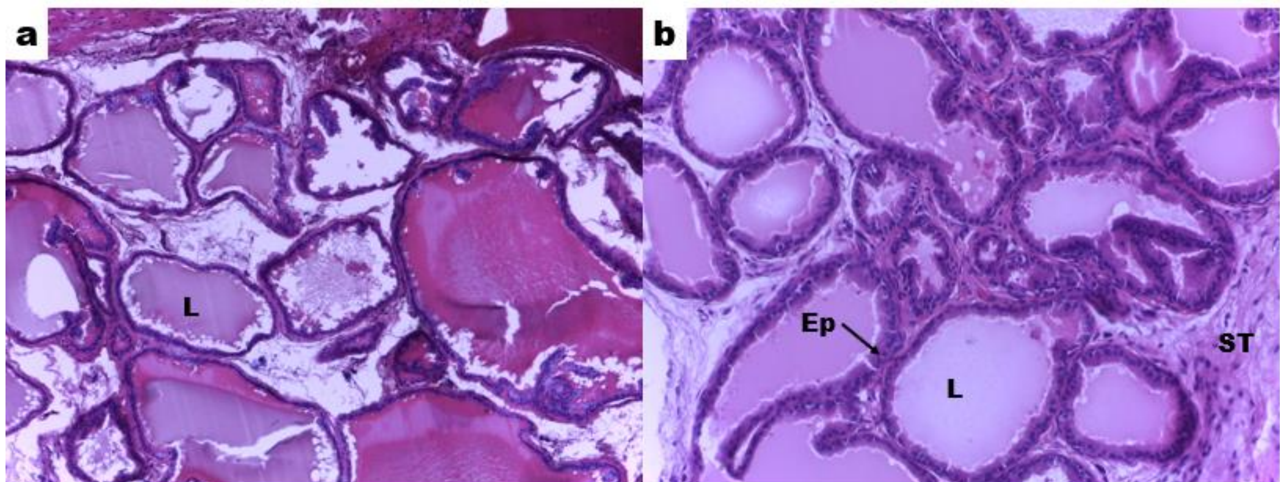


Figure 20: Photomicrograph of P-rat prostate showing normal secretory epithelium and stroma, (**a**) Section taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf, (**b**) Section taken from control group. **L**: Lumen, **Ep**: Epithelium, and **ST**: Stroma; H and E stain, (**a**) 100x, (**b**) 200x total magnification.

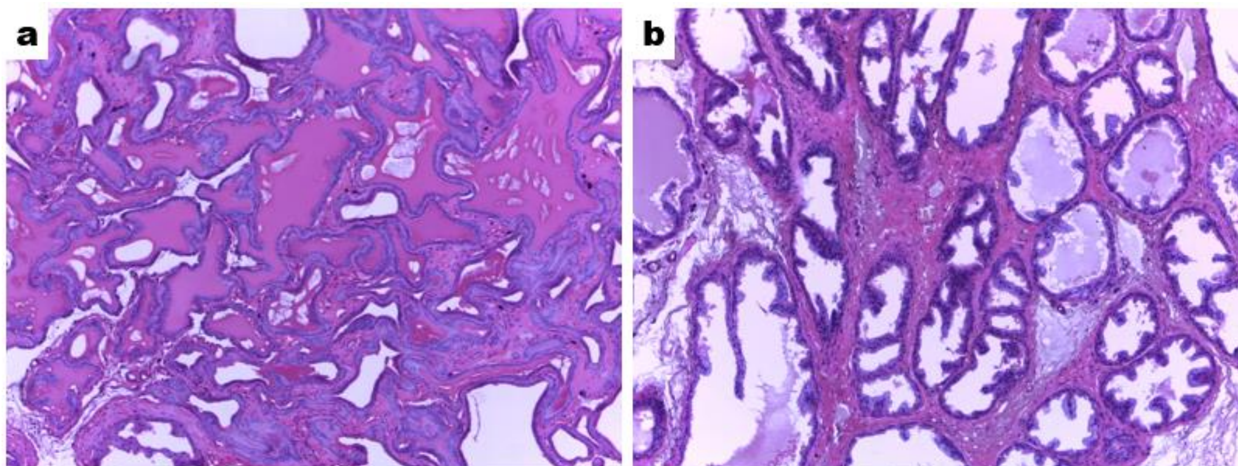


Figure 21: Photomicrograph of P-rat seminal gland showing normal secretory epithelium and stroma, (**a**) Section taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf, (**b**) Section taken from control group; H and E stain, 100x, total magnification.

Female reproductive organs, mainly ovaries, uterus, and vagina were microscopically examined following the routine tissue processing. The result of this examination showed that there were no significant microscopic changes in the above reproductive organs of the treated rats.

As presented in Figure 22, the ovaries appeared normal, containing ovarian follicles of different stages, corpus luteum, vascular stroma, and covering germinal epithelium. No follicular cyst, interstitial stromal cell hyperplasia/hypertrophy, or increased/decreased number of follicular cells were observed in the ovaries of the treated groups compared to the control group.

Moreover, in the uteri of the experimental and control group rats, the epithelial lining, endometrial glands, and the myometrium were not significantly affected by the treatment. Thus, no metaplastic change, hypertrophy, or hyperplasia of the epithelial lining, neutrophil infiltration in the endometrial stroma, cystic changes of endometrial glands were seen in any of the uteri from all groups (Figure 23).

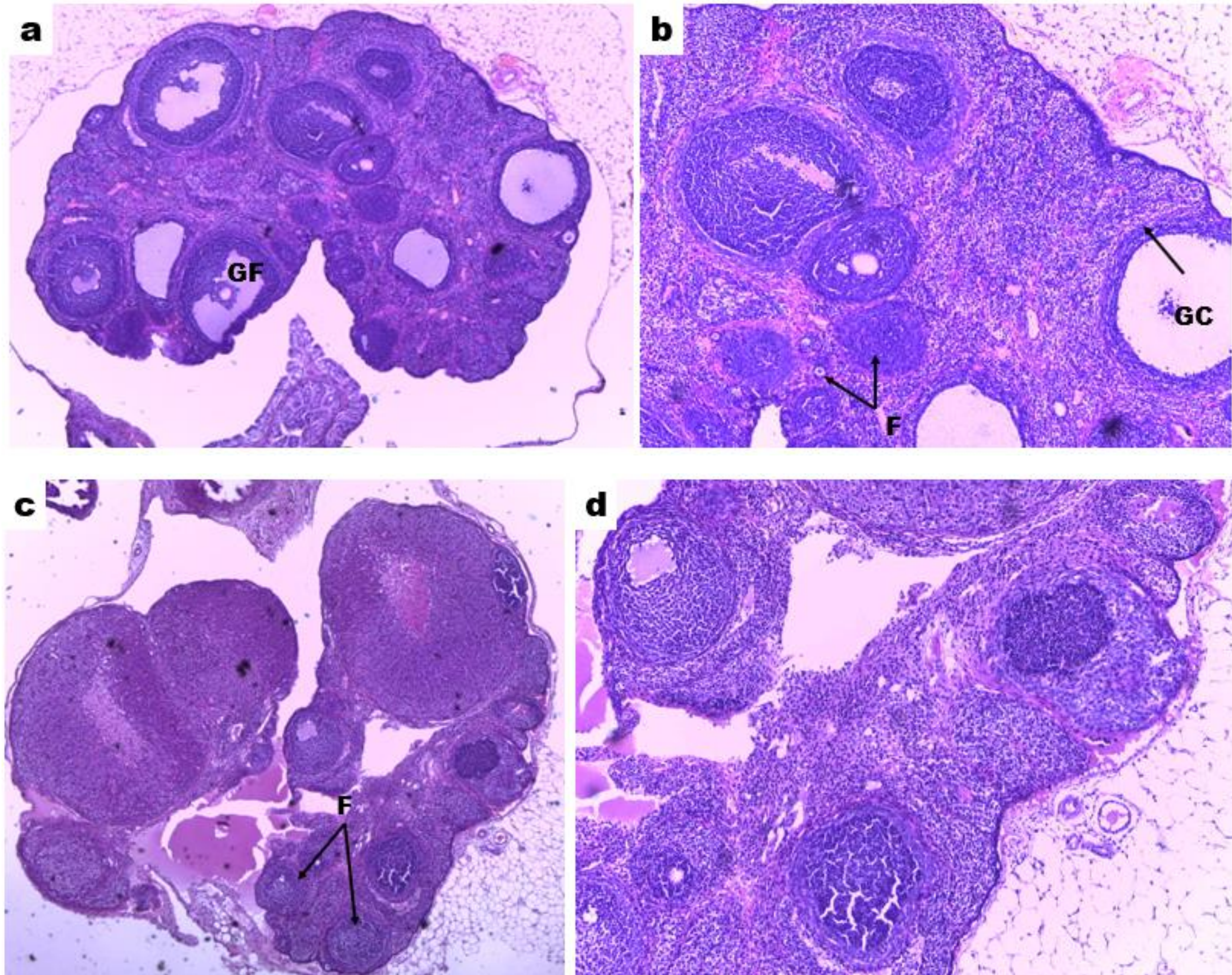


Figure 22: Photomicrograph of P-rat ovary showing normal ovarian cortex, (**a &b**) Sections taken from the rat treated with 1000 mg/kg of 70% ethanol extract of *Syzygium guineense* leaf, (**c & d**) Sections taken from control group. **F**: Different stage follicular cell, **GF**: Graafian follicle, **GC**: Granulosa cells; H and E stain, a & c 40x, b & d 100x total magnification.

In the microscopic examination of the vagina, only one, from twenty rats treated with 1000 mg/kg of the plant extract showed fibroma in the vagina (Figures 24 a & b). The rest of the rats in all groups revealed normal histological appearance of the vagina in both the epithelium and the other supporting tissues (Figures 24 c & d).

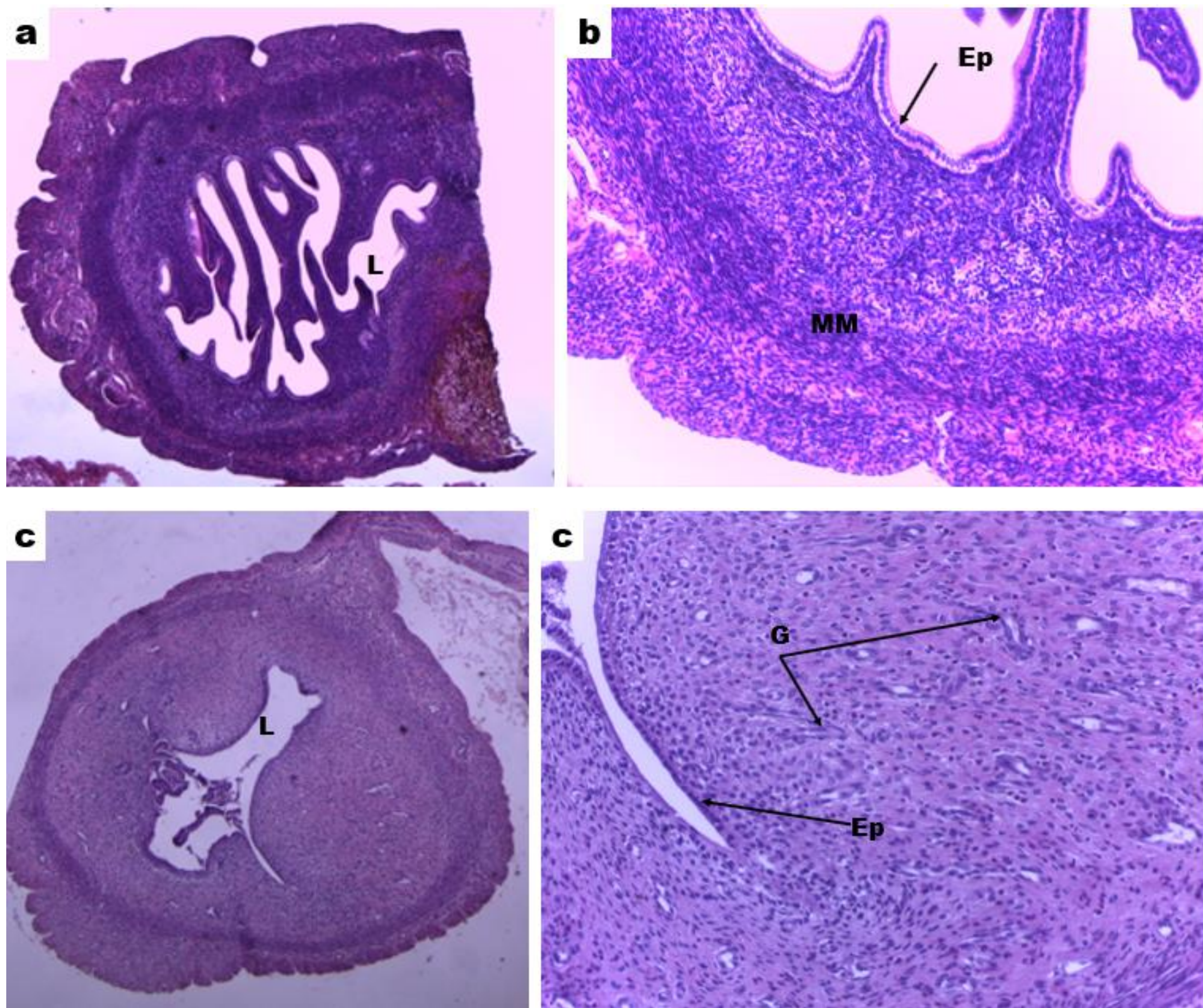


Figure 23: Photomicrograph of P-rat uterus showing normal uterine epithelium and musculature, (**a & b**) Sections taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf, (**c & d**) Sections taken from control group. **L**: Uterine lumen, **Ep**: Epithelium, **G**: Uterine glands, and **MM**: Muscle; H and E stain, a & c 40x, b & d 100x total magnification.

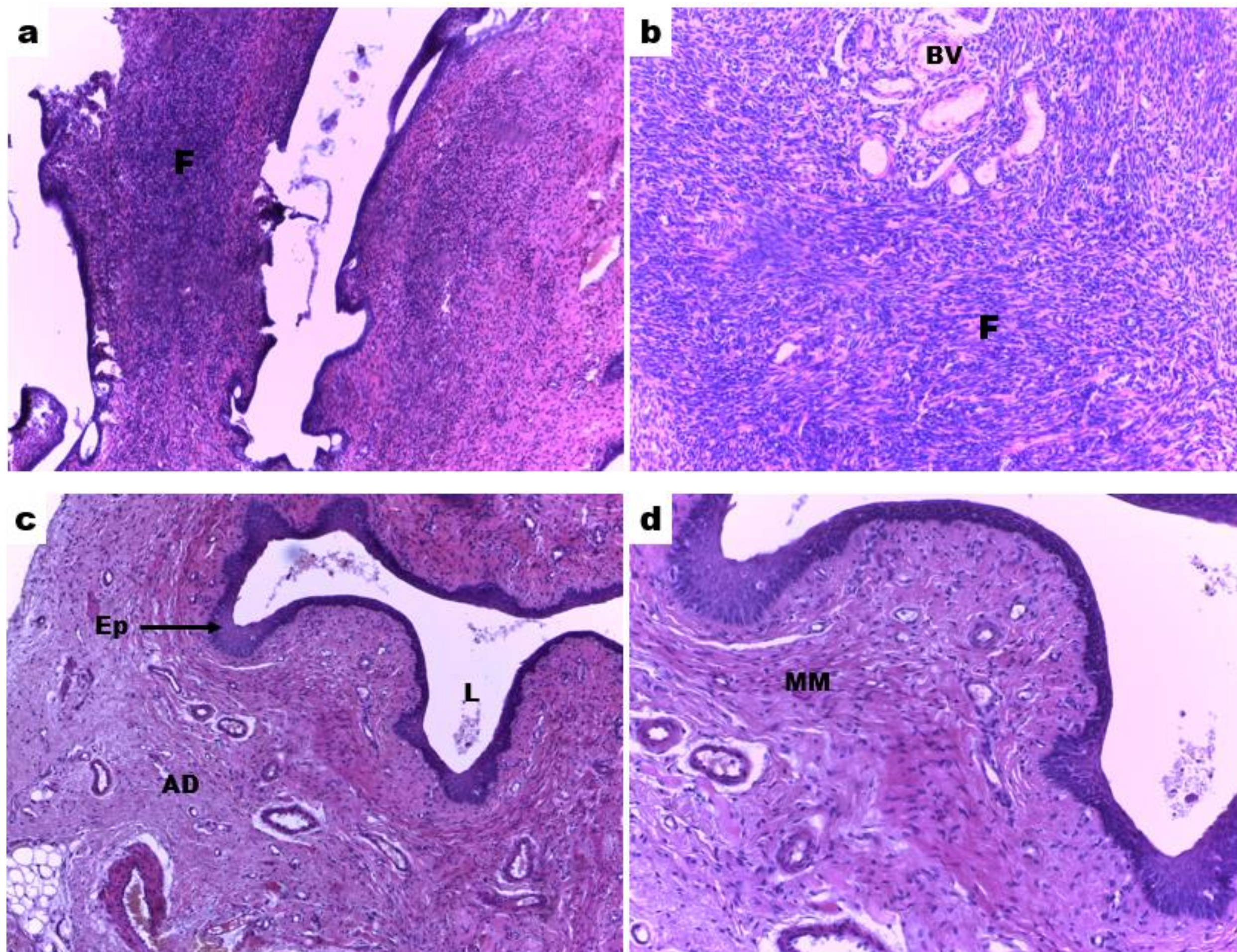


Figure 24: Photomicrograph of P-rat uterus, (**a & b**) Sections taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf, showing fibroma (**F**) in the vaginal musculature, (**c & d**) Sections taken from control group showing normal vaginal epithelium, musculature, and adventitia. **L**: Vaginal lumen, **Ep**: Epithelium, **BV**: Blood vessels, and **MM**: Muscle; H and E stain, a & c 100x, b & d 200x total magnification.

5.2.4 Hepato-renal Toxicity Assessment Results

Parallel to the reproductive toxicity assessment, parental rats were evaluated for the presence of hepato-renal toxicity. It was assessed by measuring biochemical parameters and conducting a gross and histopathological examination of the liver and kidneys. At necropsy, a macroscopic examination of the liver and kidneys was performed. No gross abnormalities in the color, texture, weight, size, and shape of the liver and kidneys were observed across the experimental groups (Figure 25).

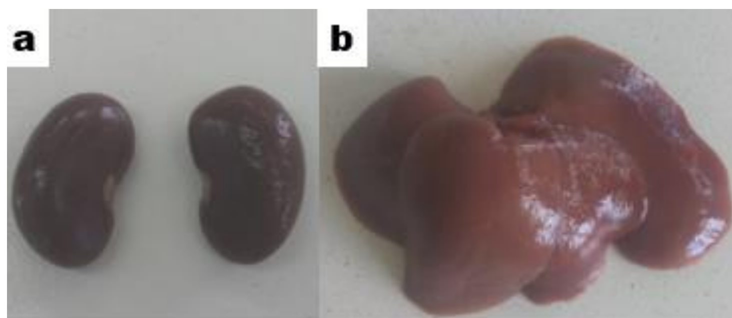


Figure 25: Photograph of rat kidneys (**a**) and liver (**b**) showing normal gross structures following administration of 70% ethanol extract of *Syzygium guineense* leaf (1000 mg/kg body weight).

5.2.4.1 Effects on the Biochemical Profile of the Parental Rats

The biochemical profile of the rats (clinical chemistry) is displayed in Tables 26 & 27. Male rats treated with a high dose *S. guineense* extract (1000 mg/kg) had significantly elevated levels of ALT (66±9.7) and AST (179.5±20.2) compared to those in the control group (ALT, 44.3±7.5 and AST, 132.8±22.7). AST was also significantly elevated compared to those in the rest of the groups. Serum levels of ALP in rats treated with 500 and 1000 mg/kg body weight of the extract were significantly higher compared to those in low dose treated as well as the control groups. In addition, the serum glucose level was reduced in rats treated with 1000 mg/kg body weight of the plant extract compared to that in the control group. However, there was no significant variation between the treatment and control groups in terms of the other liver and kidney function tests (Table 26).

In female rats, there was a significantly higher level of ALT (82.9±5.9) in rats treated with 1000 mg/kg body weight of the plant extract compared to that in the control group (58.5±16.8) as well as in the low dose (250 mg/kg) treated group. In addition, significantly higher levels of serum AST, ALP, and urea were detected in the middle and high dose treated groups compared to those in the control and low dose treated groups. The blood glucose level was significantly lower (87.0±29.0) in rats that received 1000 mg/kg of the test substance compared to the control group (129.0±25.0) (Table 27).

Table 26: Biochemical profile of P-male rats treated with 70% ethanol extract of *Syzygium guineense* leaf

Tests	Group			
	Group I 250 mg/kg	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV Control
ALT (U/L)	44.9±13.3	44.8±5	66±9.7*	44.3±7.5
AST (U/L)	124.3±12.9	113.5±9.4	179.5±20.2**	132.8±22.7
ALP (U/L)	70.3±1.53	103±7*!	100.3±6.5*!	72.3±2.08
Urea (mg/dL)	52.7±6.2	40.1±3.6	41.6±8.5	47.8±4.1
Creatinine (mg/dL)	0.447±0.04	0.443±0.03	0.403±0.07	0.41±0.00
Albumin (g/dL)	3.98±0.45	4.14±0.12	4.14±0.21	4.22±0.11
Total protein (g/dL)	6.09±0.56	6±0.17	6.28±0.19	6.12±0.43
Total cholesterol (mg/dL)	42.8±2.43	36.6±2.1	42.9±2.6	36.8±1.7
Glucose (mg/dL)	101.8±10.5	91.5±14.8	88.8±12.6*	125.6±12.8

- Results are expressed mean ± standard deviation of mean,
- * Significantly different from control group (Dunnett test),
- ** Significant difference with all the other groups,
- *! Significant difference with group I and IV,
- For all p-value was <0.05, One-Way ANOVA,
- ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase.

Table 27: Biochemical profile of P-female rats treated with 70% ethanol extract of *Syzygium guineense* leaf

Tests	Group			
	Group I 250 mg/kg	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV Control
ALT (U/L)	59.4±9.5	67±6	82.9±5.9**	58.5±16.8
AST (U/L)	164.1±9.9	190.1±2.2**	219±5.1*!	162.9±11.4
ALP (U/L)	114±3.5	130±8.5***	171±6.9*!	105±5
Urea (mg/dL)	55.1±3.3	80.3±7.8**	72.2±13*	53.9±7.6
Creatinine (mg/dL)	0.41±0.02	0.46±0.01	0.46±0.03	0.39±0.06
Albumin (g/dL)	3.9±0.2	3.5±0.2	3.6±0.2	4.0±0.2
Total protein (g/dL)	5.9±0.5	5.4±0.1	5.4±0.07	5.9±0.6
Total cholesterol (mg/dL)	66.8±4.5	67.4±6.8	67.5±3.7	85.8±27.4
Glucose (mg/dL)	107.5±8.8	98.2±10.2	87.0±29*	129±25

- Results are expressed as mean ± standard deviation of mean,
- *Significantly different from control group (Dunnett test),
- **Significant difference with group one and four,
- *! Significant difference with all the other groups,
- For all p-value was <0.05, One-Way ANOVA,
- ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase.

5.2.4.2 Effect on Histology of the Liver and Kidneys

Administration of ethanol leaf extract of *S. guineense* did not produce a significant change in the histology of the liver. Only some fatty changes were observed in the liver of one rat treated with 1000 mg/kg body weight of the plant extract (Figures 26 a & 26 b). In all the other groups, there were no structural alterations observed. Its microscopic structures: the portal triad, bile duct system, the hepatocytes, and the sinusoids appeared normal (Figures 26 c & 26 d). In addition, any

dose of the plant extract did not affect the histology of the kidney. The glomerular capillaries, the Bowman's capsule, the afferent and efferent arterioles, and the renal tubes did not show any structural alterations across all experimental groups (Figure 27).

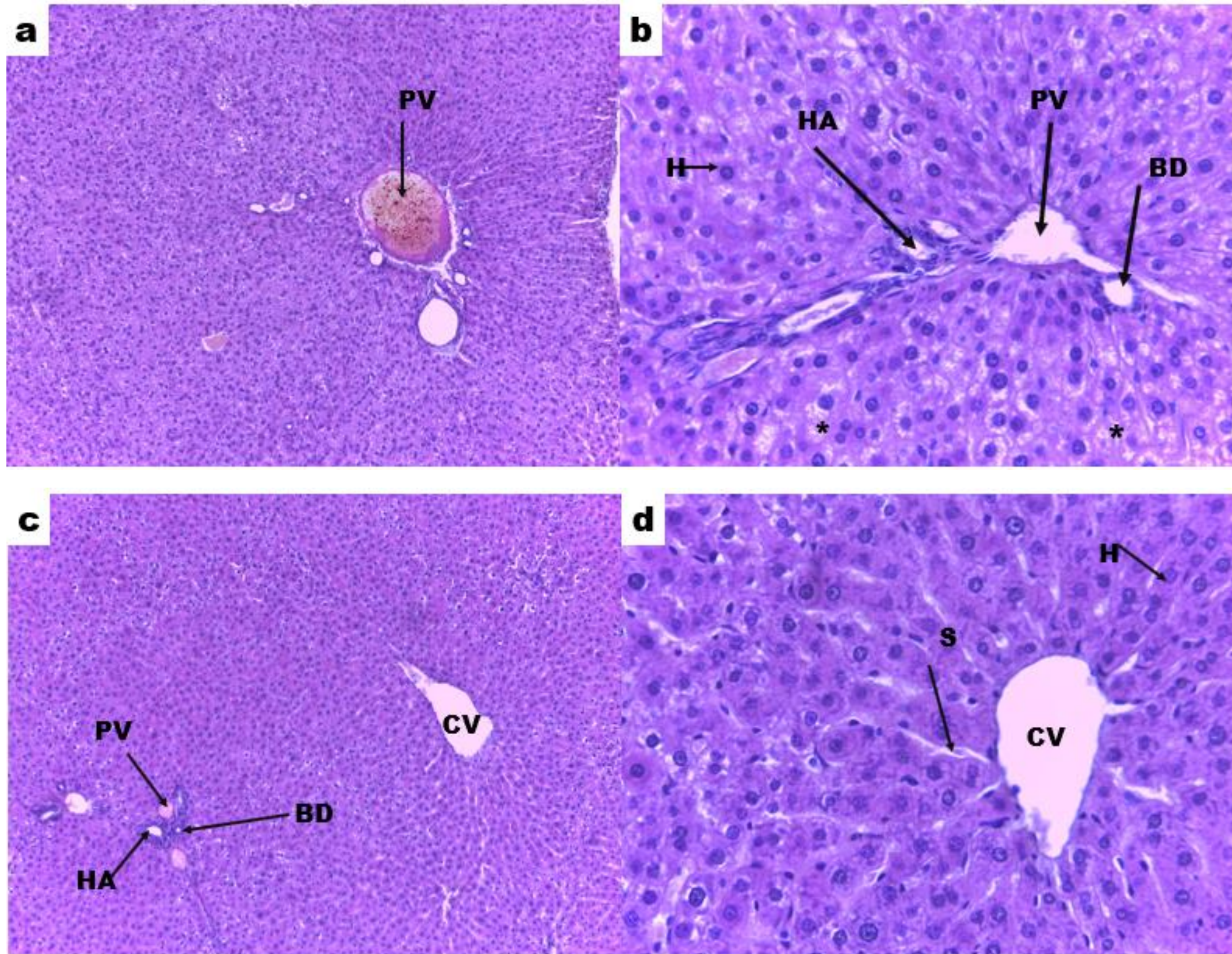


Figure 26: Photomicrograph of rat liver, **a & b** Sections taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf showing fatty change (*), **c & d** Sections taken from control group showing normal liver. **PV**: Portal vein, **HA**: Hepatic artery, **BD**: Bile duct, **CV**: Central vein, **H**: Hepatocyte, **S**; Sinusoid. H and E stain, 100x (a &c) and 400x (b & d) total magnification.

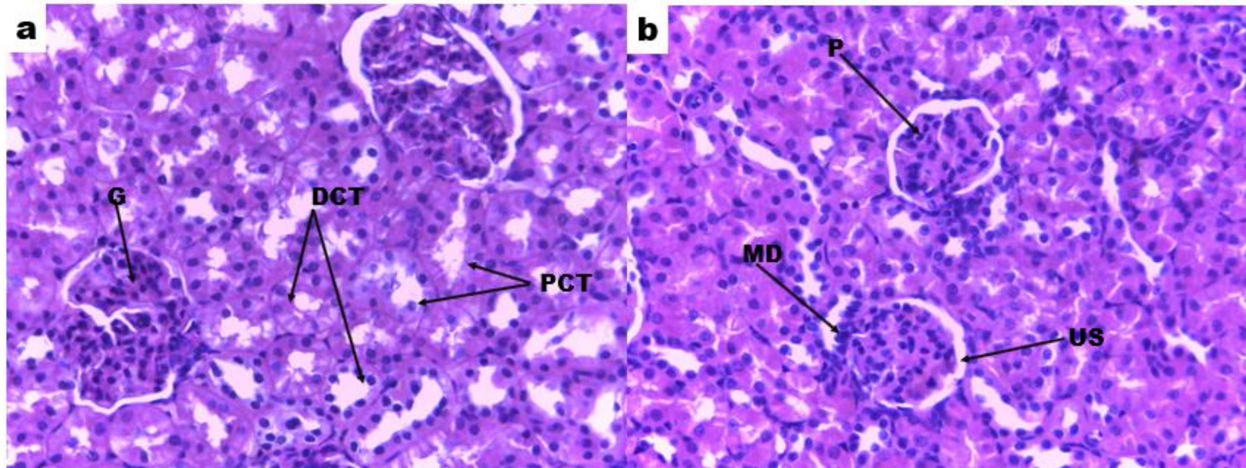


Figure 27: Photomicrograph of rat kidney showing normal microscopic structures, (**a**) Section taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf, (**b**) Section taken from control group. **G**: Glomerulus, **PCT**: Proximal convoluted tubule, **DCT**: Distal convoluted tubule, **MD**: macula densa cells, **P**: podocytes, **US**: Urinary space; H and E stain, 400x total magnification.

5.2.5 Effects on the Endocrine Endpoints

Gross examination results of the thyroid and adrenal glands did not show treatment related morphological changes (Figure 28).

Figure 28: Photograph of rat brain (**a**), adrenal gland (**b**), and thyroid gland (**c** arrow) showing normal gross structure following the administration of 70% ethanol extract of *Syzygium guineense* leaf (1000 mg/kg).

5.2.5.1 Effect on the Thyroid Hormone

Generally, compared to the control group, *S. guineense* treated rats showed decreased concentration serum of thyroid hormone (T4). The 500 and 1000 mg/kg doses treated male rats had significantly reduced level of T4 compared to the control group, p-value <0.05. Similarly, the female rats treated with 500 and 1000 mg/kg doses of the extract showed significant reduction of T4 hormone (Table 28).

Table 28: Thyroid hormone (T4) of P-rats treated with 70% ethanol extract of *Syzygium guineense* leaf

Test	Group			
	Group I 250 mg/kg	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV Control
Male T4 (µg/dl)	3.8±0.23	3.1±0.38*	3.41±0.46*	4.39±0.45
Female T4 (µg/dl)	4.1±0.4	3.1±0.7*	3.2±0.8*	4.6±0.7

Results are expressed as mean ± standard deviation of mean, *Significantly different from control group (p-value <0.05), One-Way ANOVA.

5.2.5.2 Histopathological Findings in Thyroid and Adrenal Glands

To evaluate the effect of the plant extract on the endocrine endpoints, thyroid and adrenal glands were processed for microscopic examination. This investigation did not find treatment related changes on the histology of thyroid and adrenal glands. In the thyroid gland, the spherical thyroid follicles were lined by simple cuboidal epithelium and filled with colloid (Figure 29). The adrenal glands showed well depicted adrenal cortex and medulla with their cellular components (Figure 30).

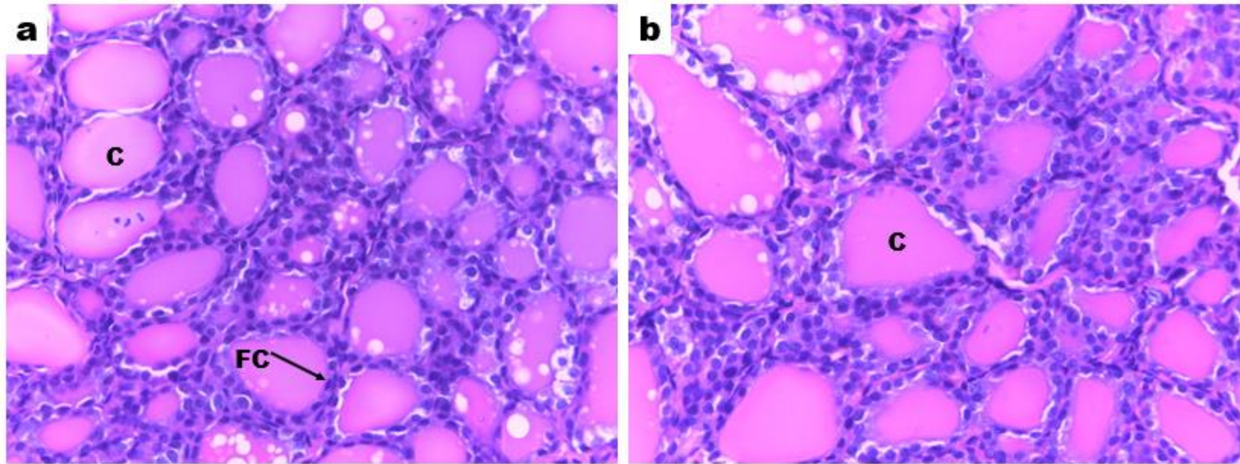
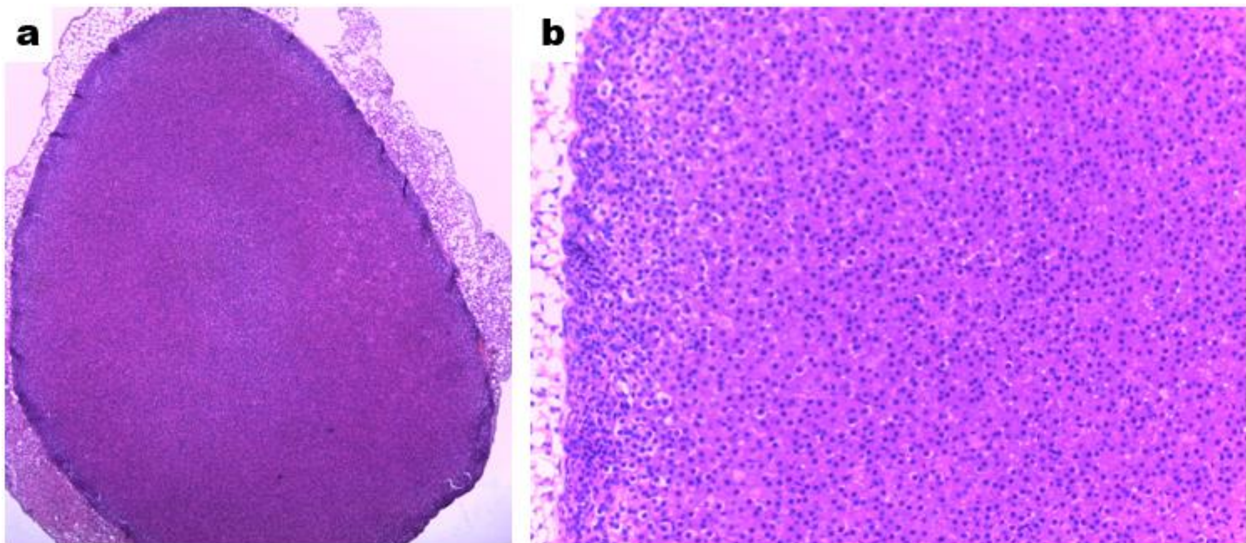


Figure 29: Photomicrograph of rat thyroid gland showing normal microscopic structures thyroid gland, (**a**) Section taken from rat treated with 1000 mg/kg of 70% ethanol extract of *Syzygium guineense* leaf, (**b**) Section taken from control group; **FC**: Follicular cell, **C**: Colloid; H and E stain, 400x total magnification.



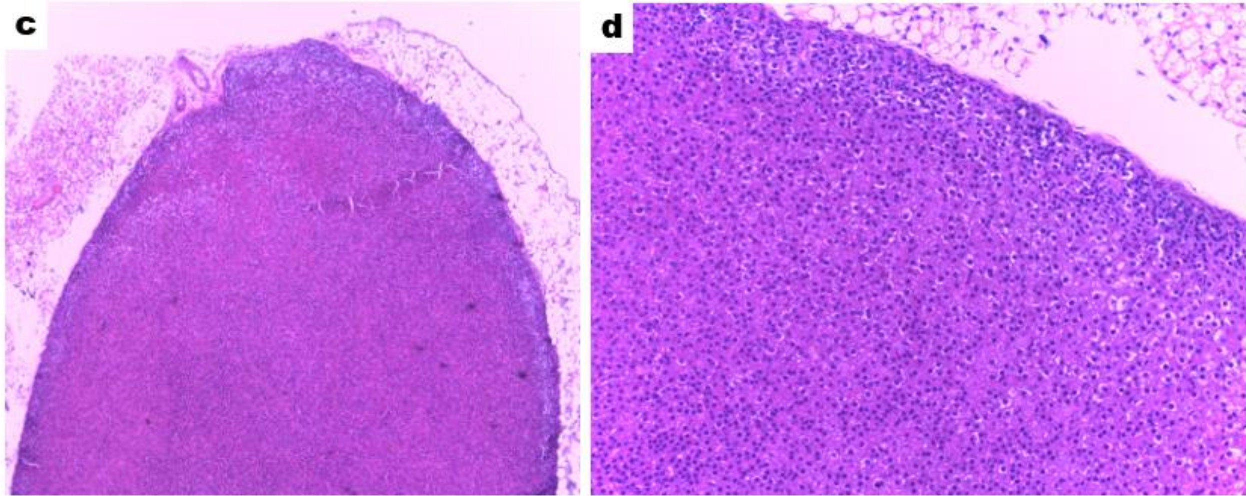


Figure 30: Photomicrograph of rat adrenal gland showing normal microscopic structure, (**a & b**) Sections taken from rat treated with 1000 mg/kg of 70% ethanol extract of *Syzygium guineense* leaf, (**c & d**) Sections taken from control group; H and E stain, 40x (a &c) and 200x (b & d) total magnification.

5.2.6 Effects on the First-Generation Offspring

5.2.6.1 The Results of F1 Set 1 Rats

5.2.6.1.1 Result of Weight Gain and Daily Food Intake

Generally, there was significant and dose dependent reduction in food consumption in both male and female F1 set-1 rats, p-value<0.05. Male and female rats treated with 500 mg/kg body weight of *S. guineense* extract had consumed significantly low amount of food compared to those in the control group. Similarly, significant food intake reduction was observed in male rats treated with 1000 mg/kg body weight of the plant extract compared with all the other groups. Female rats in the high dose treated group consumed significantly low amount of food compared with the control and low dose treated groups. Post weaning weight gain (weight at termination minus weight at PND 21) in all doses treated F1 set-1 female rats showed statistically significant reduction compared with the control group. Male rats treated with high dose of the test substance had reduced weight gain compared with those in the control group (Table 29).

Table 29: Weight gain and food intake of F1 set-1 rats treated with 70% ethanol extract of *Syzygium guineense* leaf

		Group			
		One (250 mg/kg)	Two (500 mg/kg)	Three (1000 mg/kg)	Four (Control)
Food intake	Male	361.2±29.5	314±16.5*	276±20.4**	402.3±45.7
n=20	Female	347.5±29.8	316.7±12.7*	242.3±13.9*-	379.7±22.2
Weight gain	Male	120.6±7.2	119.7±4.3	111.5±12.5*	131.6±8.8
(g)	Female	111.8±13.7	107.9±11.1	99.2±20.9	130.2±5.1*-

- Results are expressed as mean ± standard deviation of mean,
- *Significant difference with control group,
- **Significant difference with group I and IV,
- *- Significant difference with all the other groups,
- For all p-value was <0.05, One-Way ANOVA.

5.2.6.1.2 Effects on Preputial Separation and Vaginal Opening

The time of preputial separation (the day in which the prepuce was completely retracted from the glans penis and folded beneath the glans) (Figure 31) did not show significant treatment related delay. The body weight at preputial separation indicated non-significant variation between the treatment and control groups (Table 30).

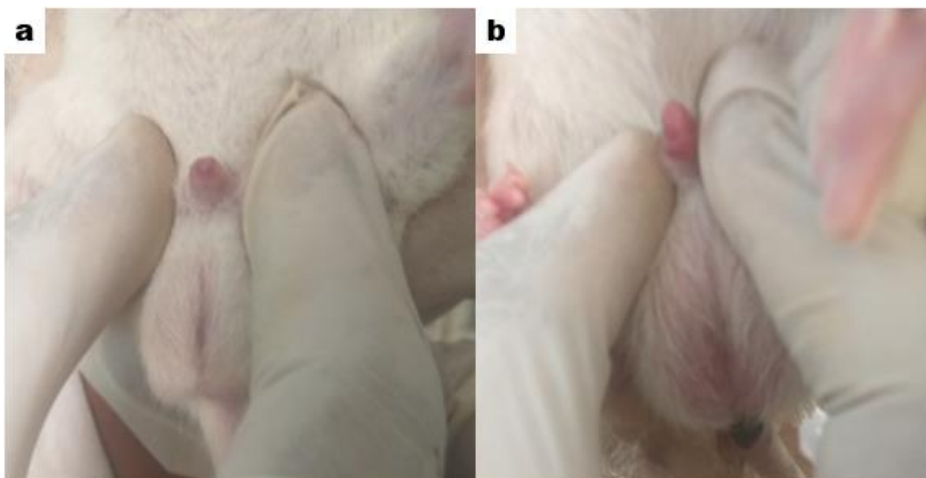


Figure 31: Penis of F1 rats before (**a**) and after (**b**) preputial separation following administration of 70% ethanol extract of *Syzygium guineense* leaf (1000 mg/kg). Preputial separation (the prepuce was completely retracted from the glans penis and folded beneath the glans).

Regarding the day of vaginal opening (when the membranous sheath covering the vaginal orifice was completely broken) (Figure 32), no significant variation was detected between the treatment and control groups. The weight at vaginal opening did not show significant difference between the treatment and control groups (Table 30).

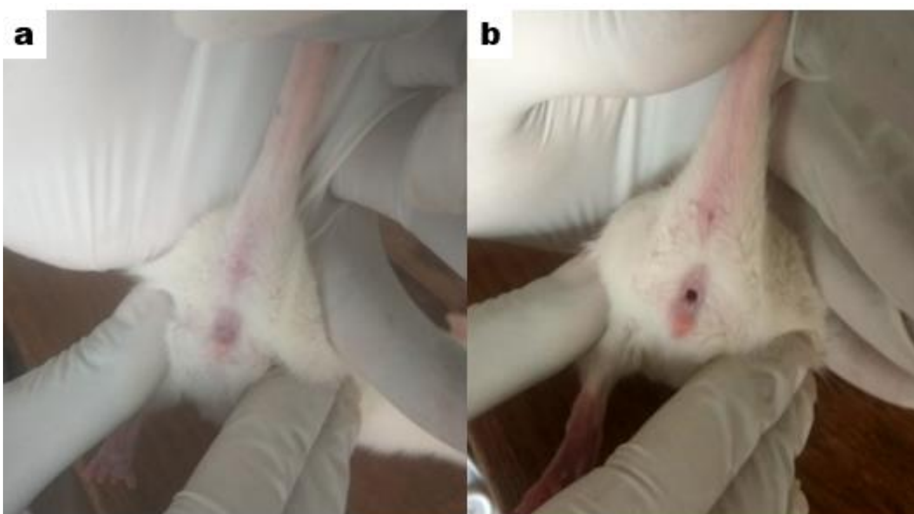


Figure 32: The vagina of F 1 rats before (**a**) and after (**b**) opening following administration of 70% ethanol extract of *Syzygium guineense* leaf (1000 mg/kg). The vagina was considered open when the membranous sheath covering the vaginal orifice was completely broken.

Table 30: Preputial separation/vaginal opening of F1 set-1 rats treated with 70% ethanol extract of *Syzygium guineense* leaf

	Group			
	One (250 mg/kg)	Two (500 mg/kg)	Three (1000 mg/kg)	Four (Control)
Preputial separation days/rats	47±1	49.8±3.5	49±2.7	47.8±3.3
Weight at preputial separation (g)/rats	133.9±13	125.4±12.2	132.1±8.6	131.4±16.3
Vaginal opening days/rats	37.8±3.2	39.9±3	40.4±3.2	38.1±5.8
Weight at vaginal opening (g)/rats	130.8±7.7	118.8±23.2	124.7±9.8	131.9±4.9

Results are expressed as mean ± standard deviation of mean, One-Way ANOVA.

5.2.6.1.3 Effects on Estrous Cycle of F1 Set One Rats

The length of estrous cycle in F1 rats was measured from the day of vaginal opening to the end of the treatment period (PND 70). Significantly longer duration of estrous cycle was observed in high dose treated rats compared to those in the control group. The effect of the plant extract on the first appeared cornified vaginal epithelium (estrus phase of the estrous cycle which is the period of ovulation) following complete vaginal opening was recorded. Even though it was not statistically significant, rats that received 1000 mg/kg body weight of the plant extract took a longer period to show the first cornified epithelium in the vaginal smear (Table 31).

Table 31: Length of estrous cycle and day of first appeared cornified vaginal epithelium following vaginal opening in F1 set-1 rats treated with 70% ethanol extract of *Syzygium guineense* leaf

	Group			
	Group I 250 mg/kg	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV Control
Duration of estrus cycle (days/rats)	4.8±0.84	4.6±0.55	5.4±0.55*	4.2±0.45
Day of vaginal epithelial cornification following VO (days/rats)	1.4±0.55	1.8±0.84	2.4±0.89	1.2±0.45

Results are expressed as mean ± standard deviation of mean, *Significant difference with control group, One-Way ANOVA; VO: vaginal opening.

5.2.6.1.4 The Result of Organ Weight (Reproductive Organ) of F1 Set-1 Rats

The relative organ weight of male and female rats at necropsy is presented in Table 32. Significantly decreased relative weight of uterus was observed in rats treated with 1000 mg/kg body weight of the plant extract compared with the control group. The weight of the ovaries in the high dose treated group also was significantly reduced compared to the low dose treated and control groups.

The relative weight of testis, epididymis, and prostate gland (whole) did not show significant variation between treatment and control groups. However, the weight of seminal gland of rats in the middle and high dose treated groups was significantly reduced compared to the control group, p-value <0.05.

Table 32: Relative reproductive organ weight of F1 set-1 rats treated with 70% ethanol extract of *Syzygium guineense* leaf

Relative organ weight (g)	Group			
	One (250 mg/kg)	Two (500 mg/kg)	Three (1000 mg/kg)	Four (Control)
Uterus	0.22±0.13	0.125±0.089	0.109±0.022*	0.244±0.079
Ovary	0.025±0.009	0.015±0.004	0.012±0.004**	0.021±0.004
Testis	0.529±0.095	0.54±0.19	0.456±0.21	0.546±0.11
Epididymis	0.141±0.027	0.124±0.038	0.106±0.034	0.159±0.017
Prostate	0.207±0.0.033	0.185±0.083	0.175±0.038	0.249±0.053
Seminal gland	0.165±0.061	0.132±0.066*	0.138±0.077*	0.244±0.066

- Results are expressed as mean ± standard deviation of mean,
- *Significant difference with control group,
- **Significant difference with group one and four,
- For both p-value was <0.05, One-Way ANOVA.

5.2.6.1.5 Results of Hepato-renal and Endocrine Toxicity in F1 Set-1 Rats

5.2.6.1.5.1 Organ Weight of F1 Set-1 Rats

There were no significant treatment-related changes observed in the gross examination and relative weight of the liver, kidneys, and adrenal glands (Table 33).

Table 33: Relative organ weight of F1 set-1 rats treated with 70% ethanol extract of *Syzygium guineense* leaf

Relative organ weight (g)		Group			
		One (250 mg/kg)	Two (500 mg/kg)	Three (1000 mg/kg)	Four (Control)
Female	Adrenal gland	0.019±0.002	0.016±0.004	0.014±0.005	0.02±0.005
	Liver	3.84±0.035	3.45±0.38	3.7±0.47	4.24±0.45
	Kidney	0.359±0.02	0.322±0.023	0.341±0.051	0.385±0.046
Male	Adrenal gland	0.015±0.002	0.025±0.024	0.013±0.003	0.011±0.003
	Liver	3.28±0.22	3.71±0.43	3.62±0.63	3.66±0.16
	Kidney	0.346±0.011	0.338±0.02	0.33±0.029	0.35±0.01

Results are expressed as mean ± standard deviation of mean, One-Way ANOVA.

5.2.6.1.5.2 Clinical Chemistry and Thyroid Hormone Assessment Results of F1 Set 1 Rats

In male rats, from the liver function tests, serum ALT level was significantly higher in rats treated with 1000 mg/kg body weight, while serum AST and ALP levels were significantly increased in rats treated with 500 and 1000 mg/kg body weight of the extract. Moreover, there was a significant decrement of thyroid hormone (T3) in high dose treated rats compared to the control one (Table 34).

Female rats treated with middle and high doses of the extract showed increased serum AST and ALP levels. The serum glucose level was reduced by the treatment of 500 and 1000 mg/kg body weight of the plant extract, compared to the control group. In addition, the serum level of thyroid hormone was significantly lowered by treatment with high dose of *S. guineense* extract. The other liver and kidney function tests did not show significant difference between the treatment and control groups. The results are presented in Table 35.

Table 34: Clinical chemistry and thyroid hormone analysis result of F1 Set-1 male rats treated with 70% ethanol extract of *Syzygium guineense* leaf

Tests	Group			
	Group I 250 mg/kg	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV Control
ALT (U/L)	58.9±6.7	67.2±4.6	78.6±2.8*	53.3±6.5
AST (U/L)	213.2±7.0	221.8±9.6**	248.8±9.2***	199.1±2.7
ALP (U/L)	177.7±14.2	241.3±12.6*	250.3±6.5*	200.3±3.5
Urea (mg/dL)	39.2±8	52.6±9.5	52.7±9.4	49.9±6.1
Creatinine (mg/dL)	0.297±0.021	0.32±0.026	0.31±0.02	0.323±0.03
Albumin (g/dL)	4.1±0.062	4.13±0.172	4.17±0.122	3.96±0.081
Total protein (g/dL)	6.02±0.12	6.35±0.07	6.35±0.42	5.75±0.14
Total cholesterol (mg/dL)	55.37±5.89	56.97±3.88	55.83±4.52	48.43±3.21
Glucose (mg/dL)	79.63±17.05	76.93±41.05	69.43±13.84	79.77±13.7
T3 (µg/dL)	1.01±0.097	0.985±0.092	0.832±0.056**	1.127±0.04

- Results are expressed as mean ± standard deviation of mean,
- *Significant difference with group I and IV,
- **Significant difference with control group,
- ***Significant difference with all the other groups,
- For all p-value <0.05, One-Way ANOVA.

Table 35: Clinical chemistry and thyroid hormone analysis result of F1 Set-1 female rats treated with ethanol extract of *Syzygium guineense* leaf

Tests	Group			
	Group I 250 mg/kg	Group II 500 mg/kg	Group III 1000 mg/kg	Group IV Control
ALT (U/L)	54.6±6.05	57.2±11.92	60.6±8.93	53.7±9.12
AST (U/L)	183.57±5.15	242.4±9.64*	231.67±10.65*	188.97±6.65
ALP (U/L)	149.33±6.03	190.33±4.51*	208.33±6.66*	147.67±7.01
Urea (mg/dL)	36.13±5	45.5±8.7	49.5±15.48	55.2±8.1
Creatinine (mg/dL)	0.307±0.029	0.337±0.035	0.313±0.012	0.31±0.026
Albumin (g/dL)	4.08±0.19	3.94±0.032	3.87±0.12	3.99±0.061
Total protein (g/dL)	6.44±0.52	5.97±0.23	5.68±0.14	5.71±0.13
Total cholesterol (mg/dL)	62.9±4.4	57.37±6.29	61.23±0.93	66.73±3.51
Glucose (mg/dL)	102.6±13.46	84.43±8.86**	80.07±8.38**	116.63±14.63
T3 (μg/dL)	0.987±0.093	0.97±0.142	0.914±0.047**	1.17±0.102

- Results are expressed as mean ± standard deviation of mean,
- *Significant difference with group I and IV,
- ** Significant difference with control group,
- For all p-value <0.05, One-Way ANOVA.

5.2.6.1.5.3 Effects on the Histopathology of Organs in F1 Set-1 Rats

In F1 set-one rats, microscopic examinations of reproductive organs, (uterus, ovary, vagina, testis, epididymis, prostate, and seminal gland), liver, kidney, thyroid, and adrenal glands were performed. However, no significant microscopic alteration in the structure of the aforementioned organs was observed.

5.2.6.2 Developmental Neurotoxicity Results

5.2.6.2.1 Daily Food Intake and Post-Weaning Weight Gain of F1 Set-2 Rats

There was a significant reduction in food intake in both male and female F1 set-2 rats treated with 500 and 1000 mg/kg body weight of ethanol leaf extract of *S. guineense* compared with the low dose treated and control groups. Post-weaning weight gain did not show a significant variation among the four groups (Table 36).

Table 36: Weight gain and food intake of F1 Set-2 rats treated with 70% ethanol extract of *Syzygium guineense* leaf

		Group			
		One (250 mg/kg)	Two (500 mg/kg)	Three (1000 mg/kg)	Four (Control)
Food intake (g) n=10	Male	217.7±39.8*	142.3±6.4	135±6.2	189.5±39.8*
	Female	169.3±14.9*	144.5±7.1	137.2±2.7	182.8±15.2*
Weight gain (g)	Male	106.2±21.2	105.8±19	103.8±13.8	116.6±20.7
	Female	110.4±18.5	102.8±9.6	101.3±7.7	107.8±19.1

Results are expressed as mean ± standard deviation of mean; *Significant difference with group two and three (p-value was <0.05), One-Way ANOVA.

5.2.6.2.2 Effects on the Gross Examination Results and Weight of the Brain

The gross morphological observation conducted on different regions of the brain did not reveal any pathological alterations (Figure 28). The relative brain weight is presented in Table 37. There was no significant change in the relative brain weight observed between treatment and control groups.

Table 37: Relative brain weight of F1 Set-2 rats treated with 70% ethanol extract of *Syzygium guineense* leaf

Relative brain weight (g)	Group			
	One (250 mg/kg)	Two (500 mg/kg)	Three (1000 mg/kg)	Four (Control)
Male	1.272±0.212	1.284±0.111	1.292±0.053	1.156±0.154
Female	1.161±0.179	1.292±0.215	1.283±0.171	1.308±0.1

Results are expressed as mean ± standard deviation of mean, One-Way ANOVA.

5.2.6.2.3 Effects on the Histology of Brain and Spinal Cord

The histology of the central nervous system (brain and spinal cord) was investigated on PND 70 rats for possible developmental neurotoxic effects of the plant extract. For microscopy, tissue samples were taken from various regions of the brain namely: the cerebrum, cerebellum, brainstem, and diencephalon. The cervical, thoracic and lumbar regions of the spinal cord were also sampled. The microscopic examination did not reveal any abnormal findings related to the treatment either in the brain or the spinal cord. The H and E stain of the brain appeared normal cytoarchitecture of neurons, glial cells, and associated blood vessels (Figure 33). The arrangement of neuronal cell bodies in gray matter and the tracts (ascending and descending) in the white matter of the spinal cord of treated and control rats did not vary (Figure 34).

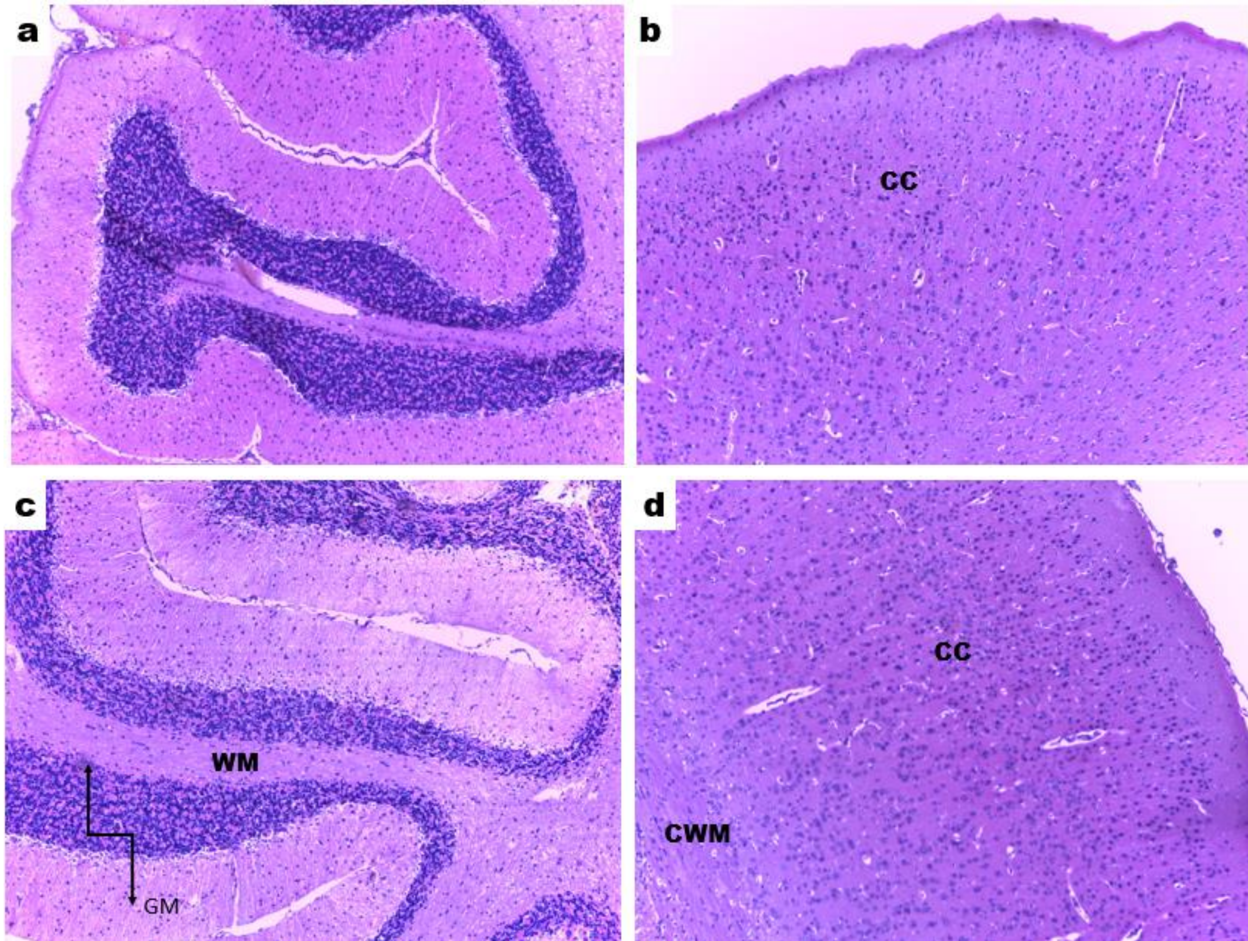


Figure 33: Photomicrograph of rat brain showing normal microscopic structure cerebrum and cerebellum, (**a & b**) Sections taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf, (**c & d**) Sections taken from control group. **CC**: Cerebral cortex, **CWM**: cerebral white matter, **GM**: Cerebellar gray matter, and **WM**: Cerebellar white matter; H and E stain, 100x total magnification.

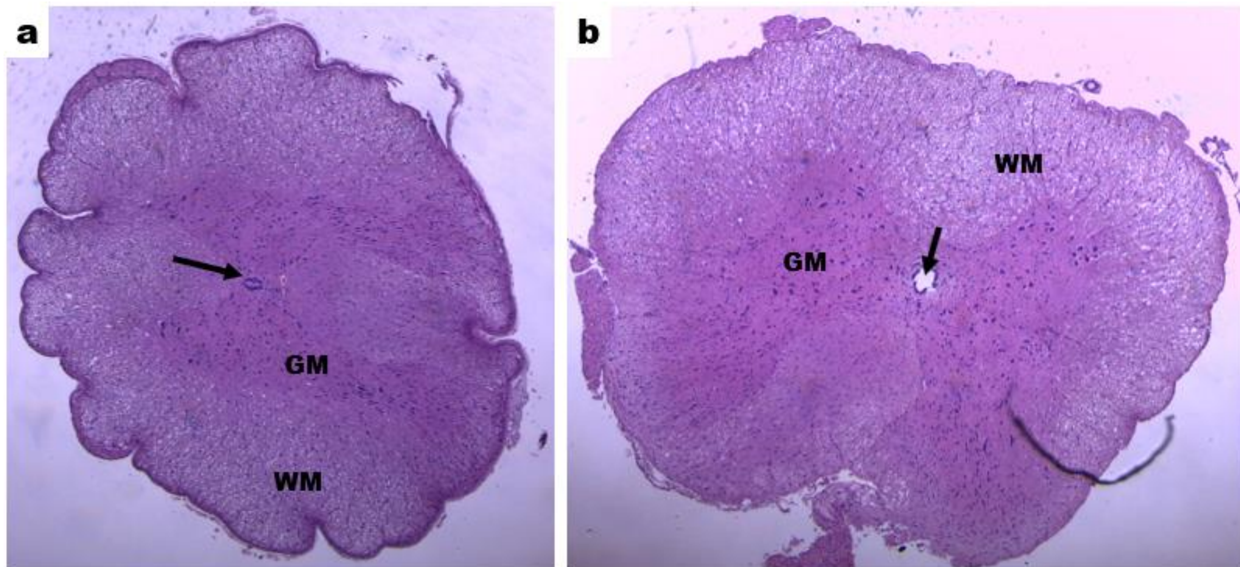


Figure 34: Photomicrograph of rat spinal cord showing normal central canal (arrow), gray matter (**GM**), and white matter (**WM**) of the spinal cord. (**a**) Section taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf, (**b**) Section taken from control group; H and E stain, 40x total magnification.

5.2.6.3 Developmental Immunotoxicity Study Results

5.2.6.3.1 Daily Food Intake and Weight Gain of F1 Set-3 Rats

Decreased food intake was observed across all treatment groups compared to the control one. Post-weaning weight gain of male rats in the high dose treated group was significantly reduced by the treatment of the test substance compared to the control group. Significant weight reduction in female rats treated with middle and high doses of the plant extract was recorded (Table 38).

Table 38: Weight gain and food intake of F1 Set-3 rats treated with 70% ethanol extract of *Syzygium guineense* leaf

		Group			
		One (250 mg/kg)	Two (500 mg/kg)	Three (1000 mg/kg)	Four (Control)
Food intake (g) n=10	Male	169.5±18.5	156±5.4	158.2±8.3	216.5±18.1*
	Female	141.5±11.9	141.8±6.2	144.5±7.1	161.7±17.4*
Weight gain (g)	Male	114.4±23.3	110.3±14.8	98±15.9**	114.1±22.2
	Female	111.9±17.5	94.6±14**	90.6±6.2***	128.5±6.6

- Results are expressed as mean ± standard deviation of mean,
- *Significant difference with all the other groups,
- **Significant difference with control group,
- ***Significant difference with group one and four,
- For all p-value <0.05, One-Way ANOVA.

5.2.6.3.2 Effects on the Gross Examination Result and Weight of Lymphoid Organs

On gross examination of the thymus, spleen, and lymph nodes, no pathological alterations were seen (Figure 35). Lymphoid organ weight of male and female F1 Set-3 rats is presented in Table 39. No significant difference was observed.

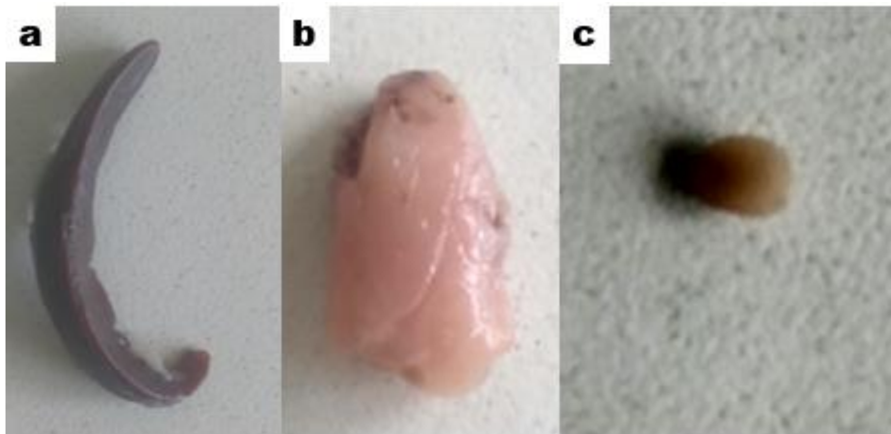


Figure 35: Photograph of rat spleen (**a**), thymus gland (**b**), and lymph node (**c**) showing normal gross structures following administration of 70% ethanol leaf extract of *Syzygium guineense* leaf (1000 mg/kg).

Table 39: Organ weight of F1 Set-3 rats treated with ethanol extract of *Syzygium guineense* leaf

Organ Weight (g)		Group			
		One (250 mg/kg)	Two (500 mg/kg)	Three (1000 mg/kg)	Four (Control)
Female	Thymus	0.385±0.077	0.326±0.033	0.3±0.033	0.36±0.041
	Spleen	0.667±0.439	0.736±0.097	0.762±0.383	0.473±0.043
	Lymph node	0.017±0.003	0.018±0.003	0.02±0.005	0.02±0.004
Male	Thymus	0.237±0.004	0.269±0.049	0.307±0.103	0.295±0.07
	Spleen	0.579±0.327	0.724±0.246	0.748±0.215	0.538±0.164
	Lymph node	0.016±0.001	0.017±0.004	0.015±0.002	0.014±0.003

Results are expressed as mean ± standard deviation of mean, One-Way ANOVA.

5.2.6.3.3 Effects on Histopathology of Lymphoid Organs

Histopathological examination in the lymphoid organs was conducted based on the criteria described by Elmore [112], as enhanced histopathology evaluation of lymphoid organs. In H & E stain of the thymus, the two morphologically distinct cortex and medulla were separated by a vascular corticomedullary zone. The outer cortex stained more densely and occupied predominantly by T-lymphocyte, sparse epithelial reticular cell, and some macrophage. The inner

medulla, which was pale staining, had more epithelial reticular cell, and fewer lymphocyte. No sign of immunotoxicity like, increased number of apoptotic, necrotized, pigmented, or inflamed cells were observed in either the cortex or medulla of the thymus (Figure 36).

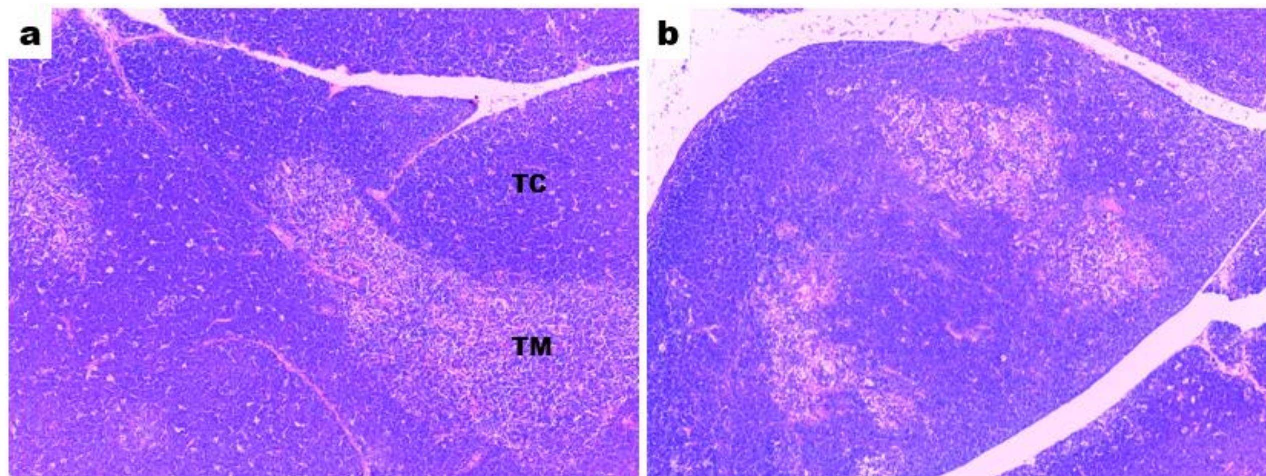


Figure 36: Photomicrograph of rat thymus gland showing normal thymic cortex (**TC**) and thymic medulla (**TM**). (**a**) Section taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf, (**b**) Section taken from control group; H and E stain, 100x total magnification.

The white and red pulp of the spleen appeared normal. The white pulp was occupied by periarterial lymphatic sheath (many T-cell), lymphoid nodules (B-cell reach region), and marginal zone (macrophage and B-cell rich area). The red pulp had splenic cord with many blood cells supported by reticular cells and splenic sinusoid lined by elongated endothelial cells. No indicator of toxicity such as, increased number of tingible body macrophages, apoptotic cell, granuloma/macrophage aggregation, pigmented macrophage, fibrosis, and necrosis were observed (Figure 37).

4

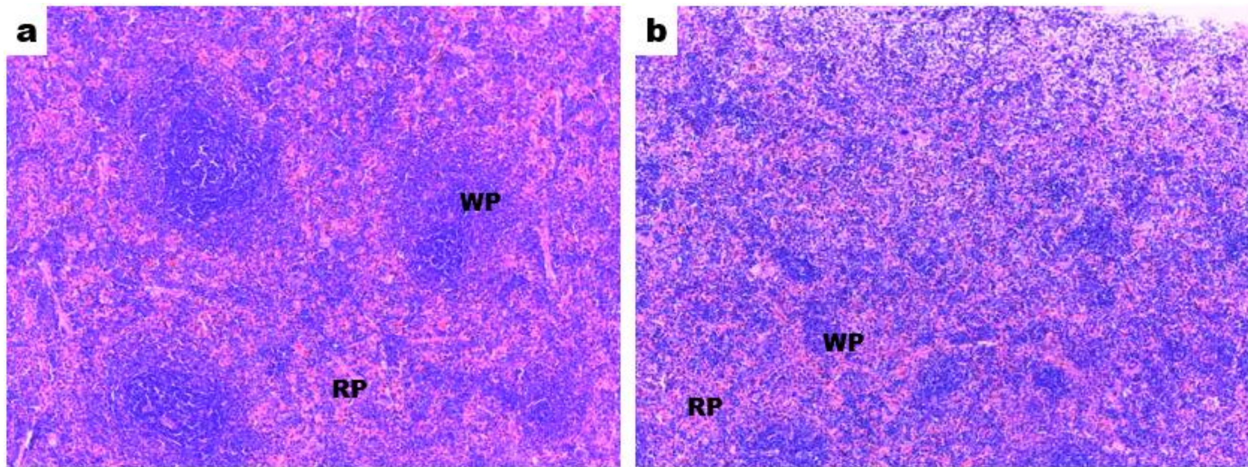


Figure 37: Photomicrograph of rat spleen showing normal white pulp (**WP**) and red pulp (**RP**), (**a**) Section taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium guineense* leaf, (**b**) Section taken from control group; H and E stain, 100x total magnification.

A representative sample of lymph nodes were taken from different regions: submandibular, cervical, and mesenteric regions. The cortex, paracortex, and medulla of the lymph nodes did not show abnormally increased lymphoid follicles, germinal centers, or cells (Figure 38).

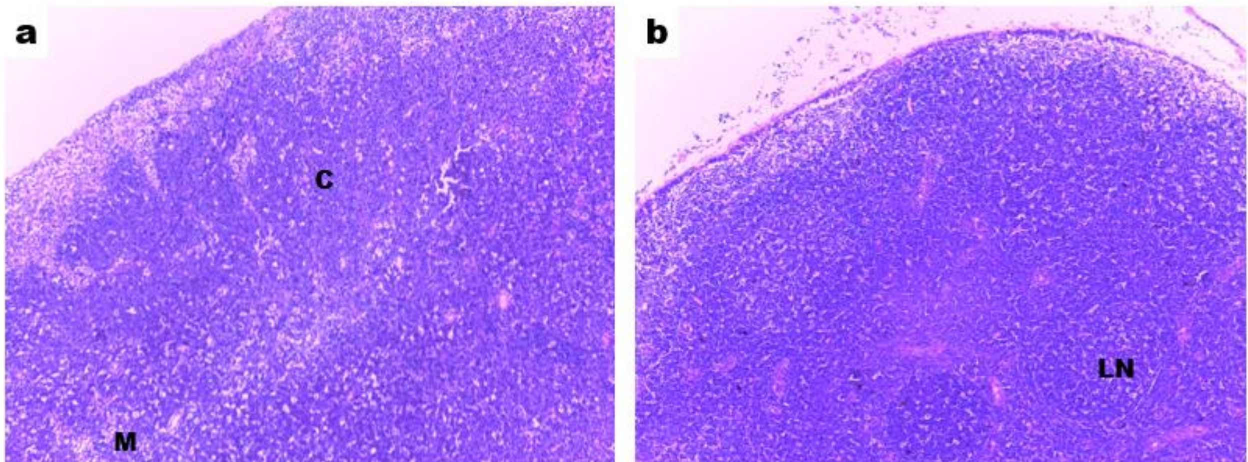


Figure 38: Photomicrograph of rat lymph node showing normal cortex (**C**) and medulla (**M**), (**a**) Section taken from rat treated with 1000 mg/kg body weight of 70% ethanol extract of *Syzygium 11guineense* leaf, (**b**) Section taken from control group. **LN**: Lymphoid nodule with germinative center; H and E stain, 100x total magnification.

6. Discussion

6.1 Potential Teratogenicity

Various plant products claimed to have therapeutic significance are obtaining acceptance in the global health market irrespective of their toxicity information [113]. On the other hand, several phytochemical constituents of medicinal plants may be potentially teratogenic. We commonly use these substances in our daily lives [114]. Toxic agents can directly or indirectly affect embryos/fetuses. When the toxic agents cross the placental membrane, they directly damage the developing embryonic/fetal tissue. Indirectly, toxicants damaging the placental tissue and compromise placental function might impede the development of embryos/fetuses [115]. There has been no published study conducted on the teratogenicity of *S. guineense*. The present study investigated the teratogenic potential of 70% ethanol leaf extract of *S. guineense* in rats. The time of extract administration was day 6-12 of gestation which is the critical period of organogenesis. The assessment of teratogenic potential of the plant extract was conducted involving two separate experiments: Day-12 and Day-20 experiments.

In the current teratogenicity study, administration of the plant extract to the pregnant rats during the crucial period of organogenesis resulted in reduced food intake and weight gain of the dams and CRL of 20 days old rat fetuses in the high dose group. It also reduced the CRL, number of somites, and overall morphological scores of 12 days old rat embryos.

Maternal weight gain during the treatment period (day 6-12 of pregnancy) was affected by the administration of the plant extract despite similar intake of food. It was evidenced by significant and dose dependent reduction in weight gain in the rats treated with higher doses of the test plant in day-12 and day-20 experiments. Reduction in weight gain was also reported by other researchers: Abba *et al* [52], Loha *et al* [54], and Amare [53], who conducted toxicity studies on non-pregnant rats and mice following administration of *S.guineense* extract. Moreover, the food intake of pregnant rats in the treatment group decreased in both the day-12 and day-20 experiments. The difference was statistically significant in day-20 experiment. A study conducted by Rogers and Kavlock [116] reported that decreased food intake can induce weight loss and other clinical signs. Therefore, this could be the reason for the decrease in weight gain during gestation.

In day-12 rat embryos, the circulatory system, musculoskeletal systems, and craniofacial developments were assessed. No significant developmental delays were observed across the various groups. Pregnancy outcomes also were not affected by treatment of rats with *S. guineense*. However, rats treated with 1000 mg/kg body weight of the test substance showed significant reduction in the number of somites compared with the *ad libitum* control group but not with the control group treated with distilled water. Rats in the *ad libitum* control group were untouched, unrestricted, and were not treated as well. Therefore, they were free from stress due to manipulations during intragastric administration of the extract or distilled water. Previous research results indicated that maternal stress can increase the level of stress hormone (cortisol), by which this hormone can pass the placental membrane and affect the development of the embryo [117, 118]. This may be the reason for the significant difference observed between the high dose treated group and *ad libitum* control but not with the control group.

Retarded fetal development *in vivo* is manifested by decreased fetal weight, placental weight, and CRL [119]. In the current experiment, treatment with 1000 mg/kg body weight of the plant extract significantly reduced the CRL of the fetuses and decreased fetal and placental weights dose-dependently. Similar to the fetuses, the CRL in the high dose treated embryos showed a statistically significant decrement. The average morphological scores also were significantly reduced in the high dose treated embryos compared with the control group. The leaf of *S. guineense* contains active components like: terpenoids, anthraquinones, flavonoids, tannins, saponins, glycosides, triterpenes, and phenols [76]. These phytochemical constituents of *S. guineense* might be responsible for the decrease in the CRL and average morphological scores of the rat embryos/fetuses. Research report indicates that secondary metabolites of plant such as alkaloids can pass the placental membrane and affect development of the embryo/fetus [120].

The other pregnancy outcomes: number of implantations, fetal resorptions, live births, and stillbirths were not affected by treatment with the plant extract. This suggested that the test plant may not have a significant detrimental effect on the progression of pregnancy in rats.

In rodents including rats, many of the bones get ossified in the late fetal period. Therefore, in teratogenicity studies, the extent of bone ossification is an important indicator of fetal maturity [93, 121]. The current study investigated the degree of bone ossification in 20 days old rat fetuses. The ossification centers of fetal skull, vertebrae, hyoid, forelimb, and hindlimb bones did not vary

significantly across treatment and control groups. However, treatment with the higher doses (1000 mg/kg and 500 mg/kg body weight) of the plant extract reduced the number of ossification centers of the sternum, caudal vertebrae, metatarsal, metacarpal, and phalanges. Nonetheless, none of these reductions were statistically significant suggesting that the test plant may not have adverse effect on fetal maturity.

The placenta plays a central role in the exchange of nutrients, gases, metabolic wastes, drugs, and immunoglobulins between the mother and the embryo/fetus. It also allows the passage of toxicants, mycotoxins, plant alkaloids, and many others [122-124]. Due to its function, the placenta is highly sensitive to toxicant influences [125]. Succeeding maternal exposure to chemicals, many of these chemicals can pass the placental membrane and reach the embryo/fetus. Thus, the chemicals can affect the fetus, the placenta, even the mother itself [126]. Administration of chemical agents in the early embryonic period, since the trophoblast cell differentiation is not complete to make the placental membrane [127], can affect the development of embryonic tissue and the placenta [128].

In the present study, decidual cystic degeneration, hemorrhage, and trophoblast proliferation was seen in both treatment and control groups. A greater frequency of decidual cystic degeneration was prevalent in rats treated with 1000 mg/kg body weight of *S. guineense*. However, these alterations in the microscopic structures of the placenta were not statistically significant. However, care should be taken when consuming the plant. The decidua basalis is found at the base of the placenta and it contains newly formed vasculatures. The decidua basalis mainly develops during early pregnancy, and it undergoes regression after gestational day 11 in rats. As a result, decidua is less sensitive to chemical exposure in the embryogenesis period than the other part of the placenta. Besides, hemorrhage can be seen when the decidual regression occurs [129]. This might be the justification for the presence of decidual cystic degeneration and hemorrhage in both treated and control groups. On the other hand, spongiotrophoblasts, glycogen cells, and trophoblastic giant cells were not noticeably affected by treatment with the plant extract.

6.2 Reproductive Toxicity

In the present investigation, the extended one-generation reproductive toxicity study was conducted to evaluate the potential toxic effect of 70% ethanol extract of *S. guineense* leaves on

the reproductive system, nervous system, immune system, endocrine system, liver, and kidney of parental and first-generation male and female rats.

The parental rats have received the plant extract for ten weeks. Throughout the experiment, a significant sign of toxicity or death was not recorded. This finding is in agreement with the sub-acute toxicity study done on methanol leaf extract of *S. guineense* [54]. However, a six-week toxicity study on mice reported the death of two mice treated with 200 and 600 mg/kg of aqueous extract of *S. guineense* leaves [53]. This disparity may be due to the difference in the test animals and method of extraction used in the respective studies.

In the current study, food intake was significantly reduced by treatment with middle (500 mg/kg) and high doses (1000 mg/kg) of *S. guineense* extract. Weight gain during the treatment period also was significantly lower in rats treated with high dose of the test substance. Body weight change is an important indicator of toxicity, disease progression and response to treatment [130]. The mechanism of how the plant extract reduces food intake is not understood yet. As reported by Chung *et al* [131], tannins damage the epithelial lining of the gastrointestinal tract and reduce food intake. *S. guineense* is a tannin-rich plant [71, 77], and thus the presence of tannins in high amount can be the reason for the reduced food intake in the *S. guineense* treated rats. It has been reported that decreased food intake can affect weight gain [116] and this may have been the reason for the decrease in weight gain observed in this study. Similarly, reduced weight gain caused by the plant extract has been reported by other investigators as well [52-54]. Studies conducted on *Syzygium cumini* also revealed decreased weight gain following long-term administration [130, 132].

The effect of the test plant on the reproductive system of rats can be manifested by changes in the normal morphology of the reproductive tract, alteration in the phases or duration of the estrous cycle, and sperm cell synthesis. These in turn determine the mating performance, pregnancy outcomes, and postnatal survival of litters [133]. In the current study, the reproductive toxicity of the test plant was investigated by measuring the length of the estrous cycle, time taken for mating/insemination, and duration of pregnancy. Moreover, pregnancy outcomes, sperm count and morphology, and histopathologic investigation of reproductive organs also were evaluated.

Rats are ideal for the assessment of reproductive toxicity since they are polyestrous and their cycle takes 4-5 days [99]. The estrous cycle of rats, as it is in humans, is controlled by hypothalamic-

pituitary-ovarian hormones. Changes in the hormones secreted by the above glands, specifically the ovarian hormones, are manifested as epithelial changes in the uterus and vagina [99]. The phases of the estrous cycle can be easily studied by the proportion of the three type of cells in the vaginal smear. The phases are divided into four: proestrus (many nucleated epithelial cells), estrus (predominantly cornified cells), metestrus (proportionate number of nucleated epithelial cells, cornified cells, and leukocytes), and diestrus (mainly leukocytes) [134]. In the current study, the length of estrous cycle was measured based on vaginal smear test.

The length of the estrous cycle was affected by administration a high dose of *S. guineense*. Rats that received 1000 mg/kg body weight of the extract of *S. guineense* had longer duration of estrous cycle. This was also supported by delayed pre-coital interval (time to insemination since they were mated) in the high dose treated group. The pre-coital interval was 7±3.6 days for 1000 mg/kg dose treated rats while that of the control was 2.8±1.2 days. This observation indicated that the plant extract affected the estrous cycle of rats. These findings can be a benchmark for investigating the anti-fertility potential of *S. guineense*. The exact mechanism of how the plant extract delayed the estrous cycle still needs further investigation. However, other researchers have reported that the presence of active components like alkaloids, tannins, and flavonoids in the crude extract exert an antigonadotrophic effect and suppress ovulation [135, 136]. Alkaloids, tannins, and flavonoids are predominant components of *S. guineense* leaves [76, 77]. Therefore, this may have been the reason for the delay in estrous cycle observed in the current study. The other reproductive indices: mating index, fertility index, and pregnancy duration were not significantly affected by treatment with the plant extract.

In the pregnancy outcomes, generally, the number of total and live births was reduced by treatment with *S. guineense* leaf extract. *S. guineense*'s secondary metabolites might have been responsible for the reduced number of total and live births. As reported by other investigators [137], plant's alkaloids cross placental membrane and affect development of the embryo.

In the current study, there was no significant variation in the number of postnatal deaths across all groups. The pup's weights measured at PND 0, 4, 7, 14, and 21 were not different in the treated and control groups. There was also no significant difference in the AGD of male and female pups recorded at PND 4. Moreover, there was no nipples/areola developed in male pups. Therefore, it may be possible to conclude that *S. guineense* does not affect the development of survived pups.

Male rats were treated for 70 consecutive days, a time that covers a full period of spermatogenesis in rats [96]. Mating performance, sperm count, sperm morphology, and weight of reproductive organs were not significantly affected by administration of *S. guineense* at different doses. In the female rats, however, uterine and ovarian weight was significantly reduced by treatment with 500 and 1000 mg/kg doses of the plant extract. As reported by Wolfsegger *et al* [138], alteration in organ weight can be due to the direct toxic effect of the test substance even in the absence of structural change in the organ. Therefore, the observed weight changes of the uterus and ovaries may be attributed to the toxic effect of the *S. guineense*. *S. guineense*'s alkaloids may be responsible for this effect since other research findings have reportedly indicated that alkaloids in the plant were toxic [139, 140].

Histopathologic data are essential part of toxicological studies that investigate hazards and elucidate toxic mechanisms [141, 142]. In male rats treated with *S. guineense* for 10 weeks, the mating performance, sperm morphology, and count were not affected by the plant extract. Similarly, gross and microscopic examination of the testis, epididymis, prostate, and seminal gland did not reveal any significant adverse effect. This finding suggested that the 70% ethanol extract of *S. guineense* did not have adverse effects on the normal structure and function of male reproductive system of Wistar rats.

In female parental rats, in contrary to the observed reduction in weights of the ovary and uterus in the high dose treated group, pathological microscopic changes in the uterus or ovaries were absent in any of the treatment groups. A vaginal fibroma is a very rare benign, noninfiltrating, and smooth muscle derived tumor [143]. The presence of fibroma in one of the rats treated with 1000 mg/kg body weight of the extract was not statistically significant and may not have been treatment-related. It has been reported by another investigator that tannins present in the *S. guineense* suppress Wnt signaling and Wnt-dependent tumor cell proliferation [74].

6.3 Hepato-Renal Toxicity

The best-registered organ toxicity because of herbal therapy is hepatic toxicity [144]. Abnormal liver function tests are the primary manifestation of hepatic toxicity. The liver is highly vulnerable to drug-induced damage due to its drug concentration and metabolic function [144, 145]. Another organ that is affected by the toxic insults is the kidney. The high blood flow to the kidney, its

function in urine concentration and metabolic activation of xenobiotics predispose it to the blood-born toxicant to a greater extent than the other organs [146]. The present hepato-renal toxicity study was aimed at investigating any toxic effect of *S. guineense* on the structure and function of the liver and kidneys following long-term administration in rats.

The results of this study indicated that administration of 70% ethanol extract of *S. guineense* leaves increased serum levels of ALT, AST, ALP, and urea. It also resulted in significant reduction in serum glucose level. These effects were noticeable especially in the rats treated with the high dose of *S. guineense*.

The weight of the liver and kidneys was not significantly altered by the treatment with test substance. This observation is supported by the findings of other researchers [54] who reported no significant change in the weight of the liver and kidneys of rats after treatment for 28 days with methanol leaf extract of *S. guineense*. However, increased liver and kidney weight have been reported in mice treated for 42 days with 200 and 600 mg/kg, respectively of aqueous leaf extract of *S. guineense* [53]. Another six weeks study on mice also reported increased liver weight at the doses of 200 and 400 mg/kg of the aqueous extract of *S. guineense* [52]. This discrepancy may be due to the differences in the test animals and the type of extraction methods used in the respective studies.

The presence of tissue damage in the liver can be evaluated by measuring serum level of enzymes like AST, ALT, and ALP. Tissue damage results in the release of extra AST and ALT to the blood circulation and thus, the blood level of such enzymes is increased. Therefore, these enzymes are important biomarkers of liver damage. ALT is the most sensitive liver enzyme that indicates liver cell damage [147].

In the current study, the serum level of ALT was significantly increased in male and female rats treated with 1000 mg/kg of the plant extract. Although increased AST level may not be specific for liver damage, the current study revealed an increased serum AST level in the high dose group (1000 mg/kg). The serum level of ALP can be increased due to bile duct obstruction, liver damage, or bone disease [148]. Our finding indicated an increased serum level of ALP in rats treated with 500 and 1000 mg/kg of *S. guineense* extract. Nevertheless, these increments in serum levels of AST, ALT, and ALP were not followed by tissue damage in the liver. Administration of *S. guineense*, even at the high dose, did not produce a significant inflammation or cellular alteration

in the liver. Administration of *S. guineense* leaves even at a high dose only affected some functions of the liver. This finding implied that may be a chronic administration of *S. guineense* extract is required to observe cellular alteration in the liver. Moreover, short-term (4 weeks) administration of *S.guineense* in rats did not result in a significant change in the liver enzymes [54]. This difference may have been due to the longer duration of the treatment period employed in the current study.

Serum urea and creatinine levels are the commonly employed renal function tests. Creatinine level indicates glomerular filtration rate and serum urea level indicates renal excretion capacity [149]. In the present study, the serum level of creatinine was not altered by treatment with *S. guineense*. There was no significant difference observed in both male and female rats of treatment and control groups. This is consistent with the histological finding of the kidney in which no structural abnormalities were observed in any of the treatment groups. Similar findings are also reported by other researchers [54, 130].

The serum urea level in male rats did not vary significantly between the treatment and control groups. However, elevated serum urea levels were recorded in female rats treated with 500 mg/kg (80.3 ± 7.8) and 1000 mg/kg (72.2 ± 13) of the plant extract.

In the current study, serum levels of total protein, albumin, and cholesterol did not change significantly in all the treated and control rats. Similar findings have been reported in short-term studies [54, 75].

Reduction of blood glucose level was observed in male and female rats, treated with 1000 mg/kg of *S. guineense* extract. This finding supported the hypothesis that *S. guineense* has hypoglycemic effect. The anti-diabetic effect of *S. guineense* leaves has been reported by many researchers [54, 75, 82]. This may be due to secondary metabolites of *S. guineense* such as flavonoids and terpenoids [77]. It has been reported that flavonoids lower the blood glucose level by modulating a glucose transporter protein [150].

In the present investigation, treatment of animals with of *S. guineense* leaves extract did not show significant microscopic changes on the normal architecture of the liver and kidney. This finding is in line with Loha *et al.* [54] and Amare [53]. On the contrary, a study conducted by Abba *et al*. [52] reported the presence of hemorrhagic necrosis and cytoplasmic vacuolations in mice liver

following six weeks of administration of 600 mg/kg body weight of the aqueous extract of *S.guineense*. In this study, fatty changes were observed in one male rat treated with 1000 mg/kg body weight of the extract. This disparity may have been due to the difference in the type of extraction methods and test animals used in the respective studies. Steatosis (fatty change) is an abnormal accumulation of fat in the liver cells. This accumulation can be due to an imbalance in fat intake, synthesis, breakdown, and secretion [151].

6.4 Endocrine Toxicity

In the present study, treatment with higher doses of *S. guineense* resulted in significant reduction in serum level of thyroid hormone (T4) compared to the control group. One of the phytochemical components of *S. guineense* is flavonoids [77]. Flavonoids have been reported to impair the function of the thyroid gland.

Research findings indicate that oral administration of flavonoids results in accumulation of this class of compounds in the thyroid and other glands [152]. There are also evidences that show flavonoids can cross the placenta and accumulate in the fetal tissues [137, 153]. Many researchers have reported the mechanisms by which flavonoids can reduce thyroid hormones. Spanka *et al*. [154] found out that flavonoids decrease thyroid hormones by inhibiting hepatic T4 50-deiodinase. Divi and Doerge [155] reported that the reduction of thyroid hormone by flavonoids is due to the inhibition of thyroperoxidase iodination activity. Another study [156] also has demonstrated inhibition of flavonoids on thyroid type-1 deiodinase activity. In the present study, it might be the flavonoids content of *S. guineense* that caused in reduction of thyroid hormone. However, the above treatment-related functional change of the thyroid gland was not supported by microscopic alteration of either the parenchyma or stroma of the thyroid gland. There was no histological detectable alteration of the thyroid gland in any of the treatment doses.

The weight of adrenal glands of male and female rats did not show significant variation between treatment and control groups. In addition, no microscopic alteration of the adrenal cortex and medulla observed in either the control or treatment groups.

6.5 Reproductive Toxicity in F1 Set-1 Rats

In the current study, F1 set-one rats received the treatment indirectly during intrauterine life and lactation, and directly through administration of *S. guineense* extract on PND 21 to PND 70. Administration of *S. guineense* extract on PNDs was aimed at assessing the extended effects of the plant extract on the postnatal development of F1 rats, mainly the effects on the reproductive, hepato-renal, and endocrine systems.

As observed in the parental rats, male and female F1 set-one rats treated with 500 and 1000 mg/kg body weight of the *S. guineense* extract showed reduction in food consumption. This indicated that long-term administration of the 70% ethanol extract of *S. guineense* leaves consistently affected food consumption of parental and F1 rats, which may be attributed to *S. guineense*'s phytochemical constituents, mainly tannins. Similarly, the post-weaning weight gain was lower in the high dose treated F1 set-1 rats which might be related to the reduced food consumption as reported by other researchers [116].

Separation of the prepuce from the glance penis is initiated by the cornification of epithelium lying between the glans penis and prepuce [157]. Complete preputial separation is assumed when epithelial cornification comes to the ventral end of the glans penis. It is an androgen dependent process and used as an external sign of puberty [157, 158]. Nutritional status and body weight also are associated with the onset of puberty [157, 159]. In the current study, administration of *S. guineense* extract did not adversely affect either the day of preputial separation or the weight of the rats at preputial separation.

The age of vaginal opening and the first occurrence of cornified epithelial cells in the vagina have been used as a markers of puberty onset in female rats. They are initiated by the rising level of estradiol and are usually associated with the first wave of developing ovarian follicles [160]. The current study investigated the age at first time of vaginal opening and determined the weight at vaginal opening. Following the opening of the vagina, vaginal fluid was collected daily by inserting a plastic pipette, thereupon, the date of the first appeared cornified epithelial cells and the length and normality of the estrous cycle was measured until the end of the treatment period (PND 70).

Exposure of F1 rats to the 70% ethanol extract of *S. guineense* did not produce significant variation in either the age of vaginal opening or the first appearance of cornified vaginal epithelial cells between the treatment and control groups. In addition, weight at the day of vaginal opening was not varied between the treated and control groups. Similar to the parental rats, high dose administration of the plant extract significantly prolonged the length of estrous cycle. This suggested that the high dose of *S. guineense* extract may delay the estrous cycle, as reported in the parental rats also.

Similar to the finding in the parental rats, in the F1 rats, the relative weight of the uterus and ovaries were significantly reduced in rats treated with 1000 mg/kg of body weight of the extract. The relative weight of the seminal gland also was reduced in male rats treated with 500 and 1000 mg/kg body weight of the extract, indicating the direct toxic effect of the plant. This toxic effect could be the result of the secondary metabolites *S. guineense*, mainly the alkaloids. Plant alkaloids have several toxic effects on animals and humans [139, 140]. But alkaloid toxicity depends on the sensitivity of the target animal [161]. However, the weights of other reproductive and non-reproductive organs were not affected by the treatment with the plant extract.

This study also investigated the effect of *S. guineense* extract on the structure and functions of the liver and kidneys in F1 set-1 rats. Consistent with the finding in the parental rats, liver function tests (ALT, AST, and ALP) were increased by treatment with the plant extract. However, microscopic examination of the liver and kidney tissues did not reveal any treatment-related abnormalities. Like in the parental rats, the hypoglycemic effect of the plant was observed also in the F1 set-1 rats. In addition, as seen in the parental rats, the serum level of the thyroid hormone was significantly reduced by the treatment with *S. guineense*.

6.6 Developmental Neurotoxicity in F1 Set-2 Rats

The developing brain is inherently more vulnerable and sensitive to injury due to neurotoxicant than adult brain [27]. Exposure to toxicants in utero and early postnatal life can cause permanent brain damage at low doses that have insignificant or no adverse effect in adults [33]. Researches conducted on herbs, have reported that some herbs such as *Artemisia absinthium* [162], *Cinnamomum camphora* [163], *Cannabis sativa* [164], *Myristica fragrans* [165], and many others have neurotoxic effects. The current study was conducted to investigate the developmental

neurotoxicity of *S. guineense* on PND 70 rats. The rats indirectly received the plant extract during intrauterine life and lactation via their dams, and directly at weaning.

Administration of the 70% ethanol extract of *S. guineense* leaves lowered the food intake of rats in mid (500 mg/kg) and high (1000 mg/kg) dose groups compared to the low dose treated and control groups. The relative brain weight was not significantly different among the experimental groups. Histopathological examinations of the brain and the spinal cord did not reveal any abnormal findings. These findings suggested that administration *S. guineense* leaves extract at the tested doses may not have toxic effect on the developing brain and spinal cord.

6.7 Developmental Immunotoxicity in F1 Set-3 Rats

The immune system of the fetus or newborn is different in many ways from the adults. Several studies indicate that in early neonatal period, there is decreased serum complement activity [40], low neutrophil reserves in the bone marrow [41], diminished ability of neutrophil in adherence and chemotaxis [42], low antibody production capacity [43], and reduced cytotoxic activity of lymphocyte [44]. These and many other factors contribute to the low immune status of the fetuses and newborns. In the current study, the developmental immunotoxicity of *S. guineense* leaves was assessed by way of organ weight and histopathology of the spleen, thymus, and lymph nodes. The duration of treatment for F1 set-3 rats was during pregnancy and lactation through their dam and from weaning to PND 60 through direct oral administration.

The findings of the current study showed that the administration of *S. guineense* extract reduced the food intake of treated rats. Moreover, postweaning weight gain of rats, treated with 500 and 1000 mg/kg was lower than the control rats. This finding is consistent with parental rats and other F1 cohort groups.

The first manifestation of immune toxicity is atrophy or decreased organ weight, especially in the thymus, as it is the first lymphoid organ that indicates structural change following exposure to immune-toxicants [166]. However, in the relative organ weight and gross examination of the lymphoid organs, no significant variation or sign of toxicity was observed. Histopathologic evaluation of the lymphoid organs is a cornerstone in the assessment of immunotoxicity [167]. Microscopic examination of the thymus, spleen, and lymph nodes did not reveal any cellular

changes in the treated as well as the control groups. This indicated that *S. guineense* extract administration did not affect structures of the developing immune system.

7. Conclusion

Administration of 70% hydroethanolic leaf extract of *S. guineense* during pregnancy may not be safe. It may delay development of the embryos, as the current study identified reduced CRL and average morphological score of 12 days old rat embryos. It may also affect growth of the fetuses, as evidenced by decreased CRL of 20 days old rat fetuses and the number of live births.

Treatment with *S. guineense* leaf extract may delay the reproductive cycle of rats, since this study reported prolonged duration of estrous cycle and pre-coital interval in the parental and F1 rats. Moreover, prolonged use of *S. guineense* leaf may be toxic to the reproductive organs, liver, kidney, and thyroid glands. Increased serum level of liver and kidney function tests as well as decreased level of thyroid hormones and relative organ weight were observed in the parental and first-generation rats. The current study finding also suggests that the test plant may not have a significant adverse effect on the development nervous and immune systems of Wistar rats. No significant detrimental effect on relative organ weight or histopathology of the brain, spinal cord, spleen, thymus, and lymph nodes was observed. Moreover, consumption of *S. guineense* leaves could have antidiabetic effect.

8. Strength and Limitations of the Study

The current study provided a number of evidences regarding teratogenicity and toxicity of *S. guineense*, it examined 17 organs. This study also fills a known gap and contribute to the knowledge of science in terms of teratogenicity and reproductive toxicity of *S. guineense*. However, it is not without limitations. The first limitation is, due to financial constraints, advanced tests like immunohistochemistry and electron microscopy were not included. Secondly, functional tests (enzymes and hormones) were measured only once, at the time of necropsy. It was good to measure more than once to check whether the changes are permanent or transient.

9. Recommendations

Based on the findings of this thesis research, the followings are recommended:

- Teratogenic effect of *S. guineense* leaves should be further evaluated by administering the plant extract during the whole duration of gestation.
- Teratogenic effect of *S. guineense* leaves should be further investigated by advanced tests.
- Teratogenic and toxic effects of *S. guineense* leaves should be tested at higher doses (greater than 1000 mg/kg body weight).
- Chronic toxicity investigation of *S. guineense* leaves is recommended.
- The toxic effect of *S. guineense* leaves should be evaluated on the functional aspects of the nervous and immune systems using advanced tests such as immunohistochemistry.
- Teratogenicity and toxicity studies should be carried out on other test animals other than rats.
- Further toxicity studies should be conducted by including additional organs such as: intestine, lung, stomach, and others.
- Investigating the toxic effects of *S. guineense* on the ultrastructure (by using electron microscope) of organs included in this research and others is recommended.
- Further investigation should be conducted on the specific secondary metabolites of *S. guineense* leaf with serum level determination to identify the toxic nature and mechanism of actions.
- The community should be restricted from using high dose of *S. guineense* leaves, especially during pregnancy.
- Ministry of health and EPHI should develop legislative guideline & therapeutic doses.

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11.Appendix

11.1 Preparation of Solutions Used in the Experiment

1. Bouin's solution:
    - 75% picric acid
    - 25% formalin
    - 5% glacial acetic acid
2. Mall's solution:
    - 79% distilled water
    - 20% glycerol
    - 1% potassium hydroxide (KOH)
3. Formalized saline
    - Sodium bicarbonate (5 g) in 99 ml normal saline
    - 1 ml formalin (40%)
4. Acid alcohol
    - 1% HCl in 70% alcohol

11.2 Tissue Processing Procedures

Tissue processing was done with Leica (TP 1020) automatic tissue processor.

1. **Fixation**:
    - 10% formalin overnight
2. **Dehydration**:
    - 40% alcohol- 1:30hrs
    - 70% alcohol- 1:30hrs
    - 80% alcohol- 1:30hrs
    - 90% alcohol- 1:30hrs
    - Absolute alcohol I- 1:30hrs
    - Absolute alcohol II- 1:30hrs
    - Absolute alcohol I- 1:30hrs
3. **Clearing**:
    - Xylene I- 1:30hrs
    - Xylene II- 1:30hrs
4. **Infiltration**:
    - Paraffin wax I-1:30hrs
    - Paraffin wax II-1:30hrs
    - Paraffin wax III-1:30hrs
5. **Embedding**:
    - With melted paraffin wax in embedding cassette, temperature (58-62 ^{o}C)
6. **Sectioning**:
    - With Leica rotatory microtome, 5 µm section was made
    - Ribbons were allowed to float in warm water (45 ^{o}C) and spread on the forested slide. The slides were dried on the hot plate (50-55 ^{o}C) for 20-30 minutes.

11.3 Hematoxylin and Eosin (H & E) Staining Protocol

1. Dewaxing:
   - Xylene I- 5minutes
   - Xylene II- 5minutes
   - Xylene III- 5minutes
2. Rehydration:
   - Absolute alcohol I- 2minutes
   - Absolute alcohol II- 2minutes
   - 90% alcohol I- 2minutes
   - 80% alcohol I- 2minutes
   - 70% alcohol I- 2minutes
   - Running tap water- 2minutes
3. Staining with Hematoxylin:
   - 5-12 minutes with frequent agitation
4. Bluing:
   - Running tap water- 8-10minutes
5. Differentiation:
   - 1% acid alcohol
6. Counterstain with eosin:
   - Eosin Y solution – 1-2 minutes
   - Washing with running tap water- 2 minutes
7. Dehydration:
   - 95% alcohol I- 2minutes
   - Absolute alcohol I- 2minutes
   - Absolute alcohol II- 2minutes
8. Clearing:
   - Xylene I and II, 2 minutes in each

11.4 Morphological Scoring System

No	Criteria	0	1	2	3	4	5	Score
A	Yolk sac circulatory system	No visible or scattered blood islands	corona of blood islands with or without anastomoses	Vitelline vessel with few yolk sac vessels	Full yolk sac plexus of vessels	Yolk stalk obliterated: Vitelline artery and vein well separated		
B	Allantois	Allantois free in the exocoelome	Allantois fused with chorion	Umbilical vessels	Separate aortic origin of umbilical and vitelline vessels			
C	Flexion	Ventrally convex	Turning	Dorsally convex				
D	Heart	Endocardial rudiment not visible or visible but not beating	Beating S shaped cardiac tube	Convoluted cardiac tube	Bulbus cordis, atrium commune and ventriculus communis	Dividing atrium commune		
E	Caudal Neural tube	Neural plate or neural folds	Closing, but unfused neural folds (groove)	Neural fold fused at the level of somites 4/5	Posterior neuropore formed but open	Posterior neuropore closed		
F	Hind brain	Neural plate	Rhombomeres A and B	Anterior neuropore formed but open	Anterior neuropore closed rhombencephalon formed	Pronounced pontine flexure with transparent roof of 4th ventricle		
G	Mid brain	Neural plate	Mesencephalic brain folds	Closing or fusing mesencephalic folds	Completely fused mesencephalon	Visible division between mesencephalon and diencephalon		
H	Fore brain	Neural plate or no visible procencephalon	Procencephalic brain folds	Completely fused procencephalon	Visible telencephalic evaginations	Well elevated telencephalic hemispheres		
J	Otic system	No sign of otic development	Flattened or indented otic primordium	Otic pits	Otocyst	Otocyst with dorsal recess	Otocyst with endolymphatic duct	
K	Optic system	No sign of optic development	Sulcus opticus	Elongated optic primordium	Primary optic vesicle with open optic stalk	Indented lens plate	Lens pockate or lens vesicle	
L	Olfactory system	No sign of olfactory development	Olfactory plate	Olfactory plate with rim	Distinct olfactory ridges	Lateral nasal process and medial rim		
M	Branchial Bars	No visible	I visible	I and II visible	I, II and III visible	II overgrowing and obscuring III		
N	Maxillary Process	No sign of maxillary development	Maxillary process demarcated. Visible cleft anterior to bar I	Maxillary process fused with nasal process				
P	Mandibular process	No sign of mandibular development from bar I	First branchial bar fused and forming mandibular process					
Q	Fore limb	No sign of fore limb development	Distinct evagination of wolfian crest at the level of somite 9-13	Fore limb bud	Paddle shaped fore limb bud	Distinct apical ridge on fore limb bud		
R	Hind limb	No sign of hind limb development	Distinct evagination of wolfian crest at the level of somite 26-30	Hind limb bud	Paddle shaped hind limb bud			
S	Somites	0-6	7 to 13	14-20	21-27	28-34	35-41	

A morphological scoring system for quantifying the development rat embryo (Brown and Fabro 1981)