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PhD Dissertation

Acute, sub-chronic and developmental toxicity effect of crude extract, fixed oil, and embelin isolated from *Embelia schimperi* Vake fruit on albino Wistar rats: An *in vivo* and *in silico* study

By: Zelalem Animaw Entewow

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Addis Ababa, Ethiopia

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Abstract

Background: *Embelia schimperi* Vake is a widely known medicinal plant under family Myrsinaceae. The fruit is the most commonly utilized part of the plant to treat various aliments by the community. Its famous traditional claim is against intestinal parasitic infections, primarily tapeworm. Moreover, the main chemical composition of the plant called embelin, a hydroxyl-benzoquinone compound, has a wide range of therapeutic value including anticancer, antibacterial, antimalaria, anti-inflammatory and anti-helminthic. The fixed oil extracted from the plant has also a significant nutritional value since it contains various types of fatty acids. However, there is little scientific data regarding the plant's toxicity effects, including its in silico, acute, sub-chronic, and developmental toxicity despite being used extensively as an alternative medicine source.

Therefore the purpose of the current study is to investigate the *in vivo* acute, sub-chronic and developmental toxicity profile of crude, fixed oil and embelin isolated from *E. schimperi* fruit on albino Wister rats and predict the *in silico* property of embelin.

Method: The plant collection was conducted at Debre Markos localities and authenticated by a botanist. The cured was extracted by macerating the ground fruit with 80% ethanol. The crude extract of *E. schimperi* was administered to pregnant albino Wistar rats during their active embryogenesis period. Treatment groups received *E. schimperi* fruit extract at doses of 250mg/kg, 500mg/kg and 1000mg/kg, whilst the controls were pair-fed and *ad libitum* groups. The quantitative and qualitative fetal-maternal outcome variables were evaluated. Maternal food intake and weight growth, number of implantations and preceding resporption, fetal viability, fetal weight, fetal crown-ramp length, placental weight, placental gross morphology, and histopathology are the variables. A compound embelin extracted from the seed of Embelia schimperi Vake was studied both *in silico* and *in vivo* . *In silico* toxicity predictions were computed using the ProTox model. The *in vivo* experiment was done by administering 5000 mg/kg of embelin to a single female albino Wistar rat, followed by three female rats in the absence of death, to determine the mean lethal dose (LD50). Afterwards, three groups of gravid rats were treated with embelin at doses of 250 mg/kg, 500 mg/kg, and 1000 mg/kg for the developmental toxicity test. Vehicle and *ad libitum* groups were used to compare the acute and developmental toxicity variables. Acute, sub-acute and developmental toxicity of fixed oil extract was conducted according to OECD guideline. Soxhlet extractor apparatus was used to extract the fixed oil

component of the plant. The acute, sub chronic and developmental toxicity test were similar with embelin test.

Result: The crude extract of *E. schimperi* has no significant effect on the feto-maternal outcomes. However, histopathological examination of the placenta showed an inflammatory reactions and calcifications in all animals treated with *E. schimperi*. *In silico* toxicity predicted, that embelin is free from hepatotoxic, carcinogenic, mutagenic, and cytotoxic effects. No inhibitory effect on hERG channels was observed. It has an immunotoxic property and an inhibitory effect on the CYP2D6 enzyme. Since mortality and signs of toxicities were not observed after treatment with 5000 mg/kg, the mean lethal dose (LD_{50}) is determined to be >5000 mg/kg. None of the morphological scores or number of somites among experimental animals became significantly different. None of the embryonic systems possessed developmental delays. Nevertheless, the crown-rump length of the high-dose group became significantly shorter. Dams' food intake and weight gain exhibited significant dose-dependent differences between embelin-treated animals and controls. The number of implantations was significantly low in the treatment group, accompanied by a higher frequency of prior resorption. The sub-chronic administration of embelin from *E. schimperi* fruit demonstrated significant effects on various physiological endpoints in both sexes of rats. The enhanced total body weight gain in treated animals, coupled with distinct alterations in food intake patterns, suggests a potential influence of embelin on metabolic processes. Moreover, the hematological and serum biochemical analyses revealed specific changes in liver enzyme levels, indicating a possible impact on hepatic function. Notably, reproductive endpoints exhibited considerable modifications, including reduced relative weights of reproductive organs, hormonal imbalances, and histopathological alterations in male reproductive tissues, indicating potential reproductive toxicity. Fixed oil extract from *E. schimperi* Vake fruit, even at high doses, demonstrates a lack of severe toxicity and maintains the overall health and reproductive functions in experimental animals. These findings add to our understanding of *E. schimperi* fixed oil extract's safety profile, supporting its prospective usage in a variety of applications.

Conclusion: Pregnancy-related *E. schimperi* fruit consumption may have an impact on the placenta's structural integrity, as shown by calcifications and inflammatory responses in the decidua basalis of rat placentas. Embelin is predicted to have a high probability of immunotoxicity potential and affect drug metabolism by inhibiting CYP2D6. In addition, it affects food intake,

weight gain, and the number of implantations in pregnant rats. Therefore, it is highly recommended not to take embelin and embelin-rich plants during pregnancy. Further *in vitro* and *in vivo* studies need to be conducted to understand the mechanism behind the toxicity of embelin. However, Fixed oil extract is relatively safe upon sub-chronic administration and evaluation of developmental effect.

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Dedication

This Ph.D. work is dedicated to the late professor Mekonnen Assefa for his Exemplary effort and achievement of improving quality education and scientific research

Declaration

I hereby declare that this Ph.D. work titled, **Acute, sub-chronic and developmental toxicity effect of crude extract, fixed oil and embelin isolated from *Embelia schimperi* Vake fruit on albino Wistar rats: An *in vivo* and *in silico* study**, is an original and independent research carried out by me under the supervision of Professor Girma Seyoum and co-supervisor Professor Kaleab Asres. I affirm that this work has not been submitted completely or in part for any other academic degree and has not been previously published.

Zelalem Animaw Entewow	_____	_____
Name	**Signature**	**Date**

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Chapter One

1. Introduction

1.1.Background

1.1.1. Traditional medicine

Traditional medicine (TM), also referred to interchangeably as ethno medicine, folk medicine or complementary and alternative medicine, is an ancient method of maintaining health that has been useful for years furthermore practiced as an indigenous medical practice in various societies (1). The importance of traditional medicine also includes the cultivation of spiritual and cultural beliefs. Such differences in the use of traditional medicine have resulted in no globally accepted definition (2). However, according to World Health Organization's definition, traditional medicine is *"the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses"* (3). Although modern elites once ignored traditional medicine, over 80% of the developing nations uses traditional medicine to maintain their healthcare, which has led to traditional healers playing a key role in the healthcare delivery system. This situation is mainly reinforced by the very limited access to the conventional and modern healthcare system caused by economic factors, as well as by the community's deep-rooted psychosocial and spiritual attachment to traditional medicine (4).

A recent study on drug use found that 35% of the population does not receive conventional medication due to economic constraints (5). However, the trend towards utilization of traditional herbal medicine (phytotherapy) by the international community has increased significantly since the beginning of the 20th century and has become a sector from which enormous financial resources can be gained. According to WHO estimates, the annual transaction value in the traditional medicine market is approximately $83 billion. This scenario required the involvement of international regulatory authorities such as the WHO to develop guidelines for appropriate use and safety (6,7).

Currently, herbal Transcendental Meditation is used in developed and developing countries to combat various infectious and non-communicable diseases. For example, patients suffering from chronic diseases such as diabetes, hypertension, and dyslipidemia often use herbal medicines as an alternative method to maintain their health. This increase in the consumption of traditional medicine as an alternative health practice has been attributed to the alarming rise in expenditure on modern/conventional medicine which is affecting affordability and reducing the availability of healthcare facilities (8,9).

1.1.2. Traditional medicine practice in Africa

The Africa people are historically rich in various indigenous knowledge, of which, traditional medicine remain to be the key component (10). TM has been practiced as a sole mechanism of health care maintenance by the people of Africa for century's way before the conventional health care system was introduced (11).

Utilization of traditional medical practice has a long history that is intricately intertwined into the social and economic fabric of the African people (12). Diverse African societies have relied on shamans, herbalists, and traditional healers for ages because they value their knowledge of health care and welfare (13). These customs, which reflect the interdependence of people with their communities and environments, have been influenced by cultural beliefs, rituals, and values (14). Traditional medicine frequently reflects metaphysical and spiritual ideas, emphasizing balance, harmony, and the importance of ancestors (15). Traditional medicine was used in many African communities not just to treat illnesses but also to maintain social cohesiveness and individual identity (16).

Traditional medicine has historically contributed significantly to healthcare accessibility from an economic point of view, particularly in areas with a dearth or inaccessibility of contemporary healthcare systems, by offering a reasonable substitute and making healthcare accessible to all(1). Communities were able to provide for their own healthcare needs by relying on native knowledge and therapeutic plants that were readily available in the area (17). Traditional medicine, however, had to deal with issues including the necessity for integration with contemporary healthcare systems and assuring the safety and effectiveness of treatments as a result of globalization and the impact of Western medical methods (18). In African communities, striking a balance between the

growth of modern healthcare and the preservation of traditional healing methods is still a difficult task, underscoring the complex interplay of tradition, culture, economics, and changing societal requirements (4,19).

However, the safety traditional medicine utilization in Africa is a major worry that is strongly ingrained in societal well-being and cultural behaviors (20). Due to problems including variable quality, incorrect dosage, and potential health concerns connected with some traditional cures, the safety of traditional medicine based health care has recently come under examination (20). To find a balance between preserving cultural traditions and ensuring the safety and efficacy of treatments, efforts have been made to modernize and regulate traditional medicine by taking initiatives for collaboration between governing bodies, medical professionals, and traditional healers (21).

1.1.3. Traditional medicine practice in Ethiopia

A wide variety of traditional healing techniques is used in Ethiopia, reflecting the complex tapestry of Ethiopian traditions. Its unique climate and biological differences, serves as a repository for a diverse range of flora and wildlife. The country has an enormous wealth of potentially useful medicinal plants, outnumbering the diversity found in many other parts of the world. People's understanding of plants is deeply established in their cultural heritage and is the result of millennia of collected wisdom and hands-on study (22).

The ancient medical practices in this nation go beyond only treating illnesses; they also include preserving and promoting human wellness in all of its aspects—physical, spiritual, social, mental, and material. These aspects of health are addressed by a variety of conventional medical strategies, ranging from spiritual cures and preventative measures to corrective procedures and surgical interventions (8). However, challenges exist in the utilization of traditional medicine in Ethiopia. Modernization, urbanization, limited documentation of traditional knowledge, and the influence of Western medicine pose significant challenges to its preservation and development (17). Efforts are being made to adhere traditional health care practice into the conventional system, acknowledging its potential contribution (23).

1.1.4. Plant based (Herbal) traditional medicine

Herbal medicines, according to WHO guidelines, are finished pharmaceuticals that display a label and contain an active element derived from aerial, subterranean, or alternate botanical constituents of plants, or amalgams of these constituents (3,24,25). Plant-based or herbal medicine has been an important feature of indigenous healthcare systems since the dawn of time, especially in developing nations where it is readily accessible, inexpensive, and in line with cultural norms (24). There is also a dramatic increased utilization of plant based traditional medicine in the developed nations to the extent of prescribing them after proper approval from the regulatory authorities such as European Union (26).

The abundance of medicinal plants' secondary metabolites, Such as alkaloids, flavonoids, saponins, terpenoids, fixed oils, and volatile oils, which were produced to defend against microbial infections or insect invasions, gave them the potential to be clinically effective medicines to treat chronic illnesses such as cardiovascular, gastrointestinal, neurological, and cancer (27,28). They also played a therapeutic role as antioxidants, antivirals, antimicrobials, and antiparasitics (27).

Because plants are organic and have a substantial and diverse variety of therapeutic effects, there is a common misperception that plant-based traditional medicine is always safe. However, there have been reports of deleterious side effects because of the inclusion of toxic substances and poor preparation and utilization. Such negative consequences could vary from minor toxicological effects to severe systemic poisoning and death (28).

1.1.5. Toxicity of medicinal plants

Medicinal plants have long been utilized in both conventional and alternative medicine due to their perceived health advantages. However, it must be admitted that not all medicinal plants are completely harmless. Some of these plants contain compounds that can be toxic if used or ingested improperly. The potentially toxic effects of medicinal plants can manifest themselves in a variety of ways, leading to side effects, organ damage and even death (29,30).

Consuming plants containing toxic alkaloids is a common route of poisoning. For example, some types of aconite, commonly known as aconite or monk fruit, contain alkaloids that can have serious effects on the cardiovascular and nervous systems. Excessive consumption or improper

preparation of these plants can lead to symptoms such as nausea, vomiting and, in more serious cases, cardiac arrhythmias (31).

Cardiac glycosides are another group of compounds found in various plants, including foxglove and foxglove. These glycosides are used in modern medicine to treat heart disease, but can be toxic if consumed in excess. An overdose of cardiac glycosides can cause symptoms such as nausea, vomiting, confusion and life-threatening cardiac arrhythmias (32,33).

In addition, herbal laxatives such as senna alexandrine, which contain anthraquinone glycosides, can cause gastrointestinal problems and colon damage when used excessively or over a long period. *Ephedra sinica*, a plant used for its stimulant properties, contains ephedrine alkaloids, which when abused or taken in high doses can cause increased blood pressure, heart palpitations, and even cardiovascular collapse (34,35).

A study by Loha (36) indicated that both single and repeated dose toxicity studies after the administration of *S. guineense* extract in rats demonstrated to lower blood glucose levels indicating a potential hypoglycemic effect of the extract (37).

Toxicity concerns include the potential for cumulative harm. Some plants contain compounds that can cause chronic health problems or organ damage when used for long periods of time (38). *Aristolochia fangchi*, a plant used in traditional Chinese medicine, contains aristolochic acids. Long-term exposure to these compounds is associated with severe kidney damage and an increased risk of urinary tract cancer (39,40). These evidences can illustrate that while medicinal plants offer a wealth of therapeutic possibilities, they also carry the potential for toxic effects.

In silico, in vivo , and *in vitro* studies have been used to identify the mechanisms of action of medicinal plants (41). Consequently, there are ranges of pathways that medicinal plants can bring effect such as the production of reactive oxygen species, disruption of cellular membranes, and inhibition of key enzymes (42–45). Interference with DNA synthesis, modulation of cellular signaling pathways, and induction of apoptosis are some of the other mechanisms (46–49). However, the toxicity mechanisms of medicinal plants vary according to plant species, plant part used, and method of preparation or extraction (50,51). Hence, understanding these mechanisms is essential part of for developing safe and effective medicinal plant applications.

1.1.6. Teratogenicity/Developmental toxicity of medicinal plants

Because of the growing traditional medicine and natural products in healthcare, the study of toxicity from medicinal plants has received increased attention in recent years (52). While medicinal plants are generally considered safe, there is growing concern about their potential toxicity and adverse effects on human health, particularly during pregnancy and lactation (53,54). Many of these plants contain a diverse range of chemical compounds, and their potential toxicity is unknown (55).

Teratogenicity and developmental toxicity refer to the ability of substances, including medicinal botanicals, to cause abnormalities or adverse effects in the developing fetus during pregnancy {Merging Citations}. Understanding the potential teratogenic and developmental effects of medicinal plants is crucial to ensure the safe use of these plants, particularly by pregnant women or people of childbearing potential. Hence, the risks and considerations associated with teratogenicity and developmental toxicity associated with medicinal plants has to been plausibly defined. Certain medicinal plants contain bioactive compounds that, when consumed during pregnancy may interfere with the normal development of the fetus and cause congenital malformations or developmental problems. These effects may vary depending on factors such as timing and duration of exposure, dose and genetic predisposition (58).

An example is thalidomide, a synthetic compound initially produced as a sedative but later banned from utilization due to its severe teratogenic consequences. It serves as a reminder of the possible harm that substances, including those from medicinal plants, can cause during pregnancy (59,60).

Several medicinal plants are known to have possible teratogenic or developmental effects. For example, studies have suggested that high doses of certain plant alkaloids, such as those found in some *Veratrum* and *Nicotiana* species, can cause developmental defects in animal models (61,62)

In addition, exposure to certain plant substances, such as: B. Pyrrolizidine alkaloids, found in several plant species, have been linked to developmental toxicity and venous liver disease (63–65). Given these risks, caution should be exercised when using medicinal plants during pregnancy or in women who wish to have children.

Several indices can be used to assess the developmental toxicity or teratogenicity of research chemicals. Among these the developmental toxicity profile of medicinal herbs are commonly evaluated based on embryonic toxicity parameters from 12 day old rat embryos and fetal toxicity parameters from 20 day old rat fetuses (66,67).

During developmental toxicity experiments, embryonic toxicity indices are one of the major targets to look for. These parameters were targeted in assessing the potential toxic effect of test substances during the period of embryogenesis, day-6 through day-12 in case of rat development. At the end of the 12^{th} day, pregnant animals were sacrificed using recommended euthanasia medication (preferably, intraperitonial injection of pentobarbitol) and embryonic parameters were explored. The following factors were considered indicators of embryonic toxicity: daily food intake, rise in maternal weight, number of implantations and resorptions, length of the crown-rump region, somites and morphological score, embryonic circulatory system, embryonic neurological system and sensory organs, and embryonic musculoskeletal system. (68).

Fetal toxicity characteristics, on the other hand, were assessed in near-term rat fetuses (20 day old) in order to investigate the probable developmental impacts that can be exhibited in late gestational age. Furthermore, these tests may be used to determine if the effects of test chemicals during the embryonic phase were physiologically resolved in late gestational age. Fetal toxicity parameters from 20 day old rat fetuses were food intake and maternal weight gain, fetal weight, placental weight and crown-rump length, gross morphology and histopathology of placenta, external visceral morphology, skeletal evaluation and number of live and dead birth (69).

Therefore, the possible teratogenic and developmental effects of medicinal plants underline the importance of careful use, particularly during pregnancy. Awareness of the risks associated with specific plants and their components, together with informed decision-making and professional advice, besides conducting rigorous experimental tests, can contribute to the safe and responsible use of medicinal plants and minimize potential harm to the developing fetus.

1.1.7. Teratogenicity/Developmental toxicity of medicinal plants in Ethiopian folk

***Syzygium guineense*:** *S. guineense* was discovered to have significant impacts on female reproduction, reducing the weight of the uterus and ovaries, lengthening the time of the estrous cycle, and lowering the number of alive fetuses (70). Despite this troubling reproduction-related

discovery from *S. guineense* , only two papers turned up, both published by the same authors, that discussed the developmental or teratogenic effects of *S. guineense* (66,71).

Notable findings from the embryotoxicity tests conducted on *S. guineense* have provided valuable insights into its potential impact on embryonic development (66,71). The test results revealed significant changes, including a decrease in food intake and weight among the pregnant rats subjected to the treatment. This decline implies a potential influence of the plant extract on the gastrointestinal system that might affect the overall growth and development of the embryos. Furthermore, measurements of the crown-rump length (CRL) exhibited a reduction, indicating a possible delay in skeletal development compared to the expected timeline (72,73)

Moreover, the treated group displayed a lower number of somites, which serve as important markers of embryonic age and developmental progress, compared to control animals. This decrease suggests a potential disruption in the proper segmentation and formation of the embryonic body axis (74,75). Additionally, the morphological scores, which assess the structural integrity and normalcy of the developing rat embryos, were also lower in the treated group, indicating potential deviations and abnormalities from the typical developmental patterns (74,76).

In addition to these outcomes, the developmental toxicity test unveiled a delay in the development of the olfactory system in the treated embryos. This delay suggests that exposure to *S. guineense* may interfere with the proper formation and maturation of the olfactory structures, thereby impairing the sense of smell in developing rat.

A developmental toxicity test by Abebe and his colleagues revealed several significant indices of fetal toxicity (66,71). A reduction in crown-rump length (CRL) of near-term fetuses was discovered, similar to the data from the day 12 trial, implying that the development of the bones may have been hindered. The decrease in fetal and placental weight signifies an issue with the fetus's ability to grow and obtain nutrients. These findings were corroborated by a histological investigation of the placentas, which indicated substantial abnormalities. One of these was decidual cystic degeneration, a sign of a breakdown in the maternal-fetal interface and the formation of fluid-filled sacs in the uterine lining. The presence of blood in placental tissue indicates bleeding and may indicate a problem with the growing fetus's blood supply. Furthermore,

trophoblastic proliferation—abnormal growth—was observed in the cells responsible for placental development, which may signal problems with normal placental functioning.

Over all, these findings highlight *S. guineense's* potential developmental harm. Along with the histopathological findings, the observed decreases in CRL, fetal weight, and placental weight suggest harmful effects on fetal development and maternal-fetal interaction.

***Moringa stenopetala*:** Despite the fact that Moringa is a plant having versatile pharmacological claim, limited studies are available to examine the developmental toxicity of *M. Stenopetala*. One study, led by Husain Abdu and colleagues, focused on evaluating the potential effects of hydroalcohlic extract derived from *M. Stenopetala* leaves on gravid rats. The study aimed to assess the impact of the extract on both the developing fetus and placenta throughout gestation. The findings shed light on the potential effect of *M. Stenopetala* and provide valuable insights into its safety profile during pregnancy (69).

Another study, conducted by Daniel and his team, explored the toxicity of methanolic extracts obtained from *M. Stenopetala* seeds on rat embryos and fetuses. This investigation evaluated the potential adverse outcomes of the seed extract on embryonic and fetal development, thereby contributing to our understanding of the safety of *M. Stenopetala* seed-derived products during pregnancy (77).

Additionally, Abdu and colleagues presented the potential adverse effects of a herbal tea formulation containing *M. Stenopetala* and *Mentha spicata* leaves on pregnant rats. The researchers assessed the impact of the herbal tea on the developing offspring during gestation, offering valuable insights into the safety of this herbal blend for use during pregnancy (78).

These researches add to the growing amount of scientific knowledge on the developmental toxicity of *M. Stenopetala* and its many extracts and formulations. These investigations provide crucial information to aid healthcare professionals and individuals in making informed decisions about the usage of *M. Stenopetala* during pregnancy by investigating the potential hazards and safety concerns related with its use during this critical period.

During day 12 of the experiment from one study elucidated that those high dose-treated rats exhibited significant delays in the development of crucial sensory systems, namely the otic, optic,

and olfactory systems (77). These sensory systems are essential for the proper perception of sound, light, and smell, respectively (79). The delays in their development in response to *M. Stenopetala* suggest potential interference with the intricate processes involved in the formation and maturation of these vital sensory structures (80)

Additionally, the high dose treatment brought reduced number of pharyngeal apparatus in day 12 embryos (77). Branchial bars, also known as pharyngeal arches, play a crucial role in the development of the respiratory and circulatory systems in vertebrate embryos (81,82). The reduction in pharyngeal bars in the high dose-treated rats implies a disruption in the proper embryonic development of these important anatomical structures, which could have implications for optimal physiology of the respiratory and circulatory systems in the later stages of development (83).

These findings underscore the potential developmental toxicity of *M. Stenopetala* leaf extract on day 12 embryos in rats. The observed delays in the development of sensory systems and reductions in the number of branchial bars highlight the sensitivity of the developing fetus to the phytochemicals present in *M. Stenopetala* leaf extract during critical stages of gestation.

In the context of *M. Stenopetala* leaf, a plant species with a diverse phytochemical profile, a study conducted on day 20 of gestation in gravid rats exposed to *M. Stenopetala* leaf extract revealed significant developmental toxicity effects.

The notable effect found in the high dose-treated group was a reduction in maternal daily food consumption and weight gain. This is concerning, as it may indicate potential adverse effects on maternal health, which in turn can impact the fetal development (84). Furthermore, the high dose treatment led to several adverse outcomes in fetal development. High fetal resorptions were observed, suggesting an increased number of fetal deaths, and there was a reduction in crown-rump length, indicating possible growth retardation. Additionally, both fetal and placental weights were decreased, implying potential impaired nutrient supply to the fetus and altered placental development (69). These findings highlight the sensitivity of the developing fetus to the phytochemicals present in *M. Stenopetala* leaf extract during this critical stage of gestation

Additionally, a reduced litter weight was observed, further emphasizing the negative impact of the of *M. Stenopetala* leaf on overall fetal growth and development (77)

Another concerning observation was that nearly half of the fetuses from the high dose treatment exhibited an absence of proximal hind-limb phalange (69). This absence of phalanges indicates possible limb malformation, pointing towards a potential teratogenic effect of the *M. Stenopetala* leaf extract on limb development during embryonic growth (85,86). Histopathological examinations of maternal tissues further revealed noteworthy changes associated with the high dose treatment, including hematoma, capillary dilatation, decidual necrosis, cytolysis, and apoptosis. These changes in maternal tissues may indicate potential placental and uterine damage, which can adversely affect fetal development (87,88). Moreover, there was an elevation in trophoblast proliferation, indicating potential alterations in the early stages of placental development. This increase in trophoblast proliferation might be an adaptive response to the adverse effects of the high dose treatment on the placenta.

However, no maternal fatalities or apparent adverse indications were linked to the herbal infusion. The examined parameters showed no notable herbal tea-linked alterations. Furthermore, the herbal infusion did not display any evident harmful consequences on intrautrine growth and advancement (78).

The results of these experiments showed that *M. Stenopetala* leaf extract is harmful to pregnant rats. The observed effects on maternal health, high fetal resorptions, decreased crown-rump length, fetal and placental weights, hind-limb malformations, and histopathological changes in maternal tissues highlight the potential risks associated with high dose *M. Stenopetala* leaf extract exposure during pregnancy. These studies stressed the need of using *M. Stenopetala* products with caution during pregnancy, and more research is needed to completely understand the underlying mechanisms and particular bioactive components that cause the reported developmental toxicity consequences.

***Achyranthes aspera*:** While multiple studies have highlighted the abortifacient and anti-fertility properties of *A. aspera*, only one study has looked into the plant's developmental toxicity profile (89,90). This study, conducted by Daniel and his team, serves as the primary research source in this topic (67). They investigated the potential negative effects of *A. aspera* on embryonic

development, with a particular emphasis on several developmental indicators. Their findings show that high doses of *A. aspera* can cause developmental defects and impede the growth of specific structures in the embryonic context during day 12 and day 20 experiments.

The prenatal toxicity of *A. aspera* leaves focusing on the effects observed on day 12 embryos revealed significant adverse effects associated with high dose treatment (67).

The study indicated a reduced number of implantation and somites in embryos from the high dose-treated rats. This suggests a potential disruption in the early stages of embryonic development, which can impact the overall formation and organization of tissues and organs (91–93). Furthermore, the high dose treatment resulted in retarded development of various systems in the embryos. The hind limb, forelimb, optic, and olfactory systems exhibited delays in their development. These delays may affect the proper formation and functioning of these vital systems, which are essential for locomotion, sensory perception, and overall survival (94,95).

Additionally, the number of branchial bars, also known as pharyngeal arches, was significantly reduced at higher doses. The branchial bars play a critical role in the development of the respiratory and circulatory systems in vertebrate embryos. As it was explained previously, the reduction in the number of pharyngeal bars indicates potential disruptions in the proper formation of these important anatomical structures, which can have long-term implications for the respiratory and circulatory functions of the developing embryos (83). These observations emphasize the sensitivity of developing embryos to the phytochemicals present in *A. aspera* leaves and underscore the importance of further research to understand the underlying mechanisms and specific bioactive compounds responsible for these developmental toxic effects.

A. aspera developmental toxicity assessment in day 20 fetuses produced a number of significant findings about the plant's effects on fetal development. At greater doses of A. aspera leaves, a substantial reduction fetal weight and CRL was seen. This suggests that the larger dose may have negative effects on the size and overall development of the fetuses, as well as the possibility of growth retardation. It is remarkable, nonetheless, that at all of the tested doses, no outward malformations were found in the kids. This shows that exposure to A. aspera leaves at the levels used during the study had no discernible impact on the physical structure and appearance of the progeny.

***Catha edulis*:** Despite extensive research on the general toxicity profile of *C. edulis*, animal studies produced different data about its impact: Female fertility was lowered, and sperm abnormalities, including testicular tissue deterioration and lower testosterone levels, were discovered in mice and rats given chat extract (96,97). When administered *C. edulis*, rabbits showed improved spermatogenesis and healthy Leydig cells (98). *C. edulis* medication also increased testosterone levels while decreasing prolactin and cortisol levels in olive baboons (99). On the other hand, when pregnant guinea pigs were exposed to *C. edulis*, their offspring experienced decreased placental blood flow and growth retardation (100). However, only two articles are discovered that declared the effect of *C. edulis* on the prenatal development of experimental animals (101,102).

The findings from day 12 experiment revealed a number of noteworthy effects regarding the plant's impact on both maternal health and embryonic growth (102). The treatment animal group showed a significant decrease in body weight gain, implying that the developing embryos experienced growth inhibition. The food consumption was also significantly reduced, signaling negative repercussions that can result in poor maternal health and impede correct fetal development, potentially leading to health issues in the offspring's later life (103).

Additionally, the group receiving high dose of *C. edulis* had a greater incidence of fetal resorptions, which suggested a higher risk of fetal fatalities and possible disturbances in embryonic development. As a result, the number of implantation sites significantly decreased, indicating problems with embryo implantation and establishment (104,105).

The study also found significant effects of *C. edulis* on the development of essential embryonic systems, such as yolk sac circulation and heart development, indicating potential cardiovascular impacts on the developing embryos (106).

Moreover, there were notable delays in the development of crucial systems and structures, which played vital roles in sensory and skeletal development. Furthermore, the treatment had a substantial impact on how the maxillary process, forelimb, and hind limb developed, raising the possibility of limb deformities and functional ramifications for the developing limbs. In the *C. edulis* treatment group, there were fewer somites, which are essential for embryonic segmentation and may indicate potential disturbances in normal embryonic organization (76,107)

Generally, the importance of further research to understand the underlying mechanisms and potential long-lasting effects of *C. edulis* on maternal health and fetal development was highlighted by these findings.

The *Catha edulis* trial on day 20 revealed significant effects on the health of the mother and fetus. There were fewer fetuses and less maternal weight gain in the therapy group, suggesting possible negative pregnancy outcomes. The decrease in implantation sites and live fetuses also suggests that embryo survival may be challenging. Growth retardation was shown by the drastically reduced fetal weight and crown-rump length. A placenta examination revealed lesions, and trophoblast necrosis and vascular abnormalities were visible in the labyrinth. Examinations of the fetal external, visceral, and skeletal structures revealed variances and abnormalities. These results underscore the need for more evaluation of effects of *Catha edulis* on maternal and fetal health, as well as its developmental toxicity (101,102)**.**

***Thymus schimperi*:** Despite the numerous medicinal claims made for *T. schimperi*, just one study revealed the developmental safety profile, a major toxicological endpoint, of the plant's essential oil (68). During day 12 experiment, maternal weight was considerably reduced, as were fetal resorptions and the number of implantation. Rat embryos treated with a high dose of essential oil had a considerably shorter crown-rump length (CRL), fewer somites, and a worse morphological score. In addition, significant changes in the developmental characteristics of the otic system, olfactory system, and branchial bars were found in the high-dose group. These data indicate that a high dose of essential oil has a negative impact on maternal weight gain and fetal development, influencing different developmental parameters in rat embryos (68).

Fetal toxicity end points indicated that maternal weight was dramatically reduced in the treatment group, and fetal resorption was significantly increased. Furthermore, there was treatment related decrement in terms of fetal number and viability, with the high-dose treatment group having a significantly lower mean litter weight. Furthermore, in the higher dose therapy group, placental weight was dramatically lowered. These findings suggest that the treatment's high dose had a negative impact on mother weight gain, fetal development, and placental health (68).

1.2.Statement of the problem

In spite of numerous researches showcasing the effectiveness of African herbal remedies for diverse human health care maintenance, many uncertainties persist, specifically concerning the safety of employing these botanical medications (108). Multiple inquiries have revealed that compounds extracted from plant-based sources often influence the viability and usual operations of nearly all organisms to some extent.

Toxicity studies on medicinal plants are relevant to evaluate the safety before they are used as a remedy for humans. In particular, the effects of herbs on the developmental process of embryos and fetuses need to be examined, as these stages are vulnerable to toxic insults (109).

Because of their embryonic and fetal development similarities to humans, rat embryos and fetuses are often used as models to study the impact of hazardous chemicals on prenatal development (110,111). However, there is currently a scarcity of research on the toxicity potential of many Ethiopian medicinal herbs, particularly their impact on fetal development, which is critical in detecting potential hazards and raising awareness for clinical decision-making (112,113).

In Ethiopia, where nearly 30% of pregnant women tend to use medicinal plants for various purposes, *E. schimperi*, a plant revered for its diverse pharmacological value, is deeply embedded in traditional health practices (114,115). Known for its analgesic, antimicrobial, anti-inflammatory and anthelmintic properties, and this plant has been an integral part of traditional Ethiopian medicine for generations (114–116)(117). Nevertheless, even with its extensive application and therapeutic promise, little is known about *E. schimperi's* possible harm to developing organisms. Pregnant women rely heavily on medicinal herbs, such as *E. schimperi*; hence, it is critical to assess these plants' possible risks to the growing fetus in a methodical manner.

1.3.Significance of the study

Teratogenicity studies represent a predominant part of the preclinical safety assessment of drugs and chemicals used in human therapy. Today, one of the main concerns of all those involved in drug development, since the action of thalidomide, is a systematic approach to experimental dermatology, making it a mandatory requirement that all drugs and chemicals are routinely tested for their safety during pregnancy and as Women are tested for childbearing potential. In addition to having significant personal and financial implications, reproductive problems and developmental anomalies, particularly congenital malformations, have posed a significant threat to public health (118–120).

Developmental toxicity evaluation of *E. schimperi* is of fundamental importance in the field of medical research and public health. *E. schimperi*, a plant deeply rooted in traditional medicine and known for its pharmacological properties, has gained recognition for its diverse therapeutic uses (121,122). However, despite its extensive use, there remains a critical gap in research on the potential developmental toxicity of this plant.

Understanding the potential risks of developmental toxicity is critical, particularly given that *E. schimperi* and similar herbals are utilized for health reasons by a significant portion of the population, including pregnant women (115). Adverse effects on the developing fetus, such as congenital abnormalities and growth failure, are referred to as developmental toxicity (123). Assessment of these potential risks is important to ensure the safe use of *E. schimperi*, particularly during pregnancy and in women of childbearing potential.

The results our study will not only fill the research gap but also provide evidence-based information on the safety of *E. schimperi*. Identifying the potential risks associated with its use is crucial for formulating guidelines and recommendations, thereby promoting responsible use in traditional and modern health practices. Furthermore, this research is a step towards merging traditional wisdom with modern health standards, balancing ancient practices with evidence-based knowledge.

Ultimately, the results from the current study will have far-reaching indications for health practices and aim to protect the health and well-being of mothers and their unborn children, not only in Ethiopia but also in regions where *E. schimperi* is used.

1.4.Literature review

1.4.1. Vegetation and distribution

E. schimperi is a plant from *Myrsinaceae* at usually grows up to 7 meters tall and is often found as a climbing shrub. Its bark is smooth and reddish brown. The branches are hairless but have bright, raised spots that act as breathing pores. The leaves are oval and reach dimensions of about 8 cm long and 4 cm wide, with a rounded tip and a narrower base than the central part. The central vein of the leaf is distinctly red; the stem can be up to 2 cm long. The central vein and around 15 side veins arise on the underside of the leaf. The tiny flowers, colored in shades of green, white or cream, grow on hairy stems from the leaf axil. When ripe, the fruits, which are frequently abundant on the stalks, are spherical and reach a diameter of around 6 cm. The ripe fruit turns red and contains a single seed. *E. schimperi* displays a marginal ecological footprint. As a plant indigenous to its natural surroundings, it thrives without imposing notable damage on the environment. Nevertheless, excessive harvesting of this plant for medicinal use has the potential to exhaust its numbers and influence the biodiversity of the region (124,125)

E. schimperi is found mainly in East Africa, particularly in the highlands of Ethiopia and Kenya. It grows in Ethiopian mountain forests and is commonly found in the Aberdare Mountains and other mountainous regions of Kenya. In addition, it is spreading to other East African countries, contributing to regional biodiversity (126).

1.4.2. Traditional claim of *E. schimperi*

The traditional use of *E schimperi* has a lengthy history and is well ingrained in the regional cultural landscapes. Traditional societies depended on the plant's purported medical benefits and treated various ailments using various portions of the plant (127).

Roots: Traditional medicine holds *E. schimperi* roots in high regard, particularly for gastrointestinal disorders. Root extracts are frequently used in the local populations to treat dysentery, treat diarrhea, and ease abdominal pain. The roots are a crucial component of traditional treatments because they have the ability to calm the digestive system and ease discomfort brought on by gastrointestinal issues (128,129).

Leaves: *E schimperi* leaves are yet another crucial element of conventional medicine. Leaf extracts that have been crushed or processed are thought to have anti-inflammatory effects, making them effective for treating skin disorders. These extracts are applied topically to treat dermatitis, eczema, and other skin diseases in various cultures. The leaves' potential medicinal effects are highlighted by the fact that they are made as poultices or ointments and applied directly to the affected areas (130,131).

Bark: Because of its anthelmintic qualities, the bark of *E schimperi* is highly valued in traditional medicine. Local populations for a long time to treat parasite illnesses and intestinal worms have utilized bark extracts. Usually used orally, infusions or decoctions of the bark are supposed to flush parasites from the digestive tract (132). This ancient custom reflects the conviction that the bark may effectively rid the body of unwanted germs.

Fruits: Traditional medicine makes extensive use of the berry-like fruits of *E schimperi*. Since these fruits are thought to have expectorant qualities, respiratory disorders are frequently treated with them. Fruit extracts are used to remove mucus from the respiratory tract in conventional medicine, which helps to treat illnesses including asthma and bronchitis. This traditional application is in line with the notion that the fruit may help to promote lung health (133,134).

Tonic for General Well-Being: In addition to the unique benefits for each of its components, *E schimperi* is highly regarded as a tonic for overall health. According to folk medicine, the herb fosters general health and energy. It is thought to strengthen the immune system and contains antioxidant qualities to fight oxidative stress. Against preserve their wellbeing and build resilience against various health issues, local communities incorporate *E. schimperi* into their routine health programs(135).

The traditional claim of *E schimperi* is deeply rooted in the wisdom and practice of traditional medicine. Various parts of the plant used for their perceived medicinal properties. These traditional uses underscore the cultural significance of the plant and its role in community health practices and underscore the importance of preserving traditional knowledge for future generations (136,137). However, scientific research is needed to validate these traditional claims and effectively integrate them into modern healthcare.

1.4.3. Phytochemical and pharmacological profile of *E schimperi*

E. schimperi possesses various phytochemical properties containing a variety of bioactive compounds (136,138). The plant's main constituent is embelin, which is a bright orange crystal in its purest form (121,139–141). Alkaloids and terpinoids are also prominent group of phytochemicals found in *E. schimperi* Vake fruit (141). The plant also contains flavonoids and amphiphilic phenolic lipids such as resorcinolic lipids (142,143). The fruit of plant is also rich in various types of nutritionally rich fatty acids such as linoleic, palmitic and oleic(144).Due to these plant metabolites, *E. schimperi* has been identified to have a wide range of therapeutic relevance such as: anti-helimenthic, antimicrobial, anti-inflammatory, anticancer and Anti-fertility (116,142,145,146).

1.4.3.1.Embelin

The main bioactive component of *E. schimperi* is thought to be the quinone molecule embelin. Quinones are a type of chemical compounds that have two ketone functional groups on each of their six members of the aromatic ring. Embelin, sometimes referred to by its chemical name 2,5-dihydroxy-3-undecyl-1,4-benzoquinone, has a special chemical make-up and structure (122,147).Due to its wide range of impacts on biological systems, embelin has significant pharmacological relevance. The medical and research sectors are very interested in its potential as a versatile therapeutic agent (148,149).

1.4.3.1.1. Anti-cancer activity

Embelin has powerful anti-cancer capabilities that can be used to treat different types of cancers, such as breast, prostate, hepatic, pancreatic, colon, gastric, leukemia, and multiple myeloma. Previous experiments conducted showed that embelin works against tumor cell growth and migration, triggers apoptosis, and prevents invasion, metastasis, and angiogenesis (150,151). It significantly inhibits the expression of XIAP, a crucial apoptotic protein inhibitor (IAP), and functions as an NF-B blocker. The anticancer effects of embelin are the result of numerous molecular pathways. It suppresses NF-B, a crucial regulator of tumor cell growth and progression. Embelin inhibits critical components involved in the invasion and growth of tumors by preventing the expression of anti-apoptotic and metastatic genes regulated by NF-B (152). Embelin also controls PPAR activity, upregulates P53 expression, and suppresses JAK/STAT3 signaling (153).

It also modifies the Akt/mTOR/S6K1 pathway(154). Through a multi-target approach, it also affects the TNF- pathway and inhibits osteoclastogenesis, making it a promising drug in the battle against cancer(155).

1.4.3.1.2. Anti-bacterial activity

Embelin exhibits strong antibacterial qualities, making it a possible treatment for bacterial illnesses. Studies have demonstrated its effectiveness against different bacteria, pointing to its potential use as a natural antibacterial agent. Embelin is a viable candidate for the creation of new antibacterial medications as a result of this feature (156).

Embelin inhibits various strains of Staphylococcus aureus (157). Additionally, it has bacteriostatic action toward Gram-negative organisms and bactericidal activity toward Gram-positive organisms(158). Embelin has exhibited synergistic antibacterial activity when combined with medicines like oxacillin and tetracycline, especially against strains of Staphylococcus aureus that are multi-drug resistant(139). By preventing Streptococcus mutans, a major cause of dental infections, from forming biofilms, embelin may also be effective in treating oral infections (159).

1.4.3.1.3. Anti-inflammatory activity

With a strong focus on its anti-inflammatory and antioxidant qualities, embelin, a benzoquinone derivative found in *Embelia ribes*, possesses significant therapeutic value. Embelin's capacity to block the NF-B pathway, a crucial regulator of inflammatory reactions, is what gives it its anti-inflammatory properties. Embelin efficiently reduces the generation of inflammatory mediators by modifying NF-B activity, establishing it as a potential therapeutic agent for treating inflammatory illnesses(160). Due to this quality, embelin is a potential option for the management of inflammatory diseases.

According to Bansal and his colleagues, embelin has recently shown its ability to reduce inflammation and oxidative stress in obese mice via a high-fat diet. The study elaborated on embelin capacity to control the NrF-2 and NF-B signaling pathways, offering it as a potentially effective treatment approach to combat obesity brought on by dietary variables (161). Additionally, Li et al.'s descriptions of the pharmacokinetics and oral bioavailability studies of embelin highlight its potential medical applications and clinical efficacy (162). According to Qin

et al., embelin exhibits significant effectiveness in reducing the nephrotoxicity brought on by cisplatin through its modulation of the Nr2/OH-1 signaling. This is in addition to its anti-inflammatory characteristics (163). Embelin has been proposed as a candidate for the treatment of disorders like cancer that are made worse by inflammation (164–166)

1.4.3.1.4. Anti-malarial activity

Embelin have been identified to have potential of antimalarial properties through a mechanism akin to 4-aminoquinoline and chloroquine via inhabitation of hemozoin formation (148). Embelin's probable binding to heme iron, inhibiting hemozoin formation and consequently parasite growth. Additionally, Bezu et al. conducted assessments of embelin and its derivative to evaluate the antimalarial potential, revealing superior results for the derivatives without jeopardizing precursor safety(167). Srivastava et al. reported embelin's antimalarial effect, proposing a mechanism involving PCAF/GCN5 family histone acetyltransferase inhibition, leading to reduced parasitic growth at low embelin concentrations(168). Thota et al. (2016) explored isolation, characterization, and antimalarial effect of embelin derivatives, highlighting moderate antimalarial activity of compounds such as 6-methylembelin, 4-hydroxy-2-methoxybenzamide, 5-O-ethylembelin and 5-O-methylembelin 4 compared to embelin.

1.4.3.1.5. Anthelmintic activity

Anti helementhic activity is the most commonly reported therapeutic roleof embelin, particularly against beef tapeworms and various intestinal parasites. This pharmacological role was further signified by Debebe and his colleagues through anthelmintic activity of Embelin against Hymenolepis and Hymenolepis strains that indicated a strong potential of the compound against the aforementioned helminthes (121). Embelin has exhibited anthelmintic properties against intestinal cestodes, trematodes, nematodes, and hookworm larvae *in vitro*, and its efficacy against certain parasites has been confirmed *in vivo* , underlining its potential in anthelmintic treatments (167).

1.4.3.1.6. Anti-fertility activity

In numerous investigations, the bioactive ingredient embelin has shown to have a strong antifertility effect. The disruption of female subjects' estrous cycles, which results in altered reproductive patterns, is one of its important effects. Studies have repeatedly shown that embelin

treatment significantly lowers plasma levels of important sex steroid hormones, oestradiol and progesterone (169). The delicate hormonal balance that is necessary for regular menstrual and reproductive cycles has been disturbed due to the administration of embelin.

Embelin has also been seen to reduce the ovarian synthesis of sex steroid hormones such oestradiol and progesterone, which is how it is thought to exercise its antifertility effects. The regularity and intensity of the estrous cycle are also impacted by this hormonal suppression, adding to its antifertility effects (122). Interestingly, embelin has demonstrated much stronger antifertility effects when complexed with borax, significantly changing both progesterone levels and the estrous cycle (170). Embelin has also been connected to harmful effects on male fertility, including a decrease in testosterone levels and a change in the quantity and quality of semen (171). The potential of embelin as a contraceptive drug is highlighted by these diverse effects, which call for more investigation into its processes and uses in fertility control.

1.4.3.2.Alkaloids

Alkaloids, nitrogen-containing secondary metabolites constituted in *E. schimperi* that are commonly detected using potassium bismuth iodide (Dragendorff's reagent). They have a big impact on the pharmacological profile of the plant. Specific alkaloids from *E. schimperi* have been found and researched for possible therapeutic uses on the neurological system, operate as antioxidants, or possibly have anti-inflammatory characteristics. The presence of alkaloids in *E. schimperi* underlines the plant's potential therapeutic usefulness and suggests that at least some of the traditional claims of the plant are attributed to these bioactive substances (141,172).

1.4.3.3.Flavonoids

Another class of secondary metabolites frequently found in *E. schimperi* is flavonoids, which are well-known for their antioxidant capabilities. These compounds efficiently scavenge free radicals and reduce oxidative stress, related to a number of disorders. Additionally, flavonoids exhibit anti-inflammatory qualities, suggesting that they may help to lessen inflammation and associated diseases. Additionally, they might have antibacterial properties that help fight illnesses brought on by bacteria, fungus, or other pathogens. Furthermore, flavonoids in *E. schimperi* may help explain the plant's possible antiviral and anticancer effects, making it a promising candidate for further study in contemporary medicine (143,173).

1.4.3.4.Terpinoids

Terpenoids are among the most significant secondary metabolites in *E. schimperi* with possible medicinal significance. Numerous biological effects, including anti-inflammatory, antibacterial, antioxidant, and anticancer properties, are known to be possessed by terpenoids (141). They are important for the development of anti-inflammatory medicines because their anti-inflammatory characteristics to be crucial in treating illnesses linked to inflammation (174). Terpenoids may also have antibacterial effects on different microbes, which could be useful in the fight against illnesses (175). Terpenoids found in *E. schimperi* are attributed to its antioxidant abilities, successfully working against free radicals and lowering oxidative stress. This antioxidant capability is important for proper cellular activity and is presumed to boost immunity against various communicable and non-communicable illnesses (176). Furthermore, due to the interactions of terpenoids with multiple cellular targets and signaling pathway, plants rich with these metabolites like *E. schimperi* possessed wide range of pharmacological effects that helps them to be potential as a valuable resource for both conventional and contemporary medicine (177).

1.4.4. Toxicity profile of *E. schimperi*

Various studies conducted on the toxicity of *E. schimperi*, a plant with considerable traditional and medicinal relevance has provided valuable insights into its safety profile. Acute toxicity studies involving rodents evaluating the potential toxicity effect associated following single dose administration have indicated a notably high safety margin for the hydro-alcoholic extract of *E. schimperi* fruit, suggesting mean lethal dose (LD50) exceeding 5000 mg/kg. This high LD50 value provides a reassuring indication of the plant's relatively low when administered acutely, implying that significant amounts of the extract would need to be consumed to induce lethal effects (121,178).

Moreover, chronic exposure of hydro-alcoholic extract of the plant has demonstrated a lack of adverse effects on crucial physiological indicators. In particular, the test did not lead to any discernible alterations in the hematological profile or the microstructure of vital organs such as the kidneys and liver in Wistar rats (76). This long-term assessment further emphasizes the plant's potential safety even with extended or repeated exposure, supporting its traditional use without evident chronic toxicological concerns (179).

Concordantly, a study done by Sumalatha K.R and Sreepriya M reported that embelin is a non-mutagenic plant based compound upon employing Ames test with Salmonella typhimurium strains *in vitro*. Furthermore, the same study evaluated the effect of embelin on the proliferation and viability of peripheral blood cells and murine macrophages using MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) test. The result showed that embelin has no cytotoxic effect on the aforementioned important immune cells (180).

In a study conducted by Anand O. Prakash, female rats were administered embelin for a period of 6 weeks, and the outcomes revealed significant effects on various organs from rats treated with higher dose of embelin. The adrenal gland weights were significantly increased further signified by adrenal hypertrophy upon histopathological examination. In liver histology, disintegration, necrotic changes, and perinuclear vacuolation were observed, suggesting potential hepatotoxic effects due to embelin administration. Furthermore, marked tubular damage was noted during microscopic evaluation of kidney tissues, indicating adverse effects on renal microstructure structures (181). These findings shed light on the potential physiological impacts and risks associated with the administration of embelin over a prolonged period.

1.4.5. Reproductive toxicity profile

As mentioned in the anti-fertility profile, different studies have been reporting about the fact that embelin has significant effect in the reproductive system through various mechanisms. According to E. O. Wango, female Sprague-dawley rats treated with embelin blended with corn oil exhibited notably decreased oestradiol and progesterone level both *in vivo* and *in vitro*, which is suggestive of embelin's effect in disrupting production of sex hormones(169). Experiments on male rats revealed a similar effect of embelin on sex hormones, noting that the molecule has a considerable effect in lowering testosterone levels, which is exacerbated further by extended administration (182).

Another study, by Gupta et al., found that subcutaneous administration of embelin isolated from Embelia specious caused infertility by negatively altering glucose metabolism in male rat reproductive tissues, which was reversed when the treatment was stopped (183). Furthermore, *in vivo* and *in vitro* treatment of embelin in rat epididymis sperm solution resulted in reduction of spermatozoa motility and morphological changes manifested by decapacitation and cytoplasimic derangements across the length of the sperm cell (184).

Despite the fact that embelin, a benzoquinone and an essential component of the fruit of *E. schimperi*, has been widely recognized to have a substantial effect on the reproductive system, little is known about its developmental toxicity profile, particularly how it would affect fetal and embryonic outcomes. Furthermore, the *in silico* toxicity profile, which contributes in the prediction of other toxicological profiles of plants with high embelin content, has yet to be computed. Furthermore, the developmental toxicity/teratogenicity of the crude extract has not yet been examined. Furthermore, because the fruit of *E. schimperi* contains a variety of fatty acids, its toxicity at single and repeated doses, as well as developmental toxicity/teratogenicity, is unknown.

As a result, the primary goal of this study is to determine the developmental toxicity/teratogenicity of crude extract of *E. schimperi* fruit, as well as to examine the *in silico* toxicity prediction of embelin isolated from *E. schimperi* Vake fruit and evaluate its impact on rat development. Because previous studies yielded inconsistent results, the acute toxicity of embelin was investigated further in this study. Furthermore, the acute and sub-chronic toxicity of fixed oil extract, as well as its effect on rat intrauterine development, will be investigated.

Chapter two

2. Objectives

2.1.General Objective

To evaluate the *in vivo* acute, sub-chronic and developmental toxicity profile of crude and fixed oil extract and embelin compound isolated from *E. schimperi* Vake fruit on albino Wister rats and predict the *In silico* property of embelin

2.2.Specific objectives

1. To evaluate *in vivo* developmental toxicity effect of crude extract of *E. schimperi* Vake fruit on 12-days old albino Wister rat embryos and 20-days old fetuses.
2. To assess acute toxicity effect of embelin isolated from *E. schimperi* Vake fruit on albino Wister rats
3. To assess 90 days repeated dose sub-chronic toxicity effect of embelin isolated from *E. schimperi* Vake fruit on albino Wister rats
4. To evaluate *in vivo* developmental toxicity effect of embelin isolated from *E. schimperi* Vake fruit on 12-days old albino Wister rat embryos and 20-days old fetuses.
5. To assess acute toxicity effect of fixed oil extracted from *E. schimperi* Vake fruit on albino Wister rats
6. To assess 90 days repeated dose sub-chronic toxicity effect of fixed oil extracted from *E. schimperi* Vake fruit on albino Wister rats
7. To evaluate *in vivo* developmental toxicity effect of fixed oil extracted from *E. schimperi* Vake fruit on 12-days old albino Wister rat embryos and 20-days old fetuses.

3. Methodology

3.1.Plant material collection

The fruit part of *E. schimperi* Vake fruits were collected from Debre Markos localities located 305 km away from the capital Addis Ababa, Northwest Ethiopia. The fruit, steam and root of the plant was pressed and taken to Department of Plant Biology and Biodiversity Management, Addis Ababa University to identification and authentication where a voucher specimen was given by a botanist.

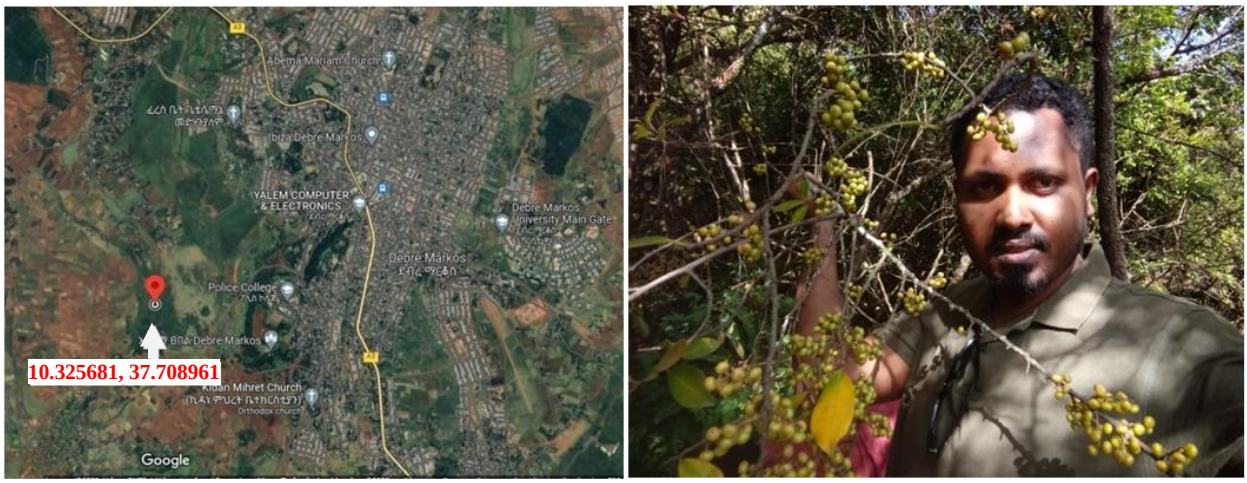

Figure 1: A photograph taken during the period of plant collection from Debre markos locality

3.2.Plant material preparation

3.2.1. Crude extract preparation

E. schimperi fruits were dried at room temperature for two weeks at the plant herbarium of Ethiopian Public Health Institute. The dried fruits were then ground. The powder was first defatted using *n*-hexane in a Soxhlet apparatus. Maceration of 100 g of the marc 1 L of 80% ethanol was conducted in a ratio of 1:10 (w/v). A yield of 12.8% dried crude extract was obtained.

3.2.2. Isolation of embelin

Embelin was isolated by modifying a method used by Belete et al. (185). Using an electric grinding mill, *E. schimperi* fruits were crushed into a coarse powder. The powder (1000 g) was macerated in ethyl acetate at a ratio of 1:10 (w/v) (plant material/solvent) for 48 hours while being shaken at 160 rpm. After that, the sample was filtered via filter paper. Two times, the marc was re-macerated.

The mixed filtrate was gathered in a glass container and kept cold for 72 hours. Bright orange-colored crystals of embelin formed at the bottom of the glass container (**Figure2**). The crystals were separated by decantation, and n-hexane was used to repeatedly wash the separated crystals until the solvent was colorless. NMR spectroscopic verification revealed that the isolated compound was a pure crystallized embelin with a chemical formula of **$C_{17}H_{26}O_4$** (**Figure 3**), and it was then refrigerated for use in later investigations.

Figure 2: Bright orange-colored crystals of embelin sedmented after macerating with ethyl acetate.

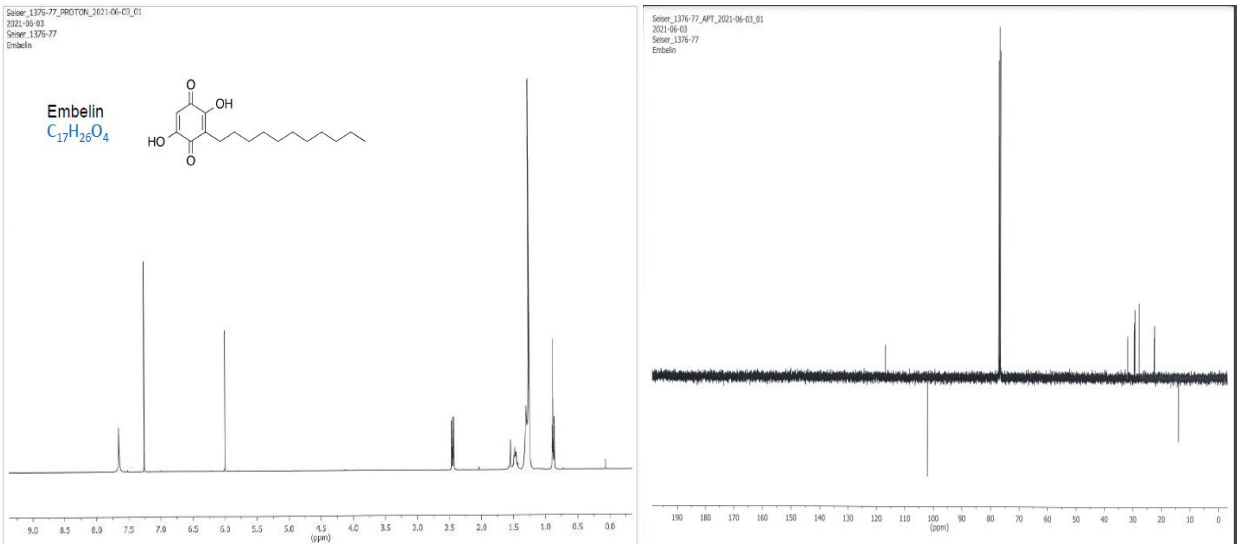

Figure 3: NMR output of embelin

3.2.3. Fixed oil extraction

Soxhlet extractor apparatus was used to extract the fixed oil of *E. schimperi* Vake. After cleaning and drying the *E. schimperi* Vake fruit, 1500ml of n-hexane (a non-polar solvent) was put to a round bottom extraction flask (distillation flask) that was placed on a heating device with a temperature control. The thimble was filled with 500g of coarsely ground plant material, which was then put into the extraction chamber. The heating device was then allowed to reach a temperature of 600°C, during which the n-hexane in the distillation flask was heated and boiled. As a result, the n-hexane evaporated through the vertical tube and reached the condenser, where it was converted into condensate and then penetrated the thimble to extract the fixed oil component of *E. schimperi* (**Figure 4**).

Figure 4: Extraction of fixed using Soxhlet extractor apparatus

3.3. *In silico* toxicity prediction

The Canonical Simplified Molecular Input Line Entry System (SMILES) [CCCCCCCCCCCC1=C(C(=O)C(C1=O)O)O] and PubChem CID (3218) of embelin were extracted from the PubChem- National institute of health (NIH) database (186). Both Canonical SMILES and the 2D structure of embelin, generated by ChemDraw, were used as inputs to generate *In silico* toxicity predictions at the ProTox II server (187). ProTox-II is a freely available *in silico* toxicity predictive model built by a combination of machine learning algorithms to predict toxicity probabilities by incorporating *in vivo* and *in vitro* toxicological assays. The reason for preferring ProTox was that it incorporates both chemical and molecular target knowledge, making it a comprehensive tool for predicting toxicological endpoints. Moreover, it stands out from other models due to its classification scheme, which divides the prediction scheme into various levels of toxicity (188,189).

3.4.Acute and sub-chronic toxicity of embelin

3.4.1. Acute toxicity

3.4.1.1.Experimental animal preparation

Organization for Economic Co-operation and Development (OECD) protocol number 425 was strictly followed for the acute toxicity experiment (190). Female albino Wistar rats of 12 weeks old weighing 220–250 grams free from previous experimental procedures were used for the test. The animals were maintained in an environmentally optimal condition and left untouched for 5 days before the experiment to let them adapt to the experimental environment, during which they were given unlimited food and water.

3.4.1.2.Dose preparation and administration

A limit test was performed using rats that were successively dosed with 5000 mg/kg of embelin dissolved in 7% tween 80 in accordance with OECD 425 guidelines. After administering 5000 mg/kg of embelin to a single female rat at first, three additional animals were dosed after 48 hours since there was no death. Five animals were used as the vehicle's control group. Prior to dosing, animals fasted for one night. Using a stomach tube, embelin and the vehicle, tween 80 were both given by gavage.

3.4.1.3.Toxicity signs and necropsy

Mortality, coma, convulsions or tremors, feces consistency, hair, itching, respiration, salivation, sleeping pattern, somato-motor activity, and behavior pattern were evaluated as signs of toxicity every 30 min for the first 4 h after administration (191). Observation was continued on a daily basis for the rest of the experimental period, until the 14th day after administration. All animals were subjected to necropsy after anesthesia with sodium pentobarbital 150 mg/kg intraperitoneal in order to assess gross pathological changes of visceral organs such as the liver, kidneys, spleen, heart, and intestines.

3.4.2. Sub-chronic toxicity

3.4.2.1.Experimental animal preparation

EPHI's animal breeding division offered albino wistar rats for both sexes weighing 200 to 250 grams and ranging in age from 10 to 12 weeks. A total of 80 rats, 40 males and 40 females were

used in the sub chronic experiment. During the acclimatization period, the animals were housed in an environment similar to that of the acute toxicity test animals.

3.4.2.2.Dose preparation and administration

The rats in the experiment were placed into four groups, each having ten rats of each sex. The first three groups, labeled Group I, Group II, and Group II, received 250 mg/kg, 500 mg/kg, and 1000 mg/kg of embelin suspended in tween 80, respectively, as well as free access to tap water and food. Tween 80 was given at a rate of one milliliter per 100 grams of body weight. The animals in Group IV were classified as the vehicle control group and were given access to tap water, food, and tween 80. The sub-chronic toxicity dosages were determined using the results of the acute toxicity test (LD_{50}>5000mg/kg). Oral gavage was used to administer embelin and the vehicle.

3.4.2.3.Cage side clinical examination

Throughout the study, experimental animals were evaluated on a regular basis for any clinical symptoms of toxicity, such as death, coma, convulsions, tremors, breathing issues, and skin abnormalities.

3.4.2.4.Food intake and body weight

For the length of the experiment, each experimental group's daily food consumption was monitored every day at the same time. Each animal's weight was recorded on the first day of the experiment and then weekly thereafter throughout the duration of the experiment. As a result, the weight gain was computed by subtracting the starting and ending weights.

3.4.2.5.Relative organ weight of vital organs

The experimental rats were allowed to stop eating for overnight before being anaesthetized with sodium pentobarbital 150 mg/kg intra-peritoneal for necropsy. An anterior abdominal incision was then made to reveal the abdominal cavity. The liver and kidneys were explanted after the surrounding tissue was removed and inspected for externally evident pathological abnormalities. A calibrated digital balance (Mettler AE160) was used to measure the weight of liver, kidneys, uterus, ovaries, testes, epididymis, and seminal vesicles. The absolute organ weight was reduced by the final rat weight and multiplied by 100 to get the relative organ weight.

3.4.2.6.Hematology and Clinical Biochemistry

Blood was collected from each experimental animal during necropsy via cardiac puncture. For hematological parameters, blood samples were collected using EDTA-containing tubes. Within 10 minutes of collection, the Sysmex-XN550 analyzed blood samples for hematological analysis. The hematological parameters evaluated were mean corpuscular volume, mean corpuscular hemoglobin, packed cell volume, total red blood cells, and mean corpuscular hemoglobin. Additionally, the assessment included red blood cell distribution width, platelet count, total white blood cells, lymphocytes, monocytes, neutrophils, and hemoglobin concentration. The clinical chemistry blood sample was placed in non-heparinized test tubes. Serum was separated after an hour by centrifuging the blood for 10 minutes and collecting it with a micropipette. COBAS 6000, Germany, analyzer was utilized for clinical chemistry analysis. Total protein, urea, total cholesterol, glucose, ALT, creatinine, AST, and albumin were assessed as end points of biochemical analysis (Figure 5). Additionally, hormone analysis was conducted through enzyme-linked immunosorbent assay (ELISA).

Figure 5: Blood sample collection via cardiac puncture and preparation for blood chemistry evaluation.

3.4.2.7.Histopathological evaluation of organs

Samples from the liver, kidneys, spleen, adrenal gland, ovary, uterus, testes, epididymis, and seminal vesicles of each experimental group were preserved for 24 hours in a 10% formalin

solution. Then, using a Leica TP 1020, tissue processing (dehydration, cleaning, and impregnation) was carried out. After that, the tissues were ready for sectioning by being covered with paraffin wax. The tissue to be sectioned by the microtome for light microscopy has a thickness of 5 m. Lastly, hematoxylin and eosin was used to stain the tissues.

3.4.2.8. Sperm Analysis

To simplify sperm analysis, semen taken from the distal end of the epididymis following surgical incision was diluted with 5 grams of sodium bicarbonate (NaHCO3) mixed in 99 ml normal saline and 1 ml 40% formalin. As a result, a microscopic assisted sperm count was performed in 5 squares using a Neubauer hemocytometer chamber (192).

In addition, the proportion of normal and abnormal sperm morphological appearances was calculated for morphological evaluation. The presence of structural traits was classified as abnormal sperm morphology (193).

3.4.2.9.Estrous cycle

Daily vaginal swab was meticulously taken from each rat during the course of the experimental period. These vaginal swabs were acquired expressly for Giemsa staining, a well-known technique for identifying and characterizing the several phases of rat estrous cycle, which include proestrus, estrus, metestrus, and diestrus via the characteristic features of cells morphology.

3.5.Acute and sub-chronic toxicity of fixed oil

3.5.1. Acute toxicity test

3.5.1.1.Experimental animal preparation

We used 12-week-old nulliparous female albino Wistar rats that had never been subjected to any previous experimental procedures. The animals were housed in a metallic cage made of stainless steel, with a controlled 12-hour light/dark cycle, ambient temperature of 22 °C, and 50%–60% relative humidity. The animals were acclimated for five days before the testing began. The animals were provided with an unlimited supply of food pellets and tap water during the acclimatization phase.

3.5.1.2.Dose preparation and administration

A limit test was performed in accordance with the OECD 425 guidelines by treating rats successively with 5000mg/kg of fixed oil dissolved in tween 80. First, a single female rat was given 5000mg/kg of fixed oil, followed by three more animals after 48 hours if no fatalities occurred. Five animals were used as the vehicle's control group. Prior to dosing, the animals were fasted for more than a night. Both fixed oil and vehicle were given via gavage through a stomach tube.

3.5.1.3.Toxicity evaluation and Necropsy

Death, coma, convulsions and tremors, eyes, faeces consistency, hair and skin, itching, mucous membrane, respiration, salivation, sleep, somato-motor activity and behaviour pattern, and urination (colour) were evaluated as symptoms of toxicity every 30 minutes for the first four hours after injection. Throughout the remainder of the experimental period, observation was performed every day. To determine macroscopic pathological alterations of organs, all animals were necropsied by administering sodium pentobarbital 150 mg/kg intra-peritoneal.

3.5.2. Sub-chronic toxicity

3.5.2.1.Experimental animal preparation

The animal breeding division of EPHI provided albino wistar rats for both sexes that ranged in weight from 200 to 250 grams and were between the ages of 10 and 12 weeks. For the sub chronic experiment, 80 rats, 40 males and 40 females, were employed. Throughout the period of acclimatization, the animals were housed in an environment that was comparable to that of the acute toxicity test animals.

3.5.2.2.Dose preparation and administration

The experimental rats were randomly divided into four groups, each with ten rats of each sex. The first three groups were labeled Group I, Group II, and Group II, and were given 250 mg/kg, 500 mg/kg, and 1000 mg/kg of fixed oil suspended in tween 80, respectively, along with free access to tap water and food. Tween 80 was administered at a dosage of 1 ml per 100 g of body weight. Group IV animals were designated as the vehicle control group and given access to tap water, food, and tween 80. The dosages for the sub-chronic toxicity were chosen based on the results of

the acute toxicity test (LD50>5000mg/kg).The fixed oil and the vehicle were given orally through oral gavage.

3.5.2.3.Cage side clinical examination

For the course of the investigation, experimental animals were monitored daily for any clinical signs of toxicity, including Death, coma, convulsions, tremors, breathing problems, and skin changes.

3.5.2.4.Food intake and body weight

Each experimental group had their daily food intake measured every day at the same time for the duration of the experiment. The weight of each animal was recorded on the first day of the experiment and then weekly after that throughout the length of the experiment. As a result, the weight gain was calculated by deducting the starting weight from the ending weight.

3.5.2.5.Relative organ weight of vital organs

At the end of the experiment, the animals were allowed to fast overnight before being anaesthetized with sodium pentobarbital 150 mg/kg intra-peritoneal for necropsy. The abdominal cavity was then revealed by making an anterior abdominal incision. After removing the surrounding tissue, the liver and kidneys were explanted and examined for externally visible pathological abnormalities. The weight of liver, kidneys, uterus, ovaries, testes, epididymis, and seminal vesicles was determined using a calibrated digital balance (Mettler AE160). The absolute organ weight was divided by the final rat weight and multiplied by a hundred to calculate the relative organ weight.

3.5.2.6.Haematology and Clinical Biochemistry

Blood samples were collected from each experimental animal during necropsy via cardiac puncture. For hematological analysis, blood samples were collected in EDTA-containing tubes. Within 10 minutes of collection, the Sysmex-XN550 analyzed blood samples for hematological analysis. The hematological parameters evaluated were mean corpuscular volume, mean corpuscular hemoglobin, packed cell volume, total red blood cells, and mean corpuscular hemoglobin. Additionally, the assessment included red blood cell distribution width, platelet count, total white blood cells, lymphocytes, monocytes, neutrophils, and hemoglobin

concentration. The clinical chemistry blood sample was placed in non-heparinized test tubes. Serum was separated after an hour by centrifuging the blood for 10 minutes and collecting it with a micropipette. COBAS 6000, Germany, analyzer was utilized for clinical chemistry analysis. Total protein, urea, total cholesterol, glucose, ALT, creatinine, AST, and albumin were assessed as end points of biochemical analysis. Additionally, hormone analysis was conducted through enzyme-linked immunosorbent assay (ELISA).

3.5.2.7.Histopathological evaluation of organs

Liver, kidneys, spleen, adrenal gland, ovary, uterus, testes, epididymis, and seminal vesicle samples from each experimental group were putted in a fixative solution (10% formalin) for 24 hours. Afterwards, tissue processing was carried out (dehydration, clearing and impregnation) using automatic tissue processor (Leica,TP 1020). Following that, the tissues were embedded in paraffin wax and prepared for sectioning. For light microscopy, the thickness of the tissue to be sectioned by the microtome was set to 5m. Finally, the tissues were stained with hematoxylin and eosin.

3.5.2.8.Sperm Analysis

In order to facilitate sperm analysis, dilution of semen, extracted from the distal end of epididymis upon surgical incision, was performed by making 5 grams of sodium bicarbonate ($NaHCO_3$) mixed in 99 ml normal saline and 1 ml 40% formalin. Consequently, microscopic aided sperm count was conducted in 5 squares by placing the suspension in a Neubauer hemocytometer chamber(192).

Furthermore, the proportion of normal and aberrant sperm morphological appearances was computed for morphological assessment. Abnormal sperm morphology was defined as the presence of structural characteristics (193).

3.5.2.9.Estrous cycle

Daily vaginal swab was meticulously taken from each rat during the course of the experimental period. These vaginal swabs were acquired expressly for the purpose of Giemsa staining, a well-known technique for identifying and characterizing the several phases of rats' estrous cycle, which include proestrus, estrus, metestrus, and diestrus via the characteristic features of cells morphology.

3.6. Developmental toxicity/ teratogenicityof crude extract, embelin and fixed oil

The experimental method for the evaluation of developmental toxicity/ teratogenicity of crude extract, embelin and fixed oil from *E. schimperi* is similar. Hence the following description of methods in these experimental procedures holds similar for all of the three components of the plant of interest demonstrated as follows.

3.6.1. Experimental animal preparation

Wistar rats, which weigh between 220 and 250 grams, were used as nulliparous female albino rats. These rats were kept in a stainless-steel metallic cage at room temperature (22 ± 3 °C) with a relative humidity of 50% to 60% and a controlled alternating 12-hour light-dark cycle. The rats were given five days to acclimate before the experiment began. During this adaptation period, all of the rats were given food (pellet) and tap water without restriction. Factors thought to bring fetal losses which were not treatment-related such as unnecessary handling of pregnant animals and stress from external factors like noise was minimized (194).

3.6.2. Mating of experimental animals

A male albino Wistar rat with demonstrated fertility was introduced into a cage with two virgin female rats in order to initiate mating. The following morning, day-1 of gestation was established following microscopic analysis of a vaginal swab to check for the presence of sperm cells. The following morning, a second vaginal smear was performed and analyzed on the female rats who had no sperm cells. Until the pregnancy was confirmed, the male rat was kept in the same cage. (195,196)

3.6.3. Dose preparation and administration

Five groups of ten pregnant rats each were created by random selection from the pool of pregnant rats. The first three groups were given 250 mg/kg, 500 mg/kg, and 1000 mg/kg of crude extract, embelin, and fixed oil from *E. schimperi* as (Group I), (Group II), and (Group III), respectively. Distilled water was used to suspend the crude extract while embelin and fixed oil were diluted with tween 80 (194) with unlimited tap water and food. Animals in Group IV were categorized as pair-fed control group and were supplied with tap water, food, and the vehicle, while Group V animals were labeled as *ad libitum* control taking food and water without restriction. The *ad libitum* group was designed to evaluate the effect of animal handling during administration o. Dose

calculation was based on prior findings (179,197) for crude extract experiment while the acute toxicity results were baselines for embelin and fixed oil test. The extracts were administered through oral gavage. These experiments were conducted on rat embryos and fetuses. The administration period in both investigations lasted from day 6 to day 12 of gestation. The justification for choosing this period for treatment was that it reflects a dynamic period of organ formation. Furthermore, the key phases of rat development are classified as embryonic days 1 to 12.

3.6.4. Cage side evaluation

Cage side clinical observation of animals was done once daily for possible signs of behavioral and physical changes throughout the experimental period.

3.6.5. Day-12 Experiment

The purpose of this experiment is to determine whether *E. schimperi* has a teratogenic effect on rat embryos. Pregnant rats were anesthetized at the end of the treatment phase (day 12 of gestation) by injecting sodium pentobarbital intra-peritoneal (198,199). Dissection was done to observe gravid uterine horns. Subsequently, the uterine horns were dissected along the antimesometrial border to divulge the developing embryos. With the aid of GXM-XTL3T101 dissecting stereomicroscope and fine forceps, the membranes were removed along with the adjacent maternal tissue to reveal the embryo surrounded by a yolk sac. Now, the yolk sac circulation became clearly visible, and was evaluated thoroughly. The yolk sac was then removed to evaluate embryonic developmental parameters. These indicators were evaluated upon the Brown and Fabro morphological scoring system (200) validated for similar test procedure by Belete *et al*. (17) and Abebe *et al.*(198,201).

3.6.6. Day-20 Experiment

These experiments were carried out in 20 days old rat fetuses. The pupose of this procedure was to evaluate the toxicity of *E. schimperi*on fetomaternal outcomes and fetal development indices in near term rat fetus. The weight of each pregnant animal was recorded on 1st, 6th, 12th and 20th day of gestation. Food intake for every 24 hours was weighed the next morning at a constant time starting from day-1 of gestation up to day of sacrification. Similarly, administration of the plant extract was done daily at a constant time(202).

On the day of sacrification (day 20 of gestation), the dams were anesthetized and dissection was done to open the gravid uterus (figure:1). Gravid uteruses were explanted immediately after the euthanasia and placed in a broad Petry dish. The antimesometrial border of the uterus was incised guided by a dissecting microscope (GXM-XTL3T101 stereo microscope) using fine forceps. The fetuses were revealed by removing the fetal membranes and detaching them from their respective placentas. After revealing the gravid uterus, the number of implantation sites and prior resorptions were counted and recorded. Alive/dead fetuses were counted after applying gentle pressure on them. Once the fetal membranes and other maternal tissues were removed, the fetuses were weighed using acalibrated digital balance (Mettler AE160). Crown-ramp length (CRL) was measured for every fetus. Placental weight was also recorded before histopathological tissue processing.

3.6.7. Placenta gross morphology and histopathology

Three placenta sample tissues were taken from each animal for gross examination and further histopathological analysis. Samples were initially fixed with 10% formaldehyde for 24 h. Then, tissue processing was carried out using an automatic tissue processor (Leica, TP 1020, Germany). The steps were arranged to start with dehydration by ascending gradient of alcohol concentration followed by clearing and impregnation by xylene and melted paraffin wax, respectively. The tissues were then embedded in paraffin wax and ready for sectioning. The thickness of the tissue to be sectioned by the microtome was adjusted to 5 µm for light microscopy. Finally, the tissues were stained by hematoxylin and eosin technique (198,203). Later the histological slides were examined by a senior pathologist under a light microscope for indices of functional as well as structural changes in the placenta (88,204).

3.6.8. External gross morphological evaluation

Fetuses were revealed by removing the fetal membranes and detaching them from their respective placentas. Afterward, each fetus was fully examined for the presence of gross structural alterationss of cranio-facial development, extrimities development, vertebral column, tail development, and external genitalia

3.6.9. Soft tissue evaluation

After fetuses were fixed in a Bouin's solution containing picric acid 75%, formalin 25%, and glacial acetic acid 5%, visceral/ soft tissue evaluation was conducted by Free-hand Razor Blade Sectioning Technique as a modified Willison's technique (198). The legs and tail were amputated at attachment to the trunk before making a transverse cut between the jaws. This will help to evaluate the palate for any cleft. Subsequently, coronal slices were made through the head to evaluate the presence of hydrocephalus, ventricular enlargement of the brain and nasal septum defect(198). Further transverse sections were made along the trunk to evaluate the possible existence of cardiovascular, respiratory and abdominal defects.

3.6.10. Skeletal staining and evaluation

This experiment was designed to evaluate *E. schimperi's* effect on the process of bone formation on 20 days old rat fetus after staining the bones of the rat fetus. Three fetus/litter were sampled and further processed based on Rigueur and Lyons method (205). The initial step was euthanizing the fetus with pentobarbital followed by tissue permeabilization and skin removal facilitated by bathing for 30 sec in 60^0C hot water. Afterwards, evisceration was done by making an abdominal incision. The eviscerated samples were immersed in a solution containing a fixative solution, 90% ethanol, for 24 h. The samples then shifted to a container filled with 1% potassium hydroxide (KOH) for a purpose of soft tissue removal. Subsequently, the samples were stained for 24 h by a solution of alizarin red (0.005%) at 4 °C to obtain an optimum level of skeletal staining. For those samples presumed to be over stained by alizarin red, Mall's solution constituted by 79% distilled water, 20% glycerin, and 1% KOH was used as a correction chemical. Finally, each sample was stored in an increasing concentration gradient of glycerol until examination. Hyoid bone, sternum, ribs, vertebrae, and bones the upper and lower limbs were assessed against Nash and Persaud's skeletal scoring chart (206).

3.7.Statistical analysis

Statistical Package for Social Science (SPSS) software version 24 was utilized for data entry and analysis. The statistical results were exhibited in terms of mean (μ) and standard deviation (SD) from the mean. One-way analysis of variance (ANOVA) with post Hoc (Turkey) test, at $P<0.05$ level of significance was employed to look over significant statistical differences among

experimental groups. Furthermore, chi square test was employed to evaluate significance of differences among proportions in different groups. Results of placental histopathology were presented qualitatively based on predefined parameters(201,207)

Chapter four

4. Result

4.1.Crude extract

4.1.1. Cage-side clinical observation

Daily cage-side clinical observation was done and recorded carefully. However, there were no significant behavioral and physical signs of toxicity observed for the whole duration of treatment period. Moreover, there was neither abortion nor maternal death report as well.

4.1.2. Day-12 Experiment

4.1.2.1.Embryonic outcomes

As illustrated in Table: 1, the embryonic developmental outcomes showed that there is no statistically significant difference in morphological score, number of somites and CRL parameters within the treatment and control groups.

Table 1: Embryonic outcome variables across experimental groups after treatment with the 80% ethanol fruit extract of *E. schimperi*

Group	Embryonic developmental outcomes		
	Morphological score/ litter	**Number of somites/litters**	**CRL(mm)/litter**
Group I (250 mg/kg)	44.71±0.7	27.91±0.4	4.6±0.3
Group II (500 mg/kg)	44.52±0.8	27.41±0.3	4.4±0.3
Group III (1000 mg/kg)	44.32±0.8	26.99±0.4	4.5±0.2
Group IV (Pair-fed)	44.44±0.6	27.23±0.5	4.4±0.4
Group V (*Ad libitum)*	44.63±0.7	27.43±0.4	4.6±0.4

Results are expressed as mean and standard deviation; One Way ANOVA, CRL: Crown-ramp length.

4.1.3. Embryonic development indices

4.1.3.1.Circulatory system

Yolk sac circulation, heart development and allantois development were analyzed as embryonic development indices of the circulatory system. The result revealed that there is no statistically significant difference concerning the aforementioned indices between the animal groups treated with *E. schimperi* and their control counterparts (Table 2) (Figure 1).

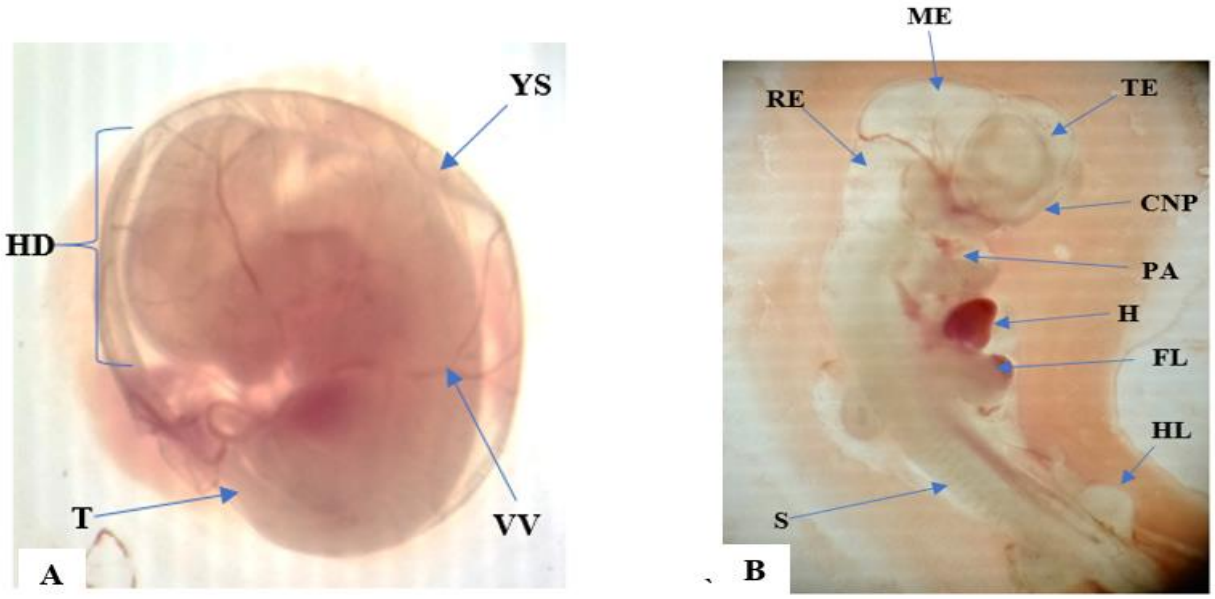

Figure 6: Rat embryos from rats dosed with 1000 mg/kg crude extract of *E. schimperi*. [A]: Embryo enclosed by its yolk sac (YS) with visible vitelline vessels (VV), Distinguishable head (HD) and tail (T) regions. [B]: CNP (Cranial neuropores/closed/), FL (Fore limb), HL (Hind limb), ME (Mesencephalon), PA (Pharyngeal apparatus), RE (Rhombencephalon), S (Somite) and TE (Telencephalon).

Table 2: Embryonic development developmental indices of the circulatory system after treated with 80% ethanol fruit extract of *E. schimperi*

Group	Proportion of retarded development		
	Yolk sac circulation	**Heart**	**Allantois**
Group IV (Pair-fed)	0	0	0
Group V (*Ad libitum*)	0	0	0
Group I (250 mg/kg)	0	0	0
Group II (500 mg/kg)	0	0	0
Group III (1000 mg/kg)	0	0	0

Results are in terms of proportions delayed development, chi-square test.

4.1.3.2.Nervous system and sense organs

As indicated in Table 3 and Figure 1, nervous system and sense organs development were assessed by examining the following indices: caudal neural tube, hindbrain, forebrain, otic system, and optic system. However, the observation found that there was no significantly associated retarded development among experimental and control groups in the nervous system and sense organs development.

4.1.3.3.Musculoskeletal System

As depicted in Table 4, musculoskeletal development indices of the experimental rats revealed that none of the parameters showed statistically significant retardation among the experimental groups compared to the controls.

Table 3: Embryonic developmental indices of the nervous system and sense organs after treatment with the 80% ethanol fruit extract of *E. schimperi*

Group	Proportion of retarded development				
	Caudal neural tube	**Hind brain**	**Fore brain**	**Otic system**	**Optic system**
Group IV (Pair-fed)	0	0	0	0	0
Group V (Ad libitum)	0	0	0	0	0
Group I (250 mg/kg)	0	0	0	0	0
Group II (500 mg/kg)	0	0	0	0	0
Group III (1000 mg/kg)	0	0	0	0	0

Results are expressed in terms of proportion of retarded development (%), chi-square test

Table 4: Embryonic developmental indices of the musculoskeletal system after treatment with the 80% ethanol fruit extract of *E. schimperi*

Group	Proportion of retarded development					
	Pharyngeal apparatus	**Maxillary process**	**Mandibular process**	**Fore limb**	**Hind limb**	**Flexion**
Group IV (Pair-fed)	0	0	0	0	0	0
Group V (*Ad libitum)*	0	0	0	0	0	0
Group I (250 mg/kg)	0	0	0	0	0	0
Group II (500 mg/kg)	0	0	0	0	0	0
Group III (1000 mg/kg)	0	0	0	0	0	0

Results are expressed in terms of proportions retarded development (%), chi-square test.

4.1.4. Day-20 Experiment

4.1.4.1.Maternal food intake and weight gain

The food intake of pregnant rats was measured daily for each group starting from day 1 up to the day of sacrification. Nevertheless, the overall weight gain was computed by deducting the initial weight (day-0) from the final weight measurement on the day of sacrification (day-20). As illustrated in Table 5, there is no statistically significant difference in both food consumption and weight gain between the groups during the period of administration and even till the day of scarification.

Table 5: Food intake and weight gain of pregnant rats treated with 80% ethanol fruit extract of *E. schimperi*

Maternal variables	**Experimental groups**				
	Group IV (Pair-fed control)	**Group V (*Ad libitum)***	**Group I (250 mg/kg)**	**Group II (500 mg/kg)**	**Group III (1000 mg/kg)**
Food intake (g)	180.26±18.08	174.57±33.92	172.94±35.26	183.09±22.36	184.74±8.63
Weight gain (g)	81.1±7.31	88.6±8.21	77.55±3.27	85.73±6.97	91.91±5.91

NB: Results are presented as mean ± standard deviation, one-way ANOVA.

4.1.4.2.Pregnancy outcomes

After exposing the uterine horns, the gravid uterus was assessed for pregnancy outcome variables. As illustrated in Table 6, the number of implantation sites was counted and turns not to have a statistically significant difference between treatment and control animals. No significant difference was observed in the number of resorption sites and live fetuses among the experimental groups. Each implantation site held alive fetus.

Table 6: pregnancy outcome of rats treated with 80% ethanol fruit extract of *E. schimperi*

Pregnancy outcomes	Experimental groups				
	Group IV (Pair-fed control)	**Group V (*Ad libitum)***	**Group I (250 mg/kg)**	**Group II (500 mg/kg)**	**Group III (1000 mg/kg)**
Number of implantation/dams	9.5±1.29	9±1.63	10±2.16	11.5±1.73	10.8±1.52
Number of prior resorptions/dams	0	0	0	0	0
Alive pups	9.5±1.29	9±1.63	10±2.16	11.5±1.73	10.8±1.52
Dead pups	0	0	0	0	0

NB: Results are presented as mean ± standard deviation from the mean, one-way ANOVA.

2.2.1.1.Fetal outcomes

Fetal weight, placental weight, and crown-ramp length were measured as parameters of fetal outcomes. However, none of them possessed a significant statistical difference among the five groups (**Table 7**).

Table 7: Fetal outcome of rats treated with 80% ethanol fruit extract of *E. schimperi*

Fetal outcomes	Experimental groups				
	Group IV (Pair-fed control)	**Group V (*Ad libitum)***	**Group I (250 mg/kg)**	**Group II (500 mg/kg)**	**Group III (1000 mg/kg)**
Fetal weight (g) Per dam	3.97±1.01	3.91±0.99	3.68±1.04	3.94±0.99	3.98±0.82
Placental weight (g)	0.61±0.06	0.59±0.07	0.61±0.08	0.62±0.04	0.54±0.19
Crown-ramp length (cm)	4.53±0.85	4.44±0.59	4.75±0.26	4.80±0.39	3.67±0.18

NB: Results are presented as mean ± standard deviation from the mean, one-way ANOVA.

4.1.4.3.Gross morphology and histopathology of placenta

As seen in **Figure 8**, light microscopic examination of placental histopathology revealed that tissues from Group I and Group II experimental groups exhibited inflammation (focal fibro-purulent exudate and hemorrhage) on the decidual layer. Moreover, animals treated with 1000 mg/kg crude extract of *E. schimperi* showed placental tissue calcification in addition to fibro-purulent exudate and hemorrhage. Placenta samples taken from the two control groups did not show any pertinent finding deviating from the normal histology. As illustrated in **Table 8**, quantitative analysis of histopathological parameters illustrated a statistically significant alteration in the occurrence of inflammatory reactions in placental tissues from *E. schimperi* treated rats when compared to the control groups. Additionally, calcification is also significantly observed in placentas from experimental animals treated with 1000 mg/kg of *E. schimperi* crude extract.

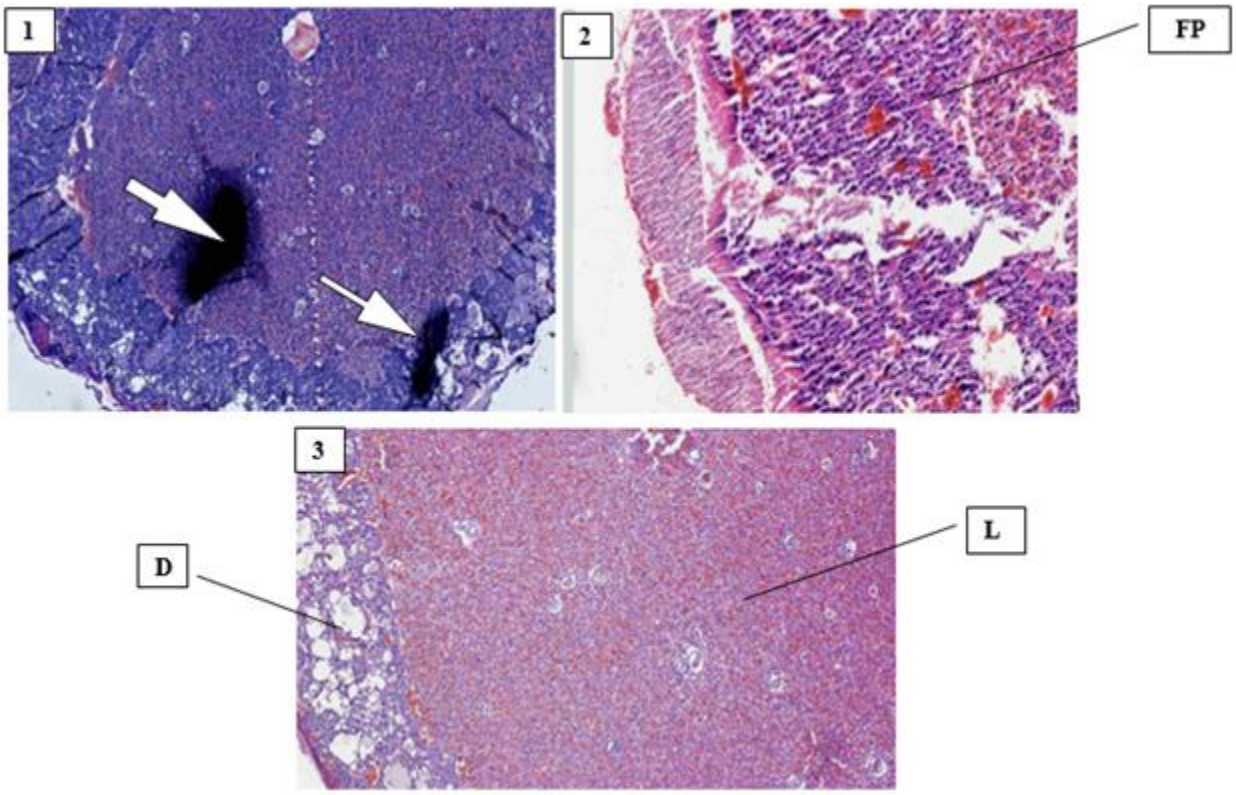

Figure 7: Photo micrograph depicting: (1) A calcified placental tissue (arrow) from rats dosed with 1000 mg/kg of *E. schimperi*, (2) Fibroprulent tissue (FP) from rats treated with 500 mg/kg and (3) Normal histology of placenta from the control rats; Decidual layer (D), Labrynzine zone (L).

Table 8 : Distribution of placental histopathological manifestations across experimental groups

Histopathological parameters of placenta	Experimental groups				
	Group IV (Pair-fed control)	**Group V (Ad *libitum)***	**Group I (250 mg/kg)**	**Group II (500 mg/kg)**	**Group III (1000 mg/kg)**
Necrosis	0	0	0	0	0
Cytolysis	0	0	0	0	0
Apoptosis	0	0	0	0	0
Inflammation	0	0	0	0	0
Calcification	0	0	0	0	0

NB: Results are presented as proportion of histopathological findings; *statistically significant difference seen from the controls at P < 0:05, chi-square test.

4.1.4.4.External and visceral morphology

Each fetus was evaluated carefully for the occurrence of external structural/morphological malformations after explanting it at the gestational age of 20 days. However, there was no significant treatment-related external morphological defect observed across the experimental groups and controls (**Table 9**). Soft tissue/visceral evaluation of fetuses fixed with Bouin's solution revealed that there were no visible abnormalities of visceral structures among fetuses born from rats treated with *E. schimperi* and controls (**Figure 8**).

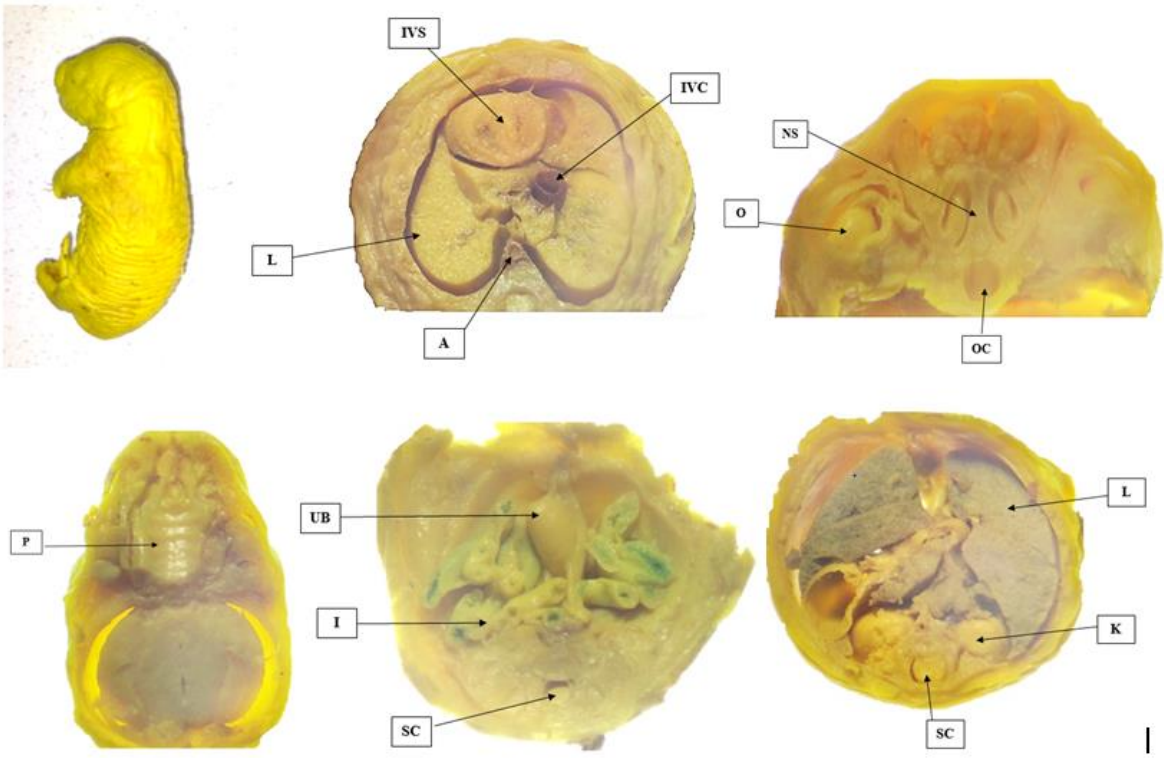

Figure 8: Visceral structures after sectioning at different level of the body based on Free-Hand Razor Blade Sectioning Technique. A: Aorta, I: Intestine, IVC: Inferior vena cava, IVS: Interventricular septum, K: Kidney, L: Lung, NS: Nasal septum, O: Orbit, OC: Oral cavity, P: Palate, SC: Spinal cord, UB: Urinary bladder.

4.1.4.5.Skeletal Evaluation

As shown in **Table 10**, **Table 11**and **Figure 9**, evaluation of skeletal ossification on rat fetuses stained with alizarin red showed that the number of ossifications in skull, sternum, hyoid, vertebral column (thoracic, lumbar, sacral and caudal vertebrae), ribs, bones of the lower limb and upper limbs possessed no statistically significant difference across experimental groups.

Table 9: External gross malformations after treatment with the 80% ethanol fruit extract of *E .schimperi*

Group	Proportion of external malformations (%)							
	Nervous system defects			Musculoskeletal defects			Others	
	EE	AE	SB	KY	SC	LD	MT	EGA
Group IV (Pair-fed)	0	0	0	0	0	0	0	0
Group V (*Ad libitum*)	0	0	0	0	0	0	0	0
Group I (250 mg/kg)	0	0	0	0	0	0	0	0
Group II (500 mg/kg)	0	0	0	0	0	0	0	0
Group III (1000 mg/kg)	0	0	0	0	0	0	0	0

Results are expressed in terms of proportions of malformations, chi-square test; EE: Exencephaly, AE: Anencephaly, SB: Spina bifida, KY: Kyphosis, SC: Scoliosis, LD: Limb defect, MT: Missed tail, EGA: External genitalia agenesis.

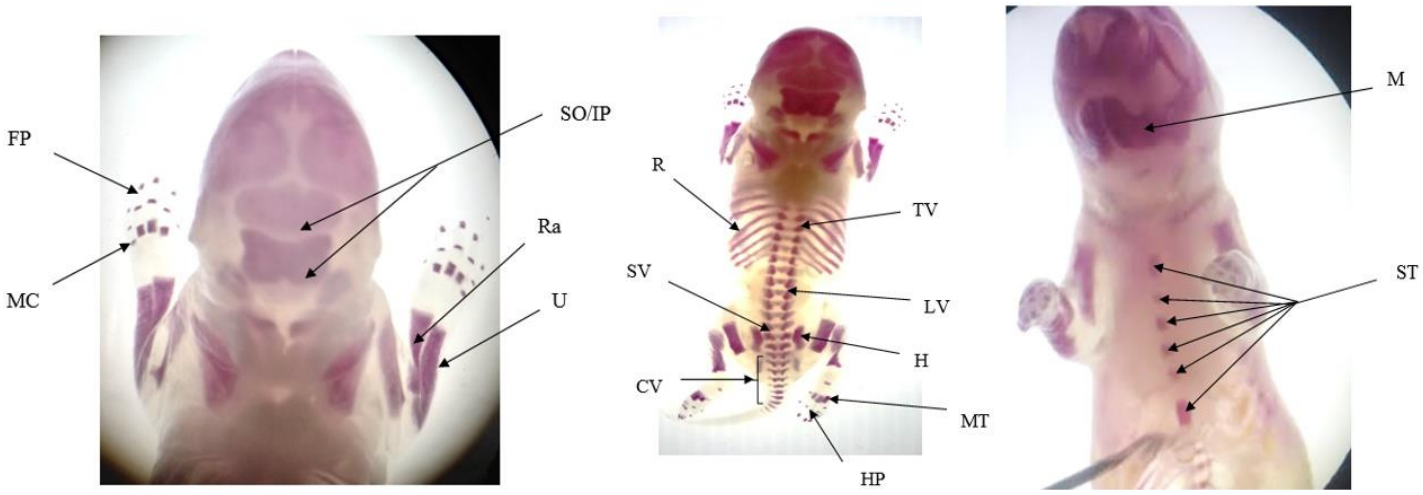

Figure 9: Ossification centers of 20 days old rat fetuses stained with alizarin red. CV: Caudal vertebrae, DP: Distal phalanges, FP; Forelimb phalanges, H: Hip bone, HP: Hindlimb phalanges, IP: Inter-parietal, L Lumbar vertebrae, M; Mandible, MC: Metacarpus, MT: Metatarsus, R: Ribs, Ra: Radius, So: Supraoccipital, ST: Sternum, SV: Sacral vertebrae, TV: Thoracic vertebrae.

Table 10: Number of ossification centers in the axial skeleton of rat fetuses from experimental groups treated with the 80% ethanol fruit extract of *E. schimperi*

Group	Sternum	Thoracic vertebrae	Lumbar vertebrae	Caudal	Ribs
Group IV (Pair-fed)	5.63±0.28	12±0	5±0	4.38±1.20	24±0
Group V (*Ad libitum)*	5.62±0.33	12±0	5±0	4.31±1.01	24±0
Group I (250 mg/kg)	5.61±0.32	12±0	5±0	4.38±0.96	24±0
Group II (500 mg/kg)	5.60±0.27	12±0	5±0	3.94±0.99	24±0
Group III (1000 mg/kg)	5.62±0.30	12±0	5±0	3.81±1.17	24±0

Results are presented in terms of mean ±standard deviation of ossification centers count, one-way ANOVA.

Table 11: Count of ossification centers in the appendicular skeleton of rat fetuses from experimental groups treated with the 80% ethanol fruit extract of *E. schimperi*

Group	Forelimb Phalanges	Hindlimb Phalanges	Metacarpus	Metatarsus
Group IV (Pair-fed)	3.91±0.28	3.61±0.33	3.93±0.24	4.13±0.18
Group V (*Ad libitum)*	3.88±0.41	3.73±0.29	3.87±0.35	4.17±0.02
Group I (250 mg/kg)	3.77±0.32	3.51±0.42	3.9±0.31	4.09±0.31
Group II (500 mg/kg)	3.83±0.26	3.47±0.37	3.8±0.41	4.02±0.22
Group III (1000 mg/kg)	3.67±0.43	3.43±0.51	3.9±0.26	3.99±0.33

Results are presented as mean ±standard deviation from the mean of ossification centers count, one-way ANOVA

4.2.Embelin

4.2.1. *In silico* toxicicty

The ProTox toxicity model revealed that embelin is predicted to be free of hepatotoxicity, carcinogenicity, mutagenicity, and cytotoxicity effects. However, the database indicated that embelin is predicted to have an immunotoxic effect and is suggested to be a CYP2D6 inhibitor. Furthermore, embelin is predicted to be devoid of a hERG-blocking effect (**Table 12**).

Table 12: *In silico* toxicicty out put of embelin after running ProTox toxicity model.

Model target		**Prediction**	**Probability**
Hepatotoxicity		Inactive	0.81
Carcinogenicity		Inactive	0.66
Immunotoxicity		Active	0.82
Mutagenicity		Inactive	0.79
cytotoxicity		Inactive	0.77
Cyp inhibitors	**1A2**	No	-
	3A4	No	-
	2D6	Yes	-
	2C19	No	-
hERG channel blocker		No	-

4.2.2. Acute toxicity and LD_{50}

The limit test of embelin at 5000 mg/kg in four animals did not exhibit mortality. Moreover, daily clinical evaluation revealed that the test animals did not show any observable signs of toxicity. As a result, the LD_{50} of embelin is declared to be >5000 mg/kg. There was no difference in weight gain or food intake between embelin-treated and vehicle-control animals. The gross necropsy also showed no gross pathological abnormalities in the experimental animals.

4.2.3. Sub-chronic Toxicity

4.2.3.1.Cage side clinical examination, food intake and body weight

During the 90 days long oral toxicity evaluation of embelin, neither death nor toxicity signs (convulsions, tremors, breathing problems, and skin changes) were observed in both sexes across test and control animal groups. As depicted in **Table 13**, total body weight gain of animals treated with embelin of *E. shimperi* fruit from both sexes is significantly higher than their counter part control groups. At all dosage levels, female rats treated with embelin and control rats have significantly different mean food intakes. Similar to this, male rats in the treatment group consumed more food than control rats over the course of the 90-day treatment period, with those treated with 1000 mg/kg of embelin consuming significantly more food.

Table 13: Effect of sub-chronic administration of embelin from *E. schimperi* Vake fruit on weight gain and food intake of rats

	Sex of Animals	**Group IV Vehicle Control**	**Group I 250mg/kg**	**Group II 500mg/kg**	**Group III 1000mg/kg**
Weight gain (gm)	**Male**	89.29±2.43	95.92±2.15*	98.42±2.7*	102.15±3.38*
	Female	37.06±2.72	45.48±1.19*	48.39±1.98*	53.75±1.56*
Food intake (gm)	**Male**	209.04±14.4	212.33±9.02	212.99±9.27	214.15±10.84*
	Female	158.8±8.94	163.05±9.94*	164.17±12.03*	164.43±9.12*

Results shown as mean ± standard deviation, one-way ANOVA.*statistically significant (P-value<0.05) compared to controls.

4.2.3.2.Effect of embelin on hematological parameters

As shown in the **Table 14** and **Table 15** below, the hematological profile parameters of experimental animals of both sexes following sub-chronic administration of embelin from *E. schimperi* Vake did not differ significantly.

Table 14: Effect of sub-chronic administration of embelin from *E. schimperi* Vake fruit on hematological profiles of male rats

Hematological Parameters	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
RBC(x 10^6/µl)	5.01±0.7	5.26 ±0.8	5.14 ± 1.1	4.92 ± 0.44
WBC (x 10^3/µl)	3.89 ± 0.41	4.21 ± 0.39	3.91 ± 0.35	3.84 ± 0.28
PLT (x 10^5)	664 ± 26.97	667 ± 22.8	659 ± 25.7	664 ± 37.27
Hgb (g/dl)	11.9 ± 0.26	12.8 ± 0.18	11.6 ± 0.37	12.44 ± 0.3
MCV (fL)	67.63 ± 0.17	68.1 ± 0.466	67.8 ± 0.39	67.0 ± 0.3
MCH (pg)	26.33 ± 3.99	26.54 ± 4.8	27.1 ± 5.2	26.44 ± 4.66
MCHC (g/dl)	37.7 ± 1.09	37.4 ± 0.37	37.0 ± 0.44	36.8 ± 0.33

Results shown as mean ± standard deviation from the mean, one-way ANOVA.

Table 15: Effect of sub-chronic administration of embelin from *E. schimperi* Vake fruit on hematological profiles of female rats

Hematological Parameters	Control tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
RBC(x 10^6/µl)	4.81±0.7	4.66±0.8	4.84 ± 1.1	4.92 ± 0.44
WBC (x 10^3/µl)	3.69 ± 0.41	4.07 ± 0.39	3.85 ± 0.35	3.94 ± 0.28
PLT (x 10^5)	604 ± 16.97	610 ± 17.8	594 ± 15.7	613 ± 17.27
Hgb (g/dl)	9.79 ± 0.42	10.5 ± 0.22	9.76 ± 0.37	9.88 ± 0.23
MCV (fL)	60.42 ± 0.17	61.61 ± 0.466	60.82 ± 0.39	62.01 ± 0.3
MCH (pg)	25.9 ± 3.63	25.34 ± 4.8	26.61 ± 4.2	24.47 ± 3.11
MCHC (g/dl)	35.67 ± 0.79	34.24 ± 0.45	35.07 ± 0.43	34.68 ±0.33

Results shown interms of mean ± standard deviation from the mean, one-way ANOVA.

4.2.3.3.Effect of embelin on hepato –renal parameters

4.2.3.3.1. Relative organ weight of liver and kidneys

Daily oral administration of embelin isolated from *E. schimperi* Vake fruit for 90 days had no influence on the relative organ weights of the liver and kidneys of from all test groups, both female and male (**Table 16**).

Table 16: Relative organ weight of liver and kidneys in male and female rats after they are treated sub-chronically with embelin isolated from *E. schimperi* Vake fruit

		Control Tween 80	**Group I 250mg/kg**	**Group II 500mg/kg**	**Group III 1000mg/kg**
Liver weight (gm)	**Male**	3.78±0.3	3.8±0.35	3.68±0.65	3.69±0.25
	Female	3.44±0.54	3.48±0.57	3.4±0.51	3.42±0.4
Kidney weight (gm)	**Male**	0.29±0.01	0.31±0.01	0.3±0.014	0.3±0.004
	Female	0.3±0.02	0.28±0.04	0.28±0.02	0.31±0.04

Results are shown as mean ± standard deviation, one-way ANOVA.

4.2.3.3.2. Effect of embelin on clinical chemistry parameters

Serum biochemical study of male experimental rats' blood found that rats administered with embelin from *E. schimperi* fruit had a statistically significant lower ALT level than control animals given the vehicle, tween 80. Male rats given embelin showed significantly lower AST levels, than control group animals. Similarly, blood ALP levels in male rats treated with embelin decreased significantly when compared to their control counterparts. However, other clinical chemistry indicators, on the other hand, did not reveal a significant difference between treatment groups and control animals (**Table 17**).

Table 17: Clinical chemistry profile of male rats after they are treated sub-chronically withembelin from *E. schimperi* Vake fruit

Hematological Parameters	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
ALT (U/L)	59.01 ± 8.6	48.44 ± 6.12*	46.54 ± 13.5*	44.35 ± 3.24*
AST (U/L)	192.4 ± 10.15	141.4 ± 9.4*	149.2 ± 9.58*	162.7 ± 20.5*
ALP (U/L)	106.2 ± 5.4	86.0 ± 7.0*	76.44 ± 1.4*	72.2 ± 2.0**
Urea (mg/dL)	48.8 ± 3.8	47.6 ± 5.1	43.1 ± 3.1	41.7 ± 7.4
Creatinine(mg/dL)	0.34 ± 0.0	0.32 ± 0.0	0.31 ± 0.0	0.32 ± 0.0
Albumin (g/dL)	4.3 ± 0.2	4.4 ± 0.1	4.4 ± 0.1	4.4 ± 0.1
Total protein (g/dL)	6.0 ± 0.1	6 ± 0.1	5.9 ± 0.11	6.1 ± 0.2
Total cholesterol (mg/dL)	39.6 ± 1.4	42.6 ± 2.3	43.5 ± 2.2	44.7 ± 2.5
Glucose (mg/dL)	113.5 ± 9.7	105.9 ± 7.43	100.4 ± 12.8	103 ± 11.6
Total triglyceride	21.64 ± 3.4	22.77 ± 2.87	23.88 ± 4.9	24.47 ± 5.1
HDL	22.66 ± 4.6	23.79 ± 5.4	24.11 ± 3.9	25.37 ± 4.12
LDL	13.57 ± 2.72	11.57 ± 3.44	12.36 ± 1.75	12.94 ± 3.16

Results are expressed as mean ± standard deviation from the mean, one-way ANOVA.*statistically significant (P-value<0.05), ** p-value ≤ 0.001 compared to vehicle controls.

Table 18 depicted the clinical chemistry profiles of female rats after 90 days treatment of embelin from *E. schimperi* fruit. Significant lowering of serum ALT, AST and ALP levels were observed from female rats treated with 1000 mg/kg of embelin. The rest of biochemical parameters didn't exhibited difference within the experimental animals

Table 18: Clinical chemistry profile of female rats after they are treated sub-chronically with embelin isolated from *E. schimperi* Vake fruit.

Clinical chemistry Parameters	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
ALT (U/L)	54.65 ± 7.46	50.46± 4.83*	47.84 ± 7.1*	45.12± 5.83*
AST (U/L)	167.45 ± 20.5	159.2 ± 7.5*	154.4 ± 9*	152.4 ± 11.21*
ALP (U/L)	116.2 ± 5.4	97.56 ± 7.0*	97.44± 1.4*	92.2 ± 3.4 *
Urea (mg/dL)	49.8 ± 4.2	44.86 ± 4.4	44.6 ± 5.6	43.55 ± 4.77
Creatinine(mg/dL)	0.4 ± 0.0	0.42 ± 0.0	0.41 ± 0.0	0.41 ± 0.0
Albumin (g/dL)	4.1 ± 0.1	4.1 ± 0.1	4.1 ± 0.1	4 ± 0.1
Total protein (g/dL)	5.77 ± 0.1	5.7± 0.11	5.81 ± 0.12	5.92 ± 0.1
Total cholesterol (mg/dL)	46.5 ± 2.6	43.6 ± 2.6	4.3 ± 3.2	45.8 ± 2.5
Glucose (mg/dL)	106.7 ± 8.7	101.88 ± 6.3	102.4 ± 7.31	105.75 ± 6.77
Total triglyceride	29.7 ± 2.5	29 ± 3.23	30.11 ± 5.1	32.71 ± 3.48
HDL	32.54 ± 3.74	34.58 ± 2.83	33.12 ± 2.6	35.44 ± 3.7
LDL	14.37 ± 1.2	14.68 ± 2.1	15.44 ± 1.35	16.8 ± 1.4

Results are expressed as mean ± standard deviation from the mean, one-way ANOVA.*statistically significant (P-value<0.05), compared to vehicle controls.

4.2.3.3.3. Effect of embelin on histopathology of liver and kidneys

Ninety days administration of embelin isolated from *E. schimperi* Vake fruit did not elucidate any significant alteration of the microscopic structure of liver tissues examined from all groups of animals from both sexes. As shown in **Figure 10 and 11**, the central veins along with their radiating hepatocytes and canaliculi appear normal across all liver tissues evaluated. Furthermore, the contents of portal triads; portal vein, hepatic artery and bile duct, arranged didn't show any significant abnormal histological change

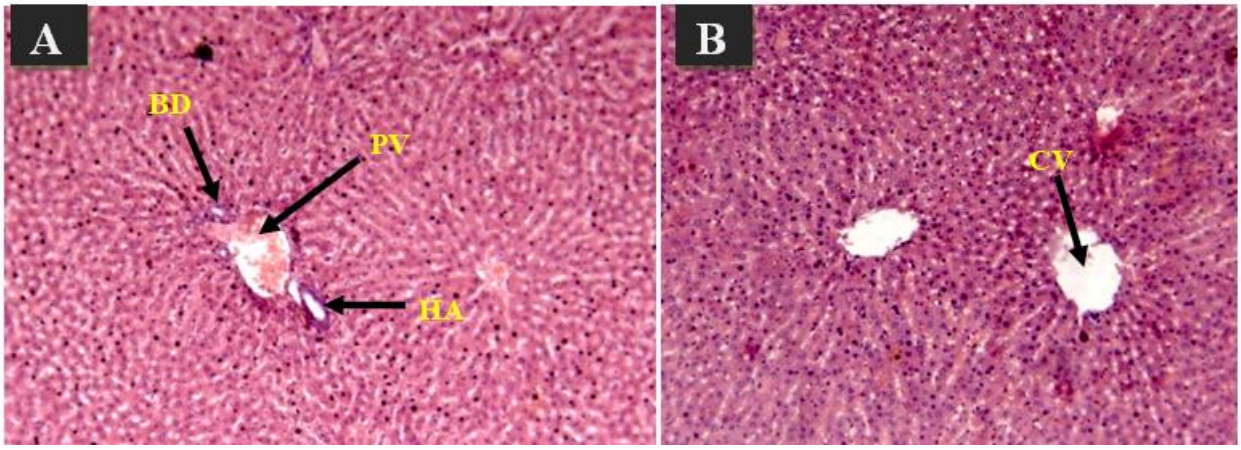

Figure 10: Photomicrograph of normal microstructure of liver tissues from male rats treated with 1000 mg/kg of embelin from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B). BD:** bile duct; **CV:** central vein; **HA**: hepatic artery; **PV:** portal vein. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

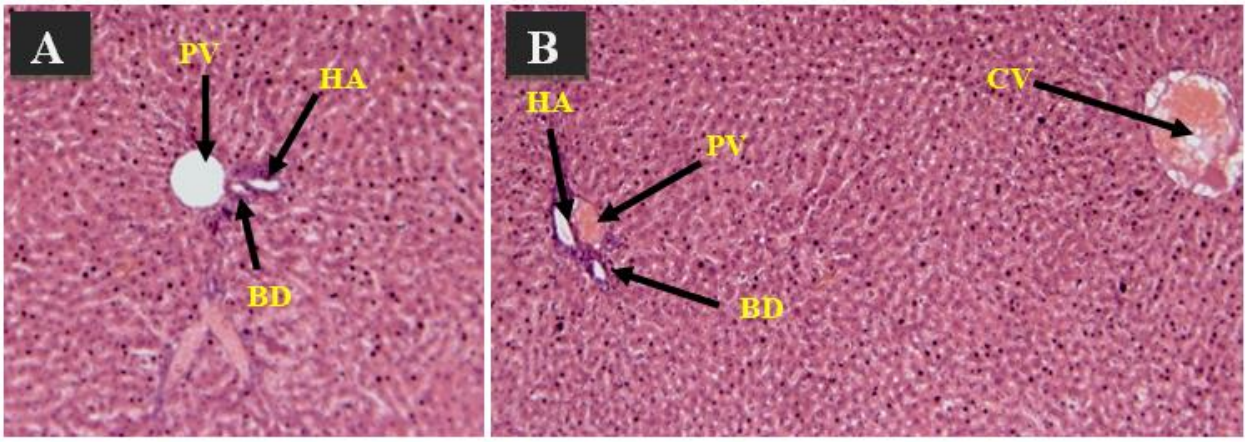

Figure 11: Photomicrograph of normal microstructure of liver tissues taken from female rats treated with 1000 mg/kg of embelin from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B). BD:** bile duct; **CV:** central vein; **HA**: hepatic artery; **PV:** portal vein. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

Histological analysis of samples from all experimental groups revealed no treatment-related structural abnormalities in the microstructure of kidney tissues. As shown in the photomicrographs (**Figure 12 & 13**), the glomerulus and tubules had normal structural integrity in both sexes.

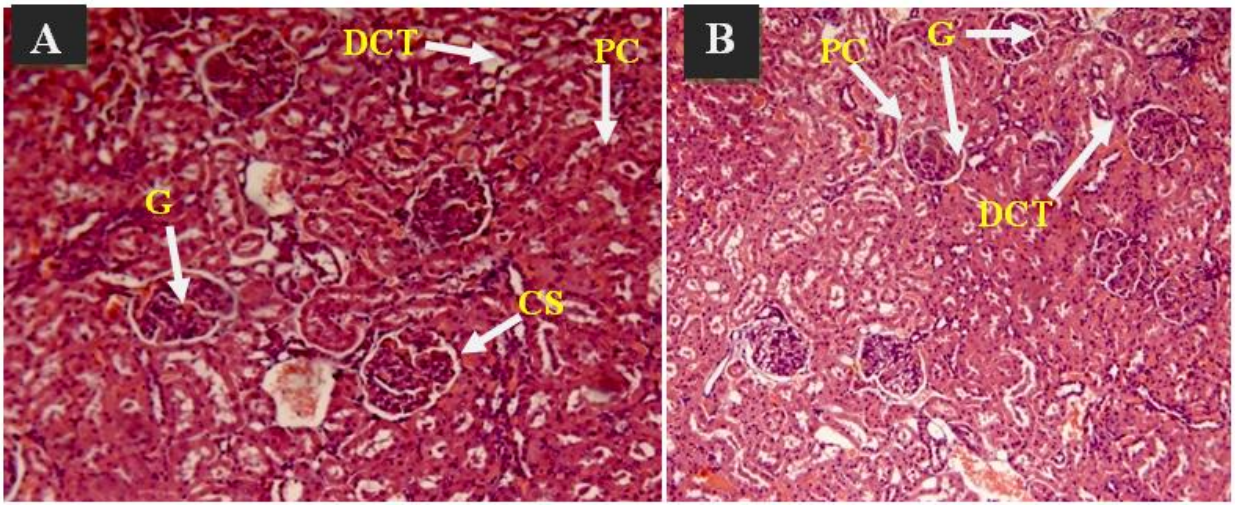

Figure 12: Photomicrograph of normal microstructure of kidney tissues from male rats treated with 1000 mg/kg of embelin from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B).BV**: blood vessel; **CS**: capsular space; **DCT**: distal convoluted tubule, **G**: glomerulus; **PCT**: proximal convoluted tubule. Hematoxylin-Eosin (H&E) stain, 400x magnification.

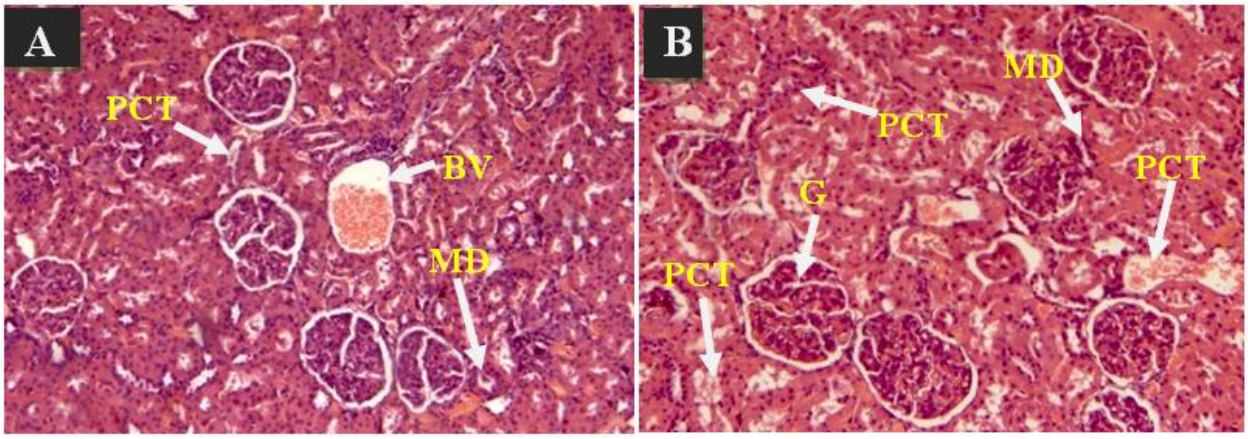

Figure 13:Photomicrograph of normal microstructure of kidney tissues taken from female rats treated with 1000 mg/kg of embelin from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B).BV**: blood vessel; **CS**: capsular space; **DCT**: distal convoluted tubule, **G**: glomerulus; **MD:** macula densa **PCT**: proximal convoluted tubule. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

2.2.1.2.Effect of embelin on histopathology of spleen

Histopathological evaluation of spleen tissues from both sexes revealed that sub-chronic administration of embelin did not bring any microscopic structural alteration in the arrangement of central arterioles, red pulb and white pulb (**Figure 14 & 15**).

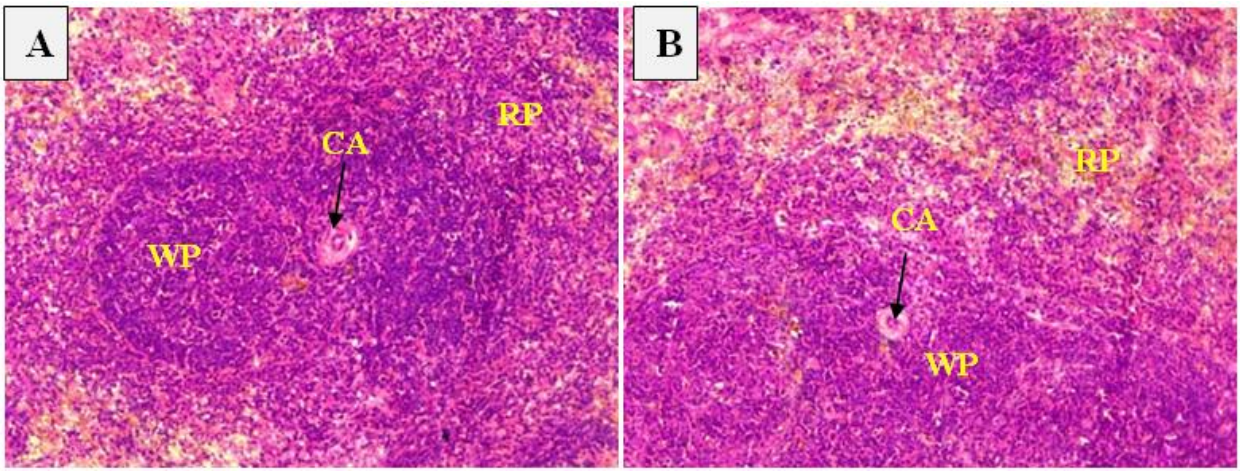

Figure 14: Photomicrograph of normal microstructure of spleen tissues from male rats treated with 1000 mg/kg of embelin from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B). CA**: central arteriole; **RP**: red pulb; **WP**: white pulb. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

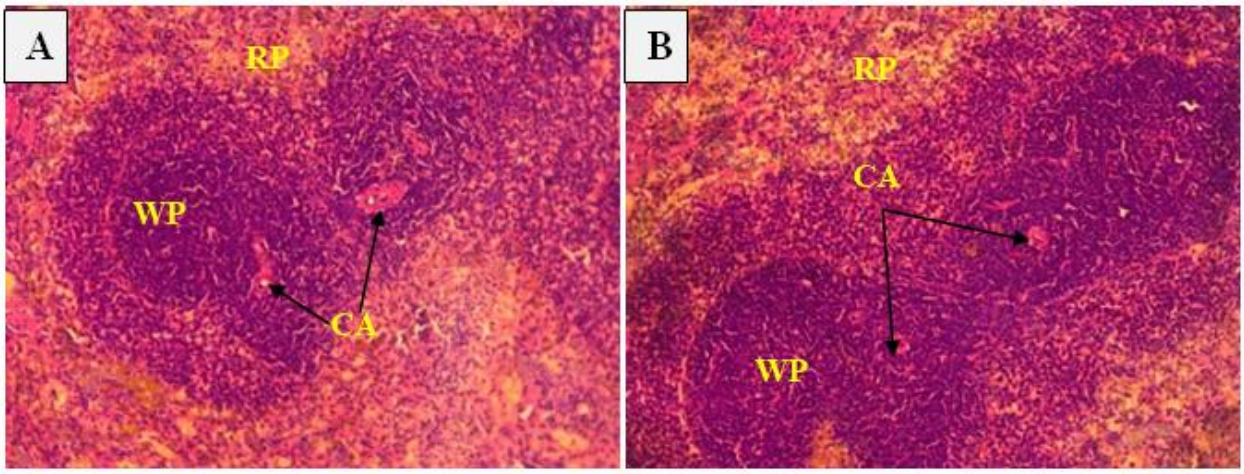

Figure 15: Photomicrograph of normal microstructure of spleen tissues from female rats treated with 1000 mg/kg of embelin from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B). CA**: central arteriole; **RP**: red pulb; **WP**: white pulb. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

2.2.1.3.Effect of embelin on histopathology of Adrenal gland

As illustrated in the photomicrographs (**Figure 16& 17**), the micro-structural arrangement of cortical layers of adrenal gland namely zona glomerulosa, zona fasiculata and zona reticularis along with the adrenal medulla did not change significantly in all embelin treated animals compared to those rats treated with tween 80 as a control vehicle. Furthermore, the sub-chronic administration of embelin didn't alter normal histological composition of the adrenal medulla.

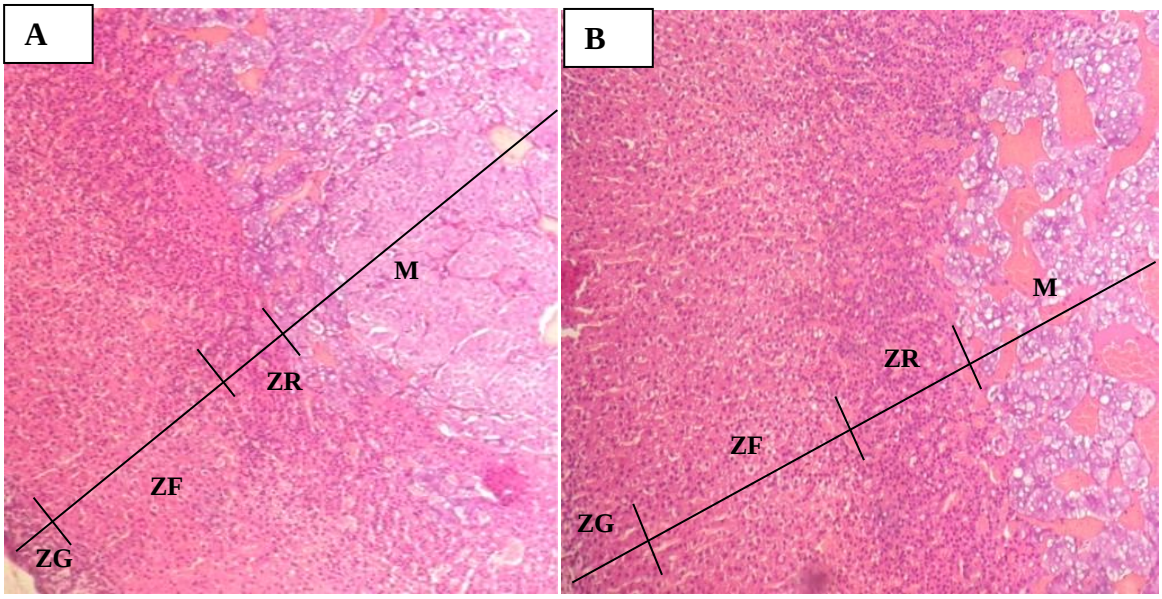

Figure 16: Photomicrograph of normal microstructure of adrenal tissueadrenal gland from male rats treated with 1000 mg/kg of embelin from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B). M:** medulla; **ZF**: zona fasiculata; **ZG**: zona glomerulosa; **ZR**: zona reticularis. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

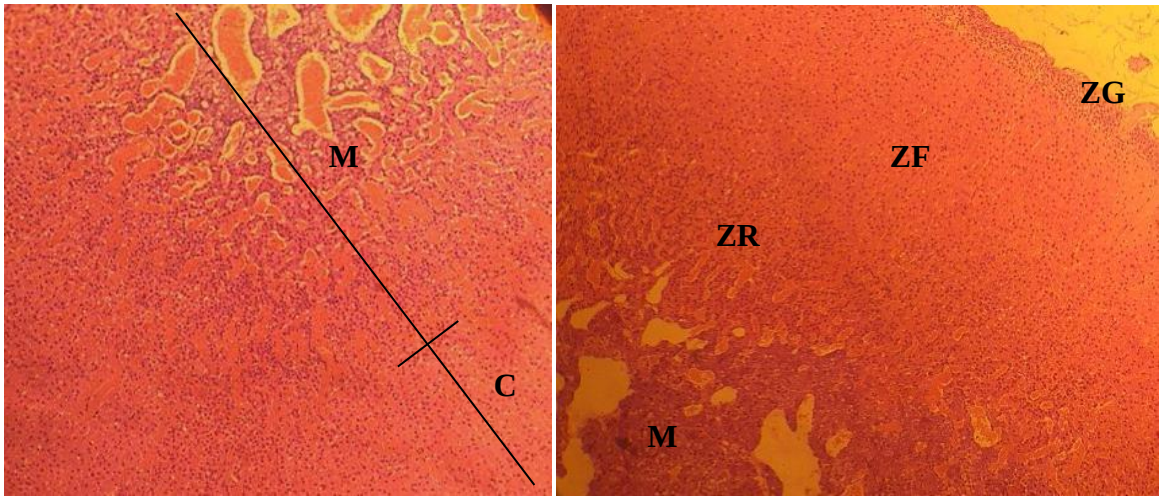

Figure 17: Photomicrograph of normal microstructure of adrenal gland taken from female rats treated with 1000 mg/kg of embelin from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B).C:** cortex, **M:** medulla; **ZF**: zona fasiculata; **ZG**: zona glomerulosa; **ZR**: zona reticularis. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

2.2.1.4.Effect of embelin on male reproductive indices

4.2.3.3.4. Relative reproductive organ weight

Relative weight evaluation of testes, epididymis, and seminal vesicles was performed as an integral measure in reproductive organs toxicity following sub-chronic administration of embelin from *E. schimperi* fruit. The results, as shown in **Table 19**, revealed that the mean relative weight of testes and epididymis from all embelin treated animals is significantly low when compared to their counterpart from the control group. However, the relative weight of seminal vesicles showed no significant change across all groups of rats.

4.2.3.3.5. Sperm count and morphology

Microscopically guided enumeration of sperm cells from the dilution of semen illustrated that there is a statistically significant abatement of sperm count in those sample examined from rats treated with all three dose level of embelin from *E. schimperi* fruits when compared to rats treated with tween 80 **(Table 20) (Figure 18)**.

Table 19: Relative organ weight of testes, epididymis, and seminal vesicles in rats after they are treated sub-chronically with embelin from *E. schimperi* Vake fruit

Reproductive organ	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
Testis	1.19 ± 0.047	0.51 ± 0.061*	0.42 ± 0.031*	0.36 ± 0.062*
Epididymis	0.54 ± 0.021	0.32 ± 0.014*	0.31 ± 0.033*	0.26 ± 0.013*
Seminal vesicles	0.87 ± 0.021	0.85 ± 0.046	0.84 ± 0.026	0.81 ± 0.037

Results are shown in terms of mean ± standard deviation, one-way ANOVA.*statistically significant (P-value<0.05) compared to controls.

Table 20: Effect of sub-chronic administration of embelin on sperm count and proportion of aberrant sperm cells

	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
Sperm count (x106/ml)	281± 11.55	219 ± 15.83**	192 ± 16.44**	165 ± 17.47**
Proportion of aberrant sperm cell (%)	7.97 ± 2.57	10.77 ± 3.6*	12.32 ± 4.51**	13.48 ± 3.11**

Results are expressed as mean ± standard deviation from the mean, one-way ANOVA.*statistically significant (P-value<0.05), ** p-value ≤ 0.001 compared to vehicle controls

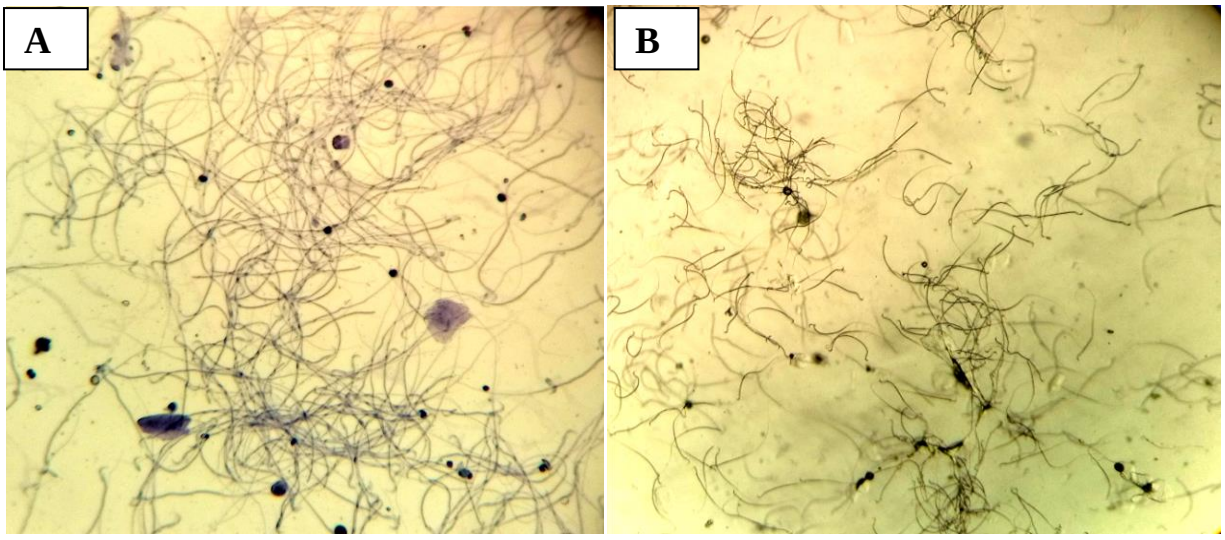

Figure 18: Photomicrograph of sperm cells from rats treated with 1000 mg/kg of embelin from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B).**400 x magnifications

4.2.3.3.6. Effect of embelin on male sex hormones

Serum levels of sex hormones, which are involved in the regulation of reproduction, significantly deviated after receiving daily dosage of embelin extracted from *E. schimperi* Vake fruit for 90 consecutive days. In this regard, it was discovered that all animals treated with embelin had considerably lower levels of testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) than rats from the control group.

Table 21: Effect of sub-chronic administration of embelin on serum levels of sex hormones

Male sex hormones	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
FSH (ng/ml)	211 ± 21.43	134.84 ± 16.08**	127 ± 18.37**	108 ± 14.22**
LH (ng/ml)	2.74 ± 0.34	1.72 ± 0.31**	1.63 ± 0.17**	1.17 ± 0.14**
Testosterone (ng/ml)	3.34 ± 0.42	2.14 ± 0.07**	1.55 ±0.05**	1.37 ± 0.04**

Results are shown as mean ± standard deviation from the mean, one-way ANOVA.*statistically significant (P-value<0.05), ** p-value ≤ 0.001 compared to vehicle controls

4.2.3.3.7. Effect of embelin on histopathology of epididymis

As depicted in **Figure19**, the histopathological analysis of epididymal tissue under light microscopy revealed notable histological alterations in rats subjected to a 90-day oral administration of embelin isolated from *E. schimperi* fruit at doses of 500 mg/kg and 1000 mg/kg, as compared to the control group. Specifically, the epididymal tissues in these dosage groups exhibited characteristics such as a reduction in the thickness and sloughing of the epididymal epithelium and the presence of cytoplasmic vacuolization.

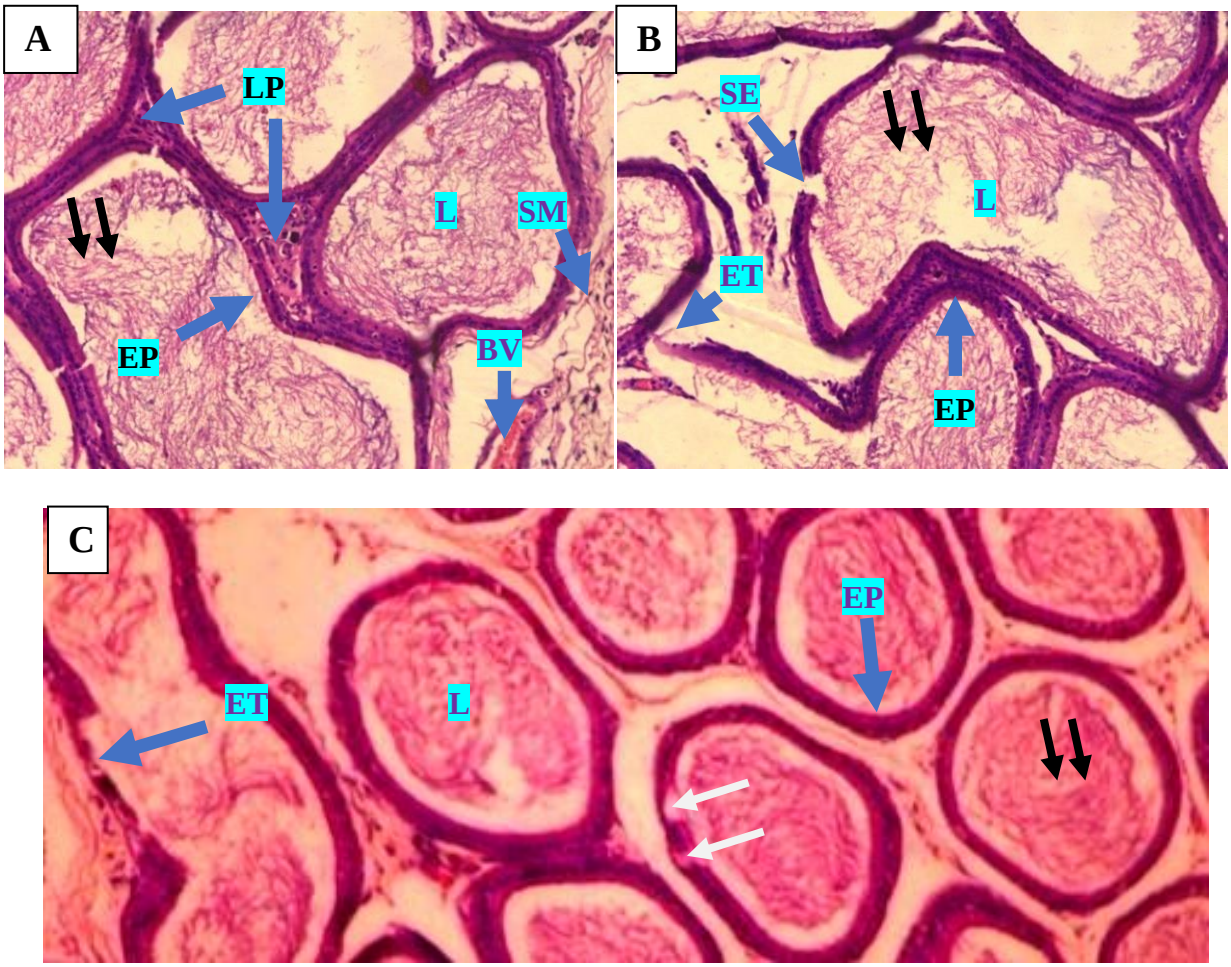

Figure 19: Photomicrograph of epididymis from; **(A):** Rats treated with tween 80 depicting normal microstructure with intact epididymal epithelium (EP), blood vessels (BV) within the lamina propria (LP), and visible spermatozoa (black arrows) inside the lumen (L); **(B):** Rats subjected to 500 mg/kg of embelin indicating thinning (ET) and sloughing (SE) epithelium; **(C)** Rats given 1000 mg/kgof embelin showing cytoplasmic vacuolization (white arrows) in addition to epithelial thinning (ET). Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

4.2.3.3.8. Effect of embelin on histopathology of testes

Upon morphological examination of the testicular tissues, sub-chronic embelin tretment resulted in a variety of structural alterations. Rat testes tissues exposed to 500 mg/kg of embelin, as shown in **Figure 20**, exhibited notable basal lamina detachments, lumenal indentation indicative of beginning lumenal atrophy, and vacuolization inside the seminiferous tubule wall. Moreover, testicular tissues from the group receiving a high dose treatment (1000 mg/kg) showed both vacuolization and luminal sloughing of germ cells.

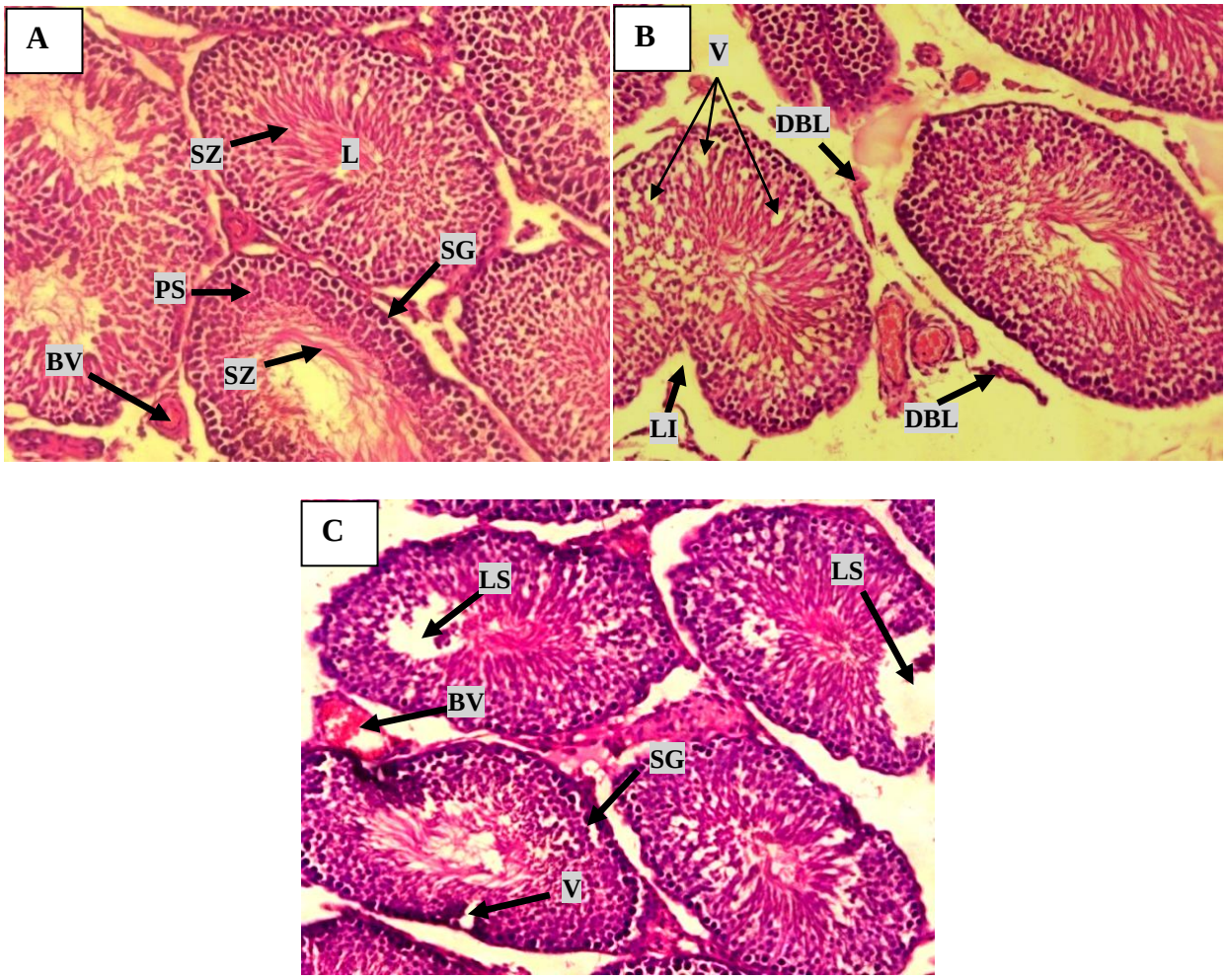

Figure 20: Photomicrograph of seminiferous tubules of testes sampled from rats sub-chronically treated with tween 80 **(A)**, 500 mg/kg **(B)** and 1000mg/kg **(C)** of embelin. **BV**: blood vessels, **DBL**: detached basal lamina, **L:** lumen, **LI:** luminal indentation, **LS:** luminal slough, **PS:** primary spermatocytes, **SG**: spermatogonia, **SZ:** spermatozoa. Hematoxylin-Eosin (H&E) staining, 400 x magnifications

4.2.3.3.9. Effect of embelin on histopathology of seminal vesicles

Upon examining seminal vesicles in the context of evaluating the sub-chronic toxicity of embelin extracted from *E. schimperi* Vake fruit, it was observed that the microstructure of all tissue samples, regardless of whether they were treated with embelin or not, was normal. The epithelium is clearly intact and has sufficiently filled luminal content, as seen in **Figure 21**.

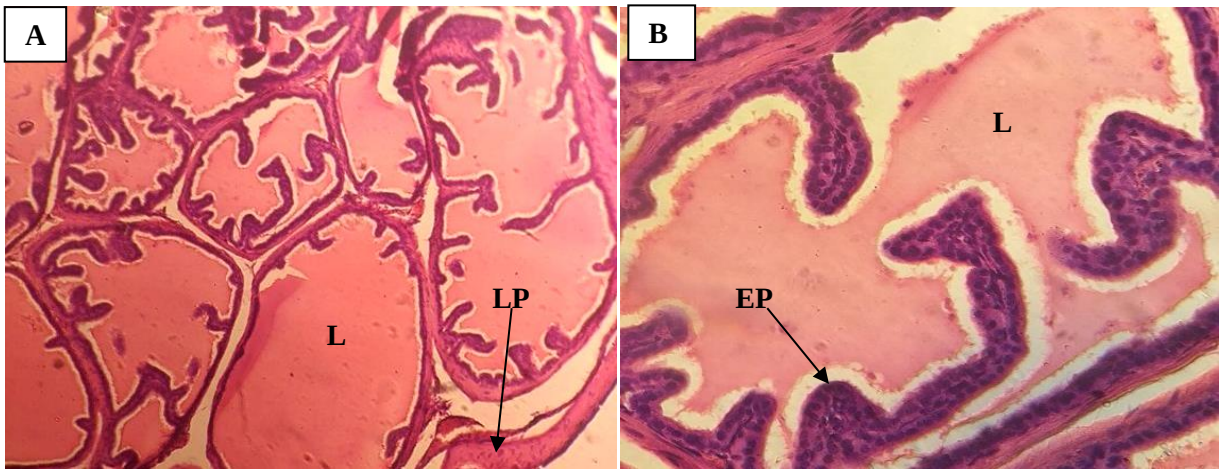

Figure 21: Photomicrograph of seminal vesicles sampled from rats treated with 1000 mg/kg showing normal microstructure. **EP**: epithelium, **L**: lumen and **LP**: lamina propria. Hematoxylin-Eosin (H&E) staining; (A) 100x and (B) 400x magnifications.

2.2.1.5.Effect of embelin on female reproductive indices

4.2.3.3.10. Relative reproductive organ weight

The sub-chronic treatment with embelin from *E. schimperi* Vake fruit bring a notable decrease in the relative weight of the uterus and ovary, the female reproductive organs, as indicated in **Table 22**. All animals treated with embelin had significantly lower relative uterine and ovarian weights than mice treated with tween 80 as a vehicle control.

Table 22: Figure: Effect of sub-chronic administration of embelin on the relative weight of female reproductive organs

Reproductive organs	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
Uterus	0.32 ± 0.021	0.21 ± 0.004*	0.18 ± 0.004**	0.11 ± 0.003**
Ovary	0.036 ± 0.0013	0.022 ± 0.0021*	0.019 ± 0.0031**	0.015 ± 0.002**

Results are shown in terms of mean ± standard deviation from the mean, one-way ANOVA.*statistically significant (P-value<0.05), ** p-value ≤ 0.001 compared to vehicle controls.

4.2.3.3.11. Effect of embelin on female sex hormones

After administering embelin for 90 days, a serum ELISA assay of female sex hormones revealed significant effects. When compared to the controls, the FSH and LH levels of female rats treated with all dosage levels of embelin showed a substantial decline. Progesterone and estrogen levels were also noticeably lower than those of respective controls (**Table 23).**

Table 23: Effect of sub-chronic administration of embelin on the serum level of female sex hormones

Female sex hormones	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
FSH (ng/ml)	18.21 ± 1.45	15.32 ± 1.97*	13.57 ± 2.04**	12.37 ± 2.18**
LH (ng/ml)	12.16 ± 1.63	11.54 ± 1.07*	10.25 ± 1.63*	9.88 ± 1.43**
Estradiol (ng/ml)	92.37 ± 6.47	89.45 ± 8.57*	86.76 ± 7.23**	84.41 ± 9.34**
Progesterone (ng/ml)	78.64 ± 9.4	73.45 ± 7.56	70.78 ± 9.74**	68.47 ± 8.67**

Results are shown as mean ± standard deviation, one-way ANOVA.*statistically significant (P-value<0.05), ** p-value ≤ 0.001 compared to vehicle controls.

4.2.3.3.12. Effect of embelin on the estrous cycle

Cytological analysis of vaginal smears from female rats treated by 250 mg/kg, 500 mg/kg, and 1000 mg/kg of embelin for 90 days showed significantly shorter estrous cycles, with mean ± standard deviations of 3.6 ± 0.48, 3.47 ± 0.75, and 3.22 ± 0.63 days, respectively, whereas the control group's mean estrous cycle was 4.35 ± 0.66 days.

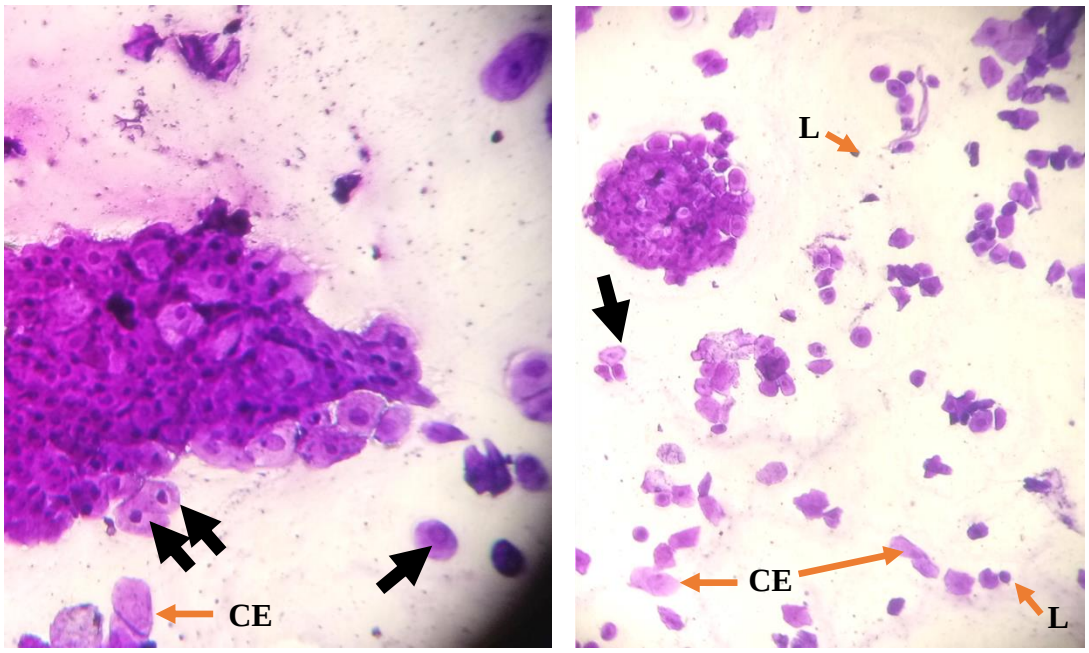

Figure 22: Photomicrograph of vaginal cytology for estrous cycle evaluation from rats treated with tween 80 control group (A) and 1000 mg/kg of embelin both during proestrous phase. **CE**: cornified epithelial cells; Nucleated epithelial cells (black arrows) and **L**: leukocytes. Eosin (H&E) staining, 100x magnifications.

4.2.3.3.13. Effect of embelin on histopathology of ovaries

Light microscopic evaluation of ovarian tissue from experimental rats after sub-chronic administration of embelin for 90 days elucidated that ovaries from high and middle dosage group, 1000 mg/kg and 500 mg/kg, possessed immature oocytes despite the fact that the tissues were taken during the late proestrous phase. However, ovarian tissues taken from control animals showed follicles at different developmental stages ranging from primary follicle up to antral follicle and corpus luteum (**Figure 23)**.

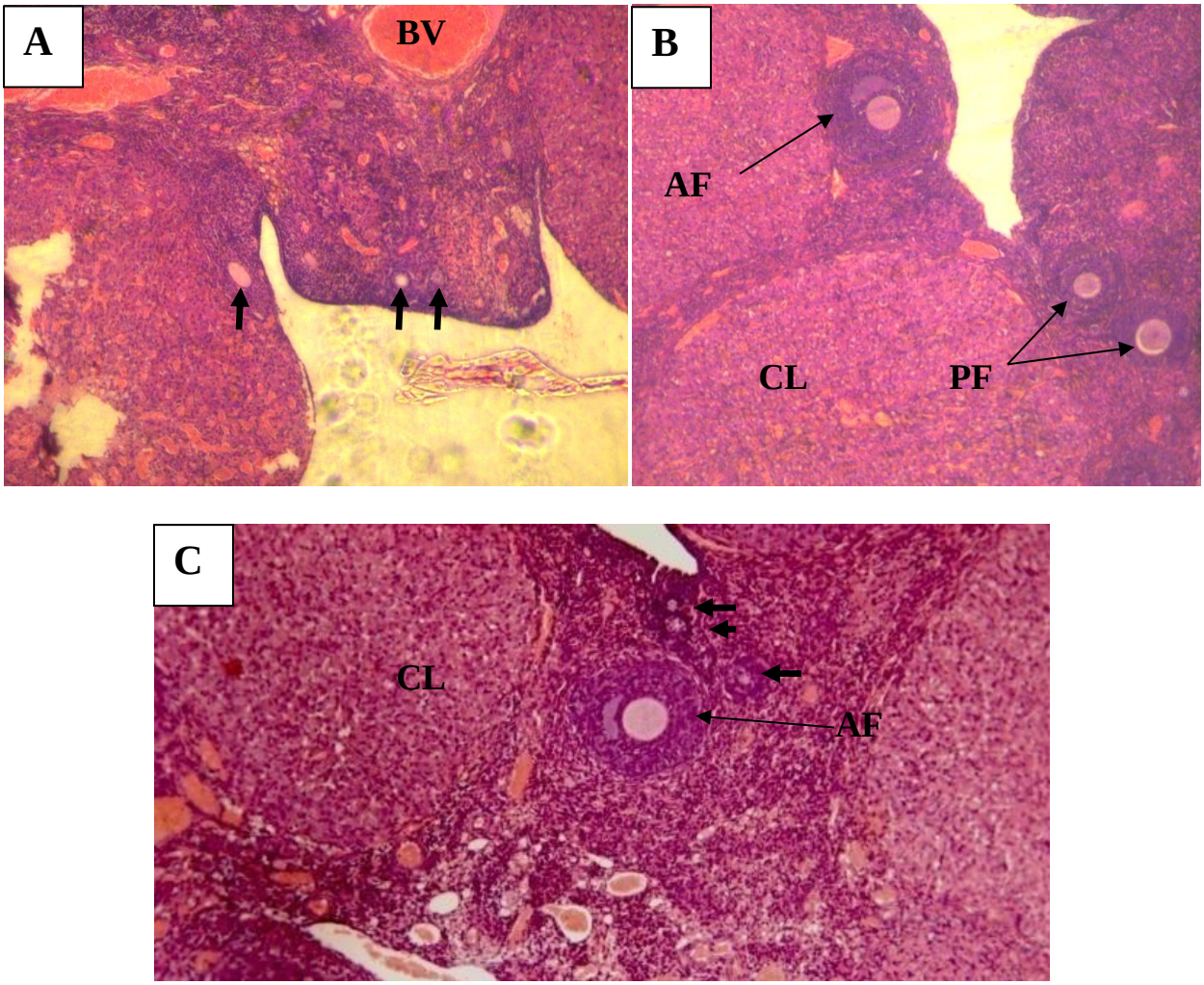

Figure 23: Photomicrograph of ovary histology after administration of 1000 mg/kg (**A**) and 500 mg/kg (**C**) of embelin and Tween 80 (**B**). AF: antral follicle; **BV**: blood vessel; **CL**: corpus luteum; **PF**: primary follicle and immature follicles (Black arrows). Eosin (H&E) staining, 400x magnifications

4.2.3.3.14. Effect of embelin on Histopathology of uterus

As illustrated in **Figure 25**, microscopic evaluation of uterine tissues explanted from experimental rats revealed that there is no significant variation in microstructure of uterus from animals treated with all dose level of embelin and controls.

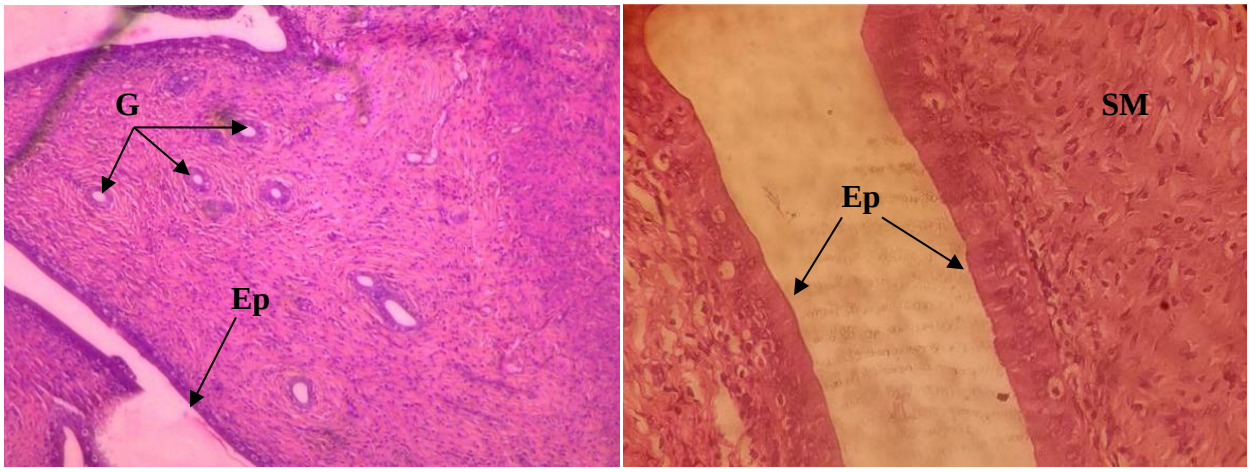

Figure 24: Photomicrograph of uterus from experimental animals treated with 1000 mg/kg of embelin for 90 days depicting normal microstructure. Ep: epithelium; G: uterine glands and SM: uterine smooth muscle. Eosin (H&E) staining, 400x magnifications.

4.2.3.3.15. Effect of embelin on histopathology of brain tissue

The result of microscopic examination of sub-chronic toxicity test revealed that administration of embelin did not induce any significant alterations in the microstructure of brain tissue. This observation holds true across all treatment doses, as depicted in **Figure 25**

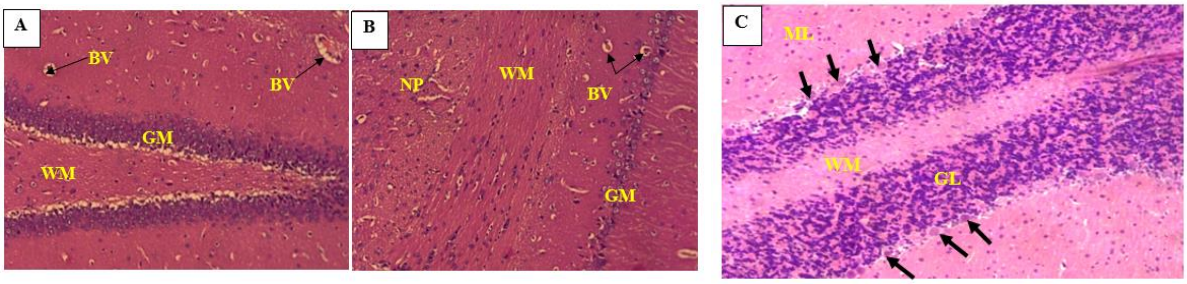

Figure 25: Photomicrograph depicting normal microstructures of brain tissue sampled from animals treated with daily administration of embelin at a dose of 1000 mg/kg (**A: cerebrum &C: cerebellum**) and control animals (**B**). **BV**: blood vessels; **GL**: granular layer; **GM**: gray mater; **ML**: molecular layer; **NP**: neuropil and **WM**: white mater.

2.2.2. Developmental toxicity

2.2.2.1.Clinical observation

The daily cage-side clinical evaluation revealed that there was neither an abortion nor a maternal

death report. Pregnant animals were also free from any sign of toxicity.

2.2.2.2.Embryonic outcomes

As embryonic outcome indicators, morphological scores and the number of somites did not exhibit significant differences across the experimental groups and their control counterparts (**Table 24**). Nevertheless, the embryonic crown-rump length (CRL) was found to be significantly shorter in the higher dose group, 1000 mg/kg, than in vehicle and *ad libitum* control animals.

Table 24: Developmental characteristics of embryos in the experimental group of pregnant rats following embelin treatment

Group	**Embryonic developmental variables**		
	Morphological score/ litter	**Number of somites/litters**	**CRL(mm)/litter**
Group IV (vehicle)	45.54±1.95	29.08±1.1	4.86±0.49
Group V (*Ad libitum)*	45.35±1.17	29.09±1.11	4.96±0.42
Group I (250 mg/kg)	44.84±1.7	28.44±1.23	4.86±0.13
Group II (500 mg/kg)	44.52±1.5	28.52±1.24	4.79±0.14
Group III (1000 mg/kg)	44.5±1.45	28±0.78	4.51±0.35*

Data were shown as mean ± standard deviation (μ±SD), CRL: Crown-rump length.*statistically significant (p-value<0.05)

2.2.2.3.Embryonic developmental indices

4.2.3.3.16. Circulatory system

The current study revealed that the yolk sac circulation and heart development showed no sign of developmental delay across all experimental groups (**Table 25; Figure 26**).

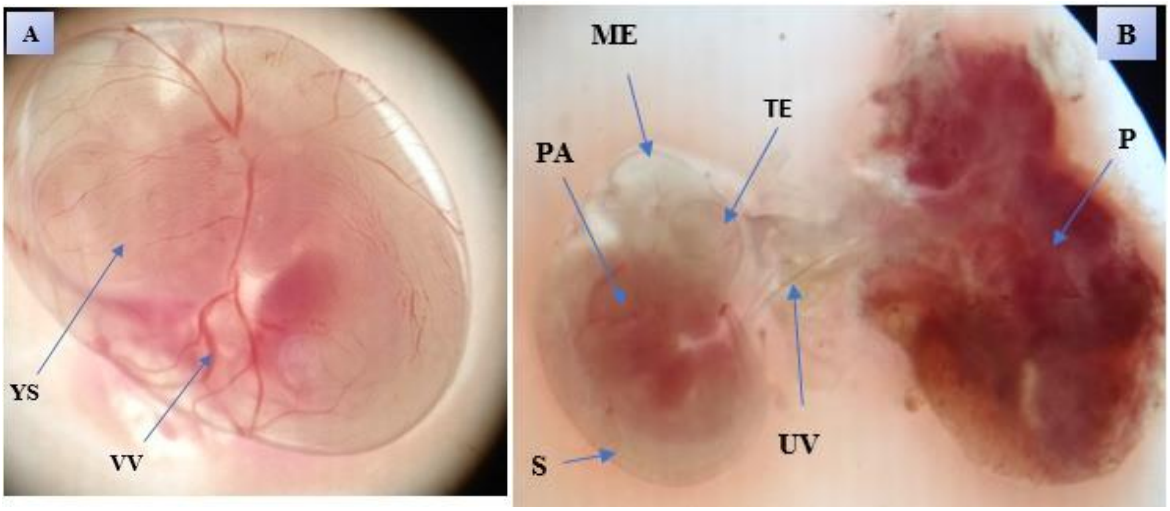

Figure 26: 12-Day-old embryos from 1000 mg/kg. [A]: Embryo inside its yolk sac (YS) with vitelline vessels (VV); [B]: ME (Mesencephalon); P (placental tissue); PA (Pharyngeal apparatus); S (Somite); TE (Telencephalon); and UV (umbilical vessels)

Table 25: Developmental characteristics of the circulatory system of embryos in the experimental group of pregnant rats following embelin treatment

Group	**Proportion of delayed development**		
	Yolk sac circulation	**Heart**	**Allantois**
Group IV (vehicle)	0	0	0
Group V (*Ad libitum*)	0	0	0
Group I (250 mg/kg)	0	0	0
Group II (500 mg/kg)	0	0	0
Group III (1000 mg/kg)	0	0	0

Results: Proportion, Chi-square test

4.2.3.3.17. Nervous system and sense organs

As indicated in **Table 26**, the caudal neural tube, hindbrain, forebrain, otic system, and optic system were not subjected to developmental delay after treating pregnant rats with a high dose of embelin.

Table 26: Nervous system and sense organs characteristics of the embryos in the experimental group of pregnant rats following embelin treatment

Group	**Proportion of delayed development**				
	Caudal neural tube	**Hind brain**	**Fore brain**	**Otic system**	**Optic system**
Group IV (vehicle)	0	0	0	0	0
Group V (*Ad libitum*)	0	0	0	0	0
Group I (250 mg/kg)	0	0	0	0	0
Group II (500 mg/kg)	0	0	0	0	0
Group III (1000 mg/kg)	0	0	0	0	0

Results: Proportion (%). Chi-square test.

4.2.3.3.18. Musculoskeletal System

As depicted in **Table 27**, the current experiment showed that musculoskeletal development parameters were not significantly delayed in the treatment groups compared to the controls.

Table 27: Musculoskeletal system characteristics of the embryos in the experimental group of pregnant rats following embelin treatment

Group	Proportion of retarded development					
	Pharyngeal apparatus	**Maxillary process**	**Mandibular process**	**Fore limb**	**Hind limb**	**Flexion**
Group IV (vehicle)	0	0	0	0	0	0
Group V (*Ad libitum)*	0	0	0	0	0	0
Group I (250 mg/kg)	0	0	0	0	0	0
Group II (500 mg/kg)	0	0	0	0	0	0
Group III (1000 mg/kg)	0	0	0	0	0	0

Results: Proportion (%), Chi-square test

4.2.3.3.19. Food intake and weight gain

The mean food consumption of animals in Group III (1000 mg/kg treatment group) showed a statistically significant increment, (p-value<0.001), compared to both pair-fed and *ad libitum* control animals. Similarly, the mean food intake of Group I (250 mg/kg) and Group II (500 mg/kg) animals was also higher than that of vehicle and *ad libitum* control animals (p-value<0.001). As illustrated in **Table 28**, experimental pregnant animals in all three treatment groups showed a significant dose-dependent decrease in the mean of their weight when compared to animals in both control groups.

4.2.3.3.20. Pregnancy outcomes

As shown in **Table 29** and **Figure 27** the number of implantations is significantly low in all of embelin-treated groups when compared to vehicle control and *ad libitum* groups. Similarly, the number of resorption sites and live fetuses were also possessed significant difference between experimental groups and the controls.

Table 28: Food intake and weight gain of pregnant rats treated with embelin

Maternal variables	Experimental groups				
	Group IV Vehicle control	**Group V *Ad libitum***	**Group I 250 mg/kg**	**Group II 500 mg/kg**	**Group III 1000 mg/kg**
Food intake (g)	171.07±14.45	167.54±21.01	183.87±23.49*	185.24±10.16*	198.09±13.62**
Weight gain (g)	87.81±5.48	89.73±4.31	71.72±3.27**	68.4±7.64**	67.7±7.31**

Data were shown as mean ± standard deviation (μ±SD).*statistically significant (p-value<0.05) and **statistically significant (p-value<0.001).

Table 29: Pregnancy outcomes in the experimental group of pregnant rats following embelin treatment

Pregnancy outcomes	Experimental groups				
	Group IV (vehicle control)	**Group V (*Ad libitum*)**	**Group I (250 mg/kg)**	**Group II (500 mg/kg)**	**Group III (1000 mg/kg)**
Number of implantation/dams	10 ± 1.58	10.4 ± 1.14	5.4 ± 0.55*	5.4 ± 1.14*	4.2 ± 0.84*
Number of prior resorptions/dams	0	0	0.4 ± 0.55*	0.8 ± 0.45*	1.4 ± 0.55*
Alive pups	10 ± 1.58	10.4 ± 1.14	5 ± 1*	4.6 ± 1.52*	2.8 ± 1.09*
Dead pups	0	0	0	0	0

Data were shown as mean ± standard deviation of the means (μ±SD). *Statistically significant (p-value<0.001).

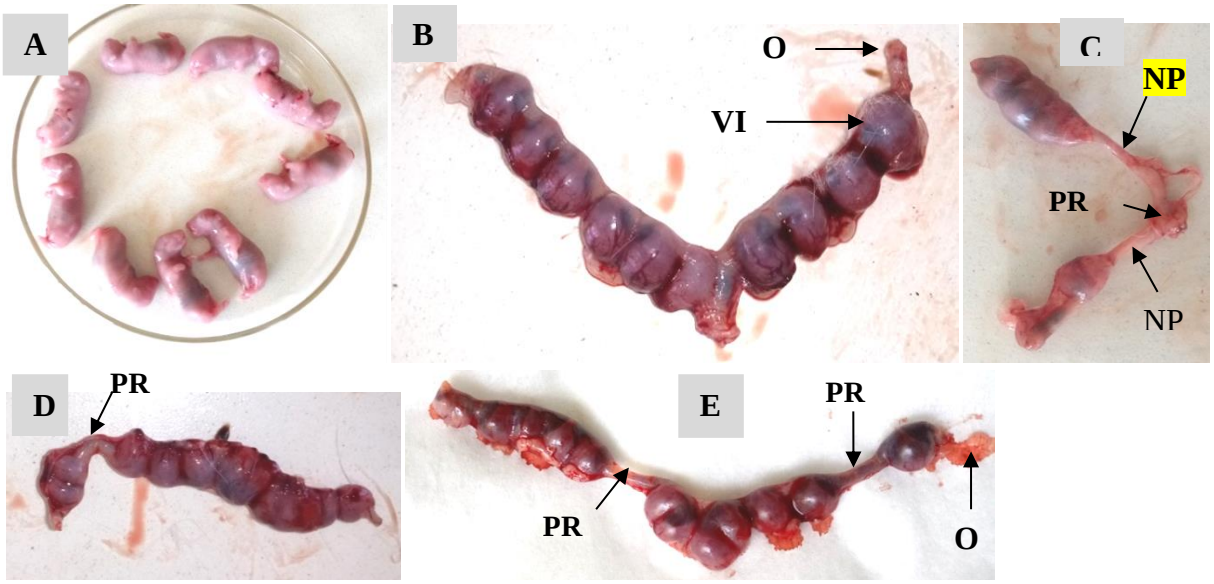

Figure 27: Implantation on a gravid uterus of rats treated with embelin. A: Alive near term fetuses, B: a gravid uterus from rats treated with tween 80 showing the ovary (O) and a viable implantation site (VI); C: A gravid uterus from high dose treatment group (1000 mg/kg) with visibly impaired implantation sites designated as NP and prior resorption (PR); D: Gravid uterus from rats treated with 250 mg/kg of embelin; E: uterus from rats treated with 500 mg/kg of embelin.

2.2.2.4.Fetal outcomes

The three pregnancy outcome markers, fetal weight, placental weight, and crown-rump length, showed no statistically significant variation in mean weight across the experimental groups (**Table 30**).

Table 30: Fetal outcomes in the experimental group of pregnant rats following embelin treatment

Fetal outcomes	**Experimental groups**				
	Group IV (vehicle control)	**Group V (*Ad libitum*)**	**Group I (250 mg/kg)**	**Group II (500 mg/kg)**	**Group III (1000 mg/kg)**
Fetal weight (g) per dam	3.47±1.00	3.66±0.76	3.78±1.02	3.98±0.87	4.02±0.86
Crown-ramp length (cm)	4.7±0.41	4.87±0.21	4.86±0.11	4.7±0.41	4.67±0.21
Placental weight (gm)	0.59±0.04	0.6±0.085	0.6±0.064	0.59±0.061	0.58±0.14

Data were shown as mean ± standard deviation of the means (μ±SD), CRL: Crown-rump length.

4.2.3.3.21. Histopathology of placenta

When the placenta tissues of the experimental animals were inspected under a microscope, there was no visible difference between the control and embelin-treated groups (**Figure 28, Table 31)**.

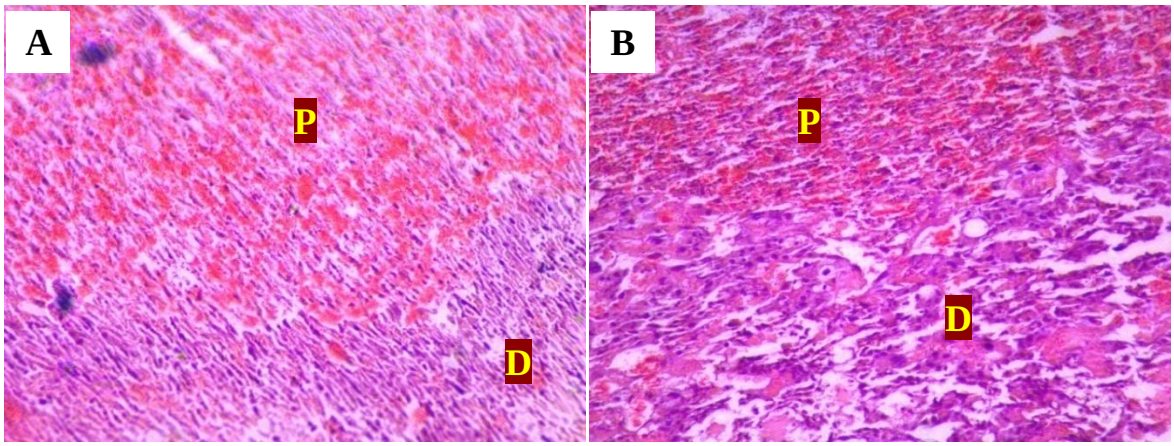

Figure 28: Normal histology of placenta from pregnant rats treated with 1000 mg/kg of embelin isolated from *E. schimperi* fruit and control; Decidual and layer (D), Labrynzine zone (L).

Table 31: Distribution of placental histopathological manifestations across experimental groups

Histopathological parameters of placenta	Experimental groups				
	Group IV (Pair-fed control)	**Group V (Ad *libitum)***	**Group I (250 mg/kg)**	**Group II (500 mg/kg)**	**Group III (1000 mg/kg)**
Necrosis	0	0	0	0	0
Cytolysis	0	0	0	0	0
Apoptosis	0	0	0	0	0
Inflammation	0	0	0	0	0
Calcification	0	0	0	0	0

NB: Results are presented as percentages of histopathological findings, chi-square test.

4.2.3.3.22. External and visceral morphology

Fetuses from both treatment and control groups did not exhibit significant external morphological defects or malformations (**Table 32**). Furthermore, there were no visible structural defects in visceral structures among all groups of animals (**Figure 29**).

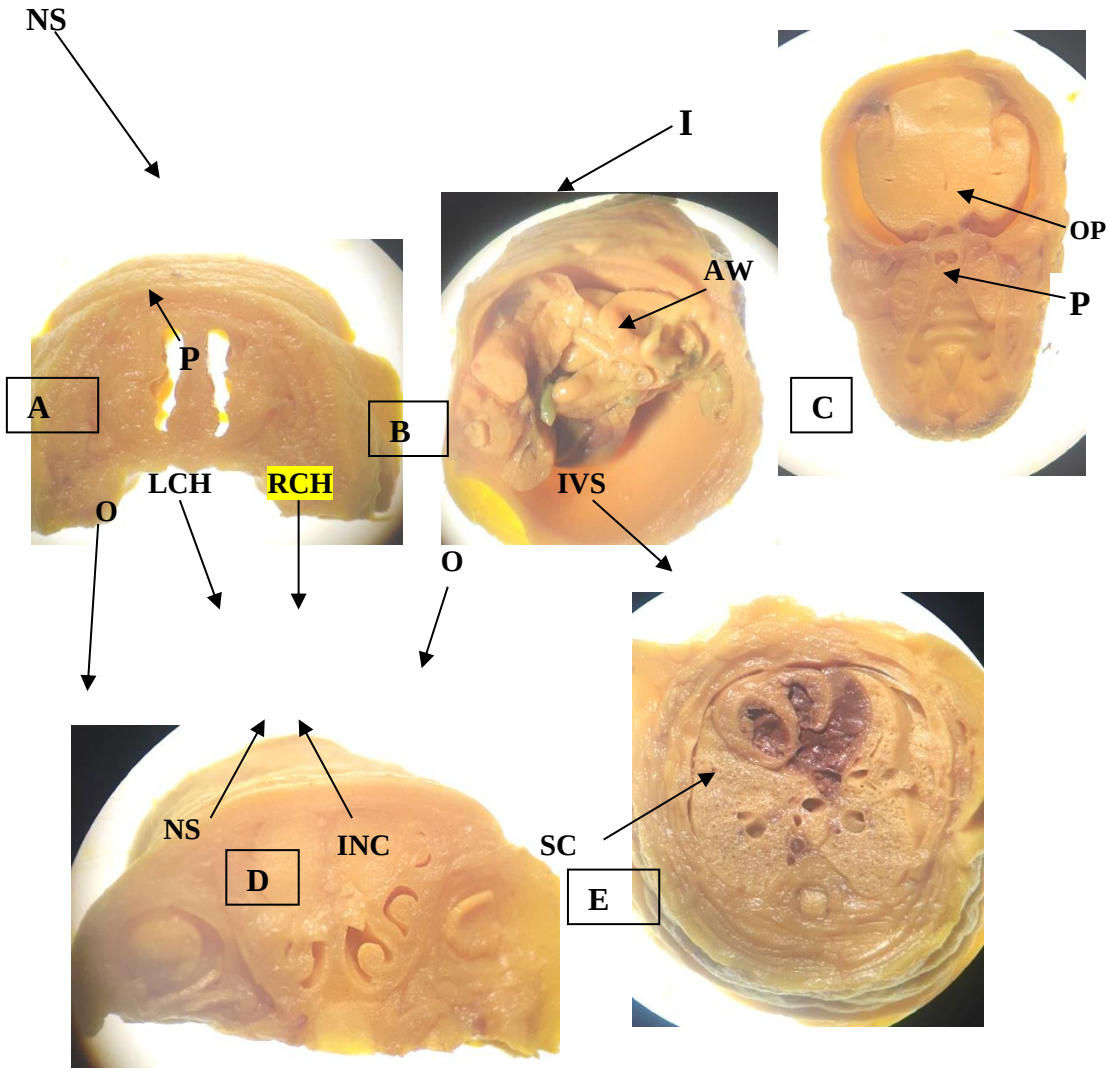

Figure 29: (A) (NS: nasal septum, P: palate); (B) (AW: abdominal wall, I: intestinal content); (C) (OP: oropharynx, P: palate); (D) (INC: inferior nasal conchae, LCH: left cerebral hemisphere, O: optical tissue and RCH: right cerebral hemisphere); (E) (IVS: interventricular septum, SC: spinal cord).

Table 32: External gross malformation characteristics in the experimental group of pregnant rats following embelin treatment

Group	**Proportion of external malformations (%)**							
	Nervous system defects			Musculoskeletal defects			Others	
	EE	AE	SB	KY	SC	LD	MT	EGA
Group IV (Pair-fed)	0	0	0	0	0	0	0	0
Group V (*Ad libitum*)	0	0	0	0	0	0	0	0
Group I (250 mg/kg)	0	0	0	0	0	0	0	0
Group II (500 mg/kg)	0	0	0	0	0	0	0	0
Group III (1000 mg/kg)	0	0	0	0	0	0	0	0

Results: Proportion, Chi-square test. EE: Exencephaly, AE: Anencephaly, SB: Spina bifida, KY: Kyphosis, SC: Scoliosis, LD: Limb defect, MT: Missed tail, EGA: External genitalia agenesis.

4.2.3.3.23. Skeletal Evaluation

As shown in **Table 33, Table 34, and Figure 30**, there is no significant difference in the number of ossification centers in both the axial and appendicular skeletons.

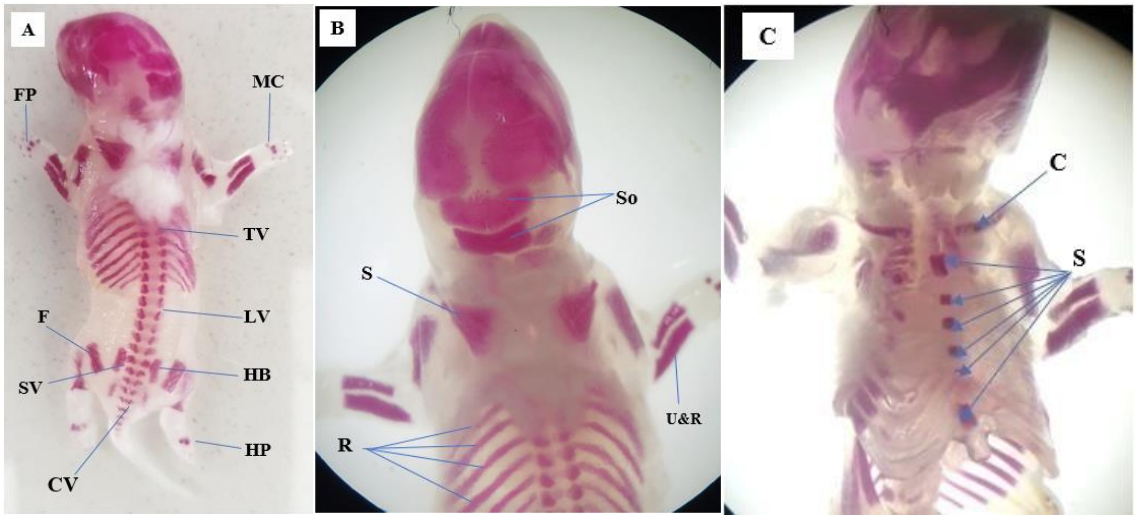

Figure 30: Skeletal ossification with alizarin red. C: clavicle, CV: Caudal vertebrae, F: femur, FP: Forelimb phalanges, HB: Hip bone, HP: Hindlimb phalanges, LV: Lumbar vertebrae, MC: Metacarpus, R: Ribs, So: Supraoccipital, S: Sternum, SV: Sacral vertebrae, TV: Thoracic vertebrae, U & R: ulna and radius

Table 33: Number of ossification centers in the axial skeleton of rat fetuses from experimental groups treated with embelin

Group	**Sternum**	**Thoracic vertebrae**	**Lumbar vertebrae**	**Caudal**	**Ribs**
Group IV (vehicle)	5.77±0.14	12±0	5±0	4.21±0.23	24±0
Group V (*Ad libitum)*	5.69±0.18	12±0	5±0	4.31±0.22	24±0
Group I (250 mg/kg)	5.68±0.12	12±0	5±0	4.22±0.24	24±0
Group II (500 mg/kg)	5.48±0.21	12±0	5±0	4.11±0.12	24±0
Group III (1000 mg/kg)	5.64±0.2	12±0	5±0	4±1.38	24±0

Data were shown as mean ± standard deviation of the means (μ±SD)

Table 34: Number of ossification centers in the appendicular skeleton of rat fetuses from experimental groups treated with embelin

Group	**Forelimb phalanges**	**Hind limb phalanges**	**Metacarpus**	**Metatarsus**
Group IV (vehicle)	3.8±0.28	3.51±0.32	3.89±0.29	4.14±0.27
Group V (*Ad libitum)*	3.85±0.32	3.44±0.31	3.87±0.31	4.08±0.14
Group I (250 mg/kg)	3.81±0.42	3.49±0.39	3.87±0.31	4.07±0.27

Group II (500 mg/kg)	3.84±0.26	3.48±0.41	3.81±0.38	4±0.35
Group III (1000 mg/kg)	3.82±0.31	3.42±0.51	3.88±0.31	4.01±0.43

Data were shown as mean ± standard deviation of the means (μ±SD)

4.3.Fixed oil

4.3.1. Acute toxicity and LD_{50}

All female animals treated with 5000mg/kg fixed oil extract limit test survived without showing any clinical signs of toxicity. As a result, the LD50 of *E. schimperi* Vake fruit fixed oil extract can be declared greater than 5000mg/kg. Furthermore, necropsy revealed no gross pathological changes on experimental animals.

4.3.2. Sub-chronic Toxicity

4.3.2.1.Cage side clinical examination, food intake and body weight

The first two days of the experimental period were marked by diarrhea in both sexes of the experimental animals from the 1000 mg/kg treatment group along the daily cage side clinical assessment of the sub-chronic oral toxicity test of fixed oil extract (**Figure 31**). However, in test and control animal groups, no mortality or other severe toxicity indicators (such as convulsions, tremors, respiratory issues, and skin changes) were seen in either sex.

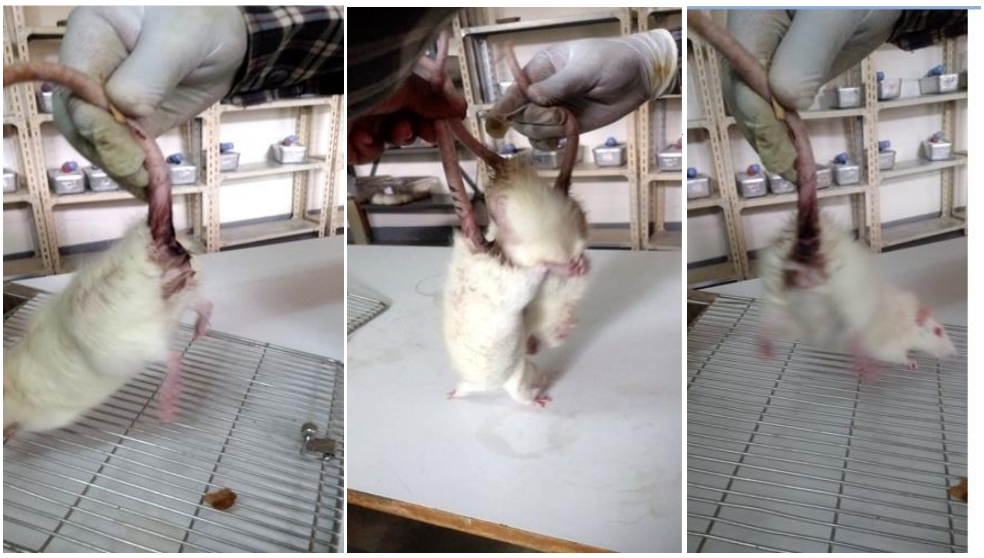

Figure 31: Diarrheal incident from experimental animals treated with 1000 mg/kg of fixed oil extract from *E. schimperi*.

4.3.2.2.Food intake and body weight

Table 35 shows that compared to the control rats in both sexes, the total mean weight increase of the animals from all fixed oil extract of *E. shimperi* treatment groups illustrated statistically significant elevation. Additionally, during the course of the experiment, the experimental animals that received the fixed oil extract also consumed a notably higher amount of food.

Table 35: Effect of sub-chronic administration of fixed oil extracted from *E. schimperi* Vake fruit on weight gain and food intake of rats

	Sex of Animals	**Group IV Vehicle Control**	**Group I 250mg/kg**	**Group II 500mg/kg**	**Group III 1000mg/kg**
Weight gain (gm)	**Male**	92.64±6.54	98.28±8.15**	109.66±5.37**	115±7.64**
	Female	39.53±3.44	45.81±1.19*	48.47±5.23**	56.45±3.58**
Food intake (gm)	**Male**	204.14±16.37	212.61±10.12*	213.49±12.77*	218.31±12.53**
	Female	149.38±9.13	158.17± 10.88*	160.23±11.4*	169.35±10.2*

Results are defined in terms of mean ± standard deviation, one-way ANOVA. *statistically significant (P-value<0.05), ** p-value ≤ 0.001 compared to vehicle controls.

4.3.2.3.Effect of fixed oil on hematological parameters

Following sub-chronic administration of the fixed oil produced from *E. schimperi* Vake, the hematological profile parameters of experimental animals of both sexes did not exhibit notable difference, as shown in the **Table 36 & 37**and below.

Table 36: Effect of sub-chronic administration of fixed oil extracted from *E. schimperi* Vake fruit on hematological profiles of male rats

Hematological Parameters	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
RBC(x 10^6/µl)	5.30 ± 0.8	5.32 ± 0.7	5.27 ± 0.9	5.34 ± 0.6
WBC (x 10^3/µl)	4.01 ± 0.31	4.05 ± 0.32	3.99 ± 0.29	4.08 ± 0.34
PLT (x 10^5)	670 ± 19.4	671 ± 18.2	669 ± 17.8	672 ± 18.7
Hgb (g/dl)	12.5 ± 0.21	12.6 ± 0.24	12.7 ± 0.22	12.8 ± 0.27
MCV (fL)	67.8 ± 0.39	67.9 ± 0.43	68.2 ± 0.38	67.7 ± 0.41
MCH (pg)	26.62 ± 4.2	26.68 ± 4.6	26.75 ± 4.4	26.80 ± 4.8
MCHC (g/dl)	36.9 ± 0.28	36.9 ± 0.29	37.1 ± 0.31	36.8 ± 0.27

Results are illustrated in terms of mean ± standard deviation from the mean, one-way ANOVA.

Table 37: Effect of sub-chronic administration of fixed oil extracted from *E. schimperi* Vake fruit on hematological profiles of female rats

Hematological Parameters	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
RBC(x 10^6/µl)	4.74 ± 0.68	4.72 ± 0.7	4.79 ± 1.0	4.88 ± 0.42
WBC (x 10^3/µl)	3.88 ± 0.40	4.12 ± 0.38	3.92 ± 0.33	4.03 ± 0.31
PLT (x 10^5)	605 ± 16.23	612 ± 16.7	596 ± 15.1	611 ± 16.97
Hgb (g/dl)	9.88 ± 0.41	10.3 ± 0.20	9.84 ± 0.36	9.96 ± 0.21
MCV (fL)	60.58 ± 0.18	61.78 ± 0.456	60.99 ± 0.38	61.89 ± 0.41
MCH (pg)	25.76 ± 3.48	25.62 ± 4.7	26.42 ± 4.1	24.88 ± 3.25
MCHC (g/dl)	35.02 ± 0.75	34.09 ± 0.42	34.95 ± 0.41	34.54 ± 0.32

Results are shown as mean ± standard deviation from the mean, one-way ANOVA.

4.3.2.4.Effect of fixed oil on hepato –renal parameters

4.3.2.4.1. Relative organ weight of liver and kidneys

The daily oral administration of fixed oil derived from *E. schimperi* Vake fruit for 90 days showed no effect on the relative organ weights of the liver and kidneys of the experimental animals from any test group, male or female (**Table 38**).

Table 38: Relative organ weight of liver and kidneys in male and female rats after they are treated sub-chronically with fixed oil extracted from *E. schimperi* Vake fruit

		Control Tween 80	**Group I 250mg/kg**	**Group II 500mg/kg**	**Group III 1000mg/kg**
Liver weight (gm)	**Male**	3.78 ± 0.3	3.8 ± 0.35	3.68 ± 0.65	3.69 ± 0.25
	Female	3.44 ± 0.54	3.48 ± 0.57	3.4 ± 0.51	3.42 ± 0.4
Kidney weight (gm)	**Male**	0.29 ± 0.01	0.31 ± 0.01	0.3 ± 0.014	0.3 ± 0.004
	Female	0.3 ± 0.02	0.28 ± 0.04	0.28 ± 0.02	0.31 ± 0.04

Results given in terms of mean ± standard deviation from the mean, one-way ANOVA.

4.3.2.4.2. Effect of fixed oil on clinical chemistry parameters

Serum biochemical analysis of male experimental rats' blood revealed that the mean ALT level from rats treated with 1000mg/kg of fixed oil from *E. schimperi* is statistically significant higher than in control animals given the vehicle, tween 80. Male rats treated with 500 mg/kg and 1000 mg/kg of fixed oil extract from the plant had significantly elevated AST levels than the control group animals **Table 39**. Similarly, serum ALP levels of male rats from 500 mg/kg and 1000 mg/kg of fixed oil possessed a significant elevation compared to their control counterparts. However, other clinical chemistry parameters did not show significant difference across treatment groups and control animals.

Table 39: Clinical chemistry profile of male rats after they are treated sub-chronically with fixed oil extracted from *E. schimperi* Vake fruit

Hematological Parameters	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
ALT (U/L)	45.2 ± 3.0	50.2 ± 12.9*	52.6 ± 6.0**	58.9 ± 8.8**
AST (U/L)	133.1 ± 20.2	140.5 ± 9.1**	141.9 ± 8.8**	193.2 ± 10.5**
ALP (U/L)	72.8 ± 2.2	76.2 ± 1.3*	86.4 ± 6.8**	105.8 ± 5.6**
Urea (mg/dL)	48.5 ± 4.0	47.2 ± 5.4	43.6 ± 3.6	42.1 ± 7.2
Creatinine(mg/dL)	0.34 ± 0.02	0.32 ± 0.01	0.31 ± 0.01	0.33 ± 0.01
Albumin (g/dL)	4.3 ± 0.2	4.3 ± 0.1	4.4 ± 0.1	4.4 ± 0.1
Total protein (g/dL)	6.1 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	6.2 ± 0.2
Total cholesterol (mg/dL)	39.8 ± 1.6	42.4 ± 2.1	43.2 ± 2.0	44.4 ± 2.3
Glucose (mg/dL)	114.1 ± 9.5	106.5 ± 7.2	100.8 ± 12.5	103.3 ± 11.2
Total triglyceride	21.9 ± 3.2	22.4 ± 2.6	23.5 ± 4.7	24.3 ± 5.0
HDL	22.3 ± 4.4	23.5 ± 5.2	24.0 ± 3.7	25.2 ± 4.0
LDL	13.2 ± 2.6	11.2 ± 3.2	12.0 ± 1.6	12.6 ± 3.0

Mean ± standard deviation from the mean, one-way ANOVA. *statistically significant (P-value<0.05), ** p-value ≤ 0.001 compared to vehicle controls.

Table 40 depicted the clinical chemistry profiles of female rats after 90 days treatment of fixed oil extracted from *E. schimperi* fruit. Significant elevation of serum ALT, AST and ALP levels were observed from female rats treated with 1000 mg/kg of fixed oil extract. The rest of biochemical parameters did not exhibited difference within the experimental animals

Table 40: Clinical chemistry profile of female rats after they are treated sub-chronically with fixed oil extracted from *E. schimperi* Vake fruit.

Hematological Parameters	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
ALT (U/L)	87.1 ± 5.6	60.9 ± 8.5**	58.7 ± 4.4**	54.8 ± 7.4**
AST (U/L)	212.3 ± 10.0	176.7 ± 9.3**	173.6 ± 9.5**	167.6 ± 20.4**
ALP (U/L)	116.1 ± 5.5	97.6 ± 7.1**	92.3 ± 3.3**	97.5 ± 1.4**
Urea (mg/dL)	49.9 ± 4.1	44.9 ± 4.3	44.7 ± 5.5	43.6 ± 4.8
Creatinine(mg/dL)	0.40 ± 0.0	0.42 ± 0.0	0.41 ± 0.0	0.41 ± 0.0
Albumin (g/dL)	4.1 ± 0.1	4.1 ± 0.1	4.1 ± 0.1	4.0 ± 0.1
Total protein (g/dL)	5.8 ± 0.1	5.8 ± 0.1	5.9 ± 0.1	5.9 ± 0.1
Total cholesterol (mg/dL)	46.3 ± 2.6	43.3 ± 2.4	43.4 ± 3.1	45.7 ± 2.5
Glucose (mg/dL)	106.6 ± 8.6	101.9 ± 6.2	102.5 ± 7.3	105.8 ± 6.7
Total triglyceride	29.8 ± 2.4	29.1 ± 3.2	30.2 ± 5.0	32.7 ± 3.5
HDL	32.6 ± 3.7	34.6 ± 2.8	33.2 ± 2.6	35.5 ± 3.6
LDL	14.4 ± 1.2	14.7 ± 2.0	15.5 ± 1.3	16.8 ± 1.4

Results are expressed as mean ± standard deviation from the mean, one-way ANOVA. *statistically significant (P-value<0.05), compared to vehicle controls.

4.3.2.4.3. Effect of fixed oil on histopathology of liver and kidneys

Ninety days of fixed oil extract from *E. schimperi* Vake fruit treatment revealed no significant changes in the microscopic structure of liver tissues evaluated from all groups of rats. The central veins, as well as their radiating hepatocytes and canaliculi, appear normal across all liver tissues examined, as illustrated in (**Figure 32 & 33**). Furthermore, the organized contents of portal triads (portal vein, hepatic artery, and bile duct) did not demonstrate any substantial aberrant histological change.

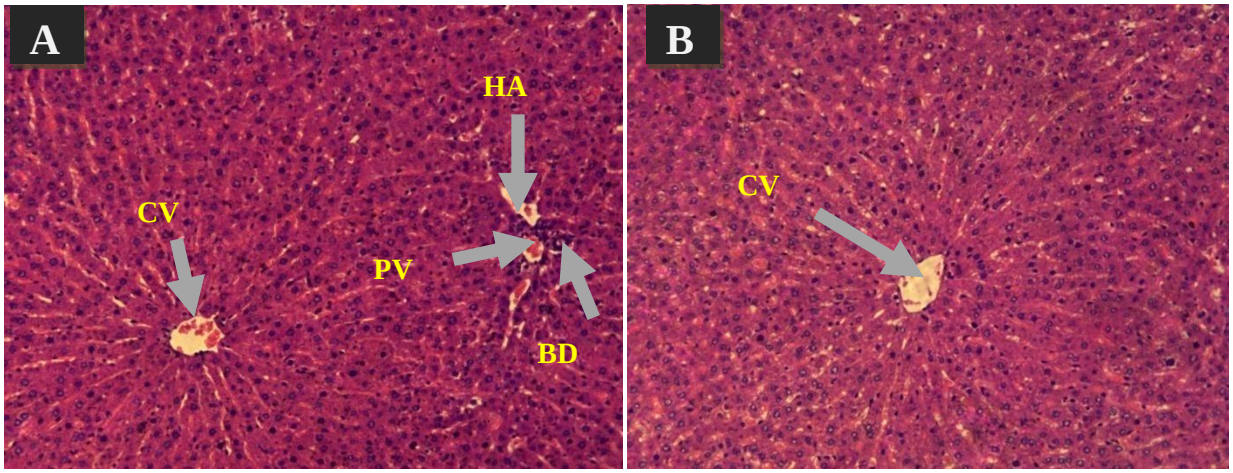

Figure 32: Photomicrograph of normal microstructure of liver tissues from male rats treated with 1000 mg/kg of fixed oil of *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B). BD:** bile duct; **CV:** central vein; **HA**: hepatic artery; **PV:** portal vein. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

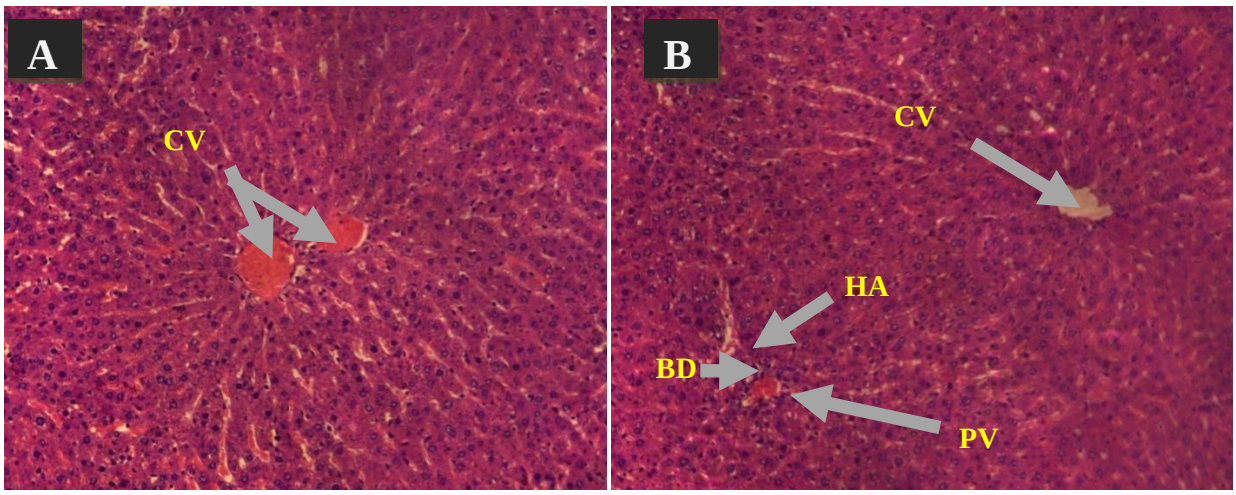

Figure 33: Photomicrograph of normal microstructure of liver tissues from female rats treated with 1000 mg/kg of fixed oil of *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B). BD:** bile duct; **CV:** central vein; **HA**: hepatic artery; **PV:** portal vein. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

The microstructure of kidney tissues did not exhibit any treatment-related structural changes, according to histological inspection of samples from all experimental groups. The photomicrograph (**Figure 34 & 35**) displays the normal structural integrity of the tubules and glomerulus

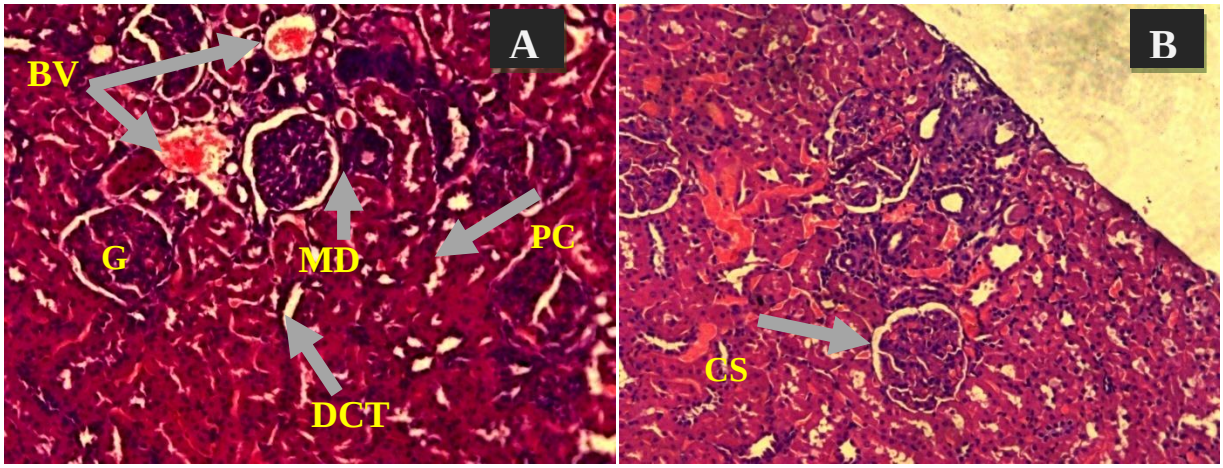

Figure 34: Photomicrograph of normal microstructure of kidney tissues taken from male rats treated with 1000 mg/kg of fixed oil extract from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B).BV**: blood vessel; **CS**: capsular space; **DCT**: distal convoluted tubule, **G**: glomerulus; **MD:** macula densa; **PCT**: proximal convoluted tubule. Hematoxylin-Eosin (H&E) stain, 400x magnification.

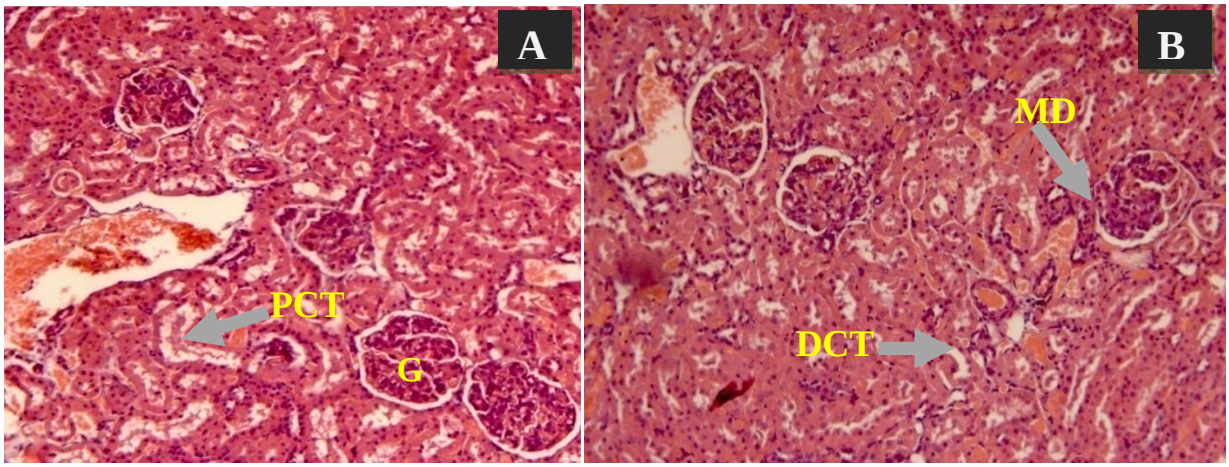

Figure 35: Photomicrograph of normal microstructure of kidney tissues taken from female rats treated with 1000 mg/kg of fixed oil extract from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B).BV**: blood vessel; **CS**: capsular space; **DCT**: distal convoluted tubule, **G**: glomerulus; **MD:** macula densa **PCT**: proximal convoluted tubule. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

4.3.2.5.Effect of fixed oil on histopathology of spleen

Sub-chronic administration of fixed oil did not result in any microscopic structural changes to the arrangement of central arterioles, red pulb, and white pulb, according to a histopathological study of spleen tissues from both sexes (**Figure 36 & 37**).

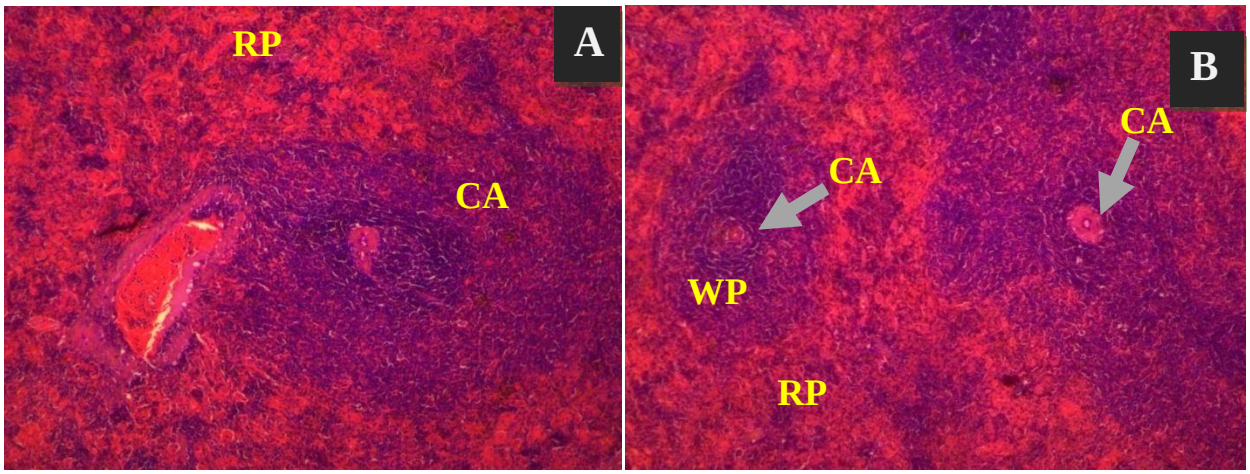

Figure 36: Photomicrograph of normal microstructure of spleen tissues taken from male rats treated with 1000 mg/kg of fixed oil extract from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B). CA**: central arteriole; **RP**: red pulb; **WP**: white pulb. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

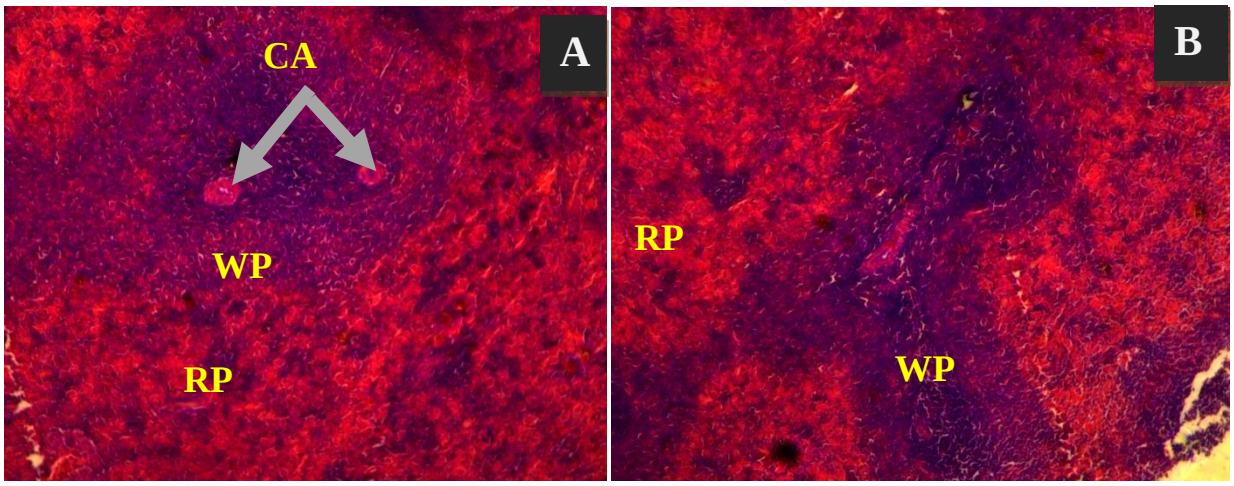

Figure 37: Photomicrograph of normal microstructure of spleen tissues taken from fe male rats treated with 1000 mg/kg of fixed oil extract from *E. schimperi* Vake fruit **(A)** and vehicle control (tween 80) **(B). CA**: central arteriole; **RP**: red pulb; **WP**: white pulb. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

4.3.2.6.Effect of fixed oil on histopathology of Adrenal gland

The photomicrograph **(Figure 38)** illustrates that, in comparison to rats treated with tween 80 as a control vehicle, the microstructural arrangement of the cortical layers of the adrenal gland, namely zona glomerulosa, zona fasiculata, and zona reticularis, as well as the adrenal medulla, did not significantly change in any of the fixed oil treated animals. Moreover, the sub-chronic administration of fixed oil did not alter the normal histological composition of the adrenal medulla.

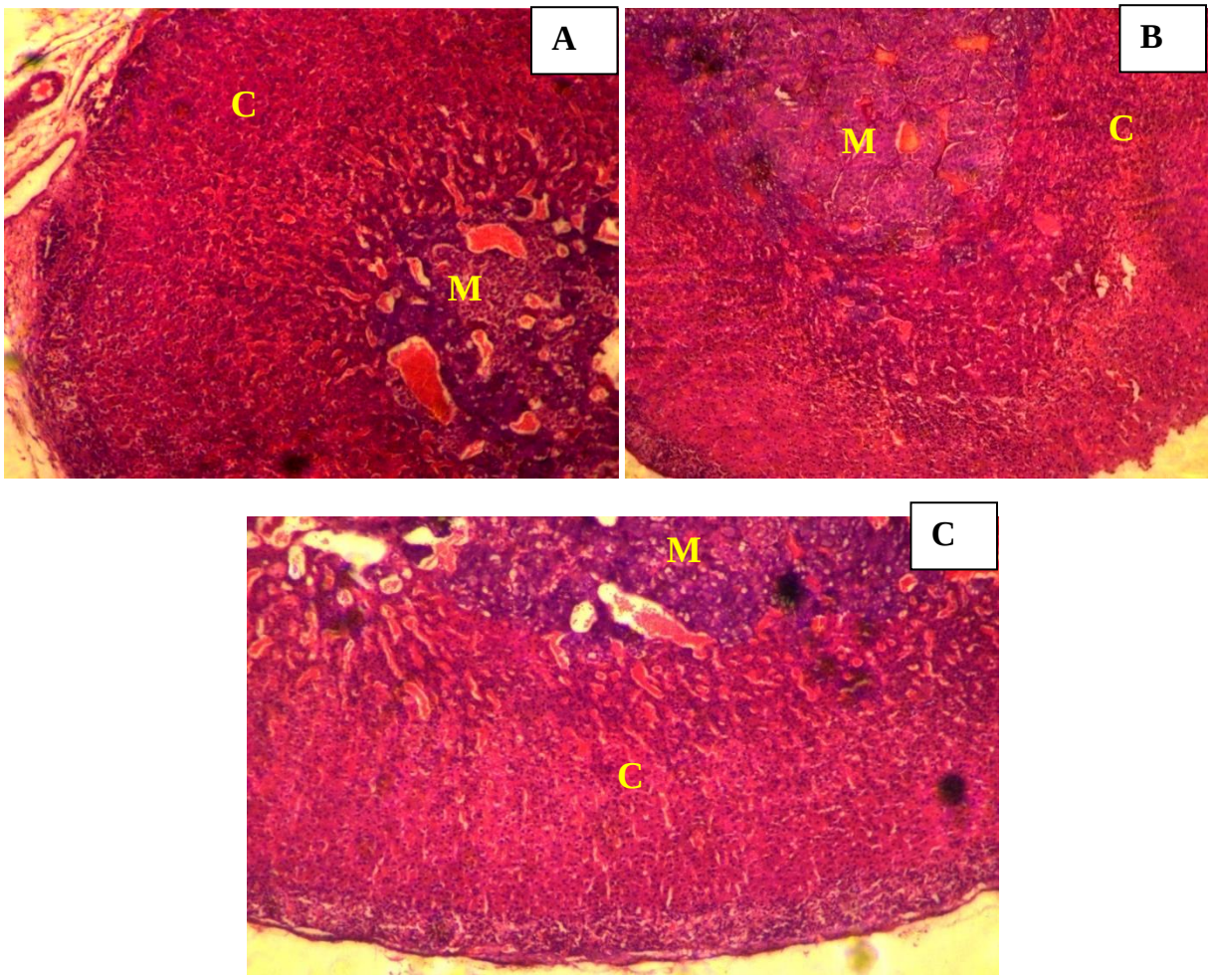

Figure 38: Photomicrograph of normal microstructure of adrenal gland taken from male**(A)**, female **(B)** rats treated with 1000 mg/kg of fixed oil extract from *E. schimperi* Vake fruit and **(C)** controm. **C:** cortex, **M:** medulla. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

4.3.2.7.Effect of fixed oil on male reproductive Indices

4.3.2.7.1. Relative reproductive organ weight

Testes, epididymis, and seminal vesicles were evaluated for relative weight as a crucial indicator of reproductive organ toxicity after receiving fixed oil derived from *E. schimperi* fruit for 90 days. The results, which are displayed in **Table 41**, indicate that there is no discernible difference between the experimental groups' mean relative weights of the aforementioned male reproductive organs.

Table 41: Relative organ weight of testes, epididymis, and seminal vesicles in rats after they are treated sub-chronically with fixed oil from *E. schimperi* Vake fruit

Reproductive organ	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
Testis	1.20 ± 0.046	1.15 ± 0.059	1.12 ± 0.030	1.17 ± 0.061
Epididymis	0.55 ± 0.020	0.53 ± 0.015	0.52 ± 0.032	0.50 ± 0.012
Seminal vesicles	0.88 ± 0.020	0.86 ± 0.045	0.85 ± 0.025	0.82 ± 0.036

Results illustrated as mean ± standard deviation from the mean, one-way ANOVA.

4.3.2.7.2. Sperm count and morphology

When rats were treated with all three dose levels of fixed oil from *E. schimperi* fruits in comparison to rats treated with tween 80, the sperm count in the samples examined from the rats did not show any difference (**Table42**) (**Figure 39**)

Table 42: Effect of sub-chronic administration of embelin on sperm count and proportion of aberrant sperm cells.

	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
Sperm count (x106/ml)	281± 11.55	282.42 ± 17.1	279.33 ± 18.63	278 ± 16.57
Proportion of aberrant sperm cell (%)	8.23 ± 1.73	8.33 ± 1.46	8.51 ± 1.57	8.48 ± 2.14

Results are evaluated as mean ± standard deviation, one-way ANOVA.

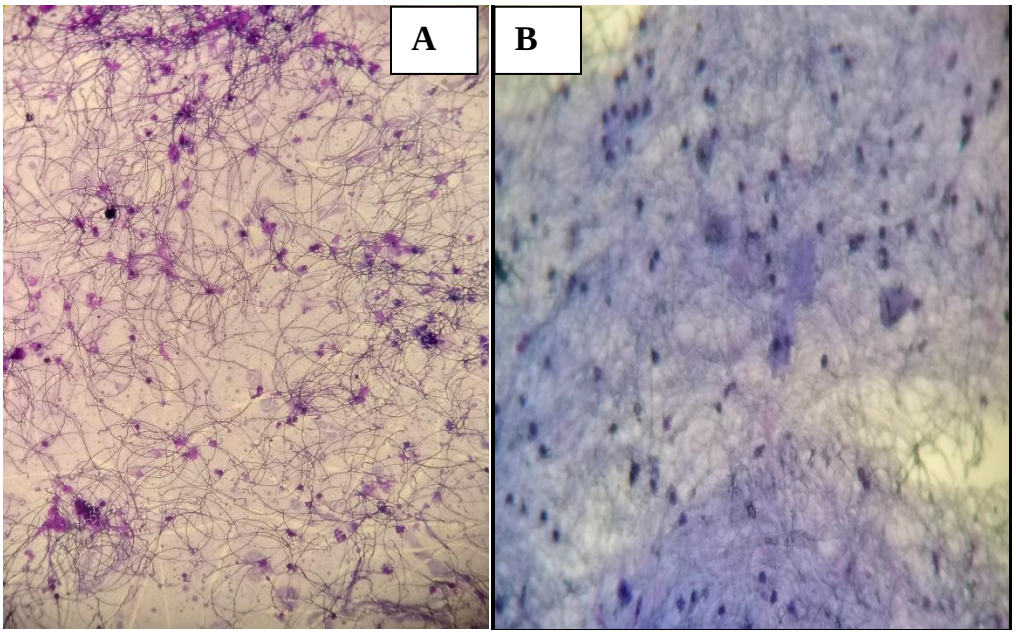

Figure 39: Photomicrograph of sperm cells from rats treated with 1000 mg/kg of fixed oil from *E. schimperi* Vake fruit **(A)**and vehicle control (tween 80) **(B).** Giemsa stain**,** 400 x magnifications

4.3.2.7.3. Effect of fixed oil on male sex hormones

After taking a daily dosage of fixed oil produced from *Emelia schimperi* Vake fruit for 90 days in a row, the levels of male sex hormones, including testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH), did not change (**Table 43)**.

Table 43: Effect of sub-chronic administration of fixed oil on serum levels of male sex hormones

Male sex hormones	**Control** **Tween 80**	**Group I** **250mg/kg**	**Group II** **500mg/kg**	**Group III** **1000mg/kg**
FSH (ng/ml)	211 ± 21.43	211.84 ± 16.37	210.5 ± 17.42	212 ± 13.1
LH (ng/ml)	2.74 ± 0.34	2.72 ± 0.31	2.68 ± 0.16	2.7 ± 0.12
Testosterone (ng/ml)	3.34 ± 0.42	3.41 ± 0.08	3.35 ±0.04	3.37 ± 0.037

Results shown as mean ± standard deviation, one-way ANOVA.

4.3.2.7.4. Effect of fixed oil on histopathology of epididymis

As rats were given fixed oil extracted from *E. schimperi* fruit orally for 90 days, there were no significant histological changes observed in their epididymal tissue under light microscopy, as shown in **Figure40** as compared to the control group.

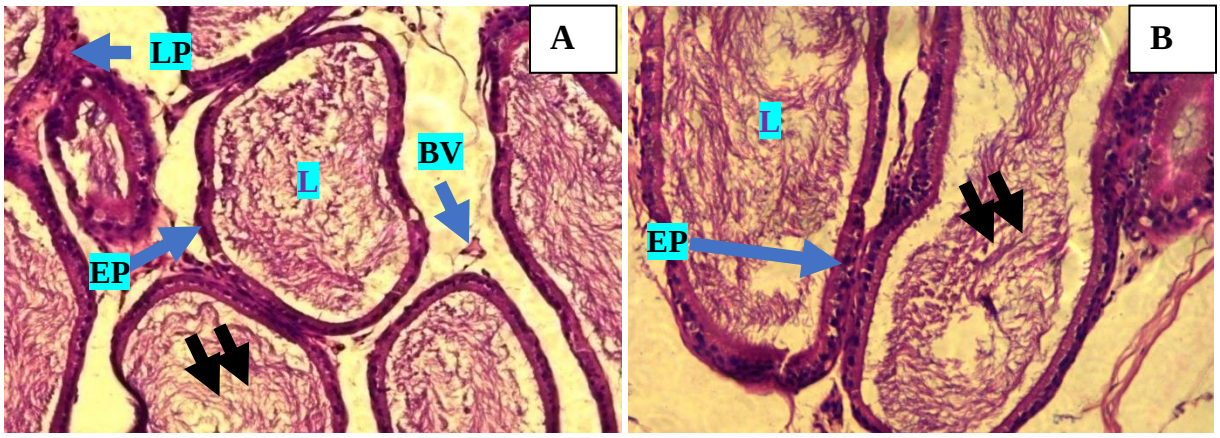

Figure 40: Photomicrograph of rats treated with tween 80 and 1000 mg/kg of fixed oil (**A & B**) showing the epididymis in normal microstructure with blood vessels (**BV**) within the lamina propria (**LP**), visible spermatozoa (black arrows) inside the lumen (**L**), and intact epididymal epithelium (**EP**). 400x magnification of Hematoxylin-Eosin (**H&E**) staining.

4.3.2.7.5. Effect of fixed oil on histopathology of testes

After sub-chronic treatment with fixed oil extract, testicular tissues under a light microscope showed no structural changes in any of the experimental animals (**Figure 41**)

4.3.2.7.6. Effect of fixed oil on histopathology of seminal vesicles

The microstructure of all tissue samples, whether or not they were treated with fixed oil, was found to be normal when seminal vesicles were examined in the context of assessing the sub-chronic toxicity of fixed oil produced from *E. schimperi* Vake fruit. As observed in **Figure 42**, the epithelium is evidently intact and has an adequately full luminal content.

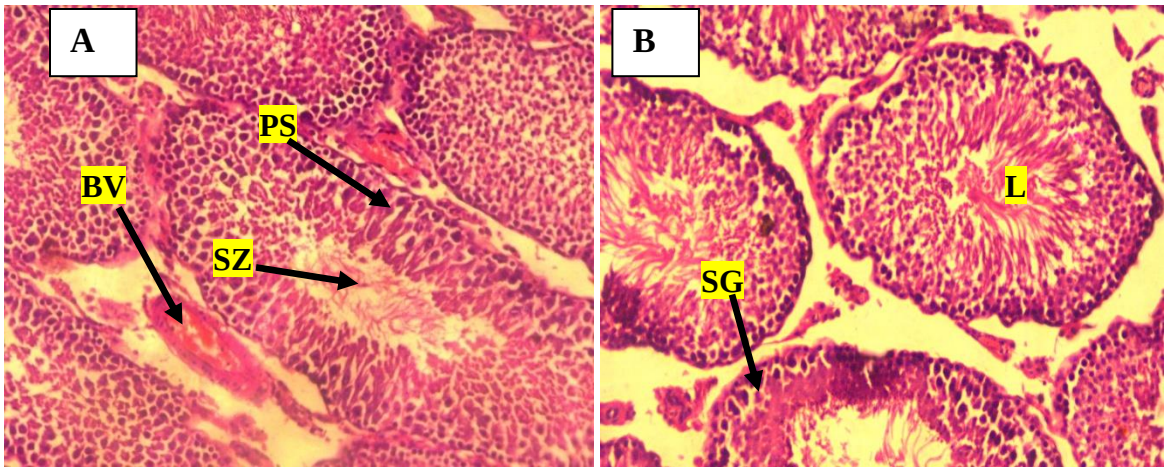

Figure 41: Photomicrograph of seminiferous tubules of testes sampled from rats sub-chronically treated with tween 80 **(A)** and 1000mg/kg **(B)** of fixed oil extract. **BV**: blood vessels, **L:** lumen, **PS:** primary spermatocytes, **SG**: spermatogonia, **SZ:** spermatozoa. Hematoxylin-Eosin (H&E) staining, 400 x magnifications.

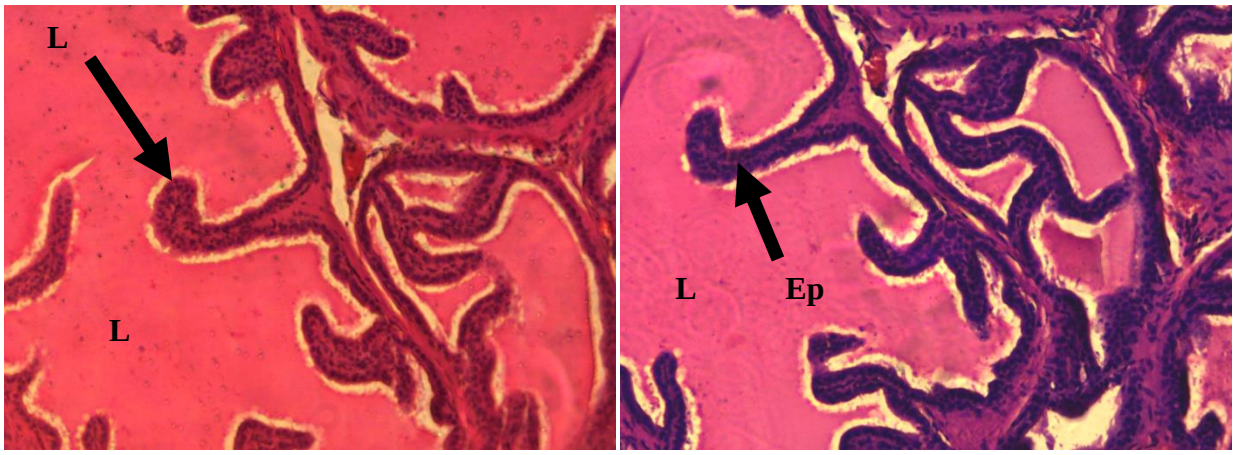

Figure 42: Photomicrograph of seminal vesicles sampled from rats treated with 1000 mg/kg of fixed oil showing normal microstructure. **EP**: epithelium, **L**: lumen and **LP**: lamina propria. Hematoxylin-Eosin (H&E) staining; (A) 100x and (B) 400x magnifications.

4.3.2.8.Effect of fixed oil on female reproductive indices

4.3.2.8.1. Relative reproductive organ weight

Table 44 shows that the relative weight of the uterus and ovaries did not exhibit any alteration after a sub-chronic treatment with fixed oil extract from *E. schimperi* Vake fruit.

Table 44: Effect of sub-chronic administration of fixed oil extract on the relative weight of female reproductive organs

Reproductive organs	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
Uterus	0.32 ± 0.021	0.31 ± 0.005	0.32 ± 0.005	0.33 ± 0.003
Ovary	0.036 ± 0.0013	0.035 ± 0.0011	0.036 ± 0.0031	0.035 ± 0.002

Mean ± standard deviation, one-way ANOVA.

4.3.2.8.2. Effect of fixed oil on female sex hormones

An ELISA analysis of female sex hormones conducted in the serum following a 90-day fixed oil administration period showed no discernible effects. Female rats treated with all dosage levels of fixed oil did not exhibit different levels of FSH and LH from the controls. The levels of progesterone and estrogen is not different from the corresponding controls (**Table 45**).

Table 45: Effect of sub-chronic treatment of fixed oil on the serum level of female sex hormones

Male sex hormones	Control Tween 80	Group I 250mg/kg	Group II 500mg/kg	Group III 1000mg/kg
FSH (ng/ml)	18.21 ± 1.45	17.91 ± 2.73	17.57 ± 1.84	17.71 ± 1.38
LH (ng/ml)	12.16 ± 1.63	12.24 ± 1.63	12.25 ± 2.01	11.88 ± 1.53
Estradiol (ng/ml)	92.37 ± 6.47	92.53 ± 8.57	91.74 ± 7.23	91.91 ± 8.74
Progesterone (ng/ml)	78.64 ± 9.4	78.75 ± 7.56	78.78 ± 2.74	78.47 ± 8.67

Mean ± standard deviation, one-way ANOVA.

4.3.2.8.3. Effect of fixed oil on the estrous cycle

Vaginal smears from female rats treated with fixed oil and tween 80 for ninety days were analyzed cytologically, and the results indicated regular estrous cycles in all experimental groups

4.3.2.8.4. Effect of fixed oil on histopathology of ovaries

After receiving fixed oil sub-chronically for 90 days, the ovarian tissue from experimental rats was examined under a light microscope, and the results revealed that the ovarian tissues from all treatment groups had a micro-structural arrangement that was comparable to the ovarian tissues from control animals (**Figure 43)**.

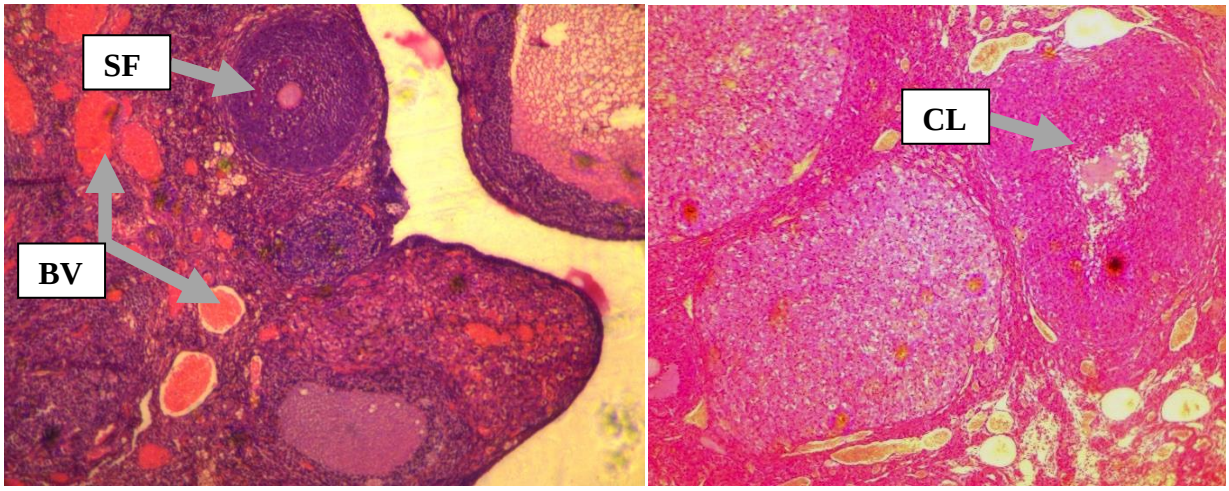

Figure 43: Photomicrograph of ovary histology after administration of 1000 mg/kg of fixed oil and Tween 80 (**B**). **BV**: blood vessel; **CL**: corpus luteum; **SF**: secondary follicle. Eosin (H&E) staining, 400x magnifications.

4.3.2.8.5. Effect of fixed oil on Histopathology of uterus

As illustrated in **figure44**, microscopic evaluation of uterine tissues explanted from experimental rats revealed that there is no significant variation in microstructure of uterus from animals treated with all dose level of embelin and controls.

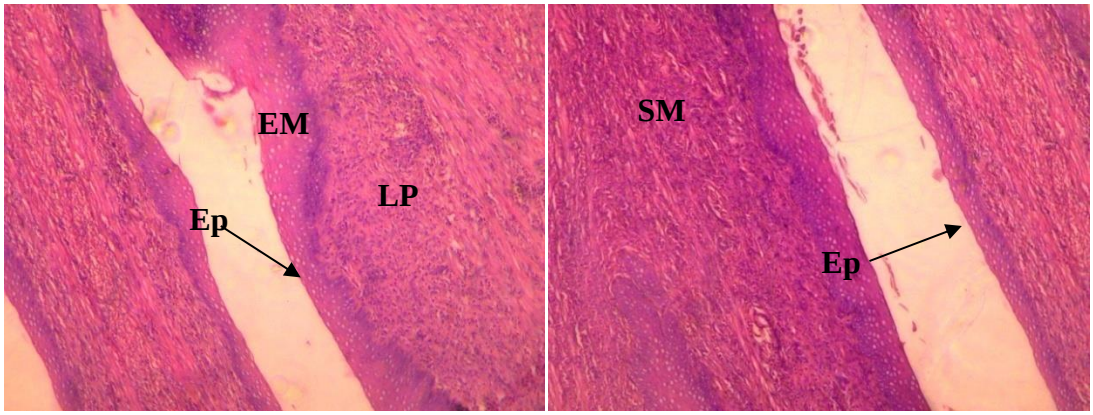

Figure 44: A photomicrograph showing the normal microstructure of the uterus taken from experimental animals given 1000 mg/kg of embelin for 90 days. Endometrium (EM) SM is uterine smooth muscle; LP is lamina propria; and Ep stands for epithelium. 400x magnification of Eosin (H&E) staining.

4.3.2.9.Effect of fixed oil on histopathology of brain tissue

The sub-chronic toxicity test's microscopic analysis indicated that oral treatment of fixed oil did not significantly change the architecture of brain tissue. This observation is consistent with all treatment dosages, as **figure 45** illustrates.

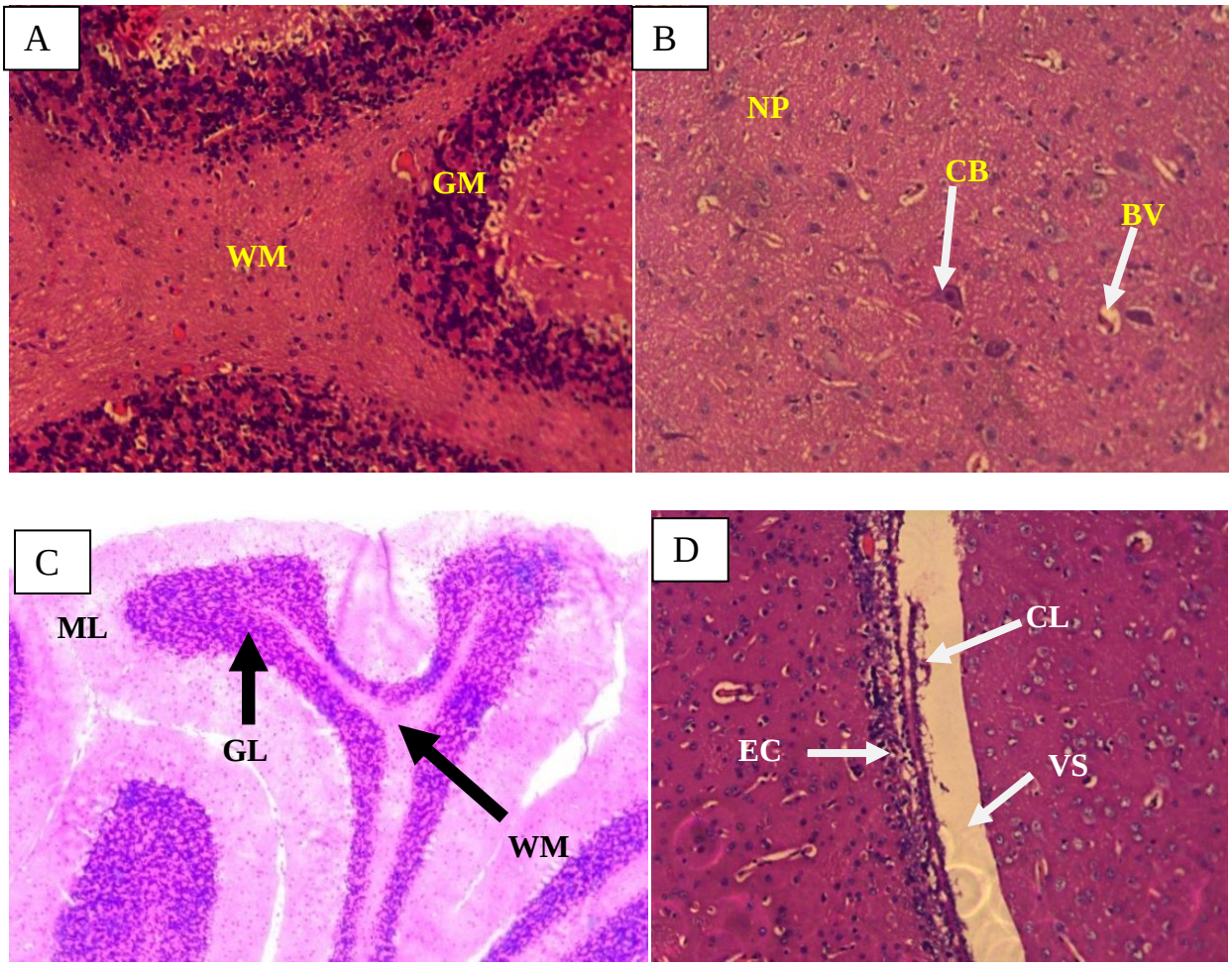

Figure: Photomicrograph depicting normal microstructures of brain tissue sampled from animals treated with daily administration of embelin at a dose of 1000 mg/kg (**A, B and D: cerebrum; C: cerebellum**) and control animals (**B**). **BV**: blood vessels; CB: neuronal cell body; **CL:** cilia**; EC:** epindymal cells**; GL**: granular layer; **GM**: gray mater; **ML**: molecular layer; **NP**: neuropil and **WM**: white mater; VS: ventricular space. 400x magnification of Eosin (H&E) staining.

4.3.3. Developmental toxicity

4.3.3.1.Clinical observation

There had been no reports of abortions or maternal deaths, according to the daily cage-side clinical evaluation. Animals who were pregnant showed no toxicity at all upon administration of the fixed oil.

4.3.3.2.Embryonic outcomes

As embryonic outcome indicators, morphological scores, number of somites and embryonic crown-rump length did not exhibit significant differences across the experimental groups and their control counterparts (**Table 46**).

Table 46: Developmental characteristics of embryos in the experimental group of pregnant rats following embelin treatment

Group	**Embryonic developmental variables**		
	Morphological score/ litter	**Number of somites/litters**	**CRL(mm)/litter**
Group IV (vehicle)	45.50 ± 1.90	29.00 ± 1.05	4.85 ± 0.48
Group V (*Ad libitum)*	45.40 ± 1.15	29.10 ± 1.10	4.95 ± 0.41
Group I (250 mg/kg)	44.75 ± 1.6	28.30 ± 1.20	4.85 ± 0.16
Group II (500 mg/kg)	44.60 ± 1.7	28.45 ± 1.22	4.78 ± 0.15
Group III (1000 mg/kg)	44.48 ± 1.55	28.05 ± 0.75	4.77 ± 0.34

Data were shown as mean ± standard deviation (μ±SD), CRL: Crown-rump length.*statistically significant (p-value<0.05)

4.3.3.3.Embryonic developmental indices

4.3.3.3.1. Circulatory system

According to the current study, none of the experimental groups displayed any signs of developmental delay in the yolk sac circulation or heart development. (**Table47**; **Figure 45**).

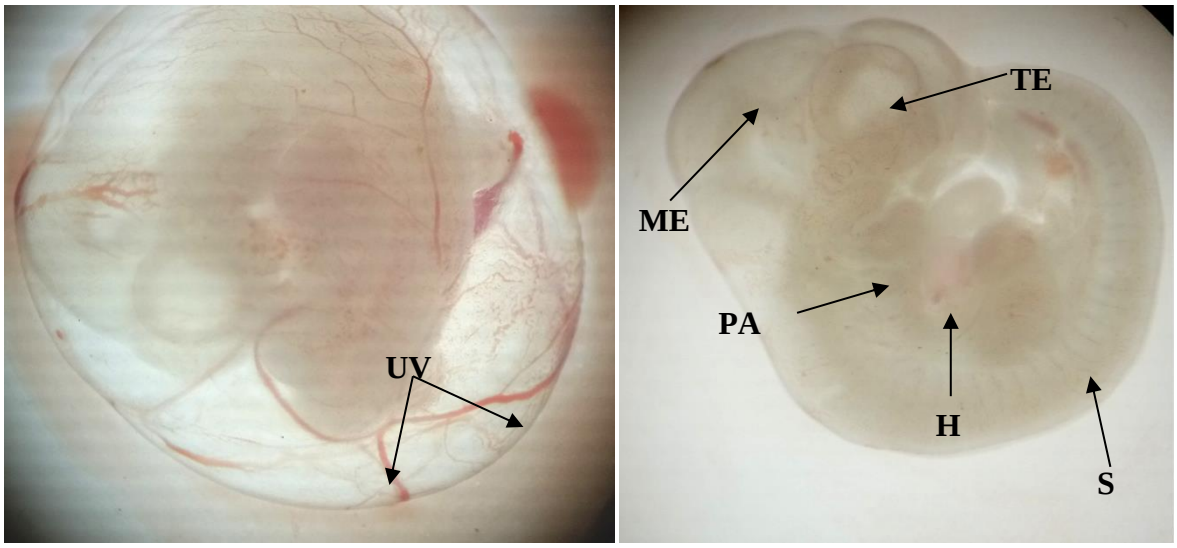

Figure 45: 12-Day-old embryos from high dose group (1000 mg/kg). [A]: Embryo inside its yolk sac (YS) with vitelline vessels (VV); [B]: ME (Mesencephalon); P (placental tissue); PA (Pharyngeal apparatus); S (Somite);TE (Telencephalon); and UV (umbilical vessels)

Table 47: Developmental characteristics of the circulatory system of embryos in the experimental group of pregnant rats following embelin treatment

Group	**Proportion of delayed development**		
	Yolk sac circulation	**Heart**	**Allantois**
Group IV (vehicle)	0	0	0
Group V (*Ad libitum*)	0	0	0
Group I (250 mg/kg)	0	0	0
Group II (500 mg/kg)	0	0	0
Group III (1000 mg/kg)	0	0	0

Results: Proportion, Chi-square test

4.3.3.3.2. Nervous system and sense organs

As indicated in **Table48**, the embryonic nervous system, otic system, and optic system were not subjected to developmental delay after treating pregnant rats with a high dose of embelin.

Table 48: Nervous system and sense organs characteristics of the embryos in the experimental group of pregnant rats following fixed oil treatment

Group	**Proportion of delayed development**				
	Caudal neural tube	**Hind brain**	**Fore brain**	**Otic system**	**Optic system**
Group IV (vehicle)	0	0	0	0	0
Group V (*Ad libitum*)	0	0	0	0	0
Group I (250 mg/kg)	0	0	0	0	0
Group II (500 mg/kg)	0	0	0	0	0
Group III (1000 mg/kg)	0	0	0	0	0

Results: Proportion (percentage). Chi-square test.

4.3.3.3.3. Musculoskeletal System

As depicted in **Table49**, the current experiment showed that musculoskeletal development parameters were not delayed in the treatment groups compared to the controls.

4.3.3.3.4. Food intake and weight gain

When compared to the control animals, the mean food intake of animals in Group III (1000 mg/kg treatment group) demonstrated a significant increase (p-value<0.001). Similarly, the mean food intake of Group I (250 mg/kg) and Group II (500 mg/kg) animals was higher compared to the vehicle and ad libitum control groups, (p-value<0.001). As illustrated in **Table50**, experimental pregnant animals in all treatment groups showed a dose-dependent increment in the mean of their weight as compared to animals in both control groups.

Table 49: Musculoskeletal system characteristics of the embryos in the experimental group of pregnant rats following embelin treatment

Group	**Proportion of retarded development**					
	Pharyngeal apparatus	**Maxillary process**	**Mandibular process**	**Fore limb**	**Hind limb**	**Flexion**
Group IV (vehicle)	0	0	0	0	0	0
Group V (*Ad libitum)*	0	0	0	0	0	0
Group I (250 mg/kg)	0	0	0	0	0	0
Group II (500 mg/kg)	0	0	0	0	0	0
Group III (1000 mg/kg)	0	0	0	0	0	0

Results: Proportion (percentage), Chi-square test

Table 50: Food intake and weight gain of pregnant rats treated with fixed oil

Maternal variables	**Experimental groups**				
	Group IV Vehicle control	**Group V *Ad libitum***	**Group I 250 mg/kg**	**Group II 500 mg/kg**	**Group III 1000 mg/kg**
Food intake (g)	171.92 ± 14.11	168.31 ± 20.64	184.12 ± 22.75**	185.75 ± 9.94**	199.01 ± 13.23**
Weight gain (g)	88.14 ± 5.36	90.01 ± 4.25	98.02 ± 3.16**	99.88 ± 7.43**	108.01 ± 7.19**

Data were shown as mean ± standard deviation of the means (μ±SD).*statistically significant (p-value<0.05) and **statistically significant (p-value<0.001).

4.3.3.4.Pregnancy outcomes

When comparing the number of implantations in experimental animals treated with fixed oil extract from *E. schimperi* fruit to vehicle control and ad libitum groups, **Table 51** and **Figure 46** demonstrate that there is no discernible difference. Comparably, it was discovered that the quantity of resorption sites or living fetuses was not altered across all experimental animals.

Table 51: Pregnancy outcomes in the experimental group of pregnant rats following embelin treatment

Pregnancy outcomes	**Experimental groups**				
	Group IV (vehicle control)	**Group V *(Ad libitum*)**	**Group I (250 mg/kg)**	**Group II (500 mg/kg)**	**Group III (1000 mg/kg)**
Number of implantation/dams	10.1 ± 1.57	10.5 ± 1.13	9.3 ± 0.56	9.5 ± 1.13	9.7 ± 0.85
Number of prior resorptions/dams	0	0	0	0	0
Alive pups	9.80 ± 1.57	9.83 ± 1.13	9.45 ± 1	9.42 ± 1.51	9.67 ± 1.08
Dead pups	0	0	0	0	0

Data were shown as mean ± standard deviation of the means (μ±SD). *Statistically significant (p-value<0.001).

4.3.3.5.Fetal outcomes

Fetal weight, placental weight, and crown-rump length—the three measures of fetal outcome indicators—showed no statistically significant variation in mean weight across the experimental groups (**Table 52**).

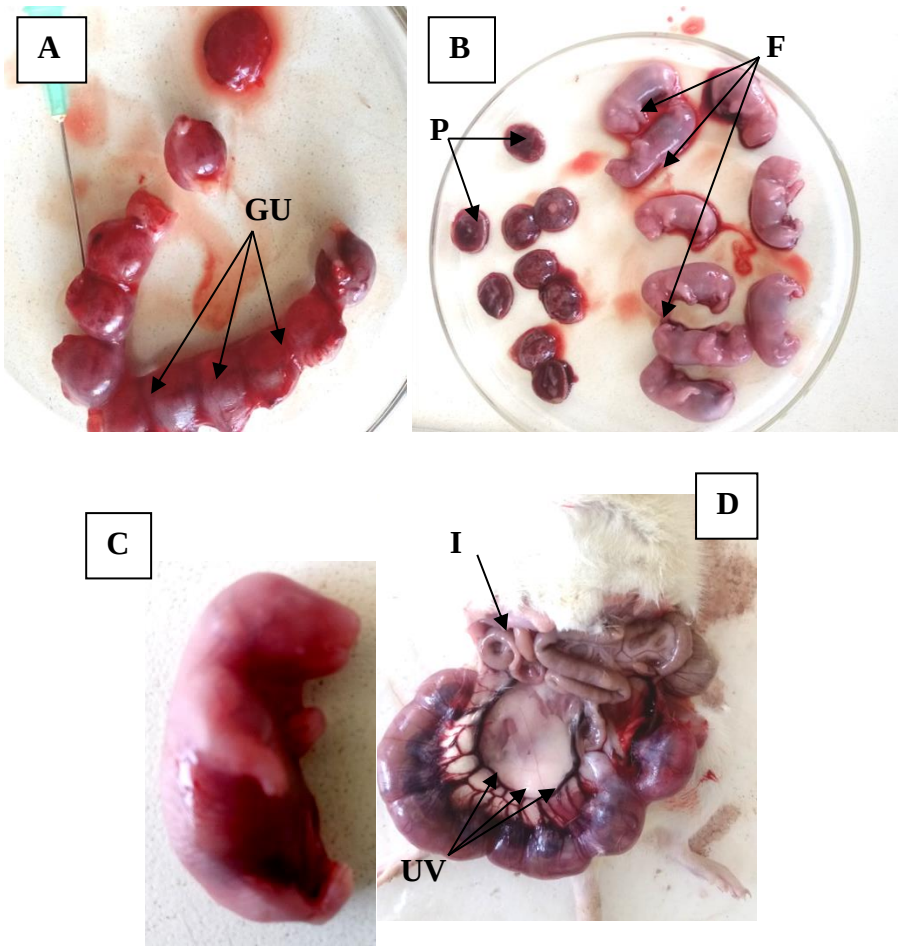

Figure 46: Pregnancy outcomesafter treatment of fixed oilextract from *E. schimperi*fruit. **A**: pregnancy outcome from rats treated with 1000 mg/kg of fixed oil depicting the intact gravid uterus (**GU**). **B**: pregnancy outcomes showing the term fetuses (**F**) with their respective placental tissue (**P**) **C**: term fetus from rats treated with the vehicle, tween 80. **D**: pregnancy outcome from the control rats illustrating the gravid uterus along with the network of uterine vessels (**UV**) and showing some intestinal contents (**I**) after incision of the abdomen.

Table 52: Fetal outcomes in the experimental group of pregnant rats following fixed oil extract treatment

Fetal outcomes	**Experimental groups**				
	Group IV (vehicle control)	**Group V (*Ad libitum*)**	**Group I (250 mg/kg)**	**Group II (500 mg/kg)**	**Group III (1000 mg/kg)**
Fetal weight (g) per dam	3.49 ± 0.99	3.68 ± 0.75	3.82 ± 1.00	3.97 ± 0.86	4.01 ± 0.85
Crown-ramp length (cm)	4.71 ± 0.40	4.86 ± 0.20	4.85 ± 0.12	4.71 ± 0.40	4.68 ± 0.22
Placental weight (gm)	0.61±0.063	0.6±0.074	0.61±0.074	0.6±0.06	0.58±0.14

Data were shown as mean ± standard deviation of the means (μ±SD).

4.3.3.6.Histopathology of placenta

When the experimental animals' placenta tissues were examined under a microscope. No discernible alteration among the controls and the fixed oil extract-treated groups (**Figure 47** & **Table 53**).

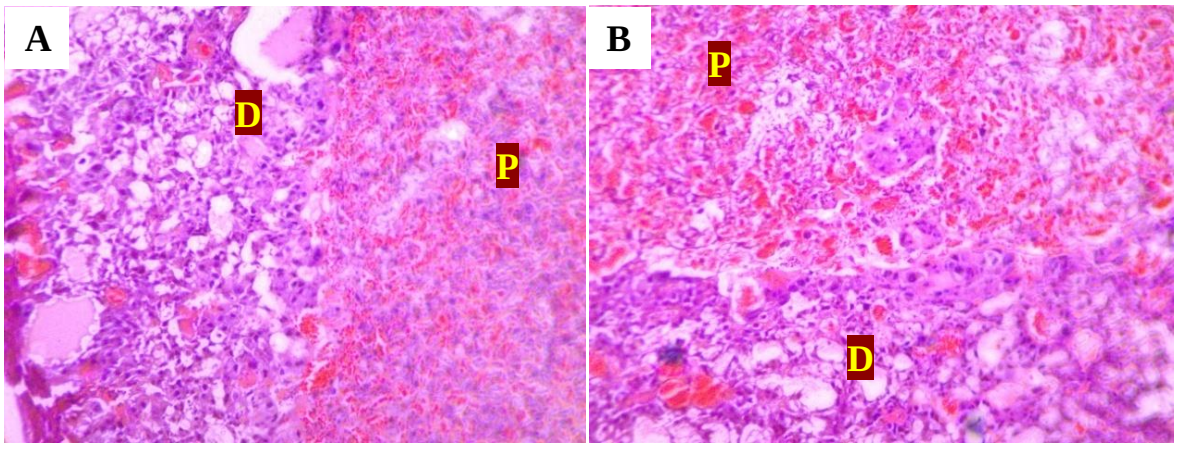

Figure 47: Normal histology of placenta from pregnant rats treated with 1000 mg/kg of fixed oil and control; Decidual and layer (D), Labrynzine zone (L).

Table 53: Distribution of placental histopathological manifestations across experimental groups

Histopathological parameters of placenta	Experimental groups				
	Group IV (Pair-fed control)	**Group V (Ad *libitum)***	**Group I (250 mg/kg)**	**Group II (500 mg/kg)**	**Group III (1000 mg/kg)**
Necrosis	0	0	0	0	0
Cytolysis	0	0	0	0	0
Apoptosis	0	0	0	0	0
Inflammation	0	0	0	0	0
Calcification	0	0	0	0	0

NB: Results are presented as percentages of histopathological findings, chi-square test.

4.3.3.7.External and visceral morphology

There were no notable external morphological deformities or malformations in the fetuses from either the fixed oil treatment group or the control group (**Table 54**). Additionally, none of the animal groups' visceral structures showed any obvious structural flaws (**Figure 48**).

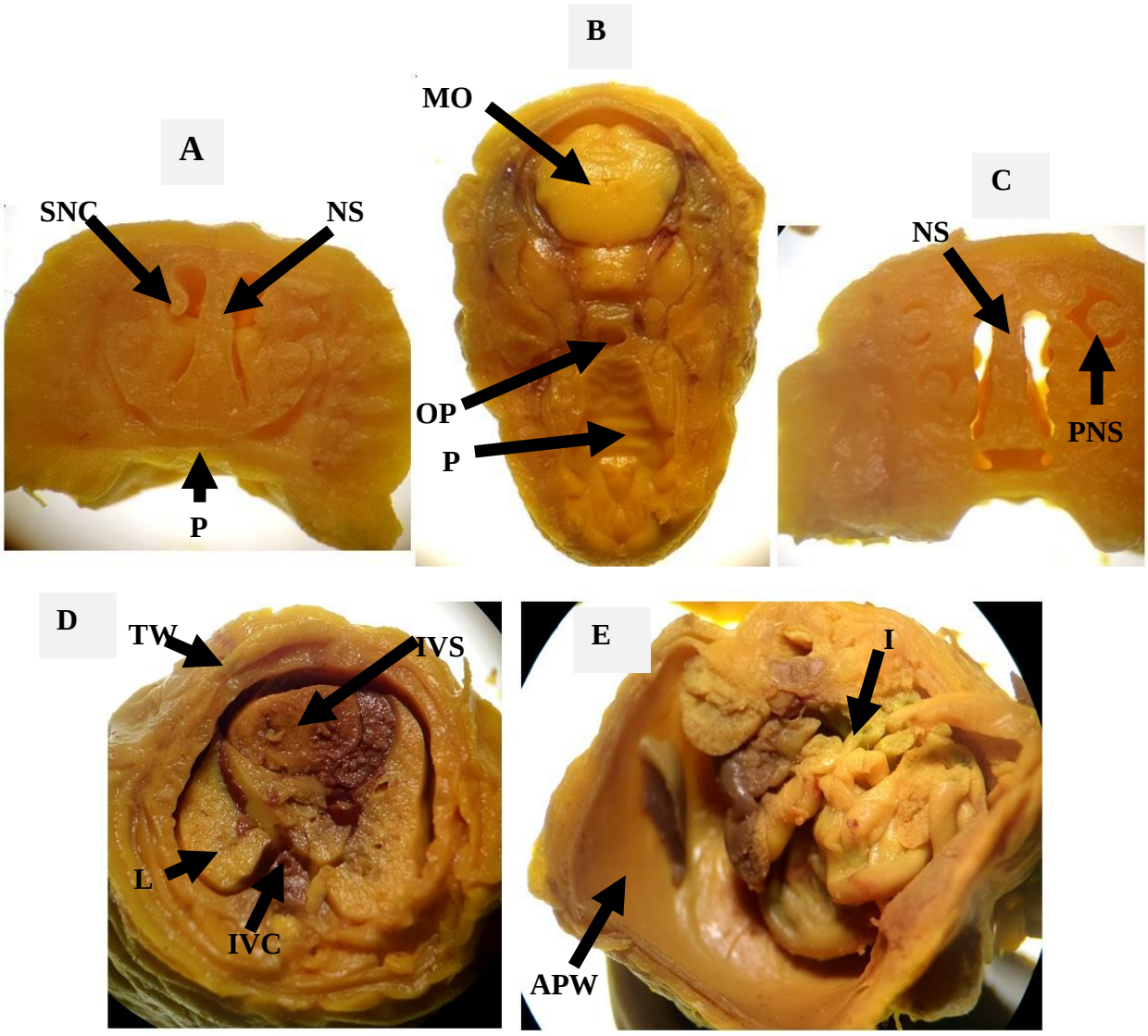

Figure 48: Visceral evaluation of term fetuses from rats treated with 1000 mg/kg of fixed oil extract (**A, B & D)** and tween 80 (**C & E)**. **SNC**: superior nasal conchae, **NS**: nasal septum, **P**: palate, **MO**: medulla oblongata, **OP**: oropharynx, **PNS**: paranasal air sinus, **TW**: thoracic wall, **L**: liver, **IVS**: interventricular septum, **IVC**: inferior venacava, **I**: intestinal content and **APW**: abdominopelvic wall.

Table 54: External gross malformation characteristics in the experimental group of pregnant rats following administration of fixed oil extracted from *E. schimperi* Vake fruit

Group	Proportion of external malformations (%)							
	Nervous system defects			Musculoskeletal defects			Others	
	ExE	AnE	SB	KY	SC	LD	MT	EGA
Group IV (vehicle)	0	0	0	0	0	0	0	0
Group V (*Ad libitum)*	0	0	0	0	0	0	0	0
Group I (250 mg/kg)	0	0	0	0	0	0	0	0
Group II (500 mg/kg)	0	0	0	0	0	0	0	0
Group III (1000 mg/kg)	0	0	0	0	0	0	0	0

Results: Proportion, Chi-square test. ExE: Exencephaly, AnE: Anencephaly, SB: Spina bifida, KY: Kyphosis, SC: Scoliosis, LD: Limb defect, MT: Missed tail, EGA: External genitalia agenesis.

4.3.3.8.Skeletal Evaluation

Rats treated with fixed oil extract of the plant did not vary from the controls in terms of the number of ossification centers in either the axial or the appendicular skeletons, as indicated by **Tables 55** & **56**, and **Figure 49**.

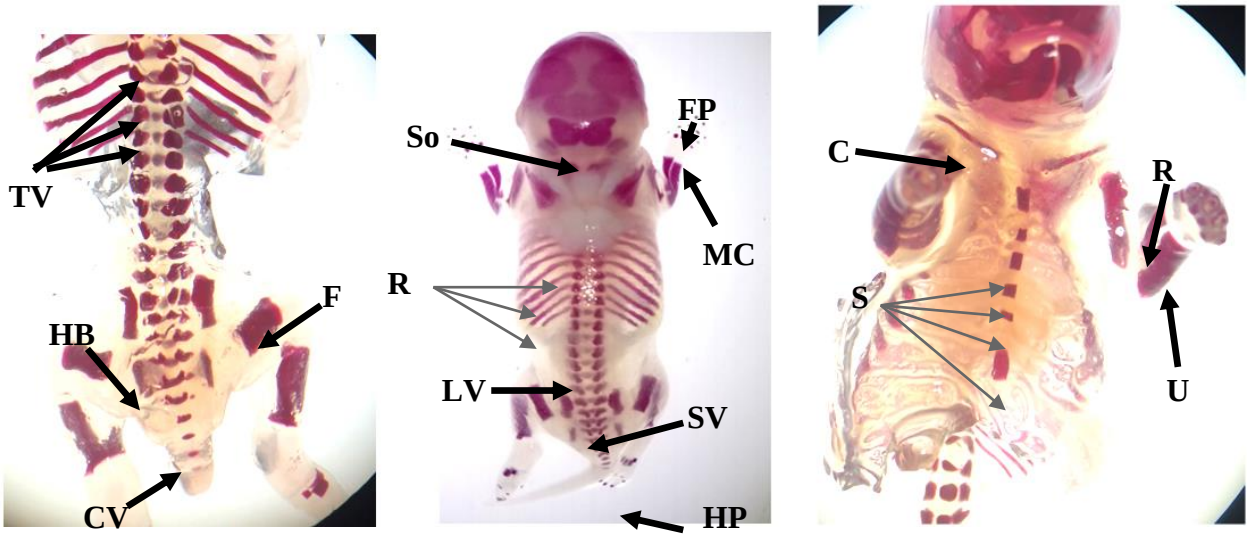

Figure 49: Skeletal ossification with alizarin red. C: clavicle, CV: Caudal vertebrae, F: femur, FP: Forelimb phalanges, HB: Hip bone, HP: Hindlimb phalanges, LV: Lumbar vertebrae, MC: Metacarpus, R: Ribs, So: Supraoccipital, S: Sternum, SV: Sacral vertebrae, TV: Thoracic vertebrae, U & R: ulna and radius

Table 55: Number of ossification centers in the axial skeleton of rat fetuses from experimental groups treated with fixed oil extract from *E schimperi* fruit

Group	Sternum	Thoracic vertebrae	Lumbar vertebrae	Caudal	Ribs
Group IV (vehicle)	5.76 ± 0.12	12 ± 0	5 ± 0	4.20 ± 0.23	24±0
Group V (*Ad libitum*)	5.70 ± 0.16	12 ± 0	5 ± 0	4.32 ± 0.21	24±0
Group I (250 mg/kg)	5.69 ± 0.11	12 ± 0	5 ± 0	4.23 ± 0.22	24±0
Group II (500 mg/kg)	5.47 ± 0.19	12 ± 0	5 ± 0	4.12 ± 0.11	24±0
Group III (1000 mg/kg)	5.66 ± 0.18	12 ± 0	5 ± 0	3.99 ± 1.39	24±0

Data were expressed as mean ± standard deviation (μ±SD)

Table 56: Number of ossification centers in the appendicular skeleton of rat fetuses from experimental groups treated with fixed oil extract from *E schimperi* fruit

Group	Forelimb phalanges	Hind limb phalanges	Metacarpus	Metatarsus
Group IV (vehicle)	3.79 ± 0.29	3.52 ± 0.31	3.88 ± 0.28	4.13 ± 0.26
Group V (*Ad libitum)*	3.84 ± 0.33	3.45 ± 0.30	3.86 ± 0.32	4.07 ± 0.15
Group I (250 mg/kg)	3.80 ± 0.43	3.50 ± 0.38	3.86 ± 0.32	4.06 ± 0.28
Group II (500 mg/kg)	3.83 ± 0.27	3.49 ± 0.40	3.80 ± 0.37	3.99 ± 0.36
Group III (1000 mg/kg)	3.81 ± 0.32	3.43 ± 0.50	3.87 ± 0.32	4.00 ± 0.42

Data were shown as mean ± standard deviation of the means (μ±SD)

Chapter Five

5. Discussion

5.1.Crude

Despite being an alternative option for health care maintenance, the main concern regarding plant-based traditional medicine remains the safety issue (208). People who use herbal medicine have a clear misconception that all plant-based drugs are safe because they come from natural sources. However, recent reports exhibited that medicinal plants can cause remarkable toxic effects on human wellbeing (209,210). Moreover, there are medicinal plants with significant genotoxic, embryotoxic, and teratogenic potentials (211,212). Yet, the majority of plants are not studied for their teratogenic effect. Given this, the current study explored the effect of *E. schimperi* on the developmental indices of rat embryos and fetuses. As long as our thorough search is concerned, there is no published article regarding the teratogenic effect of *E. schimperi* on rat developmental indices. Hence, the current study complements previous efforts to compile the toxicity effect of the plant.

The current study demonstrated that the 80% ethanol fruit extract of *E. schimperi* does not exhibit visible clinical symptoms during cage side evaluation of the experimental animals throughout the study period. In line with the current results, Debebe et al. (116) and Zewdu et al. (179) reported that mice and animals treated with a higher dose of the crude extract of *E. schimperi* show no behavioral and clinical changes when compared to their counterpart controls. This might indicate that crude extract of *E. schimperi* is not toxic enough to bring visible clinical manifestations.

Embryonic developmental indices are important indicators to assess the teratogenic effect of different substances (198,200). In this regard, the current study affirmed 80% hydro-alcoholic extract of *E. schimperi* did not affect CRL, somite's number, and the morphological score from 12 days old rat embryos from all dosage and control groups. Additionally, no difference in the development of circulatory, nervous, and musculoskeletal embryonic systems across all experimental groups. Since the aforementioned developmental indices are directly concerned to the growth and development of embryos (195), the result of the current study can be suggestive that the plant does not interfere in the process of embryogenesis.

Fetal developmental indices are also relevant touchstones that can describe the extent of developmental delays caused by different factors. These indices were measured in near-term rat fetuses (at day-20 of gestation).The present study revealed that there is no dose-related effect of *E. schimperi* fruit on food intake and weight gain o pregnant mothers throughout the pregnancy period. Concordantly, Zewdu et al. depicted that chronic treatment with 80% ethanolic fruit extract of *E. schimperi* shows no significant effect on weight gain. This might be due to a higher safety level of the plant not to interfere and affect food intake as well as weight gain of experimental animals.

According to the current study, exposure of *E. schimperi* during active embryogenesis period of pregnant albino Wistar rats did not affect the number of implantations nor did it exhibit prior resorption sites along the length of the gravid uterus. All fetuses were alive at the gestational age of 20 days, the day of sacrification. These illustrations might suggest that the crude extract of *E. schimperi* might not hinder the implantation process and fetal viability. Furthermore, the current experiment was conducted on young and virgin albino Wistar rats which could be a possible reason for the absence of resorption since the risk of having resorption in rats is higher with old maternal age and elevated body weight (213).

Regarding fetal outcome parameters, the current study disclosed that fetal weight, placental weight, and fetal crown ramp length were not affected by exposure of the mother to different doses of the crude extract of *E. schimperi*. This might indicate a higher safety level of the plant extract to influence the aforementioned fetal outcome indices. However, further experiments shall be conducted to rule out possible compensational changes during late gestational periods(195,201).

Placenta is an important fetomaternal organ that helps the developing fetus to grow properly and safely by being a barrier against some toxic chemicals in addition to its nutritional role. As a result, placenta becomes a highly susceptible target organ for drug- or chemical-induced adverse effects during pregnancy(21). In this regard, the present study revealed that histopathological analysis of placental tissues from rats treated with 250mg/kg and 500mg/kg of *E. schimperi* fruit extract exhibited significant inflammatory indices, focal fibro-purulent exudate and hemorrhage when compared to both pair-fed and the *ad libitum* groups. This might be attributed to the possible presence of metabolites causing inflammatory reactions in the placental tissue that could provoke

impairment of the microvasculature of placenta. Placental tissue calcification was observed on tissues taken from animals treated with 1000mg/kg of the fruit extract. One of the possible reasons pertaining to placental tissue calcification might be the presence of alkaloids and terpenoids that could provoke excessive expression of bone morphogenetic protein-7 (BMP7), a transforming growth factor-β (TGF-β) also known as osteogenic protein-1, near to the implantation site, in the decidua (214). Another possible hypothesis is the presence of dystrophic calcification, a physiological mechanism by which extracellular calcium combines with phosphate resulting in the formation of hydroxyapatite crystals during apoptosis and tissue perforation caused by trophoblast invasion into phagocyte epithelial and decidual cells (215,216)

Gross/external morphology, visceral morphology, and skeletal evaluations are the key fetal developmental endpoints that were investigated to determine the effect of *E. schimperi* during the fetal period of the experimental animals (198,201). The present study revealed that there are no significant dose-related differences in gross and visceral fetal morphological indices among *E. schimperi* treatment groups and controls. The previous report reinforced the current finding by revealing that chronic treatment with 80% hydro-alcoholic extract of *E. schimperi* does not exhibit any sign of toxicity (179). This indicates that the crude extract of the plant might not bring unintended birth outcomes including overt birth defects.

Another parameter to assess the teratogenic effect of substances such as herbal products in fetal rats is the number of prenatal ossification centers in both axial and appendicular skeletons (198,217–219).The current study analyzed the amount of ossification centers in the sternum, thoracic vertebrae, lumbar vertebrae, caudal vertebrae, ribs, forelimb phalanges, hind-limb phalanges, metacarpus, and metatarsus. However, no difference in the mean of ossification centers in the previously mentioned skeletons among the treatment groups and their control counterparts. Furthermore, all fetuses exhibited an adequate number of ossification centers for their age. This might indicate that the 80% hydro-alcoholic fruit extract of *E. schimperi* does not affect the osteogenesis of the rat skeleton.

5.2.Embelin

There have been shreds of evidence generated pertaining to the therapeutic relevance of embelin isolated from *Embelia* species to manage various ailments such as dermatological problems,

gastrointestinal-related diseases, pain, metabolic disorders, fertility problems, neurological disorders, and cancer (122). However, its safety profile has not yet been explicitly explored, other than a few suggested safety margins for the compound. Moreover, the *In silico* toxicity profile and its effect on the developmental process remain less known despite the fact that they are crucial parts of the toxicological endpoint, especially when dealing with such a compound of strong pharmaceutical importance. Hence, this study investigated the aforementioned toxicity profile of embelin.

In silico, studies of chemicals and compounds are becoming preferred predictive computer-based computational methods before diving into *in vivo* and *in vitro* studies. Such studies also showed the strong clinical success of drug targets (220). The current study revealed that *in silico* toxicity output predicted embelin to be a non-hepatotoxic, non-carcinogenic, non-mutagenic, and non-cytotoxic agent. In line with these findings, animal studies confirmed that embelin is safe on the liver and does not have a carcinogenic effect; rather, it has hepatoprotective and anti-cancer activity (221–223).

The hepatoprotective property of embelin is explained by its role in lowering elevated liver enzymes and boosting protein and albumin concentrations during the carbon tetrachloride-induced hepatotoxicity test (224). The same study signified the hepatoprotective role of embelin through histopathological findings in such a way that it reduced hepatic cord dilation and minimized the inflammatory response, accompanied by lowering mononuclear cellular infiltration. The anti-cancer activity of embelin is suggested based on its potency in inhibiting cell growth, inducing apoptosis and activating caspase-9 in BIR3 domains of XIAP, inhibiting programmed cell death, especially during prostate cancer (225).

The *In silico* output also indicated that embelin has an immunotoxic property. Similar reports stated that plant-based natural products have the potential to possess an immunotoxicity effect (226–229). This might be due to the possible effect of embelin on immune regulatory molecules such as PD-1 (programmed cell death 1), CTLA-4(cytotoxicity T-lymphocyte antigen 4) and galectins since there are evidences showing that medicinal plants have a potential of immune checkpoints inhibition (230,231). On the contrary, there are medicinal plants with immunostimulant activity that make them a potential treatment option against immunotoxic agents

(232). Hence, further *in vivo* and *in vitro* studies need to be conducted to validate and further understand the effect of embelin on the immune system. Another *in silico* finding from the current study is the predicted inhibitory effect of embelin against the CYP2D6 enzyme, an important polymorphic enzyme that serves as a catalyst for the metabolism of various drugs (233). In this regard, various reports emerged showing that both medicinal plant extracts and conventional drugs exhibit similar inhibitory effects against the CYP2D6 enzyme (234–236). Regarding the cardiac toxicity potential of embelin, hERG channel (K^+ channel facilitating the cardiac conducting system) inhibition is considered a key indicator (237,238). In this respect, the current *in silico* prediction reveals that embelin has no inhibitory effect on hERG channels. This is a good indication of a higher safety margin for *Embelia* extract to be free of cardiac toxicity effects as compared to *In silico* and *in vitro* evaluations of some medicinal plant extracts and chemicals that were found to have an inhibitory potential against hERG channels and hence be cardiotoxic (239,240).

The acute oral toxicity test of embelin isolated from the fruit part of *E. schimperi* showed no recorded fatality and no observed toxicity for two weeks on female rats treated with a single dose of 5000 mg/kg. Thus, the mean lethal dose (LD_{50}) is determined to be above 5000 mg/kg. This result indicated that the compound had higher margin of safety in rats during the acute oral toxicity test. This result is in agreement with reports from rats studies that demonstrated that embelin is safe when given acutely at higher doses (122).

According to the current study, embelin, isolated from *E. schimperi*, is found to be non-fatal compound upon 90 days oral administration. This is an evidence that the compound is tolerable during long term administration to rats as similar reports are also declaring that the compound has no life threatening effect on experimental animals (181,241).

Food consumption and weight gain are important endpoints during the evaluation of test chemicals for sub-chronic toxicity. In view of this, the current study depicts that the total food intake and body weight gain of rats treated with embelin are significantly higher in a dose-dependently than the control groups. This significant increment might be attributed to the potential antihelimenthic role of embelin that enhanced the appetite of experimental animals, leading to subsequent weight gain. However, a report suggested that 21 days of embelin treatment at a dose of 50 mg/kg on high-fat diet-induced obese rats helped to reduce body weight (242).

The current study looked at hematological indicators as part of hematopoietic and biochemical end goals after 90 days of oral embelin administration and found no variation between the experimental groups. This might indicate that embelin did not bring undesirable hematological effects on rat blood. However, serum biochemical analysis from the current study indicated that there is a substantial dose dependent decrement in the level of liver enzymes namely ALT, AST and ALP. In line with these findings, an article depicted that oral treatment of embelin at 25 mg/kg to rats took carbon tetrachloride (CCl_4) showed significant lowering of the aforementioned liver enzymes suggestive of the potential of embelin being antioxidant and hepatoprotectve (243). This finding is further reinforced by an experiment on mice from thioacetamide-induced acute liver injury depicted that embelin treatment helps to mitigate the progression of liver injury (244).

Histopathological examination of important organ tissues from experimental animals in this study, including the liver, kidneys, spleen, and adrenal glands, revealed that sub-chronic embelin treatment did not cause any significant microstructural changes across all experimental rats. This finding is widely supported by studies done on similar compound from other species of *Embelia* including *E. ribes* reporting that embelin isolated from these plants possessed substantial importance in maintaining normal microscopic structures of vital organs on experimental rodents via protecting injured tissue through macrophagic activation and various signaling modalities (245–247).

Reproductive endpoints are crucial in toxicological studies because they assess the presumed effects of substances on the male and female reproductive systems, as well as on the development and health of offspring. Hence understanding reproductive toxicity is essential to evaluate the effects of test substances as crucial endpoints of toxicological studies

The current study depicted that daily administration of embelin to male albino Wistar rats for 90 days brought significant dose related decrement in the mean of relative weight of testes and epididymis. This is in line with prior reports that declared subcutaneous administration of embelin from *E. ribes* brought significant decrement in the weight of male reproductive structures, especially those from higher dosage group (171,248). The reason behind this undesirable outcome of a critical end point might be due to the fact that embelin is evidenced to affect reproductive organs via affecting their hormonal regulation (245).

In the current sub-chronic test, all embelin-treated rats had significantly lower testosterone, LH, and FSH levels than the control group. Moreover, the current findings showed that there is a statistically significant reduction in sperm count in rats treated with all three dose levels of embelin. In accordance with these findings, there is substantial evidence that embelin has a significant effect on lowering the blood level of male sex hormones late alone the sperm count. While such effects may vary depending on the manner of administration, oral administration of embelin has the greatest effect (249).

In addition to the alterations in sex hormones noted in this study, histopathological evaluation of epididymal tissues revealed significant histological changes such as thinning of epithelium thickness and sloughing, as well as the presence of cytoplasmic vacuolization. Testicular tissues also had prominent basal lamina detachments, lumenal indentation indicative of early lumenal atrophy, and vacuolization inside the seminiferous tubule wall. Similar findings were observed from previous studies done upon both short and long term administration of embelin via different route (250,251). Such histological manifestations were assumed due to the previously mentioned effect of embelin in disrupting the hormonal balance that could affect the overall reproductive framework (250,251).

Regarding female reproductive endpoints, the present study found that embelin-treated animals had considerably lower relative uterine and ovarian weights than vehicle control rats accompanied by significant depletion of female sex hormones and shorter estrous cycles. In line with the current findings, similar report from *in vivo* and *in vitro* studies depicted that embelin treatment with 10 mg/kg and 20 mg/kg body weight exhibited disrupted estrous cycles, along with a significant decrease in plasma estradiol and progesterone (170,252). Similarly, a study by Simukoko illustrated that subcutaneous administration of embelin had significant effect on the aforementioned sex hormones and structural integrity of ovarian tissues (253). Furthermore, histological examination of ovarian tissues revealed that embelin administration had a substantial effect on oocyte maturation due to evidence of altering the synthesis of sex hormones that governed the process of oocyte maturation (254).

To evaluate possible neurotoxic consequences, toxicological experiments must examine brain histology. By identifying structural alterations in the brain, studies might provide information on susceptible areas and impacted cell types. Hence the knowledge of substance's effects on the

central nervous system can be improved through the identification of the relationship between reported behavioral aberrations and underlying structural changes, which help researchers, anticipate possible long-term ramifications. In this regard, the current study depicted that sub-chronic treatment of embelin didn't exhibited structural alteration in the microstructure of the brain that can be an indicative of the compound's safety pertaining to neurotoxicity. Supporting this, studies on embelin embarked that the compound has therapeutic role in treating cerebral ischemia (249). Furthermore, embelin has been reported to possess antipsychotic role via reversing elevated levels o dopamine, noradrenaline and serotonin in mice (255).

In the present study, developmental toxicity of embelin was evaluated based on the effect of the plant isolate during the early pregnancy period (Day 12 of gestational age) and the late pregnancy period (Day 20 of gestational age). During both early and late developmental toxicity experiments, embelin did not exhibit any observable signs of toxicity, including abortion and maternal death, for the whole duration of the treatment period. This might indicate that embelin is a non-abortifacient yet tolerable compound for pregnant rats, even at a higher dose of 1000 mg/kg. In line with this, a previous study revealed that administration of an 80% ethanol extract of *E. schimperi* Vake fruits elicited neither maternal mortality nor abortion (256). However, there should be no misconception that medicinal plants are non-abortifacient because there have been reports that medicinal plants may have abortifacient capacity via various mechanisms such as inducing utrine contraction, damaging placental tissue, causing multiple organ damage, and death (257–259).

Early pregnancy developmental toxicity was assessed based on morphological score, number of somites, and embryonic crown-rump length, in addition to the extent of developmental delay in the embryonic circulatory, musculoskeletal, and nervous systems. In this regard, the current study found no statistically significant difference in the mean of morphological scores or the number of somites. Furthermore, none of the embryonic systems exhibited delayed development. However, the crown-rump length of embryos from the high dose group is significantly shorter than their counterparts from vehicle and *ad libitum* controls. These findings might indicate that embelin is not an embryo-disrupting agent in rats, especially at low and middle doses. Yet, the decrement in crown-ramp length of embryos from the high dose group (1000 mg/kg) might be attributed to a transient effect of embelin in the stature of the embryo since such a difference was not appreciated

in the CRL of near term rat fetuses. This might be due to the fact that outcomes from embelin treatment usually return to normal after withdrawing the treatment (122,181).

The current study revealed that there is a significant dose-dependent difference between embelin-treated animals and controls with respect to mean maternal food intake and weight gain in pregnant rats. The mean maternal food intake of all treatment groups was significantly higher than that of the control animals. This might be explained by the anthelmintic effect of embelin, which possibly harmonizes the gastrointestinal environment to increase their appetite (181,260,261). On the other hand, rats in the treatment groups gained significantly less weight than those in the control group despite their high food intake. This might be attributed to the fact that animals in the treatment group had fewer pregnancies than those in the control group, which directly influenced the amount of weight gain measured since the more pregnancies, the more weight gained. Furthermore, embelin is reported to have a weight reduction effect in rats to the extent that it contributes to regulating metabolic disorders at a low dose of 50 mg/kg (242). Correspondingly, the current study depicts that the number of implantations is significantly lower in the experimental groups than in the controls during the late pregnancy experiments. This could be due to embelin's effect on lowering sex hormone concentrations as well as the implantation process, which can increase the risk of resorption (262–264).

According to the current study, there are no notable treatment-related changes in the number of skeletal ossifications or external or visceral morphological alterations in near-term fetuses. This finding might be attributed to the resolved effect of embelin after the treatment period since the treatment period was only during the period of organogenesis (122).

5.3. Fixed oil

Because they have potential therapeutic applications and their safety profiles need to be evaluated, fixed oils produced from medicinal plants are important subjects for toxicity investigations. These oils are frequently utilized in conventional medicine and pharmaceutical formulations. They are made up of triglycerides, fatty acids, and other bioactive substances (265,266). The assessment of fixed oils in toxicity studies includes determining how they affect several physiological systems, such as organ function, hematological parameters, and reproductive health (267,268). In view of this, the fixed oil extract from *E. schimperi* Vake fruit exhibited a high level of safety in the current toxicological assessment. The LD50 was declared to be greater than 5000mg/kg, as all female

animals treated with this dose survived without showing toxicity signs while diarrhea observed in the 1000 mg/kg group during the initial days was the only notable adverse effect. This might indicate that the non-polar content of the plant might be non-fatal. However, the diarrhea can be explained as sometimes high dose of fatty acid intake can bring mild and tolerable loose stool episode as reported by Kosuke et al. (269).

Upon sub-chronic experiment, the fixed oil extract did not induce significant changes in hematological parameters, relative organ weights, or microscopic structures of liver, spleen, adrenal, kidney, and reproductive organs. These might be favorable indications of the oil to be safe during utilization for prolonged time. Furthermore the major phytochemical contents of the oil such as linolic acid, palmitic acid and oleic acid are important fatty acids for maintaining the body metabolism (270).

Additionally, there were no alterations in sex hormones, estrous cycles, or brain tissue histopathology. The study concludes that the fixed oil extract from *E. schimperi* Vake fruit, even at high doses, demonstrates a lack of severe toxicity and maintains the overall health and reproductive functions in experimental animals. These findings contribute to the understanding of the safety profile of *E. schimperi* fixed oil extract, supporting its potential use in various applications (271).

5.4.Conclusion and recommendation

The results of the current study depicted that treatment of the 80% ethanol fruit extract of *E. schimperi* during a period of active embryogenesis of albino Wistar rats did not affect food consumption, weight gain and implantation outcomes. However, histopathological examination of the placenta showed inflammatory reactions and calcifications on the maternal part of the rat placenta. This is a redolent scenario that the extract affects the microvasculature of the placenta. Therefore, consumption of fruits of *E. schimperi* by pregnant women is not recommended.

The sub-chronic administration of embelin from *E. schimperi* fruit demonstrated significant effects on various physiological indicators in both male and female animals. The enhanced total body weight gain in treated animals, coupled with distinct alterations in food intake patterns, suggests a potential influence of embelin on metabolic processes. Moreover, the hematological and serum biochemical analyses revealed specific changes in liver enzyme levels, indicating a possible impact on hepatic function. Notably, reproductive endpoints exhibited considerable modifications, including reduced relative weights of reproductive organs, hormonal imbalances, and histopathological alterations in male reproductive tissues, indicating potential reproductive toxicity. While some organs like the liver, kidney, and brain did not show significant microscopic alterations, the observed effects on reproductive and metabolic parameters emphasize the need for further investigations to justify the underlying mechanisms and assess the overall safety of embelin administration. These findings showed the relevance of a comprehensive toxicological evaluation to ensure a thorough understanding of the potential health implications associated with the consumption or exposure to embelin-containing substances.

Fixed oil extract from *E. schimperi* Vake fruit, even at high doses, demonstrates a lack of severe toxicity and maintains the overall health and reproductive functions in experimental animals. These results contribute to the elaboration of the safety profile of *E. schimperi* fixed oil extract, supporting its potential use in various applications.

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