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Composition and Evaluation of Acute, Subacute, and Developmental Toxicity of Essential oil and Developmental Toxicity of aqueous crude extracts of Thymus schimperi in Wistar Albino rats; In vivo and In-Silico Toxicity Studies

Ph.D. Dissertation

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Addis Ababa, Ethiopia

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Composition and Evaluation of Acute, Subacute, and Developmental Toxicity of Essential oil and Developmental Toxicity of aqueous crude extracts of Thymus schimperi in Wistar Albino rats; In vivo and In-Silico Toxicity Studies.

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A ddissertation submitted to the school of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the Ph.D. Degree of Science in Human Medical Anatomy.

Doctor of Philosophy in Medical Anatomy

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ABSTRACT

Background: In Ethiopian traditional medicine, the aerial part of *Thymus schimperi* is widely used to treat diseases such as gonorrhea, cough, liver disease, kidney disease, hypertension, stomach pain, and fungal skin infections. In addition, theyhave been used as vegetables to flavor a broad variety of food products. However, there is an insufficient investigation of the toxic effect of *T. schimperi*. The aim of this study was, therefore, to evaluate the acute, sub-acute, developmental, and in-silico toxicity of the essential oil,as well as the developmental toxicity of the aqueous crude extract of *T. schimperi* on the Wistar albino rats.

Method: The aqueous extracts of *T. schimperi* leaves were prepared.Essential oil of the aerial part of *T. schimperi*was extracted by hydrodistillation and was analyzed by GC-MS. The oil was subjected to toxicity studies. In the acute toxicity study, rats were randomly divided into seven groups (n=5). The control group received the vehicle (distilled water and 2% tween 80) whereas the experimental groups received single doses of 300, 600, 900, 1200, 1500, and 2 000 mg/kg of vehicle dissolved essential oil. In the sub-acute toxicity study, rats were randomly divided into four groups (n=10). The control group received the vehicle whereas the experimental groups received vehicle-dissolved doses of 65 mg/kg, 130 mg/kg, and 260 mg/kg of oil orally for 28 days. At the end of the experiment, blood samples were collected for hematology and clinical chemistry evaluation. Gross pathology and histopathology of the liver and the kidneys were also evaluated. For the in-silico toxicity study, PubChem CID numbers of GC-MS identified bioactive compounds in essential oils of *T. schimperi* have been obtained from PubChem. Chemdraw (8.0) was used to construct the two-dimensional structure of the compounds. The Swiss ADMET web tool was used to convert the two-dimensional structures into a simplified molecular-input line input system (SMILES). Furthermore, the toxicity parameters were predicted via Protox II, vNN, and ADMET servers. For aqueous and essential oil extracts developmental toxicity experiments, five groups of Wistar albino rats, each consisting of ten pregnant rats, were used as experimental animals. For the aqueous crude extract developmental toxicity study, the rats in groups III-V were given 500 mg/kg, 1000 mg/kg, and 2000 mg/kg extract of *T.schimper*, respectively. On the other hand, in the essential oil developmental toxicity study, the doses 65 mg/kg, 130mg/kg, and 260 mg/kg of the essential extract of *T. schimperi* were administered forIII-V groups, respectively. Group I and II were negative and *ad libitum* controls for both experiments. Similarly, Embryos and fetuses were revealed on days 12 and 20 of gestation, respectively. The embryos were examined for developmental delays or growth retardation. Gross external, skeletal, and visceral anomalies in the fetuses were examined. Histopathological examination was carried out on the placenta from both the treatment and control groups.

Results: In this study, the LD_{50} of the essential oil of *T. schimperi* was found to be 1284.2 mg/kg. According to the World Health Organization, the oil is classified as moderately hazardous in its oral administration. In the subacute toxicity study, rats showed no significant changes in behavioral indices, gross pathology, body weight, biochemical, and in most hematological parameters. However, hematological profiles showed a significant decrement in

WBC counts and a significant increment of MCV in high dose (260 mg/kg) groups as compared to the control group. Furthermore, no significant differences were observed between the control and essential oil-treated groups, observed in the gross and histopathology of the liver and the kidneys.

In the in-silico toxicity study, all compounds derived from essential oil showed no cardiac toxicity (h-ERG Blocker), AMES (Ames Mutagenicity), and cytotoxicity via Pro Tox II, ADMET, and vNN-ADMET toxicity predictors. However, by using these servers, from the total 57 compounds, around 21% showed carcinogenicity, 8.8% showed hepatotoxicity, 3.5% caused drug-induced liver injury, 3.5% showed immunotoxicity, and only 1.75 % were potentially toxic to the mitochondrial membrane.

In the aqueous crude extract developmental toxicity study, on embryo day 12, the number of somites and the morphological scores in the high-dose treatment group were significantly lower than the control groups. Similarly, the number of implantation sites, fetal weight, fetal resorption, CRL, and placental weightwere also significantly lower in the high dose (2000 mg/kg) treatment group. The mean numbers of implantation sites in the pair-fed control group and the high dose(2000 mg/kg) group were 11.1 ± 0.76 and 8.01 ± 0.45, respectively. Similarly, in the middle dose (1000 mg/kg) and high dose (2000 mg/kg) groups, the developments of the otic and olfactory systems were significantly delayed. Furthermore, in the high dose group (2000 mg/kg), the developmental score of optic system, the number of branchial bars, and the maxillary and mandibular processes were significantly lower than the control groups. Treatment with the aqueous extract of the *T.schimperi*caused no skeletal or soft tissue malformations. In an essential oil developmental toxicity study, the developmental socres of fetal resorptions, crown-rump length, the number of somites, and morphological scores were significantly lower in 12-day-old rat embryos treated with 260 mg/kg of the extract. There was also a significant delay in the developments of the otic system, olfactory system, and a reduction in the number of branchial bars in day-12 embryos treated with 260 mg/kg of the oil.However, external morphological examinations of rat fetuses revealed no detectable structural abnormalities. The fetal skull, vertebrae, hyoid, forelimb, and hindlimb ossification centers did not differ significantly across all groups. Moreover, treatment with the essential oil caused no skeletal or soft tissue malformations. Although the difference was not statistically significant, fetuses of high-dose treated rats had a reduced number of ossification centers in the caudal vertebrae and hind limp phalanges.There were no significant histopathological changes in placentas in either the crude aqueous extract or the essential oil experiments. Although the difference was not statistically significant, placentas from high-dose essential oil treatment rats had increased decidual cystic degeneration, thrombosis in the intervillous spaces, and decidual cellular apoptosis. Similarly, in the essential oil experiment, capillary dilation and terminal villi proliferation increased dose-dependently.

Conclusion: From this study, oral administration of the essential oil *T. shimperi* up to a dose of 130 mg/kg is not harmful. However, in the high-dose (260 mg/kg) group, the WBC count was significantly decreased and the MCV was significantly increased. In the in-silico toxicity study,

most of the components of the oil were found to be nontoxic although a few of the compounds showed carcinogenicity, hepatotoxicity,immunotoxicity and mitochondrial membrane potential toxicity.The crude aqueous and essential oil extracts of *T. shimperi* at high doses have a detrimental effect on the development of rat embryos and fetuses. Its developmental toxicity is evidenced by significant delays in fetal and embryonic development, a decrease in the number of implantation sites, and an increase in fetal resorption. Furthermore, administration of the aqueous crude and essential oil extracts in higher doses resulted in a significant decrease in placenta weight, and litter weight. It is, therefore, essential to conducting chronic toxicity of the essential oil as well as its components which showed toxicity in the in- silico study before using preparations containing *T. schimperi* essential oil as drugs. In addition, the present study provided evidence that using the *T.schimperi* extracts in a high dose could affect the developing embryo and fetus. Thus, it is recommended to discourage the use of crude and essential oil extracts in high doses.

Keywords: *Thymus schimperi*; Aqueous crude extract;Essential oil; Acute toxicity; Sub-acute toxicity;Developmental toxicity;*Insilico toxicity*; and Wistar albino rats.

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DEDICATION

This Ph.D. dissertation is dedicated to my beloved mother, Mrs. Addis Lemeneh,she is always a source of my strength and inspiration.

STATEMENT OF DECLARATION

This is my declaration regarding this dissertation titled “Composition and Evaluation of Acute, Subacute, and Developmental Toxicity of Essential oil and Developmental Toxicity of aqueous crude extracts of *Thymus schimperi* in Wistar Albino rats; In vivo and In-Silico Toxicity Studies.” I Fentahun Adane in the Department of Anatomy, College of Health Sciences, and Addis Ababa University have conducted the work presented in this thesis in collaboration with TMMRD of EPHI. Every concept is taken from literature has been acknowledged and cited in the reference section. No part of this dissertation has been presented for any certificate in any other University.

Name: Fentahun Adane Nigat,**Signature**_____ **Date**_____

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LIST OF ABBREVIATIONS AND ACRONYMS

AAU	Addis Ababa University
EPHI	Ethiopian public health institute
T. schimperi	Thymus schimperi
TM	Traditional medicine
CT	crude extract of Thymus schimperi
EOT	Essential oil Thymus schimperi
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CK	Creatinine kinase
LDL	Low-density lipoprotein
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
OECD	Organization for Economic Cooperation and Development
WHO	World Health Organization
CRL	Crown-rump length
H&E	Hematoxylin and Eosin
OECD	Organization of Economic Co-operation and Development
DILI	Drug-Induced Liver Injury
hERG	Human Ether-à-go-go-Related Gene
HT	Hepato- Toxicity
ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
MMP	Mitochondrial Membrane Potential
DPPH	Diphenyl-picryl hydrazyl

CHAPTER ONE

1. INTRODUCTION

1.1. Background of the Study

1.1.1. Traditional Medicine

Traditional medicine (TM) is an ancient healing practice centered on the healthcare system and community that humans have used to cope with and treat a variety of ailments and diseases that have threatened their lives and existence. Traditional medicine hasvarious definitionsin different cultures and has various types of indigenous healing techniques. Thesedefinitionsinclude folk medicine, ethnomedicine, alternate medicine or complementary medicine, and native healing (1). TM is expected to be a component of the health care system although it has been underestimated. Herbalists, herb vendors, village midwives (conventional birth attendants), bonesetters, traditional therapists, and other professionals are TM practitioners(2).The most widespread TM practice is the use of herbal medicines, which are plant-based formulations that claim to have therapeutic advantages. Herbal medicines are utilized by the majority of people all over the world(3-5).Herbal medicines are comprised of herbs, herbal preparations, herbal materials, and finished herbal products that contain plant parts or other plant materials as active ingredients(6).

TM has been used as the primary health care system modality by approximately 65 % of the global population (7).Many millions of people use herbal medicines as their primary source of health care, and in some cases as their only source of care, particularly in developing countries. Most people in developing countries, especially in rural areas, useTM as their primary health care system rather than modern pharmaceutical products.The better accessibility, availability, and affordability of TM are the main reasons for choosing traditional medicine over modern medicine(8).

In African, this is a common practice ofTM although it is not well organized like it is in China and India. It is the cornerstone of primary health care for the majority of Africans, particularly those living in rural areas. According to WHO data in Africa, TM is used by up to 80% of the

population to adequately achieve their health care needs(9).The proportion of conventional health workers to the population is 1:500 while the proportion of doctors to the population is 1: 40,000(1).Therefore, indigenous therapists remain their primary health care providers for millions of individuals in rural areas of Africa.

1.1.2. Traditional Medicine in Ethiopia

In Ethiopia, as in the majority of African countries, TM is the primary source of health care for about 80% of the population (1).According to a study done in 2005, approximately 90% of Ethiopian migrants use traditional medicine to meet their basic health care needs (10). TM is in demand in Ethiopia due to its culturally related usage traditions, community belief in its values, accessibility, and low cost (10).More than 95 % of traditional medicinal preparations are derived from plantsin Ethiopia(11).

In Ethiopia, about 800 plant species have been used as traditional medicinal plants(12). The majority of medicinal herbs are prepared fresh, and the techniques for their usage include pressing, grinding, boiling, chewing, crushing, and binding. The oral route is the most commonly used mode of administration. In addition, dermatological and nasal administration are used (13).

1.1.3. Safety assessment of Traditional medicines

Traditional medicine has been used by the communities for a long period and is believed to be useful. Nevertheless, their mode of action may not be well understood in modern scientific terms, and they are usuallymixtures of active ingredients (10).The safety and effectiveness of many commonly available herbs have recently come into question due to reports of side effects and possible interactions with prescribed medications(14).The majority of scientific data demonstrate that many plants are highly toxic, mutagenic, and carcinogenic when used as food or in conventional medicine (15-17).The WHO also states that improper use of conventional medicines or practices can have harmful or dangerous consequences and that more research is needed to ensure that the various practices and medicinal plants used by traditional systems are effective and safe (18).

The increased interest in using herbal medicine around the world generally requires knowledge of the effects on the human body of the various plant preparations used in disease management (19, 20).Various ethnic groups have confirmed the occurrence of adverse effects and, in some cases, life-threatening conditions possibly caused by herbal medicines (21).There are also several examples of potential side effects associated with the most commonly used herbal medicines and other forms of alternative and complementary medicine(22, 23).As a result, determining the toxicity profiles of medicinal herbs is a concern that requires attention and intervention. An overdose of TM can cause chronic damage to the blood composition and tissue of the various organs.Certain herbs used in herbal medicine have been found to contain toxic components with a variety of potential side effects (24). For example, blindness and changes in central nervous system function have been observed in individuals who ingested *Hagenia abyssinica*(22). In patients who took herbal medicine to control body weight(25),neuropathy and coma were also reported.

Traditional therapists have no sufficient scientific understanding of medicinal plants and including the cause and prevention of toxicity(26). TM toxicity is not due to the inherent dangers of using conventional medicine, rather it occurs because of its improper use (14). It also happens because of misidentification, improper planning, or improper administration and dosage of the herbal medicine(27). Therefore, evidence on the safety and efficacy of conventional medicines is necessary (28). Side effects of herbal medicines that are difficult or impossible to detect clinically could be detected by experimental studies/tests. Immunotoxicity, genotoxicity, carcinogenicity, and, developmental toxicityare some of the tests that have been recommended (29).

1.1.4. Assessment of Potential developmental toxicity

A teratogen is any substance that can disrupt the normal developmental process and causes a congenital defect or deformity(30). Death, anatomical deformities, and functional deficits are among the possible outcomes of congenital malformations(30). Aside from supplying nutrients and oxygen, uteroplacental circulation has the potential to deliver teratogenic substances to the growing embryo or fetus. As a result, the embryo or fetus can have difficulties during pregnancy. Teratogenicity studies, also known as prenatal developmental toxicity or embryo-fetal

developmental studies, show the effects of chemicals on the mother and the fetus when they are exposed during embryogenesis(31). Before being used by humans, herbal products are examined for potential teratogenicity using a rodent model. As a result, teratogenicity testing should be done before the commercialization of herbal products (31). Developmental toxicity studies are the most reliable source of data regarding the negative effects of treatment on embryonic or fetal development that can manifest at birth. As a result, such studies aim to determine whether a test substance has the potential to induce embryo/fetal defects, embryo-fetal death, fetal weight (length) reduction, or any other negative impacts on maternal health(32). Herbal teratogenicity has been reported in various studies. For instance, a study conducted in Iran found that administering *perovskia abrotanoides* (Tinget) ethanolic extract, which is traditionally used to treat parasitic infections, caused spina bifida, aglossia gastroschisis, polydactyly, and tarsal extender, as well as skeletal abnormalities in fetus rats (33). Similarly, *Lawsonia Inermis* leaf is also used as a cosmetic drug in Iran. In mice fetuses, a seven-day administration of hydro-alcoholic *Lawsonia inermis* extract resulted in no development of parietal bones or extra ribs(33). In addition, a study conducted on plant alkaloids that cause developmental defects through the disruption of cholinergic neurotransmission has reported that exposure of a developing embryo or fetus to alkaloids from plants, plant products, or plant extracts has the potential to cause developmental defects in animals (34).

In a recent study on the teratogenic effect of high doses of *Syzygium guineense* (Myrtaceae) leaves on Wistar albino rat embryos and fetuses, the 12-day old rat embryos, crown-rump length, number of somites, and morphological scores were affected (35). Furthermore, *Moringa stenopetala* and *Achyranthes Aspera* have been shown in subacute and chronic toxicity studies to be safe.However, the herbs showed effects on prenatal growth retardation such as decreased litter weight and crown-rump length, a delay in the development of an otic, optic, and the olfactory system, and a reduction in some branchial bars in the embryo and fetus of Wistar rats at the high doses (36, 37). Even if some data support herbal products' developmental toxicity, specific evidence is scarce and inconsistent(38).

1.1.5. Insilico Toxicity

Resource-intensive and time-consumingsafety studies of herbal plants in the early phases of the preclinical process could be reduced using a computer-aided design such as Protox II and ADMET predictive models(39-41).The software Chemdraw (8.0) (42)is important to build two-dimensional structures of compounds from the phytochemical analysis. The Swiss ADME web tool is used to convert the two-dimensional structures into a simplified molecular-input line input system (SMILES) that can be analyzed by servers for toxicity prediction (43).

Toxicity profiles: hERG potassium channel inhibition (cardiotoxicity), (Hepatotoxicity), and AMES (Ames Mutagenicity) can be predicted using the ADMET (Absorption, Distribution, Metabolism, Execration, and Toxicity) server (44). In Addition, drug-induced liver injury (DILI), mitochondrial membrane potential (MMP) toxicity, and cytotoxicity parameters can alsobe predicted via vNN- ADMETserver (45). Similarly, the organ toxicity (Hepatotoxicity), toxicological endpoints (Carcinogenicity, Immunotoxicity, and Mutagenicity), and acute toxicity (LD50 (mg/Kg)) of compounds can be evaluated using the Protox II web server(http://tox.charite.de/protox_II) (46, 47).

1.2. Statement of the problem

Worldwide, traditional medicine practices differ widely from country to country and region to regionsince they are impacted by cultural, historical, personal attitudes, and philosophical reasons (48). Many traditional medicine preparations have been used extensively, and knowledge about them has been accrued by several generations of practitioners from experience, trial, and error (48).

In most disease conditions, they are given for an extended period without a proper dosage (49).The fact that herbal medicines are natural does not mean that they do not contain some toxic substances which could be harmful to humans if consumed (50). As a result, the widely held belief that anything “natural is safe” is incorrect (50, 51) andmany herbal medicinescould have toxic side effects and can be fatal in high doses (52).

Herbal medicine toxicity is becoming more widely recognized in most parts of the world (53).Herbal medicine toxic effects ranging from allergic reactions to cardiovascular, hepatic, renal, neurological, and dermatological toxicity(52). The danger associated with the potential toxicity of such herbal therapies used over a long period requires practitioners to keep abreast of reported occurrences from medicinal herb ingestion (49).

T. schimperi is one of the most commonly used traditional medicinal plants in Ethiopia (54).This plant is very common and can be found in almost every home and local market. People with gonorrhea, cough, and liver disease, renal disease, stomach pain, hypertension, kidney problems, and dermal fungi use the plant in various forms (54).

Globally, pregnant women are commonly used in traditional medicine for pregnancy-related illness, abortion, and food cravings during pregnancy (55). In Africa, mothers are more likely to use traditional medicines during pregnancy due to cultural and socio-economic factors(56). According to a recent systematic review and meta-analysis study conducted in Ethiopia, around 47.77% of pregnant women use herbal medications during their pregnancy due to their availability and accessibility(57).

In an acute and subchronic toxicity study, the crude extract of *T. schimperi* was found to have a nontoxic effect on Wistar rats in Ethiopia (58). However, no previous research has been done on the effects of *T. Schimperi* on developing embryos and fetuses.

Some medicinal plants (*Syzygium guineense (Myrtaceae),Moringa stenopetala and Achyranthes aspera*) have no toxicity in subacute and subchronic toxicity studies have shown to causes significant growth delays on certain growth parameters in embryos and fetuses(36, 37).

As a result, it is unclear whether the most commonly consumed *T.schimperi* has developmental effects on the developing embryo or not.Previous research on *T. scimperi* crude extractrevealed changes in some toxicity parameters though they were not statistically significant. As a result, it is important to fractionate the *T.scimperi* contents and assess the toxicity of the compounds.The aim of this study was, therefore, to evaluate the acute, sub-acute, developmental, and in-silico

toxicity of the essential oil, as well as the developmental toxicity of the aqueous crude extract of *T. schimperi* on the Wistar albino rats.

1.3. Significance of the study

Herbal treatments are known to have substances that help people maintain their health and heal illnesses. However, they may contain hazardous compounds that are harmful to health or even causingdeath. According to the World Health Organization (WHO), abusing standard medications or procedures might have dangerous or catastrophic outcomes.As a result, further research is needed to determine the efficacy and safety of a variety of medicinal plants used in traditional medicine.

T.schimperi has been studied for its use in the treatmentof various diseases such as gonorrhea, cough and liver disease, kidney diseases, stomach pain, hypertension, kidney problems, and dermal fungi.

Generally, this study would address a gap in acute, subacute, and developmental toxicity data that might be utilized to guide future toxicity studies in higher animals. The study's outcome will serve as a basis for further investigation of *T.schimperi* to make better use of its claimed nutritional and therapeutic values. It will also assist in the development of regulatory laws on the use of the *T.schimper*.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Thymus Schimperi

2.1.1. Ethnobotany

The large plant family in the world is represented by 236 genera and above and 7200 species are *Lamiaceae/Labiatae*(59). This family is widely used as ethnomedicine because of its diverse chemical composition(60).

Ethiopia has significantly abundant *Lamiaceae* family herb growing in different regions and possesses a variety of the wild-growing species of this family. Many species belonging to different genera of the family *Lamiaceae* have been reported to be found in different parts of the country. The genus *Thymus* contains about 350 species widely distributed in equatorial regions of the world (61).The two species *T. schimperi* Ronniger and *T. serrulatus* Hochst ex Benth, both locally in Amharic known as *Tosign*, are the endemic species represented in Ethiopia(62).These species are commonly found in Ethiopian highlands (63) and are restricted to the afromon-tane and afro-alpine zones of the country (64).

Thymus schimperi is commonly found across southern, eastern, and northern Ethiopia(65). It is also widely distributed in Amhara, Oromia, and Southern Nations Nationalities and Peoples Regions. It is found in Denkoro forest (66), Chancho(59), Ankober (67), Menz Gera Midir (Guassa)(59), Tarma Ber wereda of North Shewa and Gondar areas(62).

In Oromia Region, *T.schimperi* is found in Adaba Dodola area(68), Dinsho(69, 70); Sanetti Mountains(59); Goma(71), Asendabo(72); areas around Jimma(73); Debre Zeyit(59), Awash National Park(74), and Menagesha Suba State Forest(75).

Figure 1: Photograph of *T. schimperi*, source Tesfamaryam et al., 2015.

T. Schimperi is a small perennial herb. It is woody at the base and has 5 to 40 cm tall with prostrate stems. It is often erect ata younger age. It has quadrangular, green to purple, and blue-green or pale-green leaf blades, and is endemic to the highlands of Ethiopia. It growsnaturally on roadsides, in open grasslands, on bare rocks and slopes at altitudes between 2200 and 4000 meters above sea level(76)(**Figure 1**).

2.1.2. Phytochemical Property of *T. schimperi*

The phytochemical screening of *T. schimperi* leaves aqueous crude extract showed the presence of steroids, alkaloids, flavonoids, saponins, and tannins(77).*T. schimperi* contains about 1.0% - 2.5% essential oil. The essential oil is a pale yellow liquid with a strong floral, warming, and aromatic perfume(78). Thyme oils contain phytochemicals such as carvacrol, thymol, γ-terpinene, and p-cymene. These phytoconstituents are present in various proportions and characterized by a large number of monoterpenes which account for 90% of the oil. Thymol and carvacrol are more common, and they are accompanied by the couple of c-terpinene/p-cymene, the four monoterpenes being biogenetically closely related(79). *Thymus* is characterized by the presence of intraspecific chemotype variation. Each of the six chemotypes, thymol, carvacrol, geraniol, -terpineol, thuyanol-4, and linalool, is named after its major constituent

monoterpene(80). *T. schimperi* is a chemotype that contains thymol and carvacrol(62). The chemical composition and the isolation yield of thyme oils depend on several factors, such as the environment or region in which they are grown, the cultivation practice, the development stage of the plant, harvesting time, and habitat (79, 81). The principal uses of *T.schimperi* in dietary and food processing are identified by the activities of the chemical components of thyme, thymol, and carvacrol, present in the essence of thyme, and also flavonoids and other polyphenols, which are considered to be involved in the activities of perfume, aroma, antioxidants, and antimicrobials(82, 83).

2.1.3. Traditional Uses of *T.schimperi*

In different parts of the world, *T. schimperi* (thyme) extracts are traditionally used orally to treat dyspepsia and other gastrointestinal disturbances, bronchitis, pertussis, laryngitis, tonsillitis, and coughs due to colds (59, 84). Topical applications of thyme extracts have been used in the treatment of minor wounds, common cold, disorders of the oral cavity, as well as antibacterial agents in oral hygiene (59).

In Ethiopian traditional medicine, *T. schimperi* is used to treat different diseases like gonorrhea, cough, liver disease, renal diseases, hypertension (59), stomach pain (85), kidney problems (71), and dermal fungi (67). In Addition,*T. schimperi* is used in a variety of forms (59). The fresh or dried leaves are used locally as condiments and tea (63), in the preparation of “berbere”(pepper powder) and “shirro” (bean/pea powder) (86), and for the preparation of metata ayb (fermented cottage cheese) (87).

2.1.4. Pharmacologicalactivity of *T.schimperi*

In the study, conductedin diabetic mice, aqueousand 80% methanol extracts of *T. schimperi* leaves reduced blood glucose levels. As a result, *T. schimperi* has anti-diabetes Mellitus activity(81). In addition, the aqueous extract of *T. schimperi* leaves shows antihypertensive and diuretic action, according to a study conducted in rats(88).In another study, polyphenolic components of *T.schimperi*reduced cell proliferation and promoted cell death in human gastric adenocarcinoma (AGS) and liver hepatocellular carcinoma (HepG2) cancer cells(89). Similarly, major bioactive chemicals found in *T. schimperi* essential oil, such as carvacrol and thymol, can

cause cell cycle arrest in the sub G0/G1 phase, cellular apoptosis, and cell proliferation (90, 91). Furthermore, a previous study found that carvacrol, which is the major constituent of *T.shimberi* essential oil, inhibits DNA synthesis(92).*T.schimperi* has antimicrobial, anti-inflammatory, and antioxidant properties, as shown below.

2.1.4.1. Antimicrobial Activity

Antimicrobial properties have been stated more frequently in a wide range of plant extracts, essential oils, and natural products to find new chemical classes of antifungal and antibacterial drugs(93, 94). The natural compounds of *T.schimperi*, particularly its phenolic components, thymol, and carvacrol, have antibacterial activity against both gram-positive and gram-negative bacteria, due mainly to their effects on the membrane bacteria (95).Thymol and carvacrol have respiratory antiseptic activity because they are eliminated through the respiratory system(96). *T.schimperi*is an antiseptic agent for urinary tract, mouth, and skin wounds due to its antibacterial activity(97).

2.1.4.2. Anti-inflammatory Activity

Inflammation is considered a primary process of physiological defense and is associated with the body'sdefenses against wounds, diseases, toxic substances, allergens, and other harmful stimuli(98).The in-vivo examinations of ethanol extract from *T. schimperi* in rats demonstrate analgesic and anti-inflammatory activities(98). These practices may be associated with carvacrol and thymol, which in animal models demonstrated inhibitory effects on the cyclooxygenase enzyme as well as inhibitory effects on the supplement and the synthesis of nitric oxide(99).Carvacrol has an inhibitory impact on prostaglandin synthesis. This action encourages the use of thyme to relieve muscle and joint pain in ointments.Rosmarinic acid also displays anti-inflammatory activity in this extract. Rubefacient and providing analgesia are topical treatments of other thyme essential oil, effective in cases of bruises or sprains(99, 100).The anti-inflammatory activity of phenol compounds in *T. Shimperi*has been documented(99, 101).

2.1.4.3. Antioxidant Activity

*T. schimperi*essential oil contains thymol and carvacrol, as well as flavonoids and other polyphenols, all of which are known to be antioxidants (82).Rosmarinicacid, hydroxycinnamic derivatives, and flavonoid compounds demonstrated significant in vitro antioxidant activity by inhibiting iron-induced superoxide anion formation and lipid oxidation in microsomal and mitochondrial systems(82).Furthermore, the essential oil thymol demonstrated in vitro antioxidant activity by neutralizing the radical DPPH (diphenyl-picryl hydroxyl)(82).

2.1.5. Safety and Toxicity Studies of *T. schimperi*

Moneystudies have been done on *T.schimperi* efficacy. However, less has been done on its toxicity or safety and no research has been done on the *T.schimperi*potential effect on prenatal development. The oral dose limit of 5000 mg/kg crude extract of *T. shimperi*in the acute toxicity test done on rats showed that no clinical signs of toxicity, mortality, or behavioral changes (88). In addition, the acute toxicity study conducted by Debelo(58)showed that no signs of toxicity were found in the crude extract of *T.schimperi* and the LD_{50} was suggested to be greater than 10,000 mg/kg. Ina sub-chronic toxicity study of the *T.schimperi* at the treatment doses of 200 mg/kg and 600mg/kg, there was no sign of toxicity in the blood hematology and chemistry parameters. However, the kidneys and liver of the treatment group light microscopic examination show that a higher dose of localized mononuclear lymphocytic infiltration and moderate blood congestion occurred within the hepatic portal and central veins of the liver(58).

In another acute and sub-acute toxicity study carried out on mice, the results showed that essential oil extracted from *T. schimperi* caused no mortality up to the doses of 2000 mg/kg. There was no significant change in the serum enzyme level of the study mice caused by the essential oil of the plant. Histopathological examination of the liver and kidneys were also showed that the plant caused no major organ damage(102).However, no previous acute and sub-acute toxicity studies have been conducted on the Wistar albino rats, nor has any research been conducted on the *T.schimperi* crude extract or the essential oil developmental toxicity.

Many medicinal plant species have received little attention, and teratogenic effects cannot be ruled out (103). No previous studies have been conducted for the evaluation of teratogenic disorders associated with the administration of *T. schimperi* extracts. However, several investigations have reported the harmful teratogenic effects of some herbal medicines which havealkaloids (34).

To identify the potentially toxic effects of *T.schimperi*, overall preclinical toxicity (Acute, sub-acute, insilico, and developmental toxicity) investigations were conducted, especially the liver and kidneys are the target organs for sub acute toxicity studies of animals (104). If these organs are mildly inflamed and injured, the permeability of the cell membrane increases, releasing cytoplasmic enzymes like ALP and AST into the blood. Inflammation also causes mitochondrial ALT and AST to be released(105, 106).

2.1.5.1. Blood

Blood is a specialized body fluid that provides the cells of the body with the necessary substances such as nutrients, oxygen, and transports waste products from those of the same cells(107).It is the most significant body fluid that controls the body's vital functions such as breathing, circulation, osmotic equilibrium, the transport of metabolic material excretion. To transport gases, nutrients, minerals, metabolic products, and hormones between various organs, blood circulation within the cardiovascular system is important(107).It consists of fluid known as plasma and components produced, such as white blood cells (WBC) or leukocytes, red blood cells (RBC) or erythrocytes, and platelets or thrombocytes(108).Plasma makes up about 55% of the total volume of blood. It is an aqueous solution containing < 1% of electrolytes and ions such as calcium, potassium, sodium, and bicarbonate; about 7% of proteins like albumins, globulins, and fibrinogen; and organic compounds such as various amino acids, vitamins, hormones, lipids and cofactors(109).

The rat's blood volume is estimated to account for 7% of its body weight; younger rats typically have a higher volume of blood compared to their body weight than older rats(110).Red blood cells that have biconcave disks such as the form of the luck nucleus and never leave the circulatory system under normal conditions and their major role is to bring oxygen without their hemoglobin(111).The number of red blood cells is 7-13 million/µl. Rat erythrocytes have a

diameter of 4-7µm and are morphologically similar to those in humans(109).Erythrocytes have an average lifetime of 120 days in humans and 61 days in rats(109).

Leucocytes are full cells, with a nucleus and cell organelles comprising each cell. They contain lymphocytes, monocytes, and granulocytes.According to the staining properties of the granules, the granulocytes are classified into three types; they are called neutrophils, eosinophils, and basophils and constitute 55-65%, 1-3%, and 0.3-0.5% of white blood cells, respectively.20-30% and 3-8% of white blood cells are both lymphocytes and monocytes, respectively(112).Leucocytes are active in the body's protection against pathogens(112).

Platelets are small structures containing red-purple granules that are discoid, non-nucleate. By plugging defects in the walls of the blood vessels and leading to the initiation of the blood-clotting cascade, platelets play a critical role in the regulation of bleeding (homeostasis)(108).

Blood parameters are potentially the most rapid and measurable changes under stress and are the basis of health condition evaluation(113).The significance of hematological parameters is well known in clinical biochemistry, population genetics, and medical anthropology. Current assumptions suggest that any change in WBCs, RBCs, platelet counts, and RBC components, as well as WBC or leukocyte, may be used as useful markers of disease or stress in animals (107).

2.1.5.2. Liver Structure and Function

The liver is the largest internal organ, accounting for 2.5%of adult human body weight. It has a reddish-brown color.It is situated below the diaphragm in the right upper quadrant of the abdomen, where almost the entire right hypochondriac area, part of the epigastric region, is occupied and extends into the left hypochondriac region. In this position, the liver is well covered by a rib cage in the diaphragm dome; and this position is retained by peritoneal reflections (attachments)(114, 115). Much of the liver is protected by a thick, irregular capsule of collagenous connective tissue known as "the capsule of Glisson," which is in turn covered by the peritoneum visceral sheet. Septate from the Glisson's capsule and separate the liver into lobes and lobules(116).There are two main lobes in the liver (the smaller left and larger right) and two minor lobes (caudate and quadrate). The hepatic artery, which contributes about 20-30% of the

blood supply, and the portal vein, which contributes the remaining 70-80% of the blood supply, have the dual blood supply to the liver(116, 117).

The rat liver is the cranial organ on the right side of the abdominal cavity, comes into close relations with the diaphragm, and is a multi-lobulated structure(118).The mass of the liver of rats is approximately 5-6% of the total body weight, while the weight of the liver of rats is on average 15.5 g with a total volume of 22. 6 ml. The transverse and the dorso-ventral diameter of the rat liver is approximately 8.5-9 cm and 4.8-5.2 cm respectively, while the craniocaudal diameter is 3.2-3.5 cm(118).The liver has thousands of important functions, including the successful absorption into the bile and/or blood of amino acids, carbohydrates, bile acids, cholesterol, proteins, lipids, and vitamins for storage and metabolism(118).

When the rat abdominal cavity is opened through the lineaalba, as shown by(118), the rat liver normally has two surfaces: diaphragmatic and visceral surfaces. Since the liver of the rat is a multi-lobulated organ, it has about the same surfaces placed flat against each other as the lobes.The diaphragmic convex surface is in contact with the right abdominal wall and diaphragm.The concave visceral surface is very rough since the guts (stomach, downward duodenum, right colic flexure, jejunum, spleen, pancreas, right kidney, and suprarenal gland) are related to it.Like the human liver, the rat liver lobes are labeled after the portal branches that supply them, as the portal system is the most continuous anatomical reference system among mammals(119).The rat liver is multi-lobulated, as it is in other mammals. The liver of the rats has lobes: median (or middle), left, right, and caudate, and all but the left are subdivided into two or more sections(120, 121)**(Figure 2**).There is a gall bladder for mice and humans, but not for rodents.

Traditionally, human liver lobes have been designated as right, left, quadrate, and caudate, but recently it has been suggested that the liver can be divided into 8 segments based on the right and left sides of the vascular and ductal branching patterns(120, 122).The liver lobes of the rat match the segments of the human liver(120). Segment I is CL equivalent, while segment II is LL equivalent, and segments III, IV, and VIII are ML equivalent, while segments VI and VII are RL comparable(120).The middle one (ML) is the largest, contributing too much of the weight of the

liver. It is trapezoidal, set by the falciform ligament in the diaphragm and abdominal wall.It is following the left lateral lobe (LLL) and is differentiated into a broad right medial lobe (RML) and a smaller left middle lobe by a vertical fissure (main fissure or umbilical fissure) (LML)(123). There are both left and right hepatic vascular elements in the RML(120).

The right lobe (RL) is situated in the right hypochondria on the right side of the vena cava and subsequently in the right one and is almost entirely hidden by the medial lobe.It is divided into two pyramidal-shaped lobules by horizontal fissure: the superior (SRL, also called the right posterior lobe) and the inferior (SRL, also called the right posterior lobe) (inferior right lobe, IRL, also called the right anterior lobe)(123).

There is a rhomboid form in the left lateral lobe (LLL), flattened and positioned over the anterior part of the stomach in the epigastric and left hypochondriac regions. The left part of the medial lobe is hidden by its medial component. The upper surface is slightly convex and molded on the sides of the diaphragm(120).

Behind the LLL and on the left of the vena porta and the inferior cava are the caudate lobe (CL).It is divided into two parts: the paracaval (caudate) section that encircles the inferior vena cava and bridges the CL and the right lateral lobe, and the anterior (superior) and posterior (inferior) portions of the Spiegel lobe in the form of disks(123).The anterior portion of the CL is anterior to the esophagus and stomach and its pedicle is superior, whereas behind these structures and its pedicle is the posterior(123).

A study performed by Martins and Neuhaus in 2007(124) showed that the origin and course of the main rat liver vessels were close to that of humans and that no variability in the origin of the vessels has been found. The rat does not have a gallbladder, unlike a human.

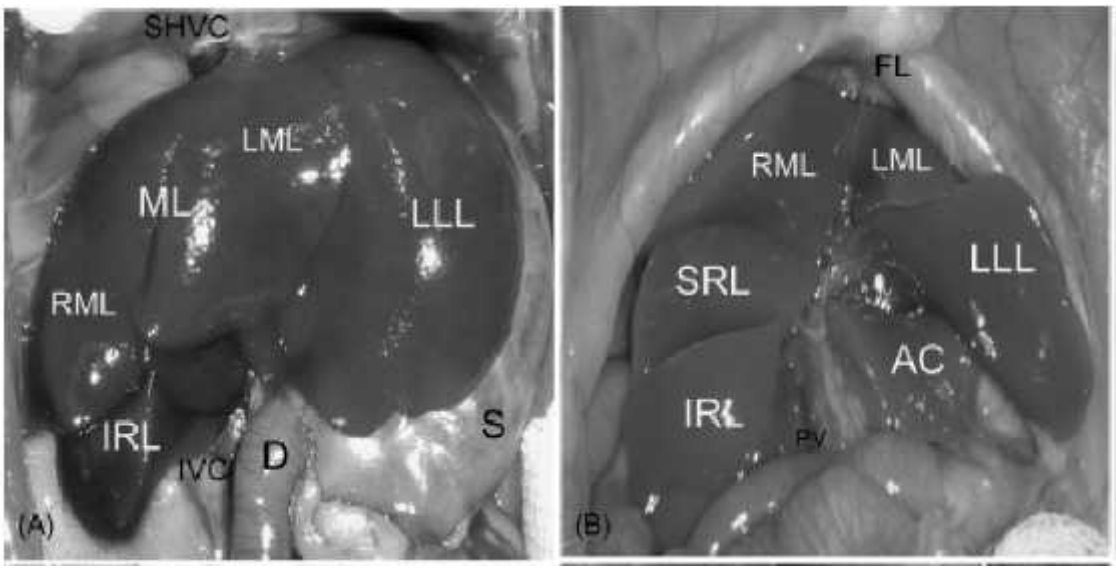


Figure 2: Rat liver in situ. (A) Anterior view, (B) Anterior view after separating the lobes (Martins and Neuhaus, 2007).

AC, anterior caudate lobe; SRL, superior right lateral lobe; IRL, inferior right lateral lobe; ML, median lobe; RML, right portion of the medial lobe; LML, left portion of the medial lobe; LLL, left lateral lobe; FL, falciform ligament; S, stomach; D, duodenum; PV, portal vein and IVC, inferior vena cava.

Irrespective of the gross differences, the microscopic characteristics of the rat and human livers are more or less similar. As seen in light-microscope sections, the liver is structurally organized into polygon-shaped classic hepatic lobules.Every lobule, as shown in (**Figure 3A**), consists of the central vein located at the middle and cords of hepatic cells peripherally radiating from the central vein. Narrow blood sinusoids are separating the cell cords. The scanty connective tissue connects each lobule.There are portal islands of connective tissue at the apices of lobules, each containing a branch of the hepatic artery, hepatic portal vein, and bile ductile forming portal triads, as shown in (**Figure 3 A & B**)(125).From the periphery to the middle of each hepatic lobule, blood still flows. Consequently, oxygen and metabolites first enter the peripheral cells of the lobule and then the more central cells, as well as all other toxic or non-toxic compounds consumed in the intestines(126).

Rat liver parenchymal cells, hepatocytes, are acidophilic cytoplasm polyhedral cells with a rounded pale stained nucleus. There are numerous microvilli on the sinusoidal surface of

hepatocytes that increase the surface area available for the exchange and absorption of substances from portal blood(125, 127).Hepatic sinusoids are capillaries that are broader and more irregular in shape than ordinary capillaries, as shown in **Figure 3A**. To slowly move the hepatocytes, these sinusoids conduct nutrient/hormone-rich venous portal blood and high-oxygenated arterial blood.Hepatic sinusoid cells include liver macrophages (Kupffer cells), endothelial cells, and stellate cells that store fat (Ito cells)(128, 129). Kupffer cells' key functions are the breakdown of aged erythrocytes, the removal of bacteria or debris from the gut that may enter the portal blood, and the clearing of foreign material, in particular endotoxin, from the circulation of the portal.Endothelial cells are long, slender cells with extended processes and are the hepatic sinusoidal lining's main cellular feature with a fenestrated endothelium lining(128)**(Figure 3 B).**

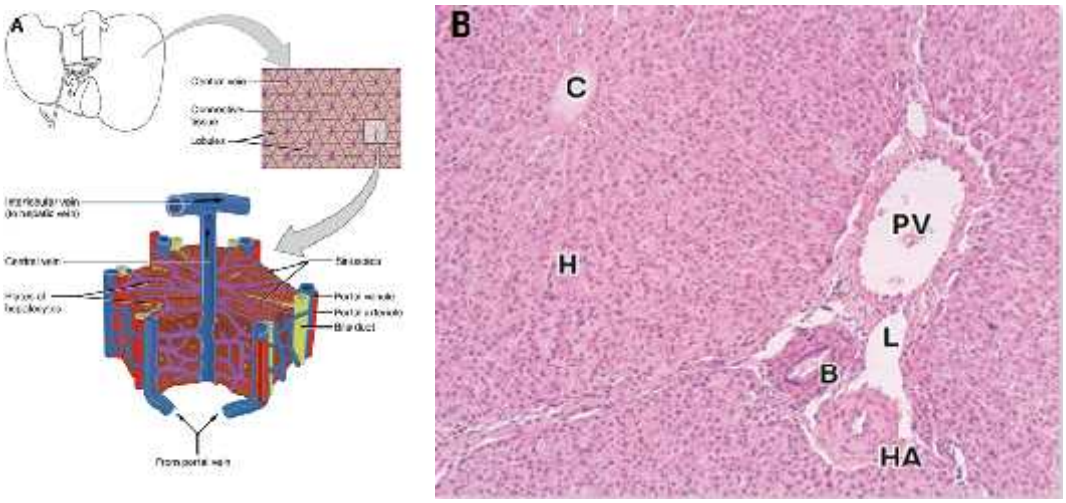


Figure 3:(A) Diagrams of the human liver Hepatic lobules, Hepatocytes, and Sinusoids (J. Gordon Betts, 2013). **(B)** Micrograph of portal triad X220. H&E (Mescher, 2013). C, central vein; H, plates of hepatocytes; L, lymphatic; PV, portal venule; HA, hepatic arteriole; and B, bile ductule.

The liver has four primary functions: secretory, storage, metabolic, and excretory; it is a vital organ. Its secretary work involves the production and releases per day of 600 to 1200 mL of bile for the emulsification into smaller particles of the large fat particles of food. The majority of plasma proteins and blood clotting factors are made(128).Metabolites such as massive quantities of glucose in the form of glycogen, amino acids, and fat-soluble diet vitamins are processed by liver cells. It also breaks down glycogen into glucose when required to supply the body with energy(130).This detoxifies and extracts drugs such as pesticides, herbicides, and poisonous

substances and, in coordination with the spleen, removes weakened red blood cells from the blood(131).

Due to the central function of the liver in xenobiotic metabolism and the position of the portal within the bloodstream, toxin accumulation within the liver is normal. Accumulation of toxins in the body faster than the liver's ability to treat and destroy them results in liver damage(132).Early proof of liver damage is generally seen by a fatty shift shown by the hepatocyte shape of cytoplasmic vacuoles. These vacuoles will pass the nucleus to one side, and hepatocytes will enlarge and appear darkly stained in their nuclei(133).Cytoplasmic vacuolation occurs predominantly as a result of severe lipid inclusion disruption and fat metabolism during pathological changes(134).The hepatocytes will undergo hydropic degeneration and become bloated when there is more serious metabolic disruption. These infected cells will experience necrosis gradually, which is an indicator of serious hepatic damage(131).

2.1.5.3. Kidney Structure and Function

The kidneys are paired, reddish-brown, bean-shaped retroperitoneal organs situated on either side of the vertebral column between the twelfth thoracic and the third lumbar vertebrae of the posterior wall of the abdominal cavity, with an adrenal gland sitting on the top or upper pole of each kidney like a small-cap(135).The right kidney lies well below the left kidney. The standard single adult human kidney has a length of approximately 11 cm to 12 cm, a width of 5.0 cm to 7.5 cm and a thickness of 2.5 cm to 3.0 cm, and a weight ranging from 125 g to 170 g for males and 115 g to 155 g for females.The kidneys are medially concave and laterally convex, and each kidney has a slit on the medial or concave surface, called the hilus, through which the renal pelvis, the renal artery and vein, the lymphatics, and the nerve plexus pass through the kidney sinus.A thick fibrous capsule that gives the fresh kidney a glistening appearance and is easily removable under normal circumstances invests the kidneys closely. The anterior and posterior surfaces, medial and lateral margins, and upper and lower poles of each kidney(114).

The rat kidneys are paired, bean-shaped, smooth, reddish-brown organs protected by a thin capsule of connective tissue that adheres to the connective tissue of the sub capsule. Rat kidneys lay retroperitoneally in the abdominal cavity on either side of the vertebral column. The right kidney of the rat is larger and heavier than the left kidney, also located more anteriorly and

cranially(136).There are dorsal and ventral plates, medial and lateral margins, and an upper and lower pole in each kidney. The lateral border is convex, while the medial border is concave, called the hilus, where major renal vessels join and exit and where the ureter originates, with indentation(137, 138).Relatively larger kidneys than females are found in male rats; kidney weight differs between inbred strains(139). Two main distinct regions are revealed in the coronal segment of the kidney: the outer pale area of the cortex, which has a light color and granular texture, and the inner medulla, which has a dark reddish-brown color. Medullas are a collection of structures that are pyramidal. In spaces between neighboring pyramids, the cortex or cortical parenchyma extends, and these extensions are known as the Bertin columns.A medullary pyramid constitutes a renal lobe with two neighboring Bertin columns plus the sub-capsular cortex (140). The medulla is split into 8 to 18 renal pyramids in humans. Each pyramid has a renal papilla of its own, extending into a minor calyx. Multiple small calyxes converge into 2-3 large calyxes. In the renal pelvis, the main calyxes converge. As in **(Figure 4),** unlike the human kidney, the rat kidney has a single renal pyramid and is thus referred to as 'unipapillate.' The rat's unipapillary kidney is surrounded directly by the renal pelvis and stretches to the ureter. The rat kidney, in its gross and microscopic appearance, resembles the human kidney(138, 140,141)**(Figure 4)**.

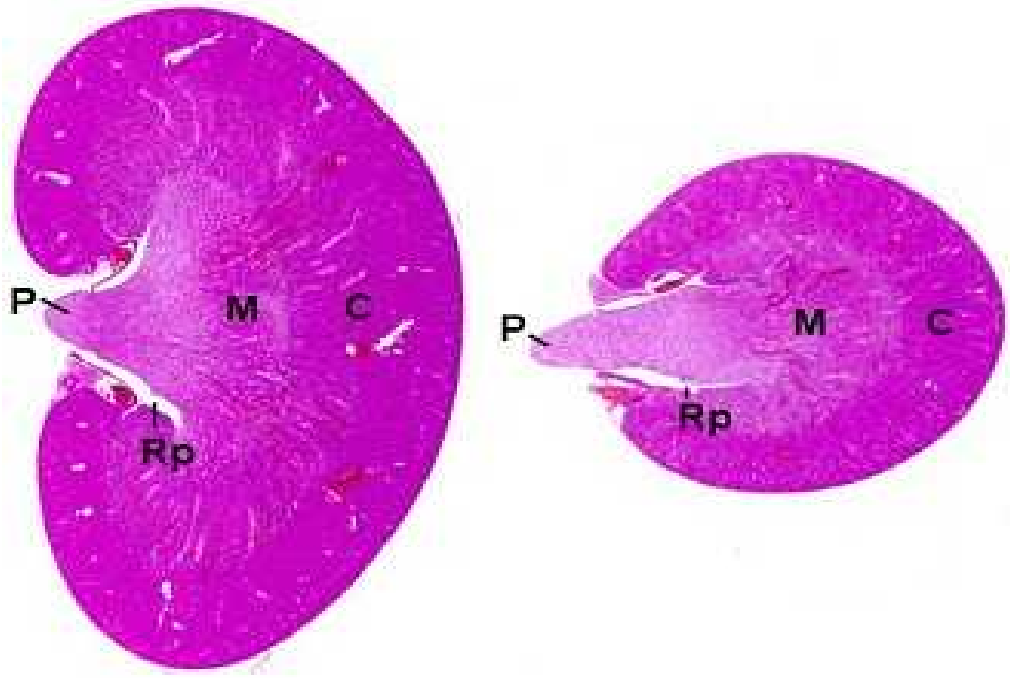


Figure 4: Photograph showing the microscopic structure of rat kidneys (Piper et al., 2012).

P, papilla; M, medulla; C, cortex; Rp, renal pelvis.

Nephrons are the kidney's essential structural and functional units. There are around 1 - 1.4 × 10^6 nephrons in a single human kidney(126).In comparison, there are about 30,000 nephrons in each adult rat kidney(141, 142).There are two sections of Nephron: the renal corpuscle where the blood plasma is filtered, and the renal tubules where the filtered fluid moves through(115).

As shown in**Figure 5B**, each renal corpuscle consists of a capillary tuft (the glomerulus) surrounded by a glomerular (Bowman's) capsule called a double-walled epithelial capsule. The inner (visceral) layer of the capsule envelops the capillaries of the glomerulus. The outer border of the renal corpuscle forms the external (parietal) layer.The urinary space, which gets the fluid filtered through the capillary wall and the visceral layer, is between the two layers of Bowman's capsule. There is a vascular pole in each renal corpuscle where the afferent arteriole enters and the efferent arteriole leaves, and a urinary pole where the proximal convoluted tubule starts.

In the cortex and medulla, the renal tubule can be subdivided into many distinct sections. That includes the proximal convoluted tubule, the Henley loop's thin and thick limbs, the distal convoluted tubule, the collecting tubules, and the collection ducts(138).The proximal convoluted tubule is lined with cells on their luminal surfaces with abundant long microvilli forming a prominent brush border for reabsorption (**Figure 5B**). Henley's loop is a U-shaped structure that interacts between the convoluted proximal and distal tubules.Simple cuboidal epithelial cells near the cortex and simple squamous epithelial cells deeper in the medulla have descending and ascending limbs (**Figure 5A**). In comparison to the cells of the proximal convoluted tubule, the distal transformed tubule is lined with plain cuboidal cells, which are flatter, smaller, and have no brush border (**Figure 5B**).The initial straight portion of the distal tubule makes contact with the vascular pole of its parent nephron's renal corpuscle and forms part of the juxtaglomerular apparatus, a specialized structure. The cells become columnar and more tightly packed at the point of contact with the arterioles, called the macula densa(116).

The nephron is not part of the collecting tubules, and these tubules are lined with simple cuboidal epithelium with rounded nuclei and basophilic cytoplasm. In contrast with the cells of the

proximal and distal convoluted tubules in progress towards the renal papilla, cell boundaries are usually obvious.The terminal part of these tubules is lined with columnar epithelium, simple or pseudostratified, and is called the papillary duct. The renal pelvis appears as a dilated cavity situated in the renal sinus at the proximal end of the ureter and facing the renal papilla(143).A simple or pseudostratified columnar epithelium lines the terminal portion of these tubules and is called the papillary duct. The renal pelvis appears as a dilated cavity situated in the renal sinus at the proximal end of the ureter, facing the renal papilla(143).

The renal interstitium is called the area between the uriniferous tubules and the blood and lymph vessels. In the cortex, it occupies a very small volume but rises in the medulla. There is a very flimsy and scant amount of loose connective tissue in the renal interstitium, containing three types of cells. Fibroblasts, macrophages, and interstitial cells are these cells(109, 126)**.**

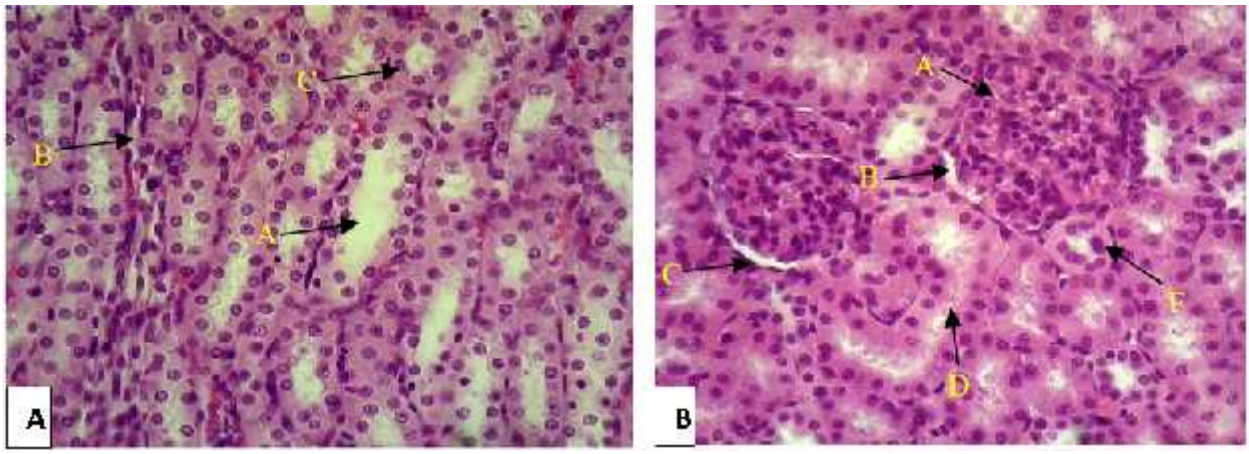


Figure 5: Photomicrograph of kidney tissue, (400× H & E), (Al-Samawy, 2012).**(A);**A-Collecting Tubule; B-Thin limb; C-thick descending limb.**(B);** A-Glomerulus.B-Visceral layer.C-Parietal layer.D-Distal tubule.E-Proximal tubule.

The kidneys remove metabolic waste from the blood plasma, control fluid osmolality and volume, preserve electrolyte balance, remove foreign chemicals, and help maintain the body's acid-base equilibrium. In addition to these, the kidneys have endocrine organ features(109).The kidneys are the main organs used to clear and excrete foreign chemicals from the body, including drugs and drug metabolites. Kidney damage can occur due to the administration of plant extracts(144).In Africa, about 35% of all cases of acute kidney failure have been implicated in the use of conventional herbal remedies(145).Nephrotoxicity can lead to systemic toxicity, reducing the ability to excrete body waste, failure to maintain the balance of body fluid and

electrolytes, and decreasing the synthesis of essential hormones(146).Therefore, an analysis of the effect on the kidney of drug extracts is important. Therefore, the evaluation of kidney tissue histology and the determination of some waste metabolic products exclusively excreted through the kidneys provide valuable information on the health status of the kidneys, including urea and creatinine metabolites(144).

2.1.5.4. Placenta Histology

Rats have hemochorialor discoid placentas. At term, the placenta weighs about one-tenth of the fetal body weight (BW); during the development of the fetus and placenta, placental growth precedes fetal development, and in the last few days of gestation, the placental weight (PW) remains constant while the fetal weight increases exponentially(147).

The cellular components of the rodent placenta are similar to those of humans, but there are some key differences; major structures are depicted in **Figures 6 and 7**. The layers that extend from the fetus include the amnion, yolk sac, reichert's membrane, placental labyrinth, basal zone (trophospongium), decidua basalis, and metrial gland. The rodent placenta is called hemotrichorial because fetal blood is separated from maternal blood by fetal endothelium, perivascular cells, fetal mesenchymal cells, and three thin layers of trophoblastic cells (cytotrophoblasts and two layers of syncytiotrophoblasts) within the labyrinth. The trophospongium is the next layer, and it is made up of spongiotrophoblasts and a deeper layer of giant cell trophoblasts. The labyrinth and trophospongium are mixed with islands of glycogen-rich cells. The decidua basalis is made up of altered maternal endometrial stromal cells. The metrial gland is the fetomaternal interface outermost layer. It is not glandular and is made up of intermixed decidual cells, specialized natural killer (NK) cells, and vessel-associated trophoblasts. This structure extends into the mesometrium from the myometrium. Human placentas, on the other hand, lack a yolk sac, are villous rather than labyrinthine, have fewer trophoblastic layers, and, exceptfor a small number of vessel-associated trophoblasts, do not cross the myometrium(148).

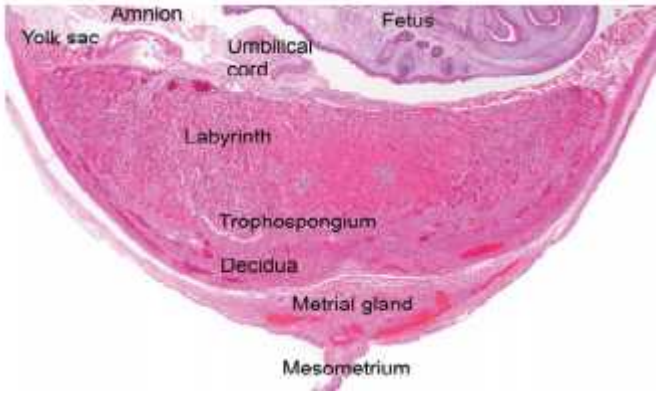


Figure 6: Subgross histologic anatomy of the rat placenta (Cline et al., 2014).

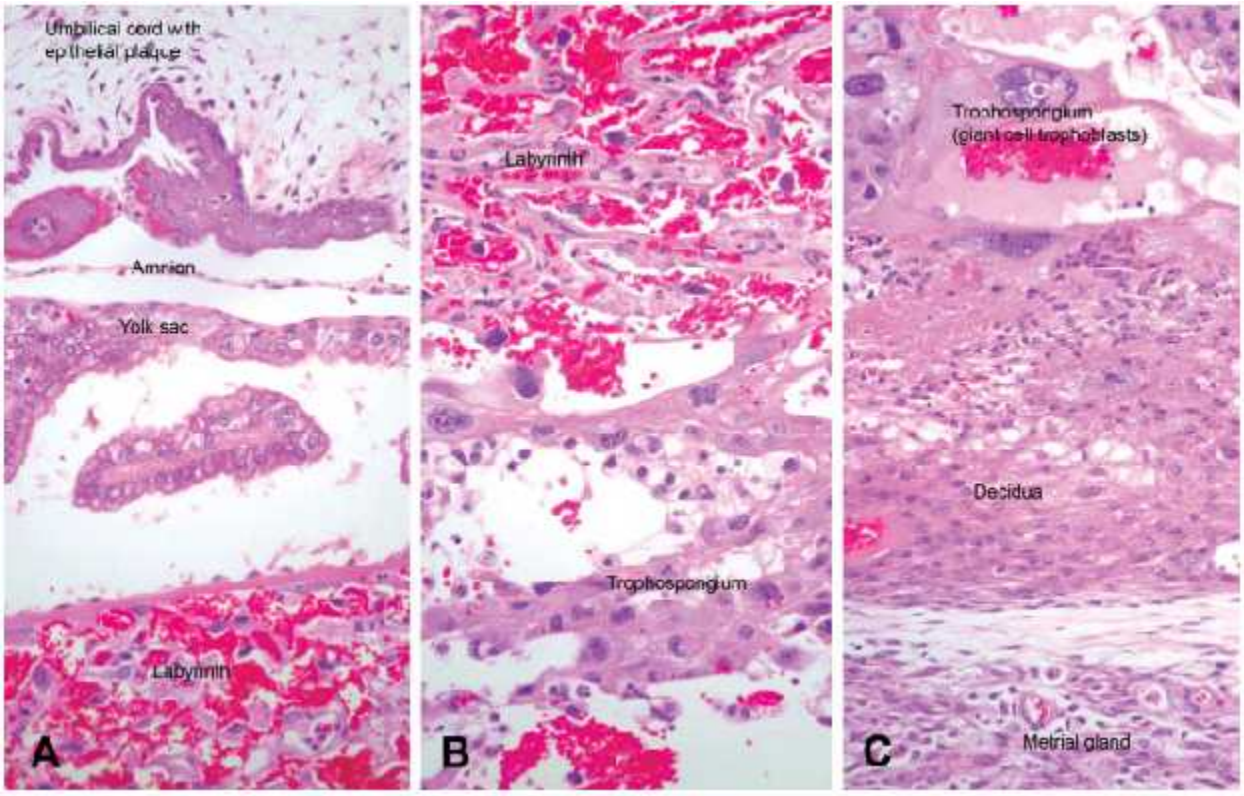


Figure 7: Higher magnification figures showing cellular components of the rat placenta(Cline et al., 2014). (A) umbilicus (bearing a focally keratinized plaque),amnion, yolk sac, Reichert's membrane, and placental labyrinth; (B) placental labyrinth to trophospongium; (C) trophospongium to metrial gland.

CHAPTER THREE

3. OBJECTIVES OF THE STUDY

3.1. General Objective

To investigate the composition and the acute, subacute, and developmental toxicity of essential oil as well as developmental toxicity of aqueous crude extracts of *T. schimperi* in Wistar albino rats; In vivo and In-Silico Toxicity Studies.

3.2. Specific objectives

- To investigate the chemical composition of essential oil of *T. schimperi*.
- To evaluate the acute toxicity of essential oil of *T. schimperi* in Wistar albino rats.
- To evaluate the sub-acute toxicity of essential oil of *T. schimperi* in Wistar albino rats.
- To assess the toxicity of compounds in the essential oil of *T.schimperi* by insilico toxicity study.
- To evaluate possible teratogenic effects of the aqueous crude extract of *T.schimperi* on 12 days old whole rat embryos.
- To assess possible teratogenic effects of the aqueous crude extract of *T.schimperi* on 20-day old rat fetuses.
- To evaluate the histopathological effects of the aqueous crude extract of *T.schimperi* on the placenta of 20 days old rat fetuses.
- To assess possible developmental toxicity of the essential oil of *T.schimperi* on 12 days old whole rat embryos.
- To evaluate possible developmental toxicity of the essential oil of *T.schimperi* on 20-day old rat fetuses.
- To assessthe histopathological effects of the essential oil of *T.schimperi* on the placenta of 20 days old rat fetuses.

CHAPTER FOUR

4. MATERIALS AND METHODS

4.1. The setting of the Experiment

In this study, there are four major experiments. The first was an evaluation of the acute and subacute toxicity of *T.schimperi* essential oil in Wistar albino rats. The second was the evaluation of the toxicity profile of the phytochemicals from the essential oil of *T.shimperi* by computational methods. The third and fourth experiments were also to assess the potential developmental toxicity of *T. schimperi* aqueous crude extract and *T. schimperi* essential oil extract after administration of the extract during the organogenesis periodof pregnant Wistar albino rats, respectively.

4.2. Study Area

The study was conducted at the Traditional and Modern Medicine Drug Research Directorate(TMMDR), Ethiopian public health Institute (EPHI), Pharmacognosy and Anatomy Departments of Addis Ababa University.

4.3. Study Period

The study was conducted from March 2019 to December 2020.

4.4. Study Design

In this study, the laboratory-based experimental study design was used. In additionto asses, the toxicity profile of the phytochemicals from the essential oil of *T.schimperi*computational method was applied.

4.5. Plant material collection and extraction

Fresh leaves of *T. schimperi* were collected in March 2019 from around Goba city, 400 km southeast of Addis Ababa, and 150 km east of Shashemene in the Oromia Region. The plant

material was authenticated by a botanist at the Ethiopian Public Health Institute (EPHI) where it was deposited (Collection number: HH-001) for future reference.

4.5.1. Crude Extraction

The areal part of *T. schimperi* wascollected and cleaned from foreign materials, dried at room temperature under shade, and ground to obtain fine powder particles using pestle and mortar.About 600g of powdered *T. schimperi* leaves were macerated in distilled water for 2 hours with continuous orbital shaker agitation. The supernatant fraction of the agitated materials was then decanted and filtered from the undissolved component of the plants using 0.1 mm^2 mesh gauze. The plant filtrates were freeze-dried at a lower temperature and pressure and then lyophilized to produce the crude extract*T. schimperi*, which yielded 60.5 g (10.08% w/w). It was then maintained at room temperature in a desiccator until it was used.

4.5.2. Essential oil Extraction

Fresh leaves of *T. schimperi* (1 kg) were extracted by hydrodistillation using a Clevenger-type apparatus. The oil obtained was stored in a sealed amber-colored vial in a refrigerator at -10 °C until it is used for the study.

4.5.2.1. Analysis of the Essential Oil

4.5.2.1.1. GC Analysis

Separation was carried out ona Shimadzu gas chromatograph; model GC-14A, fitted with a supelcowax 10 (30 m x 0.25 mm, 0.2 µm film thickness) fused silica column. The oven temperature was programmed as follows; 70 °C (5 min), 70-180 °C (5 °C/min), 180-240 °C (10 °C/min), and 240 °C (10 min). Helium was used as a carrier gas at a flow rate of 1 ml/min, and with a split ratio of 82:1. Injector and Flame Ionization Detector(FID) temperatures were 210 °C and 260 °C, respectively.

4.5.2.1.2. GC-MS Analysis

Qualitative GC-MS analyses were carried out using the MassLab VI.1 system equipped with an FI 8000GC. A Supelcowax 10 (30 m x 0.25 mm, 0.2 µm film thickness) fusedsilica column was used with oven temperature programming: 60 °C (5 min), and with the injector temperature at 210 °C. Quantitative data were obtained by flame ionization detection and electronic integration without using FID response factors. The experiments were not replicated. The compounds were identified by co-injection (GC) with authentic samples, and by computerized matching of the acquired mass spectra with library spectra (MS).

4.6. Selection and Preparation of Experimental Animals

In this study, healthy young male and female Wistar albino rats were used. The recruitment of these animals was driven by the fact that rats have recently emerged as the model organism of choice for the study of human disease, owing to their low cost, ease of handling, and physiological and genomic similarities (149).Wistar albino rats, 8 to 10 weeks of age, obtained from the Ethiopian Public Health Institute (EPHI) breeding unit, were used. The female rats were nulliparous and non-pregnant. Same-sex rats were acclimatized in a standard cage with five animals per group (n=5) and held under standard conditions (at a temperature of 20 ° C (± 2 °C), with a normal 12-hour light/12-hour dark cycle). All the experiments were conducted following the internationally accepted laboratory animal use and care guidelines (149). In addition, the institutional review board (IRB) of the College of Health Sciences, Addis Ababa University approved the protocol. Animals were acclimatized for one week before the commencement of the study and were provided with water and food pellets *ad libitum* before and until the end of the experimental period (149).

After adaptation, the animals used for developmental toxicity studies were mated overnight by placing a male Wistar albino rat in a cage containing one nulliparous female rat. The male rat was introduced to the cage at approximately 17:00 hours. After overnight mating, female rats were inspected for the presence of copulatory plugs the following morning, and vaginal smears were taken for microscopic determination of the presence of sperm. The presence of spermatozoa in the vaginal examination was considered as a day-0 of gestation(149).

4.6.1. Grouping and Dosing of Animals

In the acute toxicity study; rats were randomly divided into seven groups (n=5). The control group received distilled water with 2% of tween 80, whereas the experimental groups received a vehicle dissolved single dose of 300, 600, 900, 1200, 1500, and 2 000 mg/kg of theessential oil, respectively.

In the sub-acute toxicity study; rats were randomly divided into four groups (n=10). The control group received distilled water with 2% of tween 80, whereas the experimental groups received a vehicle (distill water with 2% of tween 80) dissolved doses of 65 mg/kg, 130 mg/kg, and 260 mg/kg of essential oil orally for 28 days, respectively.The doses specified were based on the acute toxicity report LD_{50} value of 1284.2 mg/kg.

In the essential oil of *T.schimperi*developmental toxicity study,pregnant Wistar albino rats were randomly divided into five groups, each comprising 10 animals. Two control groups were a pair-fed control group (Group I) and *ad libitum* control group (Group II). The pair-fed control group received the vehicle and the same amount of food and water as the experimental groups while the *ad libitum* control group was untouched and fed *ad libitum*. The experimental groups (Groups III-V) were given 65mg/kg, 130mg/kg, and 260 mg/kg of vehicle dissolvedessential oil of *T. schimperi*. The extract was weighed and mixed with a vehicle (distilled water and 2% tween 80) and continuously vortexed with a vortex shaker. The final volume was 2ml/100g with the vehicle and the oral gavage was used for oral administration(149).

In the aqueous crude extract of *T.schimperi*developmental toxicity study;The pregnant albino Wistar rats were randomly divided into 5 groups, each with 10 pregnant rats, and the developmental toxicity study was carried out. A pair-fed (Group I) and *ad libitum*(Group II) control groups were included in the study. The pair-fed control group received the vehicle and the same amount of food and water as the experimental groups while the *ad libitum* control group was untouched and fed *ad libitum*. The experimental groups (Groups III-V) were received 500 mg/kg, 1000 mg/kg, and 2000 mg/kg of *T. schimperi* aqueous crude extract. These various doses of the extract were selectedbased on the findings of the acute toxicity study(58). The

extract was weighed and mixed with a distilled waterand continuously vortexed with a vortex shaker. The final volume was 1ml/100g with the vehicle and the oral gavage was used for oral administration(149).

4.7. Acute Toxicity of Essential oil of *T.schimperi*

Acute toxicity evaluation was performed in compliance with OECD 425 research guidelines(150). Healthy female Wistar rats were fasted overnight but allowed access to water *ad libitum* and divided into seven groups (n=5) randomly. The vehicle (distilled water with 2% of tween 80) was provided to the first group (control group). The other six classes were treated orally with single doses of vehicle dissolved *T. schimperi* essential oil at 300, 600, 900, 1200, 1,500, and 2,000 mg/kg, respectively. Doses were selected after performing pilot studies. All the treatments were provided by force-feeding. Animals were examined for symptoms of toxicity, body weight, as well as mortality for 14 days. During the first 3 hrs after essential oil administration, toxicity signs and symptoms were observed in individual cages and then evaluated regularly throughout the study(150). The LD_{50} value was measured according to the rats'mortality observed within 14 days. On Day 15, all surviving animals were sacrificed, internal organs were excised, and organ weights were measured.

4.8. Sub-acute Toxicity Essential oil of *T. schimperi*

A subacute toxicity study was carried out in compliance with the recommendations of OECD 425 research guidelines(150). The animals were randomly divided into four groups of 10 rats per group, each group containing five male and five female rats. The oil was administered by gavage orally in doses of 65 mg/kg, 130 mg/kg, and 260 mg/kg for 28 consecutive days, whereas only the vehicle was given to the rats in the control group. The doses specified were based on the acute toxicity report LD_{50} value of 1284.2 mg/kg. Signs of toxicity and mortality were monitored regularly, with changes in body weight and weekly measurements of food and water intake. At the end of the study, animals have fasted overnight, euthanized by intraperitoneal injection of pentobarbital (150 mg/kg of body weight), and blood samples collected by cardiac puncture. Heparinized blood samples were used for the determination of hematological parameters. Non-heparinized tubes were used to analyze blood chemistry while blood glucose was determined

using fluoride tubes. The liver and the kidneys were taken and immediately weighed after dissection.

4.8.1. Hematological and Biochemical Analyses

Ethylenediaminetetraacetic acid (EDTA) was used as aprocessorof blood samples in test tubes. Hematological parameterswere determined on a hematology analyzer (Sysmex XT-1800i, SYSMEX CORPORATION, Japan). White blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration(MCHC), and platelet count (PLC) were evaluated. For biochemical analysis, blood samples were allowed to stand for 3 hrs in plain test tubes for full clotting and centrifuged for 15 min at 5000 rpm using a benchtop centrifuge (Humax-k, Human-GmbH, Germany). The plasma was drained and transferred to other clean vials and the serum was kept at -20 °C until clinical biochemistry measurements were done. The concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, albumin, and creatinine were automatically determined using Cobas Integra-400 plus Analyzer (Roche Diagnostics, Japan).

4.8.2. Organs Weight Measurements and Tissue samples

After assessing body weight, all experimental animals were sacrificed on day 29 and the target organs were taken. The organs were then kept for a few minutes in 1% normal saline to clean any extraneous tissues and weighed with precision balance. The tissue samples taken from the liver and the kidneys were placed in a test tube with 10% formalin buffered for 24 hrs and rinsed overnight under tap water. The fixed tissues were then dehydrated and washed with ethanol and xylene, respectively. In addition, it was infiltrated with molten paraffin wax and embedded in paraffin blocks. The blocks were sectioned at a thickness of 5-6 µm using Leica rotary microtome (Leica RM 2125 RT, China, checked in Germany). Ribbons of the tissue sections were gently collected using forceps and placed on the surface of a water bath at 30-40 °C before they were placed over the tissue. The slides were then mounted in slide racks and placed overnight in an oven at a temperature of 20-40 ° C to make it easy for the specimens to be fixed

on the glass slides. The thin sections then underwent different stages of xylene and alcohol treatment and were stained with hematoxylin and eosin (151).

4.8.3. Light Microscopy and Photomicrography

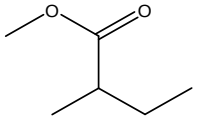
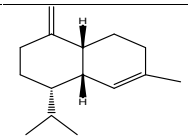
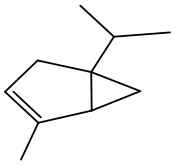
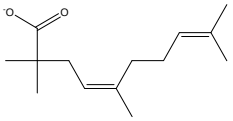
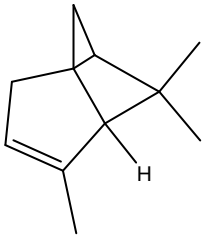
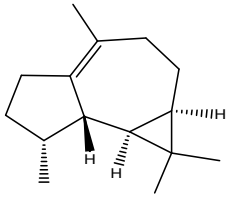
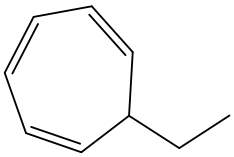
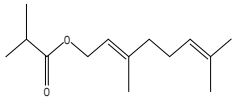
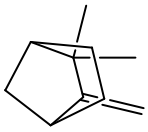
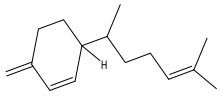
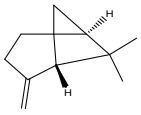
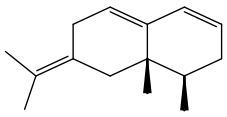
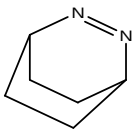
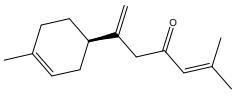
Stained tissue portions of the liver and the kidney were carefully examined in a binocular compound light microscope (Olympus CX41, Japan). Sections of tissue from the treated groups were examined for any signs of histopathological changes. Photomicrographs of selected slides from both the treated and the control group were taken using an automated digital photo camera (Evos XI, China), under a magnification of x40 and x20, respectively.

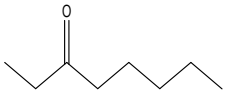
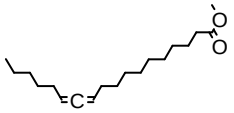
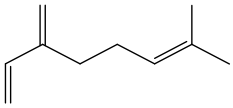
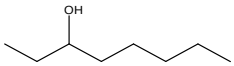
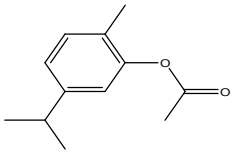
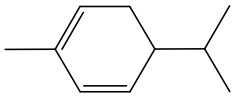
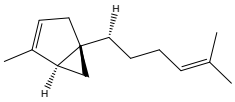
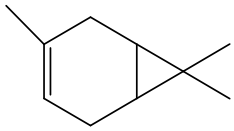
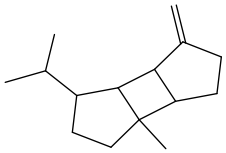
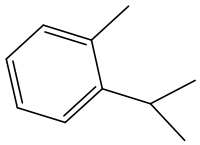
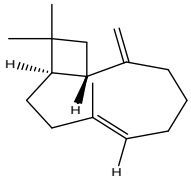
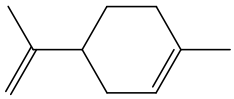
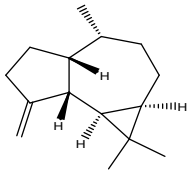
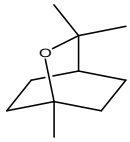
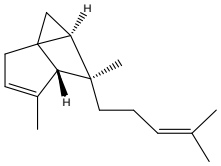
4.8.4. In-silico Toxicity Prediction

Compounds present in the essential oil of *T. schimperi* have been identified by GC-MS. The PubChem CID number was obtained from PubChem (152). Chemdraw (8.0) (42) was used to build two-dimensional structures (**Table 1**). The Swiss ADME web tool was used to convert the two-dimensional structures into a simplified molecular-input line input system (SMILES) that can be analyzed by servers for toxicity prediction (43)(**Table 2).**

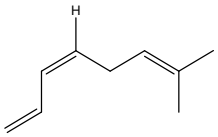
Toxicity profiles: hERG potassium channel inhibition (cardiotoxicity), H-HT (Human Hepatotoxicity), and AMES (Ames Mutagenicity) distribution were predicted using the ADMET (Absorption, Distribution, Metabolism, Execration, and Toxicity) server (44). Drug-induced liver injury (DILI), mitochondrial membrane potential (MMP) toxicity, and cytotoxicity parameters were predicted via vNN server (45). Similarly, the organ toxicity (Hepatotoxicity), toxicological endpoints (Carcinogenicity, Immunotoxicity, and Mutagenicity), and acute toxicity (LD50 (mg/Kg)) of compounds were also evaluated using the ProTox II web server(http://tox.charite.de/protox_II) (46, 47).

Table 1: The compounds of essential oil of *T.schimperi*chemical structures, drawn by ChemDraw.

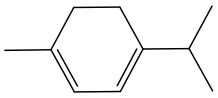
Compounds	**Chemical structure**	**Compounds**	**Chemical structure**
Butanoic acid, 2-methyl-, methyl ester		γ-Amorphene	
α- Thujene		Nerylisobutanoate	
α- Pinene		Viridiflorene	
1,3,5-Cycloheptatriene, 7-ethyl-		Geranylisobutanoate	
Camphene		β-Sesquiphellandrene	
β-Pinene		β-Vetivenene	
2,3-Diazabicyclo [2.2.2] oct-2-ene		β-Atlantol	

3-Octanone		Methyl 11,12-octadecadienoate	
Myrcene		Tetracosane	
3-Octanol		Carvacrol acetate	
Compounds	Chemical structure	Compounds	Chemical structure
Phellandrene<alpha->		Sesquithujene<7-epi->	
Carene<delta-3->		β-Bourbonene	
Cymene<ortho->		Caryophyllene(*E*-)	
D-Limonene		β-Gurjunene	
Cineole<1, 8->		*trans*-. alpha. –Bergamotene	

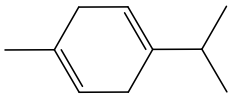
β-Z-Ocimene



α-Terpinenes



γ-Terpinene



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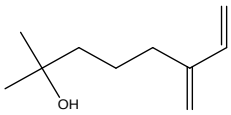
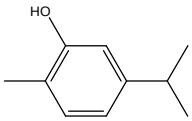
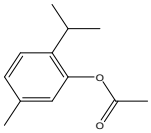


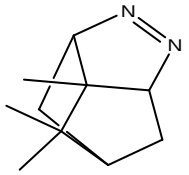
Carvacrol



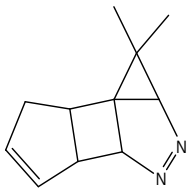
Thymolacetate



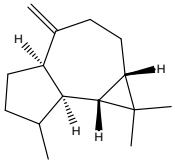
3,5-Methanocyclopentapyrazole, 3,3a,4,5,6,6a-hexahydro-3a,4,4-trimethyl-



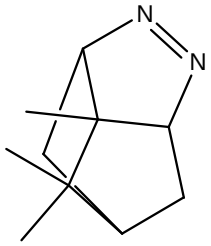
1,4-Methano-1H-cyclopenta[d]pyridazine, 4,4a,5,7a-tetrahydro-8,8-dimethyl-, (1. alpha.,4. alpha.,4a. alpha.,7a. alpha.)-



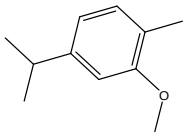
Aromadendrene<allo->



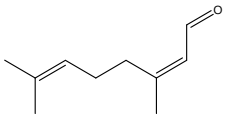
3,5-Methanocyclopentapyrazole, 3,3a,4,5,6,6a-hexahydro-3a,4,4-trimethyl-



Carvacrol, methyl ether

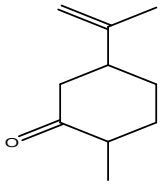


Neral

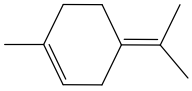


Dodecane

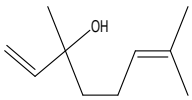
Dihydrocarvone<trans->



Terpinolene



Linalool



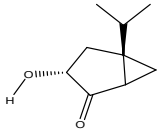
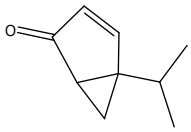
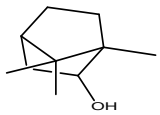
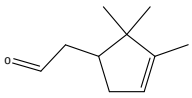
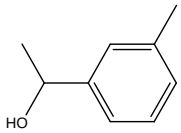
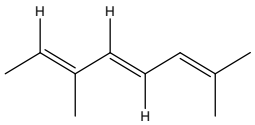
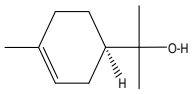
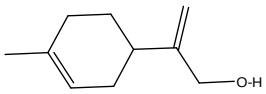
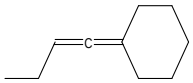
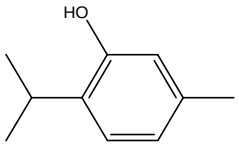
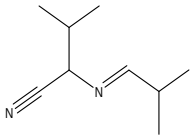
Sabinol<trans-> (trans for OH vs. IPP)		Sabina ketone<dehydro->	
Isoborneol		α-1- Campholena	
Methyl m-tolylcarbinol		Ocimene<allo->	
Terpineol<alpha->		Limonen-10-ol	
Cyclohexane, 1-butenylidene-		Thymol	
2-Isobutylideneamino-3-methylbutyronitrile			

Table 2: The compounds of essential oil of Thymus schimperi simplified molecular-input line input system (SMILES), converted by Swiss ADME web tool.

Compounds	**Simplified molecular-input line input system (SMILES)**
Butanoic acid, 2-methyl-, methyl ester	CCC(C)C(=O)OC
α-Thujene	CC(C)C12CC1C(C)=CC2
α-Pinene	[H]C12C(C)=CCC11CC1C2(C)C
1,3,5-Cycloheptatriene, 7-ethyl-	CCC1C=CC=CC=C1
Camphene	CC1(C)C2CCC(C2)C1=C
β-Pinene	[H][C]12CC11CCC(=C)[C]1([H])C2(C)C
2,3-Diazabicyclo[2.2.2] oct-2-ene	C1CC2CCC1N=N2

3-OCTANONE	CCCCCC(=O)CC
Myrcene	CC(C)=CCCC(=C)C=C
α-Phellandrene	CC(C)C1CC=C(C)C=C1
γ -3- Carene	CC1=CCC2C(C1)C2(C)C
Cymene<ortho->	CC(C)C1=CC=CC=C1C
D-Limonene	CC(=C)C1CCC(C)=CC1
Cineole<1, 8->	CC12CCC(CC1)C(C)(C)O2
B-Ocimene<(Z)	[H]\C(CC=C(C)C)=C\C=C
α-Terpinene	CC(C)C1=CC=C(C)CC1
Terpinene<gamma->	CC(C)C1=CCC(C)=CC1
Dihydromyrcenol	CC(C)(O)CCCC(=C)C=C
Terpinolene	CC(C)=C1CCC(C)=CC1
Linalool	CC(C)=CCCC(C)(O)C=C
Sabina ketone<dehydro->	CC(C)C12CC1C(=O)C=C2
α-Campholenal	[H]C(=O)C[CH]1CC=C(C)C1(C)C
Ocimene<allo->	[H]\C(C=C(C)C)=C(\[H])/C(/C)=C(\[H])C
Sabinol<trans-> (trans for OH vs. IPP)	[H]O[CH]1C[C]2(CC2C1=O)C(C)C
Isoborneol	CC1(C)C2CCC1(C)C(O)C2
Methyl m-tolylcarbinol	CC(O)C1=CC=CC(C)=C1
α-Terpineol	[H][C]1(CCC(C)=CC1)C(C)(C)O
Cyclohexane, 1-butenylidene-	CCC=C=C1CCCCC1
2-Isobutylideneamino-3-methylbutyronitrile	CC(C)\C=N\C(C#N)C(C)C
Dodecane	CCCCCCCCCCCC
Trans -Dihydrocarvone	C[CH]1CC[CH](CC1=O)C(C)=C
3,5-Methanocyclopentapyrazole, 3,3a,4,5,6,6a-hexahydro-3a,4,4-trimethyl-	CC1(C)C2CC3N=NC(C2)C13C
1,4-Methano-1H-cyclopenta[d]pyridazine, 4,4a,5,7a-tetrahydro-8,8-dimethyl-, (1. alpha.,4. alpha.,4a. alpha.,7a. alpha.)-	CC1(C)C2N=NC3C4C=CCC4C123
Carvacrol, methyl ether	COC1=C(C)C=CC(=C1)C(C)C

´Neral	CC(C)=CCC\C(C)=C/C=O
Limonen-10-ol	CC1=CCC(CC1)C(=C)CO
Thymol	CC(C)C1=C(O)C=C(C)C=C1
Carvacrol	CC(C)C1=CC(O)=C(C)C=C1
Thymolacetate	CC(C)C1=C(OC(C)=O)C=C(C)C=C1
Carvacrol acetate	CC(C)C1=CC(OC(C)=O)=C(C)C=C1
Sesquithujene<7-epi->	[H]C(CCC=C(C)C)[C]12C[C]1([H])C(C)=CC2
β-Bourbonene	CC(C)C1CCC2(C)C3CCC(=C)C3C12
Caryophyllene(E-)	[H]\C1=C(C)/CC[C]2([H])[C]([H])(CC2(C)C)C(=C)CCC1
β-Gurjunene	[H][C]12CC[CH](C)[C]3([H])CCC(=C)[C]3([H])[C]1([H])C2(C)C
Trans-. Alpha. –Bergamotene	[H][C]12CC11CC=C(C)[C]1([H])[C]2(C)CCC=C(C)C
Aromadendrene<allo->	[H][C]12CCC(=C)[C]3([H])CCC(C)[C]3([H])[C]1([H])C2(C)C
3,5-Methanocyclopentapyrazole, 3,3a,4,5,6,6a-hexahydro-3a,4,4-trimethyl-	CC1(C)C2CC3N=NC(C2)C13C
Germacrene D	CC(C)[CH]1CC[CH](C)C\C=C/[CH](C)CC1
Amorphene<gamma->	[H][C]12CCC(C)=C[C]1([H])[CH](CCC2=C)C(C)C
Nerylisobutanoate	CC(C)=CCC\C(C)=C/CC(C)(C)C(O)=O
Viridiflorene	[H][C]12CCC(C)=C3CC[CH](C)[C]3([H])[C]1([H])C2(C)C
Geranylisobutanoate	CC(C)=CCC\C(C)=C\CC(C)(C)C(O)=O
β-Sesquiphellandrene	[H]C1(CCC(=C)C=C1)C(C)CCC=C(C)C
β-Vetivenene	C[CH]1CC=CC2=CCC(C[C]12C)=C(C)C
β-Atlantol	CC(C)=CC(=O)CC(=C)[CH]1CCC(C)=CC1
Tetracosane	CCCCCCCCCCCCCCCCCCCCCCCC

4.9. Developmental Toxicity of the Aqueouscrude and Essential oil extract of *T. schimperi*

4.9.1. Day-12 Crude and Essential oil Extracts of *T. schimperi* Experiments

These experimentswere developed to assess the potential developmental toxicityof the aqueous crude and essential oil extractsof *T.schimperi* on pregnant animals in 12 days old whole rat embryos. The experimentswere intended to expose any growth and developmental abnormalities that may not have been noticeable in the near-term fetuses due to potential compensatory growth and development.

Theaqueous crude extract of *T.schimperi* experiment;after pregnancy was confirmed, pregnant rats were randomly assigned to control groups; Group I (*Pair-fed* control), Group II (*ad libitum* control), and treatment groups; Group III (500 mg/kg), Group IV (1000 mg/kg), and Group V (2000 mg/kg), respectively.

Each group consists of ten pregnant rats. Group I (*pair-fed* control) received the distilled water, Group II was untouched/unrestricted-fed (*ad libitum* control).However, Group III, IV, and V were received 500mg/kg /day, 1000mg/kg /day, and 2000 mg/kg/day of aqueous crude extract of *T.schimperi*, respectively **(Table 3).**

Table 3: Treatment schedule of Day-12 crude extract of T.schimperi experiment.

Treatment group	Number of animal	Treatment
Group I (Pair-fed control)	10	DW (1 ml/100g body weight) + diet
Group II (*Ad libitum*)	10	Diet(food and water)
Group III (CT500 mg/kg)	10	*Crude extract of Tschimperi*500mg/kg/day + diet
Group IV(CT1000 mg/kg)	10	Crude extract of *T.schimperi*1000mg/kg/day + diet
Group V(CT2000 mg/kg)	10	Crude extract of *T.schimperi* 2000mg/kg/day + diet

CT(crude extract of *Thymus schimperi*), Dw (distilled water)

The essential oil extract of *T.schimperi Experiment;* After pregnancy was verified, pregnant rats were randomly assigned to control groups, Group I (*Pair-fed* control), Group II (*ad libitum* control), and treatment groups; Group III (EOT 65 mg/kg), Group IV (EOT 130 mg/kg), and Group V (EOT 260 mg/kg), respectively.

Each group consists of ten pregnant rats. Group I (*pair-fed* control) received the vehicle (distilled water with 2% of tween 80), and Group II was untouched/unrestricted-fed (*ad libitum* control), while Group III, Group IV, and Group V were received the vehicle dissolvedessential oil65 mg/kg/day,130 mg/kg/day, and 260 mg/kg/day, respectively.

For both the aqueous crude and essential oil extracts of *T.schimperi*day 12 experiments, the treatment period was from day 6-12 of gestation. The reason for treatment from 6 - 12^{th} days of gestation is due to, this period represents an active period of embryogenesis and organogenesis. The critical developmental period in the rat is embryonic days 1 to 12(149)**(Table 4)**.

Table 4: Treatment schedule of Day-12 essential oil of T.schimperi experiment.

Treatment group	Number of animal	Treatment
Group I (Pair-fed control)	10	DW with 2% of tween 80 (2 ml/100g body weight) + diet
Group II (*Ad libitum*)	10	Diet (food and water)
Group III (EOT65 mg/kg)	10	Essential oil of *T.schimperi*65mg/kg/day + diet
Group IV(EOT130 mg/kg)	10	Essential oil of T.schimperi 130mg/kg/day + diet
Group V(EOT260 mg/kg)	10	Essential oil of *T.schimperi* 260mg/kg/day + diet

EOT; Essential oil of *Thymus schimperi*, Dw (distilled water)

At the end of the treatment period (day 12 of gestation), the gravid rats were euthanized by intraperitoneal injection of pentobarbital (150 mg/kg of body weight)(153). The uterine horns were removed and placed in Hank's balanced salt solution. They were then incised along the border of the antimesometrium to expose the embryos. To expose the underlying visceral yolk sac, the membranes covering the embryo were separated with fine forceps and a dissecting microscope.The yolk sacs circulation and growth were evaluated. The embryos were then explanted, and the development of the circulatory, nervous, visual, auditory, olfactory, and skeletal systems, as well as craniofacial development, were quantified using 17 recognizable developmental endpoints (morphological scores) based on Brown and Fabro's criteria (154). Somite numbers have also been counted.

4.9.2. Day-20 Aqueous Crude and Essential oil Extracts of *T. schimperi* Experiments

These experiments were also intended to determine the potential developmentaltoxicity of the *T. schimperi* of both aqueous crude and essential oil extracts inWistar albino rats.

The aqueous crude extract of *T.schimperi* experiment;Once pregnancy was confirmed, the animals were randomly assigned to control groups; group I (pair-fed control), group II (*ad libitum*), and treatment groups; group III (500 mg/kg), group IV (1000 mg/kg) and Group V (2000 mg / Kg). Each group consisted of ten pregnant rats. Group I (*pair-fed* control) received distilled water, and group II was untreated *ad libitum* group, whereas groups III, IV, and V were received 500 mg/kg, 1000 mg /kg, and 2000 mg/kg/day of crude aqueous extract of *T. schimperi*, respectively**(Table 5).**

Table 5: Treatment schedule of Day-20 crude extract of T.schimperi experiment.

Treatment group	**Number of animal**	**Treatment**
Group I (*Pair-fed* control)	10	DW (1 ml/100g body weight) + diet
Group II (*Ad libitum*)	10	Diet(food +water)
Group III (CT500)	10	Crude extract of *T.schimperi*500mg/kg/day + diet
Group IV(CT1000)	10	Crude extract of *T.schimperi*1000 mg/kg/day + diet
Group V(CT2000)	10	Crude extract of *T.schimperi*

	2000mg/kg/day + diet

CT (Crude extract of Thymus schimperi), Dw (distilled water)

The essential oil extract of *T.schimperi* experiment;once pregnancy was confirmed, animals were also randomly allocated to control groups; Group I (CON),Group II(*ad libitum control* group), and treatment groups; Group III (EOT 65 mg/kg), Group IV (EOT mg/kg130), and Group V (EOT 260 mg/kg). Each group consists of ten pregnant rats. The Vehicle was provided to the control Group I (CON). Group II was unregulated *ad libitum.* Thetreatment groups;Group III, IV, and V were received 65mg/kg/, 130mg/kg, and 260mg/kg/day vehicle dissolved essential oil extract of *T.schimperi*, respectively**(Table 6)**.

Table 6: Treatment schedule of Day-20 essential oil of T.schimperi experiment.

Treatment group	**Number of animal**	**Treatment**
Group I (Pair-fed control)	10	DW (2 ml/100g body weight) + diet
Group II (*Ad libitum*)	10	Diet(food + water)
Group III (EOT65 mg/kg)	10	Essential oil of *T.schimperi*65mg/kg/day + diet
Group IV(EOT130 mg/kg)	10	Essential oil of *T.schimperi* 130mg/kg/day + diet
Group V(EOT260 mg/kg)	10	Essential oil of *T.schimperi* 260 mg/kg/day + diet

EOT (Essential oil of Thymus schimperi), Dw (distilled water)

In both of the aqueous crude and essential oil extracts day- 20 experiments, the control diet of equal amounts was provided for each animal in the experimental group (Group III, IV, and V) and the control groups(Group I and II) was given the same diet and kept in the same setting

except forAqueous crude and essential oil extracts given only for the experimental groups. Every morning, every animal's daily food intake was recorded, animals were weighed and weight gain was recorded on days 1, 6, 12, and 20 of gestation(149).

Gravid females were anesthetized by intraperitoneal injection of pentobarbital (150 mg/kg of body weight) on gestational day 20; the uterine horns were exposed and examined intact. The number of implantation sites was determined by counting the metrial glands situated along the mesometrial margin of the uterine horns, which are yellowish nodules. The number of prior resorptions was measured by mitral nodules, which were not occupied by living or recently dead fetuses. Gentle pressure on them was exerted to assess the amount of live or dead fetuses. To expose the fetuses, fetal membranes, and the placenta, the uterine horns were incised along the antimesometrial border. The fetuses were then retrieved and placenta-free dissected. Following these measurements, the length of the crown-rump (CRL) and the placental weight were recorded. Fetuses were fixed for gross external inspection in Bouin's solution (aqueous saturated solution of picric acid 75 %t, formalin 25 %, and glacial acetic acid 5%)(149).

4.9.3. External Evaluation

Fetuses from both aqueous crude and essential oil experiments fixed in Bouin's solution were checked head to tail for gross external malformations under a dissecting microscope (155). The criteria to be evaluated were:

A. Craniofacial development (exencephaly, anencephaly, microphthalmia, and anophthalmia)
B. Development of the limbs (syndactyly, adactyly, polydactyly)
C. The vertebral column (neural tube defect, kyphosis, scoliosis)
D. Tail development (missing tail); and
E. External genitalia.

4.9.4. Visceral Examination

Following an external examination of the fetuses, soft tissue evaluation was done by serial sectioning. Serial sectioning was performed on the fetuses fixed in Bouin’s solution for two

weeks. The sectioning procedure was done by a surgical blade, based on the Modified Wilson technique(156). Craniocaudally, sections were done at 1-2 mm intervals under a dissecting microscope (XTL3101, 6x magnification). The first section was made through the jaw and pass posteriorly above the ear. After removing the tongue, the palate was examined for the presence of any cleft. A coronal section on the head and a transverse section on the neck and parts below were done. The following organs were assessed for any visible anomalies: brain (hydrocephalus, dilation of ventricles, microphthalmia/anophthalmia), craniofacial region (nasal septum defect, cleft palate), thoracic region (lungs: lobar defect, heart: septal defect, retroesophageal aortic arch), abdominal region (liver, stomach, and gut anomalies), and pelvic region (kidneys: agenesis, ectopic, and hydronephrosis, gonads: testes, ovarian anomalies).

4.9.5. Skeletal Staining

In both crude and essential oil experiments, skeletal staining was performed using the method Dawson(157). Depending on the size of the litter, 2 or 3 fetuses per litter have been completely eviscerated by a small midline incision in the anterior abdominal wall. Eviscerated fetuses (2 or 3) were then put in a small bottle of 95% alcohol and dehydrated for a minimum of one week. After dehydration, the specimens were cleared with 1% potassium hydroxide in a solution until the bone was cleared, normally for two days. The specimens were then moved to the new, 1% potassium hydroxide (KOH) solution and stained with a few drops of alizarin red (0.4 ml). The staining continued overnight and over-straining was fixed by storing the samples in the solution of the Mall (79 % distilled water, 20 % glycerine, and 1% KOH). Increasing glycerin concentrations (20 %, 40 %, 60 %, and 80 %) were then passed through the specimens for about one week in each concentration and finally stored for evaluation in l00% glycerine. To avoid fungal growth and contamination when stored in pure glycerin, a small thymol crystal was added.

4.9.6. Skeletal Evaluation

Using a skeletal scoring chart, developed by Nash and Persaud(158), which is a modification of the scoring system stated by Aliverti(159), the skeletal assessment was performed. Under the

microscope, ossification of the hyoid, sternebrae, metacarpal, metatarsal, and thoracic bones were studied and the number of ossified centers was counted. The primary indices of skeletal development in the rat were stated as the degree of ossification of the sternebrae, metacarpal, metatarsal and sacro-coccygeal bones (**Figure 8**).

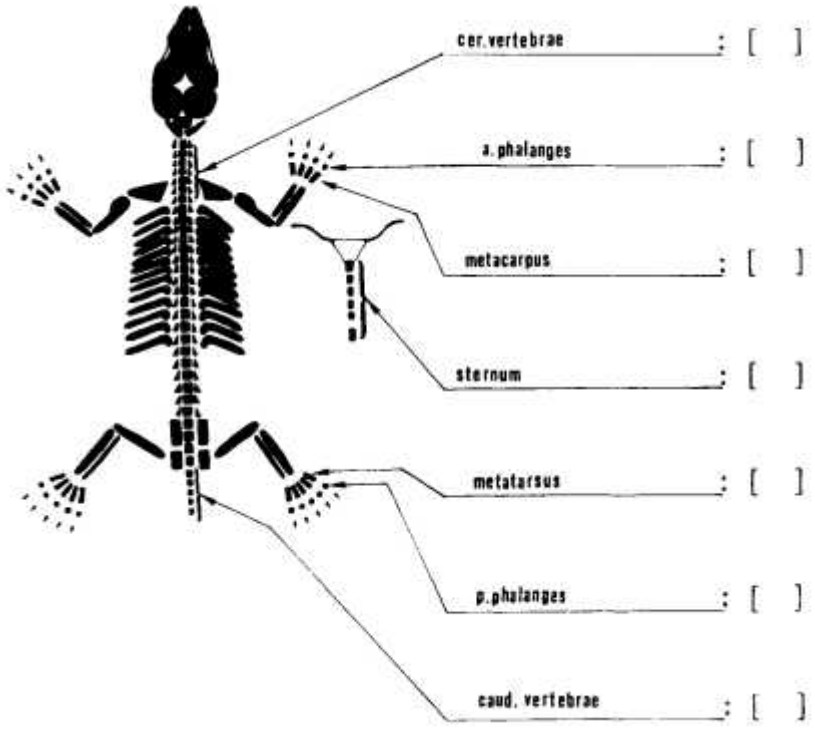


Figure 8: Skeletal Scoring chart developed by (Aliverti et al., 1979)

4.9.7. Histopathological Studies of the Placenta

In both crude and essential oil experiments, two placentas from each group were randomly selected and put into Bouin's solution. Bouin's fixed placentae have been transferred overnight to 70% ethyl alcohol. After routine treatment for light microscopy, the placenta was blocked in paraffin and cut into 4µm thick sections using a microtome. The sections were placed for microscopic inspection on glass slides, hematoxylin and eosin (H&E) stains, and coverslips. The sections were analyzed using a binocular light microscope for proof of structural and vascular changes(160).

The following structures have been investigated and used as measures of functional and structural changes in the placenta.

A. Basal zone of the placenta
B. Labyrinthine zone
C. Inter-villous spaces
D. Giant cells and trophoblasts

4.9.8. Light Microscopy and Photomicrography

A pathologist under a compound light microscope has carefully studied stained tissue sections of the placentain both crude and essential oil experiments. Tissue sections of the experimental groups were examined for any evidence of histopathological changes with the controls. Using the automated built-in digital photo camera photomicrographs of selected samples of placenta parts from both the experimental and control rats were taken under x40 objective magnification after inspection(160).

4.10. Data Processing and Analysis

Data were entered using EPI-data Version 3.02 and was exported to a statistical package for social science (SPSS) version 24for analysis. The data regarding hematological profiles and blood chemistry, maternal food intake and weight gain, pregnancy outcomes, embryonic development, fetal growth, and relative organ weight were analyzed using one-way analysis of variance (ANOVA). One-way ANOVA was used to determine whether the means of treatment and control groups were statistically different or not. Post Hoc tests (Turkey and Games-Howell) testwere applied to figure out exactly in which treatment group the difference lied. Games-Howell test was used if the assumption of homogeneity was violated, then, Turkey tests werechosen. Furthermore, the difference between the control group and each treatment group was evaluated by Dunnett's test. Dunnett's test was used to compare each treatment group with a single control group and found a statistical difference at least between the control and each treatment group. To check whether the data scores were normally distributed or not Shapiro-Wilk test of normality was applied. To meet the test of homogeneity of variance before conducting ANOVA data were exposed to Levene's test. The Chi-square test was applied to analyzethe data related to embryonic/fetal development abnormalities and placental abnormalities. The Chi-square test was used to compare the association between the frequency of

anomalies and treatment with the test substance. The experimental unit of analysis was different based on the type of experiment. Where suitable, it could be an embryo, fetus, litter, or group. The data were expressed as mean ± standard deviation of the mean (SDM) and percentages. P-value < 0.05 was considered statistically significant. The results were presented using tables and figures.

4.11. Ethical Consideration

Aletter of ethical approval was obtained before the experiment from the Department of Anatomy graduate committee and institutional review board (IRB) of College of Health Sciences, Addis Ababa University with protocol number ANAT 107/210 and IRB form AAUMF03-008 in compliance with the Organization for Economic Co-operation and Development (OECD) guidelines(149, 161) for the care and use of experimental animals. Rats used in this study were saved in the maximum standards for the humane use of animals in the biomedical research laboratory of EPHI. They were not exposed to any needless painful and terrifying conditions. Administration of the test substance was carried out by experts and maximum effort was applied to prevent them from a pathogen. Before rats were sacrificed, to avoid pain and suffering, they were anesthetized with pentobarbital. Finally, unused pups and sacrificed parental rats were disposed of humanely by the laboratory standards of EPHI.

4.12. Operational Definitions

- **Acute toxicity: -** This refers to the adverse effects that occur after oral administration of a single dose of the substance for up to 14 days.
- **Sub-acute toxicity**: - Toxicity (repeat dose toxicity) focuses on the adverse effects that occur after a single dose of a test sample is administered regularly for 28 days during the experimental period.
- **In silico toxicology: -**One type of toxicity assessment uses computational methods to evaluate, simulate, visualize or predict the toxicity of compounds via servers.
- **Developmental toxicity**: - The incidence of adverse effects of exposure before conception (either parent), during prenatal development.

- **Implantation (nidation): -**Attachment of the blastocyst, including its penetration through the uterine epithelium, to the epithelial lining of the uterus, and its integration into the endometrium.
- **Embryo: -**Any organism's early or developing stage, in particular the development of an egg fertilization product after the long axis appears and before all major structures are present.
- **Fetus: -** The unborn offspring in the post-embryonic period.
- **Resorption: -** A conceptus, which has implanted in the uterus, subsequently died and is being, or has been resorbed: Early resorption: evidence of implantation without recognizable embryo/fetus. Late resorption: dead embryo or fetus with external degenerative changes.
- **Litter: -** severalnewborns to rats at one time.
- **CRL: -** is the measurement from the vertex of the skull to the midpoint between the apices of the buttocks.
- **Apoptosis: -** is a form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area that is marked by the fragmentation of nuclear DNA.
- **Necrosis: -** is a form of cell injury, which results in the premature death of cells in living tissue by autolysis.
- **Cytolysis: -** is a pathological breakdown of a cell due to the bursting of the cell membrane caused by osmosis

CHAPTER FIVE

5. RESULTS

5.1. Chemical Composition Analysis

The percentage yield of the essential oil of the fresh leaves of *T. schimperi* obtained by hydrodistillation was 1.39% (w/w). The oil was dark yellowish with a strong spicy scent. Qualitative and quantitative analyses carried out by GC/MS and GC identified 57 compounds representing 88.75% of the total essential oil. Results of GC/MS analysis are summarized in **Table 7,** while the GC chromatogram of the major components is depicted in **Figure 9**. As shown in **Table 6,** the major constituents of the oil were carvacrol (49.90%), thymol (10.64%), o-cymene (8.54%), α-terpinene (4.5 %), linalool (2.51%), and 3-octanol (2.48%).

Table 7: Composition of the essential oil of the fresh leaves of *Thymus schimperi*.

No.	Compounds	Percent	Ret. time	Ret. index
1	Butanoic acid, 2-methyl-, methyl ester	0.05	3,787	712
	α-Thujene	0.34	7,436	921
	α-Pinene	0.12	7,585	925
	1,3,5-Cycloheptatriene, 7-ethyl-	0.03	7,861	936
	Camphene	0.03	7,939	939
	β-Pinene	0.05	8,640	974

	2,3-Diazabicyclo[2.2.2] oct-2-ene	0.02	8,818	977
2	3-Octanone	1.04	8,889	980
	Myrcene	0.58	9,048	987
3	3-Octanol	2.48	9,213	993
	α-Phellandrene	0.24	9,334	999
	δ-3-Carene	0.80	9,638	1012
4	*o*-Cymene	8.54	9,838	1020
	D-Limonene	0.30	9,935	1024
	1-8-Cineole	0.39	9,973	1026
	β-*Z*-Ocimene	0.39	10,166	1034
5	α-Terpinene	4.53	10,661	1055
	γ-Terpinene	0.28	10,860	1064
	Dihydromyrcenol	0.07	10,972	1069
	Terpinolene	0.20	11,331	1084
6	Linalool	2.51	11,633	1097
	Dehydro sabina ketone	0.03	12,099	1117
	α-Campholenal	0.03	12,132	1119
	Ocimene<allo->	0.04	12,253	1123
	Sabinol<trans-> (trans for OH vs. IPP)	0.11	12,491	1135
	Isoborneol	0.12	13,102	1165
7	Methyl *m*-tolyl carbinol	0.84	13,323	1176
	α-Terpineol	0.22	13,615	1190
	Cyclohexane, 1-butenylidene-	0.35	13,727	1195
	2-Isobutylideneamino-3-methylbutyronitrile	0.09	13,787	1198
	Dodecane	0.10	13,843	1201
	trans Dihydro carvone	0.09	13,945	1206
	3,5-Methanocyclopentapyrazole, 3,3a,4,5,6,6a-hexahydro-3a,4,4-trimethyl-	0.07	14,124	1215
	1,4-Methano-1H-cyclopenta[d]pyridazine, 4,4a,5,7a-tetrahydro-8,8-dimethyl-, (1. alpha.,4. alpha.,4a. alpha.,7a. alpha.)-	0.03	14,350	1226

	Carvacrol, methyl ether	0.15	14,687	1242
	´Neral	0.03	14,759	1248
	Limonen-10-ol	0.04	15,400	1277
8	Thymol	10.64	15,841	1298
	Carvacrol	49.90	16,130	1313
	Thymolacetate	0.08	16,859	1350
9	Carvacrol acetate	0.41	17,205	1369
	7-*epi*-Sesquithujene	0.04	17,384	1379
	β-Bourbonene	0.03	17,552	1387
10	*E*Caryophyllene	0.53	18,201	1422
	β-Gurjunene	0.02	18,358	1435
	trans. α-Bergamotene	0.09	18,454	1443
	Aromadendrene<allo->	0.08	18,560	1452
	3,5-Methanocyclopentapyrazole, 3,3a,4,5,6,6a-hexahydro-3a,4,4-trimethyl-	0.03	18,809	1473
	D-Germacrene	0.03	18,907	1482
	γ-Amorphene	0.05	19,051	1494
	Nerylisobutanoate	0.07	19,118	1500
	Viridiflorene	0.15	19,218	1508
	Geranyl isobutanoate	0.06	19,288	1515
	β-Sesquiphellandrene	0.20	19,389	1523
11	β-Vetivenene	0.49	19,700	1560
	β-Atlantol	0.13	19,951	1614
	Methyl 11,12-octadecadienoate	0.30	20,535	1819
	Tetracosane	0.09	22,097	2402
	Total (identified)	88.75 %		

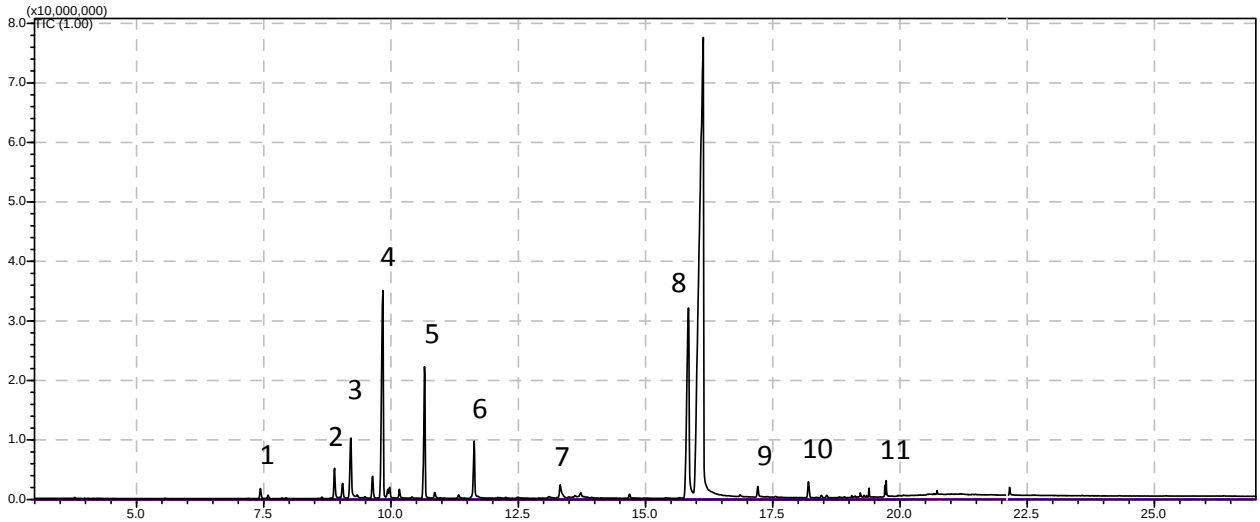


Figure 9: Gas chromatogram of the essential oil of Thymus schimperi (1: α-Thugene. 2: 3-Octanol, 3: 3-Octanone, 4: O-Cymene, 5: α-Terpinene, 6: Linalool, 7: Methyl m-tolyl carbinol, 8: Thymol and carvacrol, 9: Carvacrol acetate, 10: E -Caryophyllene, 11: β-Vetivenene.

5.2. Acute Toxicity Results

The result of acute toxicity of *T. schimperi* essential oil given orally in single doses is shown in **Table 8**and **Figure 8**. The result of acute toxicity of *T. schimperi* essential oil given orally in single doses is shown in Table 7.

Table 8: Effects of different oral single doses Thymus schimperi essential oil in rats for acute toxicity.

Conc. (C) mg/kg	**Log(C)**	**Alive (%)**	**Dead (%)**	**Prop, p**	**Corr, p**	**Logit(p)**	**Probit(p)**	**symptom**
0		100	0	0				None
300	2.5	100	0	0				None
600	2.8	100	0	0				None
900	3.0	80	20	0.2	0.2	-1.4	3.6	Hypoactivity, piloerection, Convulsion
1200	3.1	80	20	0.2	0.2	-1.4	3.6	Hypoactivity, piloerection,

								Convulsion
1500	3.2	20	80	0.8	0.8	1.4	6.4	Hypoactivity, piloerection, Convulsion
2000	3.3	0	100	1				Hypoactivity, piloerection, Convulsion
					slope:	11.9	11.9	
					intercept:	-37.0	-32.0	
					test value:	0.0	5.0	
					Log(C %)	3.1	3.1	
					LD 50	1284.2	1284.2	

After the dose, all rats treated were carefully examined for signs oftoxicity and lethality up to 14 d. Conc. (C): concentration, log (C): logarithm of the concentration, Alive (%): number of live rats in percent, dead: number of dead rats in percent.

Starting at a dose of 900 mg/kg of the essential oil, the rats showed signs of toxicity, such as hypoactivity, piloerection, convulsion including death. Mortality was observed in 900, 1200, 1500 and 2000 mg / kg groups with 20%, 20%, 80% and 100% deaths, respectively (**Table 7 and Figure 10).**The approximate LD_{50} obtained from the acute toxicity study was 1,284.2 mg/kg.

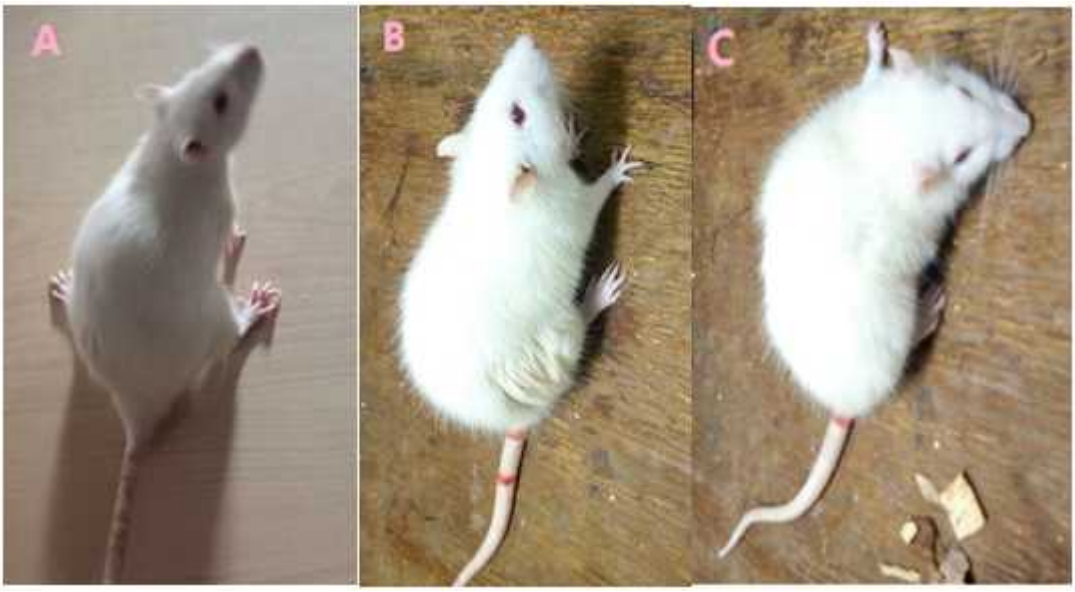


Figure 10:A: Rat within 3 hrs after treatment with 600mg/kg essential oil of *T. schimperi*, **B:** Rat within 3 hrs after treatment with 900 mg/kg essential oil of *T. schimperi* showing hypoactivity, piloerection, and convulsion, **C;** Rat within 3hrs after treatment with a single dose of 1200 mg/kg essential oil of *T. schimperi* showing hypoactivity, piloerection, and convulsion.

There was a significant decrease in body weight in 900 mg/kg and 1200 mg/kg treated groups as compared to the control group at day 7 on the acute toxicity study ($P<0.05$). In addition, on day 14, body weight has significantly decreased in 600 mg/kg, 900 mg/kg, and 1200 mg/kg treated groups ($P<0.05$) as compared to the control group. Similarly, body weight has significantly decreased in the 1200 mg/kg treated group as compared to the 300 mg/kg treated group ($P<0.05$). Nonetheless, treatment groups (1500 mg/kg and 2000 mg/kg) were not included in the analysis as these groups had too few and no living rats, respectively. Furthermore, the weights of the kidney and the liver significantly increased in treatment groups (900 mg/kg and 1200 mg/kg) as compared to the control group **(Table 9)**.

Table 9: Effects of different oral single doses of *T. schimperi* essential oil in rats for acute toxicity

Parameters		***T.Schimperi* Treatment of essential oil**			
	Control	**300 mg/kg**	**600 mg/kg**	**900 mg/kg**	**1200 mg/kg**
Bodyweight gain					
Day 7	5.78±0.07	5.00±0.05	4.71±0.03	3.43±0.03[a]	2.24±0.05[a]
Day 14	10.02±0.11	7.01±0.04	6.30±0.04[a]	5.23±0.04[a]	3.85±0.05[b]
Organs weight (g)					
Liver	6.72±1.01	6.71±1.08	7.45±1.06	7.87±0.08[b]	7.91±0.09[b]
Kidney	1.52±0.04	1.47±0.03	1.58±0.05	1.81±0.06[b]	1.94±0.06[b]
Heart	0.43±0.05	0.42±0.04	0.40±0.03	0.41±0.04	0.41±0.04
Spleen	0.52±0.09	0.55±0.08	0.54±0.07	0.55±0.08	0.62±0.08[a]

Data are expressed as mean± SDM, n=5 for each group; **a**: Significant at P<0.05 compared to the control only; **b**: Significant at P<0.05 compared to the control and 300 mg/kg group; **c**: Significant at P<0.05 compared to the control, 600 and 9 000 mg/kg; **d**: Significant at P<0.05 compared to the 900 and 1200 mg/kg.

5.3. Sub-acute Toxicity Results

In the sub-acute toxicity study, rats were randomly assigned to four groups, each of the groups with 10 rats (5M & 5F). Rats in the control group received distilled water with 2% of tween 80 while the experimental groups received 65 mg/kg, 130 mg/kg, and 260 mg/kg of essential oil orally for 28 days. Neither signs of toxicity nor deaths were observed after *T. schimperi* essential oil administration. *T. schimperi* essential oil did not result in any major changes in the body and organ weights **(Table 10)**.

Table 10: Body and organ weights of rats in the control and T.schimperi essential oil-treated groups in the sub-acute toxicity study.

		Treatment of *T. Schimperi* essential oil		
Parameters	**Control**	**65 mg/kg**	**130 mg/kg**	**260 mg/kg**
Day 0	201.00±2.78	198.00±2.22	199.00±1.98	198.00±2.02
Day 7	207.00±4.23	206.00±2.50	207.00±2.90	202.50±2.42
Day 14	210.50±4.41	210.00±2.61	210.50±3.56	205.50±2.81
Day 21	212.50±4.86	211.00±3.67	212.00±3.62	211.50±4.56
Day 28	215.50±4.89	215.00±3.45	213.00±4.71	213.50±3.65
Organs weight (g)				
Liver	5.58±0.68	6.91±0.20	6.47±0.49	5.48±0.65
Kidney	1.47±0.15	1.20±0.10	1.56±0.06	1.40±0.12
Heart	0.85±0.06	0.68±0.09	0.88±0.02	0.72±0.04
Spleen	0.56±0.08	0.47±0.09	0.46±0.10	0.64±0.07
Pancreas	1.14±0.31	1.00±0.20	1.01±0.15	1.00±0.33

The data is expressed as mean± SDM, n=10 for each group. There was no statistical difference between the control and the *T. Schimperi* essential oil treatment groups (P>0.05)

5.3.1. Hematological and Biochemical Parameters

Hematological evaluation has shown a significant decrement in WBC counts and increment in the MCV in the high dose group (260 mg/kg) as compared to the control group. There was no significant difference in RBC, HB, HCT, MCH, MCHC, and PLT levels between the control group and any of the experimental groups **(Table 11).**

There were no significant differences in liver injury markers (ALT, AST, and ALP) between the control and any of the treatment groups. In addition, there were no significant changes in levels of blood urea and creatinine, which are indicators of kidney injury.Similarly,there was no significant difference in the levels of HDL and LDL between the control and treatment groups. Finally, Electrolytes analysis revealed no significant differences in blood electrolyte levels such as sodium and potassium levels between the control and the treatment groups **(Table 12)**.

Table 11: Hematological values of rats in the control and T.schimperi essential oil-treated groups in the sub-acute toxicity study

		Treatment of *T. Schimperi*essential oil		
Parameter	**Control**	**65mg/kg**	**130mg/kg**	**260mg/kg**
RBC ($\times 10^6/\mu L$)	8.50±0.15	7.96±0.20	8.04±0.19	7.25±1.22
WBC ($\times 10^3/\mu L$)	14.09±1.37	12.94±1.04	11.80±1.50	7.40±1.79[a]
Hb (g/dl)	16.70±0.31	15.82±0.30	16.28±0.39	14.48±2.29
HCT (%)	47.73±0.84	47.98±0.69	48.37±1.36	41.97±7.03
MCV (pg)	56.13±0.49	57.57±1.70	58.93±0.80	60.15±0.39[a]
MCH (pg)	19.63±0.16	19.87±0.17	20.25±0.19	20.80±0.97
MCHC (g/dl)	34.98±0.54	32.97±0.67	33.70±0.33	35.68±1.46
PLT (($\times 10^3/\mu L$)	942.00±120.50	952.00±162.87	830.00±79.07	804.50±156.68

The data are expressed as mean ± SDM, n=10 for each group.[a]Significant difference compared to the control group ($P<0.05$). RBC: red blood cells; WBC: white blood cells; Hb: hemoglobin; HCT:

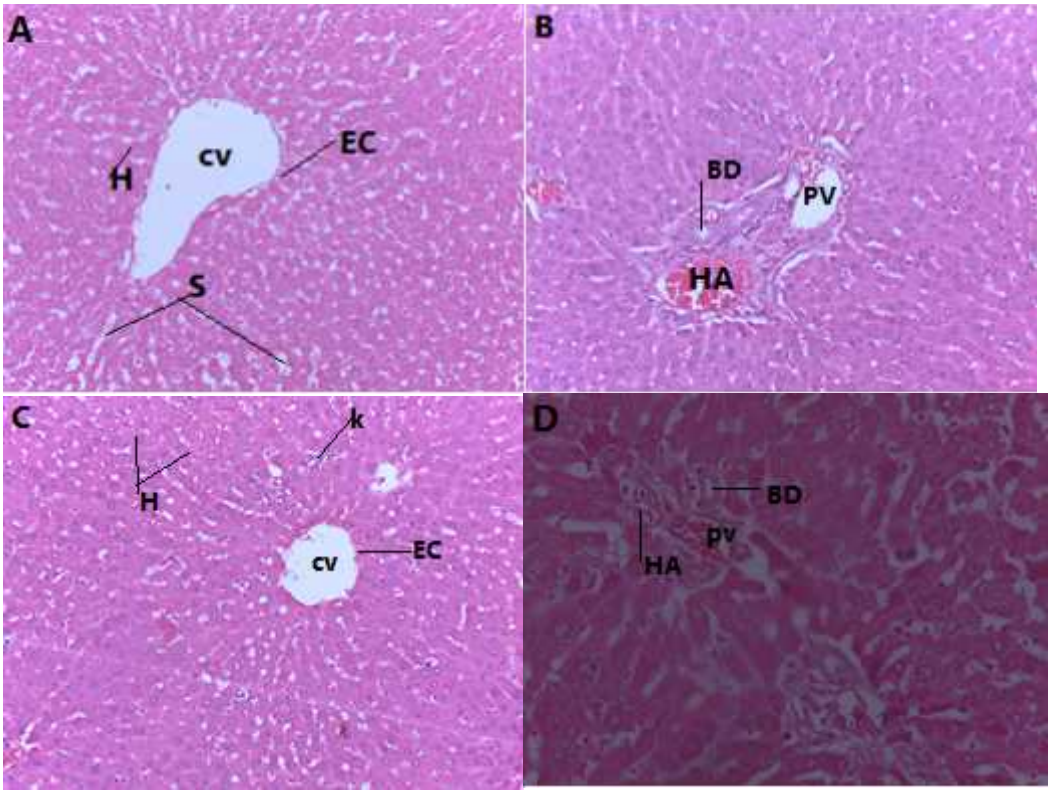
hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin;MCHC: mean corpuscular hemoglobin concentration; PLT: platelets.

Table 12: Blood chemistry values of rats in the control and Thymus schimperi essential oil-treated groups in the sub-acute toxicity study.

		Treatment of *T.Schimperi* essential oil		
Parameter	**Control**	**65mg/kg**	**130mg/kg**	**260mg/kg**
Urea (Mg/dL)	37.10±2.37	39.97±1.37	37.25±0.89	40.13±1.73
Creatinine (Mg/dL)	0.30±0.01	0.32±0.01	0.32±0.01	0.35±0.02
Sodium (mEq/L)	146.17±0.54	147.33±0.56	147.83±1.28	146.17±0.60
Potassium (mEq/LL)	4.43±0.24	3.95±0.20	4.55±0.28	4.43±0.46
Calcium (mEq/L)	2.33±0.15	2.42±0.31	2.41±0.58	2.35±0.30
Chloride (mEq/L)	105.17±0.54	104.17±0.54	105.17±0.98	104.33±0.33
Phosphate (mEq/L)	2.44±0.62	2.26±0.19	2.42±0.13	2.38±0.15
ALT (U/L)	52.38±4.56	54.07±5.78	65.60±3.55	56.67±2.15
AST (IU/L)	216.35±28.53	212.73±16.28	200.13±10.36	183.97±13.40
ALP(U/L)	76.00±10.35	73.33±11.49	91.00±7.23	88.00±7.33
Albumin (g/dL)	4.26±0.09	4.38±0.11	4.44±0.15	4.12±0.17
Total protein (g/dL)	5.76±0.09	5.85±0.12	6.12±0.18	6.01±0.10
Glucose (mEq/L)	134.45±10.68	117.18±23.90	92.82±4.70	105.72±8.23
HDL (mg/dl)	35.56±2.76	41.58±4.97	53.63±5.28	53.52±4.61
LDL (mg/dl)	20.68±3.42	26.99±2.52	19.32±2.23	18.55±1.58

5.3.2. Morphological analysis

In the selected organs, the gross pathological analysis showed no observable irregularities. Furthermore, the histopathological analysis detected any noticeable abnormalities in neither the control nor the treatment groups (**Figure 11 & 12**).



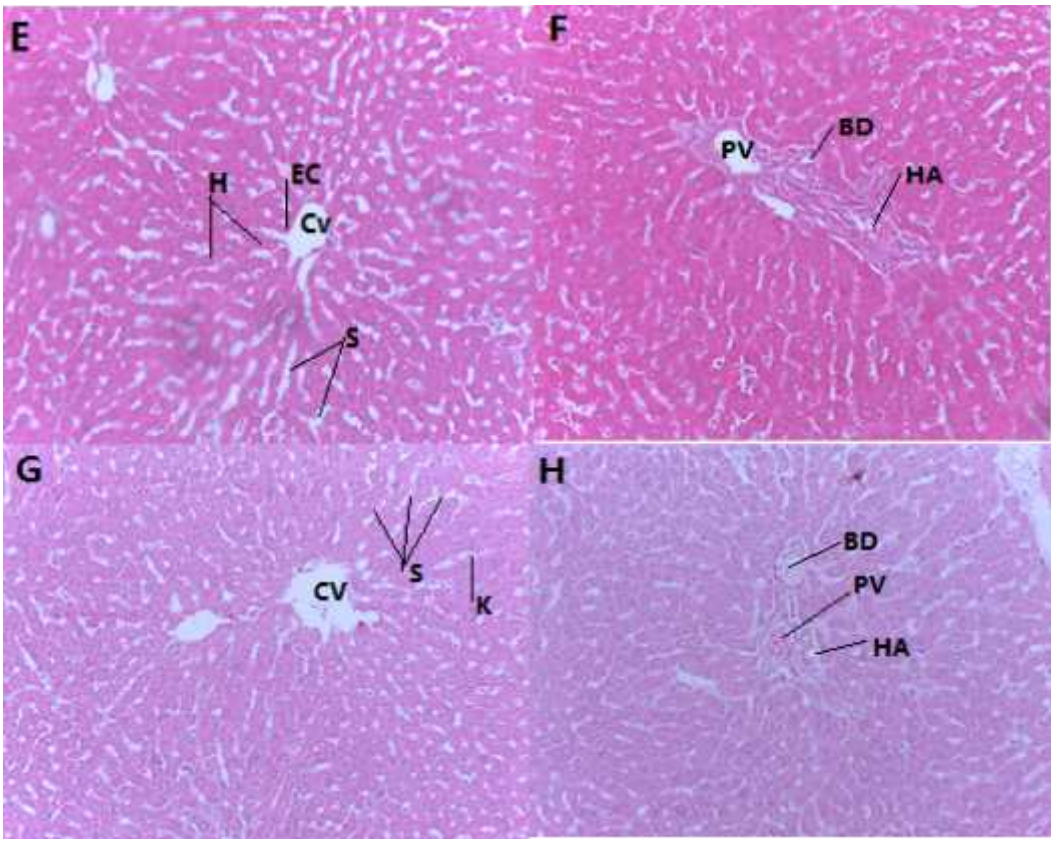


Figure 11: (A) and (B): Photomicrographs of liver sections of control rats; (C) And (D): Liver sections of rats treated with 65mg/kg of essential oil of T.schimperi; (E) and (F): Liver sections of rats treated with 130mg/kg of essential oil of T.schimperi and (H) and (G) Liver sections of rats treated with 260mg/kg of essential oil of T.schimperi. CV= central vein, EC= endothelial cells, H= hepatocytes, KC= Kupfer cells, S= sinusoids, BD=bile duct, HA= hepatic artery, and PV= portal vein.

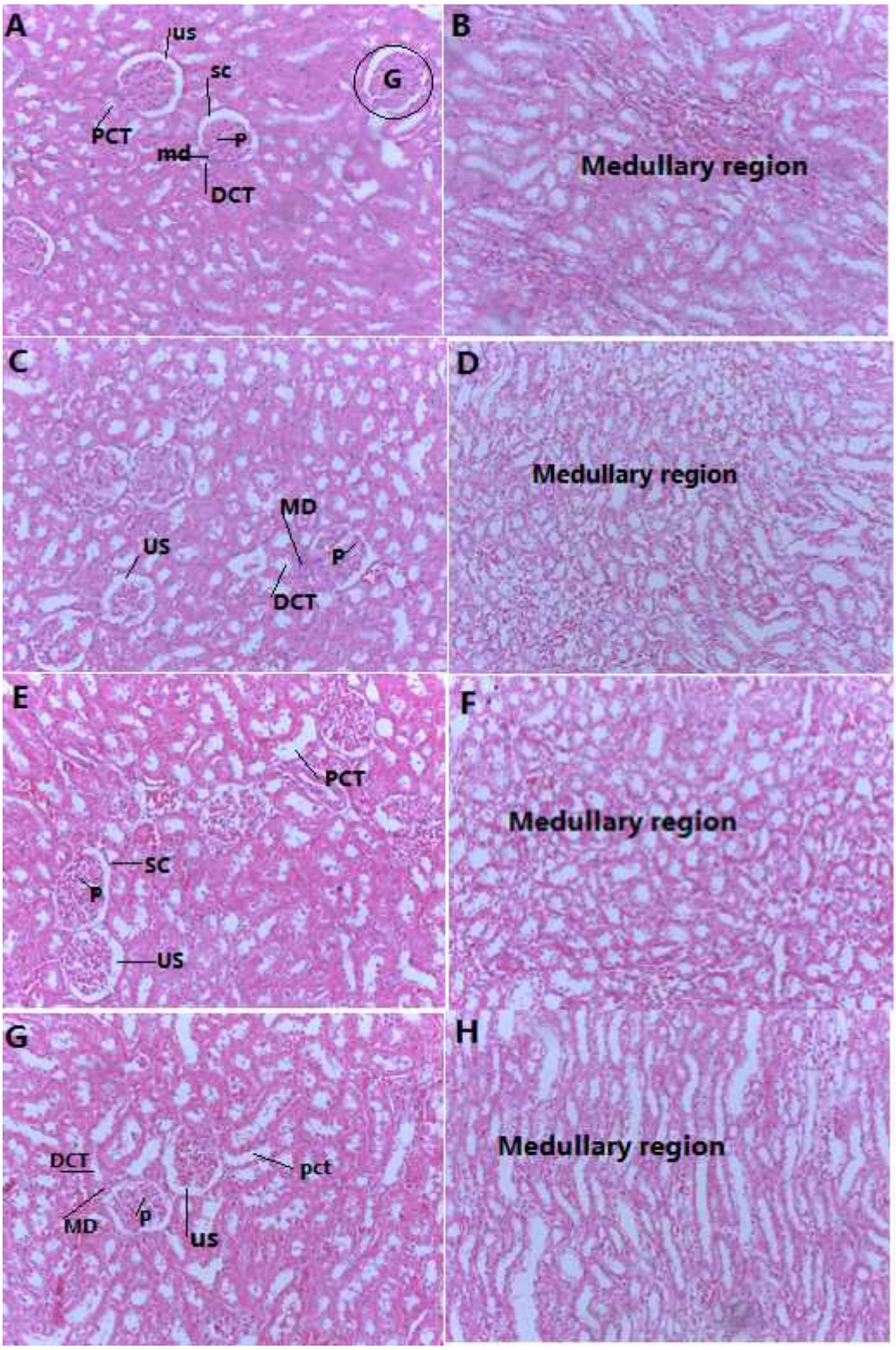


Figure 12:(A) and (B): Photomicrographs of the kidney sections of control rats, (C) and (D): kidney sections rats treated with 65mg/kg of essential oil of T.schimperi, (E) and (F): kidney sections of rats treated with 130 mg/kg of essential oil of T.schimperi and (H) And (G): kidney sections of rats treated with 260 mg/kg of essential oil of T.schimperi. PCT= proximal

convoluted tubule, DCT= distal convoluted tubule, MD= macula densa, G= glomerulus, US= urinary space, SC= squamous cell, and P= podocyte.

5.3.3. In-silico Toxicity Prediction of Compounds from the Essential oil of *T. schimperi*

Toxicities of compounds from the essential oil were also tested by Pro Tox II, ADMET, and vNN-ADMET servers. Toxicity and toxicological endpoint findings showed that all compounds derived from *T. schimperi* essential oil were free of h-ERG Blocker (cardiac toxicity), AMES (Ames Mutagenicity), and cytotoxicity. Regarding the hepatotoxicity parameters, most of the compounds (91.2%) did not show any toxicity while 8.8% of the compounds showed hepatotoxicity. Compounds that have hepatotoxicity effects are trans-sabinol, methyl m-tolylcarbinol, 2-isobutylideneamino-3-methyl butyronitrile, 10-ol limonene, and β-atlantol. Most of the compounds (96.5%) were safe for DILI (Drug-induced liver injury). However, 3.5% of compounds (thymol acetate and carvacrol acetate) have shown DILI toxicity. Similarly, 79.0% of the compounds did not show carcinogenicity while 21.0% of the compounds have shown carcinogenicity. In addition, about 3.5% of compounds (β-Bourbonene and gamma – Amorphene) are immunotoxic. Besides, most of the compounds have not shown mitochondrial membrane potential (MMP) toxicity, except thymol.From thetotal 57 compounds, predicted LD_{50} revealed that6 compounds (10.5%) have toxicity class three ($50 \leq LD50 \leq 300$), 19compounds (33.4%) have toxicity class four ($300 < LD50 \leq 2000$), 30compounds (52.6%) have toxicity class five ($2000 < LD50 \leq 5000$), and 2 compounds (3.5%) have toxicity class six ($LD50 > 5000$) **(Table 13).**

Table 13: In-silico toxicity prediction of compounds from the essential oil of *Thymus schimperi*

No	Compounds	Id	*Insilco* Toxicity								
			h-ERG Blocker	HT	DILI	Immuno toxicity	Carcinogenicity	Ames toxicity	Cyto toxicity	MMP	LD_{50} (mg/Kg)	Toxicity class
1	Butanoic acid, 2-methyl-, methyl ester	13357	No	No	No	No	Yes	No	No	No	5000	5
2	α-Thujene	17868	No	No	No	No	No	No	No	No	5000	5
3	α-Pinene	6654	No	No	No	No	No	No	No	No	4800	5
4	1,3,5-Cycloheptatriene, 7-ethyl-	561243	No	No	No	No	Yes	No	No	No	175	3
5	Camphene	6616	No	No	No	No	No	No	No	No	5000	5
6	β-Pinene	440967	No	No	No	No	No	No	No	No	5000	5
7	2,3-Diazabicyclo[2.2.2] oct-2-ene	145130	No	No	No	No	Yes	No	No	No	334	4
8	3-OCTANONE	11527	No	No	No	No	No	No	No	No	5000	5
9	Myrcene	31253	No	No	No	No	No	No	No	No	5000	5
10	3-OCTANOL	246728	No	No	No	No	No	No	No	No	5000	5

11	α-Phellandrene	443160	No	No	No	No	No	No	No	No	5700	6
12	γ -3- Carene	26049	No	No	No	No	No	No	No	No	4800	5
13	Cymene<ortho->	10703	No	No	No	No	Yes	No	No	No	1370	4
14	D-Limonene	440917	No	No	No	No	No	No	No	No	4400	5
15	Cineole<1, 8->	2758	No	No	No	No	No	No	No	No	2480	4
16	B-Ocimene<(Z)	5320250	No	No	No	No	Yes	No	No	No	113	3
17	α-Terpinene	7462	No	No	No	No	No	No	No	No	1680	4
18	Terpinene<gamma->	7461	No	No	No	No	No	No	No	No	2500	4
19	Dihydromyrcenol	29096	No	No	No	No	No	No	No	No	5300	6
20	Terpinolene	11463	No	No	No	No	No	No	No	No	4390	5
21	Linalool	6549	No	No	No	No	No	No	No	No	2200	5
22	Sabina ketone<dehydro->	527426	No	No	No	No	Yes	No	No	No	1450	4
23	α-Campholenal	249978459	No	No	No	No	No	No	No	No	5000	5
24	Ocimene<allo->	5368821	No	No	No	No	Yes	No	No	No	1900	4
25	Sabinol<trans-> (trans for	564260	No	Yes	No	No	No	No	No	No	3000	4

	OH vs. IPP)											
26	Isoborneol	64685	No	No	No	No	No	No	No	No	500	4
27	Methyl m-tolylcarbinol	110953	No	Yes	No	No	No	No	No	No	1300	4
28	α-Terpineol	442501	No	No	No	No	No	No	No	No	2830	5
29	Cyclohexane, 1-butenylidene-	556287	No	No	No	No	Yes	No	No	No	5000	5
30	2-Isobutylideneamino-3-methylbutyronitrile	573025	No	Yes	No	No	No	No	No	No	120	3
31	Dodecane	8182	No	No	No	No	No	No	No	No	750	3
32	Dihydrocarvone<trans->	24473	No	No	No	No	No	No	No	No	5000	5
33	3,5-Methanocyclopentapyrazole, 3,3a,4,5,6,6a-hexahydro-3a,4,4-trimethyl-	564375	No	No	No	No	Yes	No	No	No	880	4
34	1,4-Methano-1H-cyclopenta[d]pyridazine, 4,4a,5,7a-tetrahydro-8,8-dimethyl-, (1. alpha.,4. alpha.,4a. alpha.,7a. alpha.)-	562380	No	No	No	No	Yes	No	No	No	260	3

35	Carvacrol, methyl ether	80790	No	No	No	No	Yes	No	No	No	880	4
36	´Neral	643779	No	No	No	No	No	No	No	No	500	4
37	Limonen-10-ol	527143	No	Yes	No	No	No	No	No	No	3000	5
38	Thymol	6989	No	No	No	No	No	No	No	Yes	640	4
39	Carvacrol	10364	No	No	No	No	No	No	No	No	810	4
40	Thymolacetate	241091509	No	No	Yes	No	No	No	No	No	2000	4
41	Carvacrol acetate	80792	No	No	Yes	No	No	No	No	No	374	4
42	Sesquithujene<7-epi->	56927990	No	No	No	No	No	No	No	No	5000	5
43	β-Bourbonene	324224	No	No	No	Yes	No	No	No	No	5000	5
44	Caryophyllene(E-)	5281515	No	No	No	No	No	No	No	No	3700	5
45	β-Gurjunene	6450812	No	No	No	No	No	No	No	No	5000	5
46	Trans-. Alpha. – Bergamotene	6429302	No	No	No	No	No	No	No	No	4800	5
47	Aromadendrene<allo->	91746537	No	No	No	No	No	No	No	No	5000	5

48	3,5-Methanocyclopentapyrazole, 3,3a,4,5,6,6a-hexahydro-3a,4,4-trimethyl-	564375	No	No	No	No	Yes	No	No	No	880	4
49	Germacrene D	5317570	No	No	No	No	No	No	No	No	2760	5
50	Amorphene<gamma->	12313019	No	No	No	Yes	No	No	No	No	4400	5
51	Nerylisobutanoate	87203412	No	No	No	No	No	No	No	No	4260	5
52	Viridiflorene	10910653	No	No	No	No	No	No	No	No	5000	5
53	Geranylisobutanoate	5365991	No	No	No	No	No	No	No	No	4260	5
54	β-Sesquiphellandrene	519764	No	No	No	No	No	No	No	No	5000	5
55	β-Vetivenene	14475467	No	No	No	No	No	No	No	No	3040	5
56	β-Atlantol	181580	No	Yes	No	No	No	No	No	No	1640	4
57	Tetracosane	12592	No	No	No	No	No	No	No	No	750	3

Note: Toxicity profiles of compounds were analyzed using the Pro tox II, ADME web server (http://admet.scbdd.com), and vNN-ADMET webserver. **hERG** (human Ether-àgo-go-Related Gene), **HT** (hepato- toxicity), and **DILI** (drug-induced liver injury).**Class 3**: toxic if swallowed ($50 < LD50 \leq 300$), **Class 4**: harmful if swallowed ($300 < LD50 \leq 2000$), **Class 5**: may be harmful if swallowed ($2000 < LD50 \leq 5000$), **Class VI**: non-toxic ($LD50 > 5000$)(162).

5.5. Developmental Toxicity Resultsof Aqueous Crude Extract of *T.schimperi*

5.5.1. Day 12 experiment

5.5.1.1. Pregnancy Outcomes

Treatment of pregnant rats with the aqueous crude extractof *T.schimperi*at doses of 500 mg/kg/, 1000 mg/kg/, and 2000 mg/kg/ from days 6 to 12 of gestation resulted in a dose-dependent decrease in maternal weight gain when compared to the *ad libitum* and pair-fed control groups. However, it is not statistically significant.The maternal weight gains were4.42±0.32g and5.23±0.24g in the high dose (2000 mg/kg) and paired fed control groups, respectively. On days 1^{st}-5^{th} and 6^{th}-12^{th}, there was no significant difference in daily food intake between the groups. Regarding the implantation sites, there was a significant reduction in implantation sites in the high dose(2000 mg/kg) treated group as compared to pair-fed control and *ad libitum*control groups. Furthermore, as compared to the pair-fed and *ad libitum* control groups, thenumber of resorption sites in the high dose (2000 mg/kg)*T.schimperi*aqueous crude extract-treated group was significantly higher **(Table 14 and Figure 13).**

Table 14: Maternal weight gain, daily food intake, and outcomes of pregnancy after administration of aqueous leaf extracts of *T. schimperi* on day-12 experiment

Groups	Maternal Weight Gain Per Dam (g)	Daily Food Intake (g/day)		Outcomes of Pregnancy	
		Day 1–5	Day 6–12	Implantation Sites Per Litter	Resorptions Per Litter
G-I (Pair fed control)	5.23±0.24	15.62±0.33	16.84±0.21	11.1±0.76	0.54±0.30
G-II (*ad libitum*)	4.91±0.71	15.57±0.34	16.82±0.22	11.3±0.72	0.56±0.42
G-III (500mg/kg)	4.78±0.43	15.55±0.31	16.71±0.19	10.13±0.47	0.65±0.46
G-IV (1000mg/kg)	4.62±0.32	15.61±0.32	16.68±0.30	10.12±0.54	0.55±0.35
G-V (2000mg/kg)	4.42±0.32	15.41±0.30	16.66±0.29	8.01±0.45[b]	0.8±0.35[b]
F statistics	21.2	4.02	8.20	2.13	6.24
P value	0.06	0.31	0.06	0.03	0.02

The data are expressed as mean ± SDM. [a]Significant difference compared to the pair-fed control group ($p<0.05$), [b]Significant difference compared to the *pair-fed control* and *ad libitum* group ($p<0.05$), and [c] Significant difference compared to the Ad libitum group ($p<0.05$).

Figure 13: Number of implantation and resorption sites: A (paired fed control), B (500mg/kg), C (1000mg/kg) and E (2000mg/kg); I (Implantation site and R (Resorption site).

5.5.1.2. Embryonic Growth

In the present study, the crown-rump length(CRL)of the embryos decreased in a dose-dependent manner. The CRL of rat embryos treated with 2000 mg/kg of crudeaqueous extractof *T.schimperi*was significantly loweras compared to the control groups. The mean CRL was5.1±0.6 and 5.2±0.4in Pair fed,and *ad libitum* control groups, respectivelywhile it was 4.4±0.7in the high dose treatment group(2000mg/kg). In addition, the mean number of somites in the high dose group (2000 mg/kg) was significantly lower ($p < 0.04$) as compared with the control groups. In addition, the mean morphological score of the rats treated with 2000 mg/kg of the extract was 42.34 ± 1.55. The morphological scores of the pair-fed, and *ad libitum* control groups were 44.30±1.88 and 44.30±1.88, respectively. The mean morphological score of pregnant rats treated with 2000mg/kg of the aqueous crude extract was significantly lower than that of the control groups, $p < 0.05$**(Table 15).**

Table 15: Embryonic growth following administration of the aqueous crude extract of *T. schimperi.*

Groups	**Growth of the Embryo**		
	Number of Somites	**Morphological Score**	**CRL of the embryo (mm)**
G-I (Pair fed control), n=122	30.24±0.64	44.30±1.88	5.1±0.6
G- II (*ad libitum*), n=118	29.84±0.64	44.30±1.88	5.2±0.4
G-III (500mg/kg), n=114	30.32±0.72	44.74±1.91	4.9±0.5
G-IV (1000mg/kg), n=110	29.88±0.34	43.72±1.47	4.7±0.6
G-V (2000mg/kg), n=88	26.46±0.78[b]	42.34±1.55[b]	4.4±0.7[b]
F statistic	49.20	14.20	24.3
P value	0.04	0.01	0.02

CRL: Crown-rump length; the data are expressed as mean ± SDM. [a]Significant difference compared to the pair-fed control group ($p<0.05$), and[b] Significant difference compared to the pair-fed control and ad *libitum group* ($p<0.05$). n: number of embryos.

5.5.1.3. Embryonic Body System Development

The mean morphological score was determined from the seventieth scoring method developed by Brown and Fabro (163) and adopted for use by Seyoum and Persaud(155). As shown in **Table 16 and Figure 14**, the mean growth scoresof the otic system of rat embryos were significantly lower in the middle dose(1000 mg/kg) group and high dose (2000 mg/kg) groupas compared tothe control groups. Pair-fed and *ad libitum* control groups had a mean growth score of 3.7±0.52 and 3.6±0.51, respectively. However, the mean growth score in the middle dose (1000mg/kg) and high dose (2000mg/kg)treatment groups were 3.22±0.43 and 3.10±0.42, respectively.

Furthermore, the mean growth score of the optic system of rat embryos from the treatment group of 2000 mg/kg of *T.schimperi* aqueous crude extract was significantly lower than in the control groups. The growth scores were 3.68±0.53 and 3.67±0.51 in the pair-fed and *ad libitum* control groups, respectivelywhereas it was 3.10±0.43 in the high dose (2000mg/kg) treated group.

In the current study, rat embryos treated with 1000 mg/kg and 2000 mg/kg aqueous crude extract had significantly lower olfactory system mean developmental scores than the control groups. In the *ad libitum* and pair-fed control groups, the scores were 0.95±0.60 and 0.88±0.52, respectively.On the other hand,in the middle and high dose (1000 mg/kg and 2000 mg/kg) treatment groupsthe scores were 0.59±0.50 and 0.53±0.32, respectively.

Themean number of branchial barsshowed a significant reduction in the high dose group (2.91±0.33) as compared to the pair-fed (3.69±0.56) and *ad libitum* control groups (3.5±0.52). Similarly, the mean number of maxillary processes of the embryos was significantly lower in the high dose group (1.09±0.48)than in Pair-fed (1.67±0.54) and *ad libitum*(1.55±0.51) control groups. In addition, the mean number of mandibular processes of the embryos was significantly lower in the high dose group (0.44±0.40) than in Pair-fed (0.79±0.48) and *ad libitum* (0.88±0.51) control groups.However, there was no significant difference between the control and treatment groups in the developmental parameters of the yolk sac circulation, embryo flexion, heart, caudal neural tube, hindbrain, midbrain, forebrain, forelimb, and hind limb.

Table 16: In vivo development of rat embryo following treatment of aqueous crude extract of *T.schimperi*: Day 12 experiment.

Morphological end point	**G-I (Pair-fed control)**	**G-II (*ad libitum*)**	**G-III(500mg/kg)**	**G-IV(1000mg/kg)**	**G-V(2000mg/kg)**
Number of fetus/group	122	118	114	110	88
Yolk sac circulation	3.71±0.50	3.60±0.49	3.59±0.48	3.61±0.50	3.65±0.50
Flexion	2.7±0.32	2.6±0.31	2.7±0.32	2.6±0.31	2.7±0.32
Heart	3.6±0.50	3.6±0.50	3.5±0.49	3.5±0.49	3.6±0.50
Caudal neural tube	4±0.00	4±0.00	4±0.00	4±0.00	4±0.00
Hind brain	3.7±0.51	3.7±0.51	3.6±0.49	3.6±0.49	3.6±0.49
Mid brain	3.66±0.54	3.66±0.53	3.55±0.50	3.44±0.48	3.44±0.48
Fore brain	3.8±0.49	3.8±0.49	3.8±0.49	3.7±0.47	3.7±0.47
Otic system	3.7±0.52	3.6±0.51	3.5±0.50	3.22±0.43[a]	3.10±0.42[b]
Optic system	3.68±0.53	3.67±0.51	3.56±0.48	3.45±0.47	3.10±0.43[b]
Olfactory system	0.95±0.60	0.88±0.52	0.87±0.52	0.59±0.50[a]	0.53±0.32[b]
Branchial bars	3.69±0.56	3.58±0.52	3.48±0.50	3.47±0.57	2.91±0.33[b]
Maxillary process	1.67±0.54	1.55±0.51	1.47±0.49	1.37±0.48	1.09±0.48[b]
Mandibular process	0.88±0.51	0.79±0.48	0.78±0.47	0.64±0.43	0.44±0.40[b]

Fore limb	2±0.00	2±0.00	2±0.00	2±0.00	2±0.00
Hind limb	2±0.00	2±0.00	2±0.00	2±0.00	2±0.00

Statistical differences between the groups were analyzed by Duncan's multiple range tests. Results are expressed as mean ± SDM. [a]Significant difference compared to the pair-fed control group ($p<0.05$), [b] Significant difference compared to the pair-fed control and *ad libitum* group ($p<0.05$), and[c]Significant difference compared to the *Ad libitum* group ($p<0.05$). n: number of embryos.

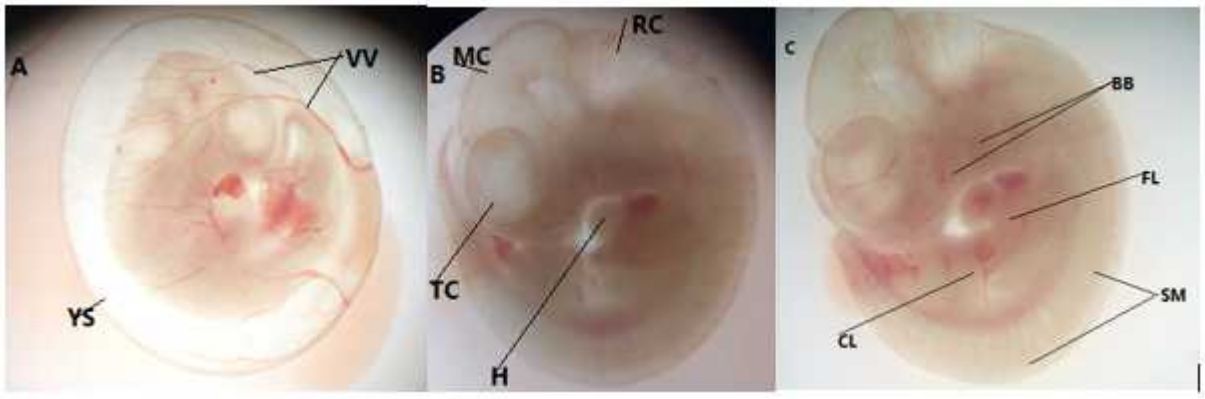


Figure 14:12-Day old rat embryos showing various organs of primordia. A: an embryo enclosed inside the intact yolk sac (YS) with vitelline vasculature surrounding it (VV). B. Yolk sac-free embryo, separated from the surrounding blood vessels, revealing the heart (H), telencephalon (TC), mesencephalon (MS), rhombencephalon (RC). C: Showing brachial bars (BB), forelimb bud (FL), somites (SM), and caudal limb bud (CL).

5.5.2. Day 20 Experiment Pregnancy Outcomes

5.5.2.1. Maternal Food Intake and Weight Gain

The maternal food intake did not significantly vary between the treatment and control groups in the pre-treatment (days 1-5), during treatment(days 6-12), and post-treatment periods(days 13-20)(**Table 17)**.

In the present study, maternal weight gain in the high-dose (2000mg/kg)treatment group and the pair-fed control groupwere 3.31± 0.47 and 5.92± 0.62, respectively, during the treatment period. Maternal weight gain was significantly lower ($p<0.04$) in the high dose (2000 mg/kg) treated

group as compared to in the pair-fed *and ad libitum*control groups during the treatment period. Similarly, maternal weight gain in the high dose group was significantly lower than the control groups in the post-treatment period(**Table 17**).

Table 17: Daily food intakes and maternal weight gains of pregnant rats onthe day-20 aqueous crude extract of *T.schimperi*experiment.

Groups	**Daily food intake (g/day)**			**Maternal weight gain (g/day)**	
	Day 1-5	**Day 6-12**	**Day 13-20**	**Day 6-12**	**Day 13-20**
G-I (Pair-fed control)	15.63± 0.17	15.95± 0.42	16.52± 0.43	5.92±0.62	16.12±0.76
G- II (*Ad libitum*)	15.42±0.08	15.74±0.14	16.46±0.52	5.87±0.73	16.73±0.81
G-III (500 mg/kg	15.29±0.18	15.56±0.12	16.21±0.23	5.70±0.82	15.81±0.51
G-IV (1000mg/kg)	15.33±0.07	15.69±0.32	16.15±0.10	5.62±0.62	15.76±0.50
G-V (2000mg/kg)	15.49±0.10	15.43±0.06	16.01±0.04	3.31±0.47[b]	10.12±0.33[b]
F statistic	3.06	8.41	53.21	9.87	10.21
P value	0.15	0.09	0.09	0.04	0.02

Results are stated as mean ± SDM. [a] the **s**ignificant difference compared to the pair-fed control group ($p<0.05$), [b]Significant difference compared to the pair-fed control and *ad libitum* group ($p<0.05$), and [c]Significant difference compared to the *ad libitum* group ($p<0.05$). n: number of embryos.

In the 500 mg/kg, 1000 mg/kg, and 2000 mg/kg aqueous crude extract of *T.schimperi* treated groups, there were 88, 86, and 80 fetuses, respectively. The number of fetuses decreased in a dose-dependent manner between groups, but this was not statistically significant.

In the current study, the mean number of implantation sites was significantly lower in the high dose treatment group(7.7±0.35) as compared to the pair-fed control group(10.2±0.63) and *ad libitum*control group (9.2±0.59). In addition,the mean number of fetal resorptions was significantly higher in the high dose treatment group (0.60±0.18) than the pair-fed control group (0.10±0.50) and *ad libitum* control group (0.20±0.52) (**Table 18**).

Table 18: Pregnancy outcomes of the day-20 experiment after administration of the aqueous crude extract of*T. schimperi*.

Groups	**No. of fetuses**	**Implantation sites**	**No. of resorptions/litter**	**No. of live fetus/dam**	**No. of dead fetus/dam**
G-I (Pair-fed control)	94	10.2±0.63	0.10±0.50	9.50 ±0.70	0.10±0.04
G- II (*ad libitum*)	96	9.2±0.59	0.20±0.52	9.60±0.76	0.20±0.05
G-III (500 mg/kg	88	8.0±0.50	0.30±0.38	8.30±0.64	0.30±0.06
G-IV(1000mg/kg)	86	8.6±1.42	0.30±0.35	7.80±0.62	0.30±0.07
G-V (2000mg/kg)	80	7.7±0.35[b]	0.60±0.18[b]	7.50±0.61	0.20±0.05
F statistic		4.34	7.01	4.21	8.23
P-value		0.03	0.04	0.13	0.15

Results are stated as mean ± SDM. [a]Significant difference compared to the pair-fed control group ($p<0.05$),[b]Significant difference compared to the pair-fed control and ad libitum group ($p<0.05$), and [c] Significant difference compared to the ad libitum group ($p<0.05$). n: number of embryos.

5.5.2.2. Fetal Growth

The high-dose treatment group meanslitter weight (2.01±0.01) was significantly lower as compared tothe mean litter weight ofthe *ad libitum*control group(2.95±0.08) and pair-fed control

group (2.88±0.06). At the high dose(2000mg/kg) of the treatment group, fetal growth at term was significantly lowerthan the pair-fed and the *ad libitum* control groups. In addition, The CRL was significantly lower in the high dose treatment group(2.21±0.16) than the pair-fed control (2.88±0.15) and the *ad libitum*control group (2.88±0.15).Similarly, the placental weight was significantly lower in the high dose treatment group (0.41±0.04) than the pair-fed control (0.50±0.07) and the ad libitum control group (0.47±0.06)(**Table 19**).

Table 19: Mean fetal growth following treatment with aqueous extract of *Thymus schimperi* in the day-20 experiment.

Groups	**Fetal growth**		
	Litter weight/fetus(g)	**CRL/fetus (cm)**	**Placental weight/fetus (g)**
G-I (Pair-fed control)	2.95±0.08	2.88±0.15	0.50±0.07
G- II (*ad libitum*)	2.88±0.06	2.78±0.13	0.47±0.06
G-III (500 mg/kg	2.74±0.05	2.73±0.12	0.45±0.05
G-IV (1000 mg/kg)	2.55±0.02	2.58±0.11	0.45±0.05
G-V (2000 mg/kg)	2.01±0.01[b]	2.21±0.16[b]	0.41±0.04[b]
P value	0.03	0.03	0.04

Results are stated as mean ± SDM. [a] Significant difference compared to the pair-fed control group ($p<0.05$), [b] Significant difference compared to the pair-fed control and *ad libitum* group ($p<0.05$), and [c]Significant difference compared to the *ad libitum* group ($p<0.05$). n: number of embryos.

5.5.2.3. External Morphological Anomalies

In the aqueous extraction developmental toxicity study, the explanted fetuses were also assessed for visible abnormalities from head to tail. The study examined craniofacial abnormalities, limb defects, vertebral column anomalies, missing tails, and external genital abnormalities. Despite

this, no treatment-related abnormalities were found in near-term rat fetuses (**Table 20 and Figure 15**).

Table 20: External malformations of rat fetuses after administration of the aqueous crude extraction of*T. schimperi.*

Group	**Fetus examined**	**Observed malformations (%)**							
		AC	**EC**	**SB**	**SC**	**KY**	**LD**	**MT**	**AEG**
G-I (Pair-fed control)	94	0	0	0	0	0	0	0	0
G- II (*ad libitum*)	96	0	0	0	0	0	0	0	0
G-III (CT500mg/kg	88	0	0	0	0	0	0	0	0
G-IV (CT1000mg/kg)	86	0	0	0	0	0	0	0	0
G-V (CT2000mg/kg)	80	0	0	0	0	0	0	0	0

The percentage of malformations is represented as a percentage of the total number of malformations (Chi-Square). AE: Anencephaly, EC: Exencephaly, SB: Spina bifida, KY; Kyphosis, SC: Scoliosis, LD: Limb defect, MT: Missed tail, AEG: Agenesis of external genitalia.

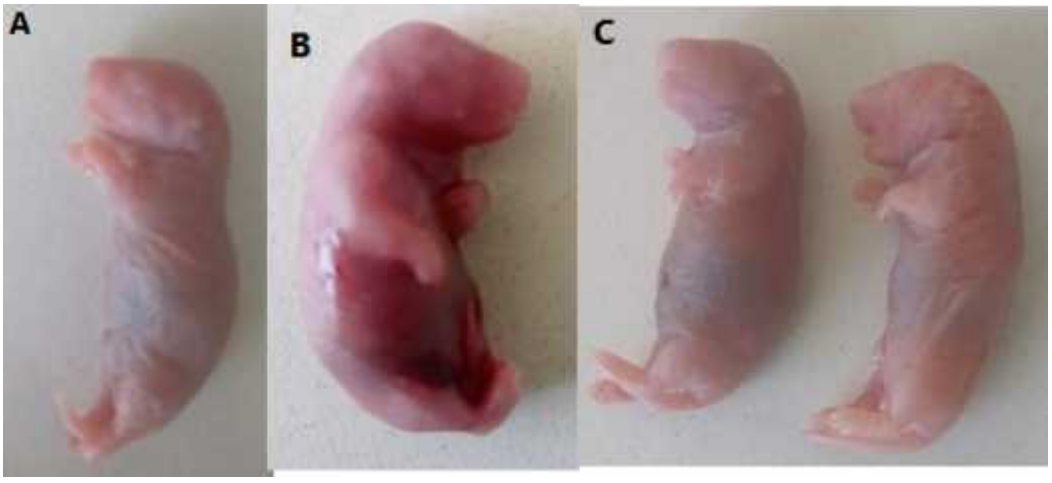


Figure 15: Live rat fetuses from control and high dosegroups: A (Pair-fed control), A (*Ad libitum* control), C (2000mg/kg).

After being fixed in Boiun's solution, the fetuses were serially sectioned for visceral soft tissue assessment. Serial incisions were made at the head, neck, thorax, and abdominal levels. Sections are evaluated for visceral abnormalities under a dissecting microscope. The head was then evaluated for cleft palate, hydrocephalus, and eye abnormalities.In addition, thyroid, thymus, trachea, and cardiac septum anomalies were also investigated in the neck and thorax. Similarly, diaphragmatic hernia, intraabdominal organ growth, and external genitalia were all investigated.In all the examinations, visceral soft tissue abnormalities have not been revealed(**Table 21 and Figure 16**).

Table 21: Visceral malformations of rat fetuses after administration of aqueous extraction of*T.schimperi.*

Group	**Fetus**	**Observed malformations (%)**										
	Examined	**HC**	**MO**	**AO**	**CP**	**NSD**	**REAA**	**VSD**	**DH**	**RA**	**HU**	**CT**
G-I (Pair-fed control)	94	0	0	0	0	0	0	0	0	0	0	0
G- II (*ad libitum*)	96	0	0	0	0	0	0	0	0	0	0	0
G-III (CT500mg/kg	88	0	0	0	0	0	0	0	0	0	0	0
G-IV (CT1000mg/kg)	86	0	0	0	0	0	0	0	0	0	0	0
G-V (CT2000mg/kg)	80	0	0	0	0	0	0	0	0	0	0	0

Results are expressed as a percentage of malformations (Chi-Square). HC-Hydrocephalus, MO-Microphthalmia, AO-Anophthalmia, CP-Cleft palate, NSD-Nasal septal defect, REAA-Retroesophageal aortic arch, VSD-Ventricular septal defects, DH-Diaphragmatic hernia, RA-Renal agenesis, HU-Hydroureters, and CT-Cryptorchid testes.

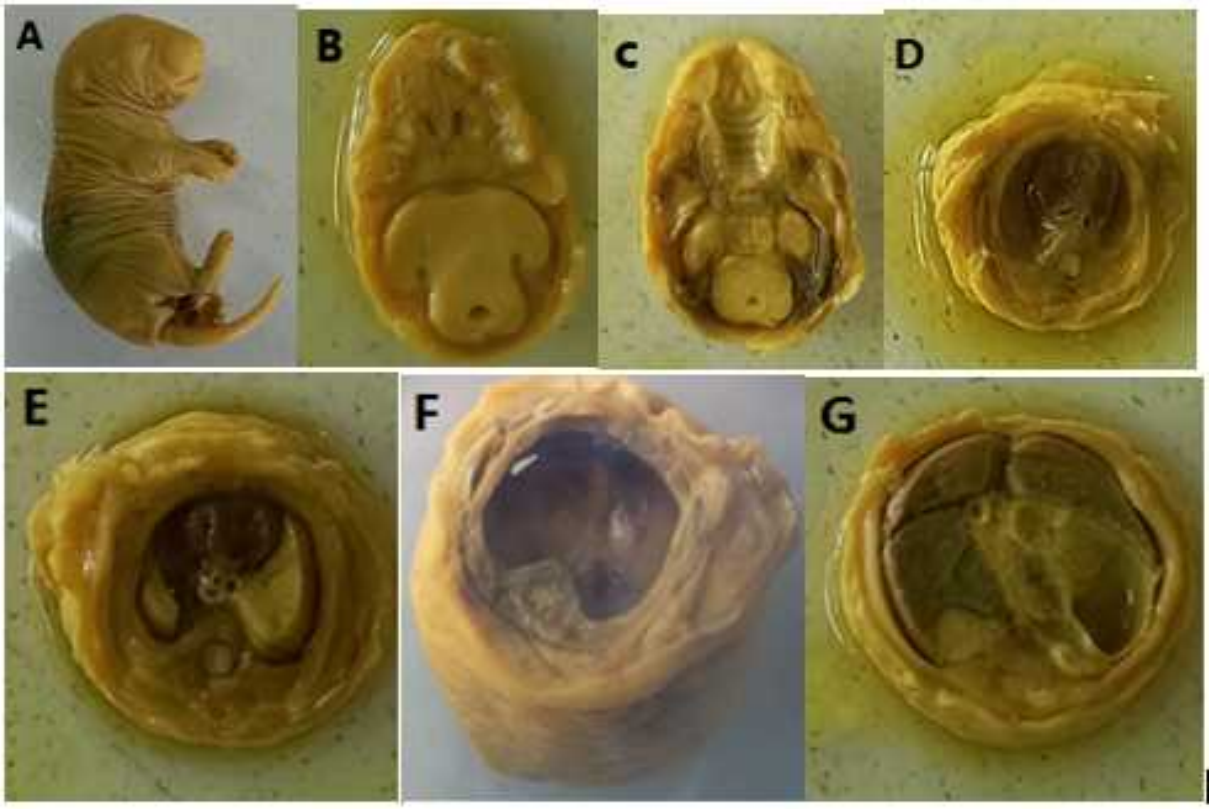


Figure 16:A 20-day old rat fetus fixed in Bouin's solution for visceral examination (2000 mg/kg). A: un-sectioned fetus; B: transverse section of the brain showing normal brain tissue; C: normal palate; D: section made through the neck showing the normal esophagus, trachea, and thyroid gland; E: section through the chest showing normal; F: intact diaphragm and G: a section made though the abdomen showing normal visceral organs.

5.5.2.4. Skeletal Malformations

The skull, thoracic vertebrae, sternum, hyoid, and metatarsals were all examined. However, neither the treatment groups nor the control groups revealed any major skeletal abnormalities. The ossification centers of the caudal/coccygeal vertebrae, as well as the phalanges of the hind limbs, differed slightly between the treatment groups and control groups. There were no hind limb phalanges in 20.1% of rat fetuses from the high dose group(**Tables 22-23 and Figure 17)**.

Table 22: Skeletal malformations of 20-day old rat fetuses following treatment with the aqueous extraction of *T.schimperi*.

Group	Percentage of skeletal malformations				
	Sternum[a]	**Hyoid[b]**	**Ribs[c]**	**Thoracic vertebrae[d]**	**Caudal vertebrae[e]**
G-I (Pair-fed control) n=50	12	0	0	0	7.5
G- II (*ad libitum* n=50	14	0	0	0	8.6
G-III (500 mg/kg n=50	16	0	0	0	11.2
G-IV (1000 mg/kg) n=50	18	0	0	0	13.4
G-V (2000 mg/kg) n=50	18	0	0	0	15.2

The percentage of skeletal malformations is calculated (Chi-Square). **[a]:**<4 ossification centers on the sternum; **[b]**: no ossification signs on the hyoid bone; **[c]**: no ossification signs on the ribs; **[d]**: <13 ossification centers on the thoracic centra; and **[e]**: <4 ossification centers on the caudal vertebrae.

Table 23: Skeletal malformations of 20 days old rat fetuses following treatment with the aqueous extraction of *T. schimperi.*

Group	Percentage of skeletal malformations of limb bones			
	Metacarpus[a]	**Metatarsal[b]**	**Forelimb phalanges[c]**	**Hindlimb phalanges[d]**
G-I (Pair-fed control) n=50	5.2	4.6	9.8	14.4
G- II (*Ad libitum*)n=50	4.1	4.1	8.7	14.8
G-III (500 mg/kg) n=50	5.5	5.8	10.2	18.2
G-IV (1000)n=50	7.2	5.6	11.8	18.4
G-V (2000)n=50	7.6	5.8	11.6	20.1

The number of skeletal malformations is expressed as a percentage (%). (Chi-Square). A: <3 metacarpus; b: <3 metatarsus; c: No forelimb proximal phalanges; d: No hindlimb proximal phalanges.

Figure 17: Alizarin red-stained rat fetuses showing different ossification centers. A: Vertebrae, B: Metatarsal, C: Hyoid, D: Sternebra, E: Supraoccipital and Inter-parietal.

5.5.2.5. Histopathological Studies of the Placenta

On the selected placentas, gross pathological evaluations of the basal zone, labyrinthine zone, inter-villous spaces, giant cells, and trophoblasts were performed. The decidual and inter-villos spaces of the placenta showed some structural changes when examined under a microscope **(Figures 18 -20)**.Histopathological alterations included decidual cystic degeneration, thrombosis in the intervillous spaces, and decidual cellular apoptosis. Although rats given a high dose of *T.schimperi* aqueous crude extract had a higher incidence of decidual cystic degeneration, none of the foregoing alterations were statistically significant **(Table 24)**.

Table 24: Placenta histopathology of 20 days old rat fetuses following treatment with the aqueous extraction of *T. schimperi*.

Group	**Percentage of placental abnormalities**		
	Decidual cystic degeneration	**Thrombosis in the intervillous spaces**	**Cellular apoptosis**
G-I (Pair-fed control)	2	2	4
G- II (*Ad libitum*)	4	0	2
G-III (500 mg/kg)	8	2	4
G-IV (1000)	8	6	6
G-V (2000)	10	8	8

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

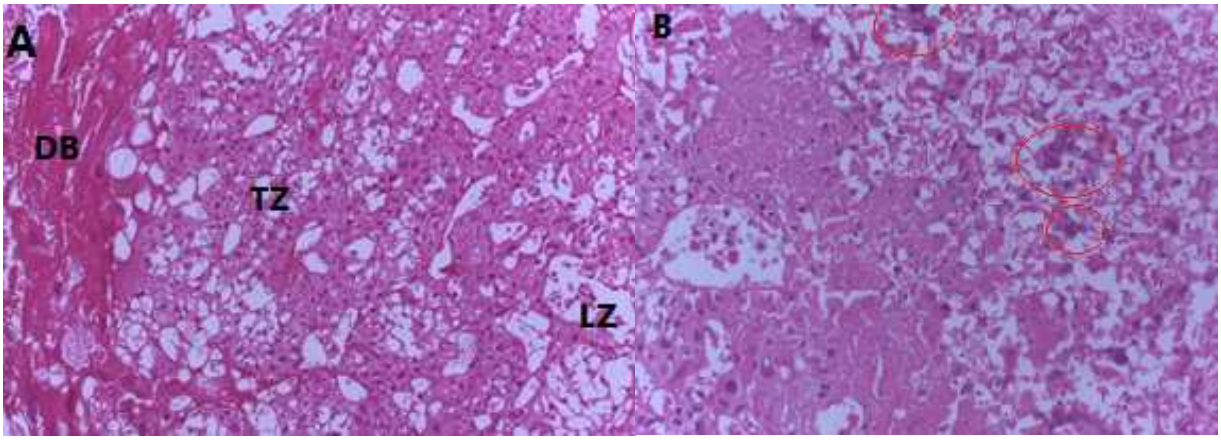


Figure 18:A; Photomicrograph showingnormal decidua in the pair-fed control placenta and **B**; foci of decidual necrosis (circles) in 2000mg/kg *T.schimperi* treated placenta. The stain used H and E, 40X.

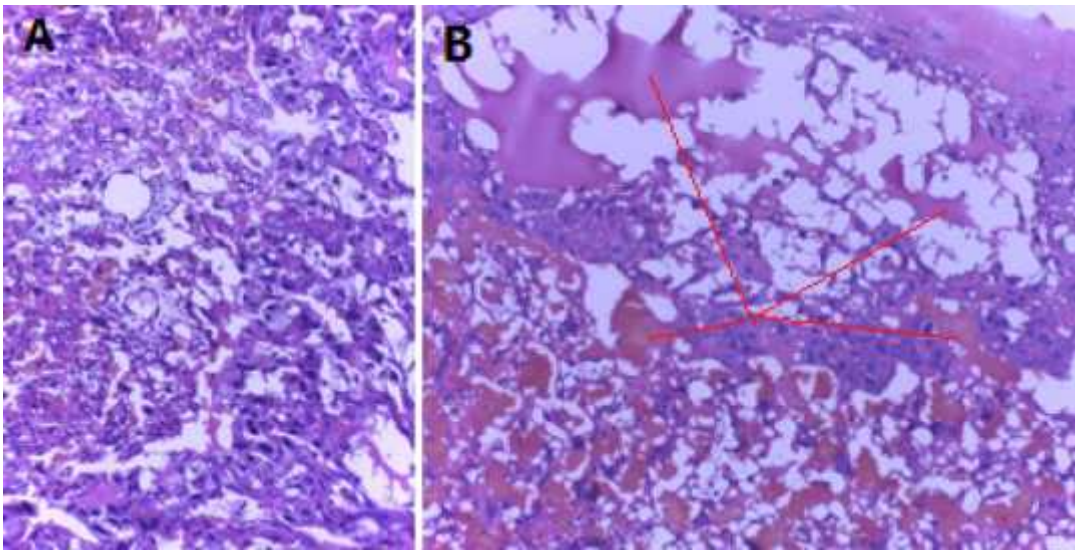


Figure 19: A; Photomicrograph showing normal intervillous space in pair-fed control and **B;**in 2000 mg/kg T.schimperi treated placentathrombosis (arrows). The stain used H and E, 40X.

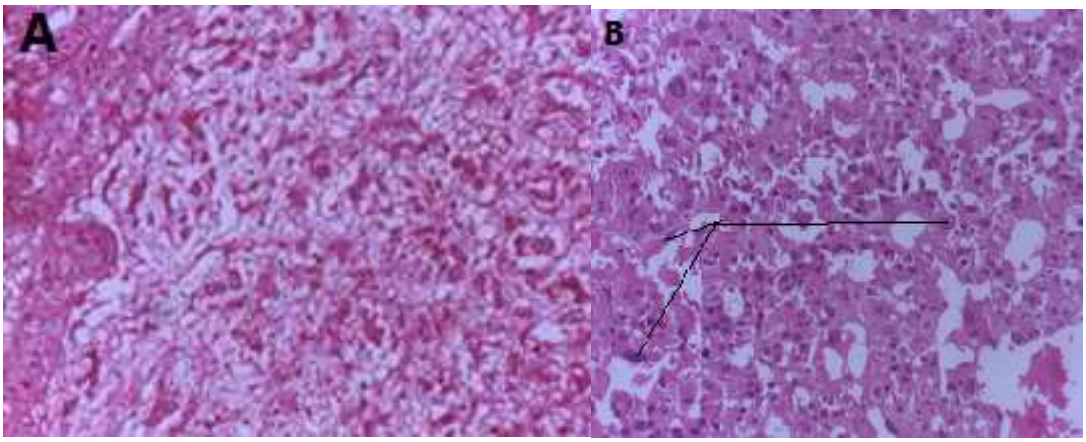


Figure 20: A; Photomicrograph showing normal decidua in pair-fed control and **B;** decidual apoptosis in 2000 mg/kg T. schimperi treated placenta. Stain used H and E, 40X

5.6. The Developmental Toxicity of *T. schimperi* Essential oil

5.6.1. Day 12 Experiment

5.6.1.1. Pregnancy Outcomes

In the current study, the maternal weight gain was significantly lower in a high-dose treatment groupas compared to *ad libitum* and pair-fed control groups.In the high dose treatment group and the *ad libitum* control group, the mean maternal weight gains were 1.73±0.411g and 3.10±0.211g, respectively. Similarly, there was a high incidence of fetal resorptions at a dose of 260 mg/kg as compared to the control groups. At a high dose treatment group (260 mg/kg) and *ad libitum*control group, the fetal resorptionswere 1.01±0.611 and 0.35±0.45, respectively. However,the number of implantation sites did not significantly varied(**Table 25 and Figure 21).**

Table 25: Pregnancy outcome following treatment of pregnant rats with essential oil extract of areal part of Thymus schimperi: Day-12 experiment.

Parameters	**Control groups**		**Treatment groups**		
	G-I (Pair fed control)	**G- II (*Ad libitum*)**	**G-III (EOT65mg/kg)**	**G-IV (EOT130mg/kg)**	**G-V (EOT260mg/kg)**
Maternal weight gain per group (g)	3.11 ±0.20	3.10±0.21	3.31±0.50	3.33±0.07	1.73±0.41[b]
Implantation sites per litter	10±0.50	10.1±0.71	9.6±0.03	9.1±0.60	10.2±0.55
Resorptions per litter	0.40±0.51	0.35±0.45	0.38±0.50	0.42±0.33	1.01±0.61[b]

The data are expressed as mean ± SDM, n=10 for each group. [a]Significant difference compared to the pair-fed control group (P<0.05),[b]Significant difference compared to the pair-fed control and*Ad libitum* group (P<0.05), and [c]Significant difference compared to the *Ad libitum* group (P<0.05). EOT= essential oil of *Thymus schimperi*.

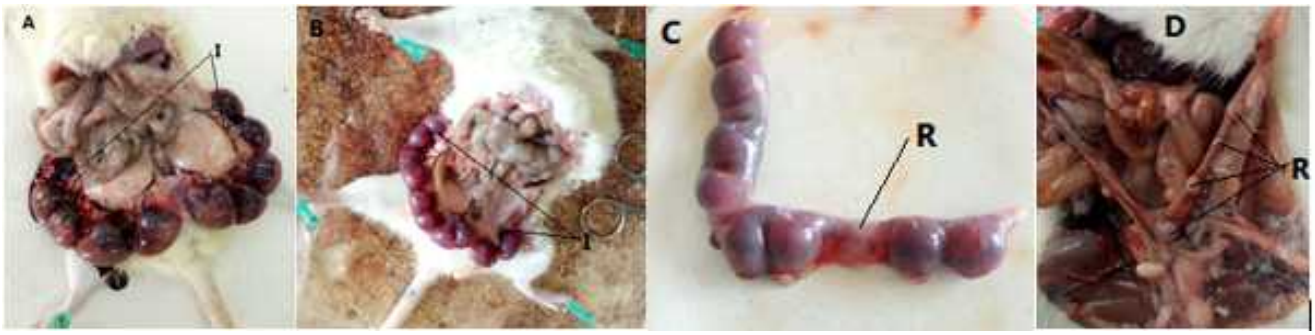


Figure 21:number of implantation and resorption sites: A (*Ad libitum* control), B,(65mg/kg), C (130mg/kg) and E (260mg/kg).I (Implantation site and R(Resorption site).

5.6.1.2. Growth of the Embryo

The CRL of rat embryos treated with 260 mg/kg of essential oil was significantly lower as compared to the pair-fed and*ad libitum* control groups. Pair-fed and *ad libitum* control groups had the mean CRL of 5.0±0.6 and 5.1±0.4, respectively, while a high dose (260 mg/kg) treatment group had the mean CRL of 4.3±0.7.Similarly, the high dose group (260 mg/kg) showed a significantly decreased mean number of somites than the control groups.Furthermore, rats given 260 mg/kg essential oil had a mean morphological score of 44.0±0.5. The scores for the pair-fed and *ad libitum* control groups were 46.1±0.2 and 45.8±0.3, respectively. The mean morphological score in pregnant rats given 260 mg/kg *T. schimperi* essential oil extract was significantly lower than the controlgroups**(Table 26)**.

Table 26: Embryonic growth following administration of the essential oil extract of Thymus schimperi.

Parameters	**Control groups**		**Treatment groups**		
	G-I (Pair fed control)n= 120	**G- II (*Ad libitum*)n= 116**	**G-III (EOT 65mg/kg)n= 109**	**G-IV (EOT 130mg/kg)n= 106**	**G-V (EOT 260mg/kg)n= 98**
Number of somites/litter	29.2±1.1	30.1±0.8	28.7±1.3	28.1±0.8	26.4±0.9[b]
CRL of the embryo (mm)	5.0±0.6	5.1±0.4	4.8±0.5	4.6±0.6	4.3±0.7[b]
Morphological score/litter	46.1±0.2	45.8±0.3	44.7±0.5	44.3±0.4	44.0±0.5[b]

CRL: Crown-rump length; Results are expressed as mean ± SDM. [b]: mean significantly different from pair-fed and *ad libitum* control, the p-value is <0.05. n (number of embryos). EOT: essential oil of Thymus schimperi.

5.6.1.3. Embryonic Body System Development

As shown in **Table 27 and Figure 22**, there was a significant change in the developmental parameters of the otic system and branchial bars.The growth score of the otic system of rat embryos in the middle dose (130 mg/kg)and high dose (260 mg/kg)treatement group of the essential oil was significantly lower as compared to the pair-fed and *ad libitum* control groups. Pair-fed and *ad libitum* control groups had a mean growth of 3.6±0.50 and 3.5±0.50, respectively. However, the score in the middle dose (130mg/kg) and high dose (260mg/kg) treatment groups were 3.1±0.41 and 3.0±0.40, respectively.Similarly, the growth score of the olfactory system of rat embryos from the middle dose(130 mg/kg) and high dose (260 mg/kg)were significantly lower than the pair-fed and *ad libitum* control groups. The scores were 0.9±0.70 and 0.8±0.62 in the pair-fed and *ad libitum* control groups, respectively. However, the scores in the middle and high dose treatment groups were 0.7±0.60 and 0.5±0.42, respectively.In addition, the high dose group (260 mg/kg) had a significantly lower mean number of branchial bars (2.9±0.33) as compared to the pair-fed (3.6±0.55) and *ad libitum* control groups

(3.5±0.52).However, there was no significant difference in the yolk sac circulation, embryo flexion, heart, caudal neural tube, hindbrain, midbrain, forebrain, optic system, maxillary process, mandibular process, forelimb, and hind limb developmental parameters between the treatment and control groups.

Table 27: In vivo development of rat embryo following treatment with essential oil extract administration of Thymus schimperi: Day 12 experiment.

Morphological endpoint	**G-I (Pair-fed control)**	**G- II (*Ad libitum*)**	**G-III (EOT 65mg/kg)**	**G-IV (EOT 130mg/kg)**	**G-V (EOT 260mg/kg)**
Number of fetus/group	120	116	109	106	98
Yolk sac circulation	3.8±0.51	3.7±0.50	3.60±0.49	3.62±0.50	3.66±0.51
Flexion	2.8±0.33	2.6±0.30	2.7±0.32	2.5±0.31	2.7±0.32
Heart	3.6±0.51	3.6±0.50	3.5±0.49	3.5±0.49	3.6±0.50
Caudal neural tube	4±0.00	4±0.00	4±0.00	4±0.00	4±0.00
Hind brain	3.6±0.50	3.6±0.50	3.5±0.48	3.5±0.48	3.5±0.48
Mid brain	3.56±0.50	3.6±0.50	3.5±0.49	3.4±0.47	3.4±0.47
Fore brain	3.7±0.47	3.7±0.47	3.7±0.47	3.6±0.46	3.6±0.45
Otic system	3.6±0.50	3.5±0.50	3.4±0.55	3.1±0.41[b]	3.0±0.40[b]
Optic system	3.60±0.50	3.6±0.50	3.5±0.47	3.4±0.46	3.5±0.47
Olfactory system	0.9±0.70	0.8±0.62	0.8±0.62	0.7±0.60[b]	0.5±0.42[b]
Branchial bars	3.6±0.55	3.5±0.52	3.4±0.50	3.4±0.57	2.9±0.33[a]
Maxillary process	1.6±0.54	1.5±0.51	1.4±0.49	1.3±0.48	1.3±0.48
Mandibular process	0.8±0.52	0.7±0.50	0.7±0.50	0.6±0.49	0.6±0.49
Fore limb	2±0.00	2±0.00	2±0.00	2±0.00	2±0.00
Hind limb	2±0.00	2±0.00	2±0.00	2±0.00	2±0.00

Statistical differences between the groups were analyzed by Duncan's multiple range tests. Results are expressed as mean ± SDM.[b]: mean significantly different from pair-fed and *ad libitum* control, the p-value is <0.05.

5.6.1.4. Percentage of Retarded Development

The percentage of developmental delays in the embryonic circulatory system, nervous system, musculoskeletal system, and craniofacial area were not significantly varied across all groups after administration of the essential oil extract (**Tables 28-30)**.However, there was a significant difference in developmental parameters such as the otic system, olfactory system, somite score, and branchial bars between the treatment and control groups.The development of the otic system was delayed by 17.3%in the high dose (260 mg/kg) treatment group, which was statistically significant as compared to the control groups. However, at the high dose (260mg/kg) treatment group, the percentages of yolk sac circulation and heart developmental retardations were 4.0% and 2.0%, respectively. However, Theyarenot statisticallysignificant **(Table 28)**.

Table 28: Embryonic circulatory and olfactorysystemsdevelopment following administration of the essential oil extract of Thymus schimperi.

	Percentage of retarded development (%)		
Group	**Yolk sac circulation**	**Heart**	**Otic system**
G-I (Pair-fed control) n=120	2.5	0.8	5
G- II (*Ad libitum*) n=116	1.7	0.8	4.3
G-III (EOT 65mg/kg n=109	1.8	2.5	6.4
G-IV (EOT 130mg/kg) n=106	3.8	0.8	11.3
G-V (EOT 260mg/kg) n=98	4.0	2.5	17.3

Results are expressed as a percentage (%) of retarded development (Chi-Square). n (number of embryos)

The development of the olfactory system was delayed by 35.7 % in the high dose(260 mg/kg) treatment group, which was statistically significant as compared to the control groups. However, the nervous system developmental parameters were not shown a statistically significant difference in the percentage of developmental retardation (**Table 29**).

Table 29: Development of the embryonic nervous system and sense organs after administration of Thymus schimperi crude extract. Experiment on Day-12

Group	**Percentage of retarded development(%)**					
	Caudal Neural tube	**Hind brain**	**Mid brain**	**Forebrain**	**Optic system**	**Olfactory system**
G-I (Pair-fed control) n=120	0	0	2.5	0.8	3.3	8.3
G- II (*Ad libitum*) n= 116	0	0	2.0	0.8	3.4	8.6
G-III (EOT 65mg/kg n= 109	0	1.8	2.7	1.8	2.8	13.8
G-IV (EOT 130mg/kg) n= 106	0	0.9	2.8	2.3	3.8	29.2
G-V (EOT 260mg/kg) n= 98	0	2.0	3.1	2.0	4.0	35.7

Results are expressed as a percentage (%) of retarded development (Chi-Square). n (number of embryos)

The development of brachial bars was delayed by 15.3 % in the high dose(260 mg/kg) treatment group, which was statistically significant as compared to the control groups. Similarly, in the high dose treatment group, the Somite score was delayed by 10.2 %, which is statistically significant as compared to the control groups.However, there was no statistically significant difference in the developmental retardation percentage of embryonic flexion, development of the maxillary process, mandibular process, forelimb, and hind limb (**Table 30**).

Table 30: Embryonic musculoskeletal system development following administration of the essential oil extract of Thymus schimperi.

Group	Percentage of retarded development (%)						
	Flexion	**Branchial Bars**	**Maxillary Process**	**Mandibular Process**	**Fore Limb**	**Hind limb**	**Somites score**
G-I (Pair-fed) n=120	0	3.3	3.3	4.1	0	0	5
G- II (*Ad libitum*) n=116	0	4.2	3.4	5.1	0	0	4.3
G-III (EOT 65 mg/kg n=109	0.9	5.5	4.6	5.5	0	2	6.4
G-IV (EOT 130mg/kg) n=106	0.9	5.5	5.7	7.5	0	2	6.6
G-V (EOT 260mg/kg) n=98	1.0	15.3	6.1	8.2	0	0	10.2

Results are expressed as a percentage (%) of retarded development (Chi-Square). n (number of embryos).

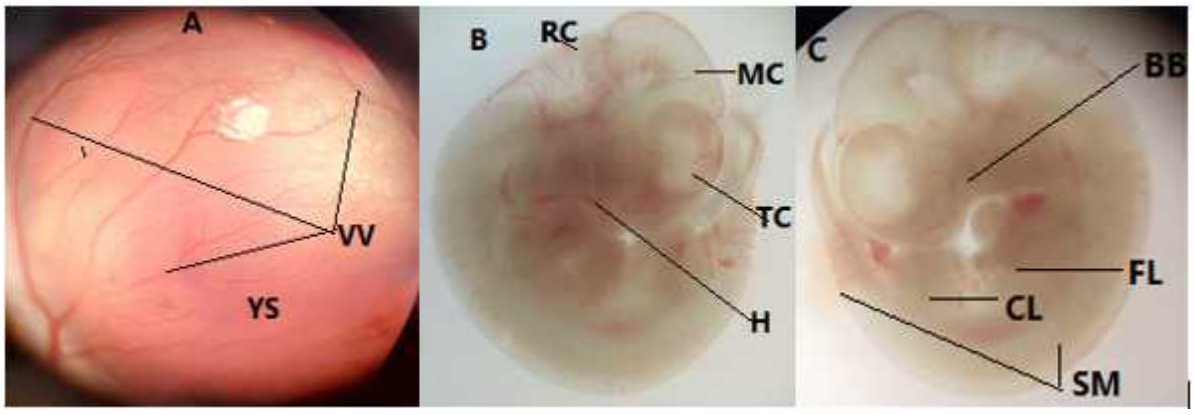


Figure 22:12-Day old rat embryos showing various organs of primordia. A: an embryo enclosed inside the intact yolk sac (YS) with vitelline vasculature surrounding it (VV). B. Yolk sac-free embryo, separated from the surrounding blood vessels, revealing the heart (H), telencephalon (TC), mesencephalon (MS), rhombencephalon (RC). C: Showing brachial bars (BB), forelimb bud (FL), somites (SM), and caudal limb bud (CL).

5.6.2. Day 20 Experiment

5.6.2.1. Pregnancy Outcomes

5.6.2.1.1. Maternal Food Intake and Weight Gain

The maternal food intake did not significantly vary between the treatment and control groups in the pre-treatment (days 1-5), during treatment (days 6-12), and post-treatment periods (days 13-20) (**Table 31)**

In the present study, the maternal weight gain in the highdose (260 mg/kg) treatement group was 3.32±0.56 g/week during the treatment period (day 6-12). The pair-fed and the *ad libitum* control groupsmaternal gain were 5.89±0.72 g/week and 5.96±0.61 g/week, respectively.During tratement (day 6-12) and post-treatment (day 13-20) periods, the maternal weight gain in the high dose (260 mg/kg) treatment group was significantly lower than in the pair-fed and *ad libitum* control groups **(Table 31).**

Table 31: Daily food intakes and maternal weight gains of rats in the day-20 experiment of essential oil of Thymus schimperi.

Groups	**Daily food intake (g/day)**			**Maternal weight gain (g/week)**	
	Day 1-5	**Day 6-12**	**Day 13-20**	**Day 6-12**	**Day 13-20**
G-I (Pair-fed control)	15.50± 0.16	15.91± 0.41	16.51± 0.42	5.96±0.61	16.01±0.75
G- II (*Ad libitum*)	15.30±0.07	15.70±0.13	16.45±0.51	5.89±0.72	16.62±0.80
G-III (65 mg/kg	15.16±0.17	15.53±0.11	16.20±0.22	5.72±0.81	15.70±0.50
G-IV (130mg/kg)	15.20±0.06	15.66±0.31	16.10±0.09	5.65±0.61	15.65±0.49
G-V (260mg/kg)	15.37±0.09	15.40±0.05	16.00±0.03	3.32±0.56[b]	9.41±0.32[b]

Results are stated as mean ± SDM. [b]: Results significantly different ($p < 0.05$) from both pair fed-control and *ad libitum* control groups.

In the current study,The fetal resorption of the high dose (260 mg/kg) treatment group was significantly increased as compared to the control groups. In the dose of 260 mg/kg, the mean

fetal resorption was 0.70±0.18. However, the mean number of fetal resorptions were 0.20±0.50 and 0.30±0.52 in the pair-fed and *ad libitum* control groups, respectively.

In this study, the number of fetuses, implantation sites, and live fetuses waslower in a dose-dependent manner across groups, but the differences were not statistically significant.Furthermore, the high dose (260 mg/kg) treatment group had a higher fetal death rate than the other groups. However, it was not statistically significant **(Table 32)**.

Table 32: Pregnancy outcomes of the day-20 essential oil of Thymus schimperi.

Groups	**No. of fetuses**	**Implantation sites**	**Number of resorptions/litter**	**Number of Live fetus/dam**	**Number of dead fetus/dam**
G-I (Pair-fed control)	96	9.8±0.74	0.20±0.50	9.40 ±0.80	0.2±0.03
G- II (Ad libitum)	98	10.1±0.89	0.30±0.52	9.50±0.86	0.30±0.04
G-III (65 mg/kg	86	9.0±0.70	0.40±0.38	8.20±0.74	0.40±0.05
G-IV (130mg/kg)	82	8.6±1.02	0.40±0.35	7.70±0.72	0.50±0.06
G-V (260mg/kg)	80	8.7±0.65	0.70±0.18[b]	7.40±0.71	0.60±0.07

Results are stated as mean ± SDM. [b]: Results significantly different ($p < 0.05$) from both pair fed-control and ad libitum groups.

5.6.2.1.2. Fetal Growth

In the current study, the high dose treatement group mean litter weight (2.71±0.11) wassignificantly lowerthan the pair-fedcontrol group (3.41±0.09g) and*ad libitum*(3.38±0.10g) control group.Furthermore, Themean growth score of the CRL was significantly lower in the high dose treatment group(2.91±0.18) than the pair-fed control (3.18±0.13) and the *ad libitum*control group (3.18±0.11).

Similarly, the placental weight was significantly lower in the high dose treatment group (0.48±0.05g) than the pair-fed control (0.57±0.06) and the *ad libitum* control group (0.57±0.06).

(**Table 33**).

Table 33: Mean fetal growth following treatment with essential oil extract of Thymus schimperi in the day-20 experiment.

Groups	**Fetal growth**		
	Litter weight/fetus(g)	**CRL/fetus (cm)**	**Placental weight/fetus (g)**
G-I (Pair-fed control)	3.41±0.09	3.18±0.13	0.55±0.07
G- II (Ad libitum)	3.38±0.10	3.18±0.11	0.57±0.06
G-II (65 mg/kg	3.34±0.09	3.13±0.15	0.55±0.06
G-III (130mg/kg)	3.25±0.12	3.08±0.13	0.55±0.06
G-IV (260mg/kg)	2.71±0.11[b]	2.91±0.18[b]	0.48±0.05[b]

Results are stated as mean ± SDM. [b]: Results significantly different ($p < 0.05$) from both pair fed-control and ad libitum groups.

5.6.2.1.3. External and visceral morphological anomalies

The explanted fetuses (**Figure 23**) were also examined for external malformations from head to tail in the essential oil developmental toxicity study. Craniofacial abnormalities, limb defects, vertebral column anomalies, missing tails, and external genital abnormalities were all investigated. Despite this, there were no treatment-related defects in near-term rat fetuses (**Table 34**).

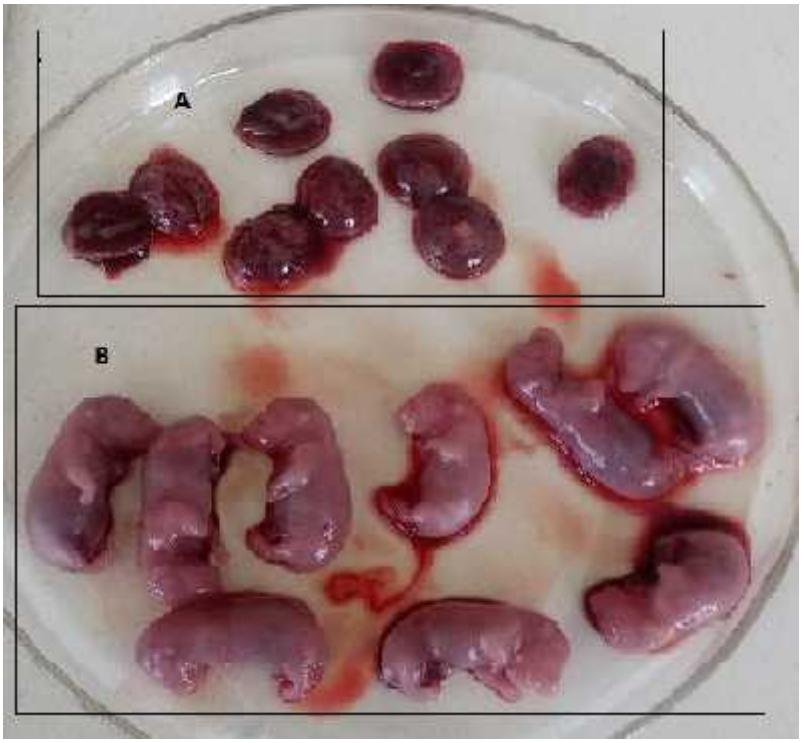


Figure 23: Placenta and Live rat fetuses from high dose rat group: A (Placenta of high dose rats) and B (fetuses of high dose groups).

Table 34: External malformations of rat fetuses after administration of essential oil extract Thymus schimperi.

Group	Fetus examined	Observed malformations (%)							
		AC	EC	SB	SC	KY	LD	MT	AEG
G-I (Pair-fed control)	96	0	0	0	0	0	0	0	0
G- II (Ad libitum)	98	0	0	0	0	0	0	0	0
G-III (65 mg/kg	86	0	0	0	0	0	0	0	0
G-IV (130mg/kg)	82	0	0	0	0	0	0	0	0
G-V (260 mg/kg)	80	0	0	0	0	0	0	0	0

The percentage of malformations is represented as a percentage of the total number of malformations (Chi-Square). AE-Anencephaly, EC-Exencephaly, SB Spina bifida, KY-Kyphosis, SC-Scoliosis, LD-Limb defect, MT-Missed tail, AEG-Agenesis of external genitalia.

After being fixed in Boiun's solution, the fetuses were serially sectioned for visceral soft tissue analysis. Serial sectioning was conducted on the head, neck, stomach, and abdomen. The parts were carefully examined under a dissecting microscope for any visceral abnormalities. The head area was investigated for cleft palate, hydrocephalus, and eye-related abnormalities. Thyroid, thymus, trachea, and cardiac septum abnormalities were also studied at the neck and chest stages. Diaphragmatic hernia, abdominal viscera agenesis, and external genitalia were also investigated. During the external morphological examination, no noticeable visceral abnormalities were identified (**Table 35 and figure 24**).

Table 35: Visceral malformations of rat fetuses following administration of the essential oil extract of Thymus schimperi.

Group	Fetus Examined	Observed malformations (%)										
		HC	**MO**	**AO**	**CP**	**NSD**	**REAA**	**VSD**	**DH**	**RA**	**HU**	**CT**
G-I (Pair-fed control)	50	0	0	0	0	0	0	0	0	0	0	0
G- II (*Ad libitum*)	50	0	0	0	0	0	0	0	0	0	0	0
G-III (65 mg/kg	50	0	0	0	0	0	0	0	0	0	0	0
G-IV (130 mg/kg)	50	0	0	0	0	0	0	0	0	0	0	0
G-V (260mg/kg)	50	0	0	0	0	0	0	0	0	0	0	0

Results are presented as a percentage of malformations (Chi-Square). HC-Hydrocephalus, MO-Microphthalmia, AO-Anophthalmia, CP-Cleft palate, NSD-Nasal septal defect, REAA-Retroesophageal aortic arch, VSD-Ventricular septal defects, DH-Diaphragmatic hernia, RA-Renal agenesis, HU- Hydroureters, and CT-Cryptorchid testes.

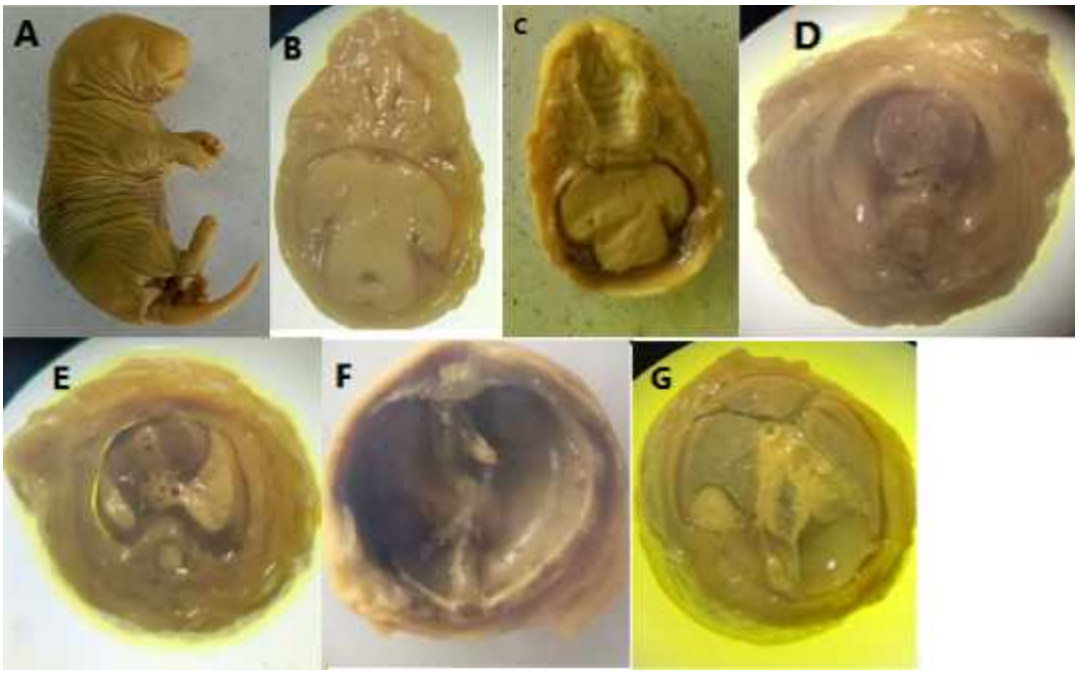


Figure 24: A 20-day old rat fetus fixed in Bouin's solution for visceral examination (260 mg/kg). A: un-sectioned fetus; B: transverse section of the brain showing normal brain tissue; C: normal palate; D: section made through the neck showing the normal esophagus, trachea, and thyroid gland; E: section through the chest showing normal; F: intact diaphragm and G: a section made though the abdomen showing normal visceral organs.

5.6.2.1.4. Skeletal Malformations

The developmental status of the skull, thoracic vertebrae, sternum, hyoid, and metatarsals were examined in this study, but no significant skeletal anomalies were found in either the treatment or control groups. However, the ossification centers of the caudal/coccygeal vertebrae and the hind limb phalanges differed slightly.The number of ossifications in the hind limb phalanges and caudal vertebrae was slightly reduced in the high-dose(260 mg/kg) group rat fetuses. It was, however, not statistically significant. There were developmental delays in the hindlimb phalanges in 28% of the rat fetuses from the high dose group (260mg/kg).However, the variation was not statistically significant. The skeletal analysis results are shown in **Tables 36, 37, and Figure 25**.

Table 36: Skeletal malformations of 20-day old rat fetuses following treatment with the essential oil extract of Thymus schimperi.

Group	Percentage of skeletal malformations (%)				
	Sternum[a]	Hyoid[b]	Ribs[c]	Thoracic vertebrae[d]	Caudal vertebrae[e]
G-I (Pair-fed control) n=50	12	0	0	0	8
G- II (*Ad libitum*)n=50	14	0	0	0	10
G-III (65 mg/kgn=50	16	0	0	0	12
G-IV (130 mg/kg)n=50	18	0	0	0	14
G-V (260 mg/kg)n=50	18	0	0	0	16

The percentage of skeletal malformations is calculated (Chi-Square). a: <4 ossification centers on the sternum; b: no ossification signs on the hyoid bone; c: no ossification signs on the ribs; d: <13 ossification centers on the thoracic centra; and e: <4 ossification centers on the caudal vertebrae.

Table 37: Skeletal malformations of 20 days old rat fetuses following treatment with the essential oil extract of Thymus schimperi.

Group	Percentage of skeletal malformations of limb bones			
	Metacarpus[a]	Metatarsal[b]	Forelimb phalanges[c]	Hindlimb phalanges[d]
G-I (Pair-fed control) n=50	6	4	10	16
G- II (*Ad libitum*)n=50	4	4	12	16
G-III (65mg/kg) n=50	6	6	12	20
G-IV (130 mg/kg)n=50	8	6	14	20
G-V (260mg/kg)n=50	8	6	14	26

The number of skeletal malformations is expressed as a percentage (%). (Chi-Square).[a]:<3 metacarpus; [b]: <3 metatarsus; [c]: no forelimb proximal phalanges; [d]: no hindlimb proximal phalanges.

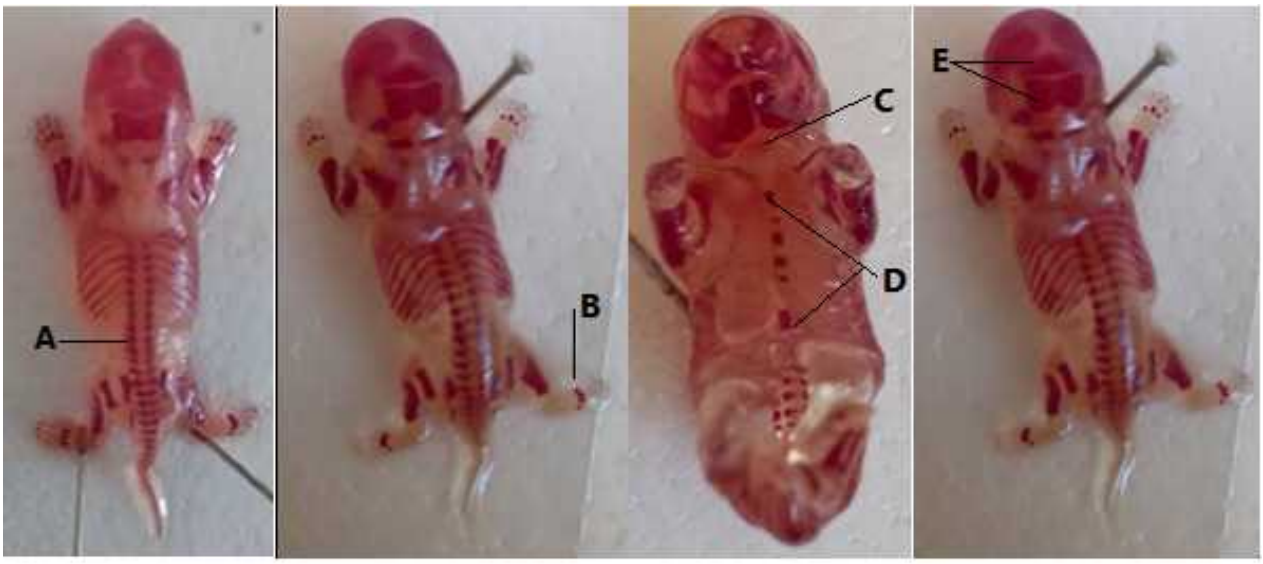


Figure 25: Alizarin red-stained rat fetuses showing different ossification centers. A-Vertebrae, B-Metatarsal, C – Hyoid, D- Sternebra, E- Supraoccipital and Inter-parietal.

5.6.2.1.5. Histopathological Analysis of Placenta

Gross anomaly was not observed during the external examination of a placenta in both treatment and control groups. But, there was a significant reduction in placental weight of pregnant rats that received a high dose (260 mg/kg) of essential oil extract of *T.schimperi* as compared to all the other groups. From the histological observation of sections of placenta after staining with Haematoxylin and Eosin, different zones of the placenta were observed. It shows that there was some structural change in the trophoblastic zone and labyrinthine zones of the placenta. Based on the table shown below, 8 % of terminal villi vessels were dilated in both 260 mg/kg essentialtreatement group, whereas in other groups there was no terminal villi dilatation in the trophoblastic zone of a placenta. In 260 mg/kg essential oiltreatment group, 10% of the placenta had an increase in a number of terminal villi vessels**(Table 38 and Figure 26).**

The placenta in high dose group whose mothers received 260 mg/kg of essential oil extract of *T.schimperi*showed multiple lesions that include decidual hypoplasia and atrophy, cytolysis, apoptosis and decidual necrosis in 5% of the placentas. Of 260 mg/kg essential oil treated

placenta, 15% had intervillous thrombosis, but it was not observed in the rest of essential oil treatment and control groups**(Figure 27).** There were no noticeable changes in the glycogen cells, spongiotrophoblast and trophoblastic giant cell in all of the treatment and control groups.

Table 38: placenta histopathology of 20 days old rat fetuses following treatment with the essential oil of Thymus schimperi.

Group	**Percentage (%) of placental abnormalities**			
	Capillary dilatation	**Terminal villi proliferation**	**Thrombosis in the intervillous spaces**	**Cellular apoptosis**
G-I (Pair-fed control)	2	0	2	2
G- II (*Ad libitum*)	0	2	4	2
G-III (65mg/kg)	5	0	0	4
G-IV (130mg/kg)	5	5	6	4
G-V (260mg/kg)	8	10	15	5

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

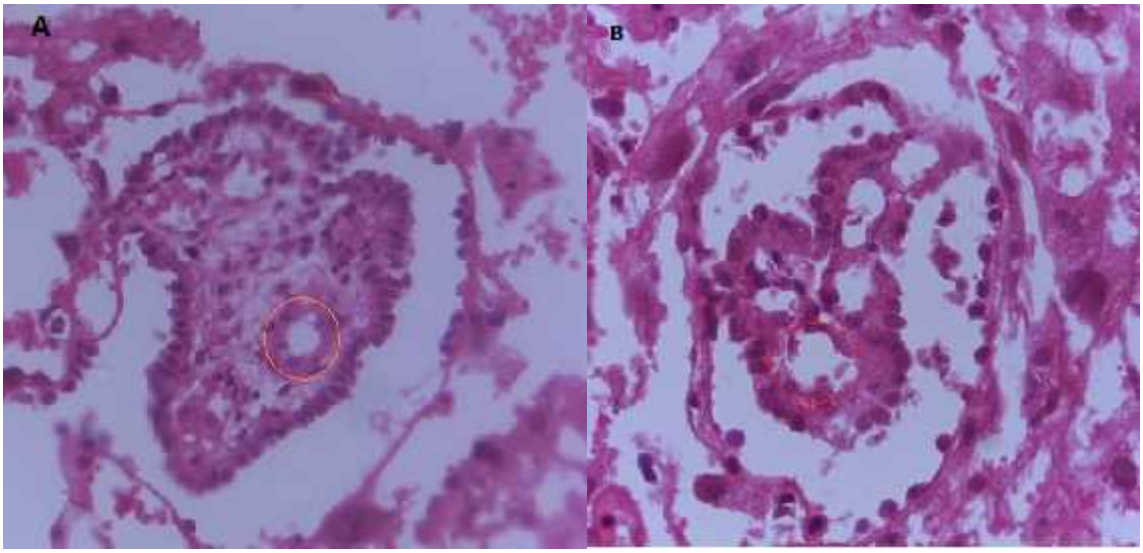


Figure 26:A; Photomicrograph showing pair-fed control and **B;** markedly increased sign of revascularization and capillary dilatation (arrows) in 260 mg/kg essential oil-treated placenta; H and E stain, 40x total magnification

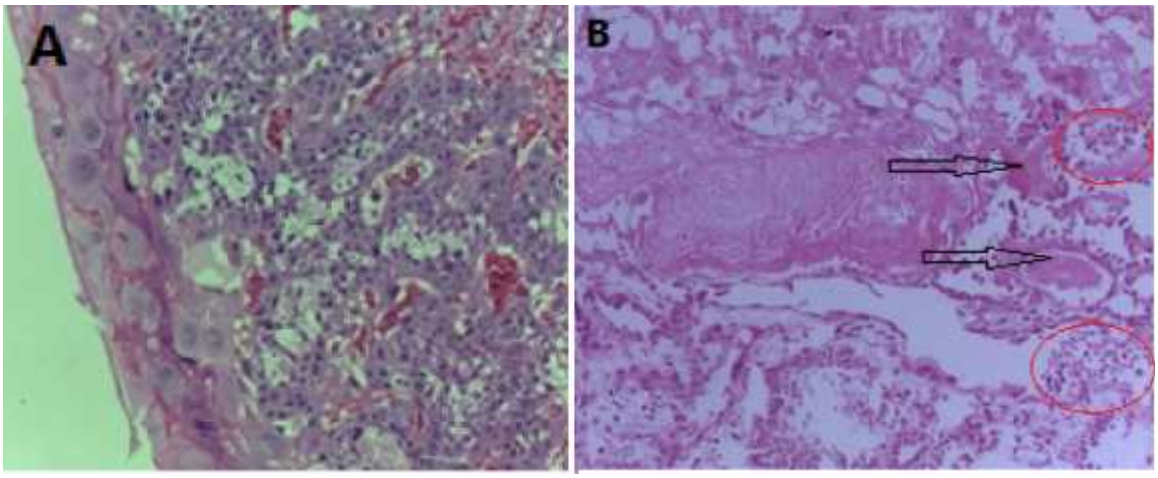


Figure 27:A; Photomicrograph showing normal intervillous space in pair-fed control and **B;** in 260 mg/kg essential oil treated placenta thrombosis (arrows) and trophoblast proliferation (circles); H and E stain, 40x total magnification.

CHAPTER SIX

6. DISCUSSION

Various medicines of herbal origin have widely been used around the world as primary therapies for various diseases (164). Safety is checked by conducting general preclinical toxicity experiments to detect potential toxic effects of any drug, primarily in the liver and kidneys of animals (104). If these organs are found to be mildly inflamed and damaged, the cell membrane permeability will significantly increase releasing cytoplasmic enzymes such as ALP and AST in the blood. Similarly, inflammation results in the release of mitochondrial ALT and AST (105, 106). Models for the toxicity screening give valuable preliminary data which can help to identify natural remedies with possible health benefits (165).

6.1. Composition of Essential oil of T. schimperi

Percentage yield of the essential oil *T. schimperi* aerial part obtained by hydrodistillation was 1.39% (v/w). The oil was analyzed by GC-MS which identified 57 compounds representing 88.75% of the total oil. The major constituents were carvacrol, thymol, *O*-cymene and α-terpinene. The chemical composition of the oil was similar to the one previously reported by Asfaw *et al*. (62), which identified p-cymene, γ-terpinene, thymol and carvacrol as major components of the oil.

6.2. Acute Toxicity of the Essential oil

In this study, using probit analysis, the LD_{50} of *T. schimperi* essential oil was found to be 1284.2 mg/kg. This level of LD_{50} is considered moderately hazardous, in oral use, as per WHO suggestions of pesticides guidelines (166). This finding was slightly lower than the report from a study conducted in Debre Berhan, Ethiopia, (LD_{50} value of 2000 mg/kg) (102). The possible justification for this slight discrepancy could be due to the animal model difference that in this study Wistar albino rats were used while mice were used in the previous study. The present acute toxicity study also revealed that *T. schimperi* essential oil-induced hypoactivity, piloerection, convulsion, and irregular body movements in the tested animals. This finding is consistent with a study done by Dires *et al.* (102), which reported that administration of a single oral dose of the

essential oil of *T. schimperi* causes signs of toxicity, such as hypoactivity, piloerection, and convulsion that may have resulted from disruptions in the activity of the autonomic nervous system (ANS) and the central nervous system (CNS). In an acute toxicity study, *T. schimperi* essential oil induced a substantial drop in body weight at higher doses which may be linked with the adverse symptoms causing the rats to become anorectic (167). In the current study, the increment in the weight of the kidneys and the liver is most likely due to edema (168).

6.3. Sub Acute Toxicity

The hematological system is susceptible to toxic chemicals and can be used as a significant index for detecting human and animal physiological changes (169). Hematological tests can quickly show physiological changes in the body, and the blood sample usually provides valuable information on the body's reaction to injury or disease, hunger, and stress (170). The extent of the toxic effect of drugs and/or plant extracts can therefore be determined by evaluation of hematological parameters (171).

In the current study, there was a significant decrease in the mean white blood cell (WBC) count at a dose of 260 mg/kg as compared to the rats in the control group. This could be due to the effects of the major bioactive compounds in *T. schimperi* essential oil, like carvacrol and thymol, and which could cause cell cycle arrest in the sub G0/G1 phase, cellular apoptosis, and cell proliferation (90, 91). There was also an increment in the mean corpuscular volume (MCV), the index that helps to determine the size of erythrocytes, at a dose of 260 mg/kg. This could be because any substance that affects cellular DNA biosynthesis, either directly or indirectly can cause macrocytic changes. MCV elevation is a sign of alterations in DNA biosynthesis (172).A previous study indicated that carvacrol inhibits DNA synthesis (92).

In the toxicological assessment, biochemical parameters play a significant role as markers due to their response to clinical signs and symptoms caused by toxicants. Assessment of liver and kidney function has paramount importance to determine the toxic properties of extracts and drugs (173). In the present study, treatment of the animals with *T. schimperi* essential oil did not result in a significant change of all biochemical parameters. Any damage to the liver causes both

ALT and AST to rise in the blood and could be taken as the first sign of the damage (173). Creatinine level is known as a strong measure of renal function. An increase in creatinine means that there is noticeable harm to functioning nephrons (173, 174). AST is primarily found in red blood cells, cardiac and skeletal muscles, and the kidneys. AST is not as specific to the liver as ALT. In the present study, the mean values of ALT and ALP in treatment groups increased, while AST decreased across treatment groups compared to control, however, the changes were not statistically significant. This result was found to be consistent with a reported data from a similar study previously conducted on the same plant from Ethiopia (102). In addition, the constituents of the essential of *T. schimperi* did not show any cardiac toxicity (h-ERG Blocker), AMES (Ames Mutagenicity), and cytotoxicity by ADMET and vNN-ADMET toxicity prediction servers. Another justification for these results might be that there are very few compounds that can cause hepatotoxicity or drug-induced liver injury, as revealed by *in-silico* toxicity studies (8.8% of the total compounds were hepatotoxic and only 3.5% were caused by drug-induced liver injury and only thymol had potential toxicity to the mitochondrial membrane toxicity).

Plasma urea measurement has been used for many years as a marker of renal function. Plasma urea is usually increased in acute and chronic kidney disease. Urea removal falls as the kidney fails and, as a result, urea tends to accumulate with diseased kidneys that are unable to excrete these substances at normal rates; this will increase the level of urea in the blood (173, 175). The average adult rat serum urea was measured approximately 15-45 mg/dl (176). In the present study, mean urea values were shown to be slightly increased at doses of 65 mg/kg and 260 mg/kg, although not significant and were not associated with histopathological changes in the kidneys.

Creatinine is formed in an endogenous manner and released at a constant rate into body fluids, and its plasma concentration is mainly controlled by glomerular filtration. As a result, both plasma concentration and its renal clearance were used as measures of the glomerular filtration rate (177). The mean amount of creatinine in the current study showed a slight increase but was not significant. In adult rats, the reference value for creatinine is around 0.2–0.8 mg / dL (178).

The measurement in this analysis was within the reference value and was supported by a lack of histopathological changes in the kidneys.

The increment of total serum protein is caused by a change in plasma water volume and an increase in plasma concentrations of one or more different proteins. Decreased plasma water volume is observed in cases of dehydration due to inadequate water intake or excessive water loss, such as severe vomiting or diarrhea (177). The standard value of total protein in adult rat serum is 5.6–7.6 mg / dL (176). Throughout the treatment groups, the overall protein levels were slightly higher as compared to the control, but it was not statistically significant. The mean total protein values for rats were within the normal range.

Lipid profile is the term given for the evaluation of total cholesterol, triglycerides, lipoproteins of high density (HDL), and lipoproteins of low density (LDL). This test is commonly used to diagnose hyperlipidemia, a risk factor for heart disease (179). However, the results of this study did not show a significant change in any of the components listed above. This finding was also supported by the result that all compounds extracted from *T. schimperi* essential oil were found to be free of h-ERG Blocker (cardiac toxicity) through ADMET and vNN-ADMET toxicity prediction servers.

The electrolytes found in blood and other body fluids are sodium and potassium. They help maintain the body's water and electrolyte balance and are also important for the proper functioning of the nerves and muscles. The hormone aldosterone controls the levels of sodium and potassium in the body. These electrolytes do not have significant changes based on the findings of this study. They are also within the normal range, in both treated as well as in control groups.

Histopathological evaluations provide information on biochemical and hematological parameters to be improved (180). Compared to controls, the general architecture of the liver, the appearance of the hepatocytes, the hepatic sinusoids, portal triads, and central veins are normal. Furthermore, compared to the control, the general histological architecture was not compromised in any of the treatment groups. The histopathological parameters of the liver had no significant change between the test animals and control animals after 4 weeks of treatment indicates that the

essential oil did not cause adverse toxic effects or hepatic damage to the liver and this result is consistent with other studies (58, 102).

In kidney histopathology analysis, rats treated with the essential oil showed no significant difference compared to controls. The sections of the treated rat kidneys displayed normal general renal structure and the regular presence of glomeruli and tubules. The proximal tubules, the distal tubules, and the macula densa were normal. The finding was further confirmed by the values of the blood's biochemical parameters (such as urea, creatinine, and total protein), which are the principal markers of kidney damage (181). This was consistent with the previous study, which stated that there was no difference in tissue morphology between the control group and treatment groups (102).

6.4. In-silico Toxicity

In addition to the *in vivo* toxicity study on animal models, the toxicity profile of all the compounds of *T. schimperi* essential oil was also evaluated by Protox II, ADMET and vNN-ADMET servers (45, 182). Toxicity and toxicological endpoint findings showed that all compounds derived from *T.schimperi* essential oil were free of h-ERG Blocker (cardiac toxicity), AMES (Ames Mutagenicity), and cytotoxicity. Regarding the hepatotoxicity parameters, most of the compounds (91.2%) did not show any toxicity while 8.8% of the compounds showed hepatotoxicity. Compounds that have hepatotoxicity effects are trans-sabinol, methyl m-tolylcarbinol, 2-isobutylideneamino-3-methyl butyronitrile, 10-ol limonene, and β-atlantol. Most of the compounds (96.5%) were safe for DILI (Drug-induced liver injury). However, 3.5% of compounds (thymol acetate and carvacrol acetate) have shown DILI toxicity. Similarly, 79.0% of the compounds did not show carcinogenicity while 21.0% of the compounds have shown carcinogenicity. In addition, about 3.5% of compounds (β-Bourbonene and gamma – Amorphene) are immunotoxic. Besides, most of the compounds have not shown mitochondrial membrane potential (MMP) toxicity, except thymol. From the total 57 compounds, predicted LD_{50} revealed that 6 compounds (10.5%) have toxicity class three ($50 \leq LD50 \leq 300$), 19compounds (33.4%) have toxicity class four ($300 < LD50 \leq 2000$), 30compounds (52.6%) have toxicity class five ($2000 < LD50 \leq 5000$), and 2 compounds (3.5%) have toxicity class six ($LD50 > 5000$).

6.5. Developmental Toxicity

Although some biologically active substances found in medicinal plants are teratogenic, the vast majority of them have often been used in various forms in the community(183). Toxic agents can either directly affect the embryo/fetus or indirectly affect the mother and enter the embryo/fetus via the placenta, or they can be a combination of both (184).

In the 20-day experiment, maternal weight gain was significantly reduced in a dose-dependent manner in the treatment and post-treatment periods in both essential oil and aqueous crude extract experiments.This finding is in line with a previous study by Abebe et al., 2021, which found that *T. shimperi* inhibits weight gain in experimental rats(102).The principal components of the thymus schimperi, carvacrol, and thymol could be a possible explanation for this finding. Dietary carvacrol and thymol were reported to inhibit weight gain in animals via affecting lipid metabolism, with carvacrol having the ability to lower plasma triglycerides(185).

In the current study, treatment groupsexposed to high doses of aqueous crude and essential oil extracts had a higher incidence of fetal resorptions than the control groups.Because this study was conducted with crude extracts, it is difficult to determine which plant components increase the resorption number. However, the increment could be due to the effects of *T. schimperi* major phytochemicals, such as carvacrol and thymolwhich could cause cell cycle arrest in the G0/G1 phase, cellular apoptosis, and inhibition of cell proliferation (90, 91).All these are the basic functions of cells for zygote growth. Similarly, the number of implantation sites in the high-dose treatment group of aqueous crude extract of *T.schimperi*was significantly lower than the control groups.This result is comparable to a previous report by Prakash et al., who noted that treating rats with some native plants has an anti-implantation activity(186).The disruption of hormone secretion, which plays an important role in embryo implantation, could be influenced by monoterpenoids components of essential oils particularly carvacrol, which can disrupt progesterone secretion by changing the neurochemical and neurobehavioral profiles of rats (187).Another possible explanation is that carvacrol, one of the most important essential oil constituents, inhibits prostaglandin synthesis(188). This is crucial for the embryo's interaction

with the endometrium such as implantation (apposition, adhesion/attachment, invasion/penetration) and decidualization(189).

The number of somites, morphological score, litter weight, and CRL were evaluated to assess the growth of embryos and fetuses. Morphological scores are used to predict the embryo's growth in the invivostudy since they have a linear relationship with embryonic age(163).In the current study, all of these parameters were significantly lower at the high doses of both the aqueous crude and essential oil extracts as compared to the control groups.In line with this study, studies conducted in Ethiopia found that high doses of *Syzygium guineense* (Myrtaceae) and *Moringa stenopetala* significantly lowerthe number of somites, morphological score, litter weight, and CRL of rat embryos and fetusesas compared to the controls(35, 36).This could be because of the fact that the plants contain alkaloids, and exposing a developing embryo or fetus to alkaloids from plants, plant products, or plant extracts has shown developmental defects in an animal study (34).

The development of the circulatory system, musculoskeletal system, nervous system, and craniofacial region in 12-day old rat embryos was assessed using a dissecting microscope in both aqueous crude extract and essential oil extract of *T.schimperi* studies. At high doses of both crude aqueous and essential oil extracts, there was a significant retardation of development in the otic system, olfactory system, and branchial bars. In addition, the maxillary and mandibular processes significantly delayed in crude aqueous extract of *T. schimperi*.This finding is similar to the study conducted in Ethiopia (36)that reported developmental delays in the otic system, olfactory system, branchial bars, maxillary and mandibular processes in pregnant rats exposed to high doses of methanolic extracts of seeds of *Moringa stenopetala.*This could be because of the presence of alkaloids in Moringa stenopetala seed extract and *T. schimperi*, which can cause developmental delays(34).Another probable explanation would be that *T. schimperi* major phytochemicals, such as carvacrol and thymol hinder cellularproliferationand migration(90, 91)which are basic cellular activity for organ system development.

External morphological analysis of 20-day old rat fetuses revealed no apparent anatomical malformations or treatment-related abnormalities in the cranial, nasal, oral, and visceral organs in both the aqueous and essential oil extract of *T.schimperi* studies. Consistent with our

findings,the previous studies on this plant acute, subacute, and chronic toxicity found to be nontoxic (58, 102).As a result, at doses administered, the *T.schimperi*aqueous and essential oil extracts have no teratogenic effect on the soft tissue of rat fetuses.

During the late fetal phase, many of the bones in rats ossify. As a result, the level of bone ossification is a significant measure of fetal maturity in developmental toxicity studies(159, 190). In the current study, the ossification centers of fetal skull, vertebrae, hyoid, and forelimb, and hindlimb bones did not differ significantly between the treatment and control groups on both the aqueous crude and essential oil extract of *Thymus schimperi*. The number of ossification centers in the caudal vertebrae and hind limb phalanges was lower when the essential oil extract was given in higher doses than then control groups. However, none of them was statistically significant, implying that the test plant has no effect on fetal skeletal growth.

The placenta is a temporary organ that exists only during pregnancy and helps to nourish and protect the fetus before it is born(191). To promote fetal growth, it obtains its metabolic, immunological(192), and secretory functions. The placenta attaches to the uterus, and the umbilical cord connects the fetus to the placenta(193).

In both crude and essential oil extracts experiments, placental weight and fetal weight were significantly decreased.The possible justification for this could be due to *T. shimperi* contains active components such as terpenoids, carvacrol, thymols, o-Cymene, α-terpineneand linalool(194)that can cross the placenta and may affect the placenta and fetal development. In addition, histopathological changes such as decidual cystic degeneration, thrombosis in the intervillous spaces, and decidual cellular apoptosis dose dependently increased. In the essential oil extract experiment, capillary dilation and terminal villi proliferation similarly increased dose-dependently. However, none of the aforementioned changes were statistically significant.

6.6. Conclusion

The yield of the essential oil from the aerial part of *T. shimperi* was found to be 1.39% v/w. GC-MS study of the oil enables the identification of 57 compounds.Carvacrol was the major

component of the essential oil, representing 49.90% followed by thymol (10.64%). Acute toxicity study showed that the LD_{50} of the oil was 1284.2 mg/kg.Similarly, sub-acute toxicity study demonstrated that the oil of *T. schimperi* does not adversely affect body weight, biochemical, and most hematological parameters at the tested doses, although the WBC count was significantly decreased and the MCV was significantly increased at a dose of 260 mg/kg. Besides, there were no signs of toxicity shown in the kidney and liver sections of the treated rats. All constituents of the essential oil of *T. schimperi* did not show any cardiac toxicity (h-ERG Blocker), AMES (Ames Mutagenicity), and cytotoxicity via Pro Tox II, ADMET, and vNN-ADMET toxicity predictors. However, by using these servers, from the total 57 compounds, around 21% showed carcinogenicity, 8.8% showed hepatotoxicity, 3.5% caused drug-induced liver injury, 3.5% showed immunotoxicity, and only 1.75 % were potentially toxic to the mitochondrial membrane.Furthermore, the results of the developmental toxicity test show that administration of a high dose (2000 mg/kg) *T. schimperi* aqueous crude extract caused significant delays in fetal and embryonic development, a decrease in the number of implantation sites, and an increase in fetal resorption, suggesting its developmental toxicity. Similarly, higher doses of *T. shimperi* crude aqueous extract resulted in a significant reduction in maternal weight gain, placenta weight, and litter weight. Moreover, at doses of 260 mg/kg, *T. schimper* essential oil extract is toxic to rat embryos and fetuses.Because of its toxicity, it was found to cause significant delays in embryonic and fetal development as well as an increase in fetal resorptions. As a result, consuming too much *T.schimperi* during pregnancy may be harmful.

6.7. Strengths and Limitations of the Study

The current study provided a variety of evidences regarding the toxicity of compounds in *T.schimperi*, assessing the toxicity profiles of 57 compounds. It also provides evidence on the developmental toxicity of *T.schimperi* in crude aqueous and essential oil extracts. This study also fills a known gap in modern science regarding *T.schimperi* insilico and developmental toxicity. However, it is not without limitations. The first limitation was that, due to time and financial constraints; chronic toxicity, the first and second-generation reproductive study were not included. The second limitation was that due to financial constraints, advanced tests such as immunohistochemistry and electron microscopy were not included.

6.8. Recommendations

The following recommendations are based on the results of this thesis study:

- Chronic toxicity study of the essential oil of *Thymus chimperi* is recommended.
- Toxicity study of toxic compounds by inslilico toxicity analysis should be tested separately.
- Further toxicity studies should be conducted by including additional organs such as: testes, ovary, pancreases, intestine, lung, stomach, and others.
- The developmental toxicity of *T. schimperi* should be investigated further by administering the plant extract throughout the entire gestation period.
- Expanded first-generation and second-generation reproductive toxicity studies should be carried out.
- Further research should be done using advanced technology to isolate and classify the active ingredient in the essential oil, as well as to investigate the mechanism of action.
- Experiments with non-rodent animals should be conducted.
- The use of high doses of *T. shimperi* in the community should be prohibited, particularly, during pregnancy.
- In collaboration with traditional therapists, the Minister of Health and EPHI should set regulatory standards and treatment doses.

CHAPTER SEVEN

7. REFERENCE

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8. APPENDIX

8.1. Preparation of Solutions Used in the Experiment

1. Bouin's solution:

• 75% picric acid

• 25% formalin

• 5% glacial acetic acid

2. Mall's solution:

• 79% distilled water

• 20% glycerol

• 1% potassium hydroxide (KOH)

3. Formalized saline

• Sodium bicarbonate (5 g) in 99 ml normal saline

• 1 ml formalin (40%)

8.2. Tissue Processing Procedures

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

1. Fixation:

• 10% formalin overnight

2. Dehydration:

• 40% alcohol- 1:30hrs

• 70% alcohol- 1:30hrs

• 80% alcohol- 1:30hrs

• 90% alcohol- 1:30hrs

• Absolute alcohol I- 1:30hrs

• Absolute alcohol II- 1:30hrs

• Absolute alcohol I- 1:30hrs

3. Clearing:

• Xylene I- 1:30hrs

• Xylene II- 1:30hrs

4. Infiltration:

• Paraffin wax I-1:30hrs

• Paraffin wax II-1:30hrs

• Paraffin wax III-1:30hrs

5. Embedding:

• With melted paraffin wax in embedding cassette, temperature (58-62 oC)

6. Sectioning:

• With Leica rotatory microtome, 5 µm section was made

• Ribbons were allowed to float in warm water (45 oC) and spread on the forested slide. The slides were dried on the hot plate (50-55 oC) for 20-30 minutes

8.3. Hematoxylin and Eosin (H & E) Staining Protocol

1. Dewaxing:

• Xylene I- 5minutes

• Xylene II- 5minutes

• Xylene III- 5minutes

2. Rehydration:

• Absolute alcohol I- 2minutes

• Absolute alcohol II- 2minutes

• 90% alcohol I- 2minutes

• 80% alcohol I- 2minutes

• 70% alcohol I- 2minutes

• Running tap water- 2minutes

3. Staining with Hematoxylin:

• 5-12 minutes with frequent agitation

4. Bluing:

• Running tap water- 8-10minutes

5. Differentiation:

• 1% acid alcohol

6. Counterstain with eosin:

• Eosin Y solution – 1-2 minutes

• Washing with running tap water- 2 minutes

7. Dehydration:

• 95% alcohol I- 2minutes

• Absolute alcohol I- 2minutes

• Absolute alcohol II- 2minutes

8. Clearing:

• Xylene I and II, 2 minutes in each

8.4. Morphological scoring chart (Brown and Fabro, 1981)

Variable type	Scores	Score descriptions	Mark
Yolk sac circulatory system	0	No visible or scattered blood island	
	1	Corona of blood islands with or without anastomosis	
	2	Vitelline vessels with few yolk sac vessels	
	3	Full yolk sac plexus of vessels	
	4	Yolk sac obliterated, vitelline artery and vein well separated	
Flexion	0	Ventrally convex	
	1	Turning	
	2	Dorsally convex	
	3	Dorsally convex with spiral torsion	
Heart	0	Endocardial rudiment not visible or visible but not beating	
	1	Beating S-shaped cardiac tube	
	2	Convoluted cardiac tube	
	3	Bulbus cordis, atrium commune and ventriculus communis	
	4	Dividing atrium commune	
Caudal neural tube	0	Neural plate or neural folds	
	1	Closing but unfused neural folds (groove)	
	2	Neural folds fused at level of somites 4/5	
	3	Posterior neuropore formed but open	
	4	Posterior neuropore closed	
Hind brain	0	Neural plate	
	1	Rhombomers A and B	
	2	Anterior neuropore formed but open	
	3	Anterior neuropore closed rhombencephalone formed	
	4	Pronounced pontain flexure with transparent roof of fourth ventricle	

Mid brain	0	Neural plate	
	1	Mesencephalic brain folds	
	2	Closing or fusing mesencephalic folds	
	3	Completely fused mesencephalone	
	4	Visible bivision between mesencephalone and diencephalone	
Forebrain	0	Neural plate or no visible procencephalone	
	1	Procencephalic brain folds	
	2	Completely fused procencephalone	
	3	Visible telencephalic evaginations	
	4	Well elevated telencephalic hemisphere	
Otic system	0	No sign of otic development	
	1	Flattened or indented otic primordium	
	2	Otic pit	
	3	Otocyst	
	4	Otocyst with dorsal recess	
	5	Otocyst with endolymphatic duct	
Optic system	0	No sign of optic development	
	1	Sulcus opticus	
	2	Elongated optic primordium	
	3	Primary optic vesicle with open optic stalk	
	4	Indented lense plate	
	5	Lense pocket or lense vessicle	
Olfactory system	0	No sign of olfactory development	
	1	Olfactory plate	
	2	Olfactory plate with rim	
	3	Distinct olfactory ridges	
	4	Lateral nasal process and medial rim	
Branchial bars	0	None visible	

	1	I visible	
	2	I and II visible	
	3	I, II and III visible	
	4	II overgrowing and obscuring III	
Maxillary process	0	No sign of maxillary development	
	1	Maxillary process demarcated, visible cleft anterior to bar I	
	2	Maxillary process fused with nasal process	
Mandibular process	0	No sign of mandibular development from bar I	
	1	First brachial bars fused and formed mandibular process	
Fore limb	0	No sign of forelimb development	
	1	Distinct evagination of wolffian crest at level of somite9-13	
	2	Fore limb bud	
	3	Paddle shaped forelimb bud	
	4	Distinct apical ridge on fore limb bud	
Hind limb	0	No sign of hind limb development	
	1	Distinct evagination of wolffian crest at level of somites 26-30	
	2	Hind limb bud	
	3	Paddle shaped hind limb bud	
Somites	0	0-6	
	1	7-13	
	2	14-20	
	3	21-27	
	4	28-34	
	5	35-41	

CRL_____

8.5. Skeletal Scoring chart developed by (Aliverti et al., 1979)