ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCE
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DEPARTMENT OF ANATOMY

Evaluation of Acute and Subacute Dermal Toxicity of Cymbopogon nardus and Eucalyptus globulus Extract Formulation on Hematological, Biochemical Parameters, and Histopathology of Skin, Kidneys, and Liver of Albino Wistar Rats

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Table of Contents

LIST OF ABBREVIATIONS AND ACRONYMS

AAU	**ADDIS ABABA UNIVERSITY**
AHRI	**ARMAUER HANSEN RESEARCH INSTITUTE**
ALB	**ALBUMIN**
ALP	**ALKALINE PHOSPHATASE**
ALT	**ALANINE TRANSAMINASE**
ANOVA	**ANALYSIS OF VARIANCE**
AST	**ASPARTATE AMINOTRANSFERASE**
CHS	**COLLEGE OF HEALTH SCIENCE**
DEET	**N, N-DIETHY-META-TOLUAMIDE**
EDTA	**ETHYLENE DIAMINE TETRA ACETIC ACID**
EPA	**ENVIRONMENTAL PROTECTIVE AGENCY**
GRAS	**GENERALLY RECOGNIZED AS SAFE**
HCT	**HEMATOCRIT**
HGB	**HEMOGLOBIN**
IGE	**IMMUNOGLOBULIN E**
LYM	**LYMPHOCYTE**
LD50	**LETHAL DOSE KILLS 50 % OF THE ANIMALS**
MCHC	**MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION**
MCV	**MEAN CORPUSCULAR VOLUME**
OECD	**ORGANIZATION OF COOPERATIVE ECONOMIC DEVELOPMENT**
Pg	**PICOGRAM**
PLT	**PLATELET**
PLC	**PLATELET COUNT**
RBC	**RED BLOOD CELL**
SEM	**SCANNING ELECTRON MICROSCOPY**
SPSS	**STATISTICAL PACKAGE FOR THE SOCIAL SCIENCES**
TM	**TRADITIONAL MEDICINE**
WBC	**WHITE BLOOD CELL**
WHO	**WORLD HEALTH ORGANIZATION**

List of Tables

List of figures

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ABSTRACT

Background: Mosquito-borne diseases, such as malaria, remain a major public health concern in Ethiopia and elsewhere globally. While synthetic repellants are effective, their repeated use has been associated with skin irritation, allergic reactions, and environmental concerns. Natural repellents, like essential oils from *C. nardus* and *E. globulus*, offer promising repellent and therapeutic advantages. However, the dermal safety of their combined use has not been scientifically evaluated.

Objectives: To evaluate the acute and subacute dermal toxicity of a combined *Cymbopogon nardus* and *Eucalyptus globulus* formulation on hematological, biochemical parameters, and histopathology of the skin, kidney, and liver of albino Wistar rats.

Methodology: The plant material was harvested about 270 kilometers south of Addis Ababa, in the Wondogenet region. The formulation was prepared by the Traditional and Modern Medicine Research Directorate of the Armauer Hansen Research Institute (AHRI). The essential oils were extracted using hydro-distillation with a Clevenger-arm apparatus. The approach outlined by the Organization for Economic Cooperation and Development (OECD) guideline 402 was used to determine acute dermal toxicity, and OECD guideline 410 was followed for the 28-day repeated dose subacute dermal toxicity study. Forty albino Wistar rats aged 8-12 weeks were utilized and randomly divided into four groups: three test groups and one control group. Each group consisted of five male and female rats (n=10 per group). Group I received 250 mg/kg, Group II received 500 mg/kg, and Group III received 1000 mg/kg of the *Cymbopogon nardus* and *Eucalyptus globulus* essential oil formulation. Group IV received only the vehicle (white petrolatum) and served as the control. The formulation was topically applied once daily to approximately 10% of the shaved dorsal skin surface for 28 consecutive days. Hematological and biochemical markers were assessed at the end of the experiment. After blood collection via cardiac puncture under sodium pentobarbital anesthesia (150 mg/kg), the liver, kidneys, and skin were harvested for histopathological examination.

Result: The dermal mean lethal dose LD_{50} of the *C. nardus* and *E. globulus* formulation was found to be above 5000mg/kg, placing the formulation in GHS category 5 (Lowest toxicity). No mortality or clinical sign of toxicity was observed in either study. There were no significant differences ($P>0.05$) in body weight gain, food intake, and water intake, hematological indices, serum biochemical markers, or relative organ weights between treated and control groups. Histopathological examination revealed normal architecture of liver, kidneys, and skin across all dose groups.

Conclusion: The formulation containing *Cymbopogon nardus* and *Eucalyptus globulus* essential oils exhibited no acute or sub-acute dermal toxicity at doses up to 1000mg/kg, suggesting a wide safety margin for short-term dermal application.

Key words: *Cymbopogon nardus*, *Eucalyptus globulus*, Acute toxicity, Subacute toxicity, Dermal, Wistar rats

1. INTRODUCTION

1.1. Background

The use of traditional plant-based repellents and medicinal products has been an integral part of human societies for centuries. These early practices laid the foundation for continued use of plant derived products as botanical insecticides. Currently, major botanical insecticides, including "pyrethrum, rotenone, neem, and essential oils" are widely utilized for pest control (1).

Essential oils are acquired through steam or hydro-distillation from different plant parts, including "leaves, flowers, fruits, roots, and wood" (2). These volatile oil compounds have broad commercial applications in "pharmaceuticals, food flavoring, fragrances, and insecticides"(3). Their broad-spectrum efficacy, minimal toxicity to mammals, and quick environmental degradation make them strong candidates as bioactive agents in insect control (3). Several studies have the effectiveness of plant extracts and essential oils as insect repellents and larvicides, with minimal adverse health effects(3). Consequently, the use of traditional repellents remains widespread among diverse cultures and communities in Africa and beyond(4).

Globally, nearly 80% of the population relies on medicinal plants, indicating their growing utilization due to the constraints of mainstream medications (5). Although medicinal plants are extensively used in traditional health care systems worldwide, their safety and toxicity profile remains insufficiently understood (6). Africa is home to more than 2000 medicinal plant species, and Ethiopia is among the most plant rich countries on the continent(7,8). More than 90% of Ethiopians depend on traditional medicine, with numerous plant species reportedly used for the prevention and control of malaria (5,8). The control of malaria and other mosquito-borne diseases is becoming increasingly difficult, as they remain a major public health problem in Ethiopia (9). *Won-Sik Choi et al.(2002)* propose that the use of repellents can be effective strategy for protecting humans from mosquito bites (4). Topical application of repellents is a common preventive approach; however, the widespread and repetitive use of synthetic repellants has led to insecticides resistance, ecological imbalance, and adverse effects on non-target organisms, including human(3).

This situation highlights the need for the development of environmentally safe, biodegradable, and species-specific mosquito control agents (3). As a result, increasing attention has been directed toward natural alternatives, particularly essential oils. Among these, *Cymbopogon nardus* essential oil has been extensively researched for its mosquito repellent effects. In addition to their insect-repelling properties, its active ingredients, such as "*citronella*, *citronellol*, and *geraniol*," have antibacterial, antioxidant, and wound-healing activities (10).

Similarly, "*1, 8-cineole*," an active component of *Eucalyptus globulus* essential oil, which is a member of the *myrtle* family, is widely used in the food, cosmetic, and pharmaceutical industries (11). Various *Eucalyptus* species are mentioned as crucial plants for tick and mosquito control in traditional ethnobotanical practices across countries (2).

Although previous studies have explored the pharmacological and toxicological profile of each plant individually, no published study has evaluated the acute and subacute dermal toxicity of a combined *Cymbopogon nardus* and *Eucalyptus globulus* formulation. Considering their proposed use in topical natural mosquito repellent formulations, it is essential to determine whether this combination poses any dermal or systemic risk following repeated topical exposure.

Therefore, the present study aimed to evaluate the acute and subacute dermal toxicity of a combined *C. nardus* and *E. globulus* essential oil formulation in albino Wistar rats, focusing on hematological, biochemical, and histopathological parameters to provide preliminary safety data for future topical application.

1.2. Statement of the problem

Cymbopogon nardus and *Eucalyptus globulus* are widely used in traditional medicine for their insect-repellent, antimicrobial, and anti-inflammatory properties, and are increasingly incorporated into modern topical formulations (12,13). In Ethiopia, where traditional medicine remains a primary healthcare resource for the majority of the population, these plants are commonly used for mosquito control because they are locally available and culturally accepted (14).

Despite their extensive traditional use and reported bioactivity, the safety profiles of these essential oils, especially when used in combination, have not been sufficiently established. While several studies have investigated the pharmacological and toxicological properties of *C. nardus* and *E. globulus* individually, available data remain limited regarding the dermal safety of their combined formulation(15).

The current study was therefore carried out to evaluate both the acute and subacute dermal toxicity of a combined formulation of *C. nardus* and *E. globulus* essential oils in albino Wistar rats, with the aim of providing basic safety data to inform its future use in topical mosquito repellent formulations.

1.3. Significance of the study

Diseases like malaria are constantly pointed out as an important public health challenge in developing countries such as Ethiopia, where malaria is among the leading causes of morbidity and mortality (16).

Synthetic repellents might be sprayed to prevent mosquito bites, but they have been reported to have side effects, including skin irritation, allergic reactions, or toxic encephalopathy, especially in sensitive populations like children (17,18). This raises interest in studying natural alternatives from medicinal plants.

This study attempts to address an important gap by providing the first scientific evaluation of acute and subacute dermal toxicity of a preparation comprising a mixture of *Cymbopogon nardus* and *Eucalyptus globulus* essential oils.

The results are expected to contribute to the generation of baseline safety data to aid in the development of natural topical mosquito repellents, support evidence-based policy for plant-based vector control strategies, and contribute to the limited scientific literature on the dermal toxicology of essential oil mixtures.

By the generation of reliable toxicological information, this research supports the careful integration of traditional medicinal plants into modern public health practice, thus ensuring that any future application of this preparation is not only effective but also safe for widespread use.

2. LITERATURE REVIEW

2.1. Cymbopogon Nardus (*C. nardus*)

Cymbopogon nardus, or *citronella,* is a tropical perennial species of the "*Poaceae*" family, extensively cultivated in tropical and subtropical countries. It is native to India and south east Asia but is known by various vernacular names, such as *lemongrass* in India, *citronella* in France, *citronella grass* in America, *sakumau* in Malaysia, and *tej-sar* in Ethiopia (10). The species prefer warm, hot climatic conditions with rainfall ranging from 250-330cm and temperature gradients of 20-30°C, with sustainable harvesting every 2-3 months making it viable for use in both medicinal and commercial sectors (10).

Cymbopogon nardus essential oil described by its high *citronella, citronellol*, and *geraniol* content, which are responsible for its aroma and broad biological activities (19). These volatile components form the basis of its antimicrobial, antifungal, antioxidant, and anti-inflammatory activities, but the oil is perhaps best known for its mosquito-repelling activity (13). A field study in Ontario, Canada, provide citronella candles and incense lowered mosquito bites by 42.3% and 24.2%, respectively, as compared to controls (20). Similarly, in Jambi Province Indonesia, a 50% *citronella* oil spray also manifested a 97.3% mortality rated against *Aedes Aegypti,* proving its high adulticidal activity (21).

Controlled laboratory tests also validate the repellent efficacy *C. nardus. Solomon et al. (2012)* reported that the essential oils from *C. citratus, C. nardus*, and *Eucalyptus citridora* all provided more than 90% immediate protection against *Anophees arabiensis* at a range of 10% to 20%, which is comparable to the established repellent DEET. Importantly, oil of *C. nardus* maintained higher than 70% repellency for 3 to 4 hours, outperforming the other oils in terms of duration of action. Combination of *C. citratus* and *C,nardus* enhanced repellency even more; however, those with *E. citriodora* reduced the overall protective values (22). Similar studies in Nepal showed 95-97% protection up to two hours after topical application, thus adding to its reliability as a natural mosquito repellent (23).

In traditional Asian and African medicine, *C. nardus* has been used to treat fever, infections, and gastrointestinal ailments (24). Ethnobotanical data point to its cultural values as domestic pest control strategy. In Eritrea, insect-repellent plants are suspended around the house to keep insects

away, whereas in Ethiopia and Kenya, dried leaves of *C.nardus* are widely burned on charcoal stoves (thermal expulsion) during the evening hours to ward off mosquitoes and other vectors (14).

Toxicological research provides evidence for the safety of *C. nardus* essential oil when applied dermally in accordance with established regulations Acute and Subacute investigations indicate a substantial margin of safety, with related species of *Cymbopogon* exhibiting oral LD_{50} values exceeding 5000mg/kg, alongside the absence of treatment related mortality over 28 day exposure periods (5). Further studies *Reduan et al., 2020; Zahi et al., 2020* affirm that no hematological, biochemical, or histopathological anomalies are present in rodents administered plant derived essential oils, thereby supporting their low systemic toxicity profile (6,25).

Consequently, *C.nardus* essential oil is deemed safe for use as a topical repellent, aligning with evaluations based on OECD guidelines.

In summary, *Cymbopogon nardus* combines cultural acceptability, proven repellent efficacy, and a favorable safety profile, making it a strong candidate for the development of natural mosquito repellents formulations. Its long lasting protective action compared to other botanicals, coupled with its proven compatibility in synergistic combinations particularly with *C.citratus* and *Eucalyptus globulus* strengthens its contribution to plant based vector control investigations (22).

2.2. Eucalyptus globulus (*E. globulus*)

Eucalyptus globulus, *blue gum*, is a popular tree species of the "*Myrtaceae"* family. Although native to Australia, the species has been extensively cultivated in Africa, Asia, and Europe, where it has been addressed by local names such as *Tasmanian blue gum* in Australia, *kumeriah* in India, and *bahirzaf* in Ethiopia (26,27).

Ecologically, the species is adaptive to a broad spectrum of climates and soils, including degraded and poor soils, thus rendering it valuable for afforestation activities (28). In Ethiopia, not only ecologically significant, but also ethnomedicinally valuable, with ancient practice of burning its leaves being undertaken to drive away mosquitoes and limit the transmission of malaria (14).

The essential oil of *Eucalyptus globulus* is characterized by a high content of *eucalyptol* (*1, 8 cineole*), which is the major bioactive constituent responsible for its antimicrobial, anti-inflammatory, analgesic, and insect repellent properties. Eucalyptol has been widely used in

medicinal preparations such as cough suppressants, inhalation products, and topical creams (28,29). It is also relevant to mention that U.S. Food and Drug Administration (FDA) recognizes it as generally recognized as safe (GRAS) when used at specific concentrations, and it is often added to food, nutraceuticals, and cosmetics preparations (30).

Toxicological evaluations confirm the relative safety of *E .globulus* essential oil under controlled applications. *Hu et al. (2014)* reported an oral LD_{50} of 3811.5mg/kg in Sprague Dawley rats, rendering it relatively nontoxic on acute exposure.(31) Although high oral doses (≥792mg/kg) caused mild hepatic and renal change, there was no systemic toxicity at lower doses, and safety pharmacology showed no adverse cardiovascular and respiratory effects (31). *Mengiste et al. (2020)* also demonstrated that acute oral doses up to 1500mg/kg and subacute dermal treatment with a 5% ointment preparation caused no mortality, clinical pathology, or histopathological change in test animals (32). Similarly, *Becker et al. (2019)* concluded that cosmetic ingredient from *E .globulus* are safe under current concentrations and good manufacturing practices, if formulations are maintained below sensitization thresholds (33). Collectively, these findings confirm a broad margin of safety for dermal and controlled applications.

Apart from safety, *E. globulus* essential oil also shows considerable insecticidal and insect repellent activity. *Choi et al. (1994*) reported strong protection against *culex pipiens pallens* in a murine topical model, with repellency >75% for over 30 minutes (4).

More recently, *Igberaese et al.(2024)* reported that *E .globulus* oil, comprising 11 bioactive compounds, induced potent insecticidal activity against *Podagrica uniforma*, whose activity at higher concentration was comparable with *cypermerthrin* (34).

Ethnobotanical research in Ethiopia also corroborates the use, with widespread traditional use of *E. Globulus* leaves as mosquito repellents (35).

Present studies highlight the synergistic potential of *Eucalyptus globulus*. *Assaggaf et al.(2022)* illustrated that its essential oil, when combined with honey, exhibited a remarkable increase in antioxidant, anti-inflammatory, antimicrobial, and dermatoprotective activity compared to their sole applications (36). These observations support its application not only as a standalone repellent but also as a promising candidate in combinatory formulations. Importantly, the combination with *Cymbopogon nardus* could potentiate the repellent activity through synergism of *eucalyptol* and

citronellal, among other analogous monoterpenes, thus enabling the development of safe, plant based formulation for topical application and vector control (37).

As a conclusion, the essential oil of *Eucalyptus globulus* possesses a very good toxicological profile in addition to its proven insect-repellent and therapeutic activities. Its long history in ethnomedicine, demonstrated safety upon application, and synergistic potential with other essential oils such as *C. nardus* present a strong rationale for the exploration of its combination formulations in dermal toxicity and repellency studies.

2.3. Combined use of *C. Nardus* and *E. Globulus* extracts

Medicinal plants are becoming increasingly recognized for their synergistic potential when combined, creating enhanced therapeutic activity, expanded bioactivity, and lowering the potential for resistance. Essential oils, specifically, are well documented to have higher biological activity when in blends compared to individual use (36). *Eucalyptus globulus* oil, which is high in *1,8-cineole*, has been shown to have added antioxidant, antimicrobial, and insect repellent activity when blended with other botanic ingredients, whereas *Cymbopogon nardus*, which is recognized for its citronella type constituents *citronellal*, *citronellol*, and *geraniol*, has been used for centuries as a mosquito repellent (38,39). The complementary chemistry of both oils supports that their blend will provide enhanced protective properties against pathogens and insect vectors alike, forming an excellent foundation for plant based topical remedies (36).

A number of studies have experimentally supported this synergism. *Soonwera* and *Sittichok (2020)* indicated that blends of *Cymbopogon* and *E. globulus* oils exhibited much greater knockdown and adulticidal activity against *Aides* mosquitoes than individual oils, with some blends causing 100% mortality within 24 hours (40). Similarly, *Singh et al. (2020)* showed that mixture of *E. globulus* and *Cymbopogon flexuosus* essential oils resulted in over 95% efficacy against the cattle tick *Rhipicephalus microplus*, with some mixtures causing total mortality at relatively low dosages, outperforming conventional acaricides in some instances (41).

In addition to mosquitoes and ticks, *Chooluck et al. (2018)* also demonstrated the powerful repellency of *Eucalyptus* and *Cymbopogon* oils, as well as their active monoterpenes, against *Blattella germanica*, further illustrating the broad spectrum prospects of such combinations in pest control (42).

Additional evidence supports their effectiveness in mosquito control. For example, *Nguyen et al. (2020)* demonstrated that blends of *Cymbopogon* and *Eucalyptus* oils caused quick mosquito knockdown in a matter of minutes and sustained high mortality rates after a period of 24 hours, while *Umar et al. (2021)* showed substantial repellent action of *Cymbopogon citratus* and *E. globulus* extracts against Anopheles species, with over 90% protection (43,44). Likewise, *Khan et al. (2017)* reported time and dose dependent insecticidal activities of *C. nardus* and *E. globulus* oils against houseflies, bedbugs, and lice, with mortality rates of over 98% at higher concentrations (45).

Together, these findings provide strong evidence that *Cymbopogon* and *Eucalyptus* essential oils possess broad spectrum insecticidal and repellent properties whose simultaneous use leads to increased efficacy through synergistic interactions. The body of evidence strongly warrants the development of combination formulations of *C. nardus* and *E. globulus*, not just for enhanced mosquito repellence but also for the safety assurance of topical preparations. As such, the determination of their toxicological profile is an important step in assessing their potential for therapeutic or cosmetic use.

2.4. Toxicology

Toxicology is the scientific study of poisons, whereby a poison can be generally considered as any substance that induces a harmful reaction when exposed to a living organism, whether by intention or accidentally. While more expansive definitions can generally define toxicology as “the study of the detection, occurrence, properties, effects, and regulation of toxic substances," such descriptive definitions generally do not aid in understanding fully the complexities of toxicity reactions(46).

Toxicity generally does not occur in a reaction at the molecular level but rather as the culmination of several steps from exposure through to metabolic changes after exposure, through absorption, distribution, and eventually metabolism. These reactions generally affect vital biomolecules in cells, usually DNA or proteins, and generally culminate in achieving a specific level of toxicity. The excretory mechanisms of the body can generally reduce adverse reactions through helping the expulsion of toxic substances(46).

Toxicology benefits society by shielding humans and the environment from harmful toxic substances and by playing a role in the creation of selective toxins such as anticancer compounds, drugs, and pesticides. One of the basic tenets of toxicology is that toxicity is always a dose-response phenomenon; in other words, most substances can be toxically poisonous at very high dosages; conversely, the same substance manifests absolutely no toxicity at low dosages. And in between these two extremes, there exist many manifestations of toxicity, from chronic subtleties to lethal manifestations(46).

The challenge of evaluating toxicity arises from the fact that there can be acute or chronic toxicities, there can be organ specificity in terms of toxicity, and there can be many other factors like age, genetic makeup, sex, dietary habits, physiological state, and overall health. Genetic factors can play especially important roles in human populations since these are largely outbred, unlike other test animals that can come from similar or exactly the same genetic stocks. So much so that many other standard toxicity parameters like the LD50 value can vary to an appreciable extent from person to person(46).

In toxicological analysis, certain classifications help in defining the kind of adverse reactions that occur due to exposure to certain substances. Acute dermal toxicity can be viewed as adverse reactions that happen as a result of direct exposure to certain test chemicals through the skin in a single, continuous process, usually not exceeding 24 hours, thereby evaluating the direct danger presented by skin exposure to such substances(47).Subacute toxicity, on the other hand, covers adverse reactions resulting from repeated exposure to a substance in a short period; these reactions are not necessarily direct but can result in significant adverse reactions in the long run(48).

Toxicity testing of new compounds represents an integral part of drug development and chemical safety evaluation. In preclinical toxicity studies, adverse reactions of new compounds to diverse biological systems are assessed, taking into consideration inter-species variation, target organs, dose-response relationships, and period of exposure(46).

In the opinion of *Parasuraman (2011),* preclinical studies are essential in determining the No Observed Adverse Effect Levels (NOAEL) of new compounds, which form a basic standard necessary before launching clinical studies of investigational compounds(49).

Toxicity evaluation can be based on analyses of accidental human exposure, in vitro evaluations using cells or cell lines, or in vivo studies using animal models. The wide range of animal models and designs in in vivo studies today continue to play important roles in current toxicity studies, giving researchers invaluable information related to the safety and biological activity of new chemical compounds(49).

2.5. Blood and its components

Blood is essential for the delivery of nutrients, hormones, metabolic waste, support for the immune system, and homeostasis (50). Blood parameters are accurate, sensitive, and reliable diagnostic indicators used in the diagnosis, prevention, and management of disease (50).

Blood is a specialized connective tissue composed of cellular elements, erythrocytes, leukocytes, and platelets scattered in a fluid medium termed plasma. *Junqueira* and *Carnerio* mention that blood plasma serves as the extracellular matrix and provides a medium for cellular components. In adult human individuals, the total blood volume is approximately 6 liters, while in rats it ranges from 5.6 to 7.1 milliliters per 100 grams of body weight (51,52).

Hematologic parameters such as hemoglobin (Hgb), red blood cell (RBC, hematocrit (Hct), mean corpuscular volume (Mcv), mean corpuscular hemoglobin concentration (Mchc), white blood cell count (WBC), lymphocyte count (L), and platelet count (Plt) are significant parameters of identifying the toxic properties of drugs, such as plant extracts. On the destruction or damage of the blood cells, the body's functioning is impaired (53).

Erythrocytes, or red blood cells, are biconcave discs without a nucleus that are, under normal circumstances, confined to the circulatory system. Human erythrocytes are 6-8μm in diameter and possess a central pallor that occupies approximately one-third of their diameter. Their function is primarily to transport hemoglobin, enabling oxygen and carbon dioxide exchange. Rat erythrocytes, being slightly smaller at 4-7μm, have a shorter lifespan of 61 days compared to 120 days in humans (37,54).

Leukocytes or white blood cells are crucial to the body's defense mechanism, targeting areas of inflammation and infection. They move through endothelial cells of venules and capillaries into connective tissue to perform protective functions (51,55).

Leukocytes are broadly classified into granulocytes and agranulocytes based on the presence or absence of cytoplasmic granules and the nature of the cytoplasmic granules. The granulocytes include neutrophils, eosinophils, and basophils, while agranulocytes consist of lymphocytes and monocytes (51).

These cells work together to give the body good immunity against bacterial, viral, and parasitic infections (56). There are five types of WBC that constitute various percentages. These include neutrophils (40-75%), eosinophils (1-6%), basophils (1%), monocytes (2-10%), and lymphocytes (20-40%) in human beings (57).

Neutrophils, which comprise 40-75% of human leukocytes, are the first cellular response to infection and tissue damage. They phagocytose debris, bacteria, and viruses. The rat's neutrophils constitute 14-20% of the leukocytes and contain hyper-segmental nuclei (37,58).

Eosinophils, 1-6% of human leukocytes, have bi-lobed nuclei and are capable of lysing parasitic worms and antigen-antibody complexes. Eosinophils of rats have band-like nuclei with range-like tendencies (51,59).

Basophils, the least common of the granulocytes, participate in allergic reactions by releasing histamine and other inflammatory mediators upon encounter with an antigen (60,61). Lymphocytes, 20-40% of leukocytes, play a central role in adaptive immunity. Certain medicinal plants have been found to control lymphocyte counts, indicating biologically active principles with potential immunosuppressive effects. Monocytes, 2-10% of leukocytes, become macrophages on invading tissues, where they perform phagocytosis and trigger an immune response (51).

Platelets are flat fragments of cells that are non-nucleated and develop from megakaryocytes in the bone marrow. Platelets play a crucial role in hemostasis by aggregating over damaged blood vessels and initiating the development of the clot. Platelet count in human beings is between 150000 to 400000 per cubic millimeter of blood, according to *Taylor et al* (59). Platelets release serotonin, which results in vasoconstriction and yet further reduces the supply of blood to the affected region (62).

Plasma, the fluid portion of blood, is made up of 90% water and 10% solutes such as proteins, electrolytes, and waste products. Plasma proteins are albumin, globulins (α, β, and γ), and fibrinogen, which are important for osmotic balance, immune function, and blood clotting (51,62).

Plasma also transports nutrients and waste products to and from tissues, and overall physiological homeostasis (51).

Blood parameters remain the optimal, most precise, and most sensitive equipment utilized for the diagnosis of disease, monitoring of treatments, and determination of physiological and pathological conditions (63). Blood ensures the delivery of materials needed, upholds immune system functions, and anticipates in homeostasis and is therefore the cornerstone of sustaining life (64).

2.6. Structure, function, and Microscopic structure of the liver

The liver is the body's largest gland and second-largest organ, preceded only by the skin. It constitutes approximately 2-3% of total adult human weight, averaging between 1.4-1.7kg (65). In the mouse, the liver-to-body weight ratio varies by strain and intrinsic traits (66).

Anatomically, there are four lobes in the liver of a mouse: the median lobe, left lateral lobe, caudate and right lobe, all of which have different sizes. The superior and inferior sections make up the caudate lobe together that adds around 10% of the whole weight of the liver. It is fully draped with the inferior omentum and is attached to the left lateral lobe via a slim interolateral ligament (66).

The median lobe, which forms about 26% of the overall weight of the liver, is fixed inferiorly to the diaphragm by the falciform ligament, a ligament originating from the diaphragm and xiphoid process and traveling to the liver, forming the interlobular fissure. The median lobe is characterized further by dividing into two, the left portion being smaller compared to the right and marked with a deep fissure (66).

The right lobe, situated beside the inferior vena cava, is subdivided into superior and inferior segments. The right inferior lobe, with a pyramidal shape, contains around 14% of the weight of the liver and dorsally lies on the diaphragm. The right superior lobe, covering around 16% of liver weight, contains a broad base extending into the paracaval area (67).

Despite the gross anatomical differences between human and rat livers, microscopic morphology is remarkably similar. Liver lobules are polygonal structures with a cover of connective tissue and are best seen in sections of rat liver. Hepatocytes are grouped radially around the central vein of each lobule, while the surrounding sinusoids extend radially from the center to the periphery (68).

Portal triads, which include portal vein branches, hepatic artery branches, and bile ducts, occur at the corners of the lobules (68).

Hepatocytes secrete bile into bile canaliculi that drain the bile from hepatic cords toward portal triads in the periphery of the lobule. Blood supply to the rat liver resembles that to human beings: the hepatic artery provides around 20-30% of the blood, and 70-80% of the blood is from the portal vein, having originated from the stomach, intestine, and spleen (68).

The main functions of the liver are detoxifying the body, metabolizing toxins and chemicals into excretable products, and synthesizing bile for digestion. Liver damage, however, occurs if the accumulation of toxins happens at a rate faster than the liver's capacity for detoxification. Some chemotherapeutic herbs are also hepatotoxic and cause damage to hepatocytes (69).

2.7. Structure, function, and Microscopic structure of the kidney

In the mouse, the kidneys are retroperitoneal on both sides of the spine at the T12 to L3 vertebral level. The sectioned kidney consists of two differentiated regions: the outer cortex and inner medulla. Cortex contains the convoluted tubules, Bowman's capsule, and initial portions of collecting ducts, all enclosed in fibrous connective tissue (69). The medulla is organized into renal pyramids, which consist of the loop of Henle, collecting ducts, and vasculature. The nephron, derived from separate embryologic origins, is the functional unit of the kidney (70).

The kidney may be broken down into three main components: cortex, medulla, and collecting system. The granular-appearing cortex, located externally, transitions into the medulla, which contains pyramidal structures. Bertin's columns, outgrowths of the cortical parenchyma between adjoining pyramids, are also implicated in the formation of renal lobes, as is also the sub-capsular cortex and medullary pyramids (70).

The collecting system is formed by major calyces that arise from the renal pelvis, with further branching into minor calyces, each of which is present with medullary papillae. Each renal pyramid has a base at the corticomedullary junction and a tip extending towards the renal pelvis (71).

Unlike humans, rats have unipapillate kidneys with a single renal pyramid emptying directly into the renal pelvis. Anatomically distinct as they are, rat and human kidneys have the same microscopic features. Each adult rat kidney contains around 30000 nephrons, while a human

kidney contains 0.6-1.4 million nephrons. Tubules like Bowman's capsules, the glomerulus in a nephron. Glomeruli in humans are roughly 200µm in diameter, while in rats, they are roughly 120µm in size, and the number is influenced by birth weight, age, and sex. Juxtamedullary glomeruli are larger compared to superficial cortical glomeruli (71).

Renal tubules consist of the proximal convoluted tubules (PCT), distal convoluted tubules (DCT), and the loop of Henle, which consists of ascending and descending limbs. The PCT, with its more intricate and longer structure, has a brush border of microvilli that aids in reabsorption. The loop of Henle is a u-shaped structure between the PCT and DCT, with the simple epithelial linings transforming from squamous in the deeper medulla to cuboidal near the cortex. The DCT is shorter and lacks a brush border, consisting of smaller and more flattened cells. A specialized structure called the juxtaglomerular apparatus is formed where the distal tubule makes contact with the vascular pole of the nephron's renal corpuscle (72).

The kidneys play a crucial role in the fluid and electrolyte balance regulation via ion level control, particularly of Na^+ and K^+. In pathological changes that occur in tubular tissues, as in nephrotic syndrome, hyperplastic tubular cells are found that contain pink granules of the cytoplasm and enlarged nuclei. Disturbances of electrolytes that manifest as high levels of ions in the blood are characteristic of this syndrome (73,74).

Herbal and chemical drug toxicities typically target the winding and collecting tubules, inducing cellular hypertrophy, cytoplasmic vacuolation, and damage to the tubules. This leads to damage of tubular cell membranes, release of cellular enzymes, and elevated blood creatinine and urea concentrations, which are evidence of impaired renal function (72).

It has been found that exposure to poisonous chemicals can result in interstitial fibrosis, extensive tubular loss, and structural changes at the cellular level, particularly in the outer cortex. Conventional medicinal plants have also been implicated in tubular damage and fibrosis in the kidney (73).

2.8. Structure, function, and Microscopic structure of the skin

The skin, which encloses the entire surface of the body externally, is the biggest body organ and an important physical barrier for the exterior world. It is very much responsible for protecting the body against infections, microorganisms, ultraviolet light, trauma, and toxins (75).

The skin also regulates body temperature, does immunological surveillance, and contributes to sensory perception, fluid loss regulation, and homeostasis. Its versatility is reflected in its variations in thickness and specialized function along various areas of the body (75,76).

The skin consists of three primary layers: the epidermis, dermis, and hypodermis, in order from outside to inside. Each of these layers holds particular structural and functional components. The epidermis, or the outermost layer, has five sublayers from deep to superficial: stratum basale (basal layer), stratum spinosum (prickle cell layer), stratum granulosum (granular layer), stratum lucidum (clear layer), and stratum corneum (cornified layer) (75).

Epidermis is composed of several cell types. Keratinocytes, which are derived from basal stem cells, are squamous epithelial cells that proliferate, migrate to the surface, and ultimately desquamate at the stratum corneum. Melanocytes, found in the basal layer, produce melanin, which imparts pigmentation and protects against ultraviolet light. Langerhans cells are antigen-presenting cells, and Merkel cells are mechanoreceptors, which aid sensory perception(76).

Under the epidermis is the dermis, a layer of predominantly connective tissue that gives structural and functional support to the epidermis. In the dermis, there is an irregular pattern of fibers, predominantly type I collagen (about 85%) giving tensile strength, and type III collagen, which is supplementary support (76).

Elastic fibers, distributed throughout the dermis, enable the skin to return to its original shape after being stretched. Type IV collagen occurs at the junction of dermo-epidermal, around Schwann cells and vascular endothelial cells, while types V, VI, and VII collagen are minimally involved in the matrix of dermis (76).

A cascade of biological reactions occurs when the skin is injured either mechanically or by toxins. It entails epidermal and dermal injury, hemorrhage, and potential contamination of deeper tissues. Wound healing in the skin is an evolutionarily conserved process that includes dynamic interactions among a variety of mechanisms such as hemostasis, inflammation, proliferation, and remodeling. Release of cytokines, growth factors, and low-molecular-weight materials from the point of injury stimulates and regulates healing shortly after trauma (77).

3. OBJECTIVE

3.1. General Objective

- To evaluate the acute and subacute dermal toxicity of a combined *Cymbopogon nardus* and *Eucalyptus globulus* formulation on hematological, biochemical parameters, and histopathology of the skin, kidney, and liver of albino Wistar rats.

3.2. Specific objectives

- To determine the LD_{50} of *Cymbopogon nardus* and *Eucalyptus globulus* formulations in albino Wistar rats.
- To assess the effects of a 28-day topical application of *Cymbopogon nardus* and *Eucalyptus globulus* formulations on the blood parameters of albino Wistar rats.
- To examine the impact of a 28-day topical application of *Cymbopogon nardus* and *Eucalyptus globulus* formulations on the microstructure of the liver in albino Wistar rats.
- To assess the effects of a 28-day topical application of *Cymbopogon nardus* and *Eucalyptus globulus* formulations on the microstructure of the kidneys in albino Wistar rats.
- To determine the effects of a 28-day topical application of *Cymbopogon nardus* and *Eucalyptus globulus* formulations on the microstructure of the skin in albino Wistar rats.

4. METHOD AND MATERIAL

4.1. Study design

An in vivo, laboratory-based experimental study was conducted to assess the acute and subacute dermal toxicity of a combined *Cymbopogon nardus* and *Eucalyptus globulus* essential oil formulation in albino Wistar rats following OECD guidelines.

4.2. Study Area

The experiment was conducted at the traditional and modern medicine research and development directorate of the Armauer Hansen Research Institute.

4.3. Collection of the plant material

First, the study plant candidates were selected based on their mosquito-repelling potential according to ethnobotanical studies. Some of the medicinal plants were subsequently collected at the Armauer Hansen Research Institute (AHRI) botanical garden, and others were collected in other locations in Ethiopia (Table 1). Identification was carried out using the flora of Ethiopia and Eritrea, and the sample voucher specimens were numbered and placed in the herbarium of the Department of Traditional and Modern Medicine Research (TMMRD) at the AHRI for further reference.

Table 1: Plant species, plant parts, and collection site

No.	***Plant species***	***Plant parts***	***Collection site***
1	C. Nardus	Leaves	Wondogenet
2	E. Globules	Leaves	Wondogenet

4.4. Preparation and extraction of the plant material

Essential oil extraction was performed by harvesting fresh plant material and conducting 3 hours of hydro-distillation for each medicinal plant separately using a Clevenger-arm apparatus. Four liters of water were added to a 500-gram quantity of chopped plant material in a round-bottom flask. The flask was then placed on a heating mantle and attached to a Clevenger arm apparatus. Cold water flowed into the condenser. During boiling, volatile oils were carried with the steam to a graduated distillate receiver, and excess water was returned to the flask.

The extracted oil was collected in sterile containers and further purified from the aqueous solution using anhydrous sodium sulfate (Na_2SO4). The essential oils were then weighed and stored in a refrigerator at 2-8^{0}C until used in experiments (78,79).

4.5. Preparation of white paraffin-based ointment mosquito repellent

An ointment of white paraffin-based mosquito repellent was prepared using essential oil from Eucalyptus globulus and Cymbopogon nardus (citronella), combined with white petrolatum. The equipment needed for this preparation included a weighing balance, heat (e.g., water bath or hot plate), mixing spatula, beakers, thermometer, and sterile storage vessels. To prepare the ointment base, white petrolatum was weighed out to 100 grams, rolled into a beaker, and melted under tap water at about 60 °C. After cooling the melted petrolatum to about 45 °C, 2 milliliters of Eucalyptus globulus and Cymbopogon nardus essential oil were added and mixed vigorously until uniformly distributed. The warm mixture obtained was then transferred into sterile vessels, allowed to cool down and solidify at normal room temperature, and labeled with product identity, constituents, and date of preparation. Finally, the ointment was kept under shade in a cool, dry place, out of direct sunlight(78,79).

4.6. Experimental animal preparation

Relatively healthy adult male and female nulliparous, non-pregnant albino Wistar rats, aged 8-12 weeks and weighing 200-300 grams, with no prior exposure to experimental procedures, were obtained from the animal facility of the Armauer Hansen Research Institute (AHRI). The rats were kept in stainless steel cages in an environmentally controlled room at a temperature of 22-23^{0}C and relative humidity between 30% and 70%, as per OECD guideline 402(47). Lighting was maintained on a 12-hour light-dark cycle to stimulate natural circadian rhythms. Standard laboratory diets were provided with *ad libitum* access to drinking water.

Before the start of the experiment, the rats were habituated to laboratory conditions in group cages for five days, as suggested by OECD guidelines. After acclimatization, animals were allotted to experimental groups randomly by an independent expert, and each rat was identified with a unique identification tag(47).

Pre-application clipping of hair was done to prepare for application, carefully removing hair from the dorsal and flank areas, covering about 10% of the total body surface area, one day before

dosing. The clipping was done carefully by an experienced laboratory technician to avoid abrasion of the skin and to reduce any effect on skin permeability that could have modified the experimental findings (47).

4.7. Grouping and dosing of experimental animals

According to OECD guideline 402 for acute dermal toxicity, a limit test was conducted using five healthy adult female albino wistar rats. The combined extract formulation of *Cymbopogon nardus* and *Eucalyptus globulus* was administered as a single dermal dose of 5000 mg/kg body weight. This was selected based on the plant origin of the test substances, their documented traditional and medicinal use, and previous studies reporting low oral toxicity, which support a low potential for systemic toxicity following dermal exposure(5,31).

For the subacute dermal toxicity study, a total of 10 healthy animals comprising 5 male and 5 females (nulliparous and non-pregnant) were used at each dose level. The animals were randomly assigned to four groups: three treatment groups and one control group, with both sexes represented in each group. The doses selected for the treatment groups were 250mg/kg, 500mg/kg, and 1000mg/kg, respectively, based on the results obtained from the acute dermal toxicity study. The control group received only the vehicle (petrolatum) for comparison

4.8. Cage side observations

Each animal was carefully observed in its cage both before and after dose administration. Observations focused on any alteration in physical appearance, including changes in the eyes, skin, and hair coat. Additionally, autonomic signs such as salivation, diarrhea, and urination were recorded, along with central nervous system (CNS) effects such as tremor or other abnormal behaviors (47).

4.9. Acute dermal toxicity study

An acute dermal toxicity test was conducted as per OECD guideline 402. A total of five albino Wistar rats were utilized for testing. The animals underwent a five-day acclimatization period prior to the trial to habituate them to lab conditions. The rats were maintained under suitable conditions in groups and were supplemented with normal laboratory food and water (47).

The preparations were applied onto the skin, with the dosage for each animal calculated based on its individual body weight. The application site was covered with a porous gauze dressing secured with non-irritating tape to ensure 24-hour exposure for extended contact with the skin. The test site was properly shielded to prevent ingestion of the formulation or dislodgment of the gauze by the animals (47).

Following the application, the animals were closely observed for any immediate response with special attention paid to the first six hours and at 24-hour intervals afterward. They were then monitored daily for 14 days. Particular attention was given to lethargy, drowsiness, salivation, convulsions, tremors, diarrhea, and other clinical signs (47).

Once the experiment was over (15 days in total), the rats were weighed, euthanized, and their internal organs, such as the liver and kidneys, were grossly examined to ascertain any pathological changes caused by the formulations

4.10. Subacute dermal toxicity test

A 28-day subacute dermal toxicity study was conducted as per OECD guideline 410. The test substance was administered in daily doses to different groups of male and female rats, with each group receiving a single dose level as established from the acute dermal toxicity experiment. Young adult healthy rats were acclimatized for two weeks to laboratory conditions before testing (80).

For the study, 40 rats were utilized and divided into four groups: three test groups and one control group. Each group consisted of five male and five female rats (n=10 per group). Test animal treatment groups were given a single daily application of the extract, while the control group received the vehicle (petrolatum). Shortly before the commencement of the test (24 hours), hair was carefully clipped from approximately 10% of the body surface area on the dorsal trunk of each rat to facilitate even application of the test material. Re-clipping on a weekly basis was performed to maintain this exposure area.

During the 28 days, rats were observed daily for signs of toxicity, including physical alterations, behavioral changes, and overall health. At the conclusion of the experiment, any remaining animals were euthanized and necropsied for the assessment of potential pathological changes (80).

4.11. Gross pathology

A comprehensive gross pathological examination was carried out in each experimental and control group to ascertain any pathological alterations of significance. Anomalies between groups of animals were carefully observed. Close attention was paid to coloration, texture, organ size of target organs, necrosis, or spots in organ analysis. Any swelling or decrease in organ size was also observed to determine potential toxic effects in treatment groups compared to control groups (80).

4.12. Blood collection for hematological and biochemical analyses

Blood samples for biochemical and hematological analysis were collected. After intraperitoneal injection of 150mg/kg of pentobarbital, 2-4 milliliters of blood were collected using heparinized syringes (5ml capacity) through quick cardiac puncture. The blood was collected in EDETA-containing or EDTA-free tubes (anticoagulant).

Hematological parameters such as hemoglobin concentration (HC), red blood cell count (RBC), white blood cell count (WBC), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), lymphocyte count, and platelet count (PLC) were estimated using an auto hematology analyzer.

Serum samples were taken after blood in non-EDETA tubes was allowed to clot and centrifuged at 3000 rpm for 20 minutes. The resulting serum was used to estimate biochemical parameters such as urea, creatinine, aspartate aminotransferase (AST), alkaline phosphate (ALP), alanine aminotransferase (ALT), and albumin (ALB) using a clinical chemistry analyzer. Control and treatment groups were compared regarding these biochemical and hematological values.

4.13. Animal dissection, tissue collection, and histological examination

At the end of the study, animals were euthanized using anesthesia via intraperitoneal injection of pentobarbital at a dose of 150mg/kg, after blood sampling. The abdomen was opened during euthanasia, and the kidneys and liver were removed. Each organ was carefully cleared of surrounding tissue, weighed using an electronic scale, and placed on clean paper for measurement.

For histological analysis, samples of the skin, liver, and kidney were taken, sectioned to a thickness of 3-5µm, and stored for analysis. Histologically stained tissue slides were examined under a microscope by a professional pathologist at AHRI and AAU.

Observations were made using light microscopes with magnifications of X10, X40, and X100. Photomicrographs of the treated and control animals' skin, liver, and kidney tissues were taken and stored digitally for comparison. Photomicrographs at X10 and X40 magnifications were used to document and highlight pathological differences.

4.14. Ethical consideration

All the research was carried out in accordance with the highest ethical standards for the humane and compassionate treatment of animals in biomedical research. Permissions were obtained from the relevant authorities in the School of Biomedicine and Laboratory Science, AAU, and AHRI before the study was initiated. All necessary care was taken to ensure that animal subjects were not subjected to unnecessary suffering or adverse conditions (OECD, 2008).

All animals were given an intraperitoneal injection of 150mg/kg of pentobarbital to relieve pain and distress during surgical procedures, which were conducted by professional and trained personnel. The animals were housed in an environment designed to meet their physiological needs and protect them from infection.

4.15. Statistical analysis

Data were entered and processed using the Statistical Package for Social Sciences (SPSS) software version 26. Statistical outcomes were represented as the mean (μ) and standard error of the mean (SEM) to reflect the variance in each group. A One-way ANOVA followed by Tukey's post hoc test was used to statistically examine differences between experimental groups, and post hoc tests were applied to identify specific group differences. Significance was considered at $P<0.05$. Histological data from liver and kidney tissue sections were qualitatively assessed, and observable structural changes were recorded to supplement quantitative findings.

5. RESULT

5.1. Acute dermal toxicity assessment

The acute dermal toxicity of the combined essential oils of *Cymbopogon nardus* and *Eucalyptus globulus* was evaluated by administering a single limit dose of 5000mg/kg body weight to five female rats. No mortality or clinical signs of toxicity were observed during the 14-day observation period. All treated Animals maintained normal activities, grooming, feeding, and water intake. Body weight changes were minimal and not statistically significant ($P>0.05$). Gross examination of the liver and kidneys revealed no observable abnormalities.

The absence of mortality at the tested limit dose suggests a dermal LD_{50} greater than 5000mg/kg, placing the formulation in a GHS category 5 or unclassified.

Behavioral and physical parameters, including skin and fur condition, eyes, mucous membranes, salivation, lethargy, sleep patterns, diarrhea, tremors, and overall behavior, remained normal throughout the observation period.

All five treated rats survived the 14 day post-treatment period without any signs of acute dermal toxicity.

For the subsequent 28-day subacute dermal toxicity study, female and male Wistar rats were divided into four groups. Groups I, II, and III received the combined essential oils at doses of 250mg/kg, 500mg/kg, and 1000mg/kg, respectively, while Group IV served as the control and received the vehicle only (Petrolatum). Female rats received doses corresponding to 70g, 140g, and 280g for Groups I, II, and III, respectively, and male rats received 75g, 150g, and 300g for the same groups, with the control group receiving vehicle only.

5.2. Subacute dermal toxicity assessment

5.2.1. General observation

Throughout the 28-day subacute dermal toxicity study, no observable signs of toxicity were recorded in female rats administered *Cymbopogon nardus* and *Eucalyptus globulus* formulation at doses of 250 mg/kg, 500 mg/kg, and 1000 mg/kg. Similarly, male rats treated with the same respective doses exhibited no behavioral abnormalities or clinical signs indicative of toxicity.

All animals remained active, alert, and exhibited normal grooming, posture, and feeding behavior throughout the observation period. Furthermore, no mortality occurred in any of the treatment groups

5.2.2. Effect of *C. nardus* and *E. globulus* formulation on weight gain

The effect of 28-day dermal application of *C.nardus* and *E. globulus* formulation on the body weight gain and relative organ weight (liver and kidney) was assessed in both female and male Wistar rats.

Body weight increased progressively in all animals over the treatment period, with no statistically significant difference observed between treated and control groups in either sex ($P>0.05$). The analysis of variance (ANOVA) showed that the p-value for mean body weight gain in female rats was 0.96, and in male rats was 0.34. Similarly, the p-value for relative liver and kidneys showed no significant difference between the treated and control groups. In female rats, p-values for relative liver and kidney weights were 0.27 and 0.39, respectively, while in males, they were 0.08 and 0.50. These values are summarized in Table 2 for females and Table 3 for males.

Gross pathological examination revealed no observable abnormalities in the liver and kidneys of both sexes. The organs appeared normal in size, shape, color, and texture, with no signs of necrosis, discoloration, or structural damage. No macroscopic difference was observed between the experimental and control groups.

Table 2: Effect of 28 days dermal application of C.nardus and E. globulus formulation on body weight gain and relative organ weights in female rats

Group	Dose in mg/kg (n=5)	Body weight gain(g) ± *SEM*	Relative organ weight	
			Liver(%)± *SEM*	**Kidney(%)±*SEM***
I.	**250mg/kg**	12.84 ± 9.75	2.41±0.07	0.66±0.02
II.	**500mg/kg**	12.86 ± 3.35	2.50±0.06	0.67±0.01
III.	**1000mg/kg**	9.48 ± 2.11	2.55±0.06	0.68±0.01
IV.	**Control**	11.22± 2.91	2.66±0.13	0.72±0.04
P- Value		**0.96**	**0.27**	**0.39**

Values are expressed as mean ± SEM (n=5 per group). Statistical significance was assessed using one-way ANOVA; P<0.05. Relative organ weight was calculated by organ weight/body weight x 100%.

Table 3: Effect of 28 days dermal application of C.nardus and E. globulus formulation on body weight gain and relative organ weights in male rats.

Group	Dose in mg/kg (n=5)	Body weight gain(g) ± *SEM*	Relative organ weight	
			Liver(%)± *SEM*	**Kidney(%)± *SEM***
I.	**250mg/kg**	9.88 ± 2.86	2.58±0.14	0.64±0.02
II.	**500mg/kg**	5.96± 1.21	2.11±0.06	0.60±0.01
III.	**1000mg/kg**	10.06± 1.80	2.48±0.16	0.65±0.03
IV.	**Control**	11.94± 2.84	2.41±0.09	0.62±0.01
P- Value		**0.34**	**0.08**	**0.50**

Values are expressed as mean ± SEM (n=5 per group). Statistical significance was assessed using one-way ANOVA; P<0.05. Relative organ weight was calculated by organ weight/body weight x 100%.

5.2.3. Effects of *C. nardus* and *E. globulus* formulation on food intake

Tables 4 and 5 present the weekly mean food consumption of female and male Wistar rats, respectively, following dermal application of *Cymbopogon nardus* and *Eucalyptus globulus* formulation over 28 days. In female rats, food intake remained stable across all treatment groups (250 mg/kg, 500mg/kg, and 100mg/kg) compared to the control group, with no statistically significant differences observed throughout the four weeks (P>0.05). Similarly, male rats exhibited consistent and stable food consumption across all dose levels, and no statistically significant differences were noted between treated and control groups during the four-week study period (P>0.05).

Table 4: Effect of 28-day dermal application of C.nardus and E. globulus formulation on food intake of female rats ((g) ± SEM).

Food Intake	Unit	Dose(mg/kg)				p-value
		250mg/kg	**500mg/kg**	**1000mg/kg**	**Control**	
Week-1	**Gram**	187.22±1.27	182.33±3.90	172.08±3.15	182.13±3.41	**0.83**
Week-2	**Gram**	186.55±2.40	176.92±3.30	181.13±4.34	185.55±2.07	**0.18**
Week-3	**Gram**	188.39±1.82	186.63±2.23	173.96±4.69	181.04±3	**0.48**
Week-4	**Gram**	185.66±2.69	177±4.42	177.45±4.93	188.73±2.14	**0.20**

Values are expressed as mean ± SEM (n=5 per group). Statistical significance was assessed using one-way ANOVA; P<0.05

Table 5: Effect of 28-day dermal application of C. nardus and E. globulus formulation on food intake of Male rats ((g) ± SEM).

Food Intake	Unit	Dose(mg/kg)			Control	p-value
		250mg/kg	**500mg/kg**	**1000mg/kg**		
Week-1	**Gram**	172.08±3.15	178.25±2.95	170.76±3.42	186.67±2.28	**0.48**
Week-2	**Gram**	181.13±4.34	172.43±3.74	184.16±4.64	184.96±1.88	**0.23**
Week-3	**Gram**	173.96±4.69	181.98±2.54	170.46±4.68	184.64±3.43	**0.06**
Week-4	**Gram**	177.45±4.93	172.83±5.02	171.01±3.28	187.91±1.19	**0.73**

Values are expressed as mean ± SEM (n=5 per group). Statistical significance was assessed using one-way ANOVA; P<0.05

5.2.4. Effects of *C. nardus* and *E. globulus* formulation on water intake

Tables 6 and 7 present the average weekly water consumption in female and male Wistar rats, respectively, during the 28-day dermal exposure period. In female rats, water intake remained relatively stable across all the treatment groups (250 mg/kg, 500 mg/kg, and 1000mg/kg) compared to the control group. No statistically significant differences were observed throughout the four-week study period ($P > 0.05$). Similarly, male rats showed consistent water intake across all treatment and control groups, with no significant differences in weekly consumption at any dose level ($P > 0.05$).

Table 6: water intake (mean ± SEM, in ml) of female rats during 28-day dermal exposure to C. nardus and E. globulus formulation.

Water Intake	Unit	Dose(mg/kg)				p-value
		250mg/kg	**500mg/kg**	**1000mg/kg**	**Control**	
Week-1	**ML**	80.22±7.69	83.46±5.75	84.32±7.42	75.14±11.23	**0.85**
Week-2	**ML**	45.30±8.13	74.92±11.51	66.14±10.53	65.30±7	**0.19**
Week-3	**ML**	45.98±12.09	35.38±4	39.86±1.65	32.82±1.34	**0.51**
Week-4	**ML**	79.74±4.37	84.52±7.51	83.22±3.92	78.88±6.93	**0.88**

Values are expressed as mean ± SEM (n=5 per group). Statistical significance was assessed using one-way ANOVA; P<0.05

Table 7: water intake (mean ± SEM, in ml) of Male rats during 28-day dermal exposure to C.nardus and E. globulus formulation.

Water Intake	Unit	Dose(mg/kg)				p-value
		250mg/kg	**500mg/kg**	**1000mg/kg**	**Control**	
Week-1	**ML**	75.30±7.1	81.40±2.73	86.56±2.18	78.30±3.33	**0.32**
Week-2	**ML**	83.18±3.38	74.02±2.63	83.86±4	81.92±2.02	**0.13**
Week-3	**ML**	83.58±2.93	70.54±3.46	77.86±3.94	78.20±5.01	**0.17**
Week-4	**ML**	90.20±3.99	96±0.70	93.7±1.03	96.02±0.77	**0.21**

Values are expressed as mean ± SEM (n=5 per group). Statistical significance was assessed using one-way ANOVA; P<0.05

5.2.5. Effects of *C. nardus* and *E. globulus* formulation on hematological parameters

Table 8 and 9 summarize the hematological profile of female and male Wistar rats, respectively, following 28 days of dermal exposure to *Cymbopogon nardus* and *Eucalyptus globulus* formulation. In both sexes, no statistically significant differences (P>0.05) were observed in any of the measured hematological parameters, including red blood cell count (RBC), white blood cell count (WBC), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), lymphocyte percentage (LYM), and platelet count (PLT), when compared to the control group.

Table 8: hematological parameters (mean± SEM) of female rats after 28-day dermal exposure to C. nardus and E. globulus formulation.

Hematologic Parameters	Units	Dose(mg/kg)				P-value
		250mg/kg	**500mg/kg**	**1000mg/kg**	**control**	
RBC	10^6/µl	7.88±0.086	7.90±0.083	7.98±0.074	7.94±0.074	**0.83**
WBC	10^3/µl	8.94±0.12	9.28±0.10	9.24±0.06	9.10±0.16	**0.22**
MCH	Pg	19.22±0.08	19.16±0.15	19.26±0.06	19.18±0.07	**0.89**
HTC	%	15.18±0.13	15.06±0.12	15.22±0.08	15.04±0.09	**0.60**
MCV	fL	56.70±0.38	56.68±0.21	56.96±0.26	56.70±0.08	**0.85**
MCHC	g/dL	33.88±0.12	33.58±0.15	34.06±0.16	33.70±0.27	**0.32**
LYM	%	76.60±0.50	78.00±0.70	77.84±0.95	78.42±0.76	**0.38**
PLT	10^3/µl	958±8.15	950±9.61	954.16±6.20	954.46±9.81	**0.93**

Values are expressed as mean ± SEM (n=5 per group). Statistical significance was assessed using one-way ANOVA; P<0.05

Table 9: hematological parameters (mean± SEM) of Male rats after 28-day dermal exposure to C. nardus and E. globulus formulation

Hematologic Parameters	Units	Dose(mg/kg)				P-value
		250mg/kg	**500mg/kg**	**1000mg/kg**	**control**	
RBC	10^6/µl	8.86±0.67	8.86±0.57	7.28±0.63	7.32±0.85	**0.20**
WBC	10^3/µl	8.06±0.56	8.18±0.78	8.70±0.83	9.60±0.92	**0.51**
MCH	Pg	18.96±0.54	19.20±0.65	19.02±0.80	19.68±0.92	**0.90**
HTC	%	15.28±0.73	16.28±0.53	13.90±0.63	15.54±0.59	**0.09**
MCV	fL	55.80±0.89	56.50±0.48	56.52±0.64	55.22±0.79	**0.54**
MCHC	g/dL	33.12±0.60	34.50±0.66	33.14±0.67	33.46±1.06	**0.56**
LYM	%	80.02±0.76	80.34±0.79	78.40±0.82	78.92±0.63	**0.26**
PLT	10^3/µl	954.08±1.04	954.92±0.98	954.18±0.63	953.86±0.49	**0.81**

5.2.6. Effects of *C. nardus* and *E. globulus* formulation on Biochemical parameters

Tables 10 and 11 present the results of serum biochemical analyses used to evaluate liver and kidney function in female and male Wistar rats, respectively, following 28 days of dermal application of *Cymbopogon nardus* and *Eucalyptus globulus* formulation. No statistically significant differences ($P > 0.05$) were observed in any of the measured biochemical parameters across treatment groups when compared to controls. These parameters include alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin (ALB), creatinine, and urea levels.

Table 10: serum biochemical parameters (mean ± SEM) of female rats after 28-day dermal exposure to C. nardus and E. globulus formulation.

Clinical parameters	Dose(mg/kg)				P-value
	250mg/kg	**500mg/kg**	**1000mg/kg**	**Control**	
ALP(IU/L)	93.18±2.34	83.70±4.87	90.14±0.90	92.72±3.01	**0.16**
AST(IU/L)	138.52±11.65	132.90±6.89	131.40±1.45	154.16±11.23	**0.28**
ALT(IU/L)	48.60±5.48	42.76±3.48	51.94±7.44	48.70±4.77	**0.69**
ALB(g/dl)	4.87±0.14	4.91±0.12	4.62±0.06	4.71±0.37	**0.74**
CREATININE(mg/dl)	0.34±0.01	0.36±0.09	0.39±0.01	0.36±0.01	**0.11**
UREA(mg/dl)	33.20±1.66	34.44±1.60	32.88±1.22	32.56±0.60	**0.77**

ALT=Alanine aminotransferase; AST=Aspartate aminotransferase; ALP=Alkaline phosphatase; ALB=Albumin. Values are expressed as mean ± SEM (n=5 per group). Statistical significance was assessed using one-way ANOVA; P<0.05

Table 11: serum biochemical parameters (mean ± SEM) of Male rats after 28-day dermal exposure to C. nardus and E. globulus formulation.

Clinical parameters	Dose(mg/kg)				P-value
	250mg/kg	**500mg/kg**	**1000mg/kg**	**Control**	
ALP(IU/L)	101.90±5.49	101.04±4.26	108.70±4.28	101.06±2.02	**0.52**
AST(IU/L)	158.46±23.29	151.14±21.17	164.46±41.58	167.84±11.87	**0.97**
ALT(IU/L)	52.42±7.86	49.16±7.88	47.02±4.17	55.64±11.94	**0.89**
ALB(g/dl)	4.59±0.14	4.55±0.15	4.35±0.03	4.37±0.02	**0.30**
CREATININE(mg/dl)	0.34±0.01	0.36±0.01	0.36±0.01	0.43±0.09	**0.58**
UREA(mg/dl)	35.216±1.17	34.62±1.02	36.96±1.47	43.00±6.99	**0.37**

ALT=Alanine aminotransferase; AST=Aspartate aminotransferase; ALP=Alkaline phosphatase; ALB=Albumin. Values are expressed as mean ± SEM (n=5 per group). Statistical significance was assessed using one-way ANOVA; P<0.05

5.2.7. The effect of 28-day dermal application of *C. nardus* and *E. globulus* on the gross pathology of organs

The visual inspection of internal organs, including the liver and kidney revealed no gross pathological changes following 28-day dermal application of *Cymbopogon nardus* and *Eucalyptus globulus* formulation at a dose of 1000 mg/kg. No sign of necrosis, discoloration, swelling, or atrophy were observed. The organs from treated rats appeared comparable in size, color, shape, and texture to those from the control group, in both sexes.

Figure 1 and 2 display representative gross morphology of organs from treated and control groups, confirming the absence of visible abnormalities.

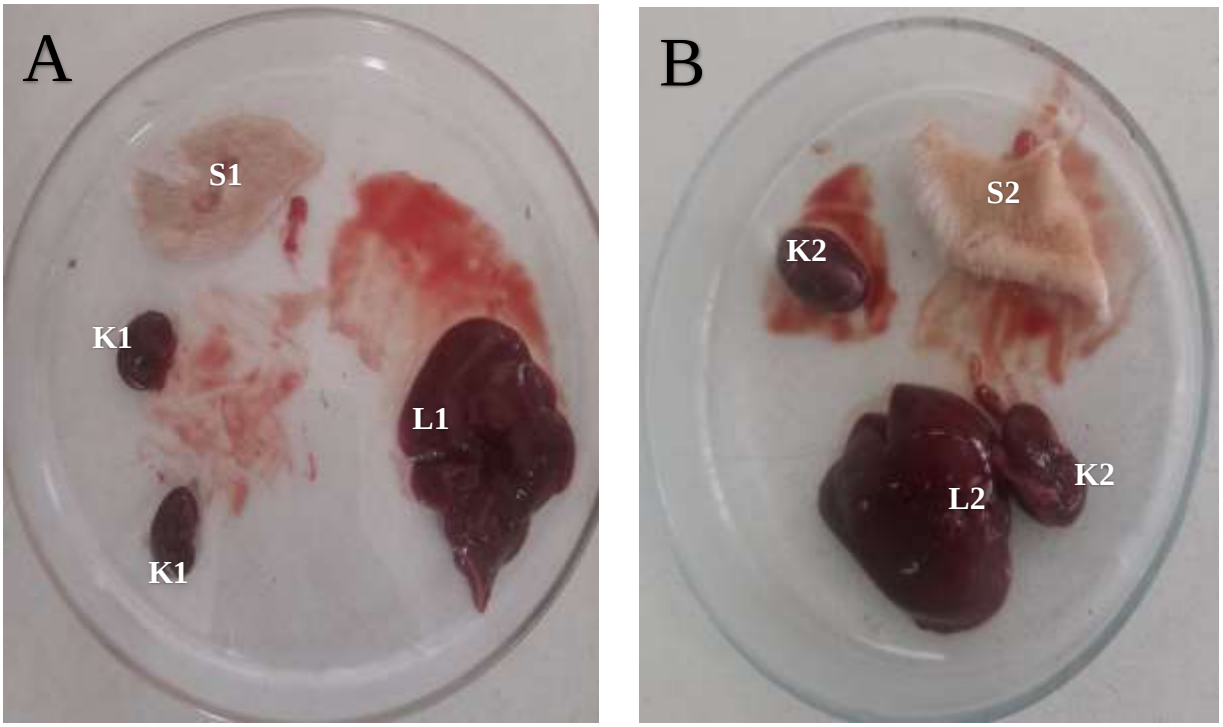

Figure 1: Gross pathological comparison of liver (L), Kidney (K), and Skin (S) tissues in female wistar rats after 28 days of dermal application of C. nardus and E. globulus formulation.

A: Tissues from high-dose group (1000mg/kg) Liver (L1), Kidney (K1), and skin (S1).

B: Tissues from control group Kidney (K2), Liver (L2), and Skin (S2).

No visible difference in color, size, or surface features were observed between treatment and control tissues.

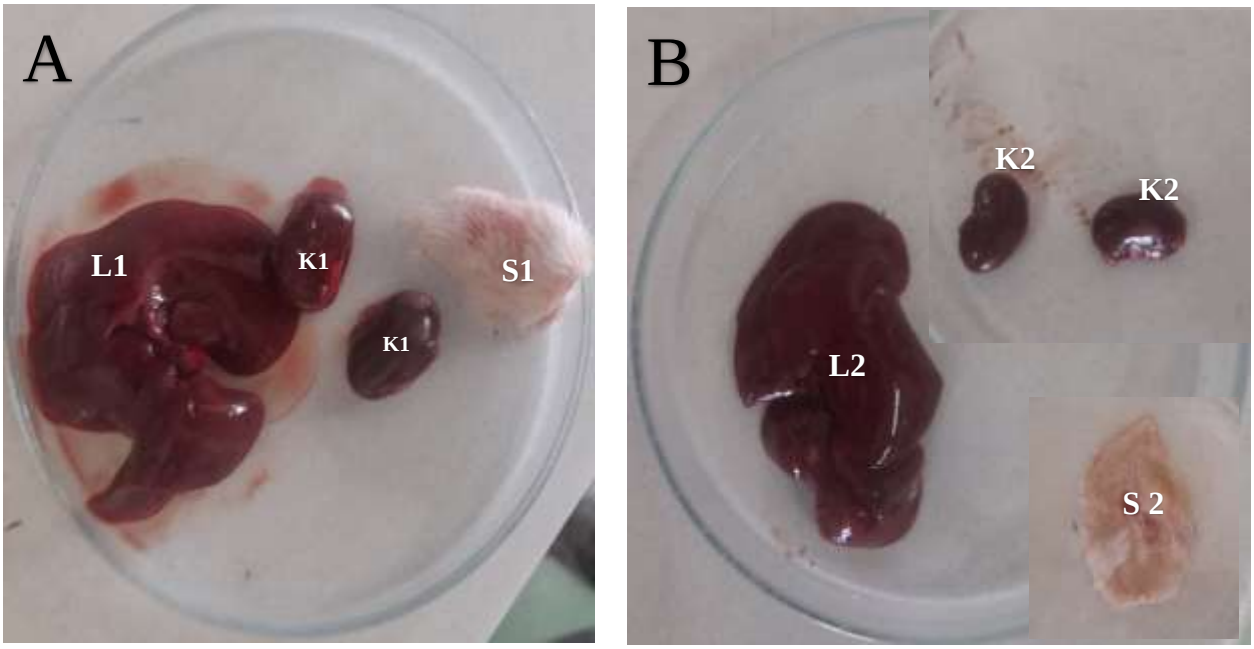

Figure 2: Gross pathological comparison of liver (L), Kidney (K), and Skin (S) tissues in Male Wistar rats after 28 days of dermal application of C. nardus and E. globulus formulation.

A: Tissues from high-dose group (1000mg/kg): Liver (L1), Kidney (K1), and skin (S1).

B: Tissues from the control group Kidney (K2), Liver (L2), and Skin (S2).

No visible differences in color, size, or surface features were observed between treatment and control tissues.

5.2.8. The effect of 28-day dermal application of *C. nardus* and *E. globulus* on the histopathology of the kidney

Histological evaluation of kidney tissues from male and female Wistar rats treated with C. nardus and E. globulus at 1000mg/kg revealed no observable pathological alterations when compared to the control group. The glomeruli were structurally intact and enclosed within well-defined bowman's capsules. The proximal convoluted tubles (PCTs) were lined with simple cuboidal epithelium exhibiting brush borders and centrally located nuclei, while the distal convoluted tubules (DCTs) displayed clear lumens with basally located nuclei, consistent with normal renal histology.

Figures 3A-D illustrate representative sections from treated and control animals, confirming structural preservation in all examined samples.

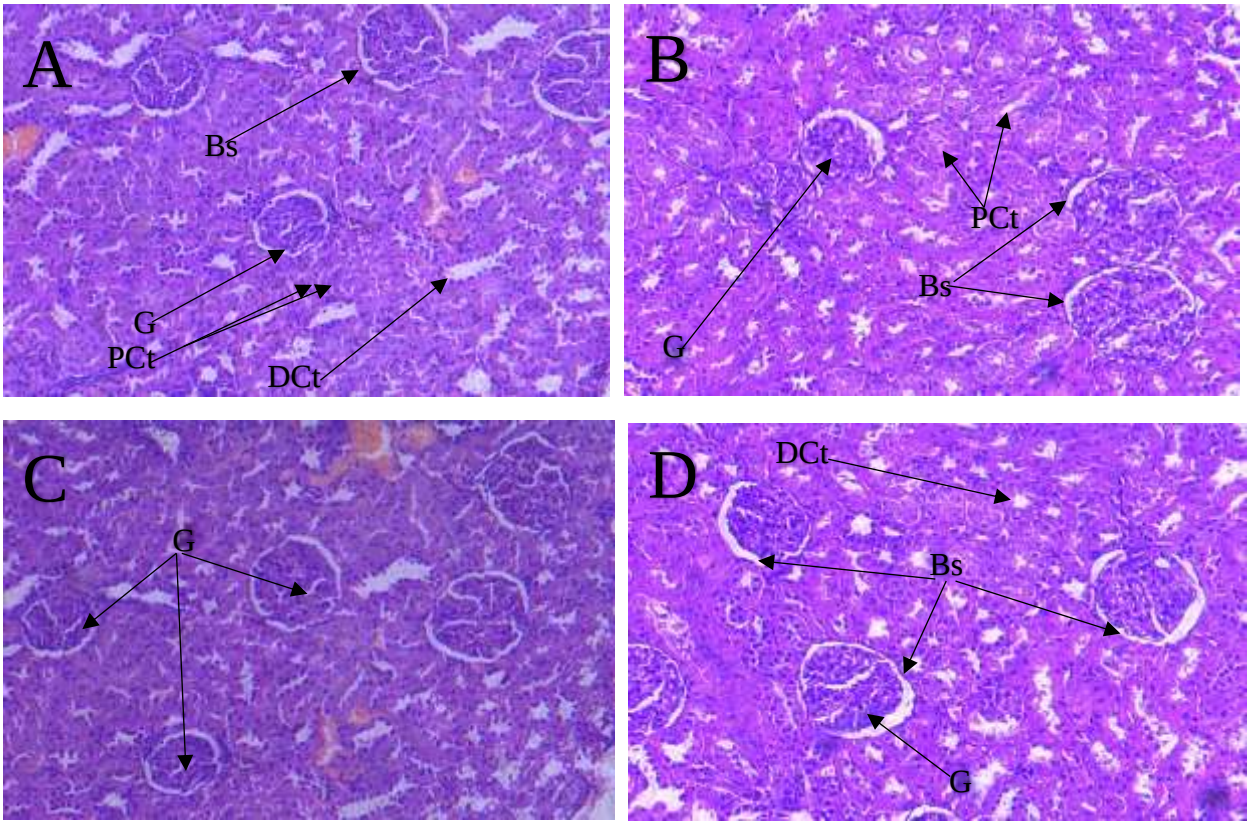

Figure 3: Kidney sections of albino Wistar rats after 28-day dermal application of C. nardus and Globulus formulation (H&E, 400x). Images taken to assess histopathological changes

A. Female rat tested with high-dose group showing normal glomeruli (G), Bowman's space (Bs), and intact proximal (PCt) and distal convoluted tubules (DCt)
B. Female rat from the control group showing proximal convoluted tubule (PCt), glomeruli (G), and bowman's space (Bs).
C. Male rat tested with high dose showing glomerulus (G)
D. Male rat from the control groups showing distal convoluted tubule (DCt), Bowman's space (Bs), and glomeruli (G).

5.2.9. The effect of 28-day dermal application of *C. nardus* and *E. globulus* on the histopathology of the liver

The microscopic examination of liver sections from both treated and control rats demonstrated preserved hepatic architecture. The central vein (CV), hepatic sinusoids (S), and radiating hepatocyte cords were clearly defined and structurally normal. The portal triad components including the portal vein (PV), hepatic artery (HA), and bile duct (Bd) were intact and exhibited no signs of degeneration or inflammation.

Treated groups at the highest dose (1000 mg/kg) showed no histological deviations from control animals, indicating the absence of hepatotoxic effects due to the dermal application of C. nardus and E. globulus.

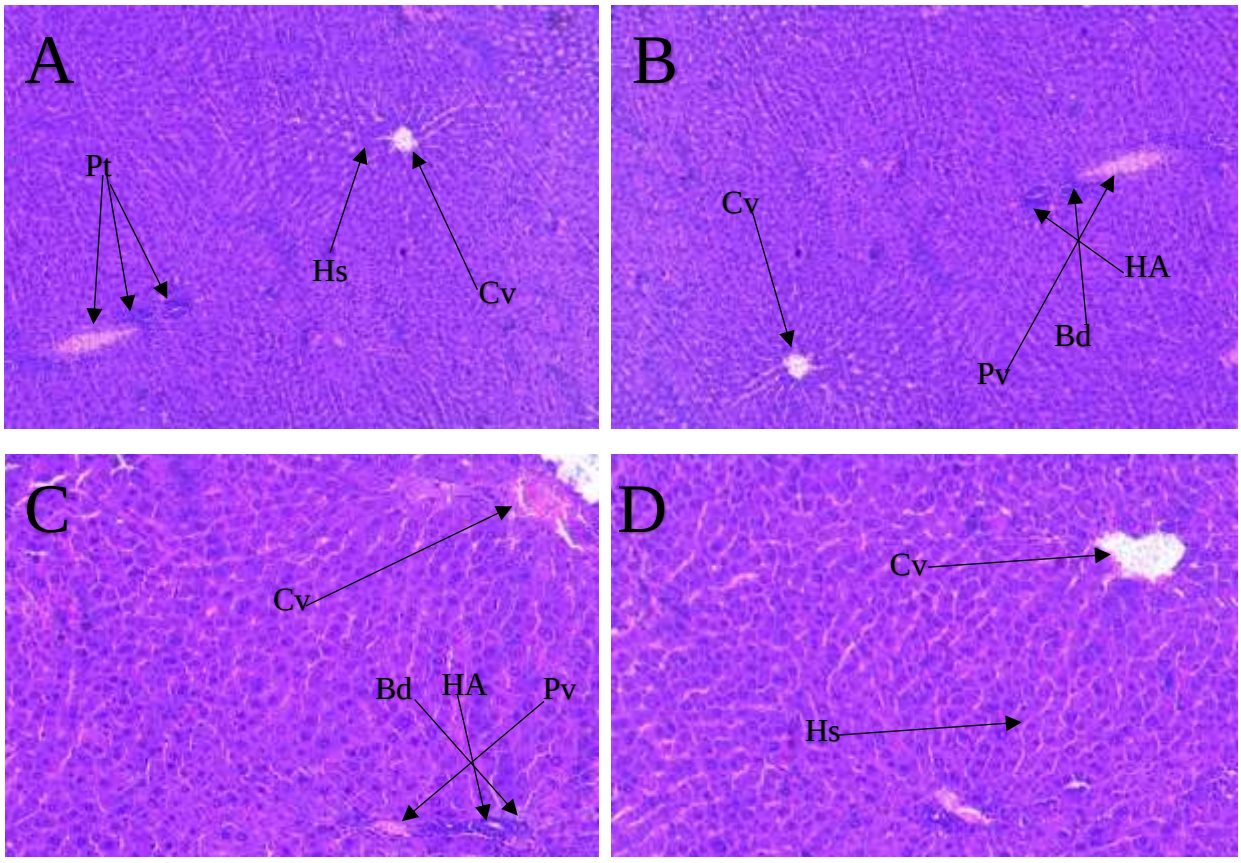

Figure 4: Liver sections of albino Wistar rats after 28-day dermal application of C.nardus and Globulus formulation (H&E, 400x). Images taken to assess histopathological changes

A. Female rat tested with high-dose group showing **Cv**: central vein, **Pt**: portal triad, **Hs**: hepatic sinusoid. **B.** Female rat from the control group showing **Cv**: central vein, **Pv**: portal vein, **Bd**: bile duct, **Ha**: hepatic artery. **C.** Male rat tested with high dose showing **Cv**: central vein, **Pv**: portal vein, **Bd**: bile duct, **Ha**: hepatic artery. **D.** Male rat from the control groups showing **Cv**: central vein, **Hs**: hepatic sinusoid

5.2.10. The effect of 28-day dermal application of *C. nardus* and *E. globulus* on the histopathology of the skin

Histopathologic evaluation of the skin section from male and female rats treated with C. nardus and E. globulus formulation at 1000mg/kg showed no detectable histopathological abnormalities when compared to the control group. The epidermis consisted of stratified squamous epithelium with normal thickness and keratinization. The dermis contained well preserved sebaceous gland (Sg) and hair follicles (Hf), while the hypodermis exhibited normal adipose tissue (At) distribution, with no signs of inflammation or degeneration.

Figures 5A-D illustrate the structural preservation of skin architecture in both treated and control groups, further supporting the formulations dermal safety.

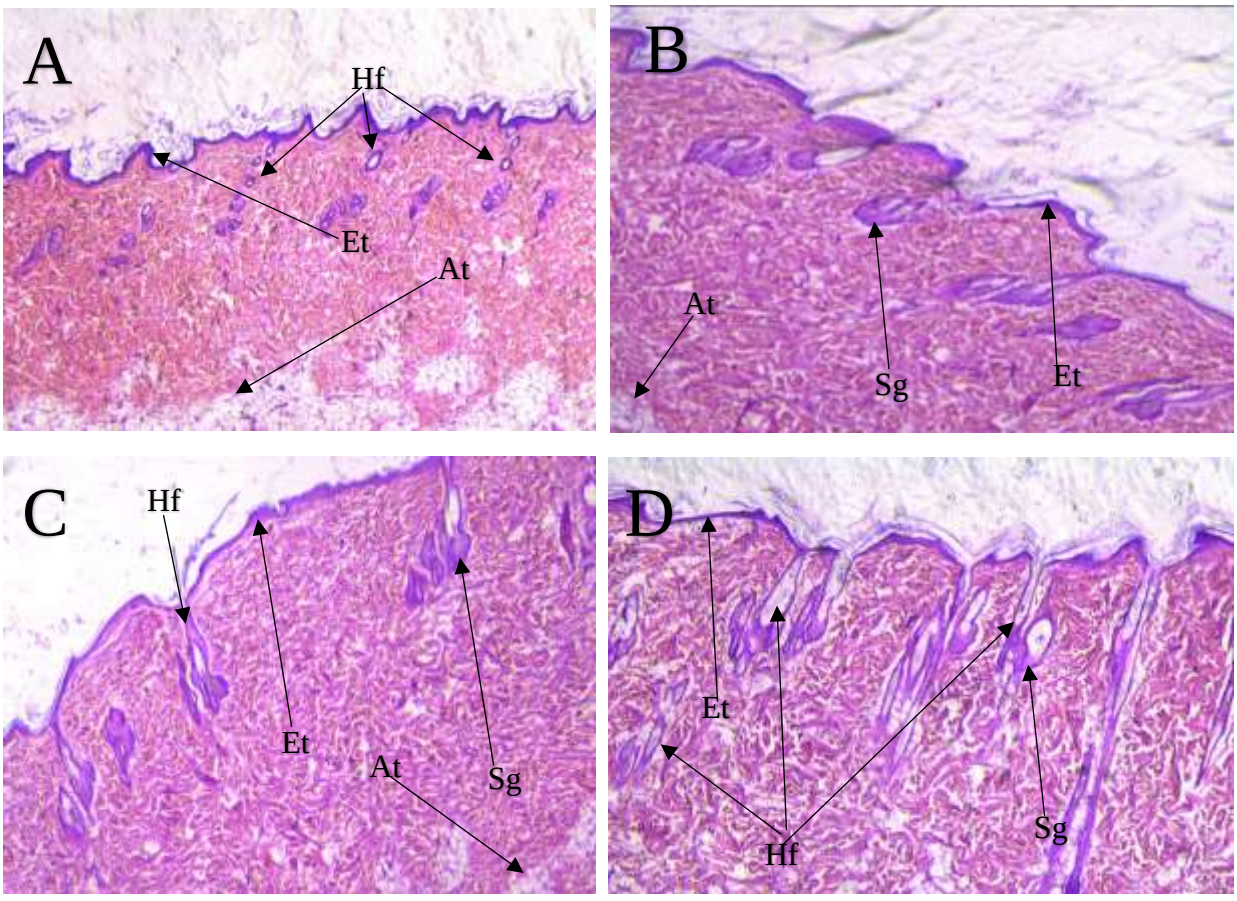

Figure 5: Skin sections of albino Wistar rats after 28-day dermal application of C.nardus and Globulus formulation (H&E, 400x). Images taken to assess histopathological changes

A. Female rat tested with high-dose group showing **Et**: epithelial tissue, **Hf**: hair follicles, **At**: Adipocyte tissue. **B.** Female rat from the control group showing **Et**: epithelial tissue, **At**: Adipocyte tissue, **Sg:** sebaceous gland. **C.** Male rat tested with high dose showing **Et**: epithelial tissue, **Hf**: hair follicles, **Sg:** sebaceous gland, and **At**: Adipocyte tissue. **D.** Male rat from the control groups showing **Et**: epithelial tissue, **Hf**: hair follicles, **Sg:** sebaceous gland

6. Discussion

Herbal remedies using plant products have seen an increasing worldwide acceptance, especially in the primary health care systems of developing countries (81). In spite of their natural nature, the assumption that plant-based medicines are inherently safe can be misleading. Many phytochemicals contain bioactive substances that, while therapeutically beneficial, can also yield toxic manifestations if incorrectly used or employed without scientific evidence (82). Hence, extensive toxicological studies, including acute and subacute studies, are crucial to determine both efficacy and safety, especially in populations where self-medication is high (82–84).

The present study was conducted to assess the acute and subacute dermal toxicity of a formulated blend of essential oils of *Cymbopogon nardus* and *Eucalyptus globulus* in Wistar rats, following the guidelines of the Organization for Economic Cooperation and Development (OECD) for chemical safety testing (47). Traditionally, these oils have been well-valued in folk medicine because of their antimicrobial, anti-inflammatory, and insect-repellent properties (39). Yet, the official use of these products in therapeutic or cosmetic formulations requires serious toxicological confirmation.

Acute Dermal Toxicity

In the acute dermal toxicity test, application of a 5000 mg/kg limit dose to female Wistar rats caused no mortality or clinical signs of toxicity throughout the 14-day observation period. Based on these findings, the dermal LD_{50} of the formulation is considered to be greater than 5000 mg/kg, classifying it as Category 5 or unclassified for dermal toxicity according to the Globally Harmonized System.(47,85,86).

This result is in close alignment with previous reports on *E. globulus*-based preparations and other plant-derived oils like *Morinda citrifolia* and *Cymbopogon martini*, which showed LD_{50} values greater than 5000 mg/kg and lack of treatment-related mortality, albeit via the oral route (5,6). Although these studies employed oral administration, the similarity in high tolerability underscores the generally low systemic toxicity of these essential oils.

Subacute Dermal Toxicity

Daily dermal treatment with the combined formulation in doses of 250, 500, and 1000 mg/kg for 28 days resulted in no mortality, abnormal clinical signs, or appreciable changes in body weight gain, food consumption, or water intake relative to controls. These parameters are generally considered sensitive markers of systemic toxicity, indicating overall metabolic and physiological integrity. The current results align with research by *Zahi et al. (2020), Reduan et al. (2020),* and *Ayenew et al. (2022),* who all noted normal weight gain and intact feeding behavior in rodents exposed dermally to essential oil extracts (5,6,25).

Weights of Organs and General Observations

Relative organ weights are primary indicators for identifying systemic or localized toxicity, and the liver and kidneys are particularly susceptible to toxicant-induced alterations (6). In the present study, there were no significant differences in the relative weights of either the liver or kidneys between treatment groups. In addition, macroscopic inspection verified the lack of lesions or gross pathological changes. These results are in strong agreement with *Ayenew et al. (2022), Reduan et al. (2020),* and *Zahi et al. (2020),* who also noted unchanged organ weights and no apparent lesions after acute or subacute dermal administration of botanical extracts. In contrast to oral routes, where metabolic stress may be greater, this congruence suggests that little systemic absorption of lipophilic chemicals like citronellal and eucalyptol through the skin barrier minimizes harmful burden on key organs (5,6,25). The present study thus adds toxicological evidence for the dermal safety of essential oil preparations at doses up to 1000 mg/kg.

Hematological Parameters

Hematological parameters are reliable markers of systemic toxicity, particularly in identifying hematopoietic suppression, inflammation, or immune impairment (25). The present study did not identify any notable alterations in red blood cell counts, hemoglobin concentration, hematocrit, white blood cell counts, or platelet counts across treatment groups, indicating that erythropoietic, hemostatic, and immune functions are intact. These results support those reported by *Ayenew et al. (2022),* who recorded hematological stability after 28 days of dermal exposure to Cymbopogon-derived oils, and those by *Reduan et al. (2020),* who also did not detect any hematological abnormalities in rats exposed to plant-derived essential oils (5,25). This further validates the

finding that subacute dermal exposure to the combined formulation does not compromise blood or immune function.

Biochemical Parameters

Serum biochemical tests also supported the lack of systemic toxicity (87). There were no changes in hepatic markers (ALT, AST) or renal parameters (urea, creatinine) of any significance. Small, non-significant variations noted at higher doses are best explained by transient adaptive responses instead of pathology. These findings are similar to those of *Hu et al. (2014),* who noted only slight hepatic and renal changes at very high oral doses of E. globulus emulsions (792–1188 mg/kg), while dermal administration was well tolerated (31). Likewise, *Mengiste et al. (2020)* found no notable biochemical changes in rats that received dermal exposure to E. globulus oil at 5% levels (32). Mechanistically, this safety can be ascribed to the barrier function of the skin, which restricts systemic absorption of lipophilic terpenoids like eucalyptol and citronellal, with a contribution from local cutaneous metabolism.

Histopathological Findings

Histopathological tests gave additional assurance of the safety of the formulation. Sections of the liver showed normal hepatocyte and sinusoidal structure, with no necrosis or steatosis, unlike the hepatocellular changes seen with high-dose oral toxicity of E. globulus *(Hu et al., 2014)* (31). Renal sections likewise indicated intact glomeruli and tubules in all groups, in accordance with reports by *Mengiste et al. (2020),* who found no renal pathology following dermal application of E. globulus (32). Skin histology showed intact epidermal and dermal structure, with no inflammatory infiltrate, ulceration, or degeneration of keratinocytes, in agreement with dermatological safety testing by *Becker et al. (2019),* who concluded that cosmetic ingredients derived from *E. globulus* are safe if formulated in a manner to prevent sensitization, while *Khairullah Zahi et al. (2015)* similarly reported that acute and sub-acute dermal exposure to *Morinda Citifolia* fruit extract in rats produced no morphological alteration in the skin (6,33).

Collectively, the present findings provide conclusive evidence demonstrating that the topical application of the blended essential oil preparation of *C. nardus* and *E. globulus*, at doses of up to 1000 mg/kg, does not cause systemic or local toxicity.

These findings strongly corroborate previous toxicological studies and extend the evidence base regarding the dermal safety profile of essential oils. The established No Observed Adverse Effect Level (*NOAEL*) of at least 1000 mg/kg/day for a period of 28 days strengthens the short-term safety of this preparation upon topical use.

Importantly, these findings highlight the protective role of the cutaneous barrier in preventing systemic toxicity related to lipophilic phytochemicals, thus highlighting the relatively safer toxicological profile of dermal application when compared with oral administration. Accordingly, the present study forms a substantial preclinical foundation for the safe integration of *C. nardus* and *E. globulus* into standardized topical preparations, including cosmetics and natural mosquito repellents, which has significant public health and future product development implications.

7. Conclusion

The present study demonstrated that dermal application of a combined C. nardus and E. globulus essential oil formulation produced no evidence of acute or sub-acute dermal toxicity in albino Wistar rats at tested doses. Acute dermal exposure to a limited dose of 5000 mg/kg caused no mortality or clinical signs of toxicity, indicating an LD_{50} greater than 5000mg/kg and classified in GHS category 5 (low toxicity). No statistically significant changes were also observed in body weight, organ weight, hematological parameters, and biochemical analyses at doses tested up to 1000 mg/kg. Histopathology of the skin, liver, and kidneys did not show any lesions or structural changes due to treatment. This suggests that the combined essential oil formulation has low dermal toxicity and poses minimal toxicological risk upon dermal exposure.

These findings establish a NO Observed Adverse Effect Level (*NOAEL*) of at least 1000mg/kg/day for 28-day dermal exposure in rats. The results suggest that the combined formulation is safe for short-term topical use under the tested conditions and provides a toxicological basis for further development as a mosquito repellent or cosmetic product.

8. Recommendation

Based on the findings of this study, it is recommended that properly designed studies be conducted to evaluate the sub chronic and chronic dermal toxicity of the Cymbopogon nardus and Eucalyptus globulus formulation, with careful monitoring of body weight, hematological and biochemical parameters, and complete histopathological investigation of the skin, liver, and kidneys. Reproductive and developmental toxicity, as well as toxicity to other organs like heart, brain, lungs, pancreas, stomach, and intestines, should also be evaluated in subsequent studies. Clinical studies in human subjects are also advocated to ascertain broad safety and efficacy profiles, thereby justifying the formulation's potential therapeutic and cosmetic applications.

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10. Appendices

10.1. Appendix I: Data Collection Tool for Food Intake and Body Weight Measurements

10.1.1. Appendix 1.1: Daily Food Intake

Daily Food In Take Of _____ Rats (g)					
Days	Group-1	Group-2	Group-3	Group-4	
Day-1					
Day-2					
Day-3					
Day-4					
Day-5					
Day-6					
Day-7					
Day-8					
Day-9					
Day-10					
Day-11					
Day-12					
Day-13					
Day-14					
Day-15					
Day-16					
Day-17					
Day-18					
Day-19					
Day-20					
Day-21					
Day-22					
Day-23					
Day-24					
Day-25					
Day-26					
Day-27					
Day-28					

10.1.2. Appendix 1.3: Weekly Body Weight

Weekly Body Weight (g) Of _____ Rats					
Group-1	Week-0	Week-1	Week-2	Week-3	Week-4
Rat-1					
Rat-2					
Rat-3					
Rat-4					
Rat-5					
Group-2					
Rat-1					
Rat-2					
Rat-3					
Rat-4					
Rat-5					
Group-3					
Rat-1					
Rat-2					
Rat-3					
Rat-4					
Rat-5					
Group-4					
Rat-1					
Rat-2					
Rat-3					
Rat-4					
Rat-5					

10.2. Appendix II: preparation of working chemicals and solutions

Solution	Components	Quantity
10% Neutral Buffered Formalin	40% formaldehyde	100 ml
	Sodium hydrogen phosphate monohydrate	4 g
	Disodium hydrogen phosphate anhydrous	6.5 g
	Distilled water	900 ml
Harris' Hematoxylin (H)	Hematoxylin crystals	2.5 g
	Absolute ethanol	25 ml
	Potassium alum	50 g
	Mercuric oxide	1.25 g
	Glacial acetic acid	20 ml
	Distilled water	500 ml
1% Acid Alcohol	70% ethanol	250 ml
	Hydrochloric acid (Concentrated)	2.5 ml
1% Alcoholic Eosin (E)	Eosin Y (water soluble)	1 g
	95% ethanol	100 ml
	Glacial acetic acid	0.5 ml
Bluing Solution	Sodium bicarbonate	2.5 g
	Distilled water	1000 ml

10.3. Appendix III: Tissue processing techniques and procedures

Step	Reagent / Method	Duration
Fixation	10% Neutral Buffered Formalin	24 hrs.
Washing	Running tap water	24 hrs.
Dehydration	70% ethanol	2 hrs.
	90% ethanol	2 hrs.
	Absolute ethanol I (99.9%)	1.5 hrs.
	Absolute ethanol II	1.5 hrs.
	Absolute ethanol III	1.5 hrs.
	Absolute ethanol IV	Overnight
Clearing	Xylene I	1.5 hrs.
	Xylene II	1.5 hrs.
Infiltration (in hot oven, 60°C)	Paraffin wax I (56°C)	1.5 hrs.
	Paraffin wax II (56°C)	1.5 hrs.
	Paraffin wax III (56°C)	Overnight

10.4. Appendix IV: Routine Hematoxylin and Eosin (H&E) Staining Procedures

Step	Reagent / Method	Duration
Deparaffinization	Xylene I	5 min
	Xylene II	5 min
Rehydration	Absolute alcohol I	4 min
	Absolute alcohol II	4 min
	95% ethanol	3 min
	70% ethanol	3 min
	Rinse in distilled water	5 min
Staining	Hematoxylin	15 min
	Rinse in running tap water	5 min
	Acid alcohol (decolorization)	1–3 sec
	Rinse in running tap water	5 min
	Sodium bicarbonate solution	3–6 sec
	Rinse in running tap water	5 min
	Eosin (counterstain)	1 min
Dehydration	70% ethanol	2 min
	95% ethanol	2 min
	Absolute alcohol II	2 min
	Absolute alcohol I	2 min
Clearing	Xylene II	4 min
	Xylene I	4 min
Mounting	DPX mounting medium	Immediate application, then allow to dry for ≥24 hrs.