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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 sub-acute 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). Essential oils were extracted by hydro distillation using a Clevenger-type apparatus. A topical formulation was prepared by incorporating essential oils of *C. nardus* and *E. globulus* (2 mL each; 1:1, v/v) into 100 g of white petrolatum, corresponding to approximately 3.5% w/w total essential oils based on estimated density. Acute dermal toxicity was evaluated following OECD guideline 402, while sub-acute (28-day) dermal toxicity was assessed according to OECD guideline 410.

Forty adult albino Wistar rats (8–12 weeks old) were randomly assigned into four groups (n=10 per group; 5 males and 5 females). The test groups received dermal applications of 250, 500, and 1000 mg/kg body weight/day of the formulation, while the control group received the vehicle (white petrolatum). The formulation was applied once daily to approximately 10% of the shaved dorsal skin for 28 consecutive days. Hematological and biochemical parameters were analyzed at the end of the study. Following euthanasia under sodium pentobarbital anesthesia (150 mg/kg), the liver, kidneys, and skin were collected for Histopathological evaluation.

Result: The dermal mean lethal dose LD50 of the *C. nardus* and *E. globulus* formulation was found to be above 5000mg/kg b.w, 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 combined *C. nardus* and *E. globulus* formulation did not produce observable acute or sub-acute dermal toxicity in Wistar rats at the tested doses. These findings indicate a favorable short-term dermal safety profile in this animal model.

Key words: Cymbopogon nardus, Eucalyptus globulus, Acute toxicity, Sub-acute toxicity, Dermal, Wistar rats

1. Introduction

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.

2. Material and Methods

2.1. Study Design

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

2.2. Study Area

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

2.3. Plant Material Collection

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	*Cymbopogon nardus*	Leaves	Wondogenet
2	*Eucalyptus globulus*	Leaves	Wondogenet

2.4. Method of Extraction

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 (12,13).

2.5. Method of white paraffin-based ointment mosquito repellent preparation

A white petrolatum-based ointment containing essential oils of *Cymbopogon nardus (citronella)* and *Eucalyptus globulus* was prepared as a topical mosquito repellent formulation.

White petrolatum (100 g) was weighed using an analytical balance and transferred into a beaker. The petrolatum was melted at approximately 60 °C using a water bath. The molten base was then allowed to cool to approximately 45 °C prior to the addition of essential oils.

Essential oils of *C. nardus* and *E. globulus* were added at 2 mL each (1:1, v/v) to the melted petrolatum and mixed thoroughly using a spatula to ensure uniform distribution. Based on an estimated essential oil density of ~0.9 g/mL, the final formulation contained approximately 3.5% w/w total essential oils.

The resulting mixture was transferred into sterile containers while still warm, allowed to cool and solidify at room temperature, and labeled accordingly. The formulation was prepared once and stored under controlled conditions in a cool, dry place, protected from direct sunlight, for the duration of the study(12,13).

2.6. Experimental Animal

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(14). 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(14).

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 (14).

2.7. 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(5,15).

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

2.8. 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 (14).

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 (14).

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 (14).

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

2.9. Sub-acute dermal toxicity test

A 28-day sub-acute 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 (16).

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 (16).

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 (16).

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 EDTA-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.

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.

2.10. 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.

3. RESULT

3.1. 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 body weight, 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 b.w, respectively, while Group IV served as the control and received the vehicle only (Petrolatum).

3.2. Sub-acute dermal toxicity assessment

3.2.1. 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 b.w. 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

3.2.2. 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%.

3.2.3. 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

3.2.4. 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

3.2.5. 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	%	40.18±0.13	40.06±0.12	40.22±0.08	40.04±0.09	**0.57**
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	%	40.46±0.73	41.50±0.53	41.15±0.63	40.42±0.59	**0.80**
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**

3.2.6. 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

3.2.7. 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.

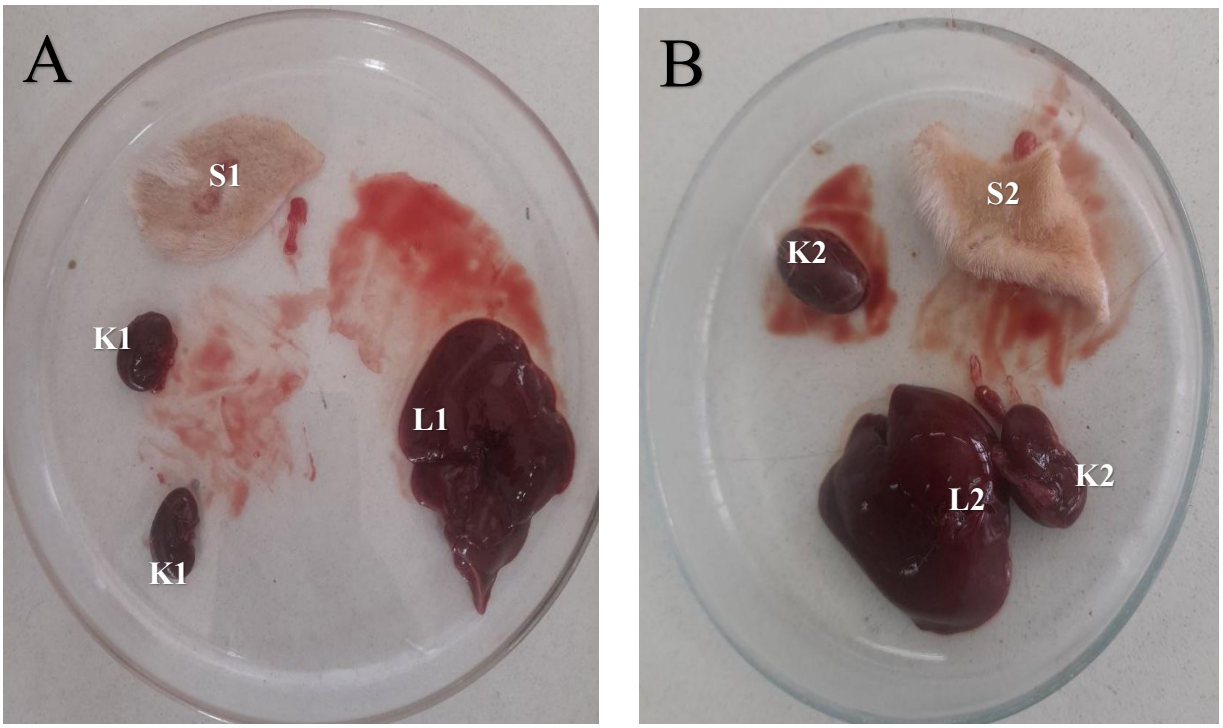

Figure 1: Gross pathological comparison of liver (L), Kidney (K), and Skin (S) tissues in female wistar rats after 28 days of dermal application of C. nardus and E. globulus formulation.

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.

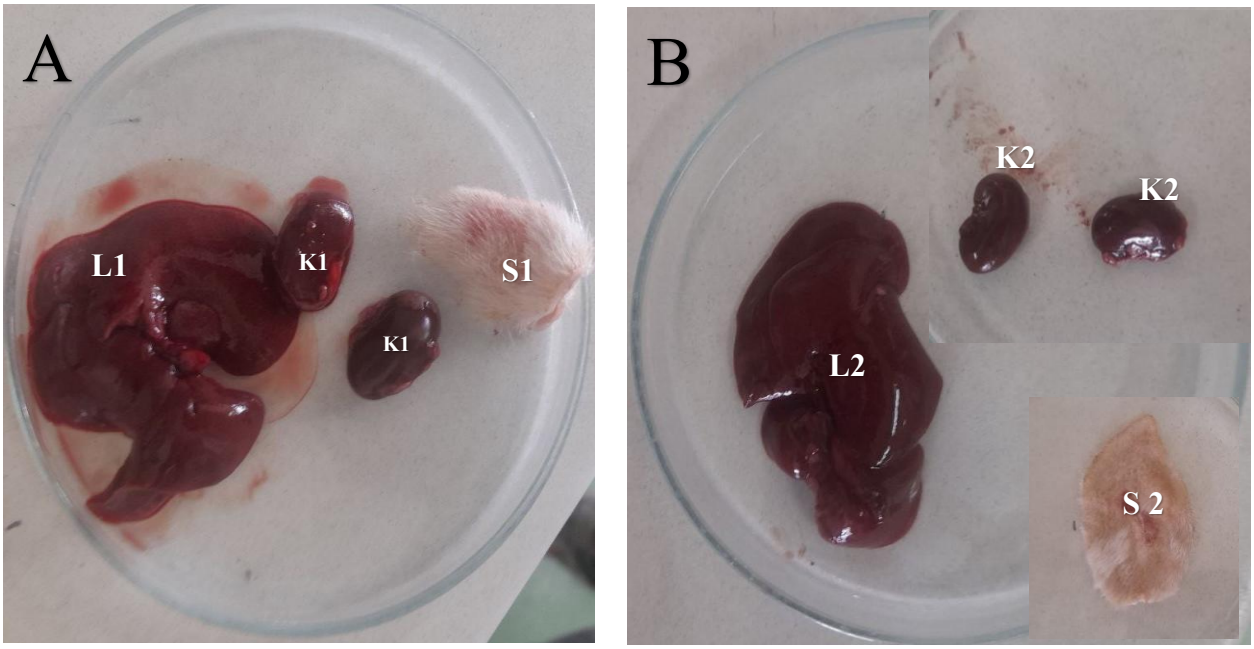

Figure 2: Gross pathological comparison of liver (L), Kidney (K), and Skin (S) tissues in Male Wistar rats after 28 days of dermal application of C. nardus and E. globulus formulation.

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.

3.2.8. 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.

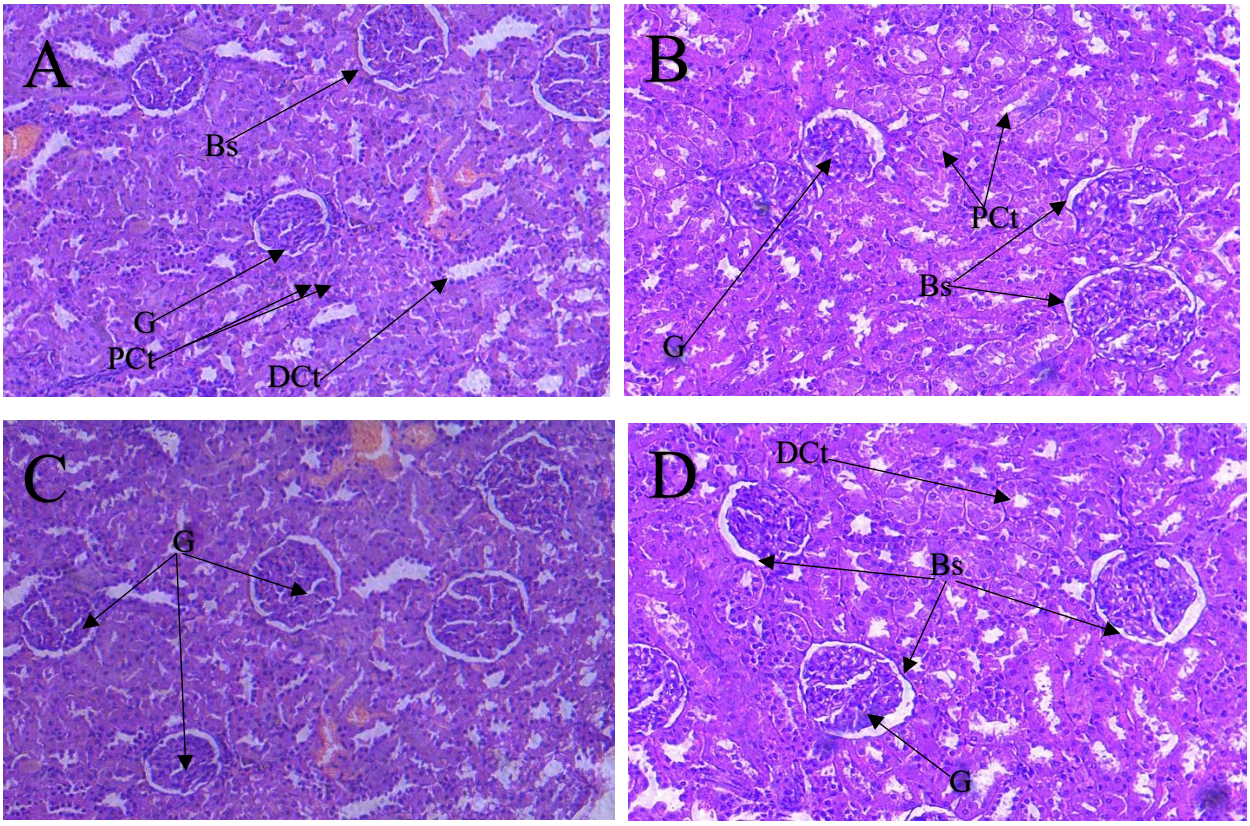

Figure 3: Kidney sections of albino Wistar rats after 28-day dermal application of C. nardus and Globulus formulation (H&E, 400x). Images taken to assess histopathological changes

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).

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

Histological evaluation of liver tissues from male and female Wistar rats treated with *C. nardus* and *E. globulus* at 1000 mg/kg revealed no observable pathological alterations compared with the control group. The hepatic architecture was well preserved, with clearly defined central veins (CV), hepatic sinusoids (S), and radiating cords of hepatocytes exhibiting normal morphology. The portal triad components, including the portal vein (PV), hepatic artery (HA), and bile duct (BD), were intact and showed no evidence of degeneration, inflammation, or other Histopathological abnormalities.

Figures 4A–D illustrate representative liver sections from control and treated albino Wistar rats, demonstrating preserved hepatic architecture and confirming the absence of treatment-related hepatotoxic effects following dermal application of *C. nardus* and *E. globulus*.

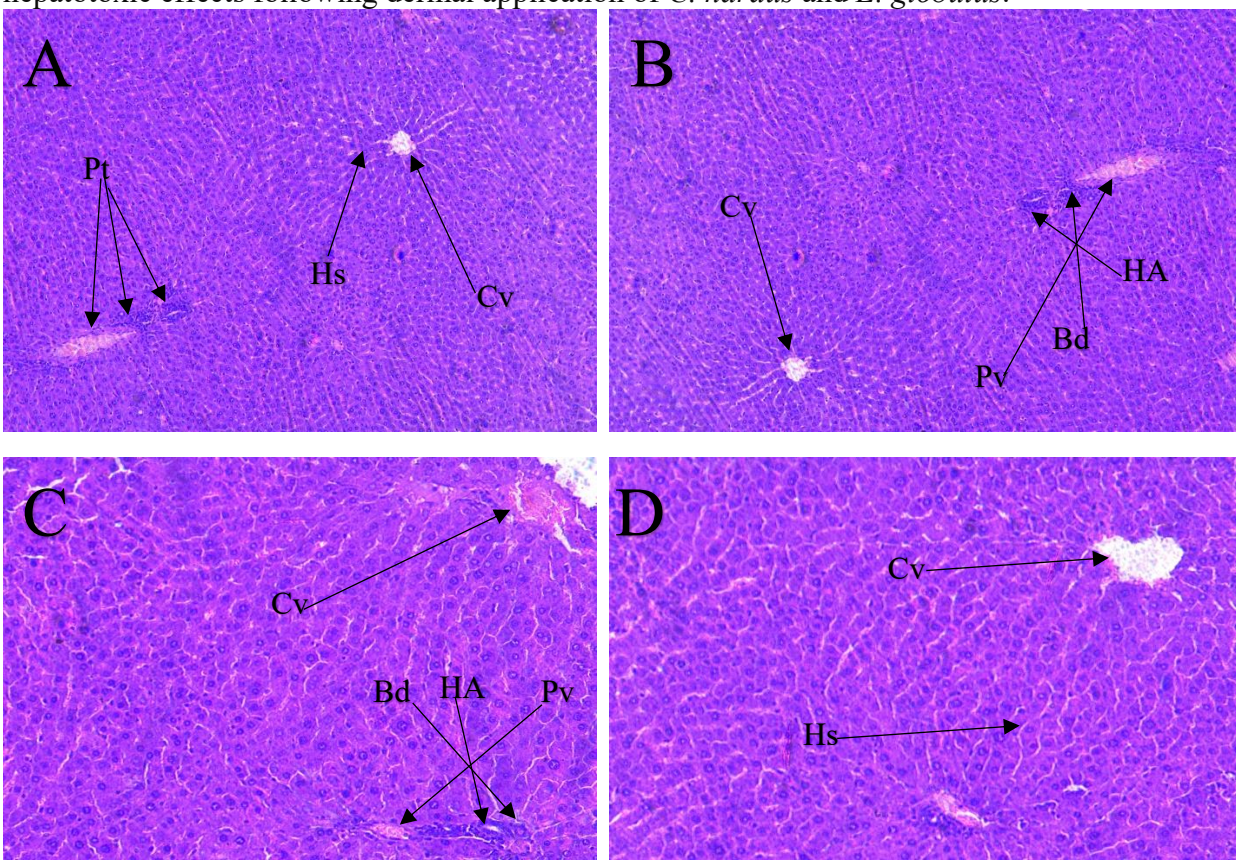

Figure 4: Liver sections of albino Wistar rats after 28-day dermal application of C.nardus and Globulus formulation (H&E, 400x). Images taken to assess histopathological changes

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

3.2.10. 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.

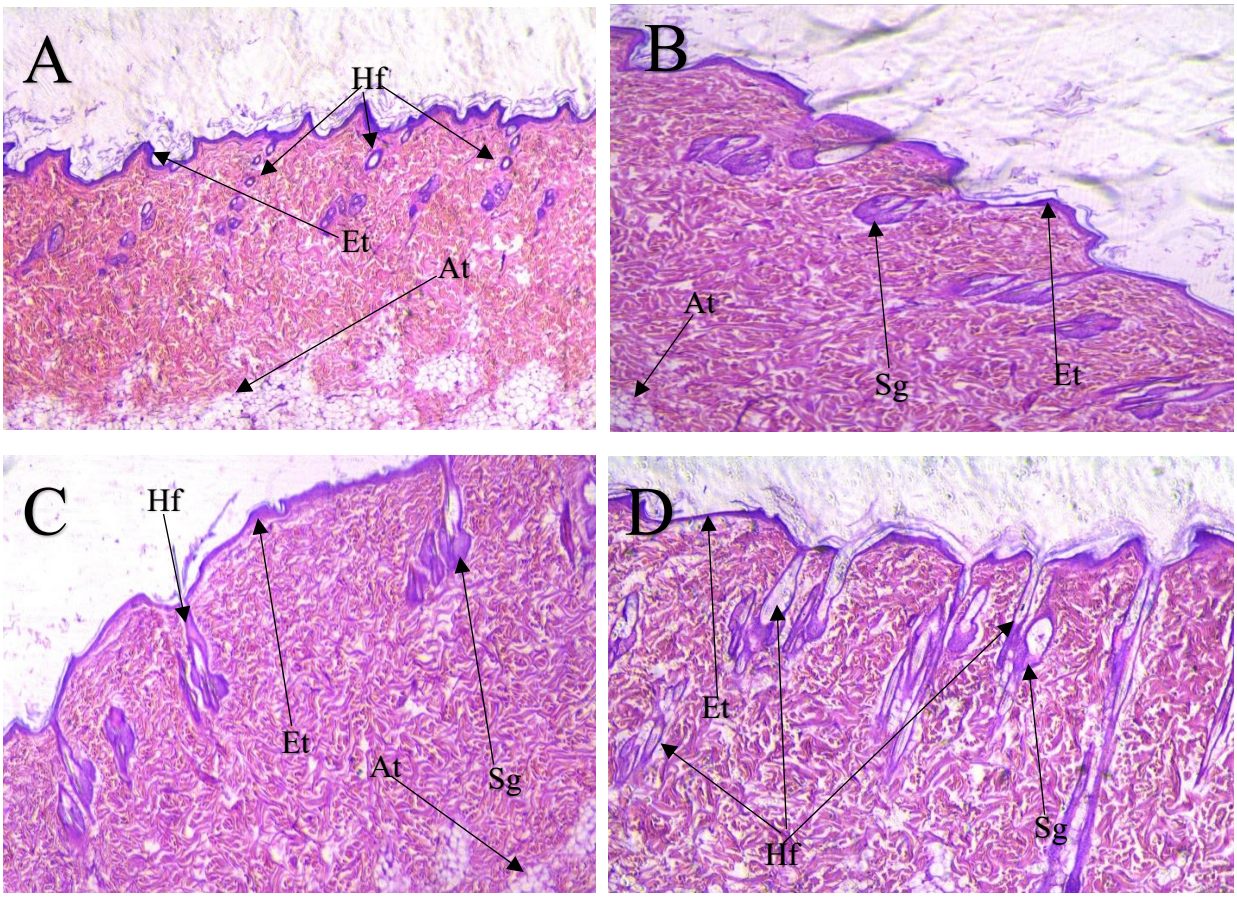

Figure 5: Skin sections of albino Wistar rats after 28-day dermal application of C.nardus and Globulus formulation (H&E, 400x). Images taken to assess histopathological changes

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

4. Discussion

Herbal remedies using plant products have seen an increasing worldwide acceptance, especially in the primary health care systems of developing countries (17). 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 (18). 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 (18–20).

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 (14). Traditionally, these oils have been well-valued in folk medicine because of their antimicrobial, anti-inflammatory, and insect-repellent properties (21). 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.(14,22,23).

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.

Sub-acute 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 (5,6,24).

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 findings are in strong agreement with *Ayenew et al. (2022), Reduan et al. (2020),* and *Zahi et al. (2020),* who similarly reported unchanged organ weights and no observable lesions following acute or subacute dermal administration of botanical extracts. In contrast to oral exposure, where first-pass metabolism may impose greater physiological stress, this consistency suggests that dermal exposure to lipophilic constituents such as citronellal and eucalyptol results in limited systemic absorption, thereby reducing toxicological burden on vital organs(5,6,24). In the assessment of general physiological parameters, no statistically significant differences in body weight gain were observed between treated and control groups. However, a reduction in mean weight gain was noted in male rats at the intermediate dose (500 mg/kg). This effect was not dose-dependent, as animals in the higher dose group (1000 mg/kg) exhibited weight gain comparable to the control group. The absence of a consistent dose–response relationship suggests that this variation is unlikely to be treatment-related and may instead reflect normal biological variability.

It is also important to consider that the relatively small sample size (n = 5 per sex per group) may limit the statistical power to detect subtle physiological differences and may contribute to variability in measured outcomes. Therefore, while the observed reduction in the mid-dose group is acknowledged, it should be interpreted with caution and not considered indicative of a

toxicologically relevant effect in the absence of corroborating clinical, biochemical, or histopathological findings.

Overall, the lack of significant changes in organ weights, absence of gross pathological alterations, and stable physiological parameters support the conclusion that the formulation did not induce observable systemic toxicity following repeated dermal exposure 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 (24). 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 (5,24). 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 (25). 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 (15). Likewise, *Mengiste et al. (2020)* found no notable biochemical changes in rats that received dermal exposure to E. globulus oil at 5% levels (26). 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)* (15). 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 (26). 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 (6,27).

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.

5. Conclusion

The present study demonstrated that dermal application of a combined Cymbopogon nardus and Eucalyptus globulus essential oil formulation produced no evidence of acute or sub-acute dermal toxicity in albino Wistar rats under the experimental conditions. A single dermal application at the limit dose of 5000 mg/kg b.w caused no mortality or treatment-related clinical signs of toxicity, indicating an LD_{50} greater than 5000 mg/kg b.w and placing the formulation in GHS Category 5 (low acute dermal toxicity). Repeated dermal application for 28 days at doses up to 1000 mg/kg/day b.w produced no statistically significant changes in body weight, relative organ weights, hematological indices, or serum biochemical parameters compared with the control group. Furthermore, histopathological examination of the skin, liver, and kidneys revealed no treatment-related structural abnormalities.

Based on these findings, the formulation exhibited a favorable dermal toxicological profile in this rat model, with a No Observed Adverse Effect Level (NOAEL) of at least 1000 mg/kg/day b.w following repeated dermal exposure for 28 days. While these results provide evidence of short-term dermal safety in rats and support further preclinical development of the formulation, they should not be interpreted as direct evidence of safety for human topical application. Additional investigations, including chronic dermal toxicity, skin sensitization and irritation studies, reproductive and developmental toxicity assessments where appropriate, and well-designed human clinical evaluations, are required to establish its safety for human use as a topical mosquito repellent or cosmetic formulation.

ABBREVIATIONS

AAU	ADDIS ABABA UNIVERSITY
AHRI	ARMAUER HANSEN RESEARCH INSTITUTE
ALB	ALBUMIN
ALP	ALKALINE PHOSPHATASE
ALT	ALANINE TRANSAMINASE
AST	ASPARTATE AMINOTRANSFERASE
DEET	N, N-DIETHY-META-TOLUAMIDE
EDTA	ETHYLENE DIAMINE TETRA ACETIC ACID
GRAS	GENERALLY RECOGNIZED AS SAFE
HCT	HEMATOCRIT
HGB	HEMOGLOBIN
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
TM	TRADITIONAL MEDICINE
WBC	WHITE BLOOD CELL

Data Availability

The majority of the data are included in this manuscript. Further data can be found from the corresponding author based on reasonable request.

Ethical Approval

The study was approved by the institutional review board of Addis Ababa University, Ethiopia, and Permissions were obtained from the relevant authorities in the School of Biomedicine and Laboratory Science, Addis Ababa University, and Armauer Hansen Research Institute before the study was initiated. A letter of clearance was obtained. In addition all possible steps were taken to avoid animal suffering during experiment

Conflict interest

The author declare that they have no conflicts of interest in this work.

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