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Research Article

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Acute Oral Toxicity Test of a Combination of Citronella (*Cymbopogon nardus*) and Neem (*Azadirachta indica*) Oils in Female DDY Mice

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ABSTRACT

Background: Increasing interest in plant-based veterinary products requires scientific evaluation of their safety. Citronella (*Cymbopogon nardus*) and neem (*Azadirachta indica*) oils contain bioactive compounds with antimicrobial and antiparasitic activities; however, data regarding the acute toxicity of their combination remain limited.

Aims: This study aimed to evaluate the acute oral toxicity and safety profile of a 1:1 combination of citronella oil (*Cymbopogon nardus*) and neem oil (*Azadirachta indica*) in female DDY mice.

Methods: A total of 24 female DDY mice were divided into four groups: one control group (aquabidest) and three treatment groups, each receiving a 1:1 mixture of citronella and neem oils at doses of 5, 10, and 15 g/kg body weight (BW) via a single oral administration. The observations were carried out for 14 days, including clinical symptoms, mortality, changes in body weight, and macroscopic examination of the liver and kidneys.

Results: No mortality or clinical signs of toxicity were observed in any group. Body weight increased normally with no significant differences ($p > 0.05$). Macroscopic examination showed normal liver and kidney features (color, size, consistency). Histopathological evaluation (H&E staining) revealed no significant abnormalities: renal glomeruli and tubules normal (degeneration score 0 in all samples); liver with no hepatocyte necrosis (score 0). $LD_{50} > 15$ g/kg BW.

Conclusion: The combination of citronella and neem did not cause any mortality or acute toxic symptoms. this material is classified as non-toxic in acute exposure ($LD_{50} > 15$ g/kg BW) and safe for veterinary herbal formulation development.

INTRODUCTION

Pet care products such as shampoos are essential in veterinary health practices because they maintain cleanliness, support skin health, and prevent ectoparasite infestations (Budreckiene et al., 2016). Regular bathing removes dirt, microorganisms, and allergens that may trigger dermatological disorders in companion animals. However, most commercial shampoos still rely on synthetic chemicals that may cause skin irritation, allergic reactions, and potential long-term toxic effects on animals and the environment. These limitations have encouraged the development of natural active ingredients that are

safer, biodegradable, and environmentally friendly (Bezerra et al., 2023).

Essential oils from tropical plants are considered promising alternatives for veterinary topical formulations. Citronella oil derived from *Cymbopogon nardus* contains citronellal, geraniol, and citronellol, which exhibit antifungal, antibacterial, and insect-repellent properties (Mahmud et al., 2022). Neem oil from *Azadirachta indica* is rich in azadirachtin, nimbin, and salannin that possess antiparasitic, anti-inflammatory, and wound-healing activities (Sidat et al., 2023). The combination of these two oils is expected to provide synergistic effects for controlling ectoparasites and maintaining the integrity of skin and hair in companion animals.

Despite traditional use, the safety of herbal ingredients requires scientific validation prior to incorporation into veterinary products. Topical shampoos risk accidental ingestion during grooming, especially in animals that frequently lick treated areas (Cope 2025). Thus, systemic toxicity assessment via acute oral toxicity testing is essential to confirm no harmful effects upon ingestion.

Acute toxicity testing evaluates intrinsic toxicity following single dose administration and estimates the median lethal dose (LD_{50}) (BPOM 2014). BPOM Regulation No. 10 of 2022 requires at least 14-day observations, including mortality, clinical signs, body weight changes, and macroscopic liver and kidney examinations (BPOM 2022). These parameters provide critical insights into the test material's safety margin. While individual citronella (*Cymbopogon nardus*) and neem (*Azadirachta indica*) essential oils exhibit low acute oral toxicity in mammals (LD_{50} >5000 mg/kg for citronella (Anggraeni et al., 2025) and >30 g/kg for neem (Deng et al., 2013), data on their combined toxicity profile remain extremely scarce. This constitutes a significant knowledge gap, as essential oil mixtures often produce synergistic, additive, or antagonistic interactions that can markedly alter toxicological outcomes relative to single exposure (You et al., 2025). The present study bridges this gap by assessing the acute oral toxicity of a 1:1 citronella neem oil blend in DDY mice, providing the first comprehensive safety data for this formulation and supporting its development as a safer active ingredient in veterinary herbal products (Maulana et al., 2025).

MATERIALS AND METHODS

This study was conducted from June to September 2025 at the Laboratory Animal Management Unit (UPHL) and the Veterinary Pharmacy Laboratory, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor, Indonesia. Ethical approval was obtained from the Animal Ethics Committee of IPB University under document No. 269/KEH/SKE/XI/2024. All experimental procedures complied with the Indonesian National Agency of Drug and Food Control (BPOM) Regulation No. 10 of 2022 concerning in vivo preclinical toxicity testing and followed the principles of laboratory animal welfare.

A total of 24 healthy female DDY mice (Mus musculus), aged 6–8 weeks and weighing 20–30 g, were used as experimental animals. The number of animals was determined using the Federer formula $(t–1)(n–1) \geq 15$, resulting in six mice per group. Animals were acclimatized for five days under controlled conditions (temperature 22–25 °C, humidity 50–60%, 12-hour light–dark cycle) with ad libitum access to standard feed and drinking water. Only clinically healthy mice without external abnormalities were included in the study.

The test materials consisted of citronella oil (*Cymbopogon nardus*, COA No. 8000-29-1) and neem oil (*Azadirachta indica*, COA No. 131w1). Both oils were mixed in a 1:1 (v/v) ratio immediately before administration to ensure homogeneity. The acute oral toxicity test was performed using a completely randomized design with four groups: a control group receiving aquabidest and three treatment groups administered the oil combination at doses of 5, 10, and 15 g/kg body weight (BW). Each group consisted of six mice. The test preparation was administered once orally via gavage using a sterile feeding needle with a maximum volume of 1 mL per animal.

Clinical observations were carried out for 14 days according to BPOM guidelines. Animals were monitored for signs of toxicity including tremor, lethargy, piloerection, salivation, diarrhea, changes in locomotor activity, and abnormal behavior. Observations were conducted twice during the first 24 hours and once daily thereafter. Mortality was recorded throughout the study for estimation of LD_{50}. Body weight was measured on days 0, 7, and 14 using a calibrated digital balance.

On day 14, all mice were anesthetized with ketamine (100 mg/kg BW) and euthanized humanely. The liver and kidneys were collected for macroscopic examination including assessment of color, surface, size, and consistency. Organs were weighed to determine absolute and relative organ weights. Tissue samples were fixed in 10% neutral buffered formalin, processed by paraffin embedding, sectioned at 4–5 µm, and stained with hematoxylin–eosin (H&E). Histopathological evaluation focused on evidence of

degeneration, necrosis, inflammatory cell infiltration, and vascular alterations.

Quantitative data of body weight and organ weight were analyzed using Statistical evaluations were performed using Minitab version 22 (Minitab Inc., State College, PA, USA). One-way ANOVA followed by Tukey's post-hoc test (where applicable) revealed no significant differences among groups ($p > 0.05$). Clinical signs, mortality, and macroscopic findings were analyzed descriptively. The entire procedure was designed to determine the intrinsic toxicity of the citronella–neem oil combination after single-dose exposure and to classify its safety based on LD_{50} estimation.

Previous studies showed that neem oil has low mammalian toxicity (Prianto et al., 2019) and essential oils from Cymbopogon did not affect body weight gain (Udayani et al., 2023). However, data on the safety of the combination of citronella and neem oils remain limited. This study was conducted to evaluate the acute oral toxicity of a 1:1 mixture of citronella (*Cymbopogon nardus*) and neem (*Azadirachta indica*) oils in female DDY mice as a scientific basis for developing safe veterinary herbal preparations.

RESULTS AND DISCUSSION

Clinical Observations and Mortality

Throughout the 14-day observation period, no mortality occurred in any experimental group administered the 1:1 mixture of citronella oil (*Cymbopogon nardus*) and neem oil (*Azadirachta indica*) at doses of 5, 10, and 15 g/kg body weight (BW). All treated animals survived until the completion of the study. According to BPOM Regulation No. 10 of 2022 and internationally recognized toxicity classification systems, substances with LD_{50} values greater than 5 g/kg BW are categorized as practically non-toxic under acute exposure conditions (BPOM 2022). Therefore, based on the observed data, the citronella–neem oil combination can be classified as having very low acute oral toxicity.

Acute toxicity testing is designed to evaluate the intrinsic toxic potential of a substance following single-dose exposure and to identify potential hazards associated with accidental ingestion. Mortality remains the primary endpoint for determining LD_{50} values and hazard classification. In this study, the lack of lethal effects at doses as high as 15 g/kg BW indicates that the bioactive constituents present in both oils did not exert life-threatening systemic toxicity (Table 1).

In addition to survival, detailed clinical observations were conducted to identify any signs of distress, toxicity, or physiological dysfunction. No abnormal clinical signs were observed during the critical first 24 hours after administration nor during the subsequent daily monitoring period. All animals maintained normal locomotor activity and exploratory behavior. Grooming patterns were consistent with typical murine behavior, and no excessive scratching, restlessness, or abnormal posture was detected. Feeding and drinking activities remained stable throughout the study, indicating preserved appetite and hydration status (Table 2).

During the 14-day observation period, no clinical signs of toxicity such as tremors, diarrhea, or lethargy

Table 1. Mortality percentage of female DDY mice

Group	Cumulative Mortality (%) After Dose Administration on Day						
	1	2	3	4	5	6	7
1A (Control)	0	0	0	0	0	0	0
1B (5 g/kg BW)	0	0	0	0	0	0	0
1C (10 g/kg BW)	0	0	0	0	0	0	0
1D (15 g/kg BW)	0	0	0	0	0	0	0
	8	9	10	11	12	13	14
1A (Control)	0	0	0	0	0	0	0
1B (5 g/kg BW)	0	0	0	0	0	0	0
1C (10 g/kg BW)	0	0	0	0	0	0	0
1D (15 g/kg BW)	0	0	0	0	0	0	0

were observed in any of the treatment groups. Daily clinical observation is an important indicator for detecting potential toxic effects on the central nervous system, digestive system, and motor functions (BPOM, 2022). The active compounds citronellal and azadirachtin are known to act selectively against arthropods without causing toxic effects in mammals, due to differences in receptor structure and metabolic pathways (Nuraida & Hariani 2022).

In addition, the absence of reactions such as salivation or diarrhea indicates that the test substance did not excessively stimulate the autonomic nervous system (Pringgodigdoyo et al., 2024). Usman and Ibrahim (2018) further stated that natural compounds with high bioactivity but low polarity tend not to cause irritation of the gastrointestinal mucosa, making them safe for oral administration. Since no clinical signs of toxicity were observed at doses up to 15 g/kg BW, it can be concluded that the test substance possesses a high safety margin and does not produce any acute effects on the nervous system or digestive tract of mice.

Body Weight Changes

Body weight increased normally across all groups, with no significant differences ($p>0.05$). Observations of clinical signs and changes in body weight are crucial in this type of test (Andriyanto et al., 2024). All groups exhibited similar body weight patterns throughout the observation period. On day 7 (D7), there were no significant differences ($p = 0.284$) in body weight between the control group (1A) receiving distilled water and the treatment groups (1B, 1C, and 1D) receiving the combination of *Cymbopogon nardus* and *Azadirachta indica* oils at doses of 5, 10, and 15 g/kg BW, respectively.

Body weight monitoring is a fundamental parameter in toxicological assessment because it reflects overall physiological integrity and metabolic stability. Toxic substances may interfere with appetite regulation, gastrointestinal absorption, endocrine balance, or energy metabolism, resulting in reduced weight gain or weight loss. Therefore, changes in body mass provide indirect yet sensitive evidence of systemic toxicity (OECD guidelines; BPOM 2022).

As shown in Table 3, body weight increased progressively in all groups, including both control and

Table 2. Observation of Toxicity Signs

Toxicity Signs	Observation Results After Day						
	1	2	3	4	5	6	7
Tremor	N	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N	N
Lethargy	N	N	N	N	N	N	N
	8	9	10	11	12	13	14
Tremor	N	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N	N
Lethargy	N	N	N	N	N	N	N

Note: N = Normal; no signs of toxicity observed.

Table 3. Average body weight of mice from day 0 to day 14 of the study

Group	Day (Mean ± SD)		
	Do (g)	D7 (g)	D14 (g)
1A	22,00± 3,22[a]	22,33± 3,01[a]	22,67±4,23[a]
1B	23,00± 2,90[a]	22,83± 2,32[a]	24,50± 2,88[a]
1C	21,00± 1,83[a]	21,00±1,90[a]	22,67±2,27[a]
1D	23,00± 3,22[a]	22,83±3,25[a]	23,83± 4,67[a]

Note: 1A (control), 1B (5 g/kg BW), 1C (10 g/kg BW), and 1D (15 g/kg BW). Identical superscripts (a) within the same column indicate no significant difference among groups based on one-way ANOVA analysis ($p > 0,05$).

treatment groups, throughout the 14-day observation period. According to BPOM (2022), a body weight loss of ≥10% during the observation period may indicate systemic toxic effects resulting from impaired function of target organs such as the liver and kidneys. When body weight gain remains within the normal range, it suggests that the homeostatic functions of the mice are not disrupted (BPOM 2022). These findings support the conclusion that the combination of citronella and neem oils possesses a high level of safety, with no evidence of adverse systemic effects following oral administration.

Citronella oil contains active compounds such as citronellal, geraniol, and citronellol, while neem oil contains azadirachtin, nimbin, and salannin, which have been reported to exert mild physiological effects but do not cause changes in body weight in mammals (Isman 2020). According to Udayani et al., (2023), administration of essential oils from the Cymbopogon genus in Wistar rats did not affect body weight gain or feed intake during a two-week acute toxicity test.

The observed increase in body weight in the treatment groups in this study indicates that the test material did not induce metabolic stress. Kamil and Syawalia (2024) reported that non-toxic natural compounds tend not to alter appetite or body fluid balance in mice, allowing body weight to increase similarly to the control group.

Based on these findings, the combination of citronella and neem oils did not exert any negative effects on energy metabolism or digestive system activity in mice. Thus, the normal pattern of body weight gain observed in all groups suggests that the test substance has high tolerability and does not produce systemic toxic effects in mice.

Organ Weights and Macroscopic Findings

Evaluation of organ weights and gross morphology provides valuable insight into potential target organ toxicity. The liver and kidneys were specifically examined because they represent the primary organs responsible for xenobiotic metabolism and excretion. Toxic insult to these organs may manifest as hypertrophy, atrophy, congestion, discoloration, or altered consistency.

Macroscopic examination revealed that the liver and kidneys of all animals displayed normal anatomical features. The liver exhibited uniform reddish-brown coloration with smooth surfaces and well-defined lobular structure. Absolute and relative organ weights are presented in Table 4.

No statistically significant differences were observed among groups ($p > 0.05$). The absolute liver weights (0.0600–0.0685 g) and relative weights (0.2585–0.3028% BW) in all groups fell within normal ranges reported for female outbred mice (including DDY) of similar body weight (20–30 g), typically 0.05–0.10 g absolute and 2.5–4.0% relative for liver (Marino 2012). Similarly, absolute kidney weights (0.0030–0.0033 g) and relative weights (0.0118–0.0145% BW) were comparable to normal values of approximately 0.10–0.15 g absolute (both kidneys combined ~0.20–0.30 g) and 1.0–1.5% relative (Marino 2012). These stable organ weights, aligned with physiological norms for the strain and age, strongly support the lack of acute toxic insult to the liver and kidneys. Organ weight variations are often considered early markers of toxicity before microscopic lesions become apparent. Hayong et al., (2019) reported that neem oil, which contains azadirachtin, exhibits hepatoprotective

Table 4. Macroscopic observation results of liver and kidney organs

Organ	Group	Mean Absolute Weight (g)	Mean Relative Weight (%)	Color	Size	Consistency
Liver	1A	0,0685	0,3028	RB	NA	Soft
	1B	0.0655	0.2675	RB	NA	Soft
	1C	0.0600	0.2585	RB	NA	Soft
	1D	0.0637	0.2605	RB	NA	Soft
Kidney	1A	0.0030	0.0133	RB	NA	Soft
	1B	0.0030	0.0118	RB	NA	Soft
	1C	0.0030	0.0133	RB	NA	Soft
	1D	0.0033	0.0145	RB	NA	Soft

Note: RB = Reddish-brown (normal organ color); NA = No Abnormalities detected.

properties due to its natural antioxidant components. Similarly, Wibowo (2022) stated that citronella oil has nephroprotective effects through the reduction of lipid peroxidation in kidney tissues. Therefore, the combination of these two oils may provide a protective effect on vital organs. The results of this study confirm that the mixture of citronella and neem oils did not induce any acute hepatotoxic or nephrotoxic effects and can thus be classified as safe, in accordance with BPOM (2022) safety criteria. Histopathological evaluation (H&E staining) of liver and kidneys showed no significant abnormalities. Renal glomeruli and tubules exhibited normal architecture (degeneration score 0 in all samples). Liver showed no hepatocyte necrosis (score 0), with variable mild to moderate fatty degeneration (scores 1–3 in some samples) but without associated inflammation or structural damage. These findings confirm the absence of acute toxic histopathological changes attributable to the test material (CoA No. 1310.22092025, IRATCO Laboratory).

CONCLUSION

The combination of citronella (*Cymbopogon nardus*) and neem (*Azadirachta indica*), administered orally to female DDY mice at doses up to 15 g/kg body weight (BW), did not cause any mortality or acute toxic symptoms. this material is classified as non-toxic in acute exposure (LD_{50} > 15 g/kg BW) and safe for veterinary herbal formulation development.

AUTHORS CONTRIBUTION

H.E.A. conceived and designed the study, conducted the experiments, analyzed the data, and drafted the manuscript. L.N.S. contributed to the formulation development and laboratory analysis. A.A.M. assisted in animal experimentation and histopathological examination. W.M. contributed to data interpretation and statistical analysis. A.A. supervised the study, critically reviewed the manuscript, and approved the final version. All authors read, discussed, and approved the final manuscript.

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"The author declares that there is no conflict of interest with the parties involved in this research".

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