

Non-clinical Safety and Toxicological Evaluation of *Rhizophora mucronata* Methanol Extract

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Abstract

This study assessed Sabah Mangrove, *Rhizophora mucronata* leave extract (RMLE) non-clinical safety and pharmacokinetic. APC/YMC count and AMES test, Acute Oral Toxicity, Sub-acute Toxicity, and RMLE Reproductive Toxicity Profiling are evaluated. RMLE's agar plate and heavy metal assay showed that bacteria and fungi, were below the National Pharmaceutical Control Bureau (NPCB) limit. RMLE's antimutagenic properties were tested in AMES experiment employing *S. typhimurium* TA98. Dose finding test (DFT) plates show no toxicity (growth suppression) for all dosages from 5000 to 313 µg/plate, with and without metabolic activation, for all five tester strains. In acute toxicity study in Sprague dawley rats, single oral dosages of RMLE (2,000 mg/kg) did not cause mortality or behavioural abnormalities. In subacute toxicity trials, RMLE (1,000 mg/kg) for 28 days did not affect body weight increase or behaviour. Hematological and biochemical markers remained unchanged. Histopathology indicated no morphological abnormalities. These findings show that RMLE is safe for medical use.

Keyword: *Rhizophora mucronata*, Mangrove, Toxicity

Introduction

Rhizophora mucronata, commonly known as Bakau Kurap, the Asian red mangrove, belongs to the Rhizophoraceae family (Grin, 2006). *Rhizophora mucronata* is distributed along the coastlines of tropical and subtropical regions (Rohini & Das,

2009). Certain plants, like *Rhizophora mucronata*, utilise various parts such as leaves, barks, and stems for extraction purposes. The leaf of *R. mucronata* contains tannins and flavonoids, which are natural compounds used in traditional medicine. These compounds have the potential to affect cell physiology. Ghosh *et al.*, (1995) found that *R. mucronata* contained various compounds including steroids, triterpenoids, alkaloids, flavonoids, tannins, and catechins. The study revealed the presence of tannins, alkaloids, flavonoids, terpenoids, and saponins in the methanol extract of *R. mucronata* leaves, as determined through phytochemical screening. Methanol extract of *R. mucronata* leaf contains condensed tannins. The leaf extracts of *Rhizophora mucronata* exhibit potent anti-diarrheal properties. Syamimiet *al.*, (2022) conducted a recent study on *R. mucronata*, a species of mangrove found in Sabah. The study revealed the potential aphrodisiac properties of this plant. The study suggests that the bioactive compound known as Catechin is responsible for the elevation of testosterone levels. Leaf extracts contain novel pharmacological compounds with potential therapeutic applications for human diseases (Swamy *et al.*, 2016).

Currently, researchers worldwide are required to conduct toxicity studies on plant-based drugs and their preparation. This is due to the growing need for assurance regarding the safety of these drugs for human consumption (Ukwuani, Abubakar, Hassan, & Agaie, 2012). Toxicological studies using animal models such as rats, mice, rabbits, monkeys, guinea pigs, and dogs are crucial for assessing the safety and efficacy of new drug formulations, in accordance with the guidelines set by the Organisation for Economic Cooperation and Development (OECD) (Ali Khairullah Zahi *et al.*, 2015). Toxicity studies are valuable in informing decision-making regarding the adoption of a new drug for clinical use. Despite the widespread utilisation of *R. mucronata* in traditional medicine, there is a dearth of systematic assessment regarding its potential toxicological impacts. The purpose of this study was to assess the safety of *R. mucronata* by conducting acute and subacute toxicity tests on RMLE. The study followed the recommended OECD guidelines for safety toxicity in rats, microbiology, heavy metal analysis, genotoxicity, and reproductive toxicity profile.

Materials and Methods

1. Plant materials and preparation of RM methanolic extract.

Young leaves of *Rhizophora mucronata* collected around the coastal areas of Sabah were authenticated at the Sabah Forestry Department, Sandakan, Sabah with a voucher specimen, *Rhizophora mucronata* (Bakau Kurap); SAN 149220. The leaves were washed, and oven dried at 40 ± 3 °C overnight. The dried leaves were ground to fine powder using grinder and successively extracted from non-polar to polar solvent using

Soxhlet apparatus for 8 hours (methanol : water ratio at 50 %). The extract was filtered, rotary evaporated at 50 °C for 8 hours to remove excess solvent and 24-hour freeze-dried process. The methanol extract was kept at 4 °C in the refrigerator until further use. Methanol has been chosen because it is the universal solvent that can dissolve all the active ingredients of secondary metabolites (Mahmiah *et al.*, 2016).

2. Animal Ethic

Prior approval was taken from SIRIM-IACUC Committee (SIRIM-IACUC/IBRC/B19-137/0023), see Appendices. Male Sprague Dawley rats, 8 weeks of age with a weight range of 200 to 300 g were selected for the study. Animals were supplied with feed and water *ad libitum*.

3. Microbial [Aerobic Plate Count (APC) and Fungi, Yeast & Mould Count (YMC)]

RMLE was diluted 10x in MLB to obtain complete dilution series from 10⁻¹ to 10⁻⁶. For each step, 1 mL mixture was diluted into 9 mL MLB. For the APC enrichment step was incubated at 30 °C ± 2 °C. For YMC enrichments, the diluted sample in 9 mL SDB was incubated at 25 °C ± 2 °C. The dilutions were thoroughly mixed, and 0.1 mL of each dilution were pipetted onto the surface of solid media of MLB for APC and SDB for YMC in pre-labelled petri dishes. Each dilution was tested on duplicate plates. Inoculum was spread over the entire surface with a sterile bent glass rod to allow the inoculum absorbed. The plates were then inverted and incubated at 30 °C ± 2 °C for 48 hours for APC and at 25 °C ± 2 °C for 7 days for YMC. (S.M. Aleid, *et al.*, 2014).

4. Bacterial Reverse Mutation Test

RMLE was assessed for its mutagenic potential using *Salmonella typhimurium* (*S. typh.*) and *Escherichia coli* (*E. coli*) strains with and without metabolic activation, following OECD Guideline for Chemical Testing technique 471, Bacterial Reverse Mutation Test. The overlay agar included 0.6% Bacto-Agar and 0.5% NaCl. *S. typh.* strains were cultured on overlay agar containing histidine and biotin, while *E. coli* strain overlay agar included tryptophan. The test involved a mixture of rat liver S9, cofactor, and buffer. RMLE was solubilized in water and used at 50 mg/mL. The dose-finding test began with 5000 µg/plate and continued with 4 geometric series concentrations. Bacterial cultures were prepared by inoculating bacterial suspension into nutrient broth and incubating until reaching target optical density. Positive and negative controls were included. Sodium azide served as the positive control mutagen, while negative controls were prepared with sterile water. RMLE, S9 Mix, and buffer were tested for contaminants. After treatment, overlay agar was

added, and plates were incubated for 48 hours. Revertant colonies were counted, and triplicate plates were used to assess variation. Bacterial colonies on minimum agar plates indicated pollution.

5. Acute Oral Toxicity

This study, in accordance with OECD guidelines No: 425, was conducted on rats to assess the potential acute oral toxicity of RMLE through a single oral dose. The study employed the up-and-down procedure (UDP) limit test, involving up to five animals. Each animal, weighing between 200 g and 210 g, was individually housed in ventilated cages for acclimatization for at least 5 days. Each rat was orally dosed with 2000 mg/kg of RMLE, at a rate of 1 mL per 100 g body weight. Oral administration utilized a stainless-steel gavage tube with a round head, measuring 2 inches in length and 18G in diameter. The first animal was dosed at 2000 mg/kg and survived the 48-hour observation. Consequently, four more animals were sequentially dosed, one at a time, with about a 24-hour interval between each dosing, leading to a total of five animals being tested. Animals were closely observed for signs of mortality, illness, injury, or abnormal behavior. Observations were made once during the initial 30 minutes after dosing, periodically within the first 48 hours (with extra focus on the initial 4 hours), and then daily for 14 days. Individual body weights were measured on Day 7 and Day 14. After 14 days, all animals were euthanized for gross necropsies.

6. Subacute Toxicity

This study, conducted in accordance with OECD Guideline No. 407, examined the toxicity of RMLE in rats through oral administration for 28 days. Approximately 15 healthy male SD rats were randomly assigned to three groups: Control, RMLE 1000 mg/kg, and Satellite. RMLE was dissolved in reverse osmosis (RO) water and administered orally daily. The control group received only RO water. The limit dose of 1000 mg/kg was determined based on the LD₅₀ value from the acute oral toxicity test, where toxicity was not expected. Rats were observed twice daily for general appearance, behavior, and signs of morbidity or mortality. Various parameters were assessed, including skin, fur, eyes, oral cavity, and more. Behavior such as tremors, convulsions, and responsiveness were monitored. Body weight, food, and water consumption were measured weekly. After 28 days, necropsies were conducted, and organ weights were recorded. Blood samples were collected for haematology and biochemistry evaluation. Necropsy involved a macroscopic examination, and selected tissues were collected, fixed, and processed for histopathological examination. Vital organs like the heart, kidneys, liver, lungs and spleen, were analyzed.

7. Statistical Analysis

Data analysis was carried out using the IBM-SPSS statistic program (Version 25.0). All the results were expressed as mean \pm S.D (standard deviation) and mean \pm SEM (standard error mean). Statistical analyses were performed using a t-test (Levene's Test for Equality of Variances) or one-way ANOVA. In one-way ANOVA, if significant differences were found, Tukey's test for parametric multiple comparisons was performed to compare treated groups with the control group. The values were considered significantly different when the P value was less than 0.05 ($p < 0.05$).

Result and Discussion

1. Microbiology Count

The utilization of the Agar plate technique aimed to assess the presence of bacterial load within RMLE. Given its derivation from plant material, RMLE is particularly susceptible to contamination. Contaminants in herbal medications not only degrade their quality but also diminish their medicinal effectiveness. Bacteria-produced toxins can render herbal medicines unsafe for consumption, potentially leading to disease instead of cure. The examination of RMLE encompassed bacterial and fungal load analysis, as outlined in Table 1. The outcome reveals the existence of bacterial and fungal load, albeit within acceptable limits. This discovery aligns with the findings of Imdadulet *al.*, (2011), who identified antimicrobial properties within RM leaf extracts. These extracts displayed the ability to inhibit the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*.

Type of Tests	Actual Result	Allowable Level
Aerobic Plate Count (cfu/mL)	105 \pm 1.0	<1000
Yeast & Mould Count (cfu/mL)	25 \pm 0.5	<1000

Table 1: Microbiology count tests results of RMLE

Result shown in mean \pm S.D

2. Bacterial Mutagenicity

The objective of this study was to evaluate the antimutagenic properties of RMLE using the TA98 tester strain of *S. typhimurium* in the Ames assay. The dose finding test (DFT) did not observe any toxic effects (growth inhibition) at concentrations ranging from 5000 to 313 µg/plate, with and without metabolic activation, in all tester strains. No precipitation of RMLE was observed on the agar surface within the concentration range of 313 to 5000 µg/plate. The DFT analysis revealed that the concentration of the main test (MT) varied between 5000 and 313 µg/plate, with and without S9 mix. These findings are summarised in the accompanying Table 2. The revertant colony count in strains TA98, TA100, TA1535, TA1537, and WP2uvrA, with and without S9. The study found no statistically significant increase in the number of revertant colonies in all bacterial strains treated with and without S9 Mix during the main test (MT) at concentrations ranging from 313 to 5000 µg/plate. The absence of microorganisms in the contaminant test suggests that the RMLE was free from contamination. This study evaluated the antimutagenic properties of RMLE at a concentration of 5000 µg/plate, with and without a metabolic activation system. Ferreira and Vargas (1999) found a correlation between elevated levels of specific phytochemicals, such as flavonoids, and their potential to exhibit antimutagenic properties. Suganthy *et al.*, (2009) detected flavonoids in RMLE through phytochemical analysis, demonstrating its antioxidant properties. The extract's flavonoids may reduce mutagenicity and enhance protective properties.

Table 2: Main test (MT) of RMLE (5000 and 313 µg/plate), with and without S9 mix.

Treatment concentration (µg/plate)	No. of his + revertant colonies/plate (mean ± SD) ^a							
	TA1535		TA1537		TA98		TA100	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Control (Water)	19 ± 8	19 ± 7	13 ± 5	15 ± 6	35 ± 10	41 ± 12	166 ± 36	171 ± 45
Positive Control	575 ± 195	186 ± 100	575 ± 195	139 ± 56	560 ± 204	193 ± 63	1146 ± 308	535 ± 156
RMLE 313	18 ± 3	15 ± 2	23 ± 2	22 ± 7	30 ± 8	42 ± 2	173 ± 8	184 ± 54
RMLE 625	17 ± 4	12 ± 1	20 ± 4	20 ± 3	33 ± 5	55 ± 5	171 ± 20	209 ± 17
RMLE 1250	16 ± 2	15 ± 3	15 ± 3	21 ± 5	27 ± 7	38 ± 7	126 ± 5	170 ± 11

RMLE 2500	16 ± 2	15 ± 2	17 ± 6	22 ± 5	25 ± 4	35 ± 5	135 ± 7	167 ± 7
RMLE 5000	17 ± 2	13 ± 1	15 ± 3	16 ± 4	27 ± 3	39 ± 8	126 ± 10	177 ± 16

3. Acute Oral Toxicity Test

Toxicity studies are valuable for assessing the therapeutic index of chemicals, drugs, and xenobiotics (Rang, 2001). In this study, all animals were individually observed for mortality, signs of gross toxicity, and behavioural changes within the first 30 minutes after administration. No deaths occurred during the 14-day procedure. All animals exhibited normal body weight and behaviour throughout the observation period. The data is presented in the Table 3.

The RMLE was classified as Category 5 for acute oral toxicity according to the Globally Harmonised System. This classification indicates that it is harmless, as the LD₅₀ value exceeded 2000 mg/kg body weight. Salawu O.A. *et al.* (2009) suggest that a substance is considered a suitable candidate for further investigation if its lowest effective dose is at least three times greater than the median lethal dose of the tested drug. RMLE demonstrates no toxicity and is deemed safe for oral administration. However, the therapeutic applicability of studies on acute toxicity is limited due to the potential for cumulative toxic effects at low doses. A subacute toxicity study (Section 5) is useful in assessing the overall safety profile of the phytomedicine. Subacute toxicity data are necessary for accurately predicting the potential harm associated with prolonged exposure to low doses of chemicals and extracts.

Table 3: Data of animal body weight (day 0, day 7 and day 14), daily observation and necropsy observation of tissue and organ

Dose (mg/kg)	Body Weight (g)			Daily Observation	Necropsy Observation of Tissue/Organ	Findings
	Day 0	Day 7	Day 14			
2000	199	218	232	Active and healthy the 14-day observation period	All tissues/organs	No gross abnormalities
2000	201	222	239		All tissues/organs	No gross abnormalities
2000	210	231	239		All tissues/organs	No gross abnormalities
2000	213	227	235		All tissues/organs	No gross abnormalities
2000	217	229	237		All	No gross

					tissues/organs	abnormalities
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4. Sub-Acute Toxicity Test

To ensure the safe utilization of plant-derived remedies, Mukinda and Syce (2007) emphasized the importance of not only relying on historical and empirical usage in humans and animals but also conducting comprehensive toxicity evaluations. Despite its historical use, there exists a gap in the understanding of the potential toxicity of RMLE, particularly the Sabah variety. This led to the present investigation, which aimed to comprehensively explore the toxicity profile of RMLE. In alignment with sub-acute toxicity guidelines, a full study encompassing three dose levels was deemed unnecessary when a single dose of 2,000 mg/kg exhibited no toxicity effects in the acute toxicity test (OECD 407, 2008). Consequently, a dose of 1,000 mg/kg/day of RMLE was selected for the sub-acute toxicity studies. The study evaluated a range of parameters in rats subjected to RMLE at this dosage, including signs of abnormality, body weight alterations, organ weights, anabolic effects, mortality rates, biochemical markers, hematological parameters and histopathological observations.

Alterations in body weight and internal organ weight are indicative of potential adverse effects, especially since animals that survive cannot lose more than 10% of their initial body weight (Raza *et al.*, 2002). In general, substances with toxic properties lead to deviations in body weight. However, rats administered RMLE showed body weight changes comparable to those in the Control group, and these changes were not statistically significant (Figure 4 (a)). This is in line with the typical physiological adaptive response to pharmacological compounds, resulting in reduced appetite (Riouani, 2008). No clinical indications of toxicity were observed among the rats in the Control (RO), RMLE and Satellite groups. This consistency aligns with a sub-acute toxicity investigation involving the methanol extract of Indian *R. mucronata* (Suganthi *et al.*, 2014). The absence of toxic symptoms and the stability of water and food consumption patterns indicated that the RM extract did not exert any adverse effects on the Wistar rats used in the study.

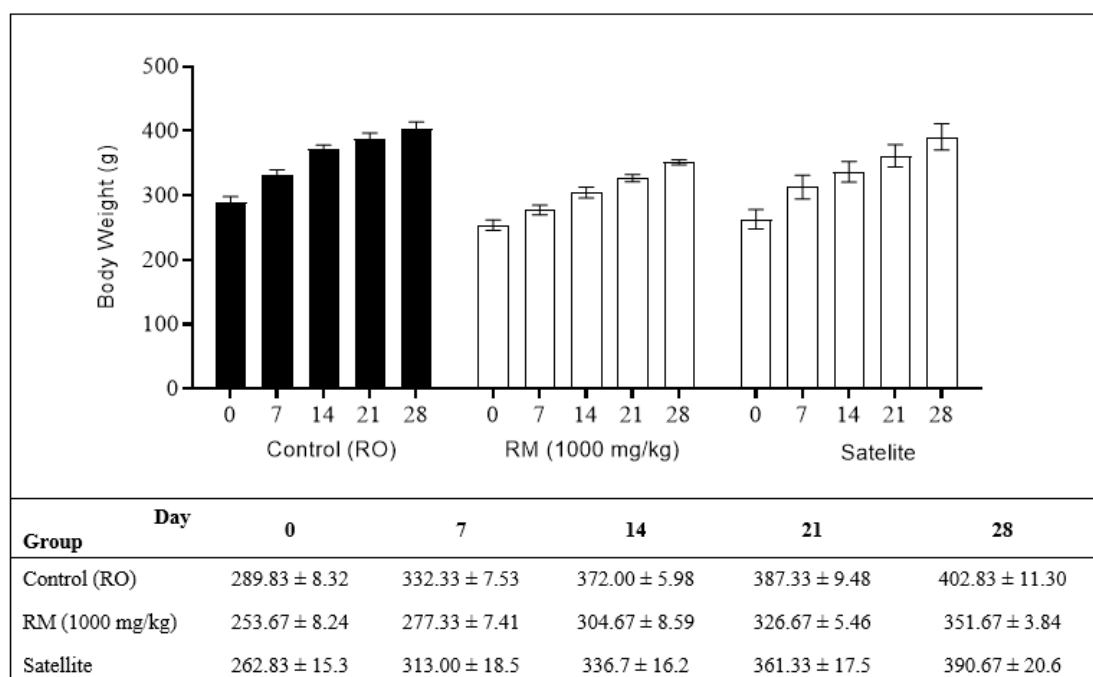


Figure 4 (a): Body weight changes (g) throughout 28 days of treatment. Values were as mean ± SEM.

4.1 Organ weight

Organ weight serves as a sensitive indicator of potential toxicity, as significant changes in organ weight might occur even in the absence of visible morphological alterations following exposure to toxic substances (Ghosh, 1995). When confronted with hazardous chemicals, the heart, liver, kidneys, spleen, and lungs are among the organs that respond metabolically in the early stages (Ara A. & Usmani J. A., 2015). Consequently, assessing relative organ weights becomes an informative approach to detecting any potential harm inflicted by toxic agents on these organs. It's common for toxic compounds to induce damage in specific target organs (Teo S., 2002).

The study's findings regarding vital and reproductive organs revealed no significant differences in organ weight between the control group and animals treated with RMLE (Table 4 (a)). Further, there were no discernible variations in the external appearance, size, colour, or microscopic characteristics of internal organs across the different groups. This collectively leads to the conclusion that RMLE demonstrates a lack of adverse effects and can be considered as virtually non-toxic. These findings provide compelling evidence of the anabolic and androgenic properties associated with RMLE.

Table 4 (a): The relative organ weights changes of rats after the single administration of RMLE (1000 mg/kg) at 28-day observation period.

Parameter	Control (RO)	RMLE (1000 mg/kg)	Satellite
Body weight (g)	402.83 ± 11.30	351.67 ± 3.84	385.22 ± 5.55
Brain weight (g)	2.10 ± 0.06	2.20 ± 0.08	2.22 ± 0.10
Heart weight (g)	1.33 ± 0.09	1.07 ± 0.09	1.08 ± 0.10
Lung weight (g)	2.20 ± 0.06	2.10 ± 0.04	2.12 ± 0.12
Liver weight (g)	12.60 ± 0.40	9.70 ± 0.35	9.92 ± 0.45
Spleen weight (g)	0.80 ± 0.10	0.57 ± 0.03	0.60 ± 0.04
Kidney weight (g)	3.00 ± 0.06	2.80 ± 0.10	2.90 ± 0.11
Epididymis weight (g)	1.24 ± 0.32	1.56 ± 0.23	1.77 ± 0.27
Testicle weight (g)	3.70 ± 0.34	4.90 ± 0.15	4.98 ± 0.18

Values were as mean ± SEM (n=6).

4.2 Anabolic Effect

The relative organ weight serves as a pivotal parameter in identifying whether an organ has undergone any injury or alteration due to the administered treatments. In cases involving toxicant exposures or drug metabolism, the liver and kidneys are the foremost organs that display responses through their metabolic processes (Vaghasiyaet *al.*, 2011). These organs play an essential role in detoxifying and processing potentially harmful substances, making them critical indicators of treatment-related effects. Presented in Table 4 (b), the results outline the relative organ-to-body weight ratios for specific organs, namely the liver, kidneys, epididymis, and testes of the rats. Remarkably, the investigation found that the relative organ weight-to-body weight ratios within the RMLE treated group did not exhibit any significant differences from those observed in the control group. The absence of significant differences in relative organ weight-to-body weight ratios between the treated and control groups underscores the relative safety of RMLE administration in terms of these crucial physiological indicators. This comprehensive understanding of the organ-level effects further supports the notion that RMLE holds promise as a potentially safe therapeutic option.

Table 4 (b): Organ weight-to-body (mg) Weight Ratio of Rats.

Organs	Control (RO)	RM (1000 mg/kg)	Satellite
Kidney	0.74 ± 0.01	0.80 ± 0.02	0.82 ± 0.05
Liver	3.13 ± 0.12	2.75 ± 0.11	2.96 ± 0.15
Testis	1.17 ± 0.05	1.31 ± 0.07	1.29 ± 0.05
Epididymis	0.31 ± 0.01	0.44 ± 0.03	0.45 ± 0.04

Result shown in mean ± S.E.M, n=6, where *P<0.05 compared to Control group.

4.3 Haematology

Haematological analyses serve as vital tools in risk assessment, as changes in the haematological system hold strong predictive value for potential human toxicity when findings from animal studies are extrapolated (Olson and Betton, 2000). Red Blood Cells (RBCs) play a pivotal role in transporting oxygen through the bloodstream due to their hemoglobin content. RBC assessments offer insights into conditions like anemia and other related disorders. The quantification of White Blood Cells (WBCs), which are responsible for immune response and infection combat, is another important aspect of haematological analysis. Neutrophils play a crucial role in guarding against infections, while lymphocytes are vital components of the immune system's effectiveness (Nathan C., 2006). Tables 4 (c) shows the mean values of haematological parameters for both control and treatment groups at the conclusion of the recovery period. The comprehensive analysis of haematological parameters, which are essential indicators of overall health, reinforces the notion that RMLE does not induce significant perturbations in the immune and blood systems of the treated animals. This further supports the premise that RMLE administration is unlikely to provoke harmful effects on this crucial physiological aspect.

4.4 Biochemistry

To assess potential impacts on liver and kidney function, biochemical tests were conducted on haematology samples from rats treated for 28 days and then allowed to recover for 14 days (Satellite group). Transaminases AST (Aspartate aminotransferase) and ALT (Alanine aminotransferase) are essential in converting crucial metabolites and act as markers of liver cell injury. Elevations in these enzymes suggest their release into the bloodstream due to injury to liver cells. A surge in plasma enzymes like AST and ALT signifies liver cell injury. The findings of the study revealed no significant changes in blood AST and ALT levels,

indicating that RMLE did not affect liver functions or metabolism. Notably, abnormal lipid accumulation in the liver leads to fatty alterations, and substances like lovastatin are used to address such changes and lower cholesterol (Babusevmet *et al.*, 2012). Functional markers like creatinine in the kidney serve as primary indicators of renal function. Elevated creatinine levels suggest impaired functioning nephrons. The investigation found no significant difference in creatinine levels between the treatment and control groups, indicating that the extract did not disrupt the kidney's ability to eliminate metabolites and didn't have negative effects on the rats' biochemical parameters. Table 4 (d) presents the mean values from the biochemical analyses performed at the end of the recovery period, grouped by treatment. While AST, total cholesterol, calcium, and total protein showed no statistically significant differences compared to the Control Group, ALT displayed a significant difference unrelated to the administered dose. The analyses conducted at the end of the recovery period (Satellite group) showed no discernible changes. The study's findings collectively suggest that RMLE didn't cause significant alterations in haematological or biochemical indicators. Therefore, RMLE, at standard therapeutic dosages, is considered safe for long-term treatment of human disorders (Babusevmet *et al.*, 2012).

Treatment	RBC (x 10 ¹² /L)	WBC (x 10 ⁹ /L)	Basophi l (%)	Neutrop hil (%)	Lymphoc yte (%)	Monocy te (%)	Eosinop hil (%)
Control (RO)	8.85 ± 0.7	9.98 ± 2.1	0.15 ± 0.2	1.58 ± 0.5	7.29 ± 1.3	1.14 ± 0.2	0.08 ± 0.01
RMLE (1000mg/kg)	7.38 ± 0.08	8.59 ± 1.4	0.09 ± 0.12	1.45 ± 0.2	6.50 ± 1.1	0.85 ± 0.05	0.02 ± 0.01
Satellite	7.54 ± 0.04	8.58 ± 3.3	0.08 ± 0.03	1.44 ± 0.5	6.48 ± 2.7	1.04 ± 0.2	0.02 ± 0.01

Table 4 (c): Haematology

Result shown in mean ± S.E.M, n=6, where *P<0.05 compared to Control group.

Treatment	Calcium (mg/dL)	ALT (U/L)	AST (U/L)	Cholesterol (mg/dL)	Creatinine (mg/dL)	Glucose (mg/dL)
Control (RO)	2.75 ± 0.25	56.68 ± 7.5	165.7 ± 39.6	1.86 ± 0.39	62.3 ± 6.59	91.0 ± 1.9
RMLE (1000mg/kg)	2.96 ± 0.41	97.48 ± 8.5*	278.23 ± 38.7*	1.53 ± 0.25	69.0 ± 5.25	59.6 ± 3.3*
Satellite	2.52 ± 0.30	57.68 ± 10.7	229.42 ± 17.7	1.08 ± 0.11	51.60 ± 1.10	84.6 ± 0.9

Table 4 (d): Biochemistry

Result shown in mean \pm S.E.M, n=6, where *P<0.05 compared to Control group.

4.5 Histopathological Observations of Vital Organs

Histopathological examination of tissue sections from rat liver, heart, kidney, lung, brain, and spleen stained with haematoxylin and eosin (H&E) was performed to complement body weight, organ weight, and biochemical analysis results. Microscopic assessment was conducted using light microscopy, and no gross pathological lesions were observed in any of the RMLE treatment or control groups (Figure 4(b)). Considering that the liver is a primary target for toxicity (Rhiovania, H. *et al.*, 2008), histopathology analysis of the liver was conducted. The liver cells' architecture, including hepatocytes and portal veins, appeared normal in both treatment and control groups. No signs of necrosis, inflammation, fibrosis, or fatty degradation were detected. Similarly, the kidneys' histology revealed no morphological changes in the RMLE treatment group. Glomerular architecture, distal and proximal tubules, remained normal. No interstitial congestion, tubular atrophy, or abnormal cell structures were observed. Microscopic examination of the lungs showed no pulmonary anomalies in treated rats, such as inflammation or cell infiltration. The spleen and heart tissues exhibited normal structure and no lesions.

Overall, histological assessments of these organs indicated no abnormalities linked to RMLE administration. This suggests that the oral use of RMLE at the studied dosage is safe and doesn't have detrimental effects on histopathological aspects. Notably, the oral dose of 1,000 mg/kg bw/day of RMLE administered for 28 days resulted in no toxicity symptoms, making it the No-Observed-Adverse-Effect Level (NOAEL) for Sprague Dawley rats. This aligns with the findings of Sughantyet *al.*, (2014) regarding Indian *R. mucronata* extract.

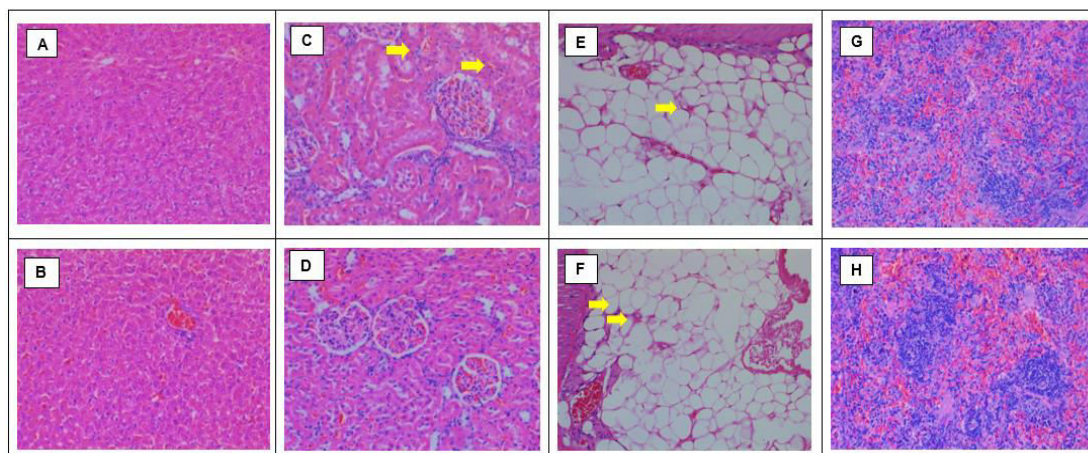


Figure 4 (b): Histology sections of vital organs rats treated in repeated dose orally with Control (RO) (A,C,E,G) and RMLE 1000 mg/kg (B,D,F,H) for 28 days. (A) & (B) Histology of liver - Both groups showed sinusoidal congestion (arrow) which is morphologically unequivocal since the vessels are conspicuously dilated and filled with blood. (C) & (D) Histology of kidney - Congested capillaries (arrow) are blood-filled and are also distended in both groups. Capillary congestion indicates impaired flow in the end-capillary bed. No necrosis and tubular degeneration were found in both groups. (E) & (F) Histology of lung - No fibrinous deposits (hyaline membrane) in air spaces, no haemorrhage, no congestion of alveolar capillaries, are one-cell thick and less than 10 leukocytes (arrow) per sample area for both groups. (G) & (H) Histology of Spleen - Both groups showed normal spleen morphology with white pulp, central arteriole surrounded by sheets of Lymphocytes and red pulp, lymphocyte surrounded by RBCs. All images are with 20× magnification (Scale: 1 unit to 50µm).

Conclusion

Plant medicine has gained recognition as a viable alternative treatment for a range of human illnesses, including testosterone therapy. The study aimed to investigate the potential contributions and advantages of *Rhizophora mucronata* as a natural aphrodisiac agent and its associated therapies.

RMLE was assessed using the TA98 tester strain of *S. typhimurium* in the Ames assay. The contaminant check confirmed that RMLE was devoid of any microorganisms, indicating it was uncontaminated. The microbiology test revealed the existence of bacterial and fungal contamination in the RMLE. Nevertheless, the quantity of bacteria and fungi falls under the acceptable threshold. The acute toxicity research indicated that RMLE is safe when administered orally at a dose of up to 2000 mg/kg of rat body weight.

No deaths or illnesses were observed in any of the dosages of RMLE examined during the acute toxicity study. The administration of the highest dosage, 2,000 mg/kg bw, resulted in changes in biochemical and histological indicators associated with liver damage. The subacute toxicity experiments showed that Sprague Dawley rats did not exhibit any symptoms of toxicity when administered a daily oral dose of 1,000 mg/kg bw/day of RMLE for 28 consecutive days. This dosage can therefore be considered the No Observed Adverse Effect Level (NOAEL) as it did not result in any biochemical, haematological, or histological abnormalities. Ultimately, this work shows significant insights into the toxicity characteristics of RMLE, both in vivo and in vitro. These findings will prove highly beneficial for conducting in vivo behavioural efficacy studies and clinical investigations of this herbal remedy.

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Disclaimer: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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