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Original Article

Sub-acute toxicity evaluation of *Ziziphus spina-christi* and *Adenanthera pavonina* leaf extracts combination in rats: an in vivo study

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A B S T R A C T

Background: The safety of combined herbal extracts remains underexplored, particularly for *Ziziphus spina-christi* and *Adenanthera pavonina*, which contain bioactive compounds with potential toxic effects.

Objective: To evaluate the sub-acute toxicity of combined leaf extracts on hematological, biochemical, and organ morphology parameters in Wistar rats.

Method: Thirty male and female rats were randomized into five groups receiving vehicle control or extract doses of 4000, 8000, and 10,000 mg/kg BW for 21 days following OECD 407. Blood hematology, renal biomarkers, bilirubin levels, organ weight, and morphology were analyzed.

Results: Mortality increased dose-dependently, reaching 20% in males and 10% in females at 10,000 mg/kg BW. Hematology and renal biomarkers showed no significant differences ($p>0.05$). Total bilirubin increased at the highest dose. Morphological abnormalities, including pale discoloration and dark foci, were observed in liver and kidneys at higher doses.

Conclusion: The combination extract is safe up to 8000 mg/kg BW, while 10,000 mg/kg BW induces organ alterations and increased mortality.

INTRODUCTION

Herbal medicines are widely used globally, with 80–85% of the population relying on plant-based therapy for health maintenance.[1,2] However, the assumption that herbal preparations are inherently safe is misleading, as many plants contain bioactive compounds with dose-dependent toxicity. Toxicity testing is therefore essential to determine the safety margin and potential adverse effects of medicinal plants.[3,4] *Ziziphus spina-christi* (bidara arabic) and *Adenanthera pavonina* (tree saga) are traditionally used for inflammatory, gastrointestinal, and systemic ailments.[5] Their leaves contain saponins, flavonoids, and tannins, which exhibit therapeutic as well as toxic potentials saponins may cause hemolysis, tannins may induce nephrotoxicity, and certain flavonoids may alter red blood cell stability.[6-9]

Previous toxicology studies primarily evaluated these plants individually, and data on renal and hepatic markers remain limited.[10-12] Existing studies also rarely assess total and direct bilirubin sensitive markers of hepatic excretory function and often use only male animals, leaving possible sex-related differences underexplored. Moreover, combinations of herbal extracts, which are common in traditional practice, may produce synergistic or antagonistic interactions that alter toxicity profile.[13-17]

No previous study has evaluated sub-acute toxicity of combined *Ziziphus spina-christi* and *Adenanthera pavonina* extracts, nor assessed bilirubin and organ morphology changes under repeated dosing.[18-21] Based on this background, this study aims to evaluate sub-acute toxicity assessment of combined extracts of these two plants, focusing on hematological parameters, renal function, bilirubin levels, and organ morphology, thereby filling an important gap related to potential interactions between their bioactive constituents.

METHOD

Study Design

This study employed an experimental design using a pretest-posttest control group only approach.[22]

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Study Site

This research was conducted at the Haematology Laboratory, Faculty of Health Sciences, Maarif Hasyim Latif University, Sidoarjo, East Java, and the Oral Biology and Biomedical Laboratory, Faculty of Dentistry, Hang Tuah University, Surabaya, East Java, for 27 days from October to November 2024.

Material

The ingredients used in this study included extracts of *Ziziphus spina-christi* and *Adenanthera pavonina*, male and female Wistar rats, feed, water, Na CMC, urea, creatinine, and bilirubin reagents, cooler packs, ketamine, and xylazine.

Extraction Process

The leaves of *Ziziphus spina-christi* and *Adenanthera pavonina* were washed, dried, and ground into powder.[16] Powder was extracted separately using 70% ethanol.[23] Each powder was separately extracted with 70% ethanol using the maceration method for 3 × 24 hours. The filtrate was filtered every 24 hours, then separated, evaporated at 40°C using a rotary evaporator, and stored in the refrigerator until use.[24]

Prosedur In Vivo

Animal Preparation

This study involved fifteen male and fifteen female Wistar rats (*Rattus norvegicus*) weighing 150-200 grams.[25] The test animals were acclimatised with access to food and water for 5 days before the study began, to adjust the rats' circumstances to the laboratory environment.[26,27] Weighing is carried out routinely before being given treatment to obtain accurate data.[28]

Sub-Acute Toxicity Test

The sub-acute toxicity test consisted of five groups: a negative control (P1), a carrier control with 1% Na CMC (P2), and treatment groups receiving 4000, 8000, and 10,000 mg/kg BW (P3–P5). Each group received 0.5 ml daily and was observed for 24 hours for signs of physical toxicity, including changes in body weight, eye condition, respiration, activity, and mortality.[29]

Haematology Parameters

Haematology analysis is performed using blood being inserted into the K_3EDTA tube.[29] As much as 1 ml was analysed using the MINDRAY BC 2800 Haematology Analyser. The parameters checked include WBC, PLT, RBC, and Haemoglobin.[30]

Clinical Chemistry Parameters

The blood is put into a 2 ml plain tube for clinical chemistry testing.[29] And let it stand for 30 minutes at room temperature. The blood is then centrifuged at 4000 rpm for 20 minutes,[31] Then the serum is separated. Serum was analysed for urea and creatinine levels,[16] total bilirubin, and direct bilirubin[30] Using *a SINOWA BS-3000P Chemistry Analyser spectrophotometer*.

Examination of Organ Morphology

Observation of organ morphology is carried out macroscopically, including changes in colour, texture,[32] And whether or not there are lesions. In addition, the weight of the organs in the treatment group was compared to that of the control group to assess the presence of morphological changes.[33]

Data Analysis

Data were expressed as mean ± SD. One-way ANOVA with Duncan post-hoc test ($p<0.05$) was used if assumptions were violated, Kruskal–Wallis test was applied.[16,34]

Ethical Considerents

The Health Research Ethics Committee of UNUSA approved the animal testing protocols under approval number 0454/EC/KEPK/UNUSA/2024.

RESULT

Haematology Parameters

Table 1 shows that in male rats, Hb levels were in the range of 9.55–14.73 g/dl, erythrocytes were 5.62–7.50 ×10⁶/µl, leukocytes were 4.30–10.20 ×10³/µl, and platelets were 451–683 ×10³/µl. In female rats, Hb levels were 8.25–14.50 g/dl, erythrocytes 4.55–7.47 ×10⁶/µl, leukocytes 7.45–13.23 ×10³/µl, and platelets 544–690 ×10³/µl. There was general variation between groups, but the statistical analysis results showed no significant difference ($p > 0.05$).

Clinical Chemistry Parameters

Table 2 shows urea levels in male rats ranged from 11.37–52.27 mg/dl and 21.17–59.03 mg/dl in females. Male creatinine levels range from 0.17–0.50 mg/dl, and females 10–0.57 mg/dl. Direct bilirubin in males is 0.03–0.17 mg/dl, and total bilirubin is 0.04–0.39 mg/dl, while in females, direct bilirubin is 0.02–0.44 mg/dl, and total bilirubin is 0.16–0.26 mg/dl. There was general variation between groups, but no consistent trends or significant differences were found ($p > 0.05$).

Relative Weight of Organs

Table 3 shows the relative liver and kidney weights in male and female rats after treatment, but these did not differ significantly. In male rats, liver weight ranged from 3.39 ± 0.91% (P4) to 4.83 ± 1.44% (P1), while kidney weight ranged from 0.61 ± 0.40% (P3) to 0.99 ± 0.68% (P1). In female rats, liver weight ranged from 3.80 ± 1.14% (P4) to 4.85 ± 0.60% (P3), and kidney weight ranged from 0.47 ± 0.13% (P4) to 0.74 ± 0.37% (P1).

Rat Mortality Rate

Figure 1 illustrates the mortality percentage of male and female rats across treatment groups. No mortality was observed in Group P1. A mortality rate of 10% occurred in female rats in Groups P2 and P4, and in male rats in Group P3. Group P5 showed the highest mortality, with 20% males and 10% females. Overall, mortality increased at higher doses, with the highest rate observed in Group P5.

Table 1. Hematological Effects of the Extract Combination

Parameter	P1	P2	P3	P4	P5
Male					
HB (g/dl)	11.40 ± 0.98	11.80 ± 2.42	9.55 ± 0.49	14.73 ± 2.37	13.40 ± 9.48
RBC (x10^6/ul)	5.62 ± 0.97	6.55 ± 1.13	6.43 ± 1.55	7.50 ± 0.75	7.30 ± 5.16
WBC (x10^3/ul)	10.2 ± 2.17	9.73 ± 6.55	9.55 ± 0.49	4.30 ± 2.42	9.00 ± 5.20
PLT (x10^3/ul)	474 ± 309.50	451 ± 269.50	683 ± 310.42	668 ± 66.27	468 ± 270.20
Female					
HB (g/dl)	11.93 ± 2.73	8.25 ± 6.29	14.50 ± 1.71	13.35 ± 0.64	13.25 ± 0.35
RBC (×10^6/ul)	5.92 ± 1.53	4.55 ± 3.18	7.08 ± 1.18	7.47 ± 0.54	6.81 ± 0.28
WBC (x10^3/ul)	13.23 ± 6.89	10.15 ± 0.64	10.47 ± 1.86	11.25 ± 5.02	7.45 ± 0.35
PLT (x10^3/ul)	544 ± 110.69	690 ± 145.66	647 ± 29.01	578 ± 57.28	569 ± 28.99

Table 2. Clinical Chemistry Effects of the Extract Combination

Parameter	P1	P2	P3	P4	P5
Male					
Urea (mg/dl)	37.90 ± 12.47	52.27 ± 1.96	21.97 ± 0.64	39.77 ± 3.02	11.37 ± 19.69
Creatinine (mg/dl)	0.40 ± 0.26	0.23 ± 0.23	0.33 ± 0.29	0.50 ± 0.10	0.17 ± 0.29
Direct bilirubin (mg/dL)	0.17 ± 0.18	0.08 ± 0.03	0.05 ± 0.06	0.11 ± 0.15	0.03 ± 0.06
Total Bilirubin (mg/dl)	0.39 ± 0.23	0.23 ± 0.11	0.17 ± 0.15	0.26 ± 0.08	0.04 ± 0.06
Female					
Urea (mg/dl)	59.03 ± 8.56	53.73 ± 47.42	32.53 ± 0.64	21.17 ± 18.47	23.47 ± 20.45
Creatinine (mg/dl)	0.30 ± 0.10	0.10 ± 0.00	0.57 ± 0.06	0.40 ± 0.35	0.33 ± 0.29
Direct bilirubin (mg/dL)	0.44 ± 0.20	0.02 ± 0.02	0.05 ± 0.02	0.06 ± 0.07	0.11 ± 0.11
Total Bilirubin (mg/dl)	0.19 ± 0.16	0.18 ± 0.21	0.26 ± 0.14	0.16 ± 0.14	0.20 ± 0.16

Table 3. Relative Organ Weight of Treated Rats

Organ	Relative Organ Weight (%)				
	P1	P2	P3	P4	P5
Male					
Liver	4.83 ± 1.44	4.38 ± 0.71	4.16 ± 0.56	3.39 ± 0.91	4.68 ± 2.10
Kidney	0.99 ± 0.68	0.84 ± .64	0.61 ± 0.40	0.69 ± 0.45	0.77 ± 0.75
Female					
Liver	4.40 ± 0.95	4.46 ± 0.67	4.85 ± 0.60	3.80 ± 1.14	4.27 ± 0.29
Kidney	0.74 ± 0.37	0.62 ± 0.04	0.63 ± 0.27	0.47 ± 0.13	0.60 ± 0.13

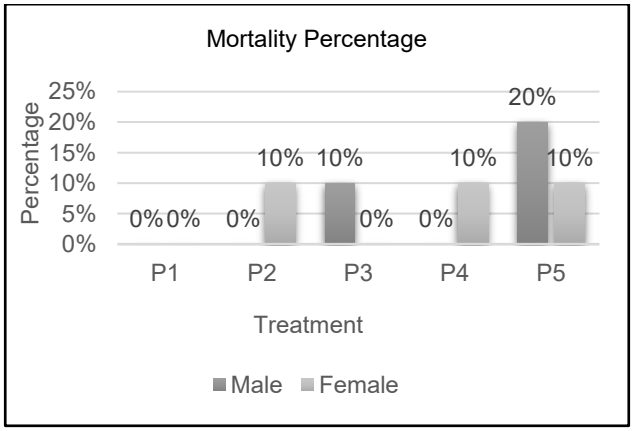
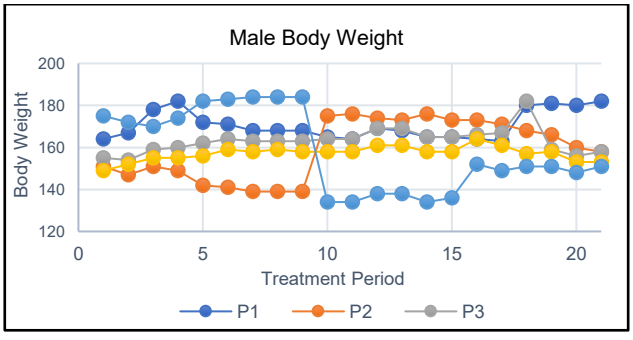


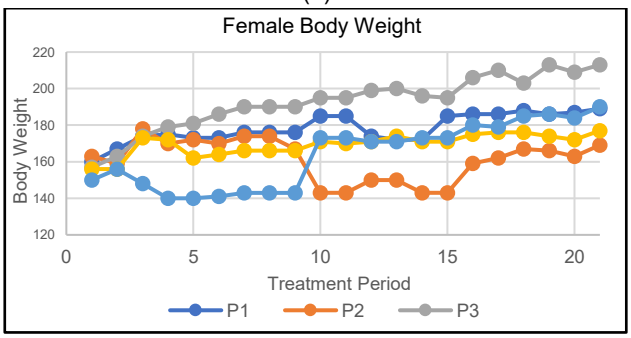
Figure 1. The rat mortality rate during the 21 days of sub-acute toxicity testing

Changes in Rat Weight

Figure 2a shows the body weight of male rats ranging from 130–190 g. The weight of the P1 group was in the range of 145–155 g, P2 at 145–170 g, P3 at 160–170 g, P4 at 150–160 g, and P5 at 150–190 g. Figure 2b shows a higher body weight of female rats, 140–210 g. The P1 group ranges between 160–180 g, P2 at 145–175 g, P3 at 170–210 g, P4 at 160–170 g, and P5 at 140–155 g.



(a)



(b)

Figure 2. Weight change of (a) male and (b) female rats over 22 days sub-acute toxicity testing of a mixture of *Zizphus spina-christi* L. and *Adenanthera pavonina* L

Organ Morphology Assessment

Figure 3 shows the macroscopic results of the rat's liver and kidneys after treatment. In the control group, the liver and kidneys appeared normal with a reddish-brown color and smooth surface. In the treatment group (P2–P5), changes in color to yellow, pale brown, or dark brown were found, as well as the presence of black spots, accompanied by morphological changes and increased texture hardness in some liver and kidney samples.

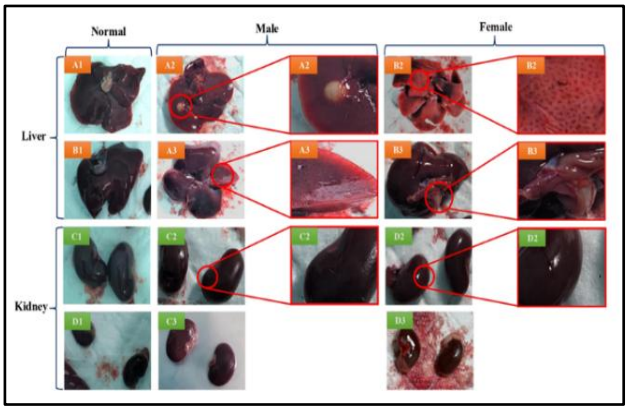


Figure 3. Macroscopic liver and kidney toxicity test of a mixture of *Ziziphus spina-christi* L. and *Adenanthera pavonina* L. leaf extract in female rats

DISCUSSION

The evaluation of the extract combination demonstrated several key findings related to its sub-acute effects. Mortality occurred only at higher doses, with a greater incidence in male rats (20%) compared to females (10%). Clinical signs such as reduced activity and respiratory changes preceding death suggest that the extract may exert physiological stress when administered at elevated concentrations. The dose-dependent pattern of mortality aligns with earlier reports showing increased mortality at higher doses of plant extracts.[35,36] Additionally, the short acclimatization period (<7 days) may have heightened susceptibility to treatment-related stress, as previous studies indicate that insufficient acclimatization can influence physiological responses.[37,38] Ther findings suggest that a longer acclimatization period, up to two weeks, results in more stable responses at comparable doses.[39,40]

Body weight changes observed throughout the 21-day treatment showed an early decrease followed by partial recovery. Although body weight is a sensitive indicator of systemic toxicity, the fluctuations in this study did not show a consistent pattern that would indicate severe or progressive toxic effects. Similar findings have been reported in studies administering plant extracts up to 5000 mg/kg without significant weight changes.[41,42] Some reports even noted only transient symptoms such as drowsiness.[43] Variability in weight responses between studies may be attributed to differences in extraction techniques, phytochemical concentrations, and plant sources, emphasizing the importance of standardization.

Hematological parameters remained within normal physiological ranges in all groups, indicating that the extract combination did not meaningfully affect hematopoiesis, consistent with previous findings on subacute exposure to plant-derived compounds.[44,45] Minor sex-related differences likely reflected normal physiological variation rather than treatment effects. Biochemical markers also showed no significant changes, with urea, creatinine, and bilirubin levels comparable across groups ($p>0.05$), supporting earlier reports of stable renal biomarkers following herbal extract administration.[46] Since elevated urea and creatinine typically indicate impaired filtration.[47] The mild fluctuations observed did not suggest renal dysfunction. Bilirubin levels varied without a clear dose–response pattern, similar to reports of neutral or hepatoprotective effects of flavonoid-rich extracts.[48] Overall, biochemical alterations were minimal and not indicative of systemic toxicity.

Macroscopic assessment of the liver and kidneys revealed visible changes at higher doses, including discoloration and dark spots, while organs from control animals appeared normal. Similar pigment alterations have been associated with anthraquinone derivatives such as emodin, which may accumulate and induce tubular changes.[49] These findings underscore that organ-level changes may occur even in the absence of corresponding serum biomarker alterations. Liver and kidney weights showed minor decreases in some groups, which may reflect early signs of tissue changes, consistent with previous findings that reductions in organ weight can indicate structural or metabolic disturbances.[44] Whereas in other cases, increased organ weight may result from edema or compensatory hypertrophy in response to toxic exposure.[50] Antinutritional compounds such as tannins and saponins have been implicated in altering nutrient metabolism and energy utilization, potentially contributing to these observations.[51]

This study has several limitations. The acclimatization period may have influenced the physiological responses of the animals, as inadequate adaptation has been reported to increase stress susceptibility.[37,38] Furthermore, the wide dosing interval limited the ability to identify precise threshold doses for toxicity. Histopathological examination was not performed in detail, restricting interpretation of early tissue alterations. Additionally, the study focused only on the liver and kidneys, without evaluating other potential target organs such as the heart, lungs, or spleen.

CONCLUSIONS AND RECOMMENDATION

The combined leaf extract of *Z. spina-christi* and *A. pavonina* is safe up to 8000 mg/kg BW, with 10,000 mg/kg BW producing toxicity evidenced by increased mortality and organ abnormalities. Future research should incorporate detailed histology, expanded organ assessment, and narrower dosing intervals to provide a more comprehensive understanding of toxicity.

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