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Toxicological Assessment and Antioxidant Profiling of an Ethanolic Extract Mixture of *Ziziphus mauritiana* and *Bauhinia racemosa* Leaves

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Abstract The current study evaluates the toxicological safety and antioxidant potential of an ethanolic extract mixture from *Ziziphus mauritiana* and *Bauhinia racemosa* leaves, two plants with significant ethnopharmacological and therapeutic value. Acute oral toxicity was evaluated in Wistar rats following the guidelines outlined by the Organization for Economic Co-operation and Development (OECD) 423 protocol, with the extract mixture administered in doses reaching 2000 mg/kg of body weight. No adverse effects or mortality were observed over a 14-day period, indicating a high margin of safety. Antioxidant activity was evaluated by measuring total phenolic content (TPC), and total flavonoid content (TFC) and through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The extract exhibited substantial phenolic (7.75 mg GAE/g) and flavonoid (6.70 mg QE/g) contents, both associated with strong antioxidant properties. The DPPH assay revealed an IC_{50} value of 75.53 µg/mL, demonstrating notable antioxidant activity, though less potent than synthetic antioxidants like ascorbic acid (IC_{50} = 24.96 µg/mL). The result indicates that the ethanolic extract mixture of *Z. mauritiana* and *B. racemosa* leaves is safe for pharmacological use, supporting their traditional application with minimal toxicity risk at standard doses. However, chronic toxicity studies are recommended to establish long-term safety. This research lays the groundwork for the development of natural antioxidant therapies, highlighting the therapeutic potential of the *Z. mauritiana* and *B. racemosa* extract mixture in combating oxidative stress-related diseases.

Keywords *Ziziphus mauritiana*, *Bauhinia racemosa*, Acute Toxicity, Antioxidant Activity, DPPH Radical Scavenging

1. Introduction

Plants with ethnopharmacological significance have played a pivotal role in drug discovery, providing bioactive compounds for diverse therapeutic applications [1,2]. Among these, the *Ziziphus* genus is widely recognized for its traditional medicinal use in managing various health conditions [3-5]. *Ziziphus mauritiana* (ber), a tropical fruit tree of the Rhamnaceae family, is characterized by its spiny branches, evergreen leaves, and small yellow flowers [4-7]. Similarly, *Bauhinia racemosa* (beedi leaf tree), a small tree native to Southeast Asia, holds therapeutic importance and typically flowers between February and May [8].

While bioactive compounds from plants offer significant pharmacological benefits, they may also pose toxicity risks, highlighting the importance of thorough safety evaluations [9]. Toxicological studies serve as a critical bridge between animal models and potential human applications, confirming safety and efficacy and the determination of appropriate dosages [10]. Standardized protocols, such as those established by the Organization for Economic Co-operation and Development (OECD), ensure reliable toxicity assessments [11]. Antioxidants are vital in neutralizing oxidative stress, a major contributor to aging and diseases such as cardiovascular (CV) disorders, diabetes, and cancer. Exploring the antioxidant properties of *Z. mauritiana* and *B. racemosa* may help mitigate these conditions and support their use in developing natural therapies for human health [12,13].

While various parts of *Z. mauritiana*, including its fruits and seeds, and *B. racemosa*, such as its bark, have documented traditional and pharmacological uses, this study focuses on the leaves due to their abundant phenolic and flavonoid content, which significantly contribute to antioxidant activity [14,15]. Extracts from *Z. mauritiana*, including its fruits, leaves, and seeds, have demonstrated antioxidant properties in previous studies [16-21]. Similarly, *B. racemosa* is distinguished by its abundant phenolic and flavonoid compounds, which play a significant role in its antioxidant properties. These properties are crucial in reducing oxidative stress linked to conditions such as CV disorders and cancer [22].

The objective of this study was to assess the toxicological characteristics of a combined ethanolic extract mixture of *Z. mauritiana* and *B. racemosa* leaves in Wistar rats, following OECD 423 guidelines for acute oral toxicity. Furthermore, the extract's antioxidant activity was evaluated by quantifying its total phenolic and flavonoid contents and performing a DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay.

Through the combined assessment of toxicological and antioxidant properties, this study offers important perspectives on the safety and pharmacological potential of the ethanolic extract mixture of *Z. mauritiana* and *B. racemosa* leaves. These findings may support the development of natural antioxidant therapies and guide future investigations into the therapeutic applications of these plants.

2. Materials and Methods

2.1. Plant Material and Extraction

2.1.1. Collection and Identification

Leaves of *Z. mauritiana* and *B. racemosa* were collected from Hatlai Ghat, a hilly region in Dharashiv (Osmanabad), Maharashtra, India. The plant material was authenticated by Dr. A. S. Linge, Head of the Department of Botany at Venkatesh Mahajan Senior College, Dharashiv, Maharashtra. Voucher specimens were deposited at the institution under herbarium number 128(B) for 2022–2023. The college is affiliated with Dr. Babasaheb Ambedkar Marathwada University, Chhatrapati Sambhaji Nagar (Aurangabad), Maharashtra, India.

2.1.2. Preparation of Extract

The leaves were rinsed using reverse osmosis-purified water and left to air dry at room temperature for 21 days until they became completely dry and brittle. The air-dried leaves were finely milled into powder using a Philips HL7756/01 750 Watt Mixer Grinder. A total of 500 g of powdered material was macerated in 2 L of absolute ethanol (99.5%) under sterile conditions and divided into four sealed glass containers. The maceration technique was chosen for its efficiency in obtaining diverse phytochemicals, encompassing both polar and non-polar constituents, while avoiding thermal degradation. The stirring frequency (4–6 times daily) and extraction period (three days) were optimized through preliminary studies to maximize the yield of active compounds. The mixture was stirred 4–6 times a day over three days and passed through Whatman No.1 filter paper for filtration. The solvent was removed via rotary evaporation, yielding a solvent-free extract of *Z. mauritiana* and *B. racemosa*. These extracts were then combined in a 1:1 ratio by weight to ensure a balanced representation of bioactive compounds from both species.

2.2. Toxicological Evaluation

2.2.1. Animal Model

The toxicological evaluation was conducted using 15 female Wistar rats, aged 6–8 weeks, selected for their physiological similarity to human metabolic processes. The rats were housed under standard laboratory conditions following OECD 423 guidelines, with provisions for environmental enrichment, proper bedding, and appropriate cage dimensions to ensure welfare [23]. Ethical clearance for the study was obtained from Scitesla Private Limited, Navi Mumbai, Maharashtra (approval number SCI/IAEC/2024-25/107, dated July 17, 2024). The rats had unrestricted access to a standard diet and water throughout the study.

2.2.2. OECD Guidelines

Acute oral toxicity was assessed using the stepwise procedure outlined in the OECD 423 guidelines. The rats were assigned to groups of three, with each group administered oral doses of 5, 50, 300, or 2000 mg/kg body weight of the extract mixture, while distilled water was given to the control group. A control group receiving distilled water was included to distinguish extract-related effects from baseline physiological variations. The control group for antioxidant tests comprised blank solutions, ensuring accurate calculation of radical scavenging activity and validating the reliability of the results. Toxicity signs were observed, including autonomic and central nervous system changes, somatomotor activity, and circulatory and respiratory system alterations. Physical characteristics such as fur, mucous membranes, and eye conditions were also assessed. Symptoms such as convulsions, salivation, diarrhea, lethargy, and tremors were recorded. Observations were conducted at 30-minute intervals post-dosing, periodically during the first 24 hours, and daily for 14 days [23].

2.3. Antioxidant Evaluation

2.3.1. Total Phenolic Content

The Folin-Ciocalteu method was employed to measure the total phenolic content (TPC) of the ethanolic extract mixture, utilizing gallic acid as the standard reference compound. A standard curve for gallic acid was generated using proportional dilutions at concentrations ranging from 20–240 μg/mL. The extract was prepared at fixed concentrations of 100 and 200 μg/mL and was tested to ensure the absorbance values aligned with the standard curve range.

The reaction mixture consisted of 0.5 mL of sample or standard, 3 mL of distilled water, and 0.25 mL of Folin-Ciocalteu reagent (diluted 1:10 with water). Following mixing, the reaction was kept in darkness for 5 minutes before adding 1 mL of a 7.5% sodium carbonate solution. The preparation was left to stand at room temperature in the absence of light for 60 minutes. The absorbance was recorded at 760 nm with a double-beam UV-Vis spectrophotometer, using a reagent blank as the reference. TPC was calculated as milligrams of gallic acid equivalents per gram of extract (mg GAE/g) using the equation y=0.0107x+0.0277 (R^2= 0.9679) [24-26].

Measurements were performed in triplicate [27]:

$$TPC = (C \times V) / M$$

Where:

C = concentration of phenolics in the extract solution, typically obtained from a standard calibration curve using gallic acid, expressed in mg/mL

V = volume of the extract solution in the assay, expressed in mL

M = mass of the extract used in the assay, expressed in grams

2.3.2. Total Flavonoid Content

The total flavonoid content (TFC) was assessed through the aluminum chloride method, using quercetin as the standard reference compound. A calibration curve was generated using proportional dilutions of quercetin that ranged from 20–240 μg/mL, while the extract was prepared at fixed concentrations of 100 and 200 μg/mL for evaluation.

The reaction mixture was composed of 0.5 mL of either the test sample or standard solution, 2 mL of distilled water, and 0.15 mL of a 5% sodium nitrate solution. After 5 minutes, 0.15 mL of a 10% aluminum chloride solution was added, followed by 1 mL of a 1 M sodium hydroxide solution after an additional minute. The mixture's final volume was brought to 5 mL with distilled water. The absorbance was recorded at 510 nm using a UV-Vis spectrophotometer, with a reagent blank serving as the reference. The TFC, expressed in milligrams of quercetin equivalents per gram of extract (mg QE/g), was calculated based on the equation y=0.0005x+0.0062 (R^2 = 0.9587) [24-26].

Measurements were performed in triplicate [27]:

$$TFC = (C \times V) / M$$

Where:

C = concentration of flavonoids in the extract solution, usually determined from a standard calibration curve using quercetin, expressed in mg/mL

V = volume of the extract solution in the assay, expressed in mL

M = mass of the extract used in the assay, expressed in grams

2.3.3. DPPH Assay

The free radical scavenging potential of the ethanolic extract mixture from *Z. mauritiana* and *B. racemosa* leaves was assessed through the DPPH assay, employing a modified protocol adapted from Khorasani Esmaeili et al. [28]. A 0.1 mM solution of DPPH was made by dissolving 19.70 mg of the compound in a small amount of ethanol, then diluting the mixture to 500 mL with ethanol. A control solution was made by combining 3 mL of ethanol with 2 mL of the DPPH solution.

A stock solution of ascorbic acid (the reference standard) was prepared by dissolving 50 mg of ascorbic acid in 50 mL of ethanol. Proportional dilutions were made to produce standard solutions at concentrations of 1, 10, 25, 50, 100, 250, 500, and 1000 μg/mL. Similarly, the ethanolic extract mixture was diluted to match these concentrations for comparison in the analysis.

For the assay, 2.4 mL of the 0.1 mM DPPH solution was combined with 1.6 mL of each sample or standard solution, covering a concentration range from 1 to 1000 μg/mL. The reaction mixtures were incubated at room temperature in the dark for 30 minutes. Following incubation, the absorbance was recorded at 517 nm using a double-beam UV-Vis spectrophotometer.

The percentage of DPPH radical scavenging activity (RSA) was calculated using the formula [24–28]:

$$RSA (\%) = (\text{Absorbance of Control} - \text{Absorbance of Sample}) \times 100 / \text{Absorbance of Control}$$

Where:

$A_{Control}$ = absorbance of the control sample (DPPH) (without the antioxidant)

A_{Sample} = absorbance of the sample (*Z. mauritiana* + *B. racemosa* extract) with the antioxidant

The IC_{50} value, representing the concentration of the extract or standard needed to neutralize 50% of the DPPH radicals, was calculated from the dose-response curve. The curve was constructed by plotting the radical scavenging activity (RSA, %) against the respective concentrations. IC_{50} values were derived from the linear section of the curve. Each concentration was evaluated in triplicate to ensure the reliability and consistency of the results. The outcomes were reported as milligrams of ascorbic acid equivalent per gram of extract (mg AAE/g) [24–28].

2.4. Statistical Analysis

The experimental data were processed using Microsoft Excel. The results for TPC, TFC, and DPPH radical scavenging activity (RSA) are presented as mean ± standard deviation (SD). IC_{50} values were determined through linear regression analysis, and the goodness of fit (R^2) was assessed to confirm the accuracy of the calculations.

3. Results

3.1. Toxicological Evaluation

3.1.1. Acute Oral Toxicity

The acute oral toxicity of the ethanolic extract mixture from *Z. mauritiana* and *B. racemosa* leaves was assessed according to OECD Guideline 423. A total of 15 female Wistar rats were allocated into five groups, with four groups receiving oral doses of 5, 50, 300, and 2000 mg/kg body weight of the extract mixture, while the control group was administered distilled water.

No significant signs of toxicity or behavioral abnormalities were observed in any dose group during the 14-day observation period. Parameters such as fur condition, skin appearance, mucous membranes, eye health, body weight, and respiratory and circulatory functions were monitored and found to be within normal physiological limits. Neurological assessment, including motor activity and responses of the central and autonomic nervous system, indicated no adverse effects across all treatment groups.

Post-mortem necropsy revealed no gross pathological abnormalities in major organs, including the liver and adipose tissues. Histological examination confirmed the absence of organ damage or toxicity-related changes in both treated and control groups. The extract mixture was well tolerated at all tested doses, including the maximum dose of 2000 mg/kg body weight, thereby establishing its safety profile in this acute toxicity study.

3.2. Antioxidant Evaluation

3.2.1. Total Phenolic Content

The TPC of the ethanolic extract mixture was evaluated through the Folin-Ciocalteu method. A concentration-dependent rise in TPC was observed, with the maximum value reaching 7.75 mg GAE/g extract at a concentration of 200 μg/mL. These findings are presented in Table 1.

Table 1. TPC of the Ethanolic Extract Mixture of *Z. mauritiana* and *B. racemosa* Leaves

Concentration (μg/mL)	GAE (μg/mL)	TPC in mg GAE/g Extract Mean ± SD
100	60.33	3.01 ± 0.0012
200	155.10	7.75 ± 0.0012

3.2.2. Total Flavonoid Content

The TFC was measured using the aluminum chloride assay. The findings also revealed a concentration-dependent increase, with the peak TFC value of 6.70 mg QE/g extract observed at a concentration of 200 μg/mL. These results are detailed in Table 2.

Table 2. TFC of the Ethanolic Extract Mixture of *Z. mauritiana* and *B. racemosa* Leaves

Concentration (μg/mL)	QE (μg/mL)	TFC in mg QE/g Extract Mean ± SD
100	27.80	1.39 ± 0.0100
200	134.00	6.70 ± 0.0100

3.2.3. DPPH Radical Scavenging Activity

The antioxidant potential of the ethanolic extract mixture was evaluated using the DPPH radical scavenging assay. The extract exhibited a concentration-dependent increase in %RSA, achieving a maximum inhibition of 95.69% at 1000 μg/mL.

The IC_{50} value for the extract mixture was 75.53 μg/mL, compared to 24.96 μg/mL for ascorbic acid, the reference standard. Figure 1 illustrates the dose-response curves, highlighting the comparative antioxidant activity of the extract mixture and ascorbic acid. Detailed % RSA values and IC_{50} results are presented in Table 3.

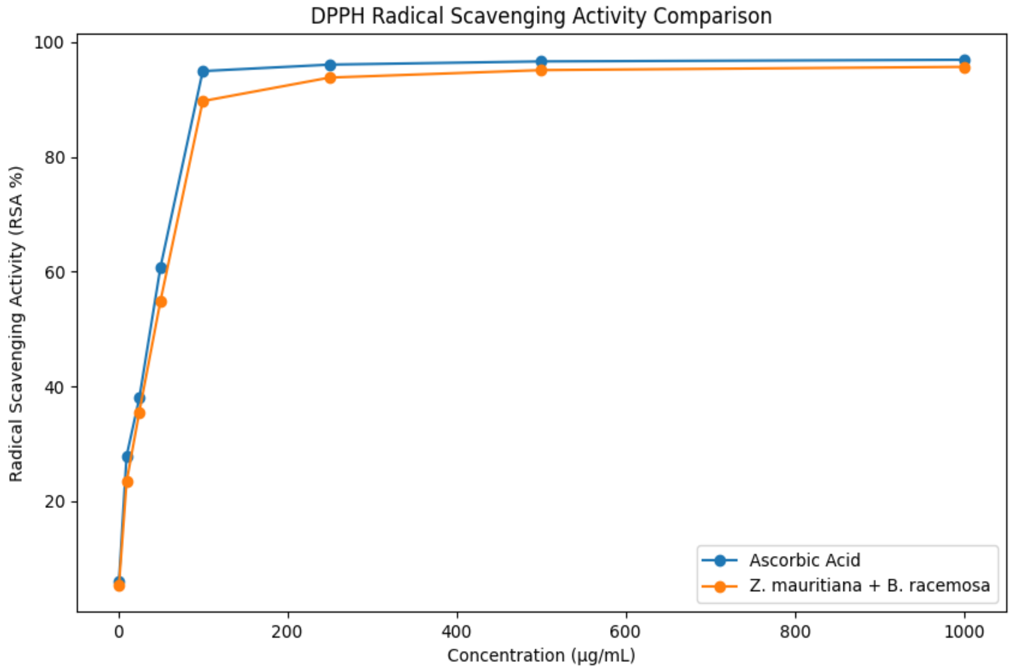

Figure 1. DPPH RSA of the Ethanolic Extract Mixture of *Z. mauritiana* and *B. racemosa* Leaves Compared to Ascorbic Acid

Table 3. DPPH RSA of the Ethanolic Extract Mixture of *Z. mauritiana* and *B. racemosa* Leaves

Concentration (μg/mL)	Ascorbic acid		*Z. mauritiana + B. racemosa*	
	RSA (%)	IC_{50} (μg/mL)	RSA (%)	IC_{50} (μg/mL)
1	6.07	24.96	5.33	75.53
10	27.87		23.37	
25	37.94		35.50	
50	60.73		54.86	
100	94.95		89.72	
250	96.08		93.82	
500	96.64		95.12	
1000	96.92		95.59	

4. Discussion

The current study evaluated the toxicological safety and antioxidant potential of an ethanolic extract mixture derived from *Z. mauritiana* and *B. racemosa* leaves, two plants widely recognized for their traditional medicinal applications.

Toxicological safety: An acute oral toxicity assessment, performed following the protocols outlined in OECD Guideline 423, indicated that the ethanolic extract mixture possesses a substantial safety margin. Throughout the 14-day observation period, Wistar rats displayed no mortality, notable adverse effects, or significant behavioral changes at doses as high as 2000 mg/kg body weight. Gross necropsy and histopathological evaluations revealed no signs of toxicity or organ damage. These findings align with earlier reports on related species. For example, Sakna et al. highlighted the safety of *Ziziphus* species, including *Z. jujuba* and *Z. spina-christi*, which are extensively used in traditional medicine [29]. Similarly, non-toxic effects have been documented for related *Bauhinia* species, such as *B. cheilantha* and *B. variegata* at comparable doses [30]. The lack of toxicity underscores the potential of the extract for subsequent pharmacological studies. However, additional studies evaluating sub-chronic and chronic toxicity are critical to establishing a comprehensive safety profile, including the potential for cumulative effects over prolonged use.

Antioxidant Potential: The extract mixture demonstrated substantial antioxidant potential, as evidenced by the TPC, TFC, and DPPH RSA assays. The TPC and TFC analyses showed concentration-dependent increases, with peak values of 7.75 mg GAE/g and 6.70 mg QE/g, respectively, at 200 μg/mL. These findings are consistent with prior research. For instance, Javed et al. documented significant antioxidant activity in methanolic extracts of *Z. mauritiana* leaves [31], and studies on *B. racemosa* have similarly highlighted its antioxidant richness in various plant parts, including leaves, flowers, and bark [32].

The DPPH assay further substantiated the extract's antioxidant capacity, with a maximum RSA of 95.69% and IC_{50} value of 75.53 μg/mL. Although the extract exhibited lower potency compared to ascorbic acid (IC_{50}=24.96 μg/mL), its natural origin makes it a promising alternative to synthetic antioxidants. The findings emphasize the potential utility of the extract mixture as a plant-based antioxidant source, suitable for applications in pharmaceuticals, nutraceuticals, or cosmetics.

Limitations and Future Directions: Despite the encouraging results, the study is limited in scope. First, the toxicological evaluation was confined to acute toxicity; chronic toxicity studies are necessary to assess long-term safety. Secondly, the antioxidant evaluations were performed *in vitro*, which does not consider factors like bioavailability, metabolic processes, or physiological interactions. Future studies should focus on *in vivo* models to confirm the extract's efficacy under physiological conditions and evaluate its impact on oxidative stress biomarkers. Additionally, exploring the extract's specific mechanisms of action and bioavailability will enhance our understanding of its therapeutic potential.

Sustainability and Broader Applications: The sustainable sourcing of *Z. mauritiana* and *B. racemosa* leaves is critical, given the potential for overharvesting to impact local ecosystems. Implementing guidelines for sustainable harvesting and exploring alternative cultivation strategies could ensure the availability of these medicinal plants for future use. Moreover, while this study focused on leaves, other plant parts such as fruits, flowers, and bark may also exhibit significant pharmacological properties. Investigating these parts could provide a broader understanding of the plant's bioactive potential and expand its applications.

5. Conclusions

The current study provides foundational evidence supporting the toxicological safety and antioxidant potential of an ethanolic extract mixture derived from *Z. mauritiana* and *B. racemosa* leaves. The extract demonstrated a high safety margin in acute oral toxicity evaluations, with no adverse effects observed at doses up to 2000 mg/kg, aligning with a favorable safety profile. This establishes a strong basis for its consideration in further pharmacological research.

The extract's substantial levels of phenolic and flavonoid compounds, along with pronounced DPPH radical-scavenging activity, emphasize its strong antioxidant potential. These characteristics indicate its promise as a natural remedy for addressing conditions associated with oxidative stress, such as chronic inflammation, neurodegenerative diseases, and cardiovascular complications. Its safety and efficacy further underscore its potential for human health applications, particularly in developing nutraceuticals and functional foods aimed at enhancing overall well-being.

In conclusion, the ethanolic extract mixture of *Z. mauritiana* and *B. racemosa* leaves represents a safe and effective natural antioxidant source with promising application in medicinal and nutraceutical development. Future research focusing on chronic toxicity studies, *in vivo* antioxidant activity, bioavailability, and elucidation of therapeutic mechanisms is necessary to further validate its potential for clinical and industrial applications, ultimately contributing to improving human health.

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