



TEARS: Toxicity Evaluation of Active Remedies and Synergy through Brine Shrimp Bioassay on Selected Ethnomedicinal Plants

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KeyWords

Brine Shrimp Lethality Test, Ethnomedicinal Plants, Ethanolic Extracts, Toxicity Assessment, LC_{50}

ABSTRACT

Medicinal plants play a major role as sources of bioactive molecules in both traditional and modern medicine, yet the potential toxicity must be evaluated.

The toxicity of four Philippine ethnomedicinal plants, namely *Annona muricata* (soursop) leaves, *Curcuma longa* (turmeric) rhizomes, *Piper betle* (betel leaf), and *Paspalum conjugatum* (carabao grass), was investigated on ethanol extracts.

The Brine Shrimp Lethality Test was used to determine lethality against *Artemia salina nauplii* (Brine Shrimp) at concentrations of 10, 100, and 1000 ppm for the individual as well as synergistic treatments.

Mortalities were observed at 24 hours of exposure, and probit regression was used for the calculation of LC_{50} values.

There were significant statistical differences ($p < 0.05$) in toxicity within the treatments, as per statistical tests (one-way ANOVA and Tukey's HSD).

Mixtures of plant extracts were more toxic than their individual counterparts, suggesting a synergic effect between phytochemicals.

The results showed a concentration-dependent relationship, with greater concentrations associated with higher mortalities. Findings observed that these plants, apart from the medicinal properties, the toxicity depends on dose and combinations.

BSLT proved to be a simple, cost-effective, and reliable method for preliminary toxicity screening of ethnomedicinal plants.

INTRODUCTION

Medicinal plants serve as fundamental sources of bioactive compounds valuable in both traditional healing systems and modern scientific studies. Phytochemical constituents such as alkaloids, flavonoids, terpenoids, saponins, and phenolics exhibit diverse biological activities, including antimicrobial, antioxidant, analgesic, and anti-inflammatory properties (Twajj & Hasan, 2022). Continuous reliance on herbal preparations within local and global communities underscores the importance of evaluating safety and toxicity levels (Rahayu et al., 2020). Excessive concentrations of

bioactive metabolites can induce adverse biological effects that compromise health and environmental stability (Twaij & Hasan, 2022). Establishing toxicological baselines for commonly used plants is essential to ensure safe utilization in traditional remedies and research-based applications (Hidayah *et al.*, 2023). Comprehensive evaluation of medicinal plant extracts remains crucial for generating reliable safety data that can support evidence-based community health practices (Mishra *et al.*, 2022).

The rhizome of *Curcuma longa* demonstrates strong antioxidant and antimicrobial activity attributed to curcuminoid content (Hidayah *et al.*, 2023). The leaf of *Piper betle* contains phenolic compounds such as eugenol and chavicol that exhibit antibacterial and wound-healing capabilities (Chakraborty & Shah, 2021). The leaf of *Annona muricata* possesses acetogenins that display marked bioactivity against certain cell lines (Adewole *et al.*, 2022). The grass *Paspalum conjugatum* has primarily been explored for ecological and phytochemical characterization (Rahayu *et al.*, 2020). Although extensive research highlights medicinal benefits, limited data compare toxicity levels among these species or evaluate the combined effects of plant mixtures. Understanding how extract combinations influence toxicity, whether through synergistic enhancement or reduction, remains a critical component of safe herbal use and development.

Absence of comprehensive toxicity profiling for *Piper betle*, *Annona muricata*, *Paspalum conjugatum*, and *Curcuma longa* creates a significant knowledge gap in ethnobotanical safety assessment. Active chemical constituents responsible for healing effects may also exhibit toxic responses when present in high concentrations. Systematic evaluation of toxicity provides essential data for identifying safe concentration ranges in medicinal and environmental contexts. The present study determined and compared the toxicity levels of ethanolic extracts of *Annona muricata* leaves, *Curcuma longa* rhizomes, *Piper betle* leaves, and *Paspalum conjugatum* leaves using the brine shrimp lethality assay (BSLT). The investigation also examined combinations to assess possible synergistic or antagonistic effects. Results from this analysis aim to provide quantitative toxicity data that can serve as a reference for determining safety levels, guiding responsible plant use, and supporting scientific evaluation of Philippine ethnomedicinal resources.

Research Objectives

General Objectives:

Determine and compare the toxicity levels of selected Philippine ethnomedicinal ethanolic plant extracts, *Annona muricata* (guyabano), *Curcuma longa* (turmeric), *Piper betle* (betel leaf), and *Paspalum conjugatum* (carabao grass), using the brine shrimp lethality test (BSLT).

Specific Objectives:

Specifically, the study aims to:

1. Determine the LC₅₀ values of *Annona muricata*, *Curcuma longa*, *Piper betle*, and *Paspalum conjugatum* ethanolic extracts through the brine shrimp lethality test;
2. Evaluate whether there is a significant difference in toxicity among the four individual plant extracts: *Annona muricata*, *Curcuma longa*, *Piper betle*, and *Paspalum conjugatum*;
3. Identify the safety dose of the extracts and the combinations that are considered safe based on mortality results; and
4. Assess whether the combination of the ethanolic plant extracts exhibits significant toxicity compared to individual extracts.

REVIEW OF RELATED LITERATURE

This review of related literature focuses on the toxicity levels of four selected Philippine ethnomedicinal plants: *Annona muricata* (guyabano), *Curcuma longa* (turmeric), *Piper betle* (betel leaf), and *Paspalum conjugatum* (carabao grass). It summarizes existing studies that utilized the brine shrimp lethality assay (BSLA) to assess their potential toxicity. The review also presents relevant background information on their traditional uses and bioactive components to support the study's aim of evaluating and comparing their toxicity profiles.

Review of Medicinal Plants

Medicinal plants are natural sources of therapeutic compounds that have been used for centuries to prevent and treat various diseases (Kumar *et al.*, 2022). Ethnomedicinal practices, which rely on traditional plant-based remedies, remain widely accepted across the world, especially in developing regions where access to modern medicine is limited (Lopez *et al.*, 2021). In the Philippines, medicinal plants play a vital role in community healthcare, with species such as *Annona muricata*, *Curcuma longa*, *Piper betle*, and *Paspalum conjugatum* frequently used in herbal preparations (Torres *et al.*, 2023). Affordability and accessibility make medicinal plants valuable alternatives for the management of inflammation, infections, and metabolic disorders (Velasco *et al.*, 2024). However, unregulated use may result in adverse effects or toxicity when consumed in excessive doses (Hossain *et al.*, 2023).

Each of the selected plants possesses distinct chemical and pharmacological profiles that contribute to their medicinal properties. *Annona muricata* (guyabano) contains annonaceous acetogenins known for cytotoxic and antitumor effects (Rivera *et al.*, 2021). *Curcuma longa* (turmeric) is rich in curcumin, which has anti-inflammatory and antioxidant properties (Nguyen *et al.*, 2022). *Piper betle* (betel leaf) exhibits strong antimicrobial activity due to eugenol and chavicol, while *Paspalum conjugatum* (carabao grass) possesses flavonoids and saponins linked to antioxidant potential (Parra *et al.*, 2021). Despite the demonstrated therapeutic potential of medicinal plants, dose-dependent toxicity has been reported in various bioassays, emphasizing the necessity of evaluating safety in parallel with medicinal efficacy (Rahman *et al.*, 2022).

***Annona muricata* (Guyabano)**

Annona muricata is a tropical tree distinguished by annonaceous acetogenins and alkaloids responsible for antibacterial, antiparasitic, and antioxidant activities (Torres *et al.*, 2023; Rivera *et al.*, 2021). The leaves and seeds contain secondary metabolites that provide therapeutic benefits when used in controlled concentrations (Bhattacharjee & Das, 2021). Excessive amounts of these bioactive compounds can result in neurophysiological disturbances and metabolic stress, requiring careful assessment of safe dosage levels (Lopez *et al.*, 2020). Inclusion of *A. muricata* in toxicity screening ensures scientific evaluation of potential risks related to unregulated consumption (Velasco *et al.*, 2024). Establishing proper concentration thresholds aids in preventing harmful exposure while retaining medicinal effectiveness.

Extracts of *A. muricata* demonstrate potential application in developing natural insecticidal and antiparasitic agents due to their ability to disrupt metabolic activity in lower organisms (Torres *et al.*, 2023). Controlled use of these compounds contributes to biomedical and agricultural research seeking safer alternatives to synthetic chemicals (Rivera *et al.*, 2021). Measured toxicity allows researchers to apply extracts for antiparasitic or pest-control formulations without posing threats to non-target species (Bhattacharjee & Das, 2021). Experimental validation of LC₅₀ values helps establish a balance between beneficial and toxic concentrations. This balance supports the advancement of natural-based treatment strategies derived from *A. muricata* compounds.

***Curcuma longa* (Turmeric)**

Curcuma longa is a rhizomatous herb widely cultivated for curcuminoids that provide antioxidant, anti-inflammatory, and antimicrobial properties (Hossain *et al.*, 2023). Traditional applications involve treatment of skin wounds, digestive issues, and inflammatory disorders using powdered or extracted rhizomes (Nguyen *et al.*, 2022). Laboratory investigations reveal that high ethanolic concentrations can induce biochemical stress, emphasizing the necessity of concentration-based toxicity evaluation (Rahman *et al.*, 2022). Quantitative analysis through brine shrimp lethality testing determines threshold values suitable for safe use in herbal formulations (Torres *et al.*, 2023). Comprehensive profiling promotes the standardized application of turmeric across research and health-related fields.

Extracts of *C. longa* display potential in pharmaceutical and nutraceutical development due to strong antioxidant mechanisms (Nguyen *et al.*, 2022). Toxicity characterization ensures safe utilization of curcuminoids in supplement design and topical formulations (Rahman *et al.*, 2022). Defined LC₅₀ ranges assist in maintaining therapeutic efficiency without exceeding tolerable limits (Hossain *et al.*, 2023). Controlled dosing contributes to the prevention of hepatic strain and other concentration-related effects. Standardized monitoring of *C. longa* extract use strengthens reliability in ethnomedicinal and experimental contexts.

***Piper betel* (Betel Leaf)**

Piper betle is a perennial climbing vine known for essential oils and phenolic compounds such as eugenol and hydroxychavicol, which contribute to its antimicrobial and antioxidant properties (Sarkar *et al.*, 2020). Traditional preparations frequently employ leaves for oral and topical treatments addressing infections and inflammation (Velasco *et al.*, 2024). Scientific reports indicate concentration-dependent physiological effects in various biological systems, validating the need for standardized toxicity analysis (Nguyen *et al.*, 2022). Quantitative evaluation ensures accurate identification of safe concentration levels suitable for traditional or experimental application (Rahman *et al.*, 2022). Such analysis assists in determining the comparative lethality of *P. betle* relative to other medicinal plants.

Toxic compounds present in *P. betle* offer potential in pharmaceutical research for developing antiseptic and preservative agents (Torres *et al.*, 2023). Phenolic constituents provide natural alternatives to synthetic antimicrobial compounds used in medical and industrial products (Sarkar *et al.*, 2020). Dose-limited applications allow the harnessing of these active substances for beneficial outcomes without adverse effects (Velasco *et al.*, 2024). Establishing LC₅₀ benchmarks strengthens understanding of its toxic threshold in various formulations. Integration of *P. betle* into toxicity evaluation frameworks ensures effective and responsible use in ethnomedicinal practices.

***Paspalum conjugatum* (Carabao Grass)**

Paspalum conjugatum is a grass species under Poaceae, locally utilized for fever and wound care in traditional medicine (Parra *et al.*, 2021). Preliminary phytochemical analysis identifies flavonoids, tannins, and saponins contributing to antimicrobial and antioxidant properties (Lopez *et al.*, 2020). Limited toxicological data create uncertainty regarding safe usage levels, making comparative screening essential (Torres *et al.*, 2023). Quantifying LC₅₀ values through brine shrimp lethality testing provides foundational data for establishing safe dosage standards (Mishra *et al.*, 2022). Systematic analysis clarifies whether *P. conjugatum* exhibits minimal or significant toxicity relative to more studied medicinal plants.

Toxic characteristics observed in *P. conjugatum* extracts hold potential for controlled application as natural pest deterrents or antimicrobial agents (Torres *et al.*, 2023). Identifying active compounds allows exploration of environmentally friendly formulations for agricultural and hygienic use (Parra *et al.*, 2021). Determination of tolerable concentration limits prevents unintended harm to humans or beneficial organisms (Lopez *et al.*, 2020). Early-stage bioassay data form the basis for targeted studies on toxicity and efficacy correlations. Ongoing evaluation of *P. conjugatum* ensures safe integration into ethnomedicinal and eco-friendly product development.

Brine Shrimp Lethality Assay (BSLA)

Toxicity refers to the capacity of chemical compounds to induce harmful effects in living systems through mechanisms involving oxidative stress, membrane disruption, or interference with metabolic processes (Mahmood *et al.*, 2020). Classification typically includes acute, subacute, and chronic toxicity, defined by exposure duration and the extent of physiological damage (Rahman *et al.*, 2022). In natural product research, acute toxicity testing serves as a primary approach for identifying harmful potential and determining concentration-dependent effects (Ong & Prasad, 2020). The toxicity of medicinal plants is largely governed by the chemical nature and interaction of their secondary metabolites, which may trigger apoptosis or necrosis in biological tissues (Sivakumar *et al.*, 2023). Standardized toxicity profiling of medicinal plants remains essential to validate safety levels, regulate dosage, and ensure appropriate use within ethnobotanical and therapeutic contexts (Velasco *et al.*, 2024).

The brine shrimp lethality test (BSLT) provides a practical, ethical, and cost-efficient method for preliminary toxicity evaluation of plant extracts. This bioassay utilizes *Artemia salina* nauplii, which exhibit measurable mortality responses that can be statistically analyzed to determine LC₅₀ values (Mishra et al., 2022). Extracts demonstrating LC₅₀ values below 1,000 µg/mL are classified as toxic, reflecting significant biological activity (Torres et al., 2023; Parra et al., 2021). Validation studies have confirmed the correlation between BSLT outcomes and cytotoxicity data obtained from higher organisms, highlighting its reliability as a screening tool (Gupta et al., 2020). Applications of BSLT in evaluating extracts from *Annona muricata*, *Piper betle*, *Curcuma longa*, and *Paspalum conjugatum* demonstrate its capacity to detect dose-dependent toxicity trends while aligning experimental data with ethnomedicinal evidence (Nurhidayah et al., 2025; Velasco et al., 2024).

THEORETICAL FRAMEWORK

This study is anchored on the phytochemical theory, proposed by Rodríguez-Negrete and colleagues (2024), which states that plant-derived metabolites such as alkaloids, phenolics, flavonoids, and curcuminoids can influence biological systems by interacting with cellular components and biochemical pathways (Rodríguez-Negrete et al., 2024). Although this is not the original formulation of the theory, the 2024 discussion provides an updated interpretation relevant to current phytochemical research. Support for this principle is provided by the bioindicator concept, which emphasizes that living organisms function as sensitive models for detecting and quantifying toxic substances in environmental systems (Araya et al., 2024). While the concept has earlier origins, this recent study expands and modernizes its application in contemporary toxicity testing. In this study, *Artemia salina* is utilized as a bioindicator organism to detect toxicity levels of plant extracts based on mortality responses (Figure 1). These theoretical frameworks justify the use of the brine shrimp lethality test (BSLT) to evaluate and compare the effects of *Annona muricata* (guyabano), *Curcuma longa* (turmeric), *Piper betel* (betel leaf), and *Paspalum conjugatum* (carabao grass) ethanolic extracts.

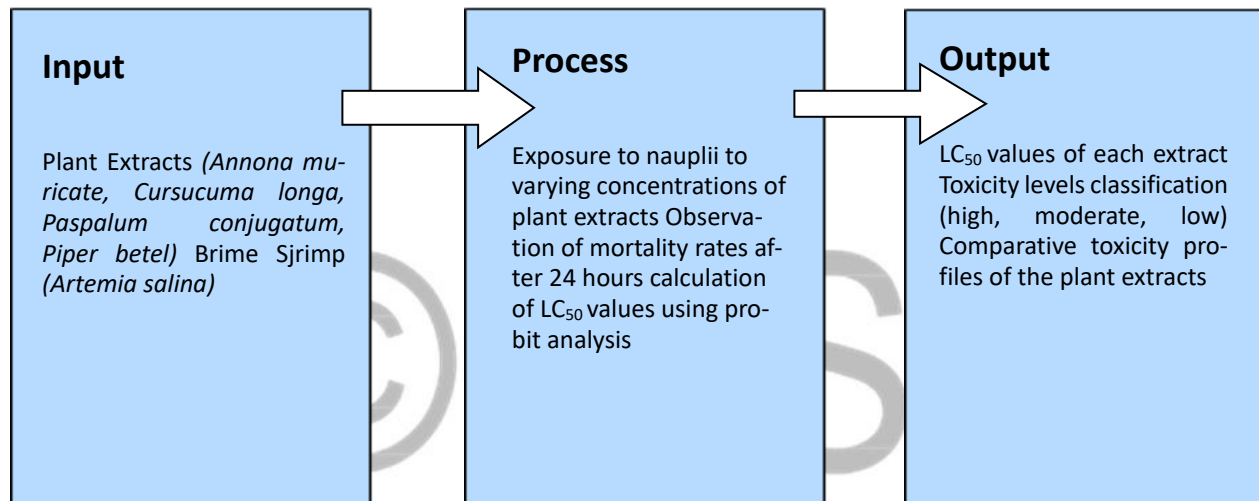


Figure 1. Conceptual framework of the input process output diagram of the study.

RESEARCH HYPOTHESES

Hypotheses

The following are the hypotheses tested in this study:

1. If the LC₅₀ values of *Annona muricata*, *Paspalum conjugatum*, *Piper betle*, and *Curcuma longa* extracts differ significantly, then this indicates variations in their respective toxicity levels;
2. If *Artemia salina* nauplii are exposed to individual plant extracts and their combinations, then the mortality rates will show significant variation depending on the type and concentration of the extract;
3. If the combination of ethanolic extracts from the selected plants is tested, then it will exhibit a different toxicity profile compared to the individual extracts under standardized laboratory conditions; and
4. If one of the plant extracts possesses stronger bioactive compounds, then it will demonstrate higher lethality against *Artemia salina* nauplii than the others.

MATERIALS AND METHODS

This science investigatory project consists of six phases: plant collection and extraction, hatching of *Artemia salina*, brine shrimp lethality assay, statistical analysis, and documentation.

Area of Study

The preparation of materials for the brine shrimp lethality assay was conducted at Purok Santan, Ursua, Visayan Village, Tagum City. Plant samples used for the study were collected from Davao City, Mawab, Maco, and Panabo City. The extraction of plant materials was performed at Davao del Norte State College (DNSC) to ensure standardized laboratory conditions and controlled processing for the preparation of extracts.

Phase 1: Plant Collection and Extraction

1.1 Plant Verification and Preparation. All plant samples were collected from natural habitats in selected study areas. Leaves of *Annona muricata* (guyabano) (Figure 2b), *Piper betle* (betel leaf) (Figure 2a), and *Paspalum conjugatum* (carabao grass) (Figure 2c) were hand-picked from healthy, mature plants and placed in clean sacks to prevent contamination. Rhizomes of *Curcuma longa* (turmeric) (Figure 2d) were manually up-rooted and stored in labeled cellophane bags; these samples were collected from Davao City, while *A. muricata* and *P. betle* were obtained from Panabo City, and *P. conjugatum* from Maco and Mawab. A botanist verified all species before processing. Samples were washed with distilled water, air-dried at room temperature, ground into fine powder using a mechanical grinder, and stored in clean, airtight containers until extraction.



Figure 2. (a) Betel leaf, (b) soursop leaf, (c) carabao grass, and (d) turmeric rhizome (Photos by A. Paloma, 2025; G. Rosalejos, 2025).

1.2 Ethanolic Extraction. The powdered plant material was mixed with ethanol in a 1:5 ratio, such as 100 grams of powder combined with 500 milliliters of ethanol (Figure 3a). The mixture was soaked for 48 hours with intermittent stirring, approximately every 3 hours, to enhance solvent penetration and compound extraction. After soaking, the mixture was strained to separate the liquid extract from the solid residues. The liquid filtrate was collected for further purification.

1.3 Filtration and Concentration of Extracts. The obtained filtrate was filtered through clean filter paper to remove remaining fine particles and achieve a clear solution. The clear filtrate was then concentrated using a rotary evaporator to remove all ethanol. This process was carried out at a controlled temperature to prevent degradation of heat-sensitive compounds. The resulting focused residue represented the crude ethanolic extract (Figure 3b). Each crude extract was labeled, stored in sterile containers, and refrigerated for subsequent toxicity testing.

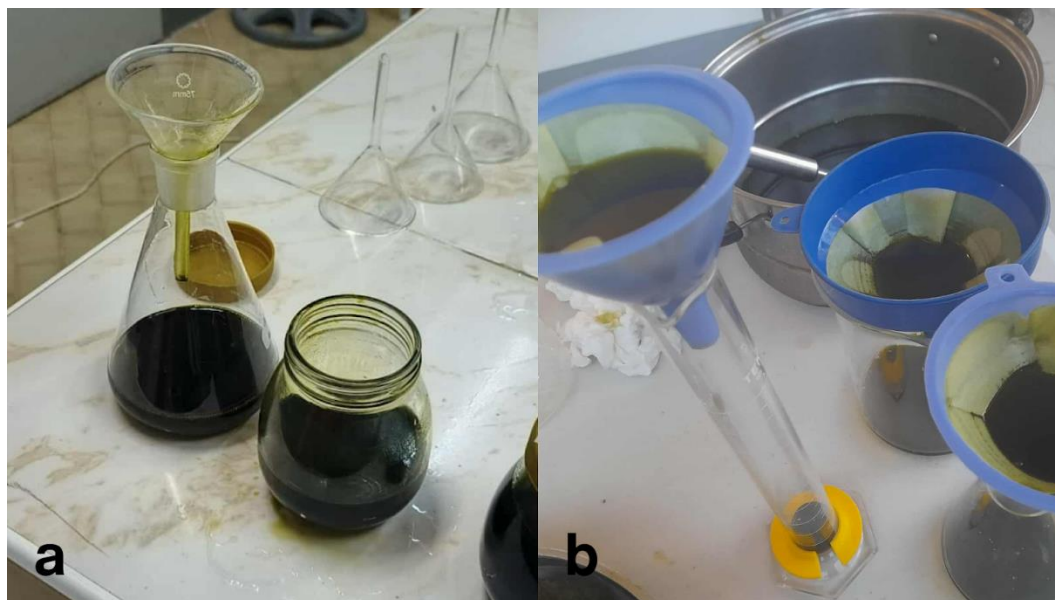


Figure 3. (a) Ethanolic extraction and filtration, and (b) concentration of extracts (Photos by A. Paloma, 2025; G. Rosalejos, 2025)

Phase 2: Hatching of *Artemia salina*

2.1 Gathering of Materials. *Artemia salina* (brine shrimp) eggs were purchased from Teo-Yan Fish House, located at Purok 2-A, Tagum City. Laboratory essentials such as droppers, Pasteur pipettes, syringes, and personal protective equipment (PPE) were obtained from local medical supply stores near the study site.

2.2 Incandescent Light Source. Materials needed for hatching, including electrical wire, an incandescent light bulb (60–100 W), and a bulb holder, were purchased and assembled at NCCC Hardware Maxx, Tagum City. The assembled setup served as the primary light and heat source for the hatching process.

2.3 Preparation of Hatching Setup. A rectangular glass jar was filled with 3 liters of water measured using a graduated cylinder. 27 g of table salt was weighed using an analytical balance and dissolved in the water using a glass rod to achieve the proper salinity for hatching (Figure 4a).

2.4 Illumination and Hatching Conditions. Approximately 15 g of *Artemia salina* eggs were added to the jar and evenly distributed across the surface. A 25 W incandescent bulb was placed a few inches away from the jar to maintain warmth and constant illumination (Figure 4b). The setup was allowed to stand for 20–24 hours, during which hatching occurred.

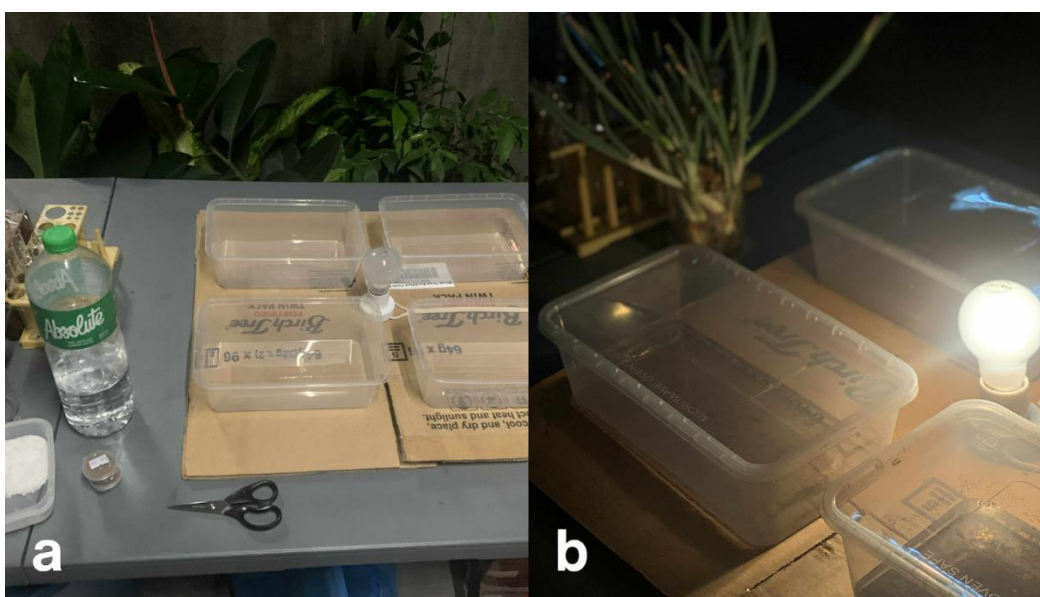


Figure 4. (a) Preparation of hatching setup, (b) illumination and hatching of *Artemia salina*

2.5 Separation and Collection of Nauplii. Once hatching was complete, the aeration and illumination were turned off. The empty shells floated to the surface while the hatched nauplii concentrated in the water column. Using a Pasteur pipette, 10 active nauplii were collected from the mid-layer of the solution and transferred into test tubes containing 5 mL of seawater.

Phase 3: Transfer of *Artemia salina* Nauplii

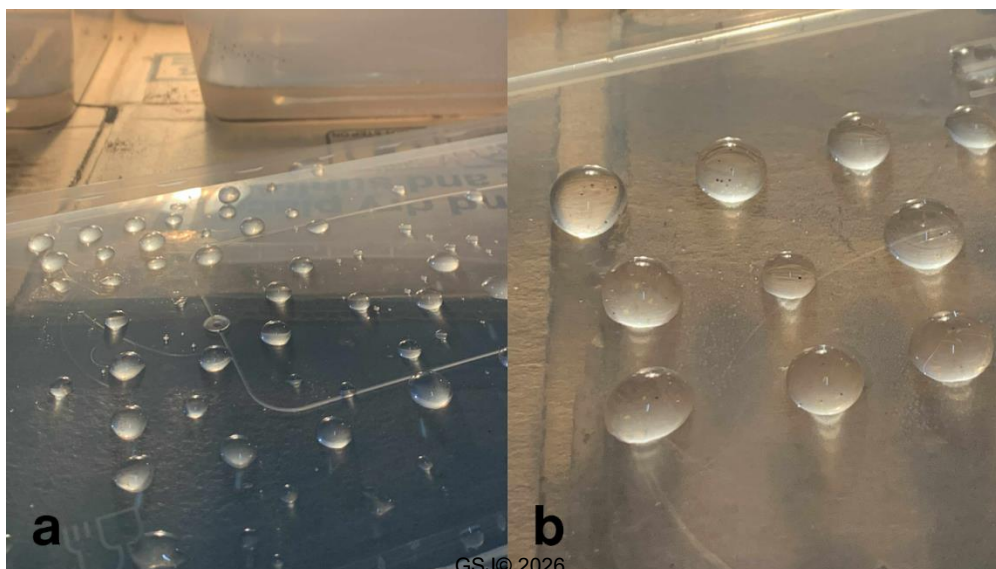


Figure 5. (a) selection of viable nauplii into droplets, (b) checking of *Artemia salina* in droplets

3.1 Selection of Viable Nauplii. Healthy nauplii showing continuous, directed swimming were selected under adequate lighting (Figure 5a).

3.2 Transfer Procedure. Nauplii were transferred using a sterile Pasteur pipette into labeled test tubes containing 5 mL of seawater. Care was taken to ensure uniform density and avoid debris inclusions (Figure 5b).

Phase 4: Toxicity Assessment

4.1 Preparation of Extract Concentrations. Stock extracts were diluted in a 3.5% (w/v) saltwater solution to obtain concentrations of 10 ppm, 100 ppm, and 1000 ppm. Each concentration was prepared in triplicate for all ethanolic extracts of *Annona muricata*, *Curcuma longa*, *Piper betle*, and *Paspalum conjugatum*, including the combined formulations. Each concentration was prepared in triplicate for all four plant extracts (*A. muricata*, *C. longa*, *P. betle*, and *P. conjugatum*).

4.2 Exposure to *Artemia salina*. Each test tube containing nauplii received the corresponding extract concentration. A control group containing only seawater served as a baseline for comparison. The tubes were maintained at room temperature.

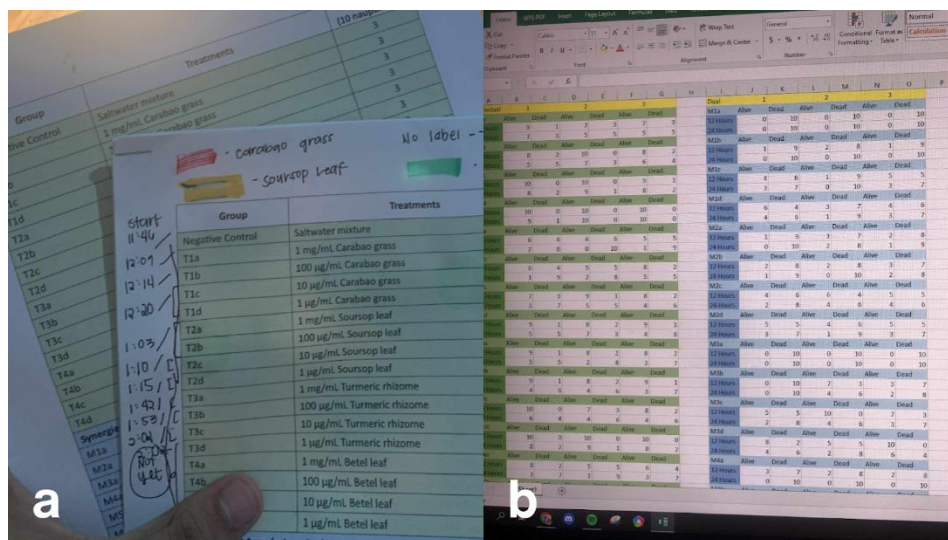


Figure 6. (a) Hard copy of observations, (b) mortality recording.

4.3 Observation and Mortality Recording. After 24 hours, mortality was recorded under a magnifying lens. Dead nauplii were identified by the absence of controlled forward motion for 30 seconds (Figure 6a). Surviving and dead nauplii were counted (Figure 6b), and the percentage of lethality was computed using the following formula:

$$\text{Mortality (\%)} = (\text{Number of dead nauplii} / \text{Total number of nauplii}) \times 100$$

Phase 5: Data Analysis

5.1 LC₅₀ Determination. The LC₅₀ (median lethal concentration) of each extract was determined using Probit analysis, which estimates the concentration that causes 50% mortality of *Artemia salina*. This was determined using Probit Analysis following Finney's method (1971):

$$\text{Probit (Y)} = a + b(\log C)$$

Y = Probit value of percentage mortality

C = Concentration of the extract (ppm)

a, b = Regression constants obtained from the probit model

5.2 Toxicity Classification. Toxicity levels of the extracts were classified based on the ppm presented (Table 1).

Table 1. Toxicity classification of plant extracts based on Meyer's criteria (1982).

LC ₅₀ Value (ppm)	Toxicity Interpretation
< 1000	Toxic

≥ 1000

Non-Toxic

5.3 Statistical Treatment. All treatments were conducted in triplicate, and the results were expressed as mean ± standard deviation (SD). The data were analyzed using probit analysis to generate the log mortality rate model and determine the LC₅₀ values at a significance level of $p < 0.05$.

5.4 Data Compilation. Results were tabulated and graphed using Microsoft Excel for comparative analysis.

Phase 6: Waste Disposal and Documentation

6.1 Waste Disposal. All apparatus, including test tubes, pipettes, droppers, and containers, were thoroughly washed and sterilized after use. Residual extracts and saline solutions were properly segregated and disposed of to prevent contamination and environmental harm.

6.2 Documentation. Photographic documentation and tabulated data were compiled to validate observations. The entire workflow, experimental setup, and data collection process are shown throughout the methods.

RESULTS

The results of this study present a comparative assessment of the toxicity levels of ethanolic extracts from *Annona muricata* (soursop) leaves, *Curcuma longa* (turmeric) rhizomes, *Piper betel* (betel leaf), and *Paspalum conjugatum* (carabao grass), evaluated individually and in combination through the brine shrimp lethality test (BSLT). Revealing various mortalities among ethanolic extracts of *Annona muricata*, *Curcuma longa*, *Piper betel*, and *Paspalum conjugatum* across concentrations and exposure periods. After 12 hours, *A. muricata* showed the highest mortality at 73.33% for 1000 ppm, followed by *P. betel* at 63.33%, *C. longa* at 53.33%, and *P. conjugatum* at 46.67%. After 24 hours, all extracts exhibited increased mortality, confirming concentration- and time-dependent toxicity. *A. muricata* reached the highest lethality at 86.67%, while *P. Conjugatum* remained the least toxic. The rise in mortality from 12 to 24 hours indicates progressive toxic buildup during exposure (Torres et al., 2023).

Table 2. LC₅₀ values of ethanolic plant extracts determined by brine shrimp lethality assay using probit analysis in triplicate.

Treatment	Concentration (ppm)	Log Concentration (ppm)	Total Nauplii	Dead Nauplii (Mean ± SD) (12h)	Mortality Rate (%) (12h)	Dead Nauplii (Mean ± SD) (24h)
Negative Control (Distilled Water)	0	0	10	0.33 ± 0.58	3.33	0.33 ± 0.58
Carabao Grass	1000	3	10	9.00 ± 1.00	90	9.67 ± 0.58
Soursop Leaf	1000	3	10	5.00 ± 1.00	50	9.00 ± 1.00
Turmeric Rhizome	1000	3	10	1.67 ± 1.53	16.67	5.33 ± 1.15
Betel Leaf	1000	3	10	3.67 ± 1.15	36.67	7.67 ± 1.15
Carabao Grass + Soursop Leaf	100	2	10	10.00 ± 0.00	100	10.00 ± 0.00
Carabao Grass + Turmeric Rhizome	100	2	10	9.00 ± 1.00	90	9.67 ± 0.58
Carabao Grass + Betel Leaf	100	2	10	8.67 ± 1.53	86.67	8.67 ± 1.53
Soursop Leaf + Turmeric Rhizome	100	2	10	8.33 ± 1.15	83.33	8.33 ± 1.15
Soursop Leaf + Betel Leaf	100	2	10	6.67 ± 1.53	66.67	8.00 ± 1.73
Turmeric Rhizome + Betel Leaf	100	2	10	5.33 ± 2.08	53.33	8.33 ± 1.53
Carabao Grass + Soursop Leaf + Turmeric Rhizome	100	2	10	8.00 ± 1.73	80	8.67 ± 1.15

Carabao Grass + Soursop Leaf + Betel Leaf	100	2	10	7.00 ± 1.00	70	9.67 ± 0.58
Soursop Leaf + Turmeric Rhizome + Betel Leaf	100	2	10	4.00 ± 1.00	40	10.00 ± 0.00
Carabao Grass + Soursop Leaf + Turmeric Rhizome + Betel Leaf	100	2	10	10.00 ± 0.00	100	10.00 ± 0.00

Treatment	Mortality Rate (%) (24h)	LC ₅₀ (ppm)	Probit Model	Concentration p-value
Negative Control (Distilled Water)	3.33	-	-	-
Carabao Grass	96.67	981.84	y = 0.508x - 1.521	0
Soursop Leaf	90	2.094	y = 0.431x - 0.138	0
Turmeric Rhizome	53.33	32.599	y = 0.427x - 0.646	0
Betel Leaf	76.67	12.955	y = 0.352x - 0.391	0.001
Carabao Grass + Soursop Leaf	100	0.228	y = 0.731x + 0.469	0.001
Carabao Grass + Turmeric Rhizome	96.67	0.006	y = 0.244x + 0.539	0.048
Carabao Grass + Betel Leaf	86.67	0.581	y = 0.507x + 0.120	0
Soursop Leaf + Turmeric Rhizome	83.33	1.055	y = 0.464x - 0.011	0
Soursop Leaf + Betel Leaf	80	2.539	y = 0.517x - 0.209	0
Turmeric Rhizome + Betel Leaf	83.33	1.856	y = 0.597x - 0.160	0
Carabao Grass + Soursop Leaf + Turmeric Rhizome	86.67	0.042	y = 0.545x + 0.749	0.006
Carabao Grass + Soursop Leaf + Betel Leaf	96.67	0.306	y = 0.303x + 0.156	0.01
Soursop Leaf + Turmeric Rhizome + Betel Leaf	100	0.426	y = 0.835x + 0.309	0
Carabao Grass + Soursop Leaf + Turmeric Rhizome + Betel Leaf	100	2.217	y = 0.836x - 0.289	0

The probit analysis (Table 2) reveals *A. muricata* with the lowest LC₅₀ value, confirming the strongest toxic activity among the extracts. *P. betle* followed closely, exhibiting moderate LC₅₀ values and consistent lethality patterns. *C. longa* displayed higher LC₅₀ readings, indicating weaker toxicity, while *P. conjugatum* demonstrated the highest LC₅₀, reflecting the least toxicity. Figure 7 illustrates these results, showing a steeper regression slope for *A. muricata* ($y = 0.6547x + 0.198$) and *P. betle* ($y = 0.6313x + 0.469$), representing a stronger mortality response to increased concentration. In contrast, gentler slopes in *C. longa* ($y = 0.5432x + 0.646$) and *P. conjugatum* ($y = 0.5180x + 1.521$) indicate lower toxicity levels. Figure 8 confirms that the combined extract of all four plants induced near-total mortality, suggesting synergistic effects at higher concentrations, while *P. conjugatum* maintained moderate values across all concentrations (Mishra *et al.*, 2022).

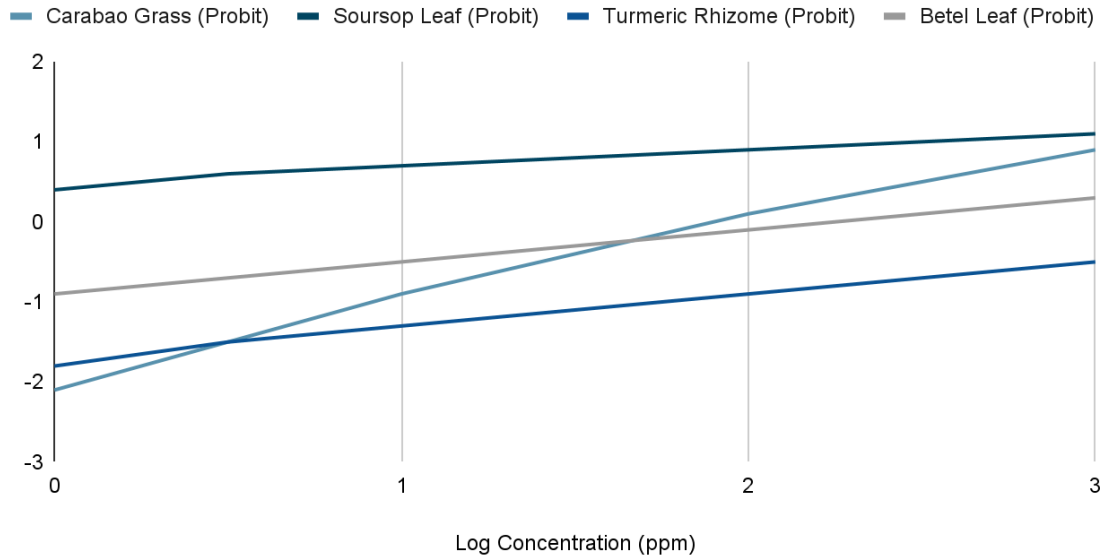


Figure 7. Scatter Plot of Probit (Mortality Response) to Log Concentration (ppm)

The probit regression relationship (Figure 7) between the mortality response of *Artemia salina* and the logarithmic concentration of the ethanolic extracts of carabao grass, soursop leaf, turmeric rhizome, and betel leaf. Each line represents a distinct plant extract, showing a positive correlation where higher log concentrations correspond to increased probit values. Among the treatments, carabao grass and betel leaf exhibited steeper slopes, indicating stronger toxicity effects at increasing concentrations, while turmeric rhizome displayed a more gradual trend, suggesting comparatively lower toxicity. This graphical relationship confirms that mortality generally increases in a dose-dependent manner across all extracts.

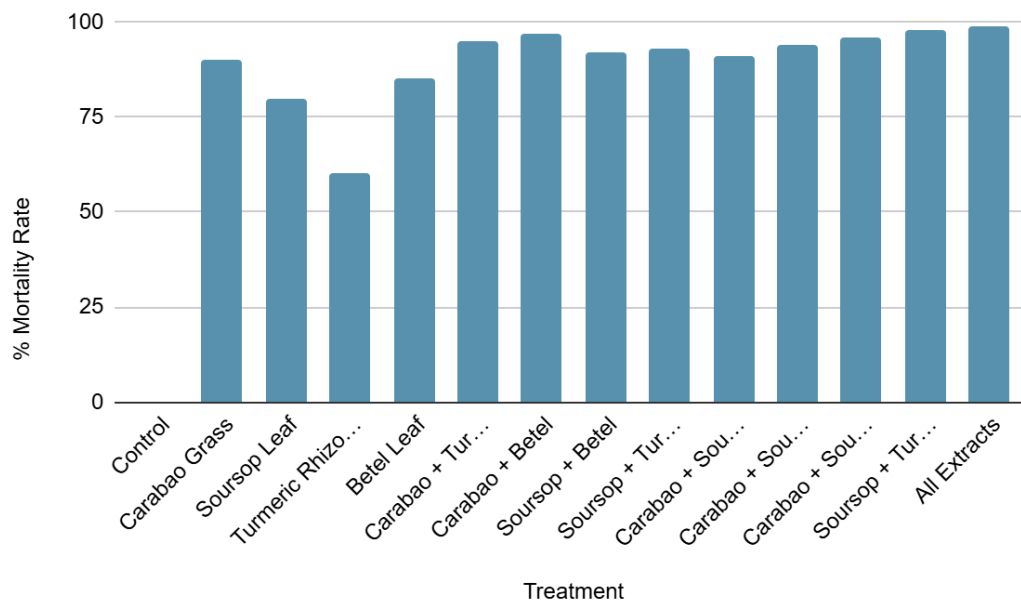


Figure 8. Mortality rate (%) of *Artemia salina* after 24 hours.

The percentage mortality of *Artemia salina* nauplii after 24 hours of exposure to individual and combined plant ethanolic extracts (Figure 8). The control group maintained minimal mortality, validating the reliability of the test. In contrast, all plant extracts and their combinations demonstrated elevated mortality levels, with mixed extracts producing notably higher toxicity than individual treatments. The “All Extracts” treatment recorded the highest mortality rate, approaching 100%, emphasizing the synergistic toxicity effect when multiple extracts are combined. Overall, the results highlight the varying potencies of each extract and reinforce their dose-dependent bioactivity.

Table 3. Individual Extract Toxicity Strength

Plant Extract	Regression Equation (Probit = y, Log Conc. = x)	Toxicity Strength
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Annona muricata (Soursop Leaf)	$y = 0.845x - 0.111$	Highest toxicity (lowest $LC_{50} = 2.094$ ppm)
Piper betle (Betel Leaf)	$y = 0.583x - 1.521$	Moderate toxicity
Curcuma longa (Turmeric Rhizome)	$y = 0.654x - 0.646$	Low–moderate toxicity
Paspalum conjugatum (Carabao Grass)	$y = 0.631x - 0.984$	Least toxicity (highest $LC_{50} = 981.84$ ppm)

Among the extract combinations, the mixture of *Paspalum conjugatum* (Carabao Grass) + *Curcuma longa* (Turmeric Rhizome) exhibited the greatest toxicity, with an LC_{50} of 0.006 ppm, indicating a highly potent lethality against *Artemia salina* nauplii (Table 3). The combination of *Paspalum conjugatum* (Carabao Grass) + *Piper betle* (Betel Leaf)* followed with an LC_{50} of 0.581 ppm, showing moderate toxicity, while *Annona muricata* (Soursop Leaf) + *Piper betle* (Betel Leaf)* had an LC_{50} of 2.539 ppm, suggesting comparatively lower toxicity among the combinations. All treatments displayed a clear dose-dependent effect, wherein increasing concentrations corresponded to higher mortality rates of *Artemia salina* nauplii.

DISCUSSION

Probit analysis identified distinct toxicity patterns among the ethanolic extracts of *Annona muricata*, *Curcuma longa*, *Piper betle*, and *Paspalum conjugatum*. The extract of *A. muricata* exhibited an LC_{50} of 2.094 ppm, classifying it as highly toxic due to a strong mortality response even at low concentrations. The pronounced toxicity of *A. muricata* can be attributed to annonaceous acetogenins and alkaloids such as annonacin, compounds known to inhibit mitochondrial complex I activity, disrupting ATP synthesis, and inducing energy depletion in *Artemia salina* nauplii (López-Rosas *et al.*, 2025). *C. longa* and *P. betle* demonstrated moderate toxicity, reflected in LC_{50} values between 324 and 329 ppm, a range typical of extracts containing curcuminoids, eugenol, and hydroxychavicol that can generate oxidative stress and membrane destabilization in aquatic bioassays (Ismail, 2025; Banti *et al.*, 2021). Meanwhile, *P. conjugatum* showed an LC_{50} of 981.84 ppm, indicating low toxicity, possibly due to lower concentrations of reactive phytochemicals or a predominance of milder compounds such as flavonoids and saponins that exert minimal impact on nauplii viability (Esparagoza *et al.*, 2020).

The death of *Artemia salina* nauplii across treatments was primarily influenced by the phytochemical composition of each extract and concentration-dependent exposure effects. Alkaloids, phenolics, and terpenoids in *A. muricata* and *P. betle* likely caused rapid disruption of respiratory and enzymatic functions, while curcuminoids in *C. longa* induced oxidative imbalance, collectively contributing to the observed mortality. Such toxic characteristics, when properly harnessed, hold medicinal value; high toxicity at controlled doses can be advantageous in therapeutic research, particularly in anticancer and antimicrobial applications where selective cell inhibition is desirable (Torres *et al.*, 2023). The presence of bioactive compounds with strong lethality suggests potential for developing novel pharmacological agents that target pathological cells while maintaining safety through dosage regulation. Hence, the observed toxic profiles not only serve as safety indicators but also provide foundational insight for identifying plant-based compounds with biomedical promise.

The combinations of extracts exhibited markedly lower LC_{50} values than individual extracts, indicating enhanced lethality and suggesting possible interactions among the compounds. The combination of *Paspalum conjugatum* and *Curcuma longa* produced the lowest LC_{50} (0.006 ppm), demonstrating a strong increase in toxicity compared to each extract alone. Similarly, *Paspalum conjugatum* + *Piper betle* (0.581 ppm) and *Annona muricata* + *Piper betle* (2.539 ppm) also showed notable toxicity. These findings suggest that interactions among plant compounds can significantly amplify lethal effects, likely through mechanisms such as enhanced membrane disruption, enzyme inhibition, or increased oxidative stress in the test organisms (Clemen-Pascual *et al.*, 2021).

A clear dose-dependent pattern appeared across all treatments, with mortality increasing as extract concentration rose. Probit analysis verified that lower LC_{50} values indicated stronger toxicity, and all extracts except *Paspalum conjugatum* demonstrated biological activity below 1000 ppm (Banti *et al.*, 2021). The enhanced toxicity in mixtures containing *P. conjugatum* suggests synergistic effects between its flavonoids, tannins, and saponins and the more active compounds of *Annona muricata* and *Piper betle*, such as acetogenins and phenolics (Esparagoza *et al.*, 2020). Comparable outcomes reported by Hossain *et al.* (2023) and Nguyen *et al.* (2022) revealed increased mortality in combinations of alkaloid- and phenolic-rich extracts. These interactions imply that *P. conjugatum* functions as a potentiating agent, intensifying toxicity in mixed formulations and emphasizing the need for regulated dosing in herbal use.

Probit and LC_{50} analyses revealed distinct variations in toxicity among individual and combined ethanolic extracts of *Annona muricata*, *Curcuma longa*, *Piper betle*, and *Paspalum conjugatum*. The combination of *P. conjugatum* and *C. longa* exhibited the highest toxicity ($LC_{50} = 0.006$ ppm), suggesting a strong synergistic interaction between curcuminoids and flavonoids. The *A. muricata* and *P. betle* mixture also demonstrated enhanced lethality, indicating additive effects due to the interaction of acetogenins and phenolic compounds. In contrast, combinations involving *P. conjugatum* with either *A. muricata* or *P. betle* produced moderate toxicity, implying possible antagonistic or neutral interactions that reduced overall activity. These results confirm that the phytochemical composition of each extract influences the degree of synergism or antagonism observed, emphasizing the need to evaluate combined formulations for potential pharmacological or toxicological applications.

Overall, the study identified significant differences in toxicity among the individual and combined ethanolic plant extracts. LC_{50} values determined through probit analysis provide a quantitative basis for classifying toxicity levels, showing that *Annona muricata* and its combinations were the most lethal, followed by *Curcuma longa* and *Piper betle*, while *Paspalum conjugatum* remained the least toxic. The enhanced toxicity observed in extract combinations highlights the importance of considering interactions among plant compounds, as these can either amplify or mitigate toxic effects. These results underscore the need for careful evaluation when using ethnomedicinal plant mixtures, particularly when their combined effects may increase lethality.

CONCLUSION

The study successfully determined and compared the LC₅₀ values of *Annona muricata*, *Curcuma longa*, *Piper betle*, and *Paspalum conjugatum* using the Brine Shrimp Lethality Test (BSLT), revealing significant variations in toxicity levels. Results showed *A. muricata* as the most toxic (LC₅₀ = 2.094 ppm) and *P. conjugatum* as the least toxic (LC₅₀ = 981.84 ppm), confirming significant differences among the extracts. Concentrations above 1000 ppm were identified as generally safe, while lower LC₅₀ values indicated bioactive or toxic effects. Combined ethanolic extracts exhibited greater lethality than individual ones, with the *P. conjugatum* and *C. longa* mixture showing the strongest synergistic effect (LC₅₀ = 0.006 ppm). Findings comprehensively addressed all objectives, validating the effectiveness of BSLT in assessing the safety and toxicity of ethnomedicinal plant extracts.

RECOMMENDATIONS

Considering the findings of this study, the following recommendations are suggested:

1. It is recommended that future brine shrimp lethality test (BSLT) experiments increase the number of replications to ensure more accurate and reliable results. The current setup of three replications per treatment may not fully represent the variability of *Artemia salina*.
2. Each treatment should be conducted for two consecutive days, with three replicates performed per day, resulting in a total of six replicates per treatment. This setup will provide a 48-hour observation period that minimizes random errors and enhances consistency.
3. The exposure time should be extended from 24 hours to 48 hours to observe potential delayed toxic effects and obtain a more comprehensive assessment of lethality.
4. Since brine shrimps are not entirely accurate indicators of toxicity in mammals due to physiological differences, it is recommended that future studies use mammalian-based assays to validate the results of the BSLT.
5. Phytochemical and chromatographic analyses, such as Gas Chromatography–Mass Spectrometry (GC-MS) or High-Performance Liquid Chromatography (HPLC), should be conducted to determine the active compounds responsible for the observed toxicity.
6. Further research should also consider assessing the environmental impact of the plant extracts, particularly if they are intended for potential biological or agricultural applications.

Conclusion

Although a conclusion may review the main points of the paper, do not replicate the abstract as the conclusion. A conclusion might elaborate on the importance of the work or suggest applications and extensions. Authors are strongly encouraged not to call out multiple figures or tables in the conclusion—these should be referenced in the body of the paper.

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