Comparative Toxicity Analysis Ethanol and Decoction Extracts of Curry Leaf (*Murraya koenigii*) Using Brine Shrimp Lethality Test

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ABSTRACT

Curry leaves (*Murraya koenigii*) are used in traditional medicine; however, their toxicity and anticancer properties remain largely unexplored. This study assessed the toxicity of ethanol and decoction extracts of curry leaves using a Brine Shrimp Lethality Test (BSLT). Curry leaves were collected, dried, and extracted with 96% ethanol using the decoction method. Artemia salina larvae were exposed to extract concentrations (62.5, 125, 250, 500, and 1000 ppm) for 24 h. The lethal concentration 50 (LC_{50}) values were determined by probit analysis. The ethanol extract showed toxicity with an LC_{50} of 263 ppm, whereas the decoction extract was non-toxic with an LC_{50} of 6,174 ppm. The ethanol extract had a higher mortality rate (93.3%) than the decoction extract (26.6%). The differential toxicity was due to the solvents and extraction techniques used. Ethanol extracts bioactive compounds, including toxic elements. The high temperature in the decoction process may break down heat-sensitive toxic compounds. This study concluded that the ethanol extract of curry leaves exhibited a more potent toxic effect than the decoction extract, warranting further research to identify toxic compounds with anticancer properties.

Keywords: toxicity analysis, brine shrimp lethality, curry leaves, decoction extract, ethanol extract

Received: April 26, 2025 **Revised:** June 13, 2025 **Accepted:** July 27, 2025

INTRODUCTION

The secondary metabolites in plants are responsible for their pharmacological and toxicological effects (Wang *et al.*, 2021). The level of toxicity in a plant is largely determined by its bioactive properties and administered dose. Additional studies are required to establish safety guidelines for herbal plant compositions by evaluating acute toxicity. This involves determining the LC_{50} values, identifying various toxic symptoms, and establishing both toxic and therapeutic dosages (Yaşar *et al.*, 2020).

Extraction techniques significantly influence the isolation of secondary metabolites from herbal plants (Sablania *et al.*, 2019). Although various methods exist, including maceration, infusion, steeping, and percolation (Bitwell *et al.*, 2023). Decoctions are frequently preferred because of their simplicity (Bommakanti *et al.*, 2023). The type of extraction method used affects the ability to isolate secondary metabolite compounds, consequently affecting the effectiveness and toxicity of the resulting extract (Abubakar and Haque, 2020).

The toxicity of herbal plants is largely attributed to their excessive use in the absence of proper supervision. Studies have indicated that the concentration of herbal compounds also plays a crucial role in determining their

toxicity (Mensah *et al.*, 2019; Firnanda *et al.*, 2021). The toxicity of herbal medicines depends on both the administered dose and toxic properties of the secondary metabolites (Wang *et al.*, 2021). These factors are equally important, and their combined effects influence therapeutic dosage in clinical applications (Anwar *et al.*, 2021). However, the toxicological properties of substances, including herbs, are dose-dependent. A non-toxic substance may become toxic if administered in sufficient quantities, whereas a highly toxic substance may be considered safe at low doses (McCarty *et al.*, 2020).

The Brine Shrimp Lethality Test (BSLT) is a simple technique to assess plant toxicity. This efficient and economical method can be used to evaluate bioactive substances or compounds with potential anticancer properties (Pitakpawasutthi *et al.*, 2021). The toxicity of a compound is determined by calculating the lethal concentration 50 (LC_{50}), which involves quantifying the mortality of *Artemia salina* larvae (Daniel *et al.*, 2023). BSLT methods indicate that an extract is classified as toxic when its LC_{50} value is below 1000 ppm (Meyer *et al.*, 1982). Additional analysis is necessary to identify secondary metabolites that may develop into anticancer medications. The LC_{50} value is a crucial indicator of the potential of a compound as an anticancer agent (Satya *et al.*, 2021).

Curry leaves (*Murraya koenigii*) are a herb belonging to the Rutaceae family. Native to India, Sri Lanka, and parts of Southeast Asia, such as Indonesia, it is known as "temurui leaf" in Acehnese. *Murraya koenigii* has several pharmacological effects. Numerous studies have reported the antidiabetic and antioxidant properties of curry leaves (Franyoto *et al.*, 2024; Kejariwal, 2020; Nandy and Das, 2023; Tabashiri *et al.*, 2022). Although *Murraya koenigii* is used in traditional medicinal practices, it may have toxic properties and potential risks to human health. The potential toxicity of *Murraya koenigii* may be indicative of its anticancer properties. Therefore, this study aimed to assess the toxicity of ethanol and decoction extracts of curry leaves by using the BSLT method.

METHODS

Samples collection and preparation

The materials used in this study were curry leaves (*Murraya koenigii*), seawater, 96% ethanol, distilled water, and *Artemia salina* larvae. Curry leaves were collected from the yard of a resident in Peukan Bada Village, Aceh Besar. The collected leaves exhibited a distinctive aroma, vibrant green hue, and reddish woody stems. After identifying the plant material as curry leaves, a 1 kg sample was collected. Subsequently, curry leaves were dehydrated at the Biomedical Laboratory of the Faculty of Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia. The drying process resulted in a final curry leaf sample weighing 200 g.

The animal material used in this study was *Artemia salina* eggs, a species of crustacean commonly known as brine shrimp. These eggs are readily available for purchase at pet stores where they are marketed as food for fish.

Production of curry leaf extract

Two hundred grams of dry powdered curry leaves (200 g) were extracted using the maceration method (Surbakti *et al.*, 2023). Then, 2000 ml of 96% ethanol was added until the simplicia was submerged. The mixture was kept in a closed vessel for 5 days, protected from direct sunlight, and occasionally stirred. After five days, it was filtered and the sediment and liquid were separated. The residue from the

maceration process was re-macerated using 2000 ml of 96% ethanol until the liquid was colorless. The extract was collected and evaporated using a rotating vacuum evaporator to obtain a concentrated extract with a sticky texture and blackish green color.

The decoction extract of the curry leaves was obtained using the heat extraction method. The extraction process was initiated by cleaning the samples to remove contaminants. Subsequently, the samples were air-dried to eliminate excess moisture from the cleaning step and maintain the integrity of the secondary metabolites. The dried sample was then sliced into smaller pieces, mashed using a blender, and placed in a pot with an adequate volume of fresh water (approximately 1 liter per 100 g of leaves). This mixture was brought to a boil over high heat, the heat was reduced to the medium, and continuing to boil the sample for 15-30 min. The pot was removed from the heat source and allowed to cool. The resulting liquid was then strained through a fine mesh sieve or muslin cloth. The final step involved transferring the filtered extract to a sterile container and storing it in a refrigerated or cool environment.

A total of 10 mg of curry leaf decoction extract and curry leaf ethanol extract were dissolved in 10 ml of seawater to obtain a stock solution concentration of 1000 ppm. Then, 5 mg of each extract was dissolved in 10 ml of seawater to obtain a 500 ppm solution. The same procedure was carried out for a 250 ppm concentration, dissolving 5 mg of each extract in 20 ml of seawater.

To produce a stock solution of 125 ppm, 25 mg of curry leaf decoction extract and ethanol extract were dissolved in 20 ml of seawater. For 62.5 ppm, 12.5 mg of curry leaf decoction extract and ethanol extract were dissolved in 20 ml of seawater. Then 0.1 ml of 1% dimethyl sulfoxide (DMSO), and seawater was added until the volume reached 10 ml. The test solution concentrations for BSLT were 62.5, 125, 250, 500, and 1000 ppm.

DMSO 1% was used because it has no toxic effect on Artemia salina and increases the solubility of the extract. The control group used only seawater to ensure that *Artemia salina* larval death was caused by chemical components in the plant extracts and not seawater.

Artemia salina **larvae hatching**

Seawater (1 liter) was introduced into the incubator, which was equipped with an aerator to elevate oxygen concentration. Subsequently, *Artemia salina* eggs (2.5 mg) were added to the incubator. The hatchery was divided into two zones: a dark area containing eggs and aerators, and a light area near light sources. This arrangement provided the necessary lighting and enabled cyst separation. An electric light source was positioned on one side of the incubator to increase the temperature in the hatching area and to facilitate the hatching process. The incubator walls were covered with aluminum foil to create a light-proof environment and the lamp was illuminated for 48 h to induce egg hatching. After hatching, the larvae naturally migrated towards the light area, and actively motile larvae were selected for use as experimental animals in this study. Larvae suitable for use as test organisms were required to meet two primary criteria: exhibiting both health and positive phototaxis and having attained an age exceeding 48 h. Morphologically, *Artemia salina* larvae at 48 h of age develop a functional mouth and digestive tract capable of ingesting specific particles (Surbakti *et al.*, 2023). *Artemia salina* larvae selected for the toxicity test were subsequently extracted using a drop pipette.

Brine shrimp lethality test (BSLT)

The BSLT method was performed using several well plates, each containing 24 wells. The wells were divided into five groups for each concentration, and one group of seawater was used as the negative control. The test included three repetitions (triplicate), with each concentration assigned to three wells.

Ten larvae were placed into each well with 1 ml of the test solution at 62.5, 125, 250, 500, and 1000 ppm, and 2 ml of seawater was used as the negative control. Each well was then mixed with 2 ml of seawater until the volume in 1 well was 3 ml (1 ml of test solution and 2 ml of seawater). *Artemia salina* shrimp larvae were then transferred to Petri dishes to simplify calculations. The larval transfer was performed using a drop pipette. The sign of a dead larva appeared immobile within a few seconds of observation. The plate was left in open air for 24 h.

Data analysis

Probit analysis was used to determine LC_{50} values (Meyer *et al.*, 1982). This value represents the concentration of the solution that resulted in 50% larval mortality. Manual analysis was used, wherein the percentage of mortality at each concentration was converted to a probit value using a probit table. The following equation was used to calculate the percentage larval mortality:

% Mortality = (Number of dead larvae)/ (Number of tested larvae) x 100%

Linear regression analysis was performed using Microsoft Excel by making a graph to obtain the linear equation: Y = mX + b.

RESULT AND DISCUSSION

The aim of this study was to evaluate the toxicity of ethanol and decoction extracts of curry leaves against *Artemia salina* larvae using the BSLT method and to determine the LC_{50} value, which is the concentration of extracts that can cause 50% death of test animals 24 h after exposure to various concentrations of solutions. The results of the toxicity test of the decoction and ethanol extracts of curry leaves using the BSLT method and the Miller Tainter Probit calculation method are shown in Table 1.

The principle of the BSLT method is that toxic compounds cause death of the tested animal (Meyer *et al.*, 1982). *Artemia salina* larvae are a beneficial model for in vivo studies, in contrast to alternative methods that are costly, time-intensive, and require specialized qualifications and facilities (Libralato *et al.*, 2016). The sensitivity of these larvae to a broad spectrum of substances, ranging from heavy metals to pharmaceutical agents, further enhances their value as model organisms in research (Lu and Yu, 2019). The toxicity assessment used *Artemia salina* nauplii at 48 h post-hatching. At this stage, the larvae exhibited peak sensitivity, with fully developed oral and digestive systems as well as sufficient resilience. These characteristics make 48-hour-old larvae ideal subjects for toxicity testing (Rasyid *et al.*, 2022).

The toxicity of herbal plants is largely attributed to their excessive use in the absence of proper supervision. Studies have indicated that the concentrations of herbal compounds play a crucial role in determining their toxic potential (Mensah *et al.*, 2019). The toxicity of herbal medicines depends on both the administered dose and the toxic properties of the secondary metabolites (Wang *et al.*, 2021). However, the toxicological properties of substances, including herbs, are dose-dependent. A

non-toxic substance may become toxic if administered in sufficient quantities, whereas a highly toxic substance may be considered safe at low doses (McCarty *et al.*, 2020).

The logarithm of the concentration against the probit value obtained from the percentage of larval mortality against the curry leaf extract is shown in Figure 1. Figure 1A shows that for the curry leaf decoction extract, a linear regression equation was produced: Y = 0.9739x + 1.3083. Based on the calculations, the curry leaf decoction extract had an LC_{50} value of 6,174 ppm, indicating that larval death reached 50% at this concentration. Figure 1B shows the linear regression curve for curry leaf ethanol extract concentration, with the equation Y = 2.3461x − 0.6774. The LC_{50} of the curry leaf ethanol extract was 263 ppm, indicating 50% death of the test animal at this concentration.

Table 1. The toxicity of curry leaf extract using the BSLT method

Extract	Concentration (ppm)	Total larvae	Mortality* (%)	Probit Value	LC_{50} (ppm)
Decoction Extract	1000	30	26.6	4.2710	6.174
	500	30	16.6	3.8877	
	250	30	13.3	3.7241	
	125	30	6.6	3.1616	
	62.5	30	6.6	3.1616	
Ethanol Extract	1000	30	93.3	6.2816	263
	500	30	86.6	5.9661	
	250	30	43.3	4.7467	
	125	30	20.0	4.0299	
	62.5	30	13.3	3.7184	

*average of triplicate.

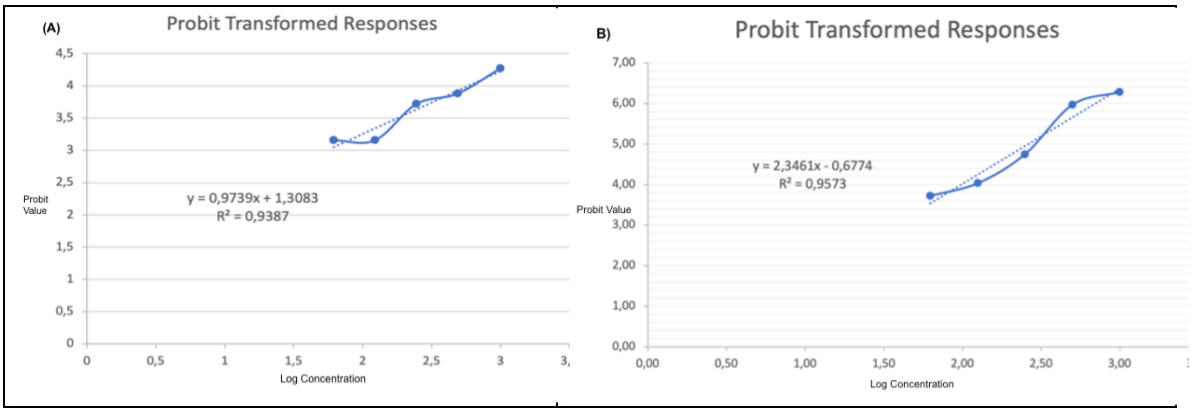

Figure 1. Probit of mortality of each concentration of curry leaf. A) decoction extract; B) curry leaf ethanol extract.

LC_{50} indicates the concentration resulting in 50% mortality of brine shrimp larvae. An inverse relationship exists between LC_{50} values and extract toxicity levels. According to Meyer, a compound is toxic and has potential as an anticancer agent in the BSLT assay if the LC_{50} value is <1000 ppm, whereas LC_{50} values above this threshold are considered non-toxic (Meyer *et al.*, 1982). In the present study, the curry leaf ethanol extract showed a level of

toxicity, with an LC_{50} value of 263 ppm. The LC_{50} value of the curry leaf decoction extract was 6174 ppm that which is non-toxic.

The differential toxicity observed between ethanol extracts and decoctions can be attributed to the different solvents and extraction techniques used. Ethanol, an organic solvent, is more effective at extracting a wide range of bioactive compounds, including polar, semi-polar, and non-polar substances, and potentially which may include toxic elements. In contrast, aqueous decoctions predominantly extract polar compounds, limiting their efficacy in isolating other bioactive constituents from curry leaves while facilitating the elimination of toxic and less water-soluble components. Moreover, the high temperatures used in the decoction process may break down the heat-sensitive toxic compounds, resulting in lower concentrations of the final extract. The decoction of plant materials in water typically leads to the decomposition or volatilization of certain toxic compounds, particularly those that are volatile or thermally unstable. This makes decoction extracts less toxic than ethanol-based extracts, which are often prepared at lower temperatures to preserve the full range of active compounds (Fauziah *et al.*, 2022; Plaskova and Mlcek, 2023).

Previous studies have established that solvent polarity affects not only the quantity and quality of crude extracts but also the secondary metabolites produced and their associated biological activities (Hanafi *et al.*, 2020; Konan *et al.*, 2022). This phenomenon may account for the fact that similar plant family compounds exhibit different levels of toxicity across different extracts. Additionally, the mean mortality rate demonstrated a concentration-dependent relationship, with the highest and lowest rates observed at 1000 and 250 ppm, respectively (Konan *et al.*, 2022).

CONCLUSION

Our findings revealed that curry leaf ethanol extracts had a higher mortality rate (93.3 %) than the decoction extract (26.6%). Therefore, we concluded that the ethanol extract of curry leaves exhibited a more potent toxic effect than the decoction extract. Further research is needed to identify and isolate potentially toxic compounds from curry leaves that could be used as anticancer drug candidates.

Acknowledgment

This work was supported by the Directorate General of Higher Education, which is part of Indonesia's Ministry of Education, Culture, Research, and Technology. (No. 393/ UN11.2.1/ PG/ 01.03/ SPK/ PTNBH/ 2024; Dated 3 May 2024).

Author Contribution

FH: Conceptualization, Methodology, Supervision, Funding, Writing- Original draft preparation, Writing- Reviewing and Editing; ZZ.: Investigation, Validation, Writing- Original draft preparation; GN: Data curation, Visualization, Writing- Original draft preparation.

Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Approval

The animals (brine shrimp and Artemia salina) used in this study were commercially sourced; thus, ethical approval was not required.

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