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Research Article

Comparative Toxicity and LC_{50} Determination of *Phaleria macrocarpa* Leaf Extracts from Sequential Maceration

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Abstract

Phaleria macrocarpa is a plant renowned for its bioactive compounds, including flavonoids, alkaloids, saponins, and tannins, which are believed to have various pharmacological properties. This study aimed to determine the lethal concentration 50 (LC_{50}) of *P. macrocarpa* leaf extracts obtained by sequential maceration in *n*-hexane, ethyl acetate, and 70% ethanol. The toxicity of the extracts was evaluated using the Brine Shrimp Lethality Test (BSLT) against *Artemia salina* leach larvae, a commonly used model organism in toxicity testing due to its sensitivity to various toxic substances. The results indicated that the 70% ethanol extract exhibited moderate toxicity with an LC_{50} of 126.981 ppm, the ethyl acetate extract was categorized as weakly toxic (LC_{50} of 691.730 ppm), and the *n*-hexane extract was classified as non-toxic (LC_{50} of 1416.537 ppm). These findings indicate that solvent polarity influences the extraction of bioactive compounds and their biological activity. This study highlights the novelty of sequential maceration as a selective extraction approach that enhances toxicity profiling and supports preliminary pharmacological screening for natural product-based drug development. Furthermore, the study underscores the importance of using selective extraction methods to isolate bioactive compounds based on their polarity. This study provides preliminary data on the toxicity profile of *P. macrocarpa* leaves and highlights the potential of its bioactive compounds for further pharmacological research, particularly in the development of natural-based therapeutic agents. Future studies should focus on isolating and identifying the active compounds responsible for the observed toxicity and on their potential for use in drug development.

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INTRODUCTION

The utilization of natural bioactive constituents as alternative therapeutic agents continues to gain significant attention due to their favorable safety profiles and minimal adverse effects when administered appropriately. Among medicinal flora, *Phaleria macrocarpa* exhibits substantial pharmacological potential[1]. While various anatomical parts of the plant possess therapeutic value, the leaves represent the most frequently utilized organ[2]. Leaves of *P. macrocarpa* contain diverse phytochemicals, including saponins, alkaloids, tannins, and flavonoids, which collectively drive its recognized antioxidant and antineoplastic activities. Flavonoids, as dominant secondary metabolites in *P. macrocarpa* foliage, play a pivotal role in mitigating various pathological conditions, including malignancy, diabetes mellitus, hypertension, cardiovascular disease, infectious processes, and metabolic disorders[3-5].

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Although previous toxicological investigations on *P. macrocarpa* primarily relied on conventional single-solvent maceration, single-solvent systems often fail to efficiently partition constituents possessing distinct polarity gradients. To overcome this limitation, the present study employs sequential multistage maceration using solvents of increasing polarity: *n*-hexane (non-polar), ethyl acetate (semi-polar), and 70% ethanol (polar)[6]. While requiring larger solvent volumes, sequential fractionation significantly enhances extraction yield, improves constituent selectivity, and enables detailed characterization of the biological activity inherent to each polarity-specific fraction. The resulting fractionated extracts were evaluated for toxicity against *Artemia salina* leach using the Brine Shrimp Lethality Test (BSLT). BSLT serves as a rapid, reliable, and cost-effective preliminary bioassay for screening cytotoxic, pharmacological, and potential anticancer properties of plant-derived compounds[7-9]. Consequently, this study aims to determine the LC_{50} values of *P. macrocarpa* leaf fractions obtained via sequential maceration to evaluate their viability for downstream pharmaceutical development.

MATERIALS AND METHODS

Materials

The fresh plant material comprised mature leaves of *P. macrocarpa* harvested from the Cilodong plantation area in Depok City, Indonesia. The collected leaves were visually characterized by an elongated lanceolate shape, dark green pigmentation, and a distinct aromatic odor. Taxonomic identity and botanical authentication of the specimen were formally confirmed at PT. Palapa Muda Perkasa, Depok, as documented in Determination Certificate No. 996/IPH.1.01/If.02/I/2025. Biological materials and analytical-grade chemical reagents utilized throughout the extraction, phytochemical screening, and bioassay procedures included *A. salina* leach cysts, marine fish salt, distilled water (aquadest), *n*-hexane (C_6H_{14}), ethyl acetate ($C_4H_8O_2$), 70% ethanol (C_2H_5OH), hydrochloric acid (HCl), and iron (III) chloride ($FeCl_3$), alongside prepared diagnostic testing solutions consisting of Dragendorff's, Mayer, Bouchard at, and Liebermann–Burchard reagents. Experimental workflows and sample processing were conducted using standard laboratory glassware and specialized equipment, including an analytical balance (LabPro-DT224C®), a mechanical grinder (ML500®), a 40-mesh sieve (ABM®), a laboratory drying oven (Memmert UN55®), a high-temperature furnace (Saftherm STM-18-12®), a rotary vacuum evaporator (IKA RV-10 Digital®), variable-volume micropipettes (Scilogex®), a dual-outlet aquarium aerator (Amara AA-999®), a specialized hatching aquarium fitted with a harvesting mesh screen, magnification lenses, extraction containers, evaporating dishes, and personal protective equipment including nitrile gloves and face masks.

Methods

Sample preparation

A 3 kg batch of fresh *P. macrocarpa* leaves were collected and manually sorted to eliminate attached stems, withered tissue, and organic debris. The selected leaves were thoroughly washed under cold running tap water to remove surface particulates, then drained and weighed to determine the initial fresh mass. The cleaned leaves were dried in a forced-air oven maintained at 45–50°C until they acquired a brownish-green coloration and became brittle upon tactile compression, indicating complete desiccation. Following drying, dry sorting was performed to remove any extraneous foreign matter or degraded leaves. The dried leaf material was pulverized using an electric laboratory grinder and then passed through a No. 40-mesh sieve to ensure a uniform particle-size distribution[10]. The resulting fine crude powder was weighed to establish the dry starting mass prior to solvent extraction.

Plant extraction

A 100 g portion of processed *P. macrocarpa* leaf powder underwent a multistage maceration process. In the first extraction stage, the powder was submerged in 1000 mL of *n*-hexane at a 1:10 (w/v) ratio in a light-resistant amber glass container. Maceration was conducted at ambient room temperature (25–30°C) for 24 hours with periodic manual agitation and shaking. The mixture was filtered through analytical filter paper to yield Filtrate I and Residue I. Residue I was subsequently re-extracted under identical volumetric, thermal, and temporal parameters using ethyl acetate to yield Filtrate II and Residue II. Finally, Residue II was extracted using the same procedure with 70% ethanol, yielding Filtrate III and Residue III. The three filtrates were individually concentrated under reduced pressure using a rotary vacuum evaporator at 30–40°C with

controlled rotation speeds to minimize thermal degradation of bioactive constituents. The concentrated extracts were further evaporated over a temperature-controlled water bath until viscous, constant-mass crude extracts were obtained.

Animal preparation

Prior to toxicity evaluations, *A. salina* leach cysts were hatched to obtain *A. salina* nauplii for bioassays[11]. Artificial seawater was prepared by dissolving commercial marine fish salt in distilled water to simulate natural oceanic salinity. Encysted eggs were introduced into the prepared saline solution within a specialized hatching aquarium under continuous aeration to maintain oxygenation and fluid circulation[12,13]. The environment was maintained at 28–30°C away from direct sunlight. Following a 24–48-hour incubation period, active nauplii were harvested by temporarily turning off aeration for approximately 15 minutes, allowing empty floating eggshells to separate from phototactic larvae that settled at the bottom of the vessel. All animal housing, handling, and experimental protocols were conducted in strict accordance with institutional ethical guidelines and approved by the Pakuan University Research Ethics Commission under Approval Certificate No. 47/KEPHP-UNPAK/10-2024.

Preparation of extract concentrations

The viscous extracts derived from 70% ethanol, ethyl acetate, and *n*-hexane were each evaluated for toxicity at target concentrations of 10 ppm, 100 ppm, and 1000 ppm, with all test groups performed in triplicate. A primary stock solution of 10,000 ppm was prepared by dissolving 1000 mg of crude extract in distilled water within a 100 mL volumetric flask and adjusting to volume. Serial working concentrations were prepared from the stock solution using standard volumetric dilution calculations, with the formula $V_1 \times C_1 = V_2 \times C_2$. The diluted extract solutions were immediately utilized in the larval toxicity assays.

Brine shrimp lethality test

Toxicological evaluations of the *P. macrocarpa* leaf extracts were conducted using the BSLT with active *A. salina* leach nauplii. Experimental setups comprised 30 individual test vials filled with 9 mL of artificial seawater. The test matrix included nine vials for the ethanol extract across three concentration levels (10, 100, and 1000 ppm in triplicate), nine vials for the ethyl acetate extract across the same concentration levels in triplicate, nine vials for the *n*-hexane extract across the same concentration levels in triplicate, and three negative control vials containing artificial seawater devoid of plant extract. Each test vial received a minor volume of yeast suspension as a nutritional source. Ten active nauplii were transferred into each vial using a fine Pasteur pipette. A 1 mL aliquot of the designated extract concentration was added to each vial to reach a final volume of 10 mL, and the test units were incubated for 24 hours under continuous artificial light[14]. Following incubation, larval mortality was quantified using an illuminated magnifying glass against a contrasting dark background. Larvae displaying complete immobility for a minimum observation period of 10 seconds were recorded as dead. Recorded mortality counts were converted to percentage mortality data to compute the median lethal concentration (LC_{50}) values for each solvent fraction.

Data analysis

Larval mortality data obtained from the BSLT were subjected to probit analysis to determine LC_{50} for each *P. macrocarpa* extract fraction. The probit modeling approach involves transforming larval mortality percentages into empirical probit units[15] and log-transforming to extract exposure concentrations[16]. Probit regression analysis was performed using IBM SPSS Statistics, incorporating log-transformed concentration metrics and 95% confidence intervals to assess statistical significance and toxicity limits. The concentration corresponding to a 50% lethal response (probit 5.00) was designated as the LC_{50} value, serving as the quantitative metric for comparative toxicity assessment across the non-polar, semi-polar, and polar fractions.

RESULTS AND DISCUSSION

The BSLT provides a rapid, reliable, and effective preliminary bioassay for evaluating the acute toxicity of botanical extracts toward living organisms. *Artemia salina* leach larvae serve as ideal model organisms owing to their compact size, low

maintenance requirements, and heightened sensitivity to toxic phytochemicals. In this investigation, *P. macrocarpa* leaf extracts were processed using systematic extraction workflows to evaluate their toxicological potential as a preliminary indicator for downstream antineoplastic drug discovery[17].

Extracts were evaluated across three polarity fractions (*n*-hexane, ethyl acetate, and 70% ethanol) at concentrations of 10, 100, and 1000 ppm. To facilitate complete dissolution of non-polar (*n*-hexane) and semi-polar (ethyl acetate) fractions in aqueous test media, 2% dimethyl sulfoxide (DMSO) was added dropwise (~1 mL)[18]. Although DMSO exhibits cytotoxicity at concentrations exceeding 7.5%, the minimal 2% concentration used here is non-toxic and does not cause background mortality in *A. salina* larvae[19,20].

Negative control treatments represent a vital baseline in toxicological investigations, ensuring that observed larval mortality is attributable solely to the active *P. macrocarpa* fraction rather than external vehicles or additives. Controls were established using 9 mL of artificial seawater and 1 mL of solvent vehicle without plant extract. This setup confirmed that neither the saline medium nor the nutrient vehicle contributed to larval baseline mortality. A standardized yeast suspension (0.6 mg/mL; 1 drop per vial) was supplied to all test units as a uniform dietary source and carrier medium[21-23]. Although *A. salina* nauplii sustain nutritional autonomy for up to 48 hours via yolk sac reserves[21], providing a controlled yeast supply helps maintain metabolic equilibrium during the 24-hour incubation period under artificial illumination[24].

Following the 24-hour exposure period, larval mortality was quantified across all treatment combinations (triplicate trials) to permit statistical evaluation. The mortality response of *A. salina* nauplii subjected to varying concentrations of *P. macrocarpa* leaf fractions is illustrated in **Figure 1**. LC_{50} values were computed via probit regression analysis using IBM SPSS Statistics at a 95% confidence level, transforming percentage mortality into empirical probits and exposure doses into logarithms[16]. Toxicological responses were evaluated using a dose-response model, in which lower LC_{50} values indicate greater biological potency.

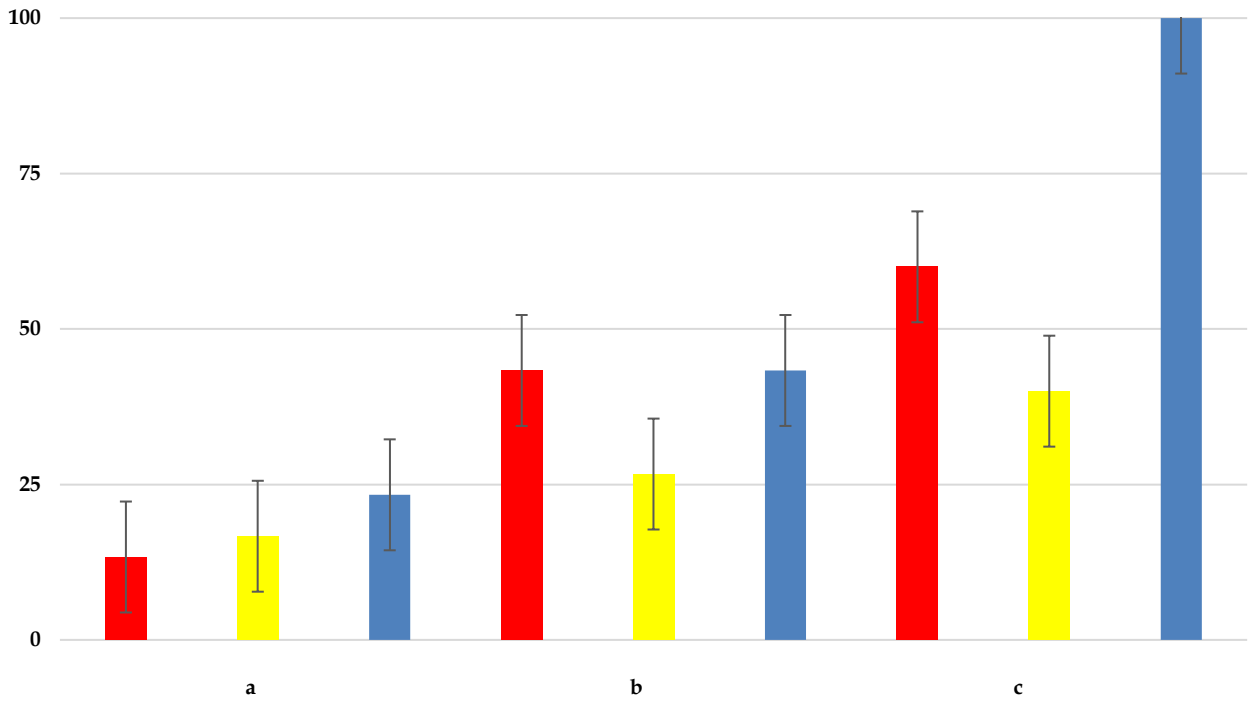

Figure 1. Larval mortality percentage across multistage extract solvents and concentrations. Note: **Yellow**: ethyl acetate; **Red**: *n*-hexane; **Blue**: ethanol. (**a**) 10 ppm, (**b**) 100 ppm, and (**c**) 1000 ppm

Prior to probit evaluation, percentage mortality data were subjected to a one-way Analysis of Variance (ANOVA) to assess homogeneity of variance and treatment effects. The ANOVA yielded a statistically significant difference among treatment groups ($p < 0.001$), rejecting the null hypothesis (H_0) at $\alpha = 0.05$[25]. Post hoc multiple comparisons were conducted using Duncan's Multiple Range Test to identify significant differences among solvent types and concentration levels, as detailed in **Table I**.

Table I. Mean mortality rate of *A. salina* leach larvae exposed to *P. macrocarpa* leaf fractions.

Treatment Fraction	Concentration (ppm)	Replication I (Dead/10)	Replication II (Dead/10)	Replication III (Dead/10)	Mean Mortality (%)
Ethyl acetate	10	1	1	2	13.33[a]
n-hexane	10	2	2	1	13.33[a]
Ethanol 70%	10	2	3	2	23.33[a,b]
Ethyl acetate	100	3	5	5	43.33[c,d]
n-hexane	100	2	3	3	26.67[a,b,c]
Ethanol 70%	100	4	4	5	43.33[c,d]
Ethyl acetate	1000	8	4	6	60.00[d]
n-hexane	1000	4	3	5	40.00[b,c]
Ethanol 70%	1000	10	10	10	100.00[e]

Note: Values sharing identical superscript letters within the mean mortality column do not differ significantly based on Duncan's Multiple Range Test ($p < 0.05$).

Duncan's post-hoc analysis confirmed a clear concentration-dependent increase in larval mortality across all solvent fractions. In the ethyl acetate fraction, mortality increased from 13.33% at 10 ppm to 43.33% at 100 ppm and 60.00% at 1000 ppm, demonstrating heightened structural disruption of larval tissue at elevated doses[26]. The *n*-hexane fraction exhibited lower mortality rates (13.33% at 10 ppm, 26.67% at 100 ppm, and 40.00% at 1000 ppm), reflecting reduced interactions between nonpolar, lipophilic constituents and larval physiological systems. Conversely, the 70% ethanol fraction exhibited the highest cytotoxic efficacy, increasing from 23.33% mortality at 10 ppm to 43.33% at 100 ppm, and reaching 100.00% mortality at 1000 ppm. The overall LC_{50} values derived from probit regression and their corresponding toxicity classifications based on McLaughlin's toxicity criteria[27] are presented in **Table II**.

Table II. Mean mortality rate of *A. salina* leach larvae exposed to *P. macrocarpa* leaf fractions.

Treatment Fraction	LC_{50} (ppm)	Toxicity Category[27]
n-hexane	1416.537	Non-Toxic
Ethyl acetate	691.730	Weakly Toxic
Ethanol 70%	126.981	Moderately Toxic

Based on standard toxicological criteria established by McLaughlin[27], crude botanical extracts exhibiting LC_{50} values below 1000 ppm are categorized as biologically toxic, whereas values exceeding 1000 ppm are classified as non-toxic. The *n*-hexane fraction yielded an LC_{50} of 1416.537 ppm, placing it in the non-toxic category due to the low biological activity of its non-polar constituents against *A. salina* nauplii. The semi-polar ethyl acetate fraction exhibited an LC_{50} of 691.730 ppm, indicating weak toxicity. This activity is attributed to semi-polar phytochemicals such as flavonoids and saponins, the latter of which possess surfactant properties that can disrupt membrane integrity and larval gut physiology.

The 70% ethanol fraction exhibited the lowest LC_{50} value at 126.981 ppm, placing it within the moderately toxic category. The pronounced toxicity of this polar fraction is driven by concentrated polar secondary metabolites, including polyphenols, tannins, and flavonoids. Flavonoids, in particular, disrupt cellular respiration, inhibit gustatory chemoreceptors, and impair digestive enzymes in *A. salina* larvae, leading to starvation and death[28].

These findings demonstrate that the bioactivity of *P. macrocarpa* leaf extract is heavily governed by solvent polarity and exposure concentration. The moderate toxicity of the polar ethanol fraction (LC_{50} = 126.981 ppm) aligns with established literature reporting LC_{50} values for *P. macrocarpa* between 100 and 800 ppm, underscoring its potential as a natural source for isolating bioactive anticancer compounds[29]. Overall, the BSLT bioassay validates the extraction sequence, offering a clear baseline for subsequent phytochemical purification, active constituent isolation, and downstream pharmacological development.

CONCLUSION

In summary, sequential leaf extracts of *P. macrocarpa* demonstrated distinct, solvent-dependent cytotoxic profiles against *A. salina* leach larvae, yielding LC_{50} values of 1416.537 ppm for the *n*-hexane fraction (non-toxic), 691.730 ppm for the ethyl acetate fraction (weakly toxic), and 126.981 ppm for the 70% ethanol fraction (moderately toxic). These outcomes confirm that the toxicological potential of *P. macrocarpa* foliage is strongly dictated by solvent polarity, highlighting the strategic advantage of sequential multistage maceration in effectively concentrating bioactive phytochemicals. Overall, the

pronounced cytotoxicity of the polar ethanol fraction supports its suitability for preliminary anticancer screening and broader phytopharmaceutical development. Nevertheless, as this investigation is constrained to preliminary bioassay screening, further research should focus on bioassay-guided isolation, structural elucidation of active constituents, and comprehensive *in vitro* and *in vivo* toxicological validations.

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AUTHORS' CONTRIBUTION

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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