Isolation and Identification Endophytic Fungi from Red Betel Leaf *(Piper crocatum)* and Its Active Compound Activity as Antioxidant

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Abstract. Previous studies have indicated that red betel leaf and endophytic fungal extracts have the ability to treat various diseases. This study aimed to investigate the antioxidant compounds of endophytic fungi isolated from red betel leaves (*Piper crocatum*). Crude extracts from these fungi were analyzed for phytochemical content, cytotoxic activity, and antioxidant capacity. The ethyl acetate crude extract (EtOAc) from endophytes fermented in Potato Dextrose Broth (PDB) medium had the highest total phenolic content (TPC) and total flavonoid content (TFC) at 55.38±2.70 mg GAE/g and 14.54±1.02 mg QE/g, respectively, with an antioxidant DPPH activity of 84.68± 2.74 µmol TE/g. Comparatively, fresh leaves yielded TPC, TFC, and antioxidant activity values of 24.71±3.15 mg GAE/g, 4.39±0.88 mg QE/g, and 27.07±2.30 µmol TE/g, respectively. This demonstrated significant differences in TFC, TPC, and antioxidant assay DPPH activities between the endophytic fungal extracts and fresh leaves. The characterization results of endophytic fungi were *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp.. This study confirmed the potential toxicity activity of isolate 1, which produced high toxicity with LC_{50} value 24.83 ppm on brine shrimp larvae. Conclusively, isolate 1 (*Aspergillus* sp.) was identified as a potential candidate for antioxidants and cytotoxic compounds for the early development of anticancer agents.

1 Introduction

Indonesia is globally recognized for its remarkable biodiversity, ranking second in terrestrial biodiversity after Brazil. The country's high level of biodiversity demands effective conservation and management strategies, ensuring that at least 30% of both land and marine ecosystems are effectively preserved. In many developing countries, people still turn to nature for healthcare practices—about 60–80% of the population depends on medicinal plants as a vital part of their everyday healthcare.

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Despite Indonesia's vast biodiversity, only approximately 7,500 plant species are utilized by local communities, and fewer than 30 species are commercially developed as raw materials for herbal medicine. The World Health Organization (WHO) states that herbal medicines include herbal plants, herbal materials, herbal preparations, and finished herbal products containing active plant parts. This broad definition encompasses a diverse range of plant-based remedies used in traditional and modern medicine. Herbal medicines play a crucial role in healthcare, particularly in regions where access to conventional pharmaceuticals is limited.

One of many herbal plants that could be cultivated for herbal medicines applicant is red betel leaf (*Piper crocatum*). Extensive research has validated the therapeutic potential of red betel leaves in the management of various diseases. Extensive research has confirmed that this traditional herbal remedy holds significant promise in disease management. These findings encourage further exploration of their pharmacological benefits. Research has demonstrated that the therapeutic efficacy of red betel leaves can be attributed to their rich bioactive compound content. The leaves of red betel are closely linked to their high concentration of phytochemicals. The phytochemical concentration of Red betel contains flavonoids, tannins, phenols, saponins, and alkaloids. These bioactive compounds impart various pharmacological properties to red betel. It exhibits tyrosinase inhibitory activity, antioxidant properties, antibacterial effects, and capacity to reduce blood glucose levels. Additionally, red betel possesses highly toxic anticancer potential.

Considering the numerous medicinal benefits derived from red betel leaves, there is a necessity for increasing the cultivation of red betel leaves. One effective method for faster and larger-scale cultivation involves utilizing endophytic microorganisms from the red betel plant itself. Red betel also serves as an excellent host for endophytic microorganisms. Endophytic fungi can produce bioactive compounds and secondary metabolites similar to those of their host plants. This phenomenon is believed to occur due to genetic transfer from the host to the endophytic fungi over time. The ability of endophytic fungi to generate bioactive compounds presents a significant opportunity for the development of herbal medicines. Endophytic fungi are microorganisms that are easy to cultivate, possess short life cycles, and can produce large quantities of bioactive compounds via fermentation.

Previous research on the efficacy of plants has largely been attributed to the presence of phytochemicals. These phytochemicals are linked to antioxidant and cytotoxic activities. Previous studies have indicated that red betel leaf extract has the ability to manage various diseases. The potential use of bioactive compounds from red betel requires extensive research to explore alternative sources of bioactive compounds. However, there is a lack of research specifically examining endophytic fungi from red betel leaves and their ability to produce phytochemicals. This study aimed to evaluate the phytochemical compounds and effectiveness of red betel leaf extract as an antioxidant for endophytic fungi. Given the substantial bioactive potential of red betel, researchers are keen to explore better alternative sources for producing bioactive compounds by isolating and identifying endophytic fungi, analyzing the secondary metabolite bioactive compounds, and evaluating the cytotoxicity of the compounds extracted from the endophytic fungi of red betel leaves.

2 Materials and methods

The materials and methods used in the study of red betel leaves and the analysis of their active compounds as antioxidants present the materials, instrumentation, and procedural frameworks implemented during the experiment, focusing on making the work easy to replicate based on methodological standards.

2.1 Isolation of endophytic fungi from red betel leaves

Endophytic fungi have been isolated from the sixth and seventh leaves of red betel. The isolation process was carried out in laminar air flow (LAF) using direct isolation techniques involving sample washing and surface sterilization. Red betel leaves were washed with distilled water for approximately 10 min, followed by a series of sterilization steps: 70% ethanol for 1 min, 5.25% NaOCl for 5 min, and 70% ethanol for 30 s. The leaves were then rinsed with sterile distilled water three times. After sterilization, the middle part of the leaves was cut and placed on Potato Dextrose Agar (PDA) medium supplemented with 200 µg of chloramphenicol. The samples were incubated at room temperature (30°C) and observed for fungal growth for approximately 7 days. The resulting mycelium from the samples was purified and cultured under sterile conditions within the LAF [1].

2.2 Purification and Identification of endophytic fungal isolates

Endophytic fungi grown on PDA isolation media are gradually purified individually on PDA without antibacterial agents. Each endophytic fungal isolate was carefully collected from the surface of the medium using an inoculation loop and transferred to sterile PDA medium for further growth. This process was repeated every two weeks to rejuvenate the fungi. Endophytic fungal isolates were identified through both macroscopic and microscopic observations. Macroscopic observations included examination of colony color, surface texture (granular, powdery, raised, or smooth), growth area, radial lines, and concentric rings. Microscopic observation was conducted using a microscope with 100x magnification. Slide culture methods used for microscopic identification. Identification involves examining hyphal structure (septate or aseptate), hyphal pigmentation, spore stalk shape, and the shape and ornamentation of asexual (generative) spores [2].

2.3 Fermentation and extraction of active compounds from endophytic fungi

Endophytic fungi were cultured in Potato Dextrose Broth (PDB) by placing four samples of actively growing culture into a 250 ml Erlenmeyer flask containing 100 ml of media. The flasks were incubated on a shaker at 120 rpm for three weeks (21 days) at 27±1°C [1]. Fungal fermentation was extracted three times using an equal volume of ethyl acetate (100 mL culture filtrate mixed with 100 mL ethyl acetate). The upper layer (ethyl acetate-containing metabolites) was collected and evaporated using a rotary vacuum evaporator at 40°C. The cell mass was separated and weighed to determine the mycelium weight. The resulting compounds were dried and concentrated to obtain the crude extract. The crude extract was dissolved in ethanol for antioxidant bioassay testing [3].

2.4 Total phenolic content determination

To measure the total phenolic content of endophytic fungi from red betel leaves, a 96-well microplate method was used. The Folin-Ciocalteu method was used to determine the phenolic content. The reagents used were 10% Folin-Ciocalteu and 10% Na_2CO_3 solutions. For the phenolic content test, 10 µL of the endophytic fungal extract from red betel leaves was added to a 96-well microplate, followed by 160 µL of distilled water and 10 µL of 10% Folin-Ciocalteu reagent. The mixture was incubated for 5 minutes at 30°C. Subsequently, 20 µL 10% Na_2CO_3 was added. The mixture was incubated for an additional 30 minutes at room temperature, and absorbance was measured at 750 nm using a nano spectrophotometer. Standard gallic acid curve concentrations of 25, 50, 75, 100, 125, 150, and 200 ppm were

used for quantification. The total phenolic content was calculated as gallic acid equivalents [4].

2.5 Total flavonoid content determination

Modifications were made to determine total flavonoid content. Quercetin was used as the standard [5]. Quercetin (10 mg) was dissolved in 10 mL of ethanol to create a 1000 mg/L solution. This solution was diluted to obtain concentrations of 10, 50, 100, 150, 200, 250, 300, and 350 mg/L. The mixture was incubated for 30 minutes at room temperature, and absorbance was measured at 417 nm using a microplate reader (nano spectrophotometer).
For flavonoid content determination, 10 µL of the extract was mixed with 120 µL of distilled water in a 96-well microplate. Subsequently, 10 µL of aluminum chloride ($AlCl_3$), 10 µL of glacial acetic acid, and 50 µL of ethanol were added to each well. The samples were incubated at room temperature in the dark for 30 minutes, followed by absorbance measurement at 415 nm using a microplate reader. The flavonoid content was expressed as milligrams of quercetin equivalent per gram of dry weight (mg QE/g dry weight) [6].

2.6 DPPH antioxidant activity assay

Antioxidant activity was assessed using the DPPH assay. In this procedure, 100 µL of the sample was pipetted into a microplate, followed by the addition of 100 µL of DPPH reagent, which was pre-incubated at 37°C. The mixture was incubated for 30 minutes, after which its absorbance was measured at 517 nm using a nano-spectrophotometer (SPECTROstarNano BMG LABTECH). Trolox was used as the standard at concentrations of 10, 30, 50, 70, and 90 µM. The Trolox equivalent (TE) in µmol/L was calculated using the equation y = ax + b, derived from the standard curve. The final results are expressed as µmol TE/gram of dry weight [7]

$$C = \frac{\mu mol - \frac{TE}{L} \times V}{m} \times FP \quad (1)$$

Explanation: C = Antioxidant activity (µmol TE/g of dry weight), V = Sample volume (L), m = Sample mass (g), FP = Dilution factor

2.7 Toxicity test using brine shrimp larvae

For the toxicity test, 10 brine shrimp larvae (*Artemia salina*) were placed in 1 mL of seawater in each well of a test plate. The control group consisted of seawater with 10 brine shrimp larvae without any test solution. Subsequently, 1 mL of the test solution was added to each well of a test plate. The plate was left undisturbed for 24 hours, after which the number of live and dead larvae was counted. The mortality of *A. salina* larvae was analyzed using Probit Minitab 19 for Windows [8].

3 Results and discussion

The results of this study on endophytic fungi from red betel leaves and the analysis of their active compounds as antioxidants included isolation and macroscopic and microscopic identification of the fungi, measurement of phenolic and flavonoid compound levels, evaluation of antioxidant activity using the DPPH assay, and assessment of their cytotoxic effects on *Artemia salina* shrimp larvae.

3.1 Characterization of Isolate 1

Isolate 1 grew rapidly. Initially, the colony was black with a white edge but quickly turned dark brown with age. The colony was circular with a distinctive black center. The texture was powdery and granular. The reverse side of the isolate was initially white, transitioning to brownish-white and potentially developing radial cracks on the agar. Microscopic observation revealed hyaline hyphae, conidiophores, phialides, and conidia; the hyphae were aseptate and lacked nuclei, and the conidia were round [9] (**Table 1**). The conidiophores originated from the basal foot cells on the substrate hyphae and ended at the vesicle, typical of the *Aspergillus* genus. Phialides were specialized for producing conidia and were dichotomously branched (biseriate). Phialides attached to vesicles via metulae cells. The round conidia were situated above the hyaline. Based on these microscopic characteristics, the pure isolate was identified as belonging to the genus *Aspergillus*, consistent with findings that *Aspergillus* was isolated from the stems of red betel leaves.

3.2 Characterization of Isolate 2

Isolate 2 showed rapid growth. Initially, the colony was light green with a white edge, but quickly turned completely green as it aged. The colony was small and circular, with a velvety surface and granular texture. The underside of the isolated plate was pale yellow, potentially developing radial cracks on the agar. Microscopic examination revealed hyaline hyphae, conidiophores, phialides, and conidia in isolate 2 (**Table 1**). The conidiophores originated from basal foot cells on the substrate hyphae and ended in vesicles. Isolate 2 had hyaline septate hyphae, complex conidiophores, and thin-walled conidia, which aligned with the typical characteristics of *Penicillium* sp.. The colony has conidiophores fully surrounding the conidial head. The phialides are usually shaped like a flask, consisting of several cylindrical bases with a distinct neck. Conidiophore stipes support a branched row of phialides at the top. Stipes are short and thin-walled. Based on morphological identification, this is consistent with findings from the Mycology Online at the University of Adelaide. The conidia form long chains, are round, green, shaped like a flask, consist of several cylindrical bases with a distinct neck, and are characteristic of *Penicillium* sp.

3.3 Characterization of Isolate 3

Isolate 3 colonies were white, but quickly turned orange with the production of conidia. The colony was circular with a filamentous or cotton-like texture. The reverse side of the isolate was pale orange, becoming dark orange as the optimal growth phase ended (Figure 9). Microscopic observations suggested that this fungus belongs to the genus *Fusarium*. This genus has distinct crescent-shaped macroconidia, which typically have septa and can develop with or without microconidia. *Fusarium* under a microscope was identified by septate hyaline hyphae, asexual pseudo-hyphae, intricate conidiophores, terminal vesicles, and long. *Fusarium* is a major plant pathogen that requires high water activity for growth. Hyaline hyphae with septate pseudo-hypha and complex conidiophore structures were observed (**Table 1**), and the characterizations shown are consistent with *Fusarium* species.

Table 1. Macroscopic and microscopic appearance of colonies from Isolates 1, 2, and 3 on PDA medium

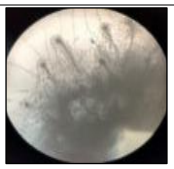
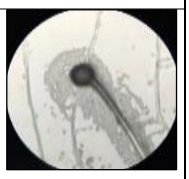
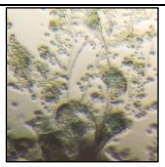
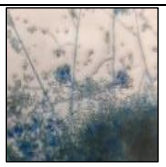
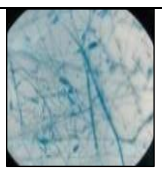
Isolate name	Front Surface	Back Surface	40x magnification	100x Magnification
Isolate 1				
Isolate 2				
Isolate 3				

Table 2. Macroscopic and microscopic characteristics exhibited by endophytic fungi of red betel leaf plants

Isolate Name		Isolate 1	Isolate 2	Isolate 3
	Surface Texture	Powdery and granular	Powdery and granular with velvet surface	Velvety and cottonlike
Macroscopic Characterization	**Front Surface Color**	The outer layer appears white, with a darker central that transitions into a blackish-brown coloration over time	Green with a white-edged colony, which then gradually becomes entirely green.	Initially white, then gradually turning cream-colored
	Back Surface Color	White with a slight cream colored and	Yellow colored with radial fissures presents	Orange colored

		radial fissures formed on the agar surface	on the agar surface	
Microscopic Characterization	**Hyphae**	Aseptate and hyaline	Septate and hyaline	Septate and hyaline
	Conidia	Round or globular	Round or globular	Crescent or hooklike-shaped
	Conidiophore	Thick walls with elongated individual stipes	Simple and branched	Branched with thin walls
	Phialides	Biseriate	Shaped like a flask, consisting of several cylindrical bases with a distinct neck	Cylindrical and individual in shape
Isolate Genus		*Aspergillus* sp.	*Penicillium* sp.	*Fusarium* sp.
Literature		[9]	(https://mycology.adelaide.edu.au)	[10].

3.4 Phenolic content measurement in endophytic fungi

The phenolic content of endophytic fungi from red betel leaves was measured using gallic acid as the standard. Folin-Ciocalteu reagent was used for the measurement. Using a nano spectrophotometer, the standard curve equation was determined to be $y = 0.004x - 0.0275$, with an R^2 value of 0.9945. The results are expressed in mg GAE/g. Based on variance analysis ($p<0.05$) and Tukey's post-hoc test (95%), the phenolic content measurements revealed significant differences between isolates 1, 2, and 3 and the red betel leaves. The findings indicated that isolate 1 exhibited the highest phenolic content at 55.38±2.70 mg GAE/g, while isolate 3 had the lowest content at 12.63±1.98 mg GAE/g, as shown in **Figure 1**.

A significant difference in phenolic content between endophytic fungal isolates and red betel leaves suggests that endophytic fungi have the potential for higher production of phenolic compounds compared to red betel leaves themselves. Endophytic fungi can serve as alternative and more efficient sources of bioactive compounds. Furthermore, the variation in phenolic content among fungal isolates highlights the differences in the metabolic capabilities of the isolates in producing phenolic compounds. Phenolic compounds in endophytic fungi play a role in defense mechanisms against pathogens and environmental stress.

Environmental variables, such as temperature, pH, humidity, and nutrient availability, play a crucial role in the growth and metabolism of endophytic fungi. Optimal conditions can

enhance the production of phenolic compounds. Genetic analysis can aid in identifying the genes involved in the biosynthesis of phenolic compounds in endophytic fungi. Endophytic fungi that reside within plant tissues often have symbiotic relationships with their hosts.

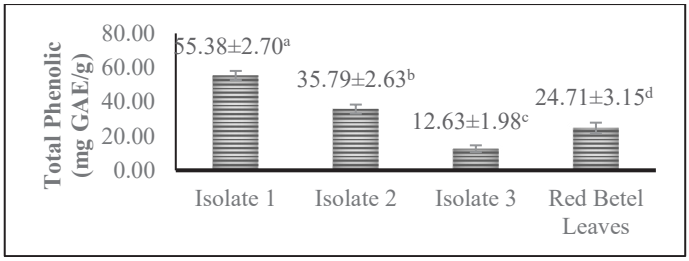


Fig 1. Total phenolic content of endophytic fungi from red betel plants. Numbers followed by the same letter indicate no significant difference based on Tukey's test (α=0.05).

3.5 Flavonoid content measurement in endophytic fungi

The flavonoid content of extracts from endophytic fungi isolated from red betel leaves was measured using quercetin as the standard and an aluminum chloride reagent. Using a nano spectrophotometer, the standard curve equation was determined to be $y = 0.0019x + 0.0023$, with an R^2 value of 0.9915. The results are expressed in mg QE/g. Based on variance analysis ($p<0.05$) and Tukey's post-hoc test (95%), the flavonoid content measurements revealed significant differences among isolates 1, 2, and 3, whereas no significant difference was observed between isolate 3 and red betel leaves. Isolate 1 exhibited the highest flavonoid content at 15.46±1.08 mg QE/g, while isolate 3 had the lowest content at 4.30±1.60 mg QE/g, as shown in **Figure 2.**

The difference observed in isolate 3, which exhibited the lowest flavonoid content, indicates a limited capacity for flavonoid synthesis or accumulation. This limitation is influenced by factors such as microenvironmental conditions and genetic variation [11]. In contrast, the high phenolic and flavonoid contents in isolate 1 suggest potential antioxidant activity. Endophytic fungi isolated from plants contain high levels of phenolic and flavonoid compounds and have significant potential for antioxidant activity.

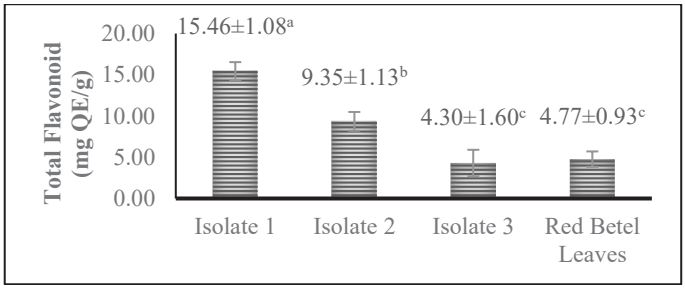


Fig 2. Total flavonoid content of endophytic fungi from red betel plants. Numbers followed by the same letter indicate no significant difference based on Tukey's test (α=0.05).

3.6 Comparative antioxidant activity of endophytic fungi and red betel leaf extracts: a DPPH assay study

The antioxidant activity of the ethyl acetate extract from endophytic fungi of red betel leaves was measured using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay method. Trolox was used as the standard for measuring DPPH antioxidant capacity. Based on variance

analysis ($p<0.05$) and Tukey's post-hoc test (95%), significant differences between each isolate and red betel leaves were confirmed. Isolate 1 demonstrated the highest antioxidant capacity, with a value of 84.68 ± 2.74 µmol TE/g, while red betel leaves showed the lowest value at 27.07 ± 2.30 µmol TE/g. The value of 84.68 ± 2.74 µmol TE/g for Isolate 1 indicates that this isolate possesses relatively high antioxidant capacity compared to the Trolox standard, as illustrated in **Figure 3.**

These values indicate that isolate 1 possesses a strong capability to neutralize free radicals, equivalent to 84.68 ± 2.74 µmol Trolox per gram of sample. Conversely, the red betel leaf extract had a value of 27.07 ± 2.30 µmol TE/g, which was significantly lower. This suggests that red betel leaves have a weaker antioxidant capacity than Isolate 1. The researchers stated that the antioxidant activity in endophytic fungal extracts of isolates 1, 2, and 3 is due to the presence of secondary metabolites such as phenolics, flavonoids, alkaloids, terpenoids, polyketides, phenylpropanoids, lignin, saponins, and phenols. The lower antioxidant capacity of red betel leaves than that of isolate 3 may be attributed to the higher concentration of other bioactive compounds in isolate 3, which were not measured in this study. Variations in compounds can be caused by various biotic and abiotic factors, as noted in some studies [11].

The high antioxidant capacity of isolate 1 due to endophytic fungi has great potential as a sustainable source of new bioactive compounds with medicinal properties. Many of the compounds found in these fungi are also present in plants, and they often show biological effects similar to those derived directly from plant sources.

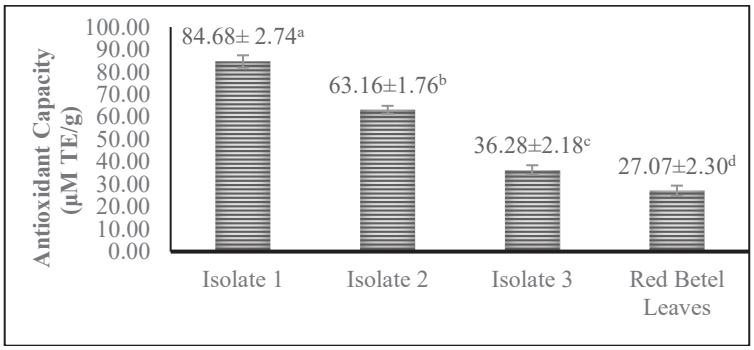


Fig 3. DPPH antioxidant capacity of endophytic fungi isolated from red betel plants. Numbers followed by the same letter indicate no significant difference based on Tukey's test (α=0.05).

3.7 Cytotoxicity of ethyl acetate extracts from endophytic fungi from red betel leaves on shrimp larvae

The toxicity of extracts is expressed by LC_{50} (Lethality Concentration), which refers to the concentration of the tested extract capable of killing 50% of the population after a 24-hour incubation period. The lower the LC_{50} value, the higher the toxicity of the extract or compound. Testing was conducted across various concentrations ranging from 10 ppm to 500 ppm, showing that at the highest concentration (500 ppm), all samples, including Isolate 1, Isolate 2, Isolate 3, and red betel leaves, achieved 100% mortality rates. This indicates that all samples exhibited full toxicity at high concentrations. However, significant differences were observed in the LC_{50} values, which reflect the concentration at which 50% mortality was reached.

Isolate 1 showed the lowest LC_{50} value of 24.83 ± 4.10 ppm, indicating its higher toxicity at lower doses compared to red betel leaves, which had the highest LC_{50} value of 38.16 ± 3.52 ppm. Meyer categorized extract toxicity based on LC_{50} values. Extracts with LC_{50} values

below 1,000 ppm are classified as toxic, whereas those above 1,000 ppm are considered non-toxic [12]. A Study explained that the endophytic fungal extract from plants also exhibited high cytotoxicity using the BSLT method. The fungal extracts and fresh red betel leaf extracts exhibited cytotoxic activity within the range of 24–38 ppm, as shown in **Figure 4**.

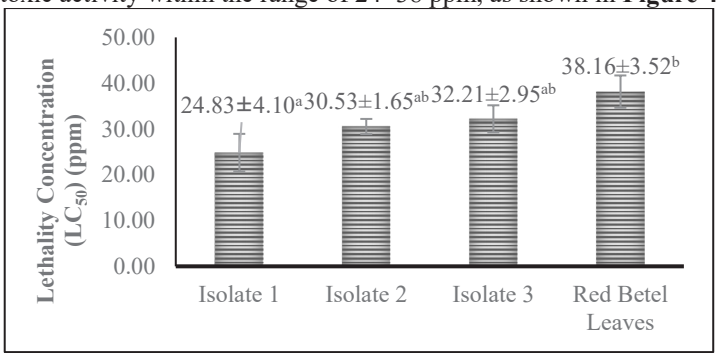


Fig 4. Lethal Concentration (LC_{50}) value of ethyl acetate extract of endophytic fungi from red betel plant leaves on shrimp larvae. Numbers followed by the same letter indicate no significant difference based on Tukey's test (α=0.05).

4 Conclusions

This study identified and isolated three different isolates from red betel plant *(P. crocatum*): isolate 1 from *Aspergillus*, isolate 2 from *Penicillium*, and isolate 3 from *Fusarium*. Isolates 1 and 2 exhibited higher levels of secondary metabolites, including phenolics and flavonoids, as well as superior antioxidant capacity and LC_{50} activity compared to the ethyl acetate extract from red betel leaves. Isolate 1 (*Aspergillus* sp.) demonstrated the highest potential for the development of natural pharmaceutical anticancer compounds that could be explored for future drug development and therapeutic applications.

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