HYDROGEN
JURNAL KEPENDIDIKAN KIMIA
https://e-journal.undikma.ac.id/index.php/hydrogen/index

February 2025. 13(1)
e-ISSN: 2656-3061
p-ISSN: 2338-6487
pp….

Antioxidant of Ethanol Extract and Toxicity of Fractions from *Aspergillus unguis* a Marine Sponge Symbiont of *Aaptos suberitoides*

Scify Bilqis Nawafi Masyerli[1], Mai Efdi[1], Muhammad Hasan Bashari[2], Mochamad Untung Kurnia Agung[3], Beginer Subhan[4], Efahmi[5,6], Yosie Andriani[6], Syafrizayanti[1*]

[1] Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Andalas, Padang, West Sumatera, Indonesia
[2] Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Bandung, West Java, Indonesia
[3] Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Padjadjaran, Bandung, West Java, Indonesia
[4] Department of Marine Science and Technology, Faculty of Fisheries and Marine Science, IPB University, Bogor, West Java, Indonesia
[5] School of Pharmacy, Bandung Institute of Technology, Bandung, West Java, Indonesia
[6] University Center of Excellence for Nutraceuticals, Bioscience and Biotechnology Research Center, Bandung Institute of Technology, Bandung, West Java, Indonesia
[*] Corresponding Author e-mail: syafrizayanti@sci.unand.ac.id

Article History
Received: 03-01-2025
Revised: 24-02-2025
Published: 28-02-2025

Keywords: antioxidant; *Aspergillus unguis*; brine shrimp lethality test; fractions; toxicity

Abstract
The marine sponge Aaptos suberitoides has been shown to have anticancer properties, with clear evidence of its capacity to suppress the growth of cancer cells. However, the pharmaceutical exploration of chemicals from marine organisms causes significant environmental concerns. Aspergillus unguis, a fungal symbiont of the marine sponge A. suberitoides, has been isolated for its potential in sustainable natural products resources. This study assesses the antioxidant activity of ethanol extract and the toxicity of four levels fractions from ethanol extracts of A. unguis mycelium and these results were reported for the first time in this study. The antioxidant of ethanol extract was determined using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method. The fractions were examined for toxicity using the Brine Shrimp Lethality Test (BSLT). The ethanol extract of A. unguis shows very strong antioxidant bioactivity (IC_{50} = 42.84 mg/L). The LC_{50} values for hexane, chloroform, ethyl acetate, and butanol fractions were determined to be 74.11 µg/mL, 93.84 µg/mL, 59.37 µg/mL, and 142.79 µg/mL, respectively. It indicates significant toxicity. These preliminary results are important knowledge for further research into the bioactivity potential of the metabolites as candidate anticancer compounds, aligning with marine pharmaceutical drug development.

How to Cite: Masyerli, S., Efdi, M., Bashari, M., Untung Kurnia Agung, M., Subhan, B., Efahmi, E., Andriani, Y., & Syafrizayanti, S. (2025). Antioxidant of Ethanol Extract and Toxicity of Fractions from Aspergillus unguis a Marine Sponge Symbiont of Aaptos suberitoides. Hydrogen: Jurnal Kependidikan Kimia, 13(1), 12-21. doi:https://doi.org/10.33394/hjkk.v13i1.14645

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INTRODUCTION

Cancer is a significant challenge in society, public health, and the economy in the 21st century, accounting for nearly one in six deaths (16.8%) globally and one in four deaths (22.8%) from noncommunicable diseases (NCDs). It is responsible for 30.3% of premature deaths from NCDs among individuals aged 30–69 years and ranks among the top three causes of death in this age group across 177 out of 183 countries (Bray Bsc et al., 2024;Bayani et al., 2024). Based on data from the Institute for Health Metrics and Evaluation (IHME) in 2019, cancer is the

second most common cause of death in Indonesia, accounting for 13.4% of total deaths, following coronary heart disease (38.2%) and preceding diabetes (8.7%) and chronic obstructive pulmonary disease (COPD) (5.9%) (Andinata et al., 2023). Cancer continues to be a leading cause of mortality globally, requiring ongoing research into effective therapeutic agents (Min & Lee, 2022).

Natural sources, including marine organisms, offer a rich repository for the discovery of novel anticancer compounds due to their high biodiversity and the diversity of bioactive compounds (Santaniello et al., 2023). According to the latest statistics, marine-derived fungi contributed nearly half (47%) of all newly reported marine natural products (NPs) in 2019 (Van Anh et al., 2021). Notably, the marine sponge *Aaptos suberitoides* has been identified in multiple studies as a promising source of anticancer compounds (Yun et al., 2019).

A study has demonstrated that the ethanol extract of *A. suberitoides* exhibits cytotoxic properties, effectively inhibiting the proliferation and migration of the HCC-1954 cell line, a HER2+ breast cancer cell line resistant to trastuzumab—at a concentration of 12 μg/mL (Bashari et al., 2021). From the same species collected in Ambon, Indonesia, the alkaloid compounds, including aaptamine (8,9-dimethoxy-1H-benzo[de][1,6]naphthyridine), and four other derivatives that have been isolated and studied for anticancer properties (Pham et al., 2013). Several investigations have confirmed the bioactivity of aaptamine within the concentration range of 1–10 μM, showing cytotoxic effects on T47D breast cancer cells, murine cancer cells, and MG63 human osteosarcoma cells by enhancing the regulation of p21 promoter expression and inducing cell cycle arrest (Aoki et al., 2006; Dyshlovoy et al., 2014; Tailor et al., 2024).

While the potential of marine organisms as sources for anticancer drug development is significant, the sustainability of marine ecosystems must be considered. Overexploitation of marine resources, including *A. suberitoides*, not only threatens marine biodiversity but also the sustainability of the organisms themselves (Varijakzhan et al., 2021). This dilemma highlights the necessity of marine conservation, which might be mitigated by exploring alternative sources such as microbial symbionts residing within marine organisms. These symbionts retain the capability to produce similarly significant bioactive compounds as their host organism (Turon et al., 2019). Among them, the genus *Aspergillus* is one of the most widespread filamentous fungi and serves as a key source of marine fungal natural products. This genus has been found to produce a wide array of structurally diverse secondary metabolites, including polyketides, alkaloids, terpenes, steroids, and peptides, many of which exhibit significant biological activities (Van Anh et al., 2021).

One example of a fungal species belonging to the genus *Aspergillus* is the marine sponge symbiont fungus *Aspergillus unguis*, associated with *A. suberitoides* collected from Harapan Island in Kepulauan Seribu, Indonesia (106°34'51.052" E, 5°38'55.462" S), shows considerable promise as a source of two novel anticancer compounds (Bashari et al., 2025). However, studies on the potential bioactivity investigation of extract and/or fractions of *A. unguis* as a source of compounds for anticancer potential candidates are still very limited and less explored. Therefore, the aim of this preliminary study is to investigate more potential from *A. unguis*, the antioxidant of ethanol extract and the toxicity of fractions from the ethanol extract of *A. unguis* for further emphasizing the value of symbiotic marine microbes in the development of potential candidate compounds for anticancer therapies.

METHOD

Materials

The experimental materials include isolate *A. unguis* from Mr. Mochamad Untung Kurnia Agung, M.Si (the Marine Sciences Program, Universitas Padjadjaran), sterilized seawater, Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), 70% alcohol, ethanol, hexane, chloroform, ethyl acetate, and butanol. Additional materials used are Whatman No. 42 filter paper, DPPH (2,2-diphenyl-1-picrylhydrazyl), ascorbic acid, 1% dimethyl sulfoxide (DMSO), and brine shrimp larvae (*Artemia salina* Leach).

Equipment and Instruments

The equipment utilized comprises general laboratory glassware, an inoculation loop, test tube racks, vial bottles, Pasteur pipettes, a laboratory shaker, and a magnetic stirrer. Precision instruments include an analytical balance (KERN ABS 220-4N), a laminar airflow cabinet (BioLab BLHZ-204), an incubator (Gallenkamp), a rotary evaporator (BUCHI R-100), and a UV-Vis spectrophotometer (Shimadzu UV-1280).

Procedures

Cultivation *A. unguis*

The isolate of the *A. unguis* was cultivated on Potato Dextrose Agar (PDA) for three days at room temperature (25-28°C). Then, culture was transferred to Potato Dextrose Broth (PDB) and maintained in 120 rpm for 30 days at 25-28°C. Each day, a 1 mL sample of the culture was extracted, diluted in a 1:1000 ratio with sea water, and its optical density measured at a wavelength of 405 nm using UV-VIS spectrophotometry, a procedure repeated twice following the methodology previously established (Nevalainen et al., 2014).

Extraction and Fractionation *A. unguis*

Approximately 5 liters of *A. unguis* culture were harvested on day 15 of incubation by filtering the mycelium using Whatman No. 42 filter paper, subsequently soaked in 500 mL of ethanol (repeated ×3 or till exhaustion) over a three-day period with continuous shaking. Then the ethanol extract evaporated under a rotary evaporator and subsequently into freeze-drying till dryness. The fractionation of this dry extract began with hexane (repeated ×3 or till exhaustion), followed in sequence by chloroform, ethyl acetate, and butanol. Each fraction of *A. unguis* was evaporated under a rotary evaporator. The dry fractions were stored at 4°C for subsequent experiments (Clark, James H., et al., 2017).

Antioxidant Assay of *A. unguis* Ethanol Extract Using DPPH Method

Two mL of ethanol extract solution with concentration were prepared in variations from 0 to 100 mg/L, and mixed with 3 mL of DPPH solution (0.1 mM) and then incubated for 30 minutes in dark conditions. Ethanol served as the control, and ascorbic acid was used as a reference in the antioxidant testing. Subsequently, absorbance was measured using a UV-Vis spectrophotometer (Shimadzu UV-1280, Japan) at a wavelength of 517 nm, then plotted as a percentage of radical scavenging to calculate the IC_{50} (Baliyan et al., 2022).

Toxicity Assay of *A. unguis* Fractions Using BSLT Method

Each fraction was tested for toxicity using the Brine Shrimp Lethality Test (BSLT). One gram of *Artemia salina* Leach eggs was hatched by soaking in 1 L of seawater with aeration in a glass box that was darkened on one side and illuminated with a 40-60 watt incandescent bulb for 48 hours. The hatched shrimp larvae migrated towards the brighter side of the glass box. Test solutions were prepared at various concentrations (0, 50, 100, 150, 250, 300 μg/mL) using the same solvent as the fraction. Control solutions were made without the addition of the

extract. 2 mL of each test solution was placed into a 30 mL vial bottle and dried until the solvent had completely evaporated. Then, 20 µL of DMSO and 5 mL of seawater were added. Subsequently, 15 shrimp larvae were introduced into each bottle. Each concentration was tested in triplicate and compared with the control solution. Observations were made after 24 hours. The number of surviving shrimp larvae was counted and the LC_{50} value of each fraction was determined (Zakwan et al., 2023).

RESULTS AND DISCUSSION

Growth Curve of *A. unguis*

The growth of the fungus *A. unguis* is depicted in **Figure 1**, which illustrates four growth phases over a 21-day culture period: the lag or adaptation phase, the log or exponential phase, the stationary phase, and the death or decline phase. The lag phase occurs from day 1 to day 2, the log phase from day 3 to day 9, the stationary phase from day 10 to day 19, and the death phase begins on day 20 and continues thereafter. The harvest of *A. unguis* is timed at the 14-day mark of incubation during the stationary phase, where the production of secondary metabolites peaks (Oliveira et al., 2024). During the stationary phase, cell growth slows due to nutrient limitations, accumulation of metabolic waste products, and environmental stress, which stimulate the active and increased production of secondary metabolites as a form of adaptation and response mechanism (Molnár et al., 2010).

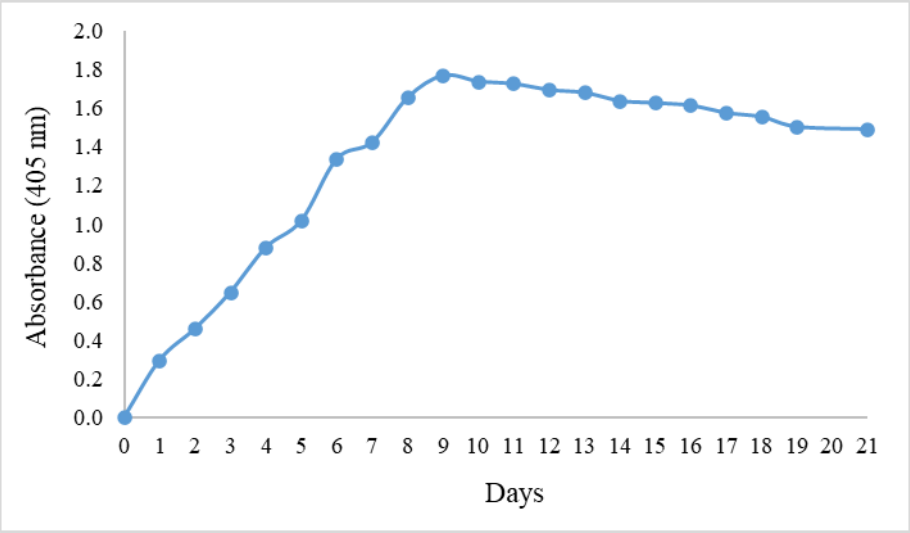

Figure 1. Growth curve of *A. unguis*

During the lag phase, fungi undergo adaptation to their environment. Fungal cells remain inactive in terms of division, while metabolic activity rises in preparation for subsequent stages. Essential molecules, including enzymes, are synthesized to facilitate growth. Following this adaptation, fungi enter the exponential or growth phase, where cellular division accelerates. At this stage, fungal growth peaks, and the cell population expands. The availability of nutrients and favorable environmental conditions supports this rapid growth (Stange et al., 2019).

However, once the optimal growth conditions are exhausted, fungi transition into the stationary phase, where growth stabilizes due to nutrient depletion and the buildup of metabolic waste. During this phase, fungal growth is balanced by the death of cells. It is also during this phase that fungi may begin to produce spores or other forms of resistance to cope with adverse conditions. Although carbon, a vital energy and nutrient source, is depleted, growth continues as dead cells undergo lysis and provide a recycled nutrient source (Oliveira et al., 2024). Ultimately, the death phase ensues, characterized by a significant reduction in biomass. In this

phase, the number of dead cells surpasses that of living cells, as nutrient availability becomes severely limited and waste product accumulation increases, resulting in a decline in fungal growth and eventual cell death (Rendowaty et al., 2017).

Moreover, growth conditions influence the growth phases; for example, the growth curve of *Aspergillus unguis* (WR8), symbiont of the sponge *Haliclona fascigera*, shows different phases when cultivated statically for 28 days and under shaking at 120 rpm for 14 days in Sabouraud Dextrose Broth (SDB). *Aspergillus unguis* (WR8) experiences its log phase from day 7 to day 14, the stationary phase from day 15 to day 21, and the death phase begins after day 21, making day 21 the optimal cultivation time for *A. unguis* (WR8). In contrast, under shaking conditions, *A. unguis* (WR8) only exhibits a log phase from day 2 to day 14 (Nursid et al., 2015; Rendowaty et al., 2017).

Extract and Fractions of *A. unguis*

The yield data for the extracts obtained from the maceration process using ethanol, as well as the weights of the *A. unguis* fractions, are presented in **Table 1**. The ethyl acetate fraction yielded the highest percentage (2.17%) compared to the other fractions. The extraction and fractionation processes were successfully conducted, resulting in yields that are considered substantial for fungal samples. Previous research has shown that the extraction of *A. unguis* (WR8), a symbiont of the sponge *Haliclona fascigera*, using a shaker resulted in a larger fungal biomass (1.45 g) due to stirring or agitation at 120 rpm, which rapidly increased fungal growth, while a smaller biomass (1.04 g) was observed under static conditions (Rendowaty et al., 2017).

Table 1. Amount and yield percentage of extract and fractions of *A. unguis*

Extract/Fraction	Weight (gram)	Yield (%)
Ethanol	72.5924	1.45
Hexane	1.2469	1.72
Chloroform	0.8752	1.21
Ethyl Acetate	1.5788	2.17
Butanol	1.3011	1.79

Agitation affects nutrient mixing, mass and heat transfer, changes in fungal morphology, variations in growth and metabolic product formation, and cell structure damage (Nursid et al., 2015; Rendowaty et al., 2017). Factors influencing the success of the extraction and fractionation processes include the methods and solvents used, environmental conditions such as temperature, sample size, media composition, culture conditions, and purification methods (Agarwal, 2018; Badar et al., 2022; Egbuna et al., 2020).

Antioxidant of *A. unguis* Etanol Extract

Nowadays, antioxidants have gained significant attention due to their beneficial effects as health promoters in the management of cardiovascular diseases, cancer, and aging, among other conditions. The growing global interest in identifying antioxidant compounds from natural sources has prompted us to evaluate the antioxidant activity of *A. unguis* extracts (Oogarah et al., 2020). The ethanol extract of *A. unguis* demonstrated the ability to scavenge DPPH radicals, as shown in **Figure 2.a**, with ascorbic acid—a synthetic antioxidant—used as a reference in **Figure 2.b**. The scavenging activity of *A. unguis* ethanol extract against free radicals increased with higher concentrations, reaching an IC_{50} of 42.84 mg/L, while ascorbic acid had an IC_{50} of 5.18 mg/L. An extract is considered to possess very strong antioxidant bioactivity if its IC_{50} value is less than 50 mg/L, and strong if the value is between 50 and 100 mg/L (Fristiohady et al., 2020). Additionally, fungi that are symbiotic with marine sponges are found to exhibit bioactivity potential comparable to their sources, underscoring the significant therapeutic potential of marine-derived fungi.

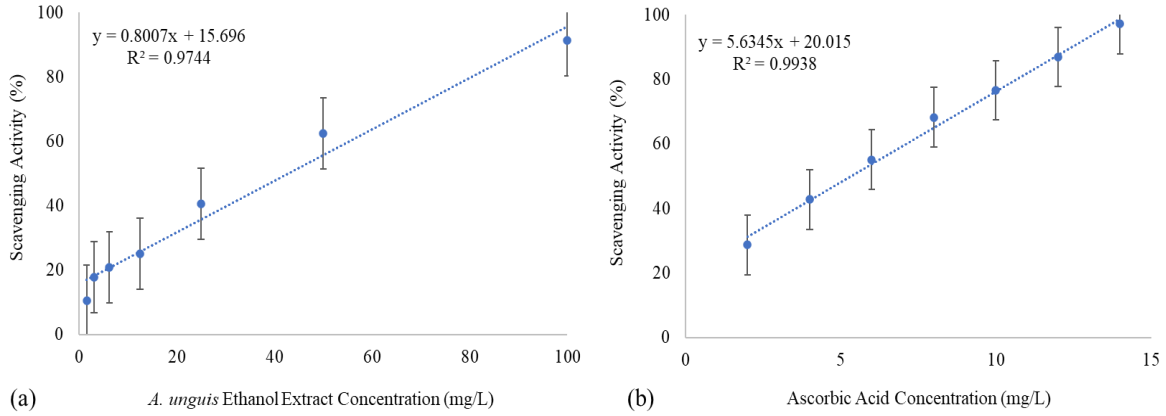

Figure 2. (a) Antioxidant activity of *A. unguis* ethanol extract and (b) Standard antioxidant ascorbic acid

There is a limited amount of research on the antioxidant effects of sponge and/or its symbiont extracts. According to existing literature, several metabolites isolated from marine sponges, including indole derivatives, aromatic alkaloids, aromatic polyketides, and phenolic compounds, have demonstrated significant antioxidant activity, surpassing that of vitamin E and ascorbic acid. A scientific study identified alkaloids, phenols, steroids, terpenoids, tannins, and saponins in extracts from the sponge *Plakortis nigra* collected from the waters of Mauritius, with total phenolic content (TPC) ranging from 2.28 ± 0.072 to 12.79 ± 0.236 mg gallic acid equivalents per gram of extract. Similarly, the findings of this study align with previously reported occurrences of phenolic compounds in extracts of *Stylissa* sp. *and Biemna tubulosa* from Mauritius, with varying polarities (Oogarah et al., 2020).

The antioxidant bioactivity of the ethanol extract of *A. unguis* was first reported in this study. A previous study reported the methanol extract from *A. suberitoides*, the marine sponge originating as a symbiont of *A. unguis*, exhibited prominent antioxidant activity with an IC_{50} of 27.42 µg/mL (Abdillah et al., 2013). Similar studies on *Aaptos* sp., utilizing acetone as a solvent, demonstrated antioxidant properties with an IC_{50} of 16.10 µg/mL (Fristiohady et al., 2020). The ethyl acetate extract from *A. suberitoides* was found to be equivalent to 286 µg of ascorbic acid per 500 µg/mL of extract, indicating strong antioxidant potential (Rivera & Uy, 2012).

Additionally, *A. unguis* sourced from diverse symbionts such as the green microalga Enteromorpha sp. showed a moderate antioxidant activity at 73.91 ± 0.91 µg/mL, contrasting with the standard ascorbic acid which exhibited an activity of 28.01 ± 0.5 µg/mL (Sajna et al., 2020). Another symbiotic variant, *Aspergillus unguis* associated with Agelas sp., inhibited acetylcholinesterase by 47.5% at a concentration of 400 g/mL and α-glucosidase by 42.3%, compared to the standard acarbose with 25% inhibition, showcasing its potential in enzymatic inhibition (Abd et al., 2015).

Toxicity of *A. unguis* Fractions

The ability of each fraction of *A. unguis* ethanol extract to induce mortality in *Artemia salina* Leach larvae is depicted in Figure 3. Determination of the toxicity of compounds or extracts acutely using shrimp larvae *Artemia salina* is a simple preliminary/prescriptive test of biological activity. This BSLT method can be used as a prescriptive test in the study of compounds that lead to cytotoxic activity tests. Each fraction exhibits varying levels of larvicidal activity, with the fastest responses and highest mortality rates occurring at a concentration of 300 µg/mL. The higher the concentration of each fraction, the greater the

number of shrimp larvae that succumb. The LC_{50} values for each *A. unguis* fraction were determined by substituting the probit value of 50 into the linear regression equation y = ax + b, using Microsoft Excel. The calculated toxicity results for the *A. unguis* fractions reveal varying LC_{50} values, as illustrated in Figure 4.

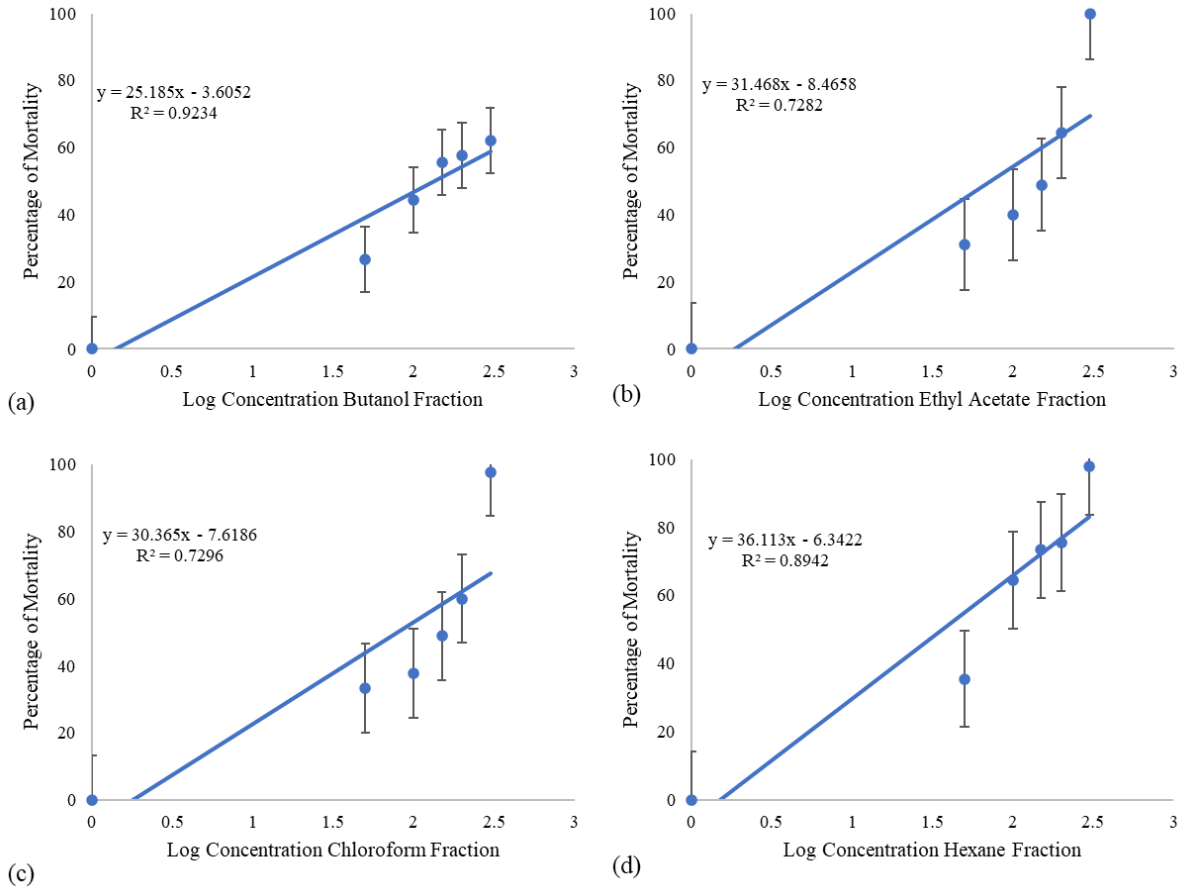

Figure 3. Percentage of mortality of *A. unguis* fractions (a) Butanol (b) Ethyl acetate (c) Chloroform (d) Hexane

The toxicity values for the hexane, chloroform, ethyl acetate, and butanol fractions of *A. unguis* were determined to be 74.11 µg/mL, 93.84 µg/mL, 59.37 µg/mL, and 142.79 µg/mL, respectively. According to the toxicity classification criteria, a compound is considered highly toxic if its LC_{50} is less than 30 µg/mL, toxic if the LC_{50} is between 30 and 1000 µg/mL, and non-toxic if the LC_{50} exceeds 1000 µg/mL (Zakwan et al., 2023). Based on these LC_{50} values, the fractions from *A. unguis* are categorized within the toxic range. These preliminary test results serve as a benchmark for further research into the bioactive potential of the metabolites contained in each fraction, particularly their anticancer properties. This study underscores the importance of evaluating the toxicological profiles of natural extracts in the development of therapeutic agents (Yun et al., 2019).

The toxicity levels of fractions derived from *A. unguis* have not been previously reported. However, the toxicity of the ethyl acetate extract from the sponge *A. suberitoides*, tested using the Brine Shrimp Lethality Test (BSLT), has been documented with an LC_{50} of 18.66 µg/ml. Fractionation of this ethyl extract through column chromatography revealed that the fourth ethyl acetate sub-fraction contains the bisdemethylaaptamine compound, an alkaloid secondary metabolite, with an LC_{50} of 2.56 µg/ml (Situmorang, Fronika, 2012). The toxicity of the acetone extract of *Aaptos* sp. sponge was also reported, with an LC50 value of 1041.5 µg/ml, which is classified as non-toxic (Fristiohady et al., 2020). The LC_{50} values are typically

obtained through statistical analysis, such as probit regression or log-logit analysis, based on the relationship between sample concentration and larval mortality percentage. A lower LC_{50} value indicates a higher toxicity of the tested sample (Fristiohady et al., 2020).

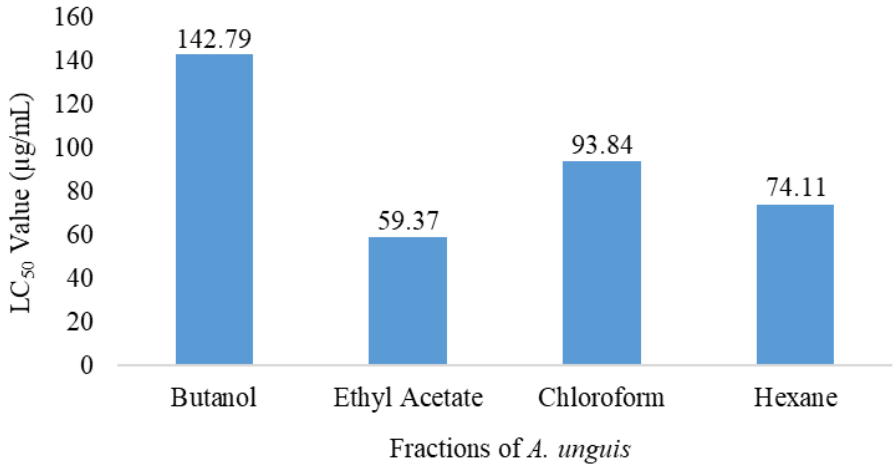

Figure 4. Fractions toxicity (LC_{50}) of *A. unguis*

This provides a basis for further research and evaluation of the potential toxicological impacts of these natural extracts, underscoring the critical role of dose-response assessments in the development of both therapeutic agents and toxicity studies.

CONCLUSION

Based on the research findings, it was discovered the ethanol extract of *A. unguis* has demonstrated very strong antioxidant activity with an IC_{50} value of 42.84 mg/L. Toxicity testing of the fractions from the ethanol extract of *A. unguis* indicates that all fractions are classified as toxic, with respective LC_{50} values of hexane (74.11 µg/mL), chloroform (93.84 µg/mL), ethyl acetate (59.37 µg/mL), and butanol (142.79 µg/mL). Therefore, the extract and its fractions from *A. unguis* show great potential for its toxicity and warrant further investigation for their anticancer capabilities against various cancer cells, which could lead to the exploration of their secondary metabolites for cancer drug development. All these investigation results related to the antioxidant of ethanol extract and the toxicity of four levels fractions from the ethanol extract of *A. unguis* marine symbiont sponge *A. suberitoides* are reported for the first time in this study.

RECOMMENDATIONS

The fractions of *A. unguis* can be further studied for their inhibitory abilities and anticancer mechanisms against various cancer cells. Additionally, the mechanisms of interaction between the compounds within these fractions and cancer target proteins can be examined to potentially develop them into structure-based drug discovery.

ACKNOWLEDGEMENTS

The authors express their gratitude to the Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Andalas, for providing the facilities for this study.

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