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Screening of antibacterial activity of Crown-of-thorns starfish (*Acanthaster* spp.) extracts using modified microtiter-plate resazurin assay

SHARON ROSE M. TABUGO[1,2,♥], TRICKSIE P. BALATERO[1,2], RODELYN M. DALAYAP[3]

[1]Department of Biological Sciences, Mindanao State University-Iligan Institute of Technology. Andres Bonifacio Avenue, Tibanga, Iligan City 9200, Lanao del Norte, Philippines. Tel.: +63-063-221-4056, ♥email: sharonrose.tabugo@g.msuiit.edu.ph

[2]Molecular Systematics and Conservation Genomics Laboratory, Center for Biodiversity Studies and Conservation, Premier Research Institute of Science and Mathematics (PRISM), MSU-Iligan Institute of Technology. Andres Bonifacio Avenue, Tibanga, Iligan City 9200, Lanao del Norte, Philippines

[3]Department of Biology, Sultan Kudarat State University. Tacurong Campus, Tacurong City 9800, Sultan Kudarat, Philippines

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Abstract. *Tabugo SRM, Balatero TP, Dalayap RM. 2025. Screening of antibacterial activity of Crown-of-thorns starfish (*Acanthaster *spp.) extracts using modified microtiter-plate resazurin assay. Biodiversitas 26: 1735-1742.* Marine organisms have emerged as promising sources for the discovery of new compounds that have medical application potential. Their ability to thrive in environments teeming with pathogenic microorganisms and predators indicates the production of unique chemical compounds with significant biological activities. This study identified and morphologically characterized *Acanthaster* spp. (Crown-of-Thorns, COT starfish) from various sites, including microhabitats in Iligan and Panguil Bays, and extracted polar and non-polar crude extracts for antibacterial activity screening. Samples were collected from three locations: Buruun, Iligan City; Samburon, Linamon; and Maigo, Lanao del Norte. Crude extracts were prepared using hexane (non-polar) and methanol (polar) solvents for all samples from the three sites. A modified microtiter antimicrobial assay was performed on all crude extracts, which exhibited antibacterial activity against *Pseudomonas aeruginosa* (a Gram-negative bacterium) and *Staphylococcus aureus* (a Gram-positive bacterium). The activity of HMCOT (hexane extract of samples from Maigo, Lanao del Norte) was comparable to Ciprofloxacin (positive control) based on the post-hoc test. Qualitative screening of the crude extracts for secondary metabolites revealed the presence of alkaloids, flavonoids, and saponins, which are likely contributors to the observed antibacterial activity. The diversity of the chemical compounds reflects the biodiversity of the source organisms, which have evolved and adapted to their specific environments. Thus, *Acanthaster* spp. shows potential for medical research, though further tests are necessary to validate these findings. The results of this study served as baseline data for future research.

Keywords: *Acanthaster* spp., antibacterial, crude extracts, microtiter plate, resazurin assay

INTRODUCTION

Many marine organisms have become essential candidates for discovering novel, medically necessary compounds. They are thought to produce novel compounds with distinctive chemical structures and remarkable biological activities, as they have adapted to survive in an environment filled with pathogenic microorganisms and key predators. The chemical diversity of the isolated compounds reflects the biodiversity of the source organisms, which have evolved and adapted to their environment. In this context, the Crown-of-Thorns Starfish (COTS) is named after the venomous, thorn-like spines that cover its upper surface. The glandular tissue around the venomous spines of the body surface produces toxins. The venomous spines can deliver stings that can provoke various pathological symptoms. The crude toxins or extracts may exhibit diverse biological activities of medical importance (Lee et al. 2014). The active compounds produced by marine organisms are often used in traditional and complementary medicine. The majority of the population prefers the use of remedies of natural origin to treat diseases as they are said to cause fewer side effects (Andriani et al. 2018). Studies show that the Crude Venom (CV) of starfishes (*Acanthaster* spp.) can be extracted, and many are believed to have a wide range of biological activities, including hemolytic, capillary permeability-increasing, myonecrotic, hemorrhagic, edema-forming, mast-cell histamine-releasing, anti-coagulant and cardiovascular activity (Lee et al. 2015; Wijanarko et al. 2018). However, the antimicrobial potential of the crude extract's preparation, respective dosage, and concentrations remains ambiguous against terrestrial, potentially pathogenic microorganisms. Notably, the production of antimicrobial activities was an indicator of the bioactive secondary metabolites (Zheng et al. 2021; Abd El Hafez et al. 2022).

Additionally, COTS are considered as native coral predators, playing an intricate role in coral reefs by acting as potential keystone species preying on fast-growing corals. As predators of fast-growing corals, they allow slow-growing corals to take its place and thrive thus, enhancing diversity. In normal numbers, they help balance and maintain biodiversity. Making them also good drivers for ecological succession in coral ecosystems. However, outbreaks of COTS due to pollution, increased in sea temperature and presence of enough nutrients sustaining their growth and reproduction may lead to significant coral damage (Hue et al. 2020; Foo et al. 2024). The adult is a corallivorous predator that preys on coral polyps. The stomach surface secretes digestive enzymes, which absorb nutrients from the liquefied coral tissue and leave a white scar on the coral skeleton, rapidly infested with filamentous algae. Among the preferred corals are *Acropora* species (table-like corals) (Birkeland 2015; Haywood et al. 2019). In the past, outbreaks have occurred in numerous locations across the Indo-Pacific region due to elevated nutrient levels resulting from anthropogenic activities and overfishing (Wijanarko et al. 2018; Ling et al. 2020). Global and local initiatives to prevent outbreaks entail manual removal of crown-of-thorns starfish to prevent corals from dying. The techniques and management strategies employed to confront such outbreaks often result in the generation of waste. After removing starfishes, they are often thrown away and become organic waste. There are no local programs to utilize such waste. Thus, this study utilized such waste and investigated the bioactivity potential of COTS found in Iligan and Panguil Bays. It also determined the presence and absence of secondary metabolites through qualitative screening.

This study takes precedence in assessing the antibacterial potential of crown-of-thorns starfish (*Acanthaster* spp.) found in Iligan and Panguil Bays. Named for its venomous, thorn-like spines, the crown-of-thorns starfish may produce crude extracts with diverse biological activities of medical significance. Additionally, the bioactivity and chemical diversity of these compounds may reflect the biodiversity of their source organisms, which have evolved and adapted to their specific environments, highlighting the importance of sampling across different locations. The study provides baseline data for further research, with the primary objective of assessing the antibacterial potential of polar and non-polar crude extracts of crown-of-thorns starfish using a modified microtiter antimicrobial assay to evaluate antibacterial activity. It also includes habitat documentation and secondary metabolite screening.

MATERIALS AND METHODS

Study area

The study areas were in Iligan and Panguil Bays. Three sites were considered, particularly in Buruun of Iligan City (8°11'13.2"N 124°10'03.4"E), Samburon of Linamon (8°11'15.4"N 124°08'18.0"E), and Maigo of Lanao del Norte (8°08'53.5"N 123°55'11.5"E) (Figure 1). Opportunistic sampling was employed, as fisherfolk and scuba diver volunteers often removed starfish during 'scubasurero' to prevent coral death. Starfish wastes were collected for identification, description, extraction, and analysis. Existing microhabitats where they were found were also documented. Identification was done through existing illustrated keys and guides. Prior informed consents were secured. Courtesy calls were made, and locals were hired as guides and field assistants. Field reconnaissance and interviews were conducted with fisherfolk.

Morphological characterization and identification of samples

Traditional morphological characterization of starfish was conducted, with identification performed using existing illustrated keys and guides. Specimens were also photographed for documentation purposes (Labnao et al. 2024).

Preparation of the starfishes

Approximately five (5) adult starfish specimens weighing between 400-500 g were collected from each site, and fresh specimens were promptly transported to the laboratory. Thick gloves were worn as a precautionary measure while handling the specimens. Since the spines can become fragile, brittle, and somewhat blunt, handling the specimens was relatively easy and manageable.

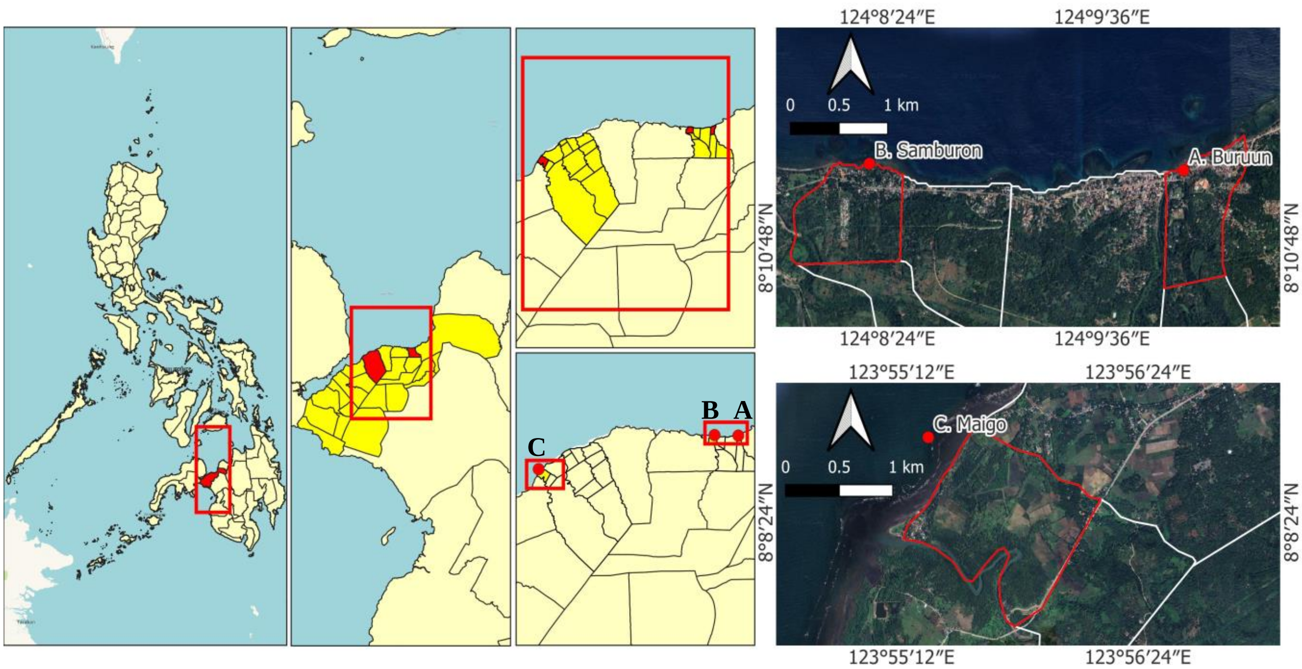

Figure 1. Map of the sampling areas: A. Buruun; B. Samburon, Linamon, Iligan Bay; C. Maigo, Panguil Bay, Philippines

Extraction

Fresh starfish samples were cut into small pieces and soaked in methanol (for one week) and hexane (for another week) before extraction. The samples were thoroughly extracted using methanol (polar) and hexane (non-polar) (Hassim et al. 2014; Sumitha et al. 2017). Then, the crude extracts were evaporated under reduced pressure at 35°C using a rotary evaporator and subsequently utilized for further analysis. The extracts were labeled as follows: methanol extracts—MBCOT (Buruun, Lanao del Norte), MMCOT (Maigo, Lanao del Norte), and MSCOT (Samburon, Linamon, Lanao del Norte); hexane extracts—HBCOT (Buruun, Lanao del Norte), HMCOT (Maigo, Lanao del Norte), and HSCOT (Samburon, Linamon, Lanao del Norte). Ciprofloxacin was used as the positive control (+).

Test organisms

The bacterial strains *Pseudomonas aeruginosa* BIOTECH 1335 and *Staphylococcus aureus* BIOTECH 1582 were obtained from the University of the Philippines-Los Baños Biotechnology Laboratory. These strains are known to be potentially pathogenic and associated with various human infections (Pitout 2008). The bacteria were sub-cultured on appropriate media as prescribed by BIOTECH and incubated overnight at room temperature. Killing/viability curves were prepared for each strain to ensure consistency in bacterial concentration. The final 5×10^5 CFU/mL concentration was used for the assay, allowing for comparisons between different strains and species (Sarker et al. 2007; Ereguero et al. 2023). These test organisms were utilized for the preliminary screening of the antimicrobial activity of *Acanthaster* spp. (crown-of-thorns) starfish crude extracts.

Modified microtiter *in-vitro* antibacterial screening

Medium

Mueller-Hinton Broth (M-H Broth) medium was used for the assay to determine the Minimal Inhibitory Concentrations (MICs). World Health Organization (WHO), Food and Drug Administration (FDA), and National Committee for Clinical Laboratory Standards (NCCLS), now known as the Clinical and Laboratory Standards Institute (CLSI), often recommend Mueller-Hinton medium for testing the most encountered aerobic and facultative anaerobic bacteria in food and clinical materials. Notably, the medium exhibits suitable batch-to-batch reproducibility, with low levels of sulfonamide, trimethoprim, and tetracycline inhibitors, and yields satisfactory growth of most non-fastidious pathogens.

Preparation of bacterial culture

Aseptic techniques were used to prepare the bacterial culture. Then, a single colony was transferred into a 100 mL bottle of Nutrient Broth (NB), capped, and incubated overnight at 35°C. After 12-24 hours of incubation, a clean sample of bacteria was prepared using aseptic techniques and the aid of a centrifuge. The broth was centrifuged for 5 minutes at 4,000 rpm while maintaining appropriate aseptic precautions. The supernatant was discarded into an appropriately labeled contaminated waste beaker. The pellet was resuspended in 20 mL of sterile normal saline and centrifuged for 5 minutes at 4,000 rpm. This step was repeated until the supernatant was clear, and then the pellet was suspended in 20 mL of sterile normal saline and labeled Bs. The optical density of the Bs was recorded at 500 nm, and serial dilutions under appropriate aseptic techniques were performed until the optical density ranged from 0.5 to 1.0. Finally, the actual number of colony-forming units was calculated from the viability graph. The dilution factor required was calculated, and subsequently, the dilution was carried out to achieve a 5×10^6 cfu/mL concentration (Sarker et al. 2007; Ereguero et al. 2023).

Preparation of resazurin solution

The resazurin solution was prepared by adapting the method used by Sarker et al. (2007), which involved dissolving a 67.5 mg tablet in 10 mL of sterile distilled water. A vortex mixer was used to ensure a well-dissolved and homogenous solution.

Preparation of the plates

The method followed in this study was adapted from Sarker et al. (2007) with slight modifications. Each sterile 96-well plate was labeled correctly and prepared under aseptic conditions. A 100 μL volume of the test extracts (COT crude extract) dissolved in 10% (v/v) DMSO or sterile water, prepared at a concentration of 0.1 mg/mL, was pipetted into the first row of columns 1-4 and 7-10. Similarly, 100 μL of Ciprofloxacin, serving as the positive control, was added to columns 6 (C6) and 12 (C12) of row A. Then, 50 μL of saline was added to rows A of C5 and C11 instead of the test extract. Next, 50 μL of sterile saline was dispensed into all wells except rows A, C6, and C12. A serial dilution was performed using a multichannel pipette (except C5 and C11), ensuring that the last 50 μL from the final row was discarded. Following this, 30 μL of Mueller Hinton Broth (MHB) was added to all wells, and 10 μL of the respective bacterial suspension (5×10^6 CFU/mL) was introduced into each well except C1 and C7, where 10 μL of saline was added instead of bacteria (this serves as the negative control).

The plates were prepared in triplicate and incubated at 37°C for 18-24 hours. Following incubation, color changes were visually assessed by adding 10 μL of resazurin dye to all wells, with observations made within 5 to 20 minutes. Notably, the dye's effectiveness diminished beyond 20 minutes, with optimal results recorded between 5 and 10 minutes. A color change from blue to pink or colorless was recorded as positive, indicating bacterial growth and no inhibition. In contrast, a blue or purple color indicated negative results, signifying bacterial inhibition. Resazurin dye is an oxidation-reduction indicator used to assess cell growth, particularly in cytotoxicity assays. It is a non-fluorescent, non-toxic dye that turns pink when reduced to resorufin by oxidoreductases within viable cells (Gross et al. 2024). The further reduced form, hydroresorufin, is uncolored and non-fluorescent (Csepregi et al. 2018; Travnickova et al. 2019). The lowest concentration at which a color change was observed was then recorded as the Minimum Inhibitory Concentration (MIC) value. The MIC value for each test extract against the bacterial strain was determined by averaging the three replicate values (Sarker et al. 2007; Ereguero et al. 2023).

Qualitative screening for secondary metabolites

Preliminary screening of secondary metabolites was done according to standard methods described by Brain et al. (1975) and Evans (2009).

Detection of alkaloids

The prepared extracts were individually dissolved in diluted hydrochloric acid and then filtered. The resulting filtrates were used for the detection of alkaloids. The filtrates were treated with Wagner's reagent. The formation of a brown or reddish-brown precipitate indicates the presence of alkaloids. Wagner's reagent solution was prepared by dissolving 2 g of potassium iodide and 1.2 g of iodine in 5 mL of distilled water and diluting the mixture to 100 mL with distilled water.

Test for flavonoids

Several dilute sodium hydroxide drops were then added to 1 mL of the crude stock extract (Yadav and Verma 2018). The crude extract appeared intense yellow, which became colorless with the addition of several drops of dilute acid, indicating the presence of flavonoids (Prakash et al. 2022).

Test for saponins (Froth test)

Next, in a test tube, a volume of 0.5 mL of the extract was added to 5 mL of distilled water. The solution was shaken vigorously and observed for the formation of a stable, persistent froth. The frothing was mixed with three drops of olive oil and shaken vigorously, after which it was observed for the formation of an emulsion.

Statistical analysis

The data was analyzed using Microsoft Office LTSC Professional Plus 2021 - Microsoft Excel (version 2108, build 14332.20791 Click-to-Run) and cross-verified with an online data analyzer available at https://astatsa.com/OneWay_Anova_with_TukeyHSD/. A One-Way Analysis of Variance (ANOVA) was used to assess the statistical difference between the different extracts and the positive control. Tukey's pairwise (post-hoc) test was subsequently employed to evaluate the comparability of the different extracts with each other and the positive control.

RESULTS AND DISCUSSION

Morphological description and microhabitats

The Crown-of-thorns starfish (*Acanthaster* spp.), found in Iligan and Panguil bays, is often characterized by long, sharp spines on the sides of the starfish's arms and upper (aboral) surface that resemble thorns and create a crown-like shape. Adult sizes range from 22 to 27 cm in diameter (across the body). They are usually of subdued colors, ranging from pale brown to greyish green, with subtle shades of blue. Morphotypes differ in color shades and hues. They are often found in microhabitats with branching corals (*Acropora* species), where they feed and devour them (Figure 2). In some areas, they were found on top of boulder corals, such as the *Porites* species, which gave them a white appearance. They can be devastating to coral reefs and often lead to high mortality rates among corals (Petie et al. 2016; Deaker and Byrne 2022).

Crown-of-Thorns Starfishes (COTS) were identified and morphologically characterized at multiple sites, along with descriptions of their associated microhabitats. Samples were collected from Iligan Bay and Panguil Bay, specifically in Buruun, Linamon, and Maigo, Lanao del Norte. Based on morphological characteristics, the specimens were confirmed as *Acanthaster* spp., displaying diverse morphotypes. The identification of *Acanthaster* spp. aligns with recent studies indicating that the Pacific group of crown-of-thorns starfish (*Acanthaster planci* (Linnaeus, 1758)) likely consists of four distinct species, collectively referred to as the *A. planci* species complex (Labnao et al. 2024). The bioactivity and chemical diversity of these compounds may reflect the biodiversity of their source organisms, which have evolved and adapted to their environments, emphasizing the significance of sampling from diverse locations.

Modified microtiter *in-vitro* antibacterial screening

Crude methanol and hexane extracts of the COT starfish were evaluated for antibacterial activity against two potentially pathogenic microorganisms, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, using a modified microtiter plate-based *in-vitro* antibacterial screening assay. This assay utilized resazurin dye as an indicator of cell viability, facilitating the determination of Minimum Inhibitory Concentration (MIC) values (Table 1) (Breznan et al. 2015; Teh et al. 2017; Ereguero et al. 2023). The crude extracts, prepared at specific concentrations based on established protocols, were stored under refrigeration until use. The antibacterial screening was conducted using a resazurin assay with Mueller-Hinton (M-H) Broth as an alternative medium to replace Isosensitest Broth. Following 24 hours of incubation, resazurin dye was added to all wells (Figure 3), initially turning them blue. Within 5–15 minutes, color changes occurred. A shift from blue to pink or colorless indicated bacterial growth (no inhibition, positive result). At the same time, the retention of blue or purple color signified the absence of bacterial growth (inhibition, negative result) (Figure 4).

The Minimum Inhibitory Concentration (MIC) values of crude methanolic extracts of *Acanthaster* spp. against *P. aeruginosa* were 1.042×10^{-3} for MBCOT, 7.8125×10^{-4} for MMCOT, and 7.8125×10^{-4} for MSCOT. Meanwhile, against *S. aureus*, the MIC for MBCOT was 4.167×10^{-3}, for MMCOT a value of 7.8125×10^{-4} and 1.042×10^{-3} for MSCOT, respectively. As for the crude hexane extracts, the MIC against *P. aeruginosa* for HBCOT was 4.167×10^{-3}, for HMCOT 4.167×10^{-3}, and 7.8125×10^{-4} for HSCOT. Moreover, against *S. aureus*, the MIC for HBCOT was 1.5625×10^{-3}; for HMCOT, it was a value of 7.03125×10^{-3} and 1.042×10^{-3} for HSCOT. Observation indicates an optimal concentration for the extracts to be effective against the specific type of bacteria. Herein, determining the MIC is crucial for obtaining more conclusive and qualitative results on the antibacterial potential of the tested extracts. MIC assays are used to evaluate the performance of all other susceptibility methods. They are used in diagnostic laboratories to provide a definitive answer in cases where other testing methods yield a borderline result or when disc diffusion methods are not suitable (Nedbalcová and Pokludová 2020).

The modified method is quite convenient. However, the results must be further validated despite being highly significant. It was apparent that both methanolic and hexane extracts yielded antibacterial activity against *P. aeruginosa* (a Gram-negative bacterium) and *S. aureus* (a Gram-positive bacterium). However, the methanolic extracts were more effective against *P. aeruginosa*, a Gram-negative bacterium. It is important to note that Gram-positive bacteria are more vulnerable to antibiotics due to the absence of an outer membrane. In contrast, Gram-negative bacteria possess an outer membrane, making them less susceptible to antibiotic treatment. Therefore, Gram-negative bacteria are more pathogenic than Gram-positive bacteria (Lakna 2017). Based on the results, the methanolic crude extracts of COT showed considerable potential against *P. aeruginosa*, a Gram-negative bacterium. The results align with the study by Abd El Hafez et al. (2022), which found that compounds from *A. planci* exhibited antibacterial activity against *P. aeruginosa*. Hence, such findings can be very promising for further study.

Table 2 shows the one-way ANOVA analysis yielding a *p*-value of 0.0135, which is less than 0.05, indicating a statistically significant difference among the groups examined. This suggests that the antibacterial activity observed varies between groups and in comparison to the positive control. However, further analysis is required to determine the specific differences between the groups.

Meanwhile, the activity of the extracts is comparable to one another but not to Ciprofloxacin (positive control), except for HMCOT, which was found to exhibit stronger inhibition. Table 3 presents Tukey's pairwise results, confirming that the antibacterial activity of the extract HMCOT is comparable to that of Ciprofloxacin, with a p-value greater than 0.05. The results suggest that HMCOT extract is highly effective against the test organisms.

Table 1. Determination of Minimum Inhibitory Concentration (MIC) using a modified microtiter antimicrobial assay of crude extracts of Crown-of-Thorns (COT) starfish (*Acanthaster* spp.)

Extract	Average MIC (mg/mL)	
	P. aeruginosa	***S. aureus***
Methanol extract		
MBCOT	1.042×10^{-3}	4.167×10^{-3}
MMCOT	7.8125×10^{-4}	7.8125×10^{-4}
MSCOT	7.8125×10^{-4}	1.042×10^{-3}
Hexane extract		
HBCOT	4.167×10^{-3}	1.5625×10^{-3}
HMCOT	4.167×10^{-3}	7.03125×10^{-3}
HSCOT	7.8125×10^{-4}	1.042×10^{-3}
Ciprofloxacin (+control)	0.025	0.0125

Notes: BCOT: Buruun, Iligan City Crown-of-Thorns; MCOT: Maigo, Crown-of-Thorns; SCOT: Samburon, Linamon Crown-of-Thorns; MIC: Minimum Inhibitory Concentration- an average of three (3) replicates

Table 2. One-way analysis of variance of crude extracts of Crown-of-Thorns (COT) starfish, *Acanthaster* spp.

Source of variation	SS	df	MS	F	p-value
Between groups	0.0005	6	0.0001	6.4530	**0.0135**
Within groups	0.0001	7	0.0000		
Total	0.0006	13			

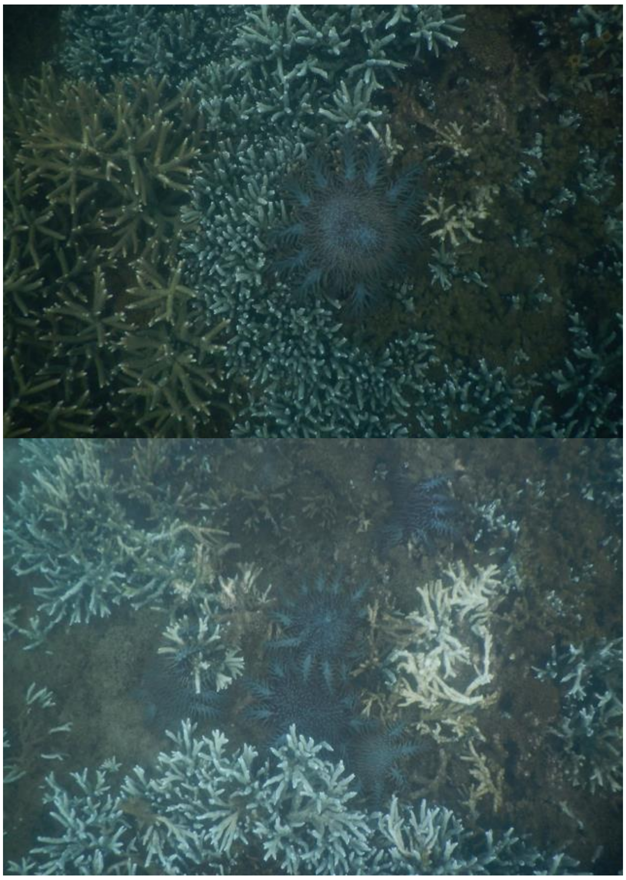

Figure 2. Crown-of-thorns (*Acanthaster* spp.), found in Iligan and Panguil bays' reef areas. They are often seen feeding on branching corals, such as the *Acropora* species, which gives them a distinctive white appearance. Pictures were taken via free diving or skin diving

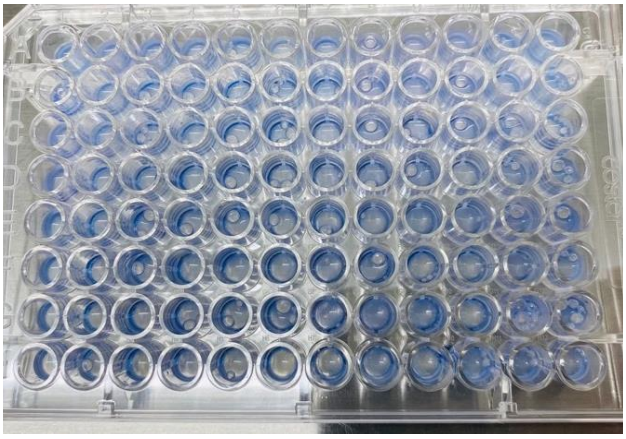

Figure 3. Plates were incubated for 24 hours; after 24 hours, the wells appeared blue when 10 μL of resazurin dye was added per well

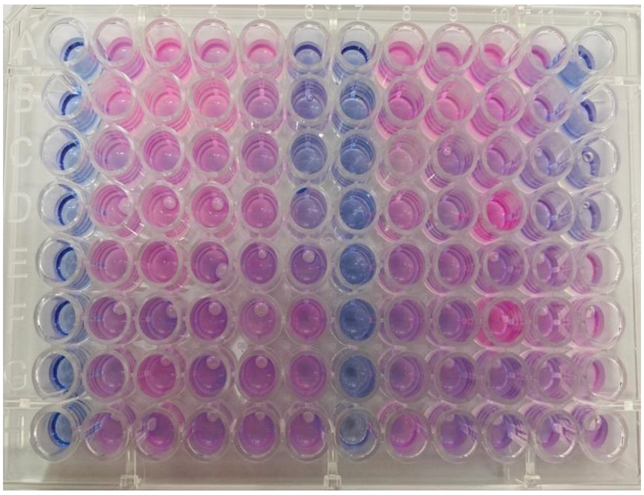

Figure 4. Plates in modified microtiter resazurin assay [pink color indicates growth and blue/purple means inhibition of growth; the test organisms were *P. aeruginosa* (C2-C6) and *S. aureus* (C8-C12); C1 and C7, sterility control (COT extract in serial dilution + broth + saline + indicator), no bacteria; C2-C4, Test compound/ COT extract (in serial dilution in wells 1–12 + broth + indicator + bacteria); C5 and C11 (control without drug (bacteria + broth + indicator); C6 and C12 positive control (Ciprofloxacin in serial dilution + broth + indicator + bacteria)

Table 3. Tukey's pairwise comparison for COT starfish extracts and positive control (Ciprofloxacin)

Treatment pairs	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inference
MBCOT vs MMCOT	0.7953	0.89999	Insignificant
MBCOT vs MSCOT	0.7438	0.89999	Insignificant
MBCOT vs HBCOT	0.0282	0.89999	Insignificant
MBCOT vs HMCOT	1.1088	0.89999	Insignificant
MBCOT vs HSCOT	0.6691	0.89999	Insignificant
MBCOT vs Cipro (+) control	6.3063	0.02835	* p<0.05
MMCOT vs MSCOT	0.0515	0.89999	Insignificant
MMCOT vs HBCOT	0.8234	0.89999	Insignificant
MMCOT vs HMCOT	1.9041	0.79754	Insignificant
MMCOT vs HSCOT	0.1262	0.89999	Insignificant
MMCOT vs Cipro (+) control	7.1016	0.01532	* p<0.05
MSCOT vs HBCOT	0.7719	0.89999	Insignificant
MSCOT vs HMCOT	1.8526	0.81498	Insignificant
MSCOT vs HSCOT	0.0747	0.89999	Insignificant
MSCOT vs Cipro (+) control	7.0501	0.01592	* p<0.05
HBCOT vs HMCOT	1.0807	0.89999	Insignificant
HBCOT vs HSCOT	0.6972	0.89999	Insignificant
HBCOT vs Cipro (+) control	6.2782	0.02899	* p<0.05
HMCOT vs HSCOT	1.7779	0.84025	Insignificant
HMCOT vs Cipro (+) control	5.1975	0.07036	Insignificant
HSCOT vs Cipro (+) control	6.9754	0.01685	* p<0.05

Table 4. Qualitative screening for secondary metabolites for the obtained crude extracts of COT starfish for both polar (methanol) and non-polar (hexane) solvents

Extract	Alkaloids	Flavonoids	Saponins
Methanol extract			
MBCOT	+++	++	++
MMCOT	++++	+	+++
MSCOT	+	++	++
Hexane extract			
HBCOT	++	+++	++
HMCOT	++++	+++	+
HSCOT	+	+++	+

Qualitative screening for secondary metabolites

Table 4 shows the results of the qualitative screening for secondary metabolites for the obtained crude extracts of COT starfishes for both polar (methanol) and non-polar (hexane) solvents. Notably, all tested extracts were positive for the presence of alkaloids, flavonoids, and saponins and may differ in the amount present. This was expected, as organisms that have adapted and evolved to their environment may exhibit different chemical diversity. Moreover, alkaloids exhibit a range of activities. It served as a painkiller, an antimicrobial, a stimulant, a muscle relaxant, an anesthetic, an antimicrobial, an anti-diabetic, an anti-cancerous agent, an anti-HIV agent, and an antioxidant, among other uses. On one hand, flavonoids possess a natural ability to influence the body's response to allergens, viruses, and carcinogens. They exhibit anti-allergic, antimicrobial, and anticancer properties, making them potential therapeutic agents for various diseases (Ibrahim et al. 2013).

On the other hand, saponins have been used in the treatment of hypercholesterolemia and hyperglycemia as an antioxidant, anticancer agents, anti-inflammatory agents, and weight loss, according to the medical field. It is a bioactive antibacterial agent (Lee et al. 2015; Kamyab et al. 2019). The production of antimicrobial activities was an indicator of the bioactive secondary metabolites (Ibrahim et al. 2013; Achmad et al. 2018; Andriani et al. 2018). The presence of these secondary metabolites may support the crude extracts' potential antibacterial activity, as observed in the modified microtiter *in-vitro* antibacterial screening. COT starfish can be promising in the field of medical research; however, its results need to be further validated through additional testing.

Starfish metabolites can be categorized into three primary groups: asterosaponins, cyclic steroidal glycosides, and glycosides of polyhydroxylated steroids. Asterosaponins, a subclass of glycosides, have been documented to possess a range of biological activities, including hemolytic, cytotoxic, antifungal, antibacterial, and antiviral properties (Lee et al. 2015: Achmad et al. 2018; Andriani et al. 2018; Abd El Hafez et al. 2022; Hillberg et al. 2023).

In conclusion, this study takes precedence in assessing the bioactivity potential of crown-of-thorns (*Acanthaster* spp.) starfish found in Iligan and Panguil Bays. It also determined the presence and absence of secondary metabolites through qualitative screening. To protect coral reefs, during outbreaks fisherfolk and scuba diver volunteers frequently remove Crown-of-Thorns Starfish (COTS) to prevent coral degradation. However, these starfish are often discarded as organic waste due to a lack of utilization programs. This study repurposed these wastes by exploring the bioactivity potential of COTS. Results reveal promising activity against Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus.* Qualitative screening for secondary metabolites revealed the presence of alkaloids, flavonoids, and saponins, which may contribute to the antibacterial properties of the extracts. The findings provide valuable baseline data for further research and potential applications.

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