 doi:10.20944/preprints202207.0085.v1

Article

Investigating the Effect of *Acanthaster planci* Extractions for Anti-enzymatic and Anti-human Pathogenic Activity

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Abstract: Crown of thorns starfish (*Acanthaster planci*) are coral predators with advantages of having toxicity in their venom and tissue regeneration capabilities. With all these characteristics, only a handful of studies have highlighted the association of microorganisms with this organism. Crown of thorns starfish are common in Fiji and their analyses of microbial diversity for secondary metabolites could be of great interest to the scientific community. This study is an attempt to investigate Fijian-based *A. planci* for their venom and associated actinomycetes antibacterial activity and further identify the type of enzymes present in the crude venom extract. The CoTS venom extract (0.192 g) harbor enzymes such as gelatinase, caesinase, and amylase. An abundant and potent actinomycete strain, represented as FJA1 showed antibacterial activity against *Enterococcus faecium* with an inhibition zone of 10 mm. Moreover, all pathogenic test microorganisms were resistant against concentrations of 500 µg and 1 mg of *A. planci* venom extract.

Keywords: Crown of thorns starfish (CoTS); actinomycetes; venom extract; anti-enzymatic; antibacterial activity

1. Introduction

Globally, newly emerging infectious diseases and evolving multidrug-resistant human pathogens arise as a major hurdle to our health sector and threatens a pre-antibiotic era (Khan & Khan, 2016). The latter invoke curiosity or urgency in finding and developing unique antibiotics through isolation of unique secondary metabolites of specific microorganisms. The ocean encompasses more than 70 % of the Earth's surface, and remain a rich reservoir of diverse marine eukaryotes and prokaryotes (Choudhary *et al.,* 2017; Sogin *et al.,* 2006). A wide range of marine habitats exists with extreme conditions which represents a treasure trove for both chemical and biological diversity compared to its terrestrial counterparts (Kijjoa & Sawangwong, 2004; Xie *et al.,* 2018). Bioprospecting for antibiotic producing marine microorganisms from unexplored marine habitats serves as an alternative of secondary metabolite discovery towards novel antibiotics. Marine ecosystem due to its ecological pressure, competition of space and nutrient, and physical parameters such as temperature, salinity, light, enables marine microorganisms to develop secondary metabolite machineries which functions in prolonging their existence (Donia & Hamann, 2003; Tortorella *et al.,* 2018). Bacteria have a versatile and malleable metabolism along with its competitive nature for resources that makes them a prominent arsenal for tangible, and uniquely bioactive compounds (Santos *et al.,* 2020). Actinomycetes are the forefront of organisms bestowed with inherent antibiotic capabilities. They are widely known as organisms of academic or industrial curiosity. Actinomycetes are an omnipresent unique group of Gram-positive bacteria that exhibits fungus-like morphology and contains sufficient content of guanine and cytosine in their genetic fabric. A higher percentage of antibiotics (> 70 %) are derived from actinomycetes, particularly *Streptomyces,* making it one of the essential contributors in clinical antibiotics (Cumsille *et al.,* 2017). Till date, actinomycetes are still one of the essential sources of new antibiotics since its discovery (Genilloud, 2017).

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The crown of thorns starfish (CoTS; *Acanthaster* spp.) is an echinoderm, classified within the family Acanthasteridae, and having distinct features such as multiple-arms and needle-like venomous spines. In tandem to this, their internal structures contain immense volume of chemicals, particularly saponin and plancitoxins which are highly toxic and unpalatable (Cowan *et al.,* 2017). Moreover, they are nocturnal and carnivorous species confined or native to Indo-Pacific region, mainly dwell among hermatypic coral reef, its primary food source. Previous taxonomic classification grouped crown of thorns sea star into a single species, *Acanthaster planci*. It is now appreciated that four species are present in the Indo-Pacific (Høj *et al.,* 2018). Here, this species is represented as *Acanthaster planci*. Despite their indurate appearance, they effortlessly maneuver around the contours of the corals on which it feeds. Individual coloration varies from red and orange to purple due to differences in diet (Ghazali *et al.,* 2017). CoTS are nocturnal organisms, however overcrowded population modulates daytime foraging behaviors (Chesher, 1969). Their feeding behavior involves external gastric digestion on a large area of coral surface tissue in a limited time period. These species normally preferred fast-growing corals than corals with slower growth rates. Limited food supply can trigger *A. planci* to devour any type of coral or potential food source resulting in higher coverage of benthic algae which leads to major devastation of coral reef ecosystem (Pratchett, 2007; Pratchett *et al.,* 2014; Rotjan & Lewis, 2008). The crown of thorns starfish has a complex life cycle. It propagates asexually via regeneration mechanism and sexually (Ghazali *et al.,* 2017), where it is developed as a planktotrophic larvae, metamorphose into herbivorous juveniles that feeds on crustose coralline algae prior to becoming corallivorous in the adult stage (Kamya *et al.,* 2018). Crown of thorns starfish has an extensive role in maintaining biodiversity and steering ecological succession within its natural habitat (Zhao *et al.,* 2013). Its overpopulation or higher densities usually correlates with widespread and long-lasting impacts of reef destruction. Majority of the studies had extensively research into the CoTS biology, but information regarding its associated microbiome is rare. A recent culture-independent study had analyzed the CoTS associated microbiome and revealed distinct microbiome within its tissue types, including the presence of actinomycetes (Høj *et al.,* 2018). Several studies have highlighted that *A. planci* possess a steadily therapeutic potential (Ghazali *et al.,* 2017; Lee *et al.,* 2014; Mutee *et al.,* 2012; Tan *et al.,* 2013). Crown of thorns starfish are a prolific source of bioactive compounds (Ghazali *et al.,* 2019). They are widely distributed in the world's ocean including Fiji's territories with clear traces of historical coral impacts (Dumas *et al.,* 2020). To date, there is unavailability of research data in terms of microbial evaluation and antibacterial activity conducted with CoTS in Fiji. This study aims to analyze the *Acanthaster planci* venom extract and its associated actinomycetes for their antibacterial and anti-enzymatic activity.

2. Materials and Methods.

2.1. Study area and sampling

Three *A. planci* specimens with body weights of 167 g - 300 g were collected from the Laucala bay reef at 18° 10′ 331′ S and 178° 25′ 148′ E. These specimens were rinsed with sterile filtered water to remove sedimentary organisms or particles, then were put on ice and immediately stored at 4 °C in the laboratory till further analysis. The study was conducted in accordance with the University of the South Pacific Research Ethics committee.

2.2. Venom extraction

The process of venom extraction was described by (Lee *et al.,* 2013). The sampled *A. planci* specimens were left to thaw at room temperature, after which they were rinsed several times with sterilized distilled water to compensate for additional unwanted microbes or substances. 78 g of *A. planci* spines were removed from the CoTS body using sterilized tweezers and dissecting scissors. The extracted spines were homogenized using a sterile mortar and pestle before being extracted with two volumes of phosphate buffered saline under room temperature. Approximately, 45 ml of collectable supernatant was

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centrifuged for 45 min at 6000 rpm and the resulting supernatant was collected as crude venom. Then the liquified crude venom was dialyzed against distilled water using the dialysis tubes to remove the salt and further lyophilized into *A. planci* spine venom powder (ASV). Approximately, 0.192 g of lyophilized venom powder is generated from 78 g of spines. A stock solution of ASV powder was prepared by dissolving 0.01 g into 1 ml of 0.01 M PBS buffer with a pH of 7.4 that was further diluted into different concentrations of ASV test solution.

2.3. Isolation of actinomycete

Actinomycete colonies were isolated using serial dilutions with dilution factors of 10^{-1} to 10^{-6}. Randomized parts of the crown of thorns starfish were selected, dissected and homogenized prior to serial dilution. Approximately 10 g of homogenized slurry was transferred into 90 ml of sterile sea water, where the suspensions undergo a vigorous mixing process. Then 0.1 ml aliquots of each dilutions were spread over onto the Actinomycete Isolation Agar (AIA) and International Streptomyces Project 2 (ISP2) media. All media were prepared with seawater rather than distilled water so as to favor actinomycetes growth. All inoculated plates were incubated in an inverted position for 7-14 days at 28 °C since actinomycetes have a sluggish growth. Colonies with distinct morphological characteristics resemblance to that of actinomycetes were purified on ISP2 medium for further antimicrobial screening analysis.

2.4. Test microorganisms

Six human pathogenic bacteria, namely Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecium, and Salmonella enterica were employed in this study. All pathogenic bacteria were cultivated in nutrient broth prior to antimicrobial assay within the USP laboratory.

2.5. Antimicrobial assay

2.5.1. Well diffusion method

The antimicrobial activity of the venom extract was determined using the well diffusion technique. Bacterial lawns were created prior to well formations on the Muller Hinton agar (MHA). Approximately, 500 µg and 1 mg of *A. planci* venom extract prepared from a stock solution were inoculated into their respective wells along with concentrations (500 µg & 1 mg) of methanol; as one of the experiment control measures. All inoculated plates are incubated at 37 °C for 24 hours and zone of inhibitions are determined.

2.5.2. Cross streak method

Separated morphological distinct colonies of actinomycetes were streaked in a straight line and incubated at 28 °C for 7 days. Pathogenic bacteria were then coated in a straight line (approximately 4 cm) perpendicular to the fully fledged actinomycete strain and incubated at 37 °C for 24 hours. Subsequently, the zone of inhibition was observed and measured. As a control measure, all six pathogenic bacteria were grown on a nutrient agar plate free of actinomycete propagules at the aforementioned cultivating conditions.

2.6. Anti-enzymatic activity

This test also employs well diffusion technique to determine the type and presence of enzymes in the venom extract. Three plates containing gelatin, starch and casein were used to prove whether enzymes are present in the sample. 0.001 g of venom powder was dissolved in 1 ml of sterile distilled water. A well was created on the media and impregnated with 100 µl of venom solution. The inoculated plates were incubated vertically at 30 °C for 24 hours before observing the zone of lysis. The Gram's iodine was added to the starch test plate after incubation to clearly view the zone of lysis. On the other hand, a chemical is also added to gelatin and casein test plates for the same purpose.

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3. Results and Discussion

3.1. Antimicrobial assay and morphological characteristics of bacterial isolate

Antimicrobial assay results are shown in Table 2 and Figures. A dominant strain of actinomycete, FJA1, was isolated from the collected crown of thorns starfish (Table 1). This isolate showed potent activity against *Enterococcus faecium* with an inhibition zone of 10 mm (Table 2 and Figure 1). The crude venom extract has no activity against all test pathogens compared to various antibiotics (control) with varied zone of lysis (Table 2 and Figure 2).

Table 1. Morphological characteristics of studied actinomycete strain.

					Colony Morphology		
CODE	**Aerial mycelium colour**	**Vegetative mycelium colour**	**Nature**	**Configuration**	**Elevation**	**Margin**	**Opacity**
FJA1	White	Brown	Powdery	Irregular	Flat	Undulate	Opaque

Table 2. Zone of inhibition of *A. planci* venom extract and its derived actinomycete .

Test pathogens	**Actinomycete strain (FJA1) zone of inhibition (mm)**	**Control: pathogen growth length (mm)**	**Venom concentration and zone of inhibition (mm)**				
			500 µg	**1 mg**	**Control**		
					Antibiotics	**Methanol**	
						500 µg	**1 mg**
Staphylococcus aureus (1)	(-)	40	(-)	(-)	48 (Penicillin) (+)	(-)	(-)
Escherichia coli (2)	(-)	40	(-)	(-)	12 (Ampicillin) (+)	(-)	(-)
Klebsiella pneumonia (3)	(-)	40	(-)	(-)	(Ceftriaxone) (-)	(-)	(-)
Salmonella enterica (4)	(-)	40	(-)	(-)	36 (Ciprofloxacin) (+)	(-)	(-)
Enterococcus faecium (5)	10 (+)	40	(-)	(-)	30 (Oxytetracycline) (+)	(-)	(-)
Streptococcus pneumoniae (6)	(-)	40	(-)	(-)	42 (Penicillin) (+)	(-)	(-)

(-) No zone of inhibition (+) zone of inhibition

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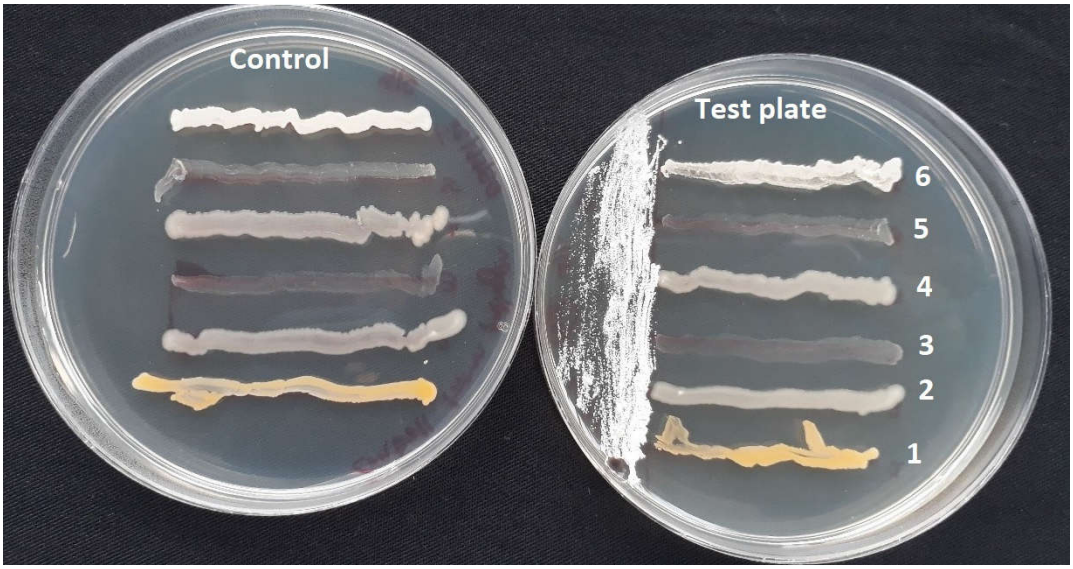

Figure 1. Antibacterial activity of the actinomycete derived from *A. planci*. **1)** *S. aureus*, **2)** *E. coli*, **3)** *K. pneumoniae*, **4)** *S. enterica*, **5)** *E. faecium*, **6)** *S. pneumoniae*.

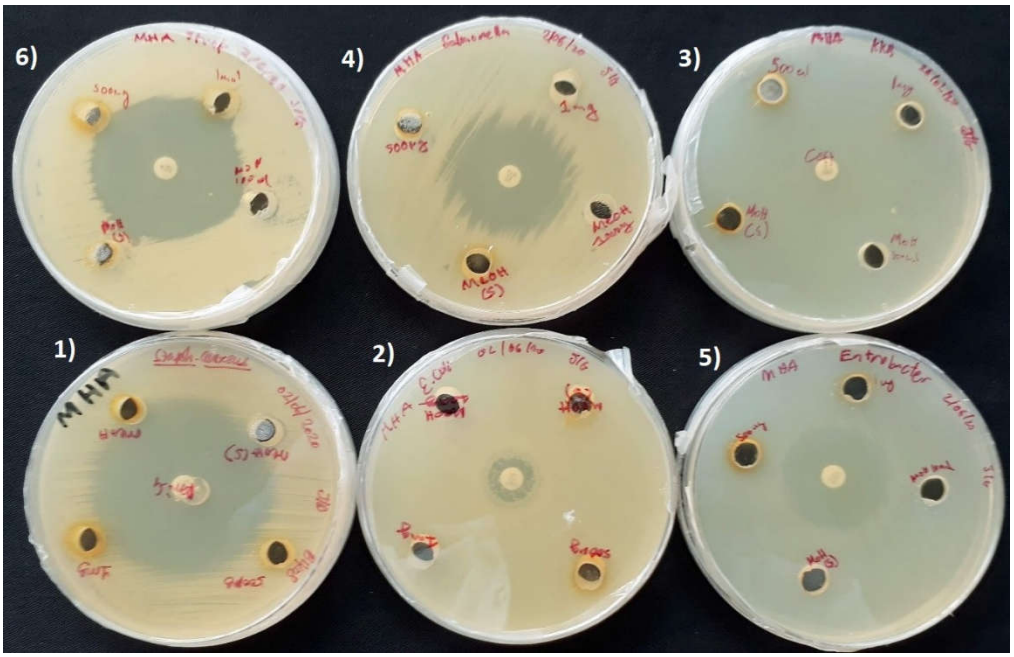

Figure 2. Antibacterial activity of *A. planci* venom extract. **1)** *S. aureus*, **2)** *E. coli*, **3)** *K. pneumoniae*, **4)** *S. enterica*, **5)** *E. faecium*, **6)** *S. pneumoniae.*

3.2. Anti-enzymatic assay

The CoTS venom comprise of proteins that are manufactured by the species acidophilic glandular cells and are stored in the epidermal layer of the organism's spines (Lee *et al.*, 2013; Luch & Butzke, 2010). Understanding the functional role of enzymes is of essence (Tyzack *et al.*, 2017). Enzymes are catalytic proteins involved in the enhancement of chemical reactions. They are categorized as intra- and extracellular enzymes with separated functions. Intracellular enzymes are necessary for cellular metabolism and survival. Whereas, extracellular enzymes are secreted to the surrounding for several purposes including the digestion of proteins into simple building blocks (Meyer-Reil, 1991). Table 3 shows the anti-enzymatic activity of the enzymes present in the *A. planci* venom. The enzymatic activity encompasses three enzymatic tests which involves casein, gelatin and starch tests. All tests were analyzed to determine the presence of proteinase, gelatinase and amylase enzymes through their ability to degrade or hydrolyze casein, gelatin

 doi:10.20944/preprints202207.0085.v1

and starch respectively. This study shows the existence of caseinase, gelatinase and amylase enzyme in the venom extract with varied zones of lysis (Table 3 and Figure 3) in descending order of activity; caseinase > gelatinase > amylase enzyme. From the given observations, it is fair to state that caseinase enzyme has an effective secretion and activity compared to others.

Table 3. Hydrolytic enzymes from *A. planci* venom.

Enzyme	Presence of enzyme	Zone of lysis (mm)
Caseinase	Positive (+)	21
Gelatinase	Positive (+)	20
Amylase	Positive (+)	15

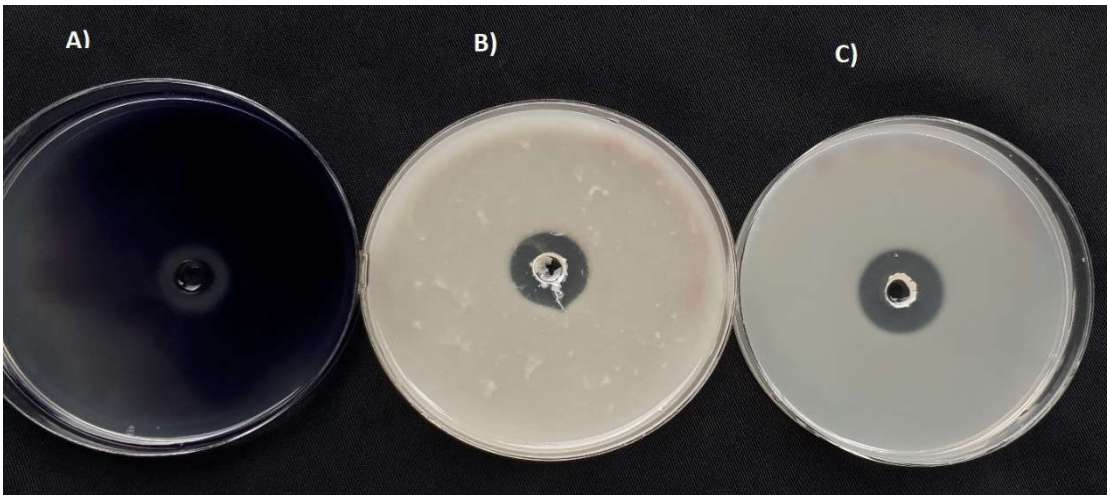

Figure 3. Anti-enzymatic zone of various enzymes. **A)** Starch, **B)** Gelatin, **C)** Casein.

4. Conclusions

To recapitulate, an actinomycete strain derived from the *A. planci* tissue showed potent activity against the pathogenic *Enterococcus faecium*. The crude venom extract obtained from *A. planci* spines had been observed to have anti-enzymatic activity against casein, gelatin and starch. The prior supports literature findings currently available. Further work may target actinomycetes genera using more selective isolation media and pretreatment methods. Additionally, isolated bioactive strains may then be used for compound elucidation, and 16S rRNA genome identification. It is also relevant to involve metagenomic studies in future research. The South Pacific Island Countries and their territories harbor a tremendous potential of mining unique or unexplored bioactive molecules for drug discovery.

Funding: The Government of Vanuatu has assisted financially to support this research project undertaken by the author.

Acknowledgement: The author wishes to acknowledge the University of the South Pacific for providing necessary facilities and support.

Conflicts of Interest: The author declares no conflict of interest.

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