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Research Article
Antibacterial and Antifouling Properties of the Horseshoe Crab *Carcinoscorpius rotundicauda*

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Abstract

Background and Objective: Horseshoe crabs are widely used in both traditional and modern pharmaceutical applications. Most of the previous studies on horseshoe crabs focused on their blood which contains hemolymph and amoebocyte lysate. This study aimed to determine the potential antibacterial and antifouling properties of different extracts from the carapace and the book gills of *Carcinoscorpius rotundicauda*. **Materials and Methods:** The crude extracts were subjected to the bioactivity tests using the disc-diffusion and the inhibition of biofilm-formation measurement assays, for both the antibacterial and antifouling activities respectively. **Results:** The results obtained indicated that the carapace extracts had stronger antibacterial and antifouling effects compared to the book gills extracts. Extracts obtained from the male displayed more activity compared to the extracts from the female with a few exceptions. Methanol and acetone carapace crude extracts showed the best overall performance. A sterol compound was isolated from the carapace acetone extracts of the male of *C. rotundicauda*. However, the compound did not display strong activity compared to the crude extract. The compound might be contributing to the observed activity with other components through a synergistic effect. **Conclusion:** The presence of antibacterial and antifouling activities in the carapace and book gills extracts could be added to the complexity of the defence mechanisms of horseshoe crabs. The results of this study, therefore, may contribute to the knowledge of the defence mechanisms of *C. rotundicauda*. Further research is needed to determine the bioactivities of other parts of the animal and to explore their potential applications.

Key words: Horseshoe crabs, *C. rotundicauda*, anti-microbial, antifouling, carapace, book gills, sterols

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Horseshoe crabs are well known as marine invertebrates which are often called living fossils. The adaptation of the animal without major physical changes to the harsh climate and pathogen threats for million years had triggered the interest to discover their defence mechanisms[1-4]. Three out of the four known horseshoe crab species are found in Malaysia. Two of these species (*Tachypleus gigas* and *Carcinoscorpius rotundicauda*) are common in the Peninsular of Malaysian shores and the third (*T. tridentatus*) is found in the east of the Malaysian coastline (Sabah).

Horseshoe crabs are widely used in both traditional and modern pharmaceutical applications. Several products from the defence molecules in the hemolymph of horseshoe crab have been identified, developed and/or biochemically characterized[5]. Several antimicrobial substances were isolated and identified from the horseshoe crabs including anti-LPS factor, Tachyplesins, Polyphemusins, Big defensin, Tachycitin and Tachystatins[6-8]. Another product known as Limulus Amoebocyte Lysate (LAL) was also developed from the plasma of the horseshoe crab Limulus *polyphemus*. It is used as a bacterial endotoxin test for medical devices and drug development studies. A potential of similar properties was also seen with plasma from *C. rotundicauda*. The plasma from *C. rotundicauda* was also reported to contain antibacterial agents and to relieve joint pain[5]. It is worth mentioning that most of the available reports on *C. rotundicauda* were focused on the blood, biology, habitat, population and breeding[3]. However, no much information was made available on the potential utilization of the body parts of horseshoe crabs such as book gills, carapace or appendage. This may be due to the overwhelming attention directed towards the identification and characterization of the hemolymph and hemocyte components of the horseshoe crab as it implies more biomedical and economical values. Based on this observation, we believe there is a need to investigate the biological activities of the other parts of horseshoe crabs such as the carapace and the book gills.

This study aimed to highlight the potential antibacterial and antifouling activities of the carapace and the book gills from the male and female of *C. rotundicauda* and to characterize some of the major chemical components of the active fraction (s).

MATERIALS AND METHODS

Collection of the samples: This study represents a part of a research project on horseshoe crabs which was conducted at the University of Malaysia Terengganu (UMT), Malaysia from the year 2012-2018. The horseshoe crab *Carcinoscorpius rotundicauda* was collected from Tanjung Dawai, Kedah, Gelang Patah, Johor and Cherating, Pahang, Malaysia. The sampling was assisted by fishermen who collected the horseshoe crab at the sampling location and transferred it to the hatchery at UMT, Malaysia. The samples were collected throughout the pre-or post-monsoon seasons due to the unfavourable weather conditions during the monsoon seasons.

Preparation of the samples and extraction: Samples were cleaned with distilled water and cut into small pieces and macerated after lyophilized (Labconco 6L, USA) to help penetration of the solvents in the extraction process from the sample. Samples were stored at room temperature until the further experiment.

Lyophilized samples of the carapace (100 g) and gill (4 g) were soaked in 10 volumes (v/w) with three different solvents, acetone (Ace), ethyl acetate (EtOAc) and methanol (MeOH) overnight at room temperature. Besides, the book gills were soaked for extraction using 60% (v/v) acetonitrile (ACN, polar aprotic with a dielectric constant of 37.5) containing 0.1% trifluoroacetic acid (TFA). The supernatant was collected and stored at 4°C while the residue was extracted once again under the same process. Supernatants were combined and then evaporated using Rotavapor R-210 (Bucchi, Germany) to obtain the crude extract. Crude extracts were collected and stored at 4°C and tested for bioassay experiment.

Bioassay screening

Antibacterial test: The antibacterial activity test was carried out by using the disc diffusion Test. The strains chosen were Gram-positive bacteria *Bacillus cereus* (ATCC 11778) and *Staphylococcus aureus* (ATCC BAA-1026), Gram-negative bacteria *Escherichia coli* (ATCC 4157) and *Salmonella typhimurium* (ATCC 29629). Nutrient broth (Oxoid, beef extract 1 g L^{-1}, yeast extract 2 g L^{-1}, peptone 5 g L^{-1}, sodium chloride 5 g L^{-1}) were prepared for growing bacteria culture in a liquid medium. Sterile 6 mm paper disc (Whatman filter paper) was saturated with extracts and left to air dry. The process was then repeated and approximately 90-150 µL of extracts in total was loaded on each disc. The diameter of the inhibition zone was measured after overnight incubation at 28-37°C for 12 hrs. The results were expressed as the diameter of the inhibition zone around the discs in mm.

Antifouling assay: The M63 medium (0.5% casamino acid, 0.2% glucose and 70% natural seawater) and de-siliconization

of glass slides were prepared for anti-fouling activity. Then, 25 mL of M63 medium was added in a 50 mL coning tube and autoclaved. The de-siliconization of the glass slides was done by soaking the glass slide overnight in Decon90. After that, tap water was used to wash the slides and then autoclaved with natural seawater. Then, one autoclaved slide was placed on each tube of the sterilized M63 medium. Prepared M-63 media was introduced with cultured test strain bacteria (*S. aureus*, *B. cereus*, *S. typhymurium* and *E. coli*). A hundred microlitre of cultured bacteria were inoculated in each tube. The cultivation process took place on a roller (STUART Roller Mixer, SRT6D) with 28 rpm for 4 days. After 4 days, 90 µL of selected crude extract was pipetted into each of the tubes and was rolled overnight. Then, the slides were removed from the corning tube and washed with running distilled water. Slides then stained using 1% crystal violet for five minutes to determine the adherence of the bacteria[9]. The slides were washed again using distilled water and 33% of acetic acid was used to de-stain the slide thoroughly[10].

Characterization of the chemical components of the crude extracts: Different analytical techniques were used to characterize the obtained extract from the horseshoe crabs. These techniques including Thin Layer Chromatography (TLC), Iodine Vapour Visualization, p-Anisaldehyde Test, Vanillin Reagent Test and the Dragendorff's reagent test.

RESULTS

Recovery of the extracted crude materials: An amount of 100 g of the lyophilized carapace and four grams of lyophilized book gills were soaked with each of the selected solvents. In general, methanol produced a higher yield of carapace extract while the lowest yield was obtained from the ethyl acetate. However, the aqueous extract had a higher yield from the book gills. Book gills yielded a higher percentage (21%) whereas carapace only 0.02%. No differences were recorded according to sex.

Activity-guided screening assay

Antibacterial activity: Results of the antibacterial activities of the crude extracts obtained from the carapace and book gills of *C. rotundicauda* are presented in Table 1. Most of the activities observed were against the tested Gram-positive bacteria. Of the crude extracts tested, carapace extracts showed more activity (29.12%) compared to book gills extracts which showed only (8.33%). In terms of male and female differences, extracts from the female carapace appeared higher in activity compared to the extracts from the male counterpart with inhibition percentages of 41 and 16%, respectively (Fig. 1a). However, no significant differences in activities were recorded between the book gills male and female crude extracts.

Most of the observed activities were recorded against *S. aureus* followed by *B. cereus* (Fig. 1b). On comparing the three solvents used as the extraction medium, acetone crude extracts showed the best overall performance followed by ethyl acetate and methanol (Fig. 1c). The acetone carapace extract also showed remarkable activity against Gram-negative bacteria *S. typhimurium* with an inhibition diameter of more than 16 mm (Fig. 1d).

Antifouling activity: Antifouling effects of the crude extracts obtained from the carapace and book gill of *C. rotundicauda* as determined by the measurement of the inhibition of biofilm-forming bacteria are presented in Table 2. Carapace

Table 1: Antibacterial activity assay using the disc-diffusion test

			Test strains			
Animal part	Sex	Solvent used	*B.c*	*S.t*	*S.a*	*E.c*
Carapace	♂	Ace	-	-	-	-
		EtOAc	-	-	+	-
		MeOH	-	-	-	-
	♀	Ace	+	+++	+	+
		EtOAc	-	-	+	-
		MeOH	-	-	-	-
Book gills	♂	Ace	-	-	-	-
		EtOAc	-	-	-	-
		MeOH	-	-	-	-
	♀	Ace	-	-	-	-
		EtOAc	-	-	-	-
		MeOH	-	-	-	-

B.c: *B. cereus*, S.t: *S. typhimurium*, S.a: *S. aureus*, E.c: *E. coli*. The activity was measured by the diameter of inhibition zone; - : No activity; + : 10 mm or less; ++: 11-15 mm; +++: 16 mm or more

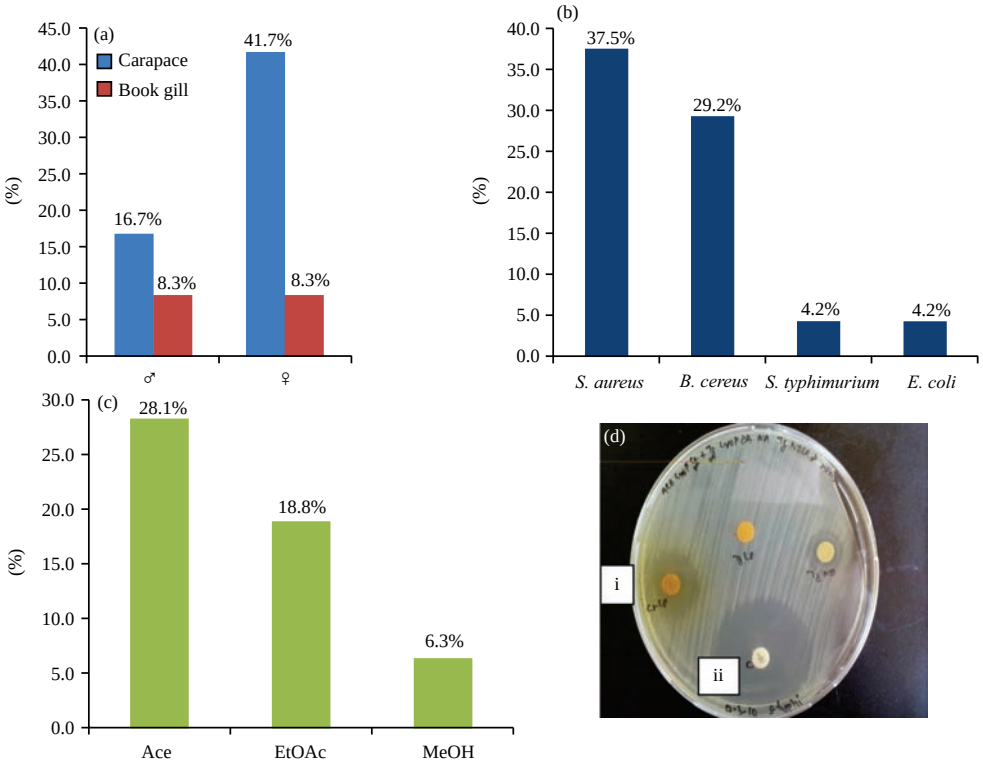

Fig. 1(a-d): Antibacterial activities observed by the extracts from the carapace and book gills of the male and female of *C. rotundicauda*. The extracts were obtained based on the body parts tested, sex and the solvent used for extraction
(a) Percentage of antibacterial activity based on male and female of horseshoe crab; (b) Percentage of antibacterial activity based on strains used; (c) Percentage of antibacterial activity based on the solvent used in extraction; (d) Inhibition zone by the crude extract of (i): *C. rotundicauda* carapace and (ii): Control (chloramphenicol) on test strain *S. typhimurium*

Table 2: Antifouling activity of the crude extracts of *C. rotundicauda*

		Antifouling activities			
		Book gill extract		Carapace extracts	
Solvent used for extraction	Test strain	♂	♀	♂	♀
Ace	*B. cereus*	-	-	++	++
	S. aureus	++	++	++	++
	S. typhimurium	-	-	++	-
	E. coli			++	++
		-	-		
EtOAc	*B. cereus*	++	-	-	++
	S. aureus	-	-	-	++
	S. typhimurium	-	-	-	-
	E. coli			++	-
		-	-		
MeOH	*B. cereus*	++	++	++	++
	S. aureus	-	++	++	-
	S. typhimurium	-	++	++	++
	E. coli	-	-	++	++
		++	++		
		++	-		
		-	++		
		++	++		
		++	++		
		++	++		
		-	++		

++: High antifouling activity, +: Mild antifouling activity, -: No antifouling activity

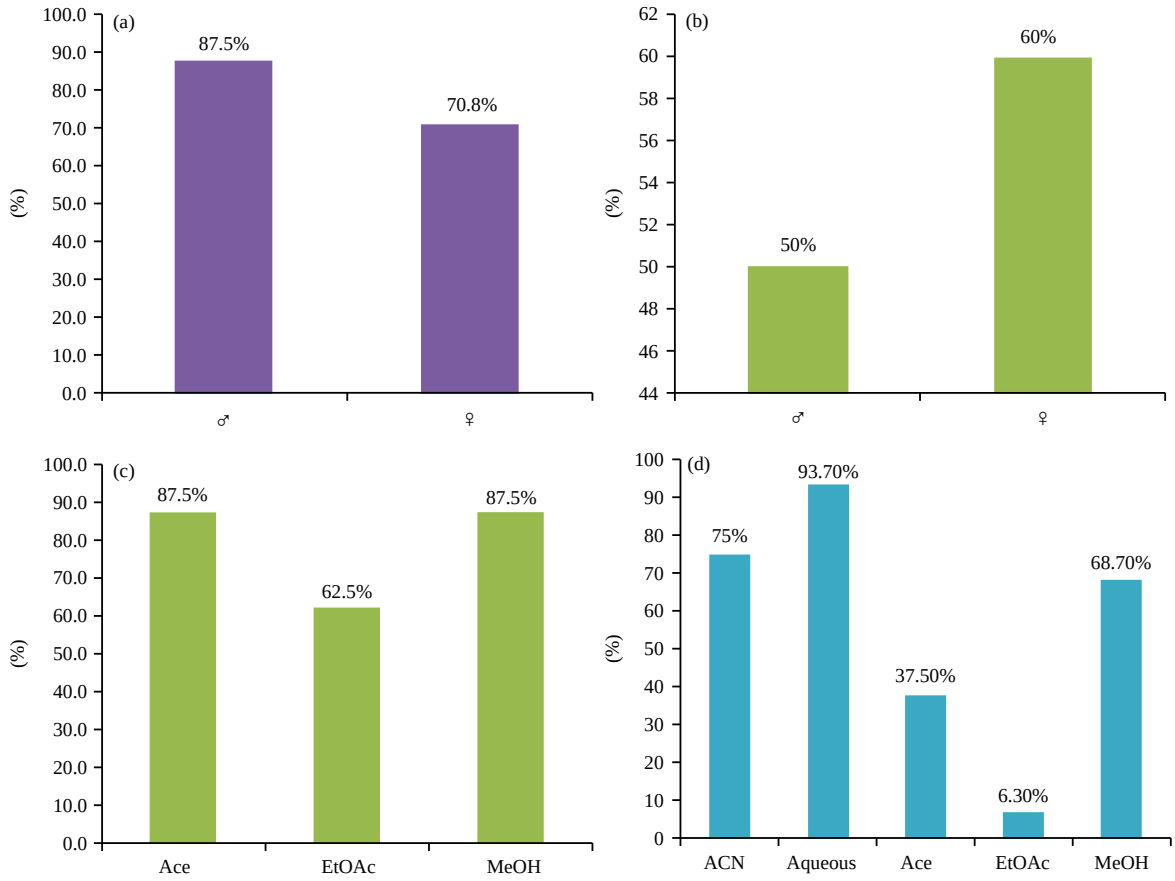

Fig. 2(a-d): Antifouling activities observed by the extracts from the carapace and book gills of the male and female of *C. rotundicauda*. The extracts were obtained based on the body parts tested, sex and the solvent used for extraction

Percentage of the antifouling activity recorded by the extracts from the carapace (a) and book gills (b) of the male and female of the horseshoe crab. Percentage of the antifouling activity recorded by the extracts from the carapace (c) and book gills (d) according to the different solvents used for extraction. ACN: Acetonitrile; Ace: Acetone, EtOAc: Ethyl acetate, MeOH: Methanol. ACN: Acetonitrile; Ace: Acetone, EtOAc: Ethyl acetate, MeOH: Methanol

crude extracts showed higher antifouling activities compared to the activities observed by the book gills extracts and most of the activities were recorded against the Gram-positive bacteria (Fig. 2a, b). Extracts obtained from the male displayed more activity compared to the extracts from the female (Fig. 2a). Methanol and acetone carapace crude extracts showed the best effects (Fig. 2c).

For the book gills, the use of 60% ACN with 0.1% TFA as an additional solvent for crude extraction produced two layers of solution which were the ACN layer and aqueous layer. Aqueous layer crude extracts showed the highest antifouling activity, followed by, ACN layer crude extracts and methanol extracts (Fig. 2d). The best performance was observed against *B. cereus* followed by *S typhimurium*. The female book gills extract appeared more active (60%) than the extracts from the same part of the male (50%). Overall extracts of both carapace and book gills displayed antifouling activities. Furthermore, if compared only between methanol, ethyl acetate and acetone crude extract of different sexes and species, carapace had higher antifouling properties (Fig. 2).

The acetone crude extract of female *C. rotundicauda* carapace was subjected to further analyses as it yielded the highest antibacterial activity in the assay. The crude extract (weight 1.423 g) was subjected to gravity column chromatography (2×5 cm) packed with silica gel 60 (0.040-0.063 mm) Merck. The column was eluted with hexane followed by gradient increasing addition of DCM, EtOAc and MeOH. The crude extract was reconstituted in hexane and loaded in the column for separation and isolation.

TLC of column fractions: The first separation process produced six fractions (CF_1). The column (CC, Si.gel 230-400

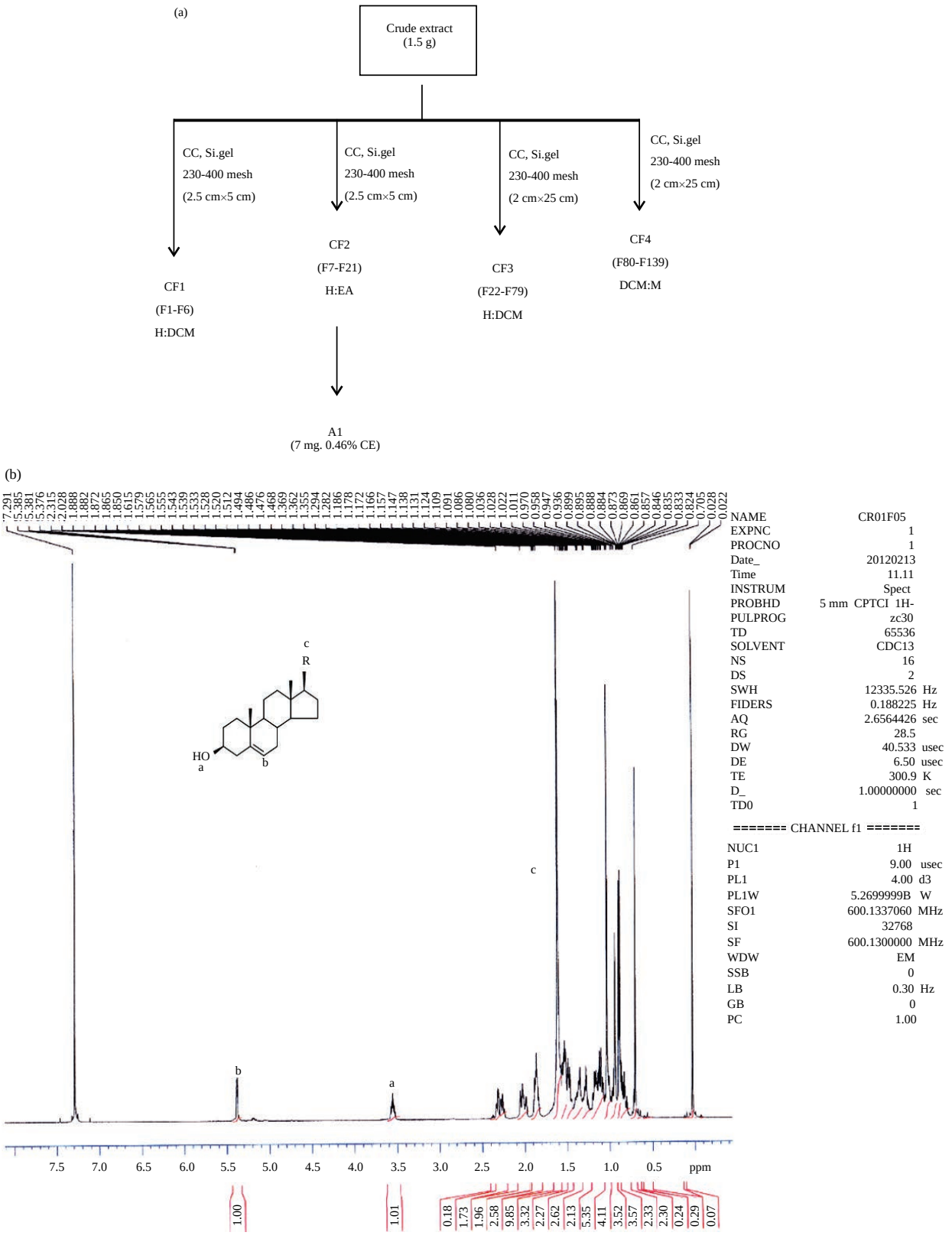

Fig. 3(a-b): Isolation of sterol compound from the acetone crude extract of the female *C. rotundicauda* carapace

(a) A summary of the isolation process; (b) 1H-NMR spectrum of crystal compound in deuterated-chloroform ($CDCl_3$)

mesh 2×5 cm) was prepared using hexane with gradual increasing addition of dichloromethane until 100% of the latter solvent. The flushed crude extract was then columned using the same column as before (CF_2) but with the gradual addition of ethyl acetate. Crystal compound was detected in fraction 11 with some impurities and was re-crystallized to remove impurities. The pure isolate (7 mg) appeared as a white and solid compound (A_1) (Fig. 3a).

The isolated compound was subjected to further analysis for identification using Proton (^{1}h) Nuclear Magnetic Resonance Spectroscopy (NMR). The proton NMR (^{1}H-NMR 600MHz, $CDCl_3$) spectra (Fig. 3b) had measured olefinic proton with chemical shift δ_H 5.38 (1H, s,b). A multiplet signal was present at δ_H 3.55 (1H, m, a) attached to a hydroxyl group which made this a hydroxy sterol. Hexagonal and pentagonal rings of carbon were attributed with a resonance that formed the sterol basic structure with a functional group (R) at δ_H 2.31-0.70 (m, c) determined the name of the sterol based on its detailed structure. Tetramethylsilane (TMS) signal was measured at δ_H 0.02 as internal standard and $CDCl_3$ resonance was detected at δ_H 7.29.

DISCUSSION

This study highlighted the potential antibacterial and antifouling activities of the carapace and the book gills from the male and female of *C. rotundicauda*. The methanolic extracts of carapace and book gills gave a high yield of crude extracts compared to other solvents used such as acetone and ethyl acetate. Besides, the book gills were also extracted by 60% ACN 0.1% TFA. Most of the solvents used for extraction showed that the book gills yield more crude extract compared to the carapace. The carapace (exoskeleton) of most arthropods primarily contains scleroprotein, polysaccharides called chitin, calcium and traces of metals, whereas the book gills are mainly consisting of tissues of thin, sac-like outgrowths from the posterior surface of opisthosoma appendages with cuticular walls[11,12]. This implies that the tissue of the book gills contains higher crude materials for extraction compared with the hard chitinous carapace.

In this study, the acetone crude extract possessed higher antibacterial activities compared with the other crude extracts. For antifouling properties, aqueous, acetonitrile and methanol extracts displayed the highest activities. This may indicate that the observed antifouling activities follow the different mechanism of actions from the antibacterial activities. The antibacterial activity might operate on a direct approach towards the bacteria while the antifouling activity might use an indirect approach such as disrupting the protective layers produced by the bacteria.

Between the two Gram-positive bacteria used, *S. aureus* showed the most vulnerability than *B. cereus*. *S. aureus* is a common mesophilic bacterium. Most of the bacterial infections are caused by mesophilic bacteria that strive in their optimum growth temperature which is around 37°C[13]. The crude extract might have a more disruptive effect on the peptidoglycan layer of the Gram-positive bacteria thus destroying the bacterial defence layer. In contrast, Gram-negative bacteria have a membrane layer that can be more protective and persistent towards the effect of the crude extracts.

The Females of the horseshoe crab seemed to produce higher antibacterial activity compared to their male counterparts. However, for antifouling, the obtained results indicated that extracts from the male had higher antifouling effects. These findings are in agreement with a previous report of Patil and Anil[14]. These authors stated that males are more conducive towards epibiosis due to its carapace surface roughness with more hydrophobic properties which may provide more surface availability and better condition for epibionts settlement. It can be assumed, therefore, that the male horseshoe crab needs to have higher antifouling properties compared with the female as it needs to be well prepared for the battle against epibionts corrosion. One of the main attentions which are significant towards the survival in the marine environment is the behaviour or the ability of the horseshoe crab to remain epibionts free from its cuticle or carapace as epibionts can cause the erosion of the cuticle and therefore increases the susceptibility of internal infections[15]. However, Harrington *et al.*[16] suggested that the defence mechanism of the animal may also due to its hypodermal glands which produce a viscous secretion for protection against epibionts and therefore prevent or the fouling process. As reported in the result the proton NMR spectrum revealed that the crystal compound isolated from the carapace of the horseshoe crab was identified as a sterol. Sterols are defined as a waxy, insoluble group of steroid alcohol which can derive either from plants or animal or in this case the marine invertebrate. It forms an important group among steroids. Sterols may occur as free sterols, acylated (sterol esters), alkylated (steryl alkyl ethers), sulfated (sterol sulfate) or linked to a glycoside moiety (steryl glycosides) which can be acylated (acylated sterol glycosides)[17]. Various marine invertebrates have been reported to contain sterols such as crustacean mollusks, echinoderms, coelenterates, *Porifera*, annelids, cnidarian and also xiphosuran[17,18]. Pakrashi *et al.*[17] reported the isolation of several sterols from the horseshoe crabs *Tachypleus gigas* and *Carcinoscorpius rotundicauda*. The isolated compounds contained C28 and C29 sterols such as campesterol (10%), stigmasterol (7%), b-sitosterol (24%) and

28-isofucosterol (3%). All of these sterols were isolated from different parts of the horseshoe crabs except the carapace. The sterol isolated in this study was from the carapace and therefore carapace can be added to the list of the sources of sterols available in the horseshoe crab.

Earlier reports also indicated the biological activities of sterols from marine resources including antimicrobials and antifouling properties amongst many others[19]. Bakar *et al.*[20] studied the biological activities of some of the sterol compounds isolated from the marine environment. The authors stated that the compounds isolated exhibited antibacterial and antifouling activities with stronger activities recorded against the Gram-negative bacteria compared to Gram-positive bacteria. However, the isolated sterol compound in this study did not show positive results. The compound might be contributing to the activity through synergistic effects[21]. It is also possible that the composition or bioactivity of the compound was altered during the routine process of the chemical analysis[22,23]. The sterol isolated in this study was from the carapace and therefore carapace can be added to the list of the sources of sterols available in the horseshoe crab. The presence of antibacterial and antifouling activities in the carapace and book gills extracts could be added to the complexity of the defence mechanisms of horseshoe crabs. However, the obtained results did not indicate the active constituent(s) that may be responsible for the observed biological effects. It is, therefore, recommended that a further study be carried out to discover the potential bioactive agents. These results contribute to the knowledge of the defence mechanisms of *C. rotundicauda* and may represent a way forward for the discovery of possible potential industrial applications.

CONCLUSION

In conclusion, the crude extracts of the horseshoe crab *C. rotundicauda* displayed antibacterial and antifouling activities in this study. The extracts inhibited the growth of both Gram-positive and Gram-negative bacteria indicating some of the complexity of the defence mechanism of the animal and its innate immunity. The crude extract recovery rate from book gills yield a higher percentage compared with carapace crude extract. Acetone solvent yielded the highest activity for the carapace crude extract. The findings of this research may contribute to the knowledge related to the defence mechanism of the horseshoe crab. Further study is needed to identify the active constituents that may be present in other parts of the animal.

SIGNIFICANCE STATEMENT

The presence of antibacterial and antifouling activities in the carapace and book gills extracts could be added to the complexity of the defence mechanisms of horseshoe crabs. These results contribute to the knowledge of the defence mechanisms of *C. rotundicauda* and may represent a way forward for the discovery of possible potential industrial applications.

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