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## Antibacterial activity of starfish *Stellaster equestris* from Southeast Coast of India

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### PEER REVIEW

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#### Comments

The present research paper comprehensively depicted the antibacterial activity and extraction of crude from the starfish *S. equestris* from southeast coast of India. They methodically carried out the analysis with authentic current techniques such as GC–MS and NMR.

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### ABSTRACT

**Objective:** To isolate and characterize the antibacterial compounds from starfish *Stellaster equestris* (*S. equestris*).

**Methods:** The starfish *S. equestris* was collected from Mudasalodai, southeast coast of India. The whole body was extracted with high polar and medium polar solvents such as methanol and ethanol. The antibacterial activity has been tested against human bacterial pathogens using standard disc diffusion method.

**Results:** The present investigation exhibited that the crude methanol extract showed the maximum zone inhibition [ $9.7 \pm 0.3$  mm] against *Escherichia coli* (*E. coli*) and *Vibrio parahaemolyticus* at 100% concentration and minimum was *Staphylococcus aureus* [ $4.0 \pm 0.6$  mm]. The crude ethanol extract showed maximum zone of inhibition in *E. coli* [ $9.70 \pm 0.33$  mm] and the lowest concentration exhibited the minimum inhibition activity against all bacterial pathogens. The fractions showed the maximum inhibition zone in *Klebsiella oxytoca* ( $5.00 \pm 0.82$  mm), *Salmonella typhi* (*S. typhi*) ( $5.00 \pm 0.82$ ) and *Staphylococcus aureus* [ $3.0 \pm 2.3$  mm]. One way ANOVA analysis of antibacterial activity showed no significant difference at 0.05% level ( $P > 0.05$ ). The crude ethanol extract showed the minimum inhibitory concentration against *E. coli* (100%), *Klebsiella pneumoniae* (75%), *S. typhi* (100%) and *Vibrio cholerae* (100%). The fraction exhibited the minimum inhibitory concentration against *Klebsiella pneumoniae*, *Proteus mirabilis*, *S. typhi*, *Staphylococcus aureus* and *Vibrio cholerae* at 100% whereas *E. coli* was at 75% concentration. No bacterial growth was observed against *Klebsiella oxytoca* at 100% concentration.

**Conclusions:** The result of the present study indicates that the crude and fractions of starfish *S. equestris* have remarkable antimicrobial activities against human bacterial pathogens. Further fraction has been characterised by using GC–MS and  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy analysis.

### KEYWORDS

Pathogens, Starfish, MIC, Polar solvents, Fraction, Characterised

## 1. Introduction

Antimicrobial peptides (AMPs) are evolutionarily conserved small molecular weight proteins of the innate immune response, with a broad spectrum of antimicrobial activities against bacteria, viruses, and fungi[1]. AMPs appear naturally throughout all three domains of life from unicellular to multicellular organisms[2–4]. The

secondary metabolites from starfish are characterised by a remarkable diversity of different steroids, including sterols, polyhydroxy steroids, mono and biosides of polyhydroxy steroids, and toxic steroid oligoglycosides named as asterosaponins[5–7]. Echinoderms are benthic organisms, which are constantly exposed to relatively high concentrations of bacteria, viruses, and fungi, of which many may be harmful to the organism.

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The survival of these organisms depends on efficient antimicrobial mechanisms to protect themselves against microbial infections and fouling.

Antibacterial potential of the echinoderms such as green sea urchin *Strongylocentrotus droebachiensis*, the common starfish *Asterias rubens*, and the sea cucumber *Cucumaria frondosa* was detected in extracts from several tissues in the coelomocyte and body wall extracts[8]. Some of the activities detected (in extracts of celomocytes and body walls) have been caused by compounds of protein nature. Several drug discovery projects have screened echinoderms for antibiotic activities. Among 83 unidentified species of echinoderms from the Gulf of California, 43% have antimicrobial activity[9]. In the same study, 58% out of 36 unidentified Caribbean species showed antimicrobial activity. Out of 22 species of echinoderms collected from the northern Gulf of Mexico, 80% had antimicrobial activity[10]. This information suggests that marine echinoderms are a potential source for new types of antibiotics for pharmaceutical use. The present study revealed that isolation of antimicrobial compounds from sea star *Stellaster equestris* (*S. equestris*) from the Mudasalodai fish landing centre.

## 2. Materials and methods

### 2.1. Sample collection

Starfish *S. equestris* was collected from Mudasalodai fish landing centre and immediately brought to the laboratory in fresh conditions and air dried for further use. The dried samples were then transferred to 10% formalin and later preserved in 70% alcohol. Taxonomical identification is done following the method of Hyman[11], and Clark and Rowe[12].

### 2.2. Extractions of crude toxins

Crude toxin was extracted following the method of Braekman *et al*[13]. For the extraction, about 1 kg of dried starfishes was initially kept in three litre of methanol, followed by 95% and 70% ethanol for 5 d each. The solvents were then removed after squeezing of specimen and filtered through Whatman No. 1 (0.4 µm) filter paper. The solvents were evaporated at low pressure by using a rotary evaporator (Lark Innovative, model LICB-7) at 35 °C. The resultant compound was finally dried in vacuum desiccators and stored at 4 °C for further use.

### 2.3. Antibacterial assay

#### 2.3.1. Test microorganisms and culture media

Test microorganisms *viz.* *Staphylococcus aureus*

(*S. aureus*) (Gram positive), *Escherichia coli* (*E. coli*), *Klebsiella oxytoca* (*K. oxytoca*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Proteus mirabilis* (*P. mirabilis*), *Salmonella typhi* (*S. typhi*), *Salmonella paratyphi* (*S. paratyphi*), *Vibrio parahaemolyticus* (*V. parahaemolyticus*) and *Vibrio cholerae* (*V. cholerae*) (Gram negative) were obtained from the Rajah Muthaiah Medical College, Annamalai University, Tamil Nadu, India. All the isolated bacteria were grown at room temperature in nutrient broth using standard procedures[14].

#### 2.3.2. Antibacterial activity

Antimicrobial activities of the starfish *S. equestris* extracts were tested by agar disc diffusion method[15]. For antibacterial activity, about 20 mL of sterile nutrient agar was poured in Petri dishes, allowed to set at 37 °C and then inoculated uniformly with 0.1 mL of 24 h broth cultures of test bacteria. Discs of Whatman No. 1 filter paper were prepared and autoclaved at 121 °C for 15 min. Each sterile disc was then dipped in 100 µL of the various concentrations of the extract and carefully placed on the agar plate using flame sterilised forceps, ensuring the discs were at least 2 cm separated from one another. After 30 min, the plates were inverted and incubated at 37 °C for 24 h. The diameter of each zone of inhibition was then measured in mm. Results were compared to the positive controls streptomycin.

#### 2.4. Minimum inhibitory concentration assay

The crude (methanol and ethanol) and fractionated extract of starfish *S. equestris* extract which showed significant antimicrobial activity, was selected for the determination of minimum inhibitory concentration (MIC) [16]. A stock solution of 1 mg/mL was prepared and serially diluted to obtain various ranges of concentrations of 25%, 50%, 75% and 100%. A total of 0.5 mL of each concentration was transferred into sterile test tube containing 2 mL of nutrient broth. To the test tubes, 0.5 mL of test organism previously adjusted to a concentration of 10<sup>5</sup> cells/mL was then introduced. A set of test tubes containing broth alone was used as control. All the test tubes and control were then incubated at 37 °C for 24 h. After the period of incubation, the tube containing the least concentration of extract showing no visible sign of growth was taken as the minimum inhibitory concentration.

#### 2.5. Gas chromatography–mass spectrography (GC–MS) analysis of crude extract

GC–MS analysis were carried out in a GC Clarus 500 PerkinElmer system and gas chromatograph interfaced to a mass spectrometer, employing the following conditions: column Elite–1 fused silica capillary column

(30 m×0.25 mm, 1D×1 µm df, composed of 100% dimethyl polysiloxane). For GC–MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate 1 mL/min, and an injection volume of 2 µL was employed (split ratio of 10:1). Injector temperature was –250 °C and ion–source temperature was 280 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min) with an increase of 10 °C/min to 200 °C/min, then 5 °C/min to 280 °C/min, ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 second and fragments from 40 to 550 Da. Total GC running time was 36 min. The relative percentage (%) of each component was calculated by comparing its average peak area to the total areas.

## 2.6. NMR analysis

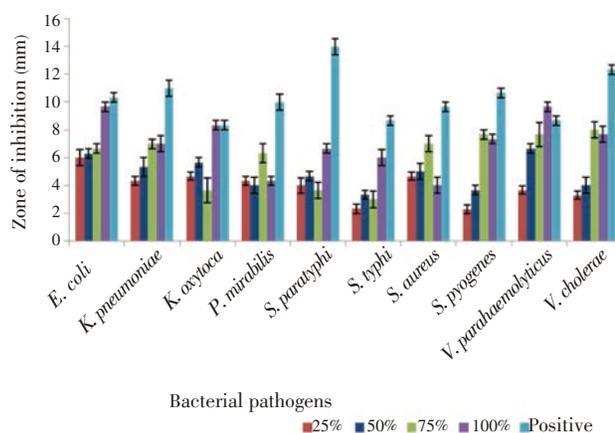
<sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded in Py–d<sub>5</sub> on a Bruker AM 500 spectrometer in 400 MHz. The sample was dissolved in deuterated DMSO solvent, and <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded using a Bruker Advance–400 MHz NMR spectrometer with cryoprobe. Spectra were referenced to residual <sup>1</sup>H and <sup>13</sup>C resonances in the deuterated solvent and recorded using standard Bruker pulse sequences.

## 3. Results

### 3.1. Antibacterial activity

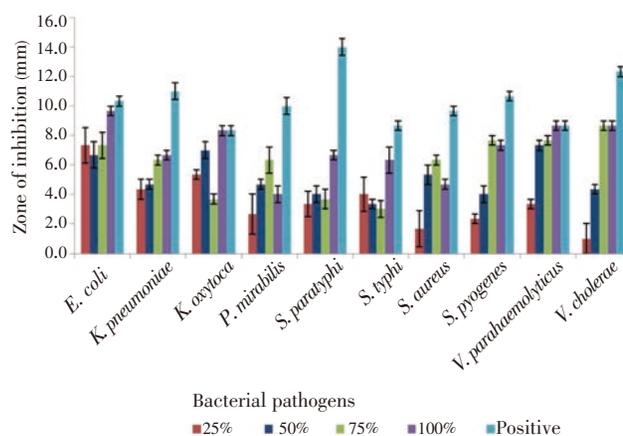
In the present study, antibacterial activities of starfish *S. equestris* crude extracts showed the zone of inhibition around the disc (Figure 1). Starfish *S. equestris* extracts showed a promising source of antibacterial activity against human bacterial pathogens. For the antibacterial activity, different concentrations such as 25%, 50%, 75% and 100% crude extract have been used. Streptomycin acted as positive control and solvent as a negative control. The 25% concentration showed the maximum zone of inhibition against *E. coli* [(6.0±0.6) mm], *K. oxytoca*, *S. aureus* [(4.7±0.3) mm each], *K. pneumoniae* and *P. mirabilis* (4.3±0.3 mm each). The 50% concentration exhibited zone of inhibition against *V. parahaemolyticus* [(6.7±0.3) mm], *E. coli* [(6.3±0.3) mm], *K. oxytoca* [(5.7±0.3) mm], and minimum was against *S. typhi* [(3.3±0.3) mm]. The 75% concentration showed the highest zone of inhibition against *V. cholerae* [(8.0±0.6) mm], *V. parahaemolyticus* [(7.7±0.9) mm], *S. pyogenes* [(7.7±0.3) mm] followed by *S. aureus* [(7.0±0.6) mm] and minimum was *S. typhi* [(3.0±0.6) mm]. The 100% concentration depicted the highest zone of

inhibition against *E. coli* and *V. parahaemolyticus* [(9.7±0.3) mm] followed by *K. oxytoca* [(8.3±0.9) mm], *V. cholerae* [(7.7±0.6) mm] and minimum was *S. aureus* [(4.0±0.6) mm]. One –way ANOVA analysis of antibacterial activity showed significant difference ( $P>0.05$ ) between the groups at 0.05% level.



**Figure 1.** Antibacterial activity of crude methanol extract of *S. equestris*.

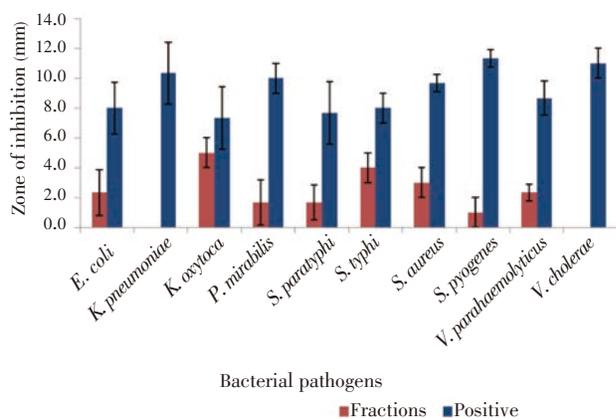
Antibacterial activity of ethanol extracts was showed in Figure 2. The maximum zone of inhibition was showed in *E. coli* [(9.70±0.33) mm] followed by *V. parahaemolyticus*, *V. cholerae* [(8.70±0.33) mm] and *K. oxytoca* [(8.30±0.33) mm], whereas the lowest concentrations exhibited the minimum zone of inhibition against all bacterial pathogens. One–way ANOVA analysis of antibacterial activity showed 0.05% level significance between the groups ( $P>0.05$ ).



**Figure 2.** Antibacterial activity of crude ethanol extract of *S. equestris*.

The antibacterial activity of purified fraction was showed in Figure 3. Streptomycin acted as positive control and solvent acted as a negative control. The maximum inhibition zone was observed against *K. oxytoca* [(5.00±0.82) mm], *S. typhi* (5.00±0.82) and *S. aureus* [(3.0±2.3) mm]. The positive control showed highest inhibition activity against *S. pyogenes* [(11.3±5.5) mm], *V. cholerae* [(11.00±5.82) mm] and *K. pneumoniae* [(10.30±5.84) mm]. One way ANOVA analysis of antibacterial activity showed no

significant difference at 0.05% level ( $P>0.05$ ).



**Figure 3.** Antibacterial activity of fraction of *S. equestris*.

### 3.2. MIC assay

The MIC was showed in Table 1–3. Ethanol extract of *S. equestris* depicted the MIC values against *E. coli* (100%), *K. pneumoniae* (75%), *S. typhi* (100%) and *V. cholerae* (100%). Whereas the methanol extract showed the MIC values of *K. pneumoniae*, *K. oxytoca*, *Salmonella paratyphi* and *V. parahemolyticus* at 100%. The 75% concentration exhibited the MIC values against *P. mirabilis*, *S. aureus* and *S. pyogenes*. No growth was observed in *P. mirabilis*, *S. typhi*, *S. aureus* and *V. cholerae* at 100% concentration. The fraction exhibited the MIC values against *K. pneumoniae*, *P. mirabilis*, *S. typhi*, *S. aureus* and *V. cholerae* at 100% whereas *E. coli* was at 75% concentration. No bacterial growth was observed against *K. oxytoca* at 100% concentration.

### 3.3. Mass spectral analysis

The GC–MS spectrum of crude ethanol extract of *S. equestris* was observed that methanol extract showed the presence of compounds with retention times at RT 17.36 (Figure 4).

**Table 1**

MIC of ethanol extract of starfish *S. equestris* against human bacterial pathogens.

Bacterial pathogens	Ethanol extract			
	100%	75%	50%	25%
<i>E. coli</i>	*	+	++	+++
<i>Klebsiella pneumoniae</i>	–	*	++	+++
<i>K. oxytoca</i>	++	++	+++	+++
<i>P. mirabilis</i>	–	*	+	+++
<i>Salmonella paratyphi</i>	+	++	+++	+++
<i>S. typhi</i>	*	+	++	+++
<i>Staphylococcus aureus</i>	–	*	++	+++
<i>Streptococcus pyogenes</i>	–	*	+++	+++
<i>V. parahemolyticus</i>	+	++	+++	+++
<i>V. cholera</i>	*	+	+++	+++

\*: MIC concentration; –: No growth; +: Cloudy solution (slight growth); ++: Turbid solution (strong growth); +++: Highly turbid solution (dense growth).

**Table 2**

MIC of methanol extracts of starfish *S. equestris* against human bacterial pathogens.

Bacterial pathogens	Methanol extract			
	100%	75%	50%	25%
<i>E. coli</i>	+	++	+++	+++
<i>Klebsiella pneumoniae</i>	*	++	++	+++
<i>K. oxytoca</i>	*	+	++	+++
<i>P. mirabilis</i>	–	*	++	+++
<i>Salmonella paratyphi</i>	*	+	+++	+++
<i>S. typhi</i>	–	+	+++	+++
<i>Staphylococcus aureus</i>	–	*	++	+++
<i>Streptococcus pyogenes</i>	–	*	+++	+++
<i>V. parahemolyticus</i>	*	+	+++	+++
<i>V. cholera</i>	–	+	+++	+++

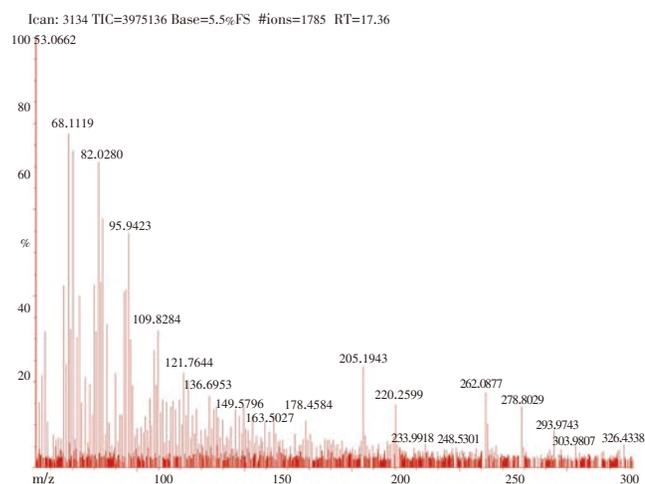
\*: MIC concentration; –: No growth; +: Cloudy solution (slight growth); ++: Turbid solution (strong growth); +++: Highly turbid solution (dense growth).

**Table 3**

MIC of fraction of starfish *S. equestris* against human bacterial pathogens.

Bacterial pathogens	Fraction			
	100%	75%	50%	25%
<i>E. coli</i>	–	*	+++	+++
<i>Klebsiella pneumoniae</i>	*	++	++	+++
<i>K. oxytoca</i>	–	++	+++	+++
<i>P. mirabilis</i>	*	+	++	+++
<i>Salmonella paratyphi</i>	+	++	++	+++
<i>S. typhi</i>	*	+	++	+++
<i>Staphylococcus aureus</i>	*	+	++	+++
<i>Streptococcus pyogenes</i>	+	++	+++	+++
<i>V. parahemolyticus</i>	+	++	+++	+++
<i>V. cholera</i>	*	+	+++	+++

\*: MIC concentration; –: No growth; +: Cloudy solution (slight growth); ++: Turbid solution (strong growth); +++: Highly turbid solution (dense growth).



**Figure 4.** GC–MS spectrum of starfish *S. equestris*.

The mass spectrum showed peak at  $m/z=262$  which is due to the loss of alkyl chain and one methyl group from

the ring. Peak at  $m/z=248$  is due to the loss of alkyl chain and two methyl group and  $m/z=223$  is due to the loss of alkyl chain, two methyl groups from ring and loss of hydroxyl groups. They are consistent with the molecular weight of the proposed structure (*i.e.*, 416.72 kDa).

Based on above informations, the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of the present compound contains signals for five ethyl groups, 6 methylheptan, 23 dimethyl groups and single protons. This data together with FT-IR and GC-MS spectrum contains  $m/z$  262, 232 suggest the molecular weight of 416.72 kDa and molecular formula of  $\text{C}_{29}\text{H}_{52}\text{O}$  corresponding to compound (3S, 5S, 8R, 9S, 10S, 13R, 14S, 17R)-17-(2R, 5R)-5-ethyl-6-methylheptan-2-yl)-10, 13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-3-ol derivatives of steroidal sulfate named as stigmastanol.

#### 4. Discussion

Marine organisms have been found to produce a great diversity of novel bioactive secondary metabolites and be a potential source for new drug discovery. Extensive investigations on starfish, chemically and pharmacologically, have been published in sufficient literatures. Several drug discovery projects have screened echinoderms for antibiotic activities<sup>[17–19]</sup>. Earlier studies have described a wide range of antibacterial compounds from echinoderms<sup>[20,21]</sup>. Antimicrobial activity has also been reported in egg extracts of echinoid *Paracentrotus lividus* (*P. lividus*)<sup>[22]</sup> and the asteroid *Marthasterias glacialis*<sup>[23]</sup>. In the northern Gulf of Mexico, 80% of 22 echinoderm species showed antimicrobial activity<sup>[10]</sup>. Body wall extracts of echinoderms displayed inhibitory activity against marine fouling bacteria and also inhibit settlement of marine larvae<sup>[24]</sup>. In the present study, the starfish *S. equestris* was extracted by using methanol and 70% ethanol solvents and was concentrated under reduced pressure and obtained a dark reddish gummy mass of 10 g. The active crude extracts was further purified and tested against the human bacterial pathogens. Some previous studies had depicted the antibacterial activity in coelomocyte/coelomic fluid of asteroids<sup>[22]</sup>, echinoids<sup>[20]</sup> and holothuroids<sup>[25]</sup>. Hence, the presence of several lysozyme or lysozyme like protein is identified from celomic fluid, celomocytes and other tissues of echinoderms<sup>[23,26]</sup>.

Several drug discovery projects have screened echinoderms for antibiotic activities. Gerardi *et al.*<sup>[27]</sup> found that the highest bacterial growth inhibition against several *Vibrio* sp. is shown by phagocytes (called amoebocytes) and red spherule cells of *P. lividus*. Stabili *et al.*<sup>[21]</sup> also reported antibacterial activity against *Vibrio alginolyticus* in celomocytes lysates and celomic fluid

of *P. lividus*. The celomocytes and body walls extracts are protein in nature. Several antimicrobial molecules (other than AMPs) have been isolated from echinoderms, including echinochrome A<sup>[28]</sup>, steroidal glycosides<sup>[29,30]</sup>, and polyhydroxylated sterols<sup>[31]</sup>. Srikumaran *et al.*<sup>[19]</sup> had reported the antibacterial activity of two starfishes, *Protoreaster lincki* and *Pentaceraster regulus* against human bacterial pathogens and biofilm bacteria. He had recorded the moderate zone of inhibition against the pathogens such as *S. paratyphi*, *K. pneumonia* [(6.100±0.124) mm] and fungal pathogen *Aspergillus flavus* [(12.00±0.63) mm] which showed most sensitive to n-butanol extracts. In fish bacterial pathogens assayed, *Streptococcus* sp., *Micrococcus* sp. [(5.00±0.33) mm] and fungal pathogen *Rhizopus* sp.1 [(8.00±0.33) mm] have been most sensitive to methanolic and n-butanol extract of *Protoreaster lincki*. Chamundeeswari *et al.*<sup>[31]</sup> have reported the antimicrobial potential of sea star *Astropecten indicus*. The maximum inhibiting zone of (13.44±0.20) mm was observed for bacteria *Pseudomonas aeruginosa* in the crude obtained from methanol followed by ethyl acetate [(11.26±0.09) mm] against *K. pneumonia* at the concentration of 1000  $\mu\text{L}$ . The results of the present study revealed that the highest concentration exhibited the maximum zone of inhibition than lowest concentrations of both methanol and ethanol extracts. The 100% concentration depicted the highest zone of inhibition against *E. coli* and *V. parahemolyticus* [(9.7±0.3) mm] followed by *K. oxytoca* [(8.3±0.9) mm], *V. cholerae* [(7.7±0.6) mm] and minimum was *S. aureus* [(4.0±0.6) mm]. One way ANOVA clearly demonstrated 0.05% significant level of activity.

Jung<sup>[32]</sup> reported that the fractions obtained from *Asterias amurensis* and *Asterina pectinifera* showed strong activity against various human bacterial and fungal pathogens and also affirmed that the pre-treated fractions with protease showed stronger result than the positive control used. Li *et al.* had reported many AMPs from echinoderms which exhibited strong antimicrobial activity against bacterial, fungal and viral pathogens<sup>[33]</sup>. In a coelomocyte extract from the starfish *Asterias rubens* showed antimicrobial activity and several protein/peptides with molecular mass around 2 kDa were isolated<sup>[34,35]</sup>.

Bauregard *et al.* first detected the presence of AMPs in the celomic fluid of the sea cucumber *Cucumaria frondosa*<sup>[25]</sup>. Villasin and Pomory reported the antibacterial activity of extracts from the body wall of Holothuroidea *Parastichopus parvimensis*<sup>[36]</sup>. The methanol-acetone extract of the body wall of the sea cucumber *Parastichopus parvimensis* had antibacterial properties. The means of the areas of inhibition for the extract and antibiotics were significantly different from that of the blank control ( $P<0.01$ ) for both *Bacillus subtilis* and *E. coli*. The

statistical analysis of present study would exhibit the 0.05% significant of both crude and fractions ( $P>0.05$ ). There was no area of inhibition around the blank control discs. The antibiotics produced areas of inhibition much greater than that of the extract.

The major components in the ethanol extract could have been responsible for the antibacterial activity. The present study depicted 100% MIC values against *E. coli*, *S. typhi*, *S. paratyphi*, *P. mirabilis*, *S. aureus*, *V. cholerae* and *K. pneumonia* in methanol, ethanol and fraction of *S. equestris* extract. Our results are in agreement with the earlier antibacterial activity of marine echinoderm, *P. lividus* against *Vibrio alginolyticus*[21]. Further, it has been reported that different body parts of the sea urchin, *Strongylocentrotus droebachiensis* possessed antibacterial activity[9]. Uma and Parvathavarthini reported the lowest (2.5 mg/L) MIC for *Bsubtilis subtilis* and *Pseudomonas aeruginosa* of the sea urchin, *Temnopleurus alexandri* extracts[37,38].

In conclusion, results obtained from the present study would suggest that the extract of sea star *S. equestris* is an interesting source of antibacterial compounds, and it could be used as lead source in the development of the potent antibacterial drugs.

### Conflict of interest statement

We declare that we have no conflict of interest.

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### Comments

#### Background

The research paper details the antibacterial activity and extraction and analysis of crude from the starfish *S. equestris* from southeast coast of India. The antibacterial studies and compound characterization from the above said organism are very few elsewhere. So the present area of research is needed.

#### Research frontiers

The drug discovery from the starfishes is an emerging

area of research. The authors have successfully carried out the antibacterial activity of the extract and analysed the same by GC–MS and NMR.

#### Related reports

The extraction and other analysis were carried out by following the published standard procedures and references.

#### Innovations and breakthroughs

The readers can be able to follow the procedures and methods quoted in the present paper for their future studies and can apply in the case of other organisms.

#### Applications

The authors can be able to move to the area of drug discovery from the extracts of the starfishes (which is being landed as by–catch) and can apply for patents in future.

#### Peer review

The present research paper comprehensively depicted the antibacterial activity and extraction of crude from the starfish *S. equestris* from southeast coast of India. They methodically carried out the analysis with authentic current techniques such as GC–MS and NMR.

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