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Research Article

# Antimicrobial activity of various extracts of the sea urchin *Tripneustes gratilla* (Echinoidea)

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**Background:** Marine invertebrates rely solely on innate immune mechanisms, the cellular component of which is characterized by hemocytes that phagocytize microbes and secrete soluble antimicrobial and cytotoxic substances. In this regard, marine invertebrates are a potential source of promising antimicrobial compounds with novel mechanisms of action.

**Objective:** The objective of this study was to evaluate extracts of the gut, gonad, spines and mouth parts of the sea urchin *Tripneustes gratilla* for antimicrobial and haemolytic activities *in vitro*.

**Methods:** Potentially bioactive metabolites were extracted using methanol and chloroform and tested for activity against *Salmonella typhi*, *Escherichia coli*, *Shigella sonnei*, *Pseudomonas aeruginosa* and *Penicillium* spp. using the agar disc diffusion method. Toxicity was determined by assaying for hemolysis against human red blood cells.

**Results:** Bioactivity against the tested bacteria was observed mainly with the methanol and chloroform extracts of the gonads and gut. Higher antibacterial activity was present in the methanol extracts compared to chloroform extracts. Activity against the *Penicillium spp* was detected only in the methanol extracts, while the chloroform extracts showed no activity. The various extracts of the sea urchin lacked any detectable hemolytic activity against human erythrocytes.

**Discussion:** These research findings suggest that marine echinoderms are a potential source of novel antimicrobial compounds.

**Key words:** *Tripneustes gratilla,* antimicrobial activity, marine invertebrates

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## 1. Introduction

The recent appearance of a growing number of bacteria resistant to conventional antibiotics has stimulated the search for novel antimicrobial agents or lead compounds from a variety of sources, including natural sources.

Microbial populations in seawater and sediments may be as high as $10^6$ and $10^9$ per milliliter, respectively (Austin, 1988). Marine invertebrates are therefore constantly exposed to high concentrations of bacteria, fungi and viruses, many of which may be pathogenic. The survival of these organisms depends on efficient antimicrobial mechanisms to protect themselves against microbial infections. During the last decade, there has been an increase in research on marine

crustaceans, molluscs and echinoderms, with particular interest on their secondary metabolites with desirable antimicrobial properties (Haug et al, 2002, Casas et al, 2010).

Antimicrobial activity in several species of echinoderms collected from Gulf of California, Mexico, Caribbean and Coast of Norway has been reported (Rinehart et al, 1981; Bryan et al, 1994; Haug et al, 2002). In addition, a variety of antimicrobial factors, including steroidal glycosides (Andersson et al, 1989), polyhydroxylated sterols (Iorizzi et al, 1995), naphthoquinone pigments (Service and Wardlaw, 1984), lysozymes (Canicatti and Roch, 1989; Stabili and Pagliara, 1994), complement-like substances (Leonard et al, 1990) and antimicrobial peptides (Beauregard et al, 2001) have also been isolated from echinoderms. These findings suggest that marine echinoderms are a potential source of new types of antibiotics for pharmaceutical development.

Unfortunately, in most of these studies on antimicrobial activity in echinoderms, whole bodies or body walls have been tested for activity. Recently, Haug et al. (2002) observed wide differences in antibacterial activities between different extracts and organs/tissues, as well as between species, of three echinoderms. Whether the same antibacterial factors are responsible for the activity in all organs or tissues remains unclear.

The present work focused on screening and comparing antimicrobial and hemolytic activities in different organs/tissues of the sea urchin *Tripneustes gratilla* (Echinoidea) collected from the Kenyan Coast.

## 2. Materials and Methods

### 2.1 Experimental animals and sample collection

Live specimens of sea urchins *T. gratilla* were randomly collected from the Indian Ocean at Bamburi area lagoon (Kenya) in August 2008. All the animals were maintained in circulating seawater and transported to the laboratory. Length, width and sex of each animal were determined before dissection. Gonads, shell, spines, mouth part and gut from 20 sea urchins were carefully dissected, pooled and preserved in 100% methanol. The samples were then stored in the dark at room temperature to avoid photolysis and thermo-degradation of secondary metabolites prior to extraction.

### 2.2 Preparation of extracts

Samples (4 g) were homogenized and extracted with 10 volumes (v/w) of 70% (v/v) methanol or chloroform in a shaker (90 rev/ min at 10 °C, 24 hr). The supernatant for each sample was collected by centrifugation (12,000 g, 5 min, 4 °C) in a Heraeus 2 minifuge (Osterode, Germany) and stored at -20 °C. The supernatant of each extract was then filtered through a 0.2 µm Millipore filter (Nalge, Rochester, N.Y. USA) and the sterile filtrate used for the antimicrobial agar disc diffusion assay.

### 2.3 Test microorganisms and culture media

Test microorganisms *Escherichia coli* (gram-negative), *Salmonella typhi* (gram-positive), *Pseudomonas auregenus* (gram-positive), *Shigella sonnei* (gram-negative), *Streptococcus aureus* (gram-positive) and *Penicillium spp.* used in these studies were obtained from the Cell Biology laboratory, Department of Biochemistry, University of Nairobi. All the isolated bacteria were grown at room temperature in nutrient broth (Difco Laboratories, Detroit, USA) using standard procedures (WHO, 1991). To test for xerophilic bacteria, test organisms were grown on 3% NaCl concentrated nutrient agar.

### 2.4 Antimicrobial assay

Antimicrobial activities of the sea urchin tissue extracts were tested by agar disc diffusion method. For antibacterial activity, 20 ml of sterile nutrient agar was poured in petri dishes, allowed to set at 37 °C and then inoculate uniformly with 0.1 ml of a 24 hr broth culture of test bacteria. For antifungal activity, 20 ml of Saboroud agar were poured and allowed to set before inoculating uniformly with 0.1 ml of test fungi.

Discs of Whatman no. 1 filter paper were cut out using an office punch and autoclaved at 121 °C for 15 min. Each sterile disc was then dipped in 100 µl of the various extracts and carefully placed on the agar plate using flame sterilized forceps, ensuring the discs were at least 2 cm separate from one another. After 30 min, the plates were inverted and incubated at 37 °C for 24 hr. The diameter of each zone of inhibition was then measured in mm. Results were compared to the positive controls penicillin, chloramphenicol, methicillin, minocyclin, coticyclin, ampicillin, erythromycin and linomycin.

### 2.5 Haemolytic assay

To test whether the sea urchin contained factors which are toxic to eukaryotic cells, the hemolytic activity of extracts from spine, mouth parts, guts and gonads was determined using fresh human red blood cells (RBC). Blood (4 ml) were collected from a healthy individual using heparin as anticoagulant and centrifuged (450 x g, 10 min) to isolate the red blood cells (RBC). The pellet containing RBCs was washed three times with phosphate-buffered saline (PBS; 50 mM $KH_2PO4$, 150 mM NaCl, pH 7·4) in order to remove the plasma and 'buffy coat'. The cell pellet was resuspended in 4 ml of PBS. The hematocrit value (Hct) was determined and the RBC suspension was further diluted to a Hct value of 10%.

The test samples were diluted to a protein concentration of 500 g/ml and the test performed in 96 well U-shaped microtitre plates (Nunc, Denmark). To each well, 50 µl extract was mixed with equal volume of PBS and 10 µl of RBC added. The plates were then incubated in a shaker at 37 °C for 1 hr and centrifuged (200 x g, 5 min). The supernatants were carefully transferred to new flat bottomed microtitre plates (Nalge Nunc, Int, Denmark) and the absorbance measured at 550 nm. Baseline hemolysis and 100% hemolysis were expressed as the amount of hemoglobin released in the presence of PBS and 0·1% Triton X-100 (Sigma), respectively.

**Table 1:** Antibacterial activity of the sea urchin (*T. gratilla*) methanol extracts

| Organ/tissue extract | Zone of inhibition (mm) | | | | | |
|---|---|---|---|---|---|---|
| | ***E. coli*** | ***Staph. aureus*** | ***Shig. sonnei*** | ***Sal. typhi*** | ***P. aeruginosa*** | ***Pen.spp*** |
| Gonads | 16 | 13 | 11 | 18 | 18 | 22 |
| Guts | 16 | 14 | 13 | 18 | 20 | 20 |
| Spine | 6 | - | 6 | - | - | - |
| Mouth parts | - | - | - | - | - | - |

*Antibacterial activity depicted by the diameter of the zone of inhibition (mm)*
*- No antimicrobial activity observed*

**Table 2:** Antibacterial activity of the sea urchin (*T. gratilla*) chloroform extracts

| Organ/tissue extract | Zone of inhibition (mm) | | | | | |
|---|---|---|---|---|---|---|
| | ***E. coli*** | ***Staph. aureus*** | ***Shig. sonnei*** | ***Sal. typhi*** | ***P. aeruginosa*** | ***Pen.spp*** |
| Gonads | 8 | 9 | 11 | 9 | 8 | 8 |
| Guts | 8 | 7 | 8 | 9 | 7 | 6 |
| Spine | - | - | - | - | - | - |
| Mouth parts | - | - | - | - | - | - |

*Antibacterial activity depicted by the diameter of the zone of inhibition (mm)*
*- No antimicrobial activity observed*

**Table 3:** Zones of inhibition of the bacteria by positive control

| Positive control | Zone of inhibition (mm) | | | | |
|---|---|---|---|---|---|
| | ***E. coli*** | ***Staph. aureus*** | ***Shig. sonnei*** | ***Sal. typhi*** | ***P. aeruginosa*** |
| Ampicillin | - | 20 | - | 7 | - |
| Chloramphenicol | 22 | 21 | 7 | 15 | - |
| Coticyclin | - | 23 | - | 30 | - |
| Methicillin | - | - | - | - | - |
| Erythromycin | - | 20 | 8 | 7 | - |
| Minocyclin | 17 | 25 | 15 | 35 | 10 |
| Penicillin | - | - | - | - | - |
| Linomycin | - | 7 | - | 7 | - |

*Antibacterial activity depicted by the diameter of the zone of inhibition (mm)*
*- No antimicrobial activity observed*

## 3. Results

Several of the extracts of the sea urchin *T. Gratilla* exhibited antimicrobial activity *in vitro.* As shown in **Table 1**, the antimicrobial activity against all the microorganisms tested was present mainly in extracts of the gonads and gut. In general, there was little or no activity detected in both the methanol and chloroform extracts of the spines and mouthparts.

The antimicrobial activity of the sea urchin extracts varied with the solvent used for extraction as well as with the bacterial or fungal strain used. The methanol extract exhibited the highest antimicrobial activity against the test organisms, specifically *P. aeroginosa*, compared to the chloroform extracts (**Tables 1** & **2**). Similarly, most of the methanolic gut and gonad extracts inhibited the growth of *Penicillium.spp* while the chloroform extracts showed much less inhibition.

For comparison, **Table 3** shows the activities of some established antibacterial drugs used as positive controls for this study: penicillin, chloramphenicol, methicillin,

minocyclin, coticyclin, ampicillin, erythromycin and linomycin.

Only *Staph. aureus, E. coli* and *Sal. typhi* grew on 3% NaCl concentrated nutrient agar; **Table 4** shows the antibacterial activity of the methanolic extracts against these three xerophilic bacterial strains. Most of the gonad and gut extracts exhibited high activity against *Staphylococcus aureus* and *Sal. typhi* compared to *E. coli* that survive at high salt concentrations.

There was no detectable hemolytic activity against human erythrocytes in all the extracts of the sea urchin tissues tested.

Images of selected experimental assay plates showing zones of inhibition can be found in **Supporting Information** (available online at http://www.uonbi.ac.ke/journals/kesobap/).

**Table 4:** Antibacterial activity of sea urchin methanol extracts against xerophilic bacteria

| Organ/tissue | Zone of inhibition (mm) | | |
|---|---|---|---|
| | *S. aureus* | *E. Coli* | *S. typhi* |
| Gonad [2] | 11 | 7 | 16 |
| Gut [2] | 10 | - | - |
| Gonad [3] | 9 | - | 12 |
| Gut [3] | 11 | - | - |
| Gonad [4] | - | 6 | 11 |
| Gut [4] | - | - | - |
| Gonad [5] | 10 | 10 | 10 |
| Gut [5] | 16 | 7 | - |
| Gonad [6] | 9 | - | 9 |
| Gut [6] | 8 | - | - |

*[2] - .immature sea urchin; [3] & [5] – female sea urchin; [4] spent; [6] – male sea urchin*
***NB:*** *Xerophilic test organisms were grown on 3% NaCl concentrated nutrient agar.*

## 4.0 Discussion

A screening for antimicrobial activity in extracts from different organs/tissues of the sea urchin *T. gratilla* was conducted. Several of the extracts of the sea urchin *T. gratilla* exhibited antimicrobial activity *in vitro.*

Antibacterial activity has previously been described in a wide range of echinoderm species (Anderson et al, 1983, 1989; Bryan et al, 1994; Ridzwan et al, 1995). In most of the species studied, the whole bodies or body walls were tested for activity.

Antimicrobial activity has also been reported in egg extracts of echinoid *Paracentrotus lividus* (Stabili et al, 1996a) and the asteroid *Marthasterias glacialis* (Stabili and Pagliara, 1994). In the latter study, the antibacterial compound was shown to be a lysozyme. The egg extracts of other marine invertebrates have also been shown to exhibit antimicrobial activity (Benkendorff et al, 2001; Haug et al, 2002). Both of these studies showed that at least some of the antibacterial compounds are not proteinaceous.

The present study shows that the antimicrobial activity of the sea urchin *T. gratilla* appears to be concentrated mainly in the gut and gonad extracts - little or no activity was observed in the spine and mouth extracts. Further study is required to establish if this observed activity is attributable to proteinaceous (including lysozyme-like) or non-proteinaceous factors.

Antimicrobial activity was observed in both the methanol as well as the chloroform extracts of the guts and gonads; however, higher inhibition was exhibited by the methanol extracts. Similarly, methanol extracts of gut and gonad inhibited the growth of *Penicillium Spp* suggesting presence of antifungal activity; activity against *Penicillium Spp,* was minimal in the chloroform extracts. As activity has been demonstrated against both gram-positive and gram-negative bacteria as well as against selected fungal species, it may therefore be reasonable to assume that multiple factors are responsible for the antimicrobial activities detected.

Furthermore, the fact that the antimicrobial activity was evaluated in non-stimulated *T. gratilla* suggests that the factor(s) responsible for the observed activities are constitutive. Whether the antimicrobial profile would change following microbial stimuli is yet to be determined.

All the tissues tested in this study did not exhibit hemolytic activity against human erythrocytes. This is an interesting observation since hemolytic activity appears to be common in extracts that show high antibacterial activity (Guzman et al, 1993; Haug et al, 2002). Nevertheless, the lack of hemolytic activity is an early indicator of potentially low toxicity of the chemical constituents of the *T. gratilla* extracts towards mammalian/human cells.

In conclusion, this study shows that extracts of the sea urchin *T. gratilla* exhibit antimicrobial activities, particularly the extracts of the guts and gonads. Differences between active extracts indicate that several different compounds could be responsible for the antimicrobial activities. Isolation and purification of the constituent active compounds is necessary in order to identify their chemical nature and to evaluate their potential as novel drugs.

## Conflict of Interest declaration

The authors declare no conflict of interest.

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