Antiseptic effect of sea cucumber (*Holothuria atra*) against multi-organ failure induced by sepsis: Molecular and histopathological study

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**Abstract.** Sepsis is a systemic inflammatory response to infection and severe sepsis patients can develop acute lung and liver injury. The aim of the present study was to evaluate the efficacy of *Holothuria atra* methanolic body wall extract (HaE), as an antioxidant and anti-inflammatory agent against induced sepsis in a cecal ligation and puncture (CLP) rat model. In total, 30 males albino rats were divided into three groups (n=10 each) as follows: Sham (Sh), which was used as negative control; sepsis (Se), which was used as a positive control and was subjected to CLP surgery; and Ho, which was subjected to CLP and fed with 200 mg/kg (body weight) of HaE, once daily for 7 days. Subsequently, the expression of various genes was detected by polymerase chain reaction, while liver and lung tissues were examined by immunohistochemistry. The expression of Caspase-3 was significantly reduced in liver and lung tissues in the Ho group, while the expression levels of Gsta2, Cat and Sod1 genes were slightly reduced in the Ho group, when compared with the Se group. In addition, expression levels of tumor necrosis factor, interferon-γ, liver interleukin (IL)1b and lung IL1a were reduced in the Ho group compared with the Se group. Furthermore, histopathological changes were observed in liver tissues of the Se group, including congestion of hepatoportal blood vessel and focal hepatic necrosis, while lung tissues showed marked edema, hemorrhage and alveolar septal thickening. The Ho group showed apparent normal hepatic parenchyma and slight interstitial pneumonia. Immunohistochemical staining of caspase-3 in liver and lung tissues showed no expression in the Sh group, strong expression in the Se group and moderate expression in the Ho group. In conclusion, HaE demonstrated beneficial effect against induced sepsis, which may be attributed to its antioxidant and antiapoptotic activities.

**Introduction**

Sepsis, one of the main issues encountered in the majority of health care centers, is a life-threatening disease that causes widespread mortality worldwide (1). The mortality rate of uncomplicated sepsis is ~25%, increasing to 80% in patients that proceed to develop multiple organ failure (2). Sepsis is associated with the presence of pathogenic microorganisms or their toxins in the bloodstream. Oxidative stress has been consistently reported in patients with sepsis, and thus antioxidants may be used as a potential therapy (3). However, the effect of antioxidants administrated in septic shock is limited (4); therefore, the expression levels of certain antioxidants (such as the glutathione S-transferases gene family), oxidative stress responsive genes (including oxidases, peroxidases, catalase and superoxide dismutase) or anti-inflammatory genes (such as interleukins) can be detected in the tissues in response to sepsis or any curative treatment (5,6). Oxidative stress is indicated by increased levels of lipid peroxides, direct detection of circulating radicals and decreased antioxidant concentrations (7).

Mitochondrial dysfunction resulting from oxidative stress has been suggested to serve a role in the development of multi-organ failure in sepsis, including liver and lung failure (3,8). Approximately 50% of patients with severe sepsis also develop acute lung injury (9,10). Pericytes in lung tissue have been shown to produce an increase in pro-inflammatory cytokines in response to bacterial lipopolysaccharide (LPS) (11).

Tumour necrosis factor (TNF) and interferon-γ (IFN-γ) are of particular importance in the development of septic shock (12-15). Therefore, the development of individual sepsis treatments has focused on the regulation of TNF expression (16,17). However, previous clinical trials investigating specific anti-TNF sepsis treatments have demonstrated the complexity of this disease and the involvement of various cytokines with overlapping functions (17). IFN-γ is also a crucial regulator of LPS-induced pathology (18,19). Treatment with IFN-γ or a neutralizing antibody against IFN-γ has been found to alter the lethal outcomes of several types of

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Gram-negative bacterial infections and endotoxins (19). IFN-γ receptor-deficient mice are relatively resistant to LPS-induced septic shock (20,21).

INF-γ serves an important role in the regulation of innate and acquired antimicrobial immunity. The expression of INF-γ is regulated by a set of complex interactions between accessory cells, such as macrophages, dendritic cells, T lymphocytes and natural killer cells (22,23). INF-γ amplifies antimicrobial immune responses by stimulating macrophage functions such as phagocytosis, respiratory burst activity, antigen presentation and cytokine secretion (18,21,24). Previous studies have suggested that IFN-γ may increase the responsiveness to LPS by altering the signal transduction pathway, in particular through the upregulation of the Toll-like receptor 4 gene expression (25), or promotion of IL-1 receptor-associated kinase expression and its association with myeloid differentiation primary response gene 88 (26).

Despite recent advances in antibiotic therapy, no significant improvements have been accomplished in the treatment of sepsis. Thus, there is an urgent demand for the development of a novel therapy for sepsis management. The increase of antibiotic-resistant pathogenic bacteria has stimulated the search for antimicrobial agents from alternative sources (27,28). Crude extract from the sea cucumber, also known as Holothuria atra (H. atra), contains cystostatic, antifungal, hemolytic, anticancer and antioxidant phenolic compounds, while it also has immunomodulatory effects. This extract has been found to have a potential hepatoprotective activity against thiocacetaamide-induced liver injury in a rat model (29). In addition, the curative effects of the sea cucumber extract were reported against DMBA-induced hepatorenal diseases in rats (30,31).

Apoptosis, another prominent feature of sepsis, involves a mechanism of closely-regulated disassembly of cells resulting from the activation of caspases, which are specialized proteases. In septic animal models, increased apoptotic cell death has also been reported in parenchymal cells, including intestinal and lung epithelial cells (32,33). Furthermore, in situ localization of cleaved caspase-3 may have an application in the histological labeling of cells in apoptosis (34). Lysoospholipids from H. atra were also shown to inhibit H2O2-induced apoptosis in macrophages (35).

Cecum ligation and puncture (CLP) is currently the most widely used animal model of sepsis (36,37). In the CLP rat model, autophagy was also induced in multiple organs, including the lung and liver (38,39). In the present study, the potent in vivo efficacy of sea cucumber body wall extract against induced sepsis in a CLP rat model was investigated at molecular and histopathological levels.

Materials and methods

Sample collection and preparation of H. atra extract (HAE).

Sea cucumbers (H. atra; n=50) were collected from the Thuwal area on Saudi Arabia's Red Sea coast. The taxonomic identity of the samples was confirmed based on the studies of Purcell et al (40). The animals were transported to the Medical Laboratory of Applied Medical Sciences, Taif University, (Turabah, Saudi Arabia), in an ice box. They were rinsed thoroughly, removing any internal organs and body fluids, and then the animals' body wall was soaked in appropriate amounts of methanol-water mixture (50:50) and stirred using a magnetic stirrer for 16 h. The mixture was filtered twice. Finally, the two extracts were pooled together and concentrated in a rotary evaporator during which the extract was evaporated at low pressure in a double boiler at 30°C using a LABROTA 4001 efficient (Heidolph Instruments GmbH & Co., Schwabach, Germany) to avoid degradation of compounds, for 2 h. The powdered extract was obtained by freeze drying and was stored at -20°C until further use.

Animals. All animal procedures were approved by the Ethical Committee Office of the Scientific Dean of Taif University (Taif, Saudi Arabia).

In total, 30 adult male albino rats (Rattus norvegicus; age, 6–8 weeks) weighing 150-170 g were obtained from the King Fahd Research Unit at King Abdulaziz University (Jeddah, Saudi Arabia). The rats were housed in polypropylene cages in an air conditioned room at a temperature of 25±2°C and under natural light cycle. They were fed standard chow pellets and had access to water ad libitum. The rats were kept for 1 week for acclimatization and then divided into three groups (10 animals in each) as follows: Sham (Sh) group, which was used as a negative control (received distilled water and underwent surgery along with cecal manipulations, but without ligation and puncture); sepsis (Se) group, which was surgically subjected to CLP [sepsis was achieved in rats by cecal ligation at a point ~1 cm from the cecal tip and punctured with a 20-gauge needle (36)], and was used as positive control; and the Ho group, in which animals were subjected to CLP and orally administered 200 mg/kg body weight HAEE, once daily for 7 days. All groups were handled under sterile and antisepctic conditions. All animals were sacrificed by inhalation of diethyl ether, and dissected after 7 days. The animals were observed daily subsequent to the surgery. The mortality rate was calculated in all groups after the incidence of the initial case of mortality. The death rate was scored each day for 7 days until the end of the experiment. The mortality rate was presented as a percentage.

The rat organs (liver and lung) were collected and applied for RNA extraction and histopathological examinations.

Mortality rate study. Following CLP surgery, the mortality rate and symptoms of sepsis were calculated over the subsequent 7 days for the 3 experimental groups. Rat mortality was recorded every 24 h until the 7th day and is expressed as a percentage.

RNA extraction and quantification. Total RNA was extracted from the liver and lungs of all rat groups according to the method described by Attia et al (42). RNA samples were diluted in diethylpyrocarbonate water to 40 µg/ml according to spectroscopy quantification using a Bio-Rad SmartSpec Plus UV/Visible Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Primers. All primers were designed (based on the gene sequences published in the GenBank database) using the Primer3Plus online software (http://primer3plus.com/). The primers were manufactured by Bioron GmbH (Ludwigshafen,
Germany). Primers sequences and polymerase chain reaction (PCR) product sizes are presented in Table I. The investigated genes included Caspase-3, glutathione S-transferase α2 (Gsta2), catalase (Cat), superoxide dismutase 1 (Sod1), TNF, IFN-γ, interleukin 1 α (Il1a) and interleukin 1 β (Il1b).

**Semi-quantitative reverse transcription-PCR (RT-PCR) analysis.** Total RNA (2 µg) was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific Inc., Waltham, MA, USA). The reaction was incubated for 60 min at 42°C and terminated by heating at 70°C for 5 min. Next, 1 ml cDNA was used for PCR analysis, which was performed using a Perkin Elmer GeneAmp 9600 system (PerkinElmer, Inc., Waltham, MA, USA). The PCR cycling conditions were as follows: Initial cycle of 10 min at 95°C, 45 sec at 54°C and 1 min at 72°C; followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 45 sec and extension at 72°C for 1 min; and then a final extension step at 72°C for 7 min. The total volume of reactions mixture was 25 µl and contained 1 unit of AmpliTaq Gold (Applied Biosystems; Thermo Fisher Scientific Inc.), 1X AmpliTaq buffer, 1.5 mM MgCl₂, 2.5 mM dNTPs and 10 pmol of forward and reverse primers. The expression of Gapdh was detected as a reference value using specific primers (Table I).

A negative control containing RNA was used to rule out genomic DNA contamination. The PCR products were confirmed by 2% agarose gel electrophoresis, and the band density was measured using ImageJ version 1.48 software (http://imagej.nih.gov/ij/).

**Histopathological examination.** Tissue samples collected from the liver and lungs of rats were fixed in 10% neutral buffer formalin solution, washed in tap water, dehydrated through an upgraded series of ethanol (50, 70, 80, 90 and 95%, followed by absolute ethanol), cleared by xylene and then embedded in paraffin. The paraffin-embedded samples were cut into 5 µm sections, which were then routinely stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA) as previously described (43).

**Immunohistochemical analysis.** The paraffin-embedded samples were cut into 3-µm sections and mounted on positively charged slides for caspase-3 Immunohistochemical examination. Sections were dewaxed, rehydrated and autoclaved at 95°C for 20 min in antigen retrieval buffer (10 mM citrate buffer, pH 6). After washing with phosphate-buffered saline (PBS), endogenous peroxidase was blocked using 3% H₂O₂ in methanol for 15 min. A primary rat-specific antibody for caspase-3 (cat.no. RB-1197-B0.-B1; Thermo Fisher Scientific Inc.) was added following dilution in PBS (1:100), cleared by xylene and then embedded in paraffin. The paraffin-embedded samples were cut into 5 µm sections, which were then routinely stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA) as previously described (43).

Table I. Primers sequence used to amplify a partial sequence of the target gene.

| Gene   | Accession no. | Primer sequence | Primer position | Band size (bp) |
|--------|---------------|-----------------|-----------------|---------------|
| Caspase-3 | NM_012922 | F: 5'-TTGGCTTTGAGCTACC-3' | 1,540 | 400 |
|         |              | R: 5'-CCAGAGCTCTGTCGTA-3' | 1,939 |     |
| Gsta2  | NM_017013  | F: 5'-GGCAAAGACAGGCACCA-3' | 432  | 231 |
|         |              | R: 5'-GGCTGAGCAACTTTCTC-3' | 662  |     |
| Cat    | NM_012520  | F: 5'-GACACATCGGTCTACAT-3' | 1,130 | 216 |
|         |              | R: 5'-GAGCCTAAGCTGAATGC-3' | 1,367 |     |
| Sod1   | NM_017050  | F: 5'-CCACTGAGGACCTCTTT-3' | 269  | 484 |
|         |              | R: 5'-CACCTTTGACCGATCATC-3' | 484  |     |
| TNF    | NM_012675  | F: 5'-ATGGGCTCCTCTCAGT-3' | 341  | 547 |
|         |              | R: 5'-GGCTGGGTAGAAGGATG-3' | 887  |     |
| IFN-γ  | AH002184   | F: 5'-TCCCTCCCACCAATTAGG-3' | 1,130 | 526 |
|         |              | R: 5'-ATTCCTCTGTCGCA-3' | 1,655 |     |
| Il1a   | NM_017019  | F: 5'-CATCGAGCTCATGCTTT-3' | 1,726 | 172 |
|         |              | R: 5'-CTTGGGTCATGAATGTTG-3' | 1,897 |     |
| Il1b   | NM_031512  | F: 5'-AGGCTTTGTCAGAGTGT-3' | 26   | 255 |
|         |              | R: 5'-TGAGTGACATGCTTCCTG-3' | 255  |     |
| Gapdh  | NM_017008  | F: 5'-AGACAGCGCCATCTTCTG-3' | 28   | 323 |
|         |              | R: 5'-TACTCAGCAGCAGCATC-3' | 350  |     |

Gsta2, glutathione S-transferase α2; Cat, catalase; Sod1, superoxide dismutase 1; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin.

Figure 1. Mortality rate of rats in the sham, sepsis group (cecum ligation and puncture) and septic group treated with *Holothuria atra* extract (Ho group).
and incubated for 30 min. The slides were then washed three times for 3 min each with PBS. Subsequently, a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (cat. no. 32230; Thermo Fisher Scientific Inc.) was applied to the tissue sections and co-incubated for 30 min.

The slides were washed three times for 3 min each with PBS, and then visualized by adding metal enhanced DAB substrate working solution (Thermo Fisher Scientific Inc.) to the tissues and incubating for 10 min. Next, the slides were washed two times with PBS (3 min each time) and then
counterstained by adding an adequate amount of hematoxylin to the slide to cover the entire tissue surface (44). The immune reactivity score used to evaluate the intensity of immunohistochemical staining and the proportion of the stained cells was classified as i) absent (0); ii) mild, 25-50%; and iii) strong, >50%.

Statistical analysis. The results are expressed as the mean ± standard error of 10 different rats per group. Statistical analysis was performed with analysis of variance and Fisher’s post-hoc test, with P<0.05 considered to indicate statistically significant differences.

Results

Mortality rate following CLP surgery. At 24 h after CLP surgery, the rats clearly displayed the sepsis symptoms, such as decreased motor activities, ocular exudates and ruffled fur. In the Sh group, the mortality rate was 10% at 24 h decreasing to 0% after 48 h. In the Se group, the mortality rate was 20% after 24 h from CLP surgery, increasing to 100% after 7 days in comparison with the negative group (Sh). Although the mortality rate was 10% at 48 h after the CLP surgery in the Ho-treated group, it decreased to 0% after 72 h and remained at this rate until the end of the experiment (Fig. 1).

Molecular detection of gene expression. Eight genes, including Caspase-3, Gsta2, Cat, Sod1, TNF, IFN-γ, Il1b and Il1a, were tested for their expression in the liver and lungs of the adult male albino rats in the three groups at 7 days after CLP (Fig. 2), with the expression of Gapdh used as an internal control. As shown in Fig. 2A, the expression of Caspase-3 was found to be significantly increased (P<0.05) in the liver and lung tissues of the Se group in response to sepsis when compared with the negative control (Sh) tissues; however, the expression was reduced in the Ho group compared with the Se group. In addition, the expression of the antioxidant Gsta2 gene was significantly increased (P<0.05) in the organs of the Se compared with the Sh group, whilst the Ho group did not display a significant difference compared with the Se group (Fig. 2B), but it was lower in the Ho group. Expression levels of the oxidative stress responsive genes, Cat and Sod1, were increased in the two organs in response to sepsis stress in the Se and Ho groups (Fig. 2C and D). Expression was significantly increased in Se group compared with the Sh group (P<0.05), whilst the Ho group did not display any significance compared with Se group. Furthermore, the expression levels of anti-inflammatory genes, TNF and IFN-γ, as well as of the liver tissue-specific Il1b and the lung tissue-specific Il1a, were significantly increased in the Se group in response to sepsis when compared with the Sh group, whereas the expression of the aforementioned genes was significantly reduced (P<0.05) in the Ho group, when compared with Se group (Fig. 2E-G). The results indicated that HaE increased the expression of oxidative stress as well as antioxidant genes, while decreased the expression of apoptotic (Caspase-3), anti-inflammatory (TNF and IFN-γ), interleukins (1a and 1b) genes in septic rats. This suggests that the extract of sea cucumber H. atra may posses antiseptic and anti-inflammatory properties.

Histopathological examination. The negative control (Sh) group showed normal architecture of the liver, with hepatic

Figure 3. Photomicrograph of rat livers in the various groups: (A) Sham group, showing the normal histological structure of hepatic lobule (bar, 11.69 µm); (B) Se group, showing the congestion of hepatoporal blood vessel, focal hepatic necrosis (short arrow) associated with inflammatory cells infiltration (long arrow; H&E; bar, 11.05 µm); (C) Se group, showing fatty change of hepatocytes (short arrow) and sporadic necrosis of hepatocytes (long arrow; bar, 11.37 µm); (D) Se group, showing hyperplasia of epithelial lining of the bile duct (short arrow) and fibroplasia in portal tract (long arrow; bar, 11.37 µm); (E) Se group, showing Kupffer cell activation (short arrow) and necrosis of sporadic hepatocytes (long arrow; bar, 12.94 µm); and (F) Sepsis and Holothuria atra extract treatment group, showing apparent normal hepatic parenchyma (bar, 13.25 µm). All stained with hematoxylin-eosin. Se, sepsis group; cv, central vein; h, hepatocyte.
lobules around the central vein and each lobule consisting of hepatic cords. The hepatocytes represented the hepatic cords and consisted of polygonal cells with centrally basophilic nuclei and clear acidophilic cytoplasm (Fig. 3A). However, the liver of the Se group presented congestion of the hepatoportal blood vessel, focal hepatic necrosis associated with inflammatory cell infiltration (Fig. 3B), fatty change of hepatocytes, sporadic necrosis of hepatocytes (Fig. 3C), hyperplasia of the epithelial lining of bile duct and fibroplasia in the portal tract (Fig. 3D). In addition, certain examined sections from the Se group showed Kupffer cell activation and necrosis of sporadic hepatocytes (Fig. 3E). By contrast, sections from the Ho group revealed apparent normal hepatic parenchyma (Fig. 3F).

The lung histological examination revealed no evidence of sepsis in the Sh group, which appeared to have a normal structure of alveoli and alveolar sacs with thin alveolar septum (Fig. 4A). By contrast, the lung tissues of the Se group showed marked edema, hemorrhage, leukocyte infiltration and alveolar septal thickening (Fig. 4B), whereas tissue sections from the Ho group presented slight interstitial pneumonia (Fig. 4C).

**Immunohistochemistry.** Immunohistochemical staining of Caspase-3 was localized in the nuclei of hepatocytes. The liver tissues of the Sh group showed no expression of Caspase-3 (Fig. 5A). By contrast, the lung tissues of the Se group showed strong expression of Caspase-3 (Fig. 5B) and moderate expression in the Ho group (Fig. 5C). Upon investigation of lung tissues, the expression of Caspase-3 was found to be localized in the nuclei of pulmonary cells. The lung tissues of the Sh group showed no expression of Caspase-3 (Fig. 5D), strong expression in the Se group (Fig. 5E) and moderate expression in the Ho group (Fig. 5F).
Discussion

H. atra is one of the most important species in the sea cucumber family, and it is found worldwide, including in the Red Sea region (40,45,46). The extract of H. atra has been evaluated for the presence of bioactive compounds and its various biological activities (47). In the present study, the administration of HaE was found to reduce the mortality rate in the CLP rats (Ho group), which was evidently elevated in the CLP rats that did not receive HaE (Se group). The decrease in sepsis-induced mortality upon HaE treatment can be explained according to the findings of Dhinakaran and Lipton (47), who stated that the extract of sea cucumber has antimicrobial activity. The Se group exhibited molecular and histopathological changes in the liver and lung tissues, which were characterized by increased expression of oxidative stress, antioxidant and anti-inflammatory genes, as well as a congestion of the hepatoporal blood vessel and focal hepatic necrosis associated with inflammatory cell infiltration. The Se rat lungs showed marked edema, hemorrhage and alveolar septal thickening, and these findings agreed with the observations of Ates et al (10), Esmat et al (48) and Baiomy and Saad (49).

The expression levels of oxidative stress and antioxidant genes can be used as markers to detect the response to microbial infection (50). The administration of HaE upregulated the expression of the antioxidant gene Gsta2, as well as of the oxidative stress responsive genes, Cat and Sod1. In addition, HaE administration downregulated the anti-inflammatory genes TNF, IFN-γ, liver Il1b and lung Il1a, and these results were in agreement with previous observation (29-31). Furthermore, these findings were confirmed by histopathological analysis in the current study, which showed apparent normal hepatic parenchyma and slight interstitial pneumonia in the Ho group.

Several studies have previously reported that TNF and IFN-γ are involved in the development of septic shock (12-20,38). Interleukins 1A and 1B, two members of interleukin-1 family (51), are also involved in sepsis. IL1A is responsible for the production of inflammation, as well as the promotion of fever and sepsis (52), while IL1B is involved in a variety of cellular activities, including cell proliferation, differentiation and apoptosis (53). Based on the present study results, we can speculate that the antimicrobial activity of HaE resulted in a decrease in sepsis, and consequently the expression levels of TNF and IFN-γ were reduced.

Various studies have investigated the effects of the sea cucumber components, including polysaccharides, which exhibited a variety of biological activities, such as anti-tumor (54), anti-oxidation (47), anti-inflammatory (51), are also involved in sepsis. IL1A and IL1B are two members of the interleukin-1 family, which may be associated with the presence of antioxidant agents. In addition, it was reported that the presence of the active phenolic compounds in the body wall of the sea cucumbers may be due to phenolic-rich materials, such as phytoplankton and particles derived from degrading marine macroalgae, which are the main sources of food for sea cucumbers (59,60). Furthermore, the body wall of sea cucumber contains chlorogenic acid, which has been found to have a potential hepatoprotective effect in several animal models of liver injury (61).

The results of the present study showed that the expression of Caspase-3 gene, which was highly elevated in Se group, was decreased in response to administration of HaE in the Ho group. This finding was supported by the immunohistochemical staining of Caspase-3 in liver and lung tissues, showing no expression of Caspase-3 in the Se group, strong expression in the Se group and moderate spression in the Ho group; these findings coincided with the observations of Hu et al (62). According to the analysis of these data at gene expression and histopathological levels, we speculate that the beneficial effect of HaE of sea cucumber against sepsis may be attributed to its antioxidant components and antiapoptotic activity. Thus, HaE may be a potential functional agent for the improvement of survival in sepsis.

In conclusion, the data presented in the current study indicated that HaE is a useful natural product that is able to mitigate the liver and lung damages resulting from induced sepsis by CLP. It possesses antioxidant, antitumor and antiapoptotic activities. The fact that the Ho group revealed apparent normal hepatic and lung parenchyma may be attributed to its antioxidant components, where necrosis and congestion decreased, which may also be attributed to its anti-inflammatory and antiapoptotic activities. Purification and identification of these structures in future studies is warranted.

It appears that with further research in this field, the extracts of marine organisms, including those of sea cucumbers, may be used as antiseptic, antioxidant, antitumor and antiapoptotic agents.

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