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***Holothuria arenicola* as a New Antiseptic Drug: *In Vitro* Antibacterial Investigation and *In Vivo* Therapeutic Role.**

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ABSTRACT

Sepsis is a fatal response accompanied by a severe bacterial infection caused by contamination. This study evaluates the antibacterial effect of the *Holothuria arenicola* (body wall and coelomic fluid extracts) *in vitro*, and explored its therapeutic potential in septic rats induced by cecal ligation and puncture (CLP) *in vivo*. The phytochemical investigations of the both extracts revealed the availability of bioactive metabolites; including alkaloids, flavonoids, tannins, phenolic compounds and protein. In the *in vivo* study, rats were divided into 4 groups (6 rats/group): sham-operated, CLP, *H. arenicola* body wall extract (200 mg/kg b.wt) and *H. arenicola* coelomic fluid (200 mg/kg b.wt). The treatment starts two hours after the CLP induction and remains for 3 days. The survival study was performed for another 24 rats. Sepsis induced significant increase in procalcitonin and some hematological disorders such as erythropenia, leukocytosis and thrombocytopenia. Both extracts of *H. arenicola* restored the procalcitonin and the hematological parameters near the control level. The effective antiseptic effect of the extracts backed to their bactericidal efficacy against the particular septic bacteria and attributed to their active antibacterial constitutes. Thus, the obtained results suggest that marine echinoderms are a potential source for the discovery of novel antibiotics.

Keywords: Sepsis - Procalcitonin - *Holothuria arenicola* extracts - Hematological parameters.

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INTRODUCTION

Sepsis is a potentially fatal response to infection that can progress from systemic inflammatory response to severe sepsis and septic shock if not recognized early and managed effectively [1]. Despite the development of numerous therapeutic approaches used in clinical settings, septic shock remains a worldwide healthcare problem [2]. With an increasing annual incidence in the developed world, mortality remains between 25 and 50% of those afflicted [3]. Sepsis is accompanied by bacterial infection that may be caused by contamination after the false surgical procedure [4]. The presence of microbial pathogens in the bloodstream triggers systemic inflammation and can lead to sepsis, which often overcomes the most powerful antibiotic therapies and causes multiorgan system failure, septic shock and death [5]. Thus, the cecal ligation and puncture (CLP) model is considered the cornerstone of sepsis research. This model of sepsis would consistently translate the real condition as it is the most closely representative of the clinical situation encountered in human beings [6].

Because of the development of antibiotic resistance in virtually all clinically important pathogens, alternatives to conventional antibiotics are urgently needed. In addition, the side effect of overuse and misuse of antibiotics which can harm vital organs like liver, kidneys and some cells such as the pancreas and spleen as well as their impact on the immune system were investigated. Natural products have proven to be highly efficient for the treatment of bacterial infections and, not surprisingly, the variety of drugs based on natural products are enormous. The marine products differ from the terrestrial natural products as the marine atmospheres are reservoirs of bioactive natural constituents, not found in terrestrial products [7]. Many of the marine natural products have antibacterial activity. These natural secondary products produced by organisms to protect themselves [8]. Several marine products (animal, algae, fungi and bacteria) were screened for their bioactivities such as antibacterial, antifungal, anticoagulant, antiviral and anti-enzyme activities [9,10]. Echinoderms (Echinodermata) are independent and quite specific branch of the animals that are not comparable with any of the other animals in terms of the body construction plan. Besides, echinoderms have the majority of pharmacologically active secondary metabolites; some of these metabolites are antimicrobial in nature [11]. Among the most dominant echinoderms in Egypt is the sea cucumber; especially the *arenicola* species "*Holothuria arenicola*" [12]. Many investigators reported the *in vitro* antibacterial and antifungal activities of *Holothuria leucospilota* body wall and coelomic fluid [13,14]. Recently, Fahmy and Fahmy *et al.* [15,16] studied the antifibrotic and antiulcerogenic effects of *H. arenicola* body wall extracts in rats. Until now the antibacterial efficacy of *H. arenicola* not studied either *in vitro* or *in vivo*. Thus, the present investigation aimed to elucidate the antibacterial effect of the *H. arenicola* body wall extract and its coelomic fluid against different gram positive and gram negative bacteria *in vitro* and also extend to study this effect against bacterial infection growth following CLP in rats.

MATERIALS AND METHODS

Sample Collection

Sea cucumber (*Holothuria arenicola*) was collected from Abu-Qir Bay in the Egyptian Mediterranean coast of the eastern Alexandrian coast (April-May 2014). To avoid damaging of animals during collection, trawling was avoided and picked by using a hand net. The sample was thoroughly washed with sea water to remove sand, mucus and overgrowing organisms. Then the collected sample was maintained in circulating sea water and transported to the laboratory of Cairo University. *Holothuria arenicola* was authenticated by invertebrate professors in the Zoology Department, Cairo University. Coelomic fluid was withdrawn immediately from the coelomocytes by puncture of the body wall with a sterile needle.

Sea cucumber preparation

Body wall extract

Body wall was soaked in appropriate amounts of methanol-water (50:50), mixing and maintained for 16 h. Then, the mixture was filtered and the process repeated for the second time. Finally, the two portions were pooled together and concentrated by rotary evaporator. The powdered extract was obtained by freeze dryer and stored at -20 °C until use [13].

Coelomic fluid

The collected coelomic fluid was homogenized with stirring using the magnetic stirrer for 15 min. and filtered using Whatman No.1. filter paper. Then, the fluid was lyophilized and the obtained cell-free coelomic fluid extract was stored at -20 °C until use [13,17].

Chemical screening of *H. arenicola* body wall extract and its coelomic fluid

The two extracts of *H. arenicola* were evaluated for the presence of different active constituents [18-21].

Antibacterial assay of *H. arenicola* body wall extract and its coelomic fluid

The potency of the body wall and coelomic fluid of *H. arenicola* to inhibit the growth of bacteria was determined by the disc diffusion method [22]. Sterile hi-sensitivity agar plates were prepared, poured into Petri dishes and allowed to solidify. The Petri plates were swabbed with 24h old cultures of the three selected bacterial strains which are *Staphylococcus aureus* (Gram positive), *Escherichia coli* and *Pseudomonas aeruginosa* (Gram negative). 100µl of each of the body wall and coelomic fluid of the *H. arenicola* (20 mg/ml) was introduced into discs (0.8 cm) and then they are allowed to dry. The discs were completely saturated with the body wall and coelomic fluid. The discs were then introduced into the upper layer of the medium at least 25mm away from the edge. The discs were then pressed lightly on the surface of the same plate. The plate was incubated at 37 °C for 72 h and then observed for clear zone of inhibition. Finally, the inhibition zone (area wherein there is no growth around the discs) was measured using the millimeter of a ruler.

Animal housing

Male albino rats (*Rattus norvegicus*) weighing 150-170 g were purchased from the National Research Center (Egypt) and used for all the experiments. The animals were housed in polypropylene cages with sawdust (five animal/cage) in a room maintained at 22±2 °C with an alternating 12-h light-dark cycle. Each animal cage was identified by a specific card. This card denotes the cage number, the number and weight of the animals it contained, name of the test substance, route of administration and dose level. The animals were fed with standard laboratory animal food pellet and drink water *ad libitum*. Procedures for animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) (CUFS/S/PHY/1714) of the Faculty of Science, Cairo University, Egypt.

Induction of sepsis by CLP

Under sterile conditions, CLP-induced sepsis was performed as described previously [23]. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p) and an abdominal midline incision was made. The cecum was exteriorized, ligated just distal to the ileocecal valve to avoid intestinal obstruction. The cecum was punctured twice at opposite ends with a 18-gauge needle and was gently squeezed to extrude fecal matter. Then, it returned into the abdominal cavity in its position. The abdominal incision was then closed in two layers using a 4-0 silk thread. Sham-operated rats underwent the same procedure, but the cecum neither ligated nor punctured. Following surgery, the rats were injected with physiologic saline solution (3 ml/kg body weight) for fluid resuscitation. Postoperatively, the rats were then housed in their cages and allowed to free access of food and water. Then, the following treatments were performed.

Animal grouping and treatment

Forty eight rats (12 rats/each group) were initially divided into 4 groups that were treated 2 hours after the CLP induction, the rat groups were divided as shown in table 1. Three days after CLP, half of the rats were euthanized and blood samples were collected, centrifuged and stored until use. On the other hand, the other remaining half of the animals (n= 24, 6/ each group) were used for survival study. The dose selected for the *H. arenicola* is considered the safe dose [16].

Table 1: Experimental animal grouping and treatment protocol.

Treatment/time Groups		CLP surgery	Administration time after CLP process	Dist.H ₂ O	<i>Holothuria arenicola</i>		Treatment time (Days)	Euthanization time (Days)	
					Body wall (200 mg/kg)	Coelomic fluid (200 mg/kg)			
Sham		-	2 h	+	-	-	3	4 th	
CLP (Sepsis)		+	2 h	+	-	-	3	4 th	
CLP+	<i>Holothuria arenicola</i>	Body wall	+	2 h	-	+	-	3	4 th
CLP+		Coelomic fluid	+	2 h	-	-	+	3	4 th

Survival study

The survival rate was observed within the following 10 days. The mortality of the rats was recorded every 24 h until the 10th day. The survival rate was expressed as a percentage.

Determination of sepsis markers

Procalcitonin

Serum procalcitonin level was determined by ELISA kit purchased from WEKA MED SUPPLIES CORP (Changchun, China).

Hematological parameters

Red and white blood cells, hemoglobin content and platelets were determined in plasma using a coulter counter machine.

Statistical analyses

Results were expressed as mean ± SEM of six animals, except for the hematological parameters (n=3). The difference between groups was analyzed by one way ANOVA followed by *Duncan's* test. Survival data were analyzed by a Kaplan-Meier and the log - rank statistic. Values of p<0.05 were considered as statistically significant. Data analysis was performed using SPSS version 15.0 software.

RESULTS

Active constituents of the body wall and coelomic fluid of *H. arenicola*

Preliminary chemical screening of body wall and coelomic fluid of *H. arenicola* showed that both of them contain most of the chemical constituents as flavonoids, alkaloids, tannins, quinones, saponins, proteins and amino acids (Table 2).

Table 2: Chemical analysis of body wall and coelomic fluid of *H. arenicola*.

Test	<i>H. arenicola</i>	
	Body wall	Coelomic fluid
Flavonoids	+	+
Alkaloids	+	+
Tannins	+	+
Quinones	+	+
Saponins	+	+
Proteins and Amino acids	+	+
Glycosides	-	-
Triterpenoids	-	-

+ = Presence, - = Absence.

Antibacterial activity of body wall and coelomic fluid of *H. arenicola*

The antibacterial activities of the body wall extract and coelomic fluid of *H. arenicola* against different gram positive and gram negative bacteria are shown in table 3. Body wall extract of the *H. arenicola* showed a broad spectrum antibacterial activity than its coelomic fluid, it showed activity against *Staphylococcus aureus* (9 mm) and *Escherichia coli* (9 mm); whereas the coelomic fluid showed activity against *Staphylococcus aureus* (9 mm) only. Both of the tested substances of the *H. arenicola* (body wall and coelomic fluid) have no antibacterial activity against the tested pathogens.

Table 3: Antibacterial activities of the body wall extract and coelomic fluid of *H. arenicola* against different gram positive and gram negative bacteria.

Pathogens	Zone of Inhibition (mm)	
	<i>H. arenicola</i>	
	Body wall	Coelomic fluid
<i>S. aureus</i>	9	9
<i>E. coli</i>	9	-
<i>P. aeruginosa</i>	-	-

Effects of body wall and coelomic fluid of *H. arenicola* on survival of septic rats

Sepsis induced by CLP causes 100% lethality within 10 days, this evidenced by a significant decrease ($p < 0.05$) in the survival rate of rats in CLP-septic rats, as compared to sham rats. Where, (0/6- 0%) rats survived in the CLP septic rats. Treatments with *H. arenicola* (body wall or coelomic fluid) markedly elevate the survival rate of the rats, as compared to the untreated CLP-septic rats. As (4/6- 66.7%) and (3/6- 50%) rats survived by the treatment of the body wall and the coelomic fluid of *H. arenicola*, respectively. No mortality was recorded in the sham group (Fig. 1).

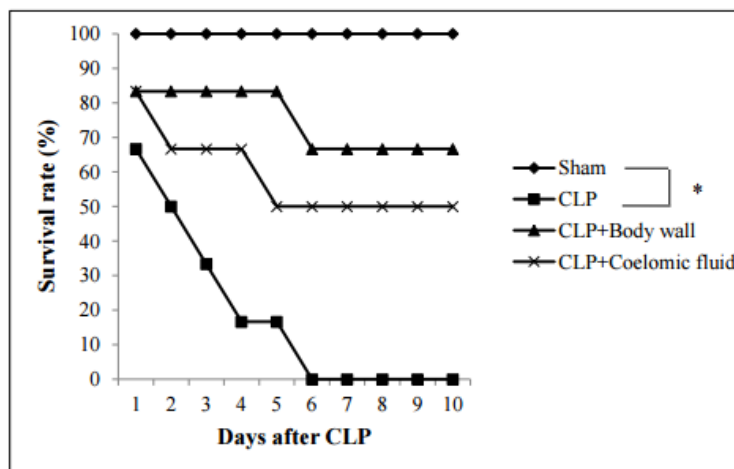


Figure 1: Effect of body wall and coelomic fluid of *H. arenicola* on 10 day survival rate of CLP-septic rats. *P < 0.05, vs sham group.

Effects of body wall and coelomic fluid of *H. arenicola* on PCT level of septic rats

The serum level of PCT increased significantly ($p < 0.05$) due to CLP process, in comparison with the sham control group. Treatment of sepsis either with body wall or coelomic fluid of *H. arenicola* significantly revert ($p < 0.05$) the PCT level to the control value, as compared to the untreated CLP-septic rats (Fig. 2).

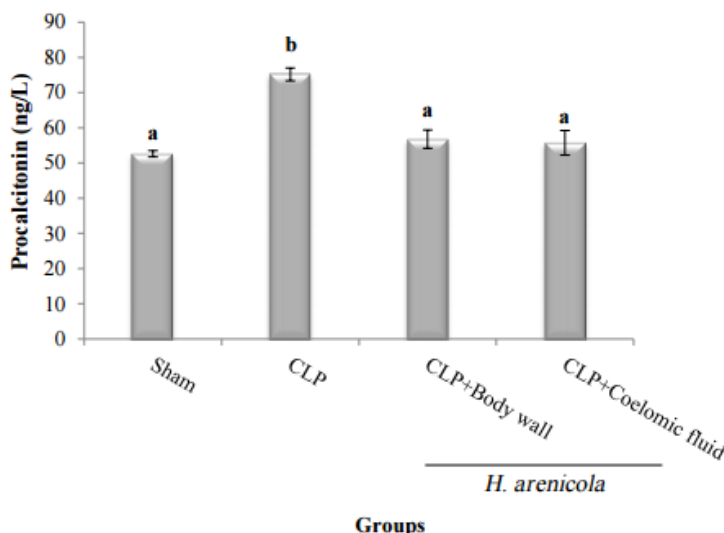


Figure 2: Effect of body wall and coelomic fluid of *H. arenicola* on PCT level of CLP-septic rats. Values are mean \pm SEM (n=6). Values with different superscript letters are significantly different (P < 0.05).

Effects of body wall and coelomic fluid of *H. arenicola* on hematological parameter of septic rats

CLP surgery causes anemia, leukocytosis and thrombopenia in the present investigation. These hematologic abnormalities were evidenced by a significant decline (p<0.05) in the Hb content, RBCs count and platelet level; as well as a significant increase (p<0.05) in the WBCs count of untreated CLP rats when compared with the normal sham values (Table 4). Post-treatment of the CLP rats with *H. arenicola* (body wall or coelomic fluid) significantly restore (p<0.05) the Hb, RBCs, WBCs and platelet levels, as compared to CLP-septic rats.

Table 4: Effect of body wall and coelomic fluid of *H. arenicola* on Hb, RBCs WBCs and platelet contents of CLP-septic rats.

Groups Parameter	Sham	CLP (Sepsis)	<i>Holothuria arenicola</i>	
			Body wall (200 mg/kg b.wt)	Coelomic fluid (200 mg/kg b.wt)
Hb (%)	12.9 \pm 0.143 ^a	8.9 \pm 0.323 ^b	12.3 \pm 0.146 ^a	11.8 \pm 0.205 ^c
RBCs x10 ⁶ (cell/mm ³)	6.5 \pm 0.89 ^a	4.2 \pm 0.21 ^b	6.1 \pm 0.87 ^a	6 \pm 0.655 ^a
WBCs x10 ³ (cell/mm ³)	8.03 \pm 0.076 ^a	15.1 \pm 0.456 ^b	8.9 \pm 0.58 ^a	9.2 \pm 0.43 ^c
Platelet x10 ³ (mm ³)	896 \pm 18.4 ^a	596 \pm 16.9 ^b	861 \pm 19.6 ^a	826 \pm 13.8 ^a

Values are mean \pm SEM (n=3). Values with different superscript letters are significantly different (P < 0.05).

DISCUSSION

The marine environment, due to its physical and chemical characteristics, is a potential source of bioactive natural products [24]. Sea cucumbers (Holothuroidea) are among the strangest members of echinoderm class regarding the structure and physiology [25]. They are important marine organism with commercial, pharmaceutical, and food value. Functional food that contains biologically-active compounds is an important source for prevention, management and treatment of chronic diseases in the modern age. In Egypt, *Holothuria arenicola* is widely distributed along the Alexandrian Mediterranean coast [12]. The ability of the body wall of sea cucumber to regenerate after being cut up reinforced the people's confidence to its use in the traditional medicine [26]. In consonance with the reports of several investigators Fahmy and Esmat *et al.* [15,27], the phytochemical investigations of the *H. arenicola* body wall extract and coelomic fluid in the present study revealed the availability of bioactive metabolites including alkaloids, flavonoids, tannis, phenolic compounds (quinine, saponins and terpenoids) and protein. It was reported that phenolic-rich materials such as phytoplankton and particles derived from degrading marine macro-algae are the main sources of food for sea cucumbers that can account for the presence of the active phenolic compounds in the body wall and coelomic fluid of sea cucumbers [28]. The present investigation affirmed the results of Bordbar *et al.* [29], who reported that the health benefits of sea cucumbers are associated with the presence of bioactive components such as saponins, glycosaminoglycans, sterols, cerberosides, peptides, sulfated polysaccharides, and essential fatty acids. Phenolic compounds are very important because of their antioxidant activity [30]. The antioxidant

activity of phenolic compounds is mainly attributable to their redox properties that play an important role as free radical scavengers, reducing agents, quenchers of singlet oxygen and complexes of pro-oxidant metals [31].

Sepsis, a systemic inflammatory response syndrome (SIRS) induced by infection, is accompanied by the presence of bacteria [32]. Abundant evidence shows that CLP-induced sepsis with acute supportive peritonitis is a typical sepsis model with G-bacteria as the predominant infection source [33]. This model, by virtue of cecal ligation and perforation, leads to the pollution of the abdominal cavity by bacteria-carrying intestinal contents, gives rise to generalized peritonitis, and induces a wide range of systemic inflammatory responses [34]. So, the CLP animal model of rats with sepsis was used in this study. The non-availability and high cost of new generation antibiotics with limited effective span have resulted in an increase in morbidity and mortality [35]. The known success of traditional medicine has guided the search for new chemotherapeutic alternatives to eliminate the infections caused by drug-resistant microbes and to reduce the harms caused by antibiotics [36,37]. Several drug discovery projects have screened for echinoderms for antibiotic activities. Moreover, anti-fungal, anti-bacterial, antithrombotic, anti-malarial, anti-protozoa and anti-virus effects have been reported from some sea cucumber isolated compounds [38]. In consistent with the findings of Adibpour et al. [13], the current work revealed that the body wall extract of the *H. arenicola* exhibited broad spectrum bactericidal activity against both gram (+ve) and gram (-ve) bacteria [*Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*] as compared to the coelomic fluid which inhibit the growth of the gram (-ve) bacteria only. The antibacterial potency of the *H. arenicola* may be due to their antimicrobial components, flavonoids, alkaloids, tannins, quinines and saponins. It was reported that these active constituents have antibacterial characteristics [39]. The current study indicates that these bactericidal components may be concentrated mainly in the body wall and little in the coelomic fluid, since the coelomic fluid exhibited moderate activity toward some species of bacteria.

Mortality rates from sepsis toxicity in developing countries vary between 25 and 50% [3]. Despite the advances in medicine, sepsis mortality rates are still increasing and survivors suffer poor quality of life [40]. The results of this study show that when *H. arenicola* body wall extract and the coelomic fluid used, the survival rates of septic rats increased by 66.7% and 50% respectively. These results are in line with many findings [23,41,42]. The death of the CLP-septic rats to the polymicrobial infection of the peritoneum, which eventually caused bacteremia and severe blood loss which cause impaired oxygen delivery leading to tissue dysfunction and finally death [41,43].

Procalcitonin (PCT), the precursor for the hormone calcitonin (CT) is a biomarker that exhibits greater specificity than other proinflammatory markers (cytokines) in identifying patients with sepsis and can be used in the diagnosis of bacterial infections [44,45]. Unlike these pro-inflammatory cytokines, whose appearance in the systemic circulation is transient, ProCT concentration remain increased for the duration of the inflammatory stimulus [46]. This feature of PCT makes it a more useful indicator for outcome prediction, as well as a potential target for therapeutic blockade. In conjunction with the previous reports [23,41,42], data from the present investigation showed significant increase in PCT level following CLP in rats. The increment of PCT in septic rats may be due to a continuous bulk-flow constitutive pathway, in which only limited conversion to mature CT occurs [47]. In this respect, a shift to constitutive secretion has been reported to occur by the experimental induction of dysfunctional prohormone convertase enzymes or by injury to the plasma membrane [48]. Moreover, PCT has been shown contributes to mortality in experimental sepsis, and that immunoneutralization of this molecule diminishes mortality in a model of hamster sepsis [49]. As PCT levels increase upon bacterial infection and decrease upon recovery, they can be used to guide antibacterial therapy as a surrogate biomarker [50]. The present study showed that treatment with *H. arenicola* body wall extract and the coelomic fluid succeed to normalize the PCT level which may be due to their antimicrobial constituents. Accordingly, antibacterial therapy represents a potential strategy to prevent septic shock. Treatment with *H. arenicola* body wall extract and the coelomic fluid normalized the PCT level through their rich of polyphenolic compound especially quinine, saponins and terpenoid that show antibacterial characteristics.

The hematological organ system is a major element in the response to a septic insult and plays a pivotal role in the resolution phase of severe sepsis [51]. Significant decrease of RBCs, Hb and platelets values of septic rats observed in this study may reflect the adverse effect induced in the hemopoietic organs. In consonance with many findings Piagnerelli and Goyette [52,53], the present investigation indicated that the

most common abnormalities of the hematologic system in sepsis are anemia, leukocytosis, thrombocytopenia, and activation of the hemostatic system. The decreased RBCs count value may be related to inhibition of erythropoiesis or decreased flow rate of RBCs from the spleen [54]. In view of the present results, it was found that treatment of septic rats with *H. arenicola* body wall extract and the coelomic fluid restored the hematological parameters near the control level.

CONCLUSION

The present study serves to present two therapeutic products from marine echinoderm, *H. arenicola* and confirm that its body wall extract or coelomic fluid showed antibacterial potency, decreased PCT, improved hematological disorders, increase the survival of animals and thereby rescue the septic rats from death. The effective antiseptic effect of the selected extracts backed to their bactericidal efficacy against the particular septic bacteria and attributed to their active antibacterial constituents. However, further studies must be carried out to elucidate the mechanisms involved during their *in vivo* antiseptic effect.

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