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Anticalculi and antibacterial activity of methanol extract of *Holothuria atra*

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ABSTRACT

Sea cucumbers are a type of marine organism with significant economic worth and potential applications as nutraceuticals. This marine organism possesses possibly useful chemicals, making it a viable alternative for the fisheries product processing businesses. The objective of this study was to assess the efficacy of the methanol extract of *Holothuria atra* in preventing the formation of calculi and its ability to inhibit the growth of bacteria. Extraction was performed using the maceration method with methanol as the solvent over a period of 72 hours The analysis revealed the presence of four bioactive components in the methanol fraction: alkaloid, triterpenoids, flavonoids, and saponins. In vitro anticalculi activity was assessed by measuring the decomposition of kidney stones (Ca) using an atomic absorption spectrophotometer (AAS). The maximum decline of calcium stones was regarded at a concentration of 500 ppm of sea cucumber extract. The disc diffusion method was employed to assess the antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. The inhibition zone diameter was measured at all concentrations (25%, 50%, 75%, and 100%), indicating their potential as antibacterial agents. The greatest level of antibacterial effectiveness against *Escherichia coli* was observed at a concentration of 50%, resulting in a zone of inhibition of 1.90 mm. In contrast, the most significant antibacterial activity against *Staphylococcus aureus* was observed at a concentration of 25%, resulting in a zone of inhibition measuring 4.50 mm.

Introduction

Nephrolithiasis is an illness in which crystal stones grow in the kidney pelvis, or calyx. Calcium oxalate and calcium phosphate in kidney stones can precipitate and obstruct the urethra, causing pain (Harianja *et al*., 2021). The frequency of kidney stone disease has a direct correlation with the prevalence of urinary system problems, impacting around 12% of the global population. (Chauhan and Joshi, 2008; Stiani *et al.,* 2019). Medical treatments for the condition include surgery, the use of wave technology (ESWL-Extracorporeal Shock Wave Lithotripsy), and laser shooting. However, these therapies are costly (Nykamp, 2014). In addition, different medications taken orally are used to treat kidney stones. However, long-term use of these medications is limited due to severe side effects and patients' lack of tolerance. As a result, several alternative therapy methods, including herbal treatments, have been used to treat urinary stones for hundreds of years without adverse reactions. The results showed that chives (*Allium schoenoprasum L.*) and celery (*Apium graveolens L.*) plants have anticalculi activity (Bahmani *et al*., 2016; Iksen *et al*., 2017; Haro *et al*., 2017). Extensive research has revealed that the marine organism *H. atra* exhibits significant antibacterial activity, effectively inhibiting pathogens such as *Pseudomonas aeruginosa* (Sukmiwati *et al*., 2020), *Bacillus paramycoides*, *Vibrio alginolyticus* (Sulardiono *et al*., 2020), and *Bacillus subtilis* (Putra *et al*., 2022). Meanwhile, the use of marine organisms for this purpose (anticalculi activity) has never been reported.

Oceans are recognized as essential sources of metabolites, which are natural compounds with pharmacological properties (Piri-Gharaghie *et al*., 2023). Marine bioresource like sea cucumber have

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pharmacological potential for developing therapeutic uses (Khotimchenko, 2018; Rasyid and Putra, 2023). Sea cucumbers (*Holothuroidea*) are invertebrate organisms with over 1700 species in the world, considerable commercial importance (food, cosmetic, nutraceutical), rich collagen and bioactive compound such as triterpene, saponin, glycosides, phenolic compound, carotenoid, peptides, vitamin and mineral (Rahael *et al.,* 2019; Xiang *et al.,* 2021; Soto *et al*., 2024).

Sea cucumbers possess a range of bioproperties, including antihypertensive, antioxidant, anticancer, wound healing, antitumor, anticoagulant, antibacterial, antifungal, hemolytic, cytotoxic, and immunomodulatory effects (Boardbar *et al*., 2011; Pangestuti and Arifin, 2018; Nugroho *et al*., 2022).

This research discusses the anticalculi and antibacterial properties of black sea cucumbers (*Holothuria atra*) from North Water Aceh, which have never been explored before. This study aims to determine the methanol extract content of *Holothuria atra* and its ability to kidney stone decay by in-vitro and its effectiveness against positive gram (*S.aureus*) and negative gram (*E.coli*) bacterial. The findings could pave the way for innovative treatments derived from marine natural products as sources anticalculi and antibacterial agents

Materials and Methods

Location and time of research

The study took place from August to November 2023 at the Laboratory of Marine Chemistry and Fisheries Biotechnology, Faculty of Marine Affairs and Fisheries, Universitas Syiahkuala. Sea cucumbers were collected at Inong Balee Fort in the Aceh Besar area.

Materials

The object this study was sea cucumber (*Holothuria atra*). The chemical such were methanol, sodium chloride, calcium chloride, ammonium oxalate, nutritional agar, dimethyl sulfoxide, aquadest, nutritional agar, and bacterial tests for *E. coli* and *S. aureus*. The tool was rotary vacuum evaporator, analytical balance, glassware, incubator, hot plate, autoclave, atomic absorption spectrophotometer (AAS) and UV-Vis spectrofotometer.

Sample preparation and extraction

The samples were collected at Inong Balee Fort in Lamreh Village, Masjid Raya sub-district, Aceh Besar district. Sea cucumbers used in studies have a length of 10-20 cm and an average mass of 200–500 grams. The sea cucumbers were washed with water, chopped into little pieces, and then dried. Sea cucumber samples were placed under maceration for a total of 72 hours, with methanol as the solvent. The ratio of solvent volume to sample was 2:1(v/w). The extraction products were separated into filtrate and residue (macerate) using Whatman paper filtration. Next, the macerate was concentrated using a rotary evaporator at a temperature of 40^0C. Finally, the resulting macerate was evaluated by phytochemical screening to identify its secondary metabolite content.

Anticalculi test

The anticalculi activity test began with the synthesis of calcium as kidney stones model by reacting calcium chloride ($CaCl_2$) and ammonium oxalate $(NH_4)_2C_2O_4$ to produce calcium oxalate precipitate (CaC_2O_4). The calcium oxalate precipitate had been heated in the oven at 105 ℃ for 2 hours to form CaC_2O_4 solid. In the in vitro anticalculi activity test, 0.1 gram of calcium was added to various concentrations of sea cucumber extract ranging from 0 ppm, 250 ppm, 500 ppm, and 1000 ppm. Then, each solution was placed in an incubator for 24 hours, stirring every 30 minutes. The solution was then filtered, and the filtrate was centrifuged at 6000 rpm for 10 minutes. Finally, the Ca concentration in the solution was determined using an atomic absorption spectrophotometer (AAS) at a wavelength of 422 nm.

Antibacterial test

Methanol extract of *Holothuria atra* was dissolved in a dimethyl sulfoxide solution to produce a stock solution with a specific concentration. In addition, the concentrated solution was diluted to obtain concentrations of 25%, 50%, 75%, and 100%. To prepare nutrient agar (NA) media, 3 grams of NA powder were dissolved in 100 ml of purified water. Lastly, all tools, such as an Erlenmeyer flask, Petri plates, and the NA media, were sterilized in an autoclave (121°C and 1 atm) for 20 minutes.

A portion of the bacterial suspension was transferred into a cuvette tube, and its absorbance was determined using a UV-Vis spectrophotometer, adopting the 0.5 McFarland standard, by calculating the optical density (OD) of 0.1 for both E. coli and S. aureus bacteria. Subsequently, a volume of 1 ml of the bacterial suspension was added to a Petri dish, along with 15 ml of NA media, and left to solidify. Next, three aseptic paper discs, each with identical dimensions in terms of diameter and thickness, were placed equidistantly on the surface. lastly, 25 µL of

Holothuria atra extract was applied onto the disc paper at four different concentrations (25%, 50%, 75%, and 100%). Additionally, a negative control (methanol) and a positive control (amoxicillin) were included. The presence of antibacterial activity is indicated by the formation of a translucent zone around the paper disc. The caliper was used to measure the diameter of the inhibition/clear area.

Data analysis

The calculation for the diameter of the inhibition zone in disc diffusion follows this formula:

Clear zone diameter = (Dv+Dh)/2

Note:

Dv = Vertical diameter (mm)

Dh = Horizontal diameter (mm)

Analysis of kidney stone (Ca) decays can be calculated by this formula:

Calcium (mg /kg) = (Creg x P x V)/G

notes

Creg : Concentration (µg/l) from AAS

P : Dissolution factor

G : Mass of sample (g)

V : Volume of sample (L)

Results

Phytochemical test

The methanol extract of the sea cucumber *Holothuria atra* contains four secondary metabolites: alkaloid, flavonoid, triterpenoid, and saponin. The results obtained from the phytochemical screening test conducted on the extract are presented in Table 1.

Table 1. Phytochemical screening result

Bioactive component	Methods	Result	Colour
Triterpenoid	Lieberman burchard	+	Brown
Flavonoid	Shinoda	+	Yellow
Saponin	Lieberman burchard	+	Orange
Alkaloid	Wagner	+	Reddish

Antibacterial test

The formation of a clear zone around the paper disc containing the extract was used to determine the level of antibacterial activity. Table 2 reveals the results of the inhibitory zone of the extract.

Table 2. Examination results of the inhibitory zone at multiple concentrations of the extract

Concentration (%)	Inhibitory Zone (mm)	
	Eschericia coli	*Staphylococcus aureus*
25	1.14	4.50
50	1.90	3.51
75	1.43	1.47
100	1.88	1.61
Control (+)	13.99	14.51
Control (-)	0	0

Anticalculi test

The calcium (Ca) decay result concentration of *H. atra* extract can be seen in Table 3.

Table 3. Calcium (Ca) decay test results of *H. atra* extract

No	Concentration (ppm)	Ca (µg/g)
1	0	7.2
2	250	16.6
3	500	35
4	1000	16.9

Discussion

Anticalculi activity

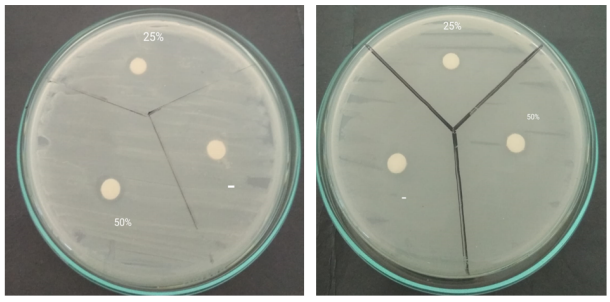
In vitro anticalculi test with *H.atra* extract measured kidney stone calcium decay. The level of calcium decay in extract is presented in Table 3. Table 3 shows how as the concentration of the methanol extract increases, so does the calcium decay. The concentration of 500 ppm exhibited the greatest percentage of calcium decay. According to Sen *et al.* (2016), calcium oxalate and flavonoids exhibit different solubility properties. At very high concentrations, the formation of complex molecules may occur, which can interfere with the binding of calcium to the bioactive molecules in the extract, thereby reducing the efficiency of calcium decay (Nicar *et al.*, 1987).

The solubility of calcium kidney stones in sea cucumber methanol extract is thought to be related to the reaction between the extract's flavonoids and calcium during the incubation phase. According to Winarti *et al.* (2014) and Sinaga *et al.*, (2019), Flavonoid substances (carboxyl group) interact with calcium in kidney stones, forming water-soluble Ca-flavonoid chelate complexes. Saponins and phenolic compounds, along with flavonoids, can act as antioxidants to prevent kidney stone formation (Harianja *et.al.*, 2021). Phytate (myo-inositol hexakisphosphate), a naturally occurring substance, has the potential to lead to the formation of calcium salts as a crystallization inhibitor throughout intrapapillary tissue and urine (Ratkalkar and Kleinman, 2011).

Antibacterial activity

The antibacterial activity was demonstrated by the presence of a clear zone of inhibition surrounding the paper disc containing the extract. According to Sari and Ma'aruf (2014), the diffusion test involved measuring the size of the clear zone. This shows how well an antibacterial component of the extract inhibits bacterial growth. The extent of the inhibitory zone can be affected by the susceptibility of the culture medium organism, incubation parameters (temperature, duration), and the rate of agar diffusion (media content and concentration).

Sea cucumber (*H. atra*) methanolic extract inhibited *S. aureus* and *E. coli* in kirby bauer tests. It is practical, easy, rapid, and sensitive to antibacterials. Test results indicated that the methanol extract from sea cucumber (*H. atra*) had modest antibacterial properties against *E. coli* and *S. aureus* bacteria (<5 mm). According Ngajow *et al.* (2013), an inhibitory zone diameter of less than 5 mm is weak antibacterial activity. At concentration 50%, the extract had the maximum inhibitory zone against *E. coli* bacteria, measuring 1.90 mm. However, S. aureus bacteria with a diameter of 4.50 mm were most inhibited at 25%. Due to its simple cell wall and structure of polysaccharides, methanol extract inhibited gram-positive bacteria (*S. aureus*) more than *E. coli* (Monte, *et al.*, 2014). According to Zunita and Batara (2023), environmental factors and bacterial neutralization of medication substances influence cell function. The inhibition zones against *S. aureus* and *E. coli* bacteria can be seen in the Figure 1. below



S. aureus *E. coli*

Figure 1. Inhibition zone of bacterial

The existence of bioactive compounds in extracts, such as alkaloids, triterpenoids, flavonoids, and saponins, can also improve activity in preventing bacterial growth. Previous research on sea cucumber extraction using methanol, ethanol, and chloroform solvents found that the presence of triterpenoid bioactive compounds possesses antifungal and antibacterial activity (Shakouri *et al.*, 2017; Ceesay *et al.*, 2019). According to Farouk *et.al.* (2007), triterpenoid compounds have antibacterial abilities, supported by the presence of polysaccharide and sulphate groups that play a role in inhibiting transportation across thicker bacterial cell membranes and DNA-binding enzyme activity. (Khattab *et al.*, 2018)

Flavonoid compounds can inhibit bacterial growth by reduce cytoplasmic membrane function, inhibiting, and disrupting energy metabolism activities (Gorniak *et al.*, 2019; Donadio *et al.*, 2021). Furthermore, flavonoids can exhibit harmful effects on bacteria by denaturing protein peptide linkages and forming molecular interactions between their hydroxyl groups and bacterial DNA, causing membrane and cell wall destruction (Liling *et al.*, 2020; Li *et al.*, 2022).

The antibacterial effect of sea cucumber extract is attributed to its saponin components, which are composed of triterpene glycosides (Aminin *et al.*, 2015). The activity of the saponin substance prevents bacterial growth by binding to lipopolysaccharides on the bacterial cell wall and lowering lipid tension. This reaction leads to bacterial lysis and death. (Amaha *et al.*, 2022).

Conclusion

Research findings indicate that the methanol extract of sea cucumber (*H. atra*) contains bioactive compounds with the potential to be used as anticalculi and antibacterial agents. Further research and extensive clinical trials are necessary to clearly understand the mechanisms of action and to formulate medically appropriate and efficient treatments.

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