Exploring the Antibacterial Potential of *Sticophus hermanii* Ethanol Extract Against *Salmonella Sp* Infection

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ABSTRACT

Background: The critical issue of bacterial resistance to antibiotics has necessitated the search for novel antibacterial agents. This study investigates the potential of the ethanol extract from the sea cucumber *Stichopus hermanni* as a source of such agents.

Methods: The ethanol extract of *Stichopus hermanni* was analyzed using Liquid Chromatography-Mass Spectrometry (LC-MS) to identify its compounds. Network pharmacology and in silico analysis were employed to predict the biological activity and toxicity of these compounds. The antibacterial effectiveness was tested using the Kirby-Bauer disc diffusion method against *Salmonella typhi*.

Results*:* LC-MS analysis identified several compounds within the extract. The in silico study suggested that compounds in the extract could have antibacterial and immunomodulatory effects. The disc diffusion tests showed the extract exhibited inhibition zones against *Salmonella typhi*.

Conclusion: The ethanol extract of *Stichopus hermanni* demonstrates potential as a source of antibacterial agents, including properties that could aid in immunomodulation. Further, comprehensive studies are needed to confirm these findings.

Keyword: Druggability, *Stichopus hermanni*, Network pharmacology, Immunomodulation, antibacterial

Abbreviations : LC-MS (Liquid Chromatography-Mass Spectrometry), CMPD (Comparative Molecular Pathobiology Database), ACTB (Actin Beta), TFR (Transferrin Receptor), MRSA (Methicillin-Resistant Staphylococcus aureus), HPLC (High-Performance Liquid Chromatography), SAR (Structure-Activity Relationship), ADME (Absorption, Distribution, Metabolism, and Excretion), CTD (Comparative Toxicogenomics Database), DAVID (Database for Annotation, Visualization, and Integrated Discovery), STRING DB (Search Tool for the Retrieval of Interacting Genes/Proteins), TNF (Tumor Necrosis Factor), NFKB1 (Nuclear Factor Kappa B Subunit 1), IL1B (Interleukin 1 Beta), TLR4 (Toll-Like Receptor 4), STAT3 (Signal Transducer and Activator of Transcription 3), MYD88 (Myeloid Differentiation Primary Response 88), GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes), PPI (Protein-Protein Interaction).

Introduction

The increasing concern worldwide regarding the resistance to antibiotics necessitates the urgent search for new sources of antibacterial agents. (Abdallah et al., 2023) This pressing issue is primarily due to the overuse and misuse of antibiotics in the medical field and the livestock industry. Such practices have led to the emergence of bacteria that are no longer affected by the current treatment options available.(Muraigiyan et al., 2023) As a result, infections that used to be straightforward to manage have now become significantly more difficult to treat. This situation elevates the risks associated with diseases, leading to a higher possibility of severe illness and mortality.(Salam, 2023) This crisis has paved the way for otherwise manageable infections to become significant health threats, underlining the importance of exploring alternative avenues for finding novel antibacterial compounds. The exploration of natural products emerges as a promising avenue for the discovery of novel antibacterial compounds. These molecules are expected to work differently from traditional antibiotics, offering a promising approach to overcome the challenges of current resistance mechanisms.(Barbosa, 2021; Ujianti, 2022)

Research has consistently revealed the antibacterial properties harbored by natural sources such as tea leaves, honey, fungi, and notably, marine organisms, presenting a promising avenue for

the discovery of new antibacterial agents. (Ndako et al., 2019)(Hemeg et al., 2020) (Ujianti et al., 2021) Specifically, the rich marine biodiversity of Indonesia has been identified as a significant reservoir of antibacterial compounds, with Nugroho (2022) showcasing the antibacterial effects of marine species endemic to the region. The gap exists in understanding the modes of action and the full potential of these marine natural compounds against bacteria that have developed resistance to conventional antibiotics, signaling an urgent need for further exploration in this area.(Ercan et al., 2023) This gap leads to investigating alternative treatment options for infections like *Salmonella Sp* that have developed resistance to the standard antibiotic treatments currently in use. Particularly relevant given the high incidence of typhoid fever in regions such as Indonesia and the broader issue of escalating antibiotic resistance worldwide. (Okeke et al., 2024)

Given the escalating issue of antibiotic resistance globally, and the high incidence of diseases like typhoid fever in regions such as Indonesia, the objective of study extends beyond confirming the antibacterial efficacy of natural substances. We aim to delve into the network of biological interactions that govern these natural sources' antibacterial properties. The use of network pharmacology stands out as a pivotal approach in our study, marking a significant stride towards innovating the development of antibacterial agents that can combat resistant bacterial infections more effectively (Noor et al., 2022) This research is particularly focused on the detailed investigation of the ethanol extract of the sea cucumber Stichopus hermanni, aiming to identify its effectiveness against bacteria resistant to current treatments. In this expanded approach, we propose a comprehensive method that combines the identification of bioactive compounds through LC-MS and in silico analysis, specifically exploring the existence of chloramphenicol and immunostimulant properties within marine natural product. We hope to unlock new, efficient, and sustainable antibacterial treatments that can address the critical health threats posed by antibiotic-resistant bacteria.

MATERIALS AND METHODS

Processing of Stichopus hermanni Ethanol Extract

Stichopus hermanni specimens were collected from Nusa Tenggara, Indonesia, processed, and extracted using ethanol. The process by extracting chopped body wall with ethanol in a proportion of 1:5 (weight/volume), adhering to established guidelines for the preparation of marine extracts. After the extraction was completed, the ethanol was meticulously evaporated using a vacuum evaporator maintained at 40°C, thereby preparing the extract for additional examinations.

Method

Liquid chromatography mass spectrometry analysis

For metabolite identification of *Stichopus herrmanni* ethanol extract, Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) was utilized. High-Performance Liquid Chromatography (HPLC) was conducted using an Agilent 1100 series pump equipped with an autosampler and vacuum degasser (Agilent, Palo Alto, CA). Separation was achieved with a fused-core C18-column (Walter, Milford, MA, USA) using an Atmospheric Pressure Chemical Ionization (APCI) source in positive ion mode. The mobile phase comprised acetonitrile and 0.1 % formic acid, flowing at 1 mL/min. The elution buffer's gradient was progressively increased from 30 to 60 % within 36 minutes. Mass spectrometry analyses were performed on an IONIC 3Q Series 200 molecular analyzer. The separated fractions were directly injected into the mass spectrometer at a flow rate of 20 µL/min. Ionization was facilitated in the electrospray mode to ensure an efficient detection and identification process of the extracted metabolites.

In silico Study

Screening of potentially active compounds in sea cucumber

Bioactive compounds in *Stichopus hermanni* were identified using liquid chromatography-mass spectrometry (LC-MS). We also obtain information from the Comprehensive Marine Natural Products Database (CMNPD) for Stichopodidae genus. The SMILE profile and 3D structure of each compound were examined using the PubChem software.

Quantitative Structure-Activity Relationship Analysis

Bioactive compounds were identified and analyzed for their potential using structure-activity relationship (SAR) analysis via the WAY2DRUG PASS prediction tool. For this particular study, we set the cut off value for Pa (Probability of being active) at > 0.7. When the Pa value exceeded the specified threshold, the compound was considered to have potent properties because its structure was similar to other compounds found in the existing drug database.

Toxicity analysis of compounds

The potential toxicity of the bioactive compounds extracted from sea cucumbers was predicted using AdmetLAB 2.0, a powerful tool for assessing drug-like properties and predicting ADME (Absorption, Distribution, Metabolism, and Excretion) profiles of chemical compounds. AdmetLAB 2.0 incorporates a diverse range of computational models and databases to analyze and understand the safety profiles of compounds. This analysis included crucial parameters, prominently the Lipinski Rule of Five, which is a benchmark in drug discovery for evaluating the drug-likeness of compounds.

Prediction of protein targets

Targets associated with the bioactive compounds from *Stichopus hermanni* were identified using the Comparative Toxicogenomics Database (CTD), selecting for targets with a scoring accuracy and probability greater than 80%. Target prediction was facilitated by the input of SMILES notation, acquired in the initial stage of the research. Relevant gene and protein information linked to cervical cancer were extracted from DisGeNet, focusing on candidates

with an overall score prediction of 0.1 or higher. The disease-related targets and those identified from the sea cucumber extract were juxtaposed via a Venn diagram to pinpoint the intersecting targets. The functional attributes of the intersecting target compound were elaborated upon with the aid of the Database for Annotation, Visualization, and Integrated Discovery (DAVID).

Network analysis

The protein targets from *Stichopus hermanni* ethanol extract were further analyzed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING DB V.12.0). The following parameters were used: Organism: Homo sapiens; network type, Full STRING network; and required core, medium confidence (0.4). The data format TSV from STRING was then further processed using CytoScape V.10.0 for network analysis.

Antimicrobial Assays

Antibacterial effectiveness was assessed using the Kirby-Bauer disc diffusion technique. Extract concentrations were tested against*Salmonella typhi* ATCC 14028 and *Staphylococcus aureus* ATCC 25923. This research took place in the Research Laboratory of the Faculty of Medicine at Muhammadiyah University Prof. DR. HAMKA. The extract concentrations were prepared at 200 µg/ml and 300 µg/ml for *Salmonella typhi* and 300 µg/ml and 600 µg/ml for *Staphylococcus aureus.* This procedure was carried out twice. We also performed a disc soaking experiment for 15 minutes. For comparison, a 30 mg chloramphenicol disc was used as the positive control, while a blank disc soaked in distilled water served as the negative control. After soaking, these discs were placed on MHA agar previously inoculated with the bacteria, then incubated at 37 °C for 24 hours. The inhibition zones were measured with a ruler and compared against each control.

Statistical Analyses

Data are presented as the mean ± standard error of the mean. When data were normally distributed, statistical analyses between two groups were performed using an unpaired Student's t-test. Differences among groups were tested using one-way analysis of variance (ANOVA). A probability value of ($p < 0.05$) was considered to be statistically significant.

RESULTS

LC-MS Analysis

LC-MS identified compounds within the *Stichopus hermanni* ethanol extract. Table 1 presents the LC-MS analysis results for the compound.

In silico Study Screening for potentially active compounds in the sea cucumber extract

Table 2 presents identification of the 16 bioactive compounds from genus *Stichopus* identified using CMNPD database. Bioactive compounds showed potential for antibacterial and immunomodulatory activities.

Quantitative Structure-Activity Relationship Analysis

Figure 3 demonstrates the potential of the genus Sticopus as a promising candidate for therapeutic applications in various conditions. Its most significant contribution is to the expression of the actin protein, which is crucial for the formation of the cell's structure. The SAR analysis highlighted the promising potentials of bioactive compounds in sea cucumbers as agents for immunostimulant (Pa Score: 5.133) and antibacterial (0.719) purposes in Figure 4.

Toxicity Analysis

Toxicity analysis of each sea cucumber sample using the AdMet Lab2.0 web server showed that all compounds found in the sea cucumber extract met the criteria for Lipinski's rule. The first compound mentioned, CMNPD13820, fulfills all of Lipinski's rule criteria and shows characteristics similar to those of a promising drug. However, there are several compounds that either exceed the molecular weight threshold (greater than 500 Dalton) or have more than five hydrogen bond donors (HBD), which means they do not comply with the rule.

Network Analysis

Construction and Analysis of Target Protein-Protein Interaction (PPI) Network

The target genes pertaining to each component were analyzed using STRING v_11 to construct and visually represent the PPI network. The data for high-confidence target protein interactions was set with a score level exceeding 0.9, ensuring the connections being analyzed. Depicts these interactions among the target proteins, encompassing an overall 117 nodes and 700 edges. Each edge in this network symbolizes a Protein-Protein Interaction (PPI). Additional parameters, including an average node degree of 12 and a local clustering coefficient of 0.599, represent the number of targets linked to the network.

In Figure 5, key targets implicated in cervical cancer—such as TNF, NFKB1, IL1B, TLR4, STAT3, MYD88—feature prominently within the network. Interestingly, these targets also play a major role in cervical cancer. TNF, TLR, IL1B and NFKB1 are centrally located within the network, underscoring their significant roles in the pathogenesis of cervical cancer. The PPI network and pathway analyses of novel genes were performed to identify critical genes related to cervical cancer

Prediction protein targets of Stichopus hermanni for Salmonella Sp

Figure 6 shows the presence of the following 2 overlapping protein targets between cervical cancer and sea cucumbers: ACTB and TFR. Figure shows the target pathway network of sea cucumber for treating *Salmonella Sp* infection.

GO gene enrichment analysis and KEGG pathway annotation

The GO and KEGG analysis identified the TLR and NfKB signaling pathways as significant in *Salmonella Sp* infection, as illustrated in Figure 7

Antimicrobial Assays

Figure 8 and 9 showed a disk diffusion resistance test, showing that the sea cucumber extract comprises chloramphenicol and various other compounds. The disk diffusion tests revealed that, after soaking the disks for 24 hours, an inhibition zone appeared for *Salmonella typhi* at concentrations of 200 ug/ml and 300 ug/ml, and for *Staphylococcus aureus* at a concentration of 600 ug/ml.

Discussion

Through LC-MS, this research reveals an interesting discovery that sea cucumber extract contains chloramphenicol, an antibiotic that has a bacteriostatic effect. This unique finding broadens the scope of research in this area. Chloramphenicol works by inhibiting the growth of bacteria. It is generally known that Chloramphenicol is an antibiotic that functions by inhibiting bacterial protein synthesis, acting on the 50S ribosome of the bacteria and stopping peptide bond formation. This action makes chloramphenicol bacteriostatic, which prevents bacteria from multiplying without directly killing them.(Smirnova et al., 2023) The unique discovery in this research is that *in silico* analysis clearly shows that the *Salmonella Sp* infection and TLR pathway play a significant role in the immune response and are particularly relevant to immunostimulant therapy for infections such as *Salmonella Typhi*. (Xu et al., 2023) TLRs signal through the recruitment of specific adaptor molecules, leading to the activation of

transcription factors like NF-κB, which in turn initiate the production of pro-inflammatory cytokines necessary for the immune response. Specifically, TLRs such as TLR4 and TLR5 have been implicated in the response to *Salmonella Sp* infections. (Lone et al., 2024) TLR4 recognizes lipopolysaccharides on the surface of Salmonella, and TLR5 recognizes flagellin, the primary protein constituent of bacterial flagella. The relevance of this pathway is underscored by studies showing that manipulating TLR signaling can alter the course of infection and provides a mechanism through which immunostimulants could potentially enhance host defenses against *Salmonella Sp*. In addition, this study emphasizes important molecules in the sea cucumber *Stichopus Sp* for its potential benefits, comparing it to the 2023 study by Ujianti (2023). At certain concentrations, this extract can create an inhibition zone against bacteria such as S. aureus, B. subtilis, and E. coli, demonstrating its effectiveness. From the CMNPD database, Sea Cucumber from genus Schistopoidea contains bioactive molecules like Stichoposides, Variegatoides, and Stichorrenosides, which might interact with pathways like TLR4 and NF-kB, crucial in immunomodulation.

Among the many proteins involved in the infection process, ACTB and TFR are proteins located at the intersection between proteins interacting with extract molecules and proteins involved in immunomodulation processes. In exploring the interactions between immunostimulants in the ACTB and TFR pathways with molecules like Stichoposides, Variegatoides, and Stichorrenosides, a potential mechanistic modulation of the immune system by the extract was discovered. The ACTB pathway, critical in the structure and function of the cell's cytoskeleton, as well as vital processes such as cell migration, cell division, and phagocytosis.(Mylvaganam et al., 2021) Extract molecules interacting with this pathway have the potential to promote actin polymerization, aiding in the reorganization of the cytoskeleton. Consequently, this can enhance the phagocytic ability of macrophages and facilitate the migration of immune cells to the infection site, speeding up and strengthening the immune

response. While the TFR pathway is essential in iron transport, energy metabolism, and the proliferation and differentiation of immune cells. Molecules interacting with this pathway have the potential to increase the availability and metabolism of iron for immune cells in fighting infections. Nevertheless, the specific roles of Stichoposides and Variegatoides in the context of the ACTB and TFR pathways are not yet fully defined, indicating a probability of complex interactions not yet revealed with cellular proteins and signaling pathways. Preliminary research opens the possibility that Stichoposides could stimulate actin polymerization or interact with TFR to modify membrane function or iron pathways. (Mesquita et al., 2021) Variegatoides might affect signaling pathways related to the expression of ACTB and TFR. Sea cucumber extract strengthens immune cells through interactions with the ACTB pathway, which assists in reorganizing the cytoskeleton for more effective phagocytosis and migration of immune cells, and the Transferrin Receptor pathway which increases the availability of iron for immune cell activation processes. (Hanna, 2017; Grander, 2022) Facilitating these two pathways, sea cucumber extract offers the potential to strengthen innate immune responses to bacterial infections, as demonstrated by the enhanced capabilities of immune cells in fighting *Salmonella Sp*. Bioactive molecules in the extract can repair or modulate cellular functions for an immune response, thereby opening opportunities for the development of immunomodulatory therapies.

In silico analysis supports the idea that this extract can enhance the immune system in *Salmonella sp* infection through a new approach, with the combination of antibiotic effects and immune stimulation.(Kanmani et al., 2020) The antibacterial and immunostimulant potential of this extract is very promising in the development of more effective antibacterial potentiation mechanisms, as well as supporting innovation in the health, pharmaceutical, and food sectors.(Vijayaram et al., 2022) To the best of our knowledge, this is the first study to discover the presence of chloramphenicol and immunostimulant effects in *Stichopus holothuroidea*

extract through LC-MS and *in silico* study. By expanding knowledge and opening opportunities for further research, this discovery offers a new, effective, and minimally invasive method for tackling *Salmonella Sp* infection, promising the development of new therapy based on sea cucumber extract.

This study identified bioactive compounds within the ethanol extract of *Stichopus hermanni* that show potential antibacterial and immunomodulatory activities. However, the detection of chloramphenicol within the extract raises questions about its origin, as chloramphenicol is a synthetic antibiotic.

Conclusion

Stichopus hermanni ethanol extract demonstrates promising antibacterial activity and potential for immunomodulation.

Acknowledgements

We extend our heartfelt gratitude to the Faculty of Medicine at Universitas Muhammadiyah Prof. DR. HAMKA for the generous support that enabled this research to be realized. We would also like to acknowledge and convey our appreciation to all individuals who have provided assistance throughout this research process.

Declaration of Interest

The authors declare no competing interests

Author contributions

Each contributor played key roles in this research: IU designed the study and contributed to data acquisition and analysis; CD assisted in data acquisition, analysis, and manuscript revision; BSL was pivotal in data analysis, result interpretation, and manuscript revision; whilst WS & TY handled manuscript preparation and figure/table design.

Funding

This study was financially supported by the Research Institute of Universitas Muhammadiyah Prof. DR. HAMKA, under contract number /F.03.07/2024

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FIGURE LEGENDS

Figure 1. Chromatogram Graph, show signal intensity in relation to the retention time in Chloramphenicol compound in sea cucumber extract

Figure 2. Mass Spectrum Graph: This graph illustrates signal intensity in relation to molecular weight. The peaks in the graph represent the detected compounds in sea cucumber extract

Figure 3. SAR from bioactive compount of genus Sticophus in whole potentiation of therapeutic based Structural analytic

Figure 4. SAR from bioactive compound of genus Sticophus that involve in candidate of therapeutic *Salmonella Sp* infection

Figure 5. Target pathway network of Sea cucumber for treating *Salmonella Sp* infection

Figure 6. Venn Diagram, intersection *Salmonella Sp* infection and *Sticophus* sp.

Figure 7. Gene ontology and KEGG pathway enrichment analysis.

Figure 8. Inhibition zones in the disk diffusion test on *Salmonella typhi* using the Kirby-Bauer disc diffusion technique. A. Inhibition zone at the Positive Control concentration, chloramphenicol 30 mg with a 24-hour incubation B. Inhibition zone at a concentration of 200 µg/ml with a 24-hour incubation C. Inhibition zone at a concentration of 300 µg/ml with a 24-hour incubation

Figure 9. Inhibition zone in the disc soaking experiment on *Staphylococcus aureus* using the Kirby-Bauer disc diffusion technique. A. Inhibition zone at the Positive Control concentration, chloramphenicol 30 mg with a 24-hour incubation B. Inhibition zone at the concentration of 300 µg/ml with a 24-hour incubation C. Inhibition zone at the concentration of 600 µg/ml with a 24-hour incubation

441 **Table 1.** LC-MS Analysis in Sea Cucumber Extract

Compound	Formula	RT (min)	Mass molecule (m/z)	Total Fragments	Signal Intensity
Chloramphenicol	$C_{11}H_{12}N_2O_5$	10.56	267.07	18	29744

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444 **Table 2.** Profile of Bioactive Compounds in Genus Stichopoidea with CMNPD database

Compound Id	Molecular Formula	Molecular Name	Molecular Mass
CMNPD13820	$C_{58}H_{106}N_2O_{18}$	SCG-1	1118.74
CMNPD13821	$C_{52}H_{95}N_2O_{20}S$	SCG-2	1099.62
CMNPD13822	$C_{62}H_{114}N_2O_{22}$	SCG-3	1238.79
CMNPD1722	$C_{43}H_{68}O_{13}$	Stichoposide A	792.47
CMNPD1723	$C_{43}H_{68}O_{14}$	Stichoposide B	808.46
CMNPD1724	$C_{69}H_{112}O_{33}$	Stichoposide C	1468.71
CMNPD1725	$C_{69}H_{112}O_{34}$	Stichoposide D	1484.70
CMNPD25648	$C_{53}H_{84}O_{22}$	Variegatuside C	1072.55
CMNPD25649	$C_{59}H_{96}O_{27}$	Variegatuside D	1236.61
CMNPD25650	$C_{66}H_{108}O_{32}$	Variegatuside E	1412.68
CMNPD25651	$C_{66}H_{108}O_{32}$	Variegatuside F	1412.68
CMNPD29857	$C_{41}H_{64}O_{13}$	Stichorrenoside A	764.43
CMNPD29858	$C_{41}H_{63}NaO_{16}S$	Stichorrenoside B	866.37
CMNPD29859	$C_{43}H_{66}O_{14}$	Stichorrenoside C	806.45
CMNPD29860	$C_{42}H_{64}O_{13}$	Stichorrenoside D	776.43
CMNPD31481	$C_{55}H_{86}O_{23}$	Stichorrenoside E	1114.56

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446 **Table 3.** Antibiotic disk diffusion for antibiotics tested

Organism	Concentration	Incubation Time	Resistance range
Salmonella typhi	Positive control	24 hours	30 mm
	Negative control		0 mm
	200 µg/ml		12 mm

	300 µg/ml		10 mm
Staphylococcus aureus	Positive control	24 hours	23 mm
	Negative control		0 mm
	300 µg/ml		0 mm
	600 µg/ml		8 mm

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