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Research Article
Bioactivity-Guided Evaluation of Extracts and Fractions of Seagrass (*Enhalus acoroides*): *In vitro* Antimicrobial and Anti-Inflammatory Activities with LC–MS/MS Characterization

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Abstract

Background and Objective: The seagrass *Enhalus acoroides* is a marine plant species widely distributed in the waters of Southeast Sulawesi. It is considered a potential source of bioactive compounds with pharmaceutical relevance. This study aimed to characterize the chemical constituents of *E. acoroides* rhizomes and evaluate their antimicrobial and anti-inflammatory activities. **Materials and Methods:** Phytochemical profiling of the extract and ethyl acetate fraction was performed using Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS). Antimicrobial activity was assessed using the microdilution method against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. Anti-inflammatory activity was evaluated using a protein denaturation assay. The statistical analysis was conducted using SPSS, with a one-way ANOVA at a significance level of $p<0.05$ (95% CI). **Results:** LC-MS/MS analysis identified twelve compounds in the extract and ethyl acetate fraction, including hecogenone, periploca side N, blumenol C glucoside, α-D-glucopyranose-1-hexadecanoate, kushenol R, procyanidin A2, physalin L, Δ5-pregnene-3β,17α,20α-diol, verticiol, quercimeritrin, pterodontoside F, and vitamin K1. Fractions D and E exhibited strong antibacterial activity against *S. aureus*, with minimum inhibitory concentrations (MICs) of 2 and 1 µg/mL, respectively, and both showed activity against *E. coli* (MIC = 2 µg/mL). Fraction E also demonstrated antifungal activity against *C. albicans* (MIC = 4 µg/mL). In the anti-inflammatory assay, fraction E exhibited the highest activity, with an IC_{50} of 29.48 µg/mL. **Conclusion:** The findings indicate that *E. acoroides* rhizomes are a promising source of bioactive compounds with significant antimicrobial and anti-inflammatory potential.

Key words: *Enhalus acoroides*, antimicrobial, anti-inflammatory, Southeast Sulawesi

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Indonesia possesses exceptionally rich marine biodiversity that is globally recognized, supported by its diverse coastal and marine ecosystems, including mangrove forests, coral reefs, and seagrass meadows[1]. Of the approximately 30-40% of seagrass species identified worldwide, around 12 species are found in Indonesian waters, namely *Syringodium isoetifolium*, *Halophila ovalis*, *Halophila spinulosa*, *Halophila minor*, *Halophila decipiens*, *Halodule pinifolia*, *Halodule uninervis*, *Thalassodendron ciliatum*, *Cymodocea rotundata*, *Cymodocea serrulata*, *Thalassia hemprichii*, and *Enhalus acoroides*[2].

Enhalus acoroides is one of the seagrass species commonly distributed in Indonesian marine environments. It typically inhabits sandy substrates subjected to high levels of biological disturbance. Previous studies have reported that extracts and isolated compounds from *E. acoroides* exhibit a wide range of pharmacological activities, including antioxidant, antibacterial, antilarval, anti-inflammatory, and antidiabetic effects. In particular, condensed extracts rich in polysaccharides and related constituents have demonstrated notable antibacterial activity[3].

Infectious diseases arise from the invasion and proliferation of microorganisms, which include bacteria, fungi, parasites, and viruses. The primary therapeutic approach to address these infections involves antimicrobial agents, encompassing antibacterial (antibiotics), antifungal, antiviral, and antiprotozoal drugs. Antimicrobials are defined as biological or chemical substances capable of inhibiting microbial growth (bacteriostatic or fungistatic) or eliminating microorganisms (bactericidal or fungicidal)[4]. However, the inappropriate and excessive use of antibiotics has contributed to the emergence of antibiotic-resistant strains, commonly referred to as multidrug resistance (MDR). Notably, *E. acoroides* contains various bioactive compounds, such as alkaloids, flavonoids, steroids, benedict, and ninhydrin, which exhibit antimicrobial properties and thus hold potential for development as natural antibiotic agents[5].

Inflammation, which often accompanies infection, can be managed using anti-inflammatory agents that function to alleviate its clinical manifestations[6]. It represents an adaptive physiological response to harmful stimuli, including infection and tissue injury, aimed at maintaining homeostasis[7]. During inflammatory processes, increased body temperature and pain may occur, potentially leading to protein denaturation[8]. Protein denaturation refers to the loss of secondary and tertiary protein structures due to external factors such as heat, chemical agents, or solvents, resulting in diminished enzymatic activity and biological function[9]. The protein denaturation assay is commonly employed as an *in vitro* method for evaluating anti-inflammatory activity. According to Hasan *et al.*[10] compounds capable of stabilizing proteins against denaturation are considered to possess anti-inflammatory potential[10]. However, prolonged use of steroidal and non-steroidal anti-inflammatory drugs is associated with adverse effects, including gastrointestinal, cardiovascular, hepatic, renal, neurological, and pulmonary complications[11]. Consequently, the exploration of natural sources for safer anti-inflammatory compounds remains an important research priority.

With the increasing complexity and diversity of bioactive compounds, there is a growing need for analytical techniques that offer high sensitivity and selectivity. Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) has become a preferred analytical tool in various stages of drug discovery due to its high separation efficiency and analytical reliability[12].

Therefore, further investigation into the metabolites of *E. acoroides* rhizomes from Southeast Sulawesi, along with their antimicrobial and anti-inflammatory activities, is warranted. In this study, the secondary metabolite profile of *E. acoroides* was analyzed using LC-MS/MS, while its antimicrobial activity was evaluated through the microdilution method, and its anti-inflammatory potential was assessed using the protein denaturation assay.

MATERIALS AND METHODS

Study area and duration: This experiment was conducted at the Laboratory of Pharmacy, Faculty of Pharmacy, from February 2023 to February 2024.

Sample preparation: Samples of seagrass rhizomes were collected from the waters of Bintang Samudra Marine Education Park, Sawapudo Village, Soropia District, Konawe Regency, Southeast Sulawesi. Species identification was carried out by an expert from the Faculty of Fisheries and Marine Science, Halu Oleo University (No. 76i.UU.29.12.1.1/PP/2024).

Extraction and fractionation: The sample (4.3 kg) was macerated in ethyl acetate for 3×24 hrs. The resulting filtrate was subsequently concentrated using a rotary vacuum evaporator, yielding 8.721 g of crude extract[13]. The extract was then fractionated by vacuum liquid chromatography (VLC) following impregnation of 25 g of the

extract onto silica gel 60 GF_{254}. Elution was carried out using a gradient system of n-hexane-ethyl acetate, followed by 100% methanol, resulting in five fractions: A (0.09 g), B (0.88 g), C (0.83 g), D (1.86 g), and E (3.26 g)[13,14].

LC-MS/MS analysis: The LC-MS/MS analysis was conducted to identify the chemicals in *E. acoroides* extract and fractions by analyzing the mass-to-charge-ratio (m/z) values and was examined by the Dictionary of Marine Natural Products[14].

Antimicrobial activity assay *in vitro*: Antimicrobial activity was evaluated using the microdilution method. Briefly, 100 µL of agar medium was combined with the extract and fractions at concentrations ranging from 0.5 to 256 µg/mL. Subsequently, 100 µL of microbial suspension (*Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*) standardized to 0.5 McFarland was added to each well. Dimethyl Sulfoxide (DMSO) was used as the negative control, meanwhile chloramphenicol and nystatin were used as positive control, respectively. The plates were then incubated at 37°C for 20 hrs. The minimum inhibitory concentration (MIC) values were determined spectrophotometrically using a microplate reader (iMark™, Bio-Rad, USA). All experiments were performed in triplicate[15].

Anti-inflammatory activity assay *in vitro*: Anti-inflammatory activity was evaluated using a protein denaturation assay. Briefly, 100 µL of the extract and fractions were added to each well of a microplate, followed by the addition of 100 µL of 0.2% bovine serum albumin (Sigma, USA). Diclofenac sodium was used as the positive control.

The reaction mixtures were heated at 71°C for 5 min and subsequently cooled at room temperature for 25 min. Absorbance was measured at 660 nm using a UV-Vis spectrophotometer (Techcomp® 2501, Shanghai). All experiments were performed in triplicate[16].

Statistical analysis: Data are presented as Mean±Standard Deviation (SD) and were statistically analyzed using either an independent t-test or one-way ANOVA followed by Tukey's *post hoc* test. Statistical analyses were performed using IBM® SPSS® Statistics for Mac OS, Version 26 (IBM Corp., Armonk, NY, USA), with a significance level set at $p<0.05$.

RESULTS AND DISCUSSION

The chemical composition of the *E. acoroides* extract and fractions A-F varied, as evidenced by the compounds identified in each sample based on LC-MS/MS analysis (Table 1).

The LC-MS profiling revealed a wide array of secondary metabolites distributed across the extract and its fractions, demonstrating that the fractionation process successfully resolved compounds based on their physicochemical characteristics. Several classes of compounds were identified in the extract and fractions, including saponins (hecogenone), steroids (periplocoside N, physalin L, and Δ5-pregnene-3β,17α,20α-diol), sesquiterpenoids and terpenoids (blumenol C glucoside, verticiol, and pterodontoside F), monosaccharides and fatty acids (α-D-glucopyranose-1-hexadecanoate), flavonoids and flavonols (kushenol R and quercimeritrin), tannins (procyanidin A2), and quinones (vitamin K1).

The crude extract was found to contain hecogenone, a steroidal sapogenin belonging to the saponin class, and periplocoside N, a steroidal glycoside. Saponins are classified based on the nature of their aglycone (sapogenin) as triterpenoid, steroidal, or alkaloid glycosides, and their presence has been documented in more than 100 plant families. *Spirostanol sapogenins* such as hecogenin have long been valued by the pharmaceutical industry and used as substrates in the production of steroid hormones and drugs. The identification of these steroidal compounds in the crude extract suggests that steroid-derived metabolites form a significant part of the plant's phytochemical profile[17,18].

Fraction A was characterized by blumenol C glucoside, a sesquiterpenoid glycoside, and α-D-glucopyranose-1-hexadecanoate, a compound in which a sugar unit is esterified to a fatty acid chain. The predominance of glycosylated compounds in this fraction is consistent with the enrichment of relatively polar metabolites during solvent partitioning and chromatographic separation. Glycosylation can alter the biological activity, water solubility, stability, intracellular transport characteristics, and subcellular localization of plant metabolites, thereby influencing many aspects of their biological behavior[19].

Fraction B displayed the greatest chemical diversity among all fractions. Its constituents included kushenol R, a prenylated flavonoid; procyanidin A2, a condensed tannin; physalin L, a steroidal metabolite; and Δ5-pregnene-3β,17α,20α-diol, another steroid-derived compound. The simultaneous presence of flavonoids, tannins, and steroids within this fraction reflects the wide range of semi-polar constituents separated during the fractionation process. Flavonoids and tannins are primarily synthesized via the phenylpropanoid pathway and perform critical functions in plants, including defense against biotic stresses such as pathogens and herbivores, mediation of ecological interactions, and protection against abiotic stresses such as UV radiation and oxidative damage[20].

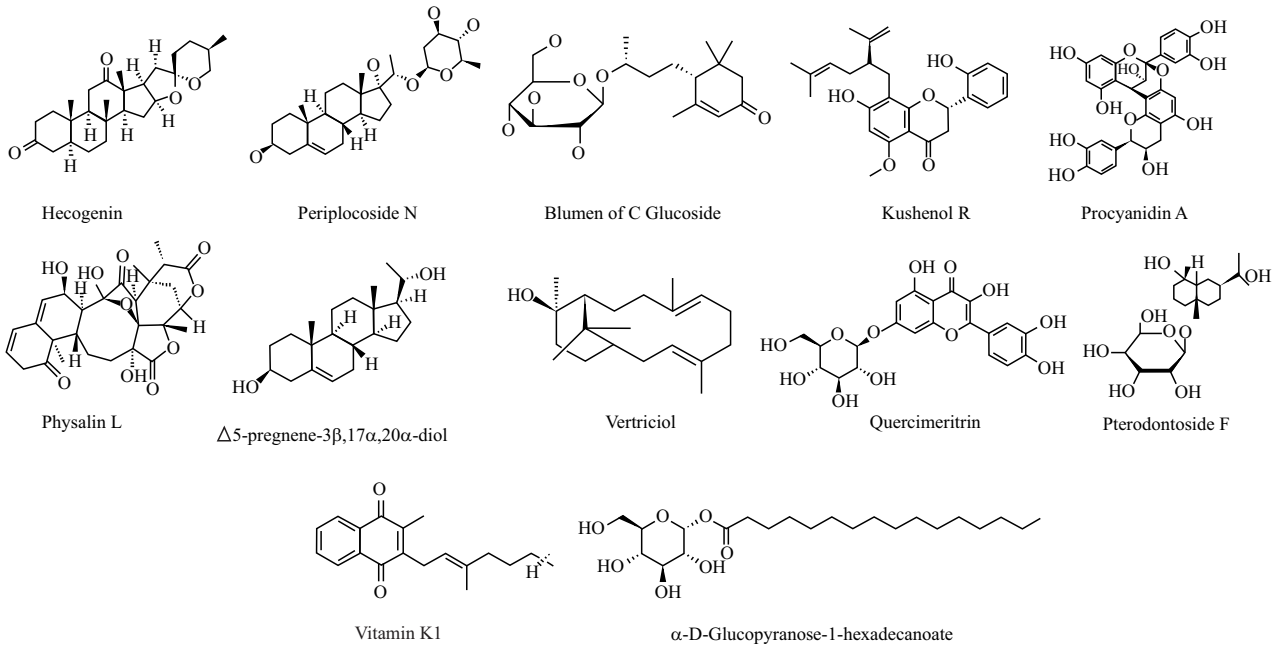

Fig. 1: Identified compound structures of the ethyl acetate extract of *E. acoroides* and its fractions

Table 1: Chemical profile of ethyl acetate extract of *E. acoroides* and its fractions

No	Sample	Rt (minute)	Observed m/z	Neutral mass (Da)	Formula	Compound name	Groups
1	Extract	9.90	429.3037	428.29266	$C_{27}H_4O_4$	Hecogenone	Saponin
		12.36	465.3232	464.31379	$C_{27}H_{44}O_6$	Periplocoside N	Steroid
2	Fraction A	8.14	395.2015	372.21480	$C_{19}H_{32}O_7$	Blumenol C glucoside	Sesquiterpenoid
		11.94	441.2841	418.29305	$C_{22}H_{42}O_7$	α-D-Glucopyranose-1-hexadecanoate	Monosaccharides and fatty acids
3	Fraction B	7.59	445.2000	422.20932	$C_{26}H_{30}O_5$	Kushenol R	Flavonoid
		7.85	599.1213	576.12678	$C_{30}H_{24}O_{12}$	Procyanidin A2	Tannin
		8.36	529.2077	528.19955	$C_{28}H_{32}O_{10}$	Physalin L	Steroid
		9.91	341.2439	318.25588	$C_{21}H_{34}O_2$	Δ5-Pregnene-3β,17α,20α-diol	Steroid
		10.64	313.2489	290.26097	$C_{20}H_{34}O$	Vertical	Terpenoid
4	Fraction C	6.57	465.1070	464.09548	$C_{21}H_{20}O_{12}$	Quercimeritrin	Flavonol
		9.55	419.2615	418.25667	$C_{19}H_{38}O_8$	Pterodontoside F	Sesquiterpenoid
5	Fraction D	9.89	473.3348	450.34978	$C_{31}H_{46}O_2$	Phytonadione (Vitamin K1)	Quinone
		10.64	313.2486	290.26097	$C_{20}H_{34}O$	Vertical	Terpenoid
6	Fraction F	10.64	313.2486	290.26097	$C_{20}H_{34}O$	Vertical	Terpenoid

These compounds, drawn using ChemDraw (Version 21.0.0.28, PerkinElmer Informatics, Inc., USA), are likely to contribute to the biological activities of *E. acoroides* shown in Fig. 1, based on LC-MS/MS data that were compared with database references from sources such as MzCloud and ChemSpider[24,25]

Fraction C contained quercimeritrin, a flavonol glycoside, and pterodontoside F, a sesquiterpenoid glycoside. The co-occurrence of glycosylated flavonoids and terpenoids in this fraction points to an enrichment of more polar compounds attributable to the presence of sugar residues. The incorporation of a sugar moiety into low-molecular-weight secondary metabolites has been shown to influence key properties of the acceptor compound, including solubility, stability, bioactivity, subcellular localization, and receptor binding behavior. Such modifications are widely observed in plant secondary metabolites and are known to affect their distribution behavior during chromatographic separation[21].

Fraction D contained phytonadione (vitamin K1), a quinone derivative, together with a terpenoid compound. Quinones are produced in organisms and utilized as electron transfer agents, pigments, and in defense mechanisms, and naturally occurring quinones can also be cytotoxins with antibacterial properties, characteristics linked to their redox properties. Terpenoids, on the other hand, represent the largest group of secondary metabolites and are highly diverse in both chemical structure and biological properties[22,23].

The same terpenoid compound was additionally detected in Fraction F, indicating that this constituent may be relatively abundant in the plant material and was distributed

Table 2: Classification of antimicrobial potency[27]

	Staphylococcus aureus		*Escherichia coli*		*Candida albicans*	
Samples	MIC (µg/mL)	Notes	MIC (µg/mL)	Notes	MIC (µg/mL)	Resistant
Extract	32	Resistant	64	Resistant	256	Resistant
Fraction A	8	Intermediate	64	Resistant	128	Resistant
Fraction B	8	Intermediate	16	Intermediate	64	Resistant
Fraction C	8	Intermediate	32	Weak	32	Resistant
Fraction D	2	Susceptible	2	Susceptible	8	Intermediate
Fraction E	1	Susceptible	2	Susceptible	4	Strong

Fraction A: Blumenol C glucoside and α-D-glucopyranose-1-hexadecanoate (glycosides), Fraction B: Kushenol R, procyanidin A2, physalin L, and Δ5-pregnene-3β,17α,20α-diol (flavonoid, tannin, and steroidal compounds), Fraction C: Quercimeritrin and pterodontoside F (flavonol and sesquiterpenoid glycosides), Fraction D: Phytonadione (vitamin K) and a terpenoid, Fraction E: Not chemically characterized in this study, Fraction F: Terpenoid compound, MIC: Minimum inhibitory concentration and Lower MIC values indicate greater antimicrobial activity

across multiple fractions during the separation process. Terpenoids are associated with a wide range of biological properties, including antioxidant, antimicrobial, anti-inflammatory, and anticancer activities, and are considered for potential application across the pharmaceutical, food, and cosmetics industries. Its repeated detection across the most active fractions underscores its potential significance within the overall phytochemical composition of the plant under study[22].

In this study, the antimicrobial activity of *E. acoroides* was evaluated using its extract and fractions against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. *Candida albicans* was included due to its role as a commensal fungus that is generally harmless under normal conditions but can cause superficial infections or severe systemic infections under certain circumstances[26].

The inhibitory activity obtained was expressed as minimum inhibitory concentration (MIC) values and subsequently interpreted according to the Clinical and Laboratory Standard Institute (CLSI) standard, as presented in Table 2[27].

As presented in Table 2, the antimicrobial properties of the extract and its fractions were assessed against *Staphylococcus aureus* (Fig. 2a), *Escherichia coli* (Fig. 2b), and *Candida albicans* (Fig. 2c), using minimum inhibitory concentration (MIC) values as the primary measure. The unfractionated crude extract showed limited antimicrobial effectiveness, yielding MIC values of 32 µg/mL for *S. aureus*, 64 µg/mL for *E. coli*, and 256 µg/mL for *C. albicans*, reflecting resistance across all three test organisms.

The fractionation process led to notable improvements in antimicrobial potency for several fractions. Fractions A, B, and C each demonstrated moderate inhibition of *S. aureus*, with MIC values of 8 µg/mL placing them in the intermediate susceptibility category. Against *E. coli*, Fraction B performed better than the other two, recording an MIC of 16 µg/mL, while Fractions A and C showed minimal or no meaningful inhibitory effects. Regarding *C. albicans*, Fractions B and C outperformed the crude extract, achieving MIC values of 64 and 32 µg/mL, respectively, though the fungus still fell within the resistant classification.

Fraction D stood out as the most potent broad-spectrum antibacterial agent among the fractions, achieving MIC values of 2 µg/mL against both *S. aureus* and *E. coli*, which placed both organisms in the susceptible range. It also showed greater antifungal activity compared to earlier fractions, with an MIC of 8 µg/mL against *C. albicans*, corresponding to intermediate susceptibility.

Fraction E proved to be the most effective fraction overall. It produced the lowest MIC against *S. aureus* at 1 µg/mL and retained potent activity against *E. coli* at 2 µg/mL, with both classified as susceptible. It also achieved the strongest antifungal performance, inhibiting *C. albicans* at an MIC of 4 µg/mL, indicative of strong antifungal activity.

Taken together, these findings indicate that the bioactive antimicrobial constituents became concentrated in Fractions D and E through the fractionation process against *S. aureus* and *E. coli*. Furthermore, fraction E demonstrated notable antifungal activity against *C. albicans*. The consistent reduction in MIC values from the crude extract to these enriched fractions reflects effective isolation of active compounds, with Fraction E demonstrating the highest overall promise as a broad-spectrum antibacterial and antifungal agent.

Natural compounds that significantly contribute to antibacterial activity include phenolics (such as flavonoids, tannins, and stilbenes), alkaloids, and terpenoids, particularly those belonging to the monoterpene and diterpene groups[28]. The moderate antibacterial activity observed in fractions A, B and C is likely associated with the presence of identified bioactive compounds (Table 1), including terpenes (terpenoids and sesquiterpenoids), flavonoids, polyphenols, and steroids.

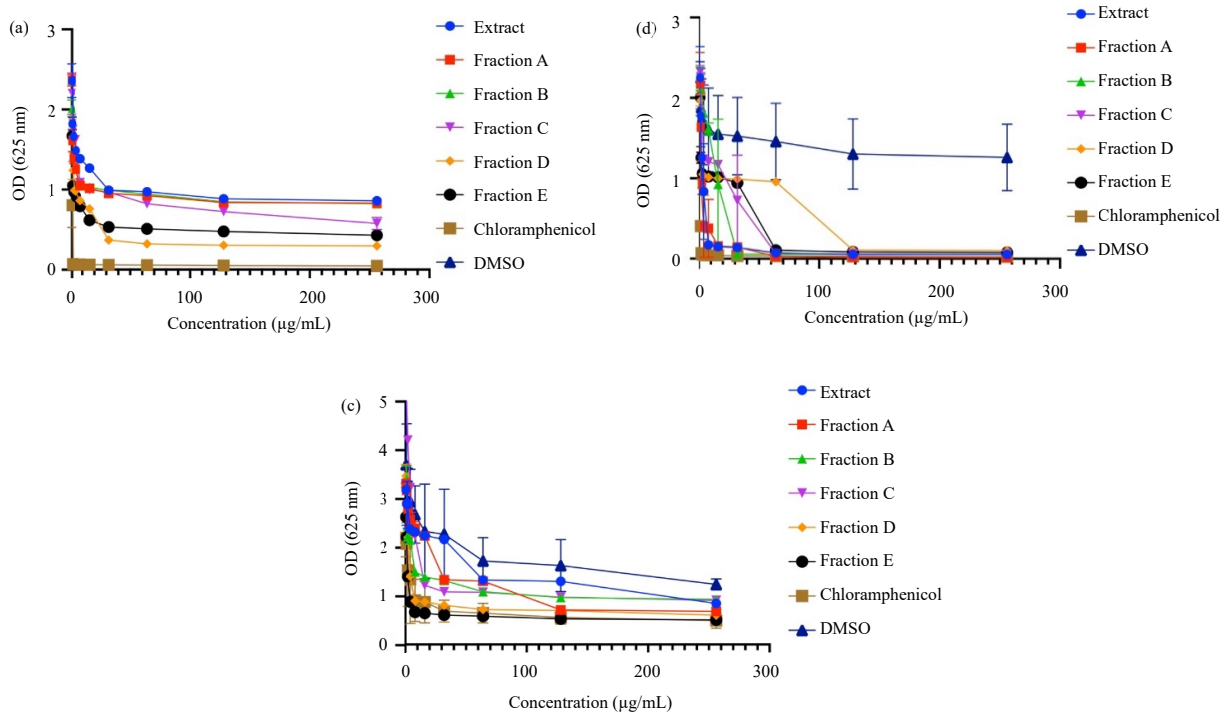

Fig. 2(a-c): Antimicrobial activity of *E. acoroides* extract and fraction on bacterial/ fungal growth, measured as optical density at 625 nm (OD_{625}, (a) Antibacterial activity in *Staphylococcus aureus*, (b) Antibacterial activity in *Escherichia coli* and (c) Antibacterial activity in *Candida albicans*

Data is presented as Mean±SD ($n = 3$)

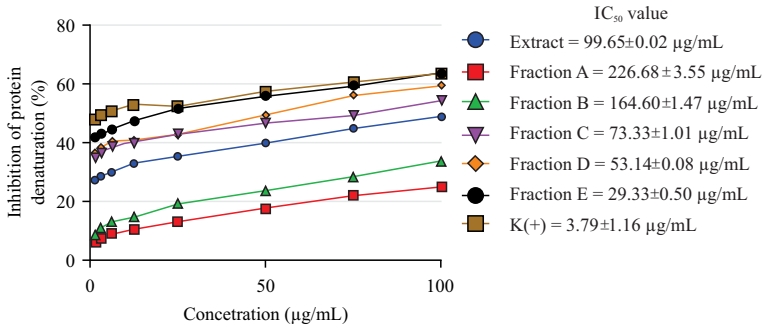

Fig. 3: IC_{50} of the extract and fractions from *E. acoroides* (K+ = diclofenac sodium)

Data is presented as Mean±SD ($n = 3$)

Terpenoids exert antibacterial effects primarily through disruption of bacterial cell membranes due to their lipophilic nature. These compounds can interact with porins (transmembrane proteins) in the outer membrane, forming stable complexes that damage porin structure and reduce membrane permeability, ultimately inhibiting bacterial growth or causing cell death[29]. Flavonoids exhibit antibacterial activity through interactions with cell membranes, where the number and position of hydroxyl groups influence their affinity for lipid bilayers. This interaction enables flavonoids to penetrate and disrupt membrane integrity, leading to cell lysis. In addition, phenolic compounds interfere with bacterial DNA synthesis and regulatory processes. Their hydroxyl groups and aromatic structures facilitate interactions with proteins, resulting in increased membrane permeability and leakage of intracellular contents. Polyphenols may also inhibit enzymes through both specific and non-specific interactions with protein moieties, with their antimicrobial potency often increasing with the degree of hydroxylation[30].

The anti-inflammatory activity of *E. acoroides* rhizome extract and its fractions is presented in Fig. 3. The IC_{50} values for the extract, fractions A-E, and the positive control were 99.65±0.02, 226.68±3.55, 164.60±1.47, 73.33±1.01, 53.14±0.08, 29.38±0.50, and 3.79±1.61 µg/mL, respectively. The result showed significantly difference among groups, which K(+) showing the lowest IC_{50} value than samples ($p<0.05$). However, among the tested samples, fraction E exhibited very strong anti-inflammatory activity, followed by the crude extract and fractions C and D, which showed strong activity. In contrast, fractions A and B demonstrated weak anti-inflammatory effects.

Based on the classification proposed by Molyneux *et al*.[31] anti-inflammatory activity is categorized as very strong when IC_{50}<50 µg/mL, strong at 50-100 µg/mL, moderate at 100-150 µg/mL, and weak when IC_{50}>150 µg/mL[31].

The compounds identified in the extract and fractions are likely to contribute to their anti-inflammatory activity through inhibition of protein denaturation. Hecogenone exhibits notable anti-inflammatory effects, which is further supported by a study reporting that hecogenone suppresses the expression of pro-inflammatory cytokines, including IL-1β, IL-6, IL-12, and TNF-α in *in vivo* models[32,33].

Similarly, periplocoside N inhibits the production of pro-inflammatory mediators such as IL-1β, IL-6, TNF-α, and COX-2, as well as suppresses B cell proliferation in autoimmune conditions, including rheumatoid arthritis[32]. In addition, palmitic acid, derived from α-D-glucopyranose-1-hexadecanoate, is suggested to exhibit anti-inflammatory activity through its inhibitory effect on phospholipase enzymes[34,35].

CONCLUSION

The findings of this study highlight *Enhalus acoroides* rhizomes as a promising source of bioactive compounds with significant antimicrobial and anti-inflammatory activities. The enhanced potency observed in specific fractions suggests that bioactive compound enrichment plays a critical role in therapeutic efficacy. These compounds hold potential as alternative antimicrobial agents to combat the increasing threat of microbial resistance, as well as safer anti-inflammatory agents with fewer side effects compared to conventional drugs.

Future studies should focus on the isolation and structural elucidation of the active constituents, as well as detailed investigations of their mechanisms of action, toxicity profiles, and *in vivo* efficacy. Such efforts are essential to support the development of *E. acoroides*-derived compounds into clinically relevant pharmaceutical agents.

SIGNIFICANCE STATEMENT

This study demonstrates that the rhizomes of *Enhalus acoroides* are a valuable source of bioactive phytochemicals with potent antimicrobial and anti-inflammatory activities. The identification of twelve bioactive compounds, together with the strong antibacterial, antifungal, and protein denaturation inhibitory effects of the ethyl acetate fractions, highlights the pharmaceutical potential of this seagrass species. These findings provide a scientific basis for the sustainable utilization of *E. acoroides* as a natural source of therapeutic agents and support further studies on the isolation, characterization, and *in vivo* evaluation of its active constituents for drug development.

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