Trop J Nat Prod Res, June 2025; 9(6): 2504 - 2512 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Secondary Metabolites of *Isis hippuris*: *In vitro* and *In silico* Studies on Antimicrobial Potential

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ARTICLE INFO

Article history:
Received 23 February 2025
Revised 16 April 2025
Accepted 05 May 2025
Published online 01 July 2025

ABSTRACT

Isis hippuris is a marine species and is abundant in Southeast Sulawesi. However, studies on its potential, particularly for medicinal development, are still limited. This research aims to explore the chemical contents and biological activities of *I. hippuris* from the waters of Bukori Island. Ethylacetate extract of *I. hippuris* (EAE) was fractionated by vacuum liquid chromatography (VLC) and analyzed through phytochemical screening and LC-MS/MS. The antimicrobial potential was evaluated against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* using the microdilution method and *in silico* analysis. The results showed that fractionation of EAE produced five fractions (A–E). Fraction E showed the highest antibacterial activity against *S. aureus* and *E. coli*, with MIC values of 2 µg/mL, thus categorized as susceptible. At the same time, Fractions C and E exhibited the highest antifungal activity against *C. albicans*, with MIC values of 4 µg/mL, thus categorized as susceptible. Additionally, 3 out of 12 major compounds in Fraction E and 14 of 21 major compounds in Fraction C were identified. *In silico* studies predicted that periplocoside M and 25(S)-ruscogenin have the highest affinity for *S. aureus*, saurufuran B and siraitic acid E for *E. coli*, and abrusoside A and periplocoside M for *C. albicans*, targeting *β*-ketoacyl-ACP synthase, tyrosyl-tRNA synthetase, and sterol-14*α*-demethylase, respectively. In conclusion, *I. hippuris* from Southeast Sulawesi shows promising potential as an antibiotic agent

Keywords: Antimicrobes, in silico study, in vitro study, *Isis hippuris*, secondary metabolites

Introduction

Southeast Sulawesi Province, Indonesia, is an archipelago comprising approximately 651 small islands. Around 74.25% of its area is oceans,[1] thus contributing to the abundance of marine resources in this region. Several marine biota of Southeast Sulawesi have been studied for their potential in medicine development, such as sponges and corals. Recent reports include the discovery of a novel compound, Clathruhoate, extracted from *Chlatria* sp.,[2] while other similar studies on sponges have also conducted chemical screening,[3] evaluating its biological activities for anti-hyperlipidemia potential,[4] anti-inflammatory property,[5] antioxidant capabilities, and acute toxicity.[6] Studies on corals have contributed other important discoveries through chemical and medicinal characteristics, such as the study of *Lobophytum* sp. and *Nepthea* sp[7-9]

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Citation: Sahidin PRS., Rahman DA., Fristiohady A, Sadarun, B., Yodha, AWM., Arfan, Manggau, MA., & Sundowo, A. Secondary metabolites of *Isis hippuris*: *In vitro* and *in silico* studies on antimicrobial potential. Trop. J. Nat. Prod. Res., 2025 9(6): 2504 - 2512 https://doi.org/10.26538/tjnpr/v9i6.22

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

The abundance of diverse coral species in the region presents opportunities for exploration, including the Sea Bamboo (*Isis hippuris*). The population of *I. hippuris* in Southeast Sulawesi, as represented by the population on Bukori Island, is approximately 142 individuals per 500 m^2 with most of the commodities being exported[10]. Indonesia exported around 420 tons of dry sea bamboo in 2013, which is generally used as jewellery, ornaments, and building materials.[11] To increase the benefits and economic value of *I. hippuris*, various aspects need to be studied, including chemical and pharmaceutical aspects, which will enrich the ongoing research in the aforementioned theme of exploration study of marine resources of this area. Sea bamboo sourced from other locations has been previously studied, such as a discovery in 1977 reported various secondary metabolites from Australian *I. hippuris*, such as Hippurin-1,12 polyoxygenated steroids, hydrocarbons, phenols, alkaloids, and fatty acids.[13-16] All previously mentioned metabolites have potential in pharmacological activities as antioxidants,[17] anticancer,[18-19] and antiviral agents.[20] Moreover, 17,20-epoxisteroids were recently found in *I. hippuris* from Taiwan, and the discovery of 24-Dehydrohippuristanol was reported to be active against DLD-1 and NoVo cancer cells.[21] Hippuristanol is a polyoxygenated steroid, similar to hippuristeron and hipposterol, which have demonstrated biological activities as anticancer agents, notably in treating leukaemia.[22] Other steroid compounds also exhibit cytotoxic activity against P-388, A549, HT29, and MEL28 cells.[23]

The chemical content of *I. hippuris* is closely affected by its environment (location) and its conservation status.[24,25] Therefore, to expand this knowledge gap, this study aims to explore the chemical compounds of *I. hippuris* from Southeast Sulawesi through extraction and fractionation techniques, Liquid Chromatography Mass Spectrometer (LC-MS/MS) analysis, evaluation of antimicrobial

potential (bacteria and fungi), and *in silico* studies. The antimicrobial mechanism of the identified chemical compounds provides new information that can support the future utilization of *I. hippuris* in the pharmaceutical industry.

Materials and Methods

Materials

A Waters Acquity UPLC I-Class was used in conjunction with a Xevo G2–X2 Quadrupole Time-of-Flight (QToF) mass spectrometer to perform the LC-MS/MS study. Methanol, ethyl acetate, and n-hexane are among the chemicals used, as well as analytical-grade distilled water. The chromatography study uses TLC plate 60 F_{254} 0.25 mm (Merck®), silica gel 60 GF_{254} p.a (Merck®), and silica 60 G (Merck®). The other materials used for the study and testing were cerium sulfate ($CeSO_4$) (Merck®), *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, Mueller Hinton Broth (MHB), Sabouraud Dextrose Broth (SDB), NaCl 0,9%, dimethyl sulfoxide (DMSO), chloramphenicol and nystatin.

Sample Collection

Samples were collected from Bokori Islands, Southeast Sulawesi, on November 6, 2023, at depths between 4-10 m (3°56'12.7"S 122°39'53.2"E). The collected samples were stored in separate ice containers and analyzed by the Indonesian Marine Ecosystem Specialist at the laboratory of the Faculty of Fisheries and Marine Science, Halu Oleo University, as *Isis hippuris* with number 5871/UN29.18.1.1/PP/2023.

Extraction and Fractionation

Fresh *I. hippuris* (1 kg) was chopped into small pieces and extracted in ethyl acetate (3 x 10 L, 24 hours each time) at room temperature. The filtrate was concentrated with a rotary evaporator (Buchi RII, Switzerland) at 4°C to obtain a thick ethyl acetate extract of *I. hippuris* (EAE). A total of 21.4 g of EAE was fractionated using Vacuum Liquid Chromatography (KVC), resulting in five fractions (A-E).

Antibacterial and Antifungal Potencies

The microbes were prepared using standard procedure. Antimicrobial potencies and minimum inhibition concentration (MIC) were evaluated using the microdilution method with positive control chloramphenicol for antibacterial and nystatin for antifungal.[26] *Staphylococcus aureus* and *Escherichia coli* were cultured on MHB at 37°C for 24 hours, while *Candida albicans* was grown on SDB at the same temperature for 72 hours. The resulting microbial cultures were suspended in sterile 0.9% NaCl, and 100 µL of each suspension was added to a 96-well plate. These suspensions were then combined with test samples at concentrations ranging from 0.5 to 256 µg/mL, reaching a total volume of 200 µL per well. Chloramphenicol and nystatin served as positive controls, respectively, whereas dimethyl sulfoxide (DMSO) was used as a negative control. The microbial suspensions were adjusted to match a 0.5 McFarland turbidity standard. Incubation was carried out at 37°C for 20 hours. The minimum inhibitory concentration (MIC) was assessed using a spectrophotometric method to measure turbidity.[27]

Phytochemical screening and LC-MS/MS Analysis

Phytochemical Screening

The presence of alkaloids, flavonoids, tannins, terpenoids, steroids, and saponins in this study is referred to a previous study in sponges, which was determined using phytochemical screening methods.[8]

LC-MS/MS Analysis

Secondary metabolites from fraction A were analyzed using a Liquid Chromatography Mass Spectrometer (LC-MS/MS) (Waters, USA) with an HSS T3 C18 column at 40°C. The mobile phases used were 0.1% formic acid in water (A) and acetonitrile (B), with a gradient elution of 0.300 mL/min. Mass spectrometry was performed in positive ESI mode, scanning 50-1200 m/z, capillary voltage 1.5 kV, cone voltage 30 V, and desolvation temperature 500°C. Data analysis was performed using UNIFI software, with further interpretation via mzCloud, ChemSpider, and PubChem.[28]

In-silico study for Mechanisms of Anti-bacteria and Antifungal Potencies

The three-dimensional structures of the target proteins in this study, *β*-ketoacyl-acyl carrier protein synthase (*β*-ketoacyl-ACP synthase) from *E. coli* (PDB 1FJ4),[29] tyrosyl-tRNA synthetase (TyrRS) from *S. aureus* (PDB 1JIJ),[30] and sterol 14*α*-demethylase from *C. albicans* (PDB 5FSA),[31] were obtained from the Protein Data Bank. The three-dimensional structures of the compounds from Fractions C and E of *I. hippuris* were retrieved from the PubChem database. Both target and compound structures preparation were performed using AutoDockTools v1.5.6 (The Scripps Research Institute, USA, released in 2011), following standard protocols.[32] The preparation of the target structures involved the removal of water molecules and bound residues, protonation, and adding Kollman charges.[33,34] The docking process was conducted using AutoDock Vina v1.1.2 software (The Scripps Research Institute, USA, released in 2011).[32] Docking simulations were performed at the binding sites of *β*-ketoacyl-ACP synthase (docked site coordinates: x = 4.302, y = -19.867, z = 0.617), TyrRS (x = -11.273, y = 13.817, z = 86.08), sterol 14*α*-demethylase (x = 191.537, y = 3.197, z = 37.951). Finally, the interactions between these target proteins and test ligands were visualized using Discovery Studio Visualizer v17.2.0.16349 (Dassault Systèmes, France, released in 2017). The best compound from each fraction was determined based on its highest affinity toward the targets *β*-ketoacyl-ACP synthase from *E. coli*, tyrosyl-tRNA synthetase from *S. aureus*, and sterol-14*α*-demethylase from *C. albicans*.

Results and Discussion

A total of 21.4 g of *I. hippuris* ethyl acetate extract (EAE) was successfully extracted from 1 kg of sample (yield 2.14%). Fractionation of EAE produced five fractions (A–E). A visual representation of *I. hippuris* and the Thin Layer Chromatography (TLC) chromatogram of fractions A–E, using silica gel as the adsorbent and an n-hexane: ethyl acetate (8:2) mixture as the eluent, is shown in Figure 1.

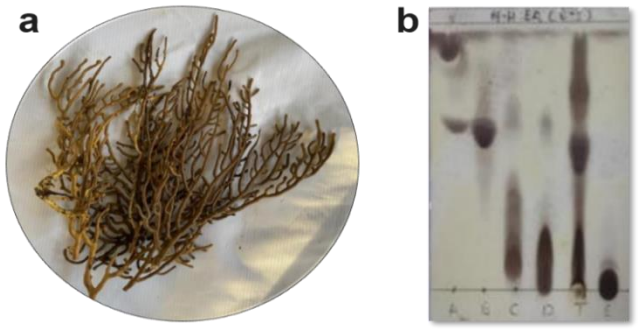

Figure 1: *Isis hippuris* (a) and TLC chromatogram of Fractions A-E (b)

Antimicrobial the testing result of samples consisting of EAE, fraction A–E of *I. hippuris* against bacteria (*E. coli* and *S. aureus*), fungi (*C. albicans*), and positive control (chloramphenicol for bacteria test and nystatin for fungi test), showed varying potential. Results of the antimicrobial screening showed that fraction E demonstrated the highest antibacterial activity with MIC values of 2±0.04 µg/mL and 2±0.03 µg/mL towards both *E. coli* and *S. aureus*, respectively (Figure 2). It was indicated as susceptible to bacteria because the MIC value obtained was 4 µg/mL. Also, Figure 2 shows the antifungal potential of EAE *I. hippuris* and their fractions against *C. albicans*. The data above shows that Fractions C and E significantly have the highest activity compared to all samples with MIC values of 4±0.09 µg/mL and 4±0.12 µg/mL, respectively, which shows the potential as antifungal towards *C. albicans*. Fractions C and E were susceptible to *C. albicans* with a MIC value was ≤ 4 µg/mL. The differences in biological activity are closely linked to the chemical content of the sample.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

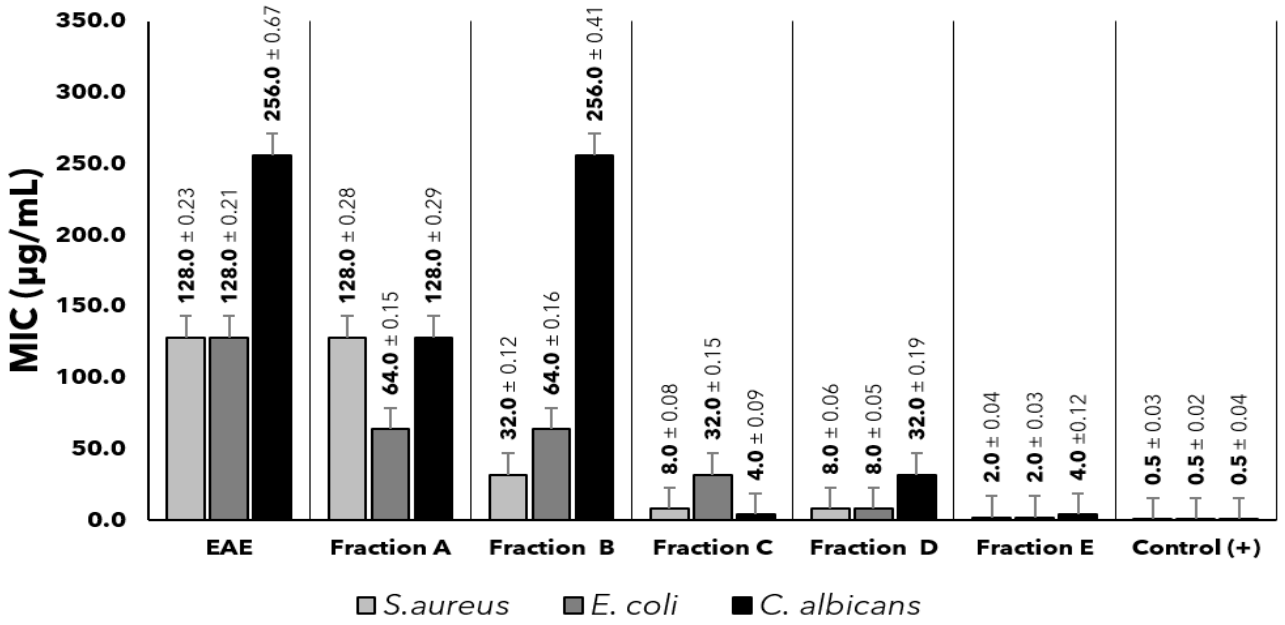

Figure 2: Antimicrobial profile of EAE and all Fraction of *I. hippuris*

Results of phytochemical screening in Table 1 indicated that the secondary metabolites consisted of alkaloids, phenols/tannins, flavonoids, terpenoids, and steroids. Secondary metabolites generally have antimicrobial potency. Alkaloids are a broad and structurally diverse group of compounds that have been the basis for developing important antibacterial and antifungal drugs, such as metronidazoles and quinolones.[35] Other secondary metabolites with potential antimicrobial activity against bacteria or fungi are steroids,[36] terpenoids,[37] and phenolic compounds with activity against Gram-negative and Gram-positive bacteria. Tannins play a crucial role in plant defence against pathogens by exhibiting direct antimicrobial properties, enhancing immune responses, interacting synergistically with other defence compounds, and influencing the plant's microbiome.[38] Some flavonoids, including sophoraflavone G and (-)-epigallocatechin gallate, are known to disrupt cytoplasmic membrane function, while licochalcone A and C inhibit energy metabolism.[35]

The LC-MS/MS analysis results support the presence of secondary metabolites (Table 1).

Table 1: Phytochemical screening of EAE and Fractions of *I. hippuris*

Sample(s)	Secondary Metabolite				
	Alkaloids	Phenols/ Tannins	Flavonoids	Terpenoids	Steroid
EAE	+	+	+	+	+
Fraction A	-	-	-	+	+
Fraction B	+	+	+	+	+
Fraction C	+	-	+	+	+
Fraction D	+	-	-	+	+
Fraction E	+	+	-	+	+

The Liquid Chromatography (LC) chromatogram in Figure 3 displays peaks indicating the presence of compounds in the sample. The height or area of the peak reflects the amount of compound detected. High intensity indicates a greater concentration of compounds, while low-intensity peaks may reflect small amounts of compounds or less efficient ionization results. In MS/MS mode, the detected compounds are broken down into smaller fragment ions. The fragmentation patterns are compared with reference spectra to confirm the chemical content (Table 2). The confirmed chemical structure is shown in Figure 4. Analysis of fractions C and E by LC-MS/MS was conducted to confirm their potential activity as antimicrobials through *in silico* studies.

Table 2: Secondary metabolites of Fraction C and E of *I. hippuris* based on LC-MS/MS data

No. Structure	Rt (min)	Observed $[M+H]^+$ (m/z)	Experimental Neutral Mass (Da)	Formula	Component Name	Group
			Fraction C			
1	4.10	197.1169	196.10994	$C_{11}H_{16}O_3$	Digiprolactone[39]	Terpenoid
2	4.84	187.0868	186.07931	$C_{11}H_{10}N_2O$	1,2,3,4-Tetrahydro-1- oxo-ß-carboline[40]	Alkaloid
3	5.17	317.2105	316.20384	$C_{20}H_{28}O_3$	Saurufuran B[41]	Terpenoid
4	5.35	301.2156	300.20893	$C_{20}H_{28}O_2$	Sugiol[42]	Terpenoid
5	5.92	335.2206	334.21441	$C_{20}H_{30}O_4$	Leoheterin[43]	Terpenoid
6	6.36	337.2365	336.23006	$C_{20}H_{32}O_4$	Siegesbeckic acid[44]	Terpenoid
7	6.92	351.2168	350.20932	$C_{20}H_{30}O_5$	14-Deoxy-11- hydro-xyandrographolide[45]	Terpenoid
8	7.12	431.3151	430.30831	$C_{27}H_{42}O_4$	25(S)-Ruscogenin[46]	Steroid
9	7.24	505.3514	504.34509	$C_{30}H_{48}O_6$	Esculentagenic acid[47]	Steroid
10	7.46	503.3349	502.32944	$C_{30}H_{46}O_6$	11a,12a-Epoxy-23ß,23-dihydroxyolean-28,13ß-olide[48]	Steroid

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ISSN 2616-0692 (Electronic)

11	7.77	461.3255	460.31887	$C_{28}H_{44}O_5$	Polyporusterone D[49]	Steroid
12	8.48	647.3792	646.37170	$C_{36}H_{54}O_{10}$	Abrusoside A[50]	Steroid
13	10.14	305.2475	304.24023	$C_{20}H_{32}O_2$	Arachidonic Acid[51]	Fatty Acid
14	5.58	355.1288	332.14124	$C_{22}H_{20}O_3$	-	-
15	5.81	347.2202	346.21441	$C_{21}H_{30}O_4$	-	-
16	8.91	687.3754	686.36661	$C_{38}H_{54}O_{11}$	-	-
17	9.35	671.3797	670.37170	$C_{38}H_{54}O_{10}$	-	-
18	9.62	729.3856	728.37718	$C_{40}H_{56}O_{12}$	-	-
19	10.29	313.2736	312.26645	$C_{19}H_{36}O_3$	-	-
20	9.96	256.2632	255.25621	$C_{16}H_{33}NO$	-	Alkaloid
			Fraction E			
21	7.27	457.2955	456.28757	$C_{28}H_{40}O_5$	Siraitic acid E[52]	Steroid
22	7.49	463.3415	462.33452	$C_{28}H_{46}O_5$	Polyporusterone F[49]	Steroid
23	7.55	605.3693	604.36113	$C_{34}H_{52}O_9$	Periplocoside M[53]	Steroid
24	6.27	274.2743	273.26678	$C_{16}H_{35}NO_2$	-	Alkaloid
25	6.91	591.3897	590.38187	$C_{34}H_{48}O_8$	-	-
26	7.17	573.3435	572.33492	$C_{33}H_{48}O_8$	-	-
27	8.06	645.3654	622.37706	$C_{40}H_{50}N_2O_4$	-	Alkaloid
28	8.63	482.3611	459.37124	$C_{29}H_{49}NO_3$	-	Alkaloid
29	9.63	510.3926	487.40254	$C_{31}H_{53}NO_3$	-	Alkaloid
30	2.59	185.0707	184.06366	$C_{11}H_8N_2O$	-	Alkaloid
31	3.20	249.1232	226.13577	$C_{16}H_{18}O$	-	
32	3.31	144.0807	143.07350	$C_{10}H_9N$	-	Alkaloid

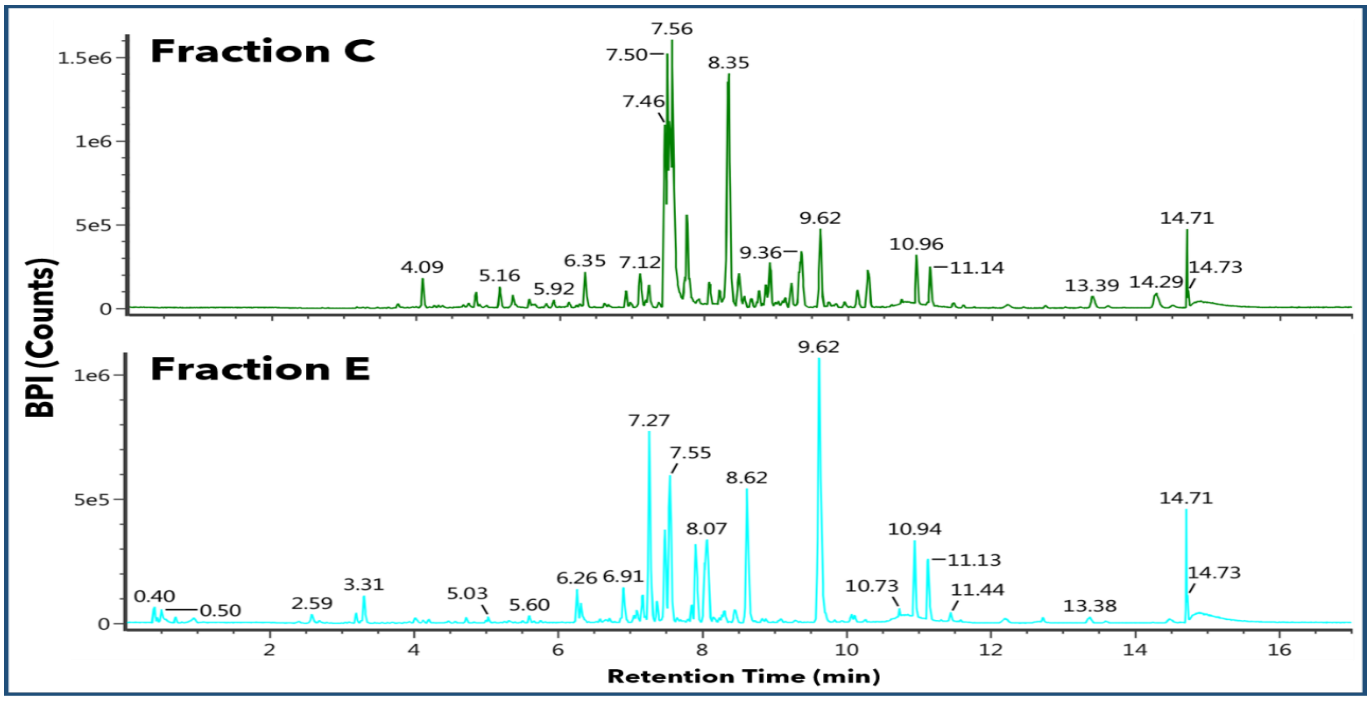

Figure 3: LC-MS/MS chromatograms of Fraction C and Fraction E

In-silico study was conducted on the LC-MS phytoconstituents of *I. hippuris* to determine their mechanisms of anti-bacteria and antifungal potencies. Determination of the most active compounds against *E. coli*, *S. aureus*, and *C. albicans* from fractions C and E of *I. hippuris* was carried out using an *in-silico* study using the docking method. The docking results from Fractions C and E of *I. hippuric* against *β*-ketoacyl-ACP synthase from *E. coli*, TyrRS from *S. aureus*, and sterol 14*α*-demethylase from *C. albicans* revealed that periplocoside M and critic acid E from Fraction E are predicted to have the best affinity towards *E. coli*, *S. aureus*, and *C. albicans*. Meanwhile, the Abrusoside A and 11*α*,12*α*-Epoxy-23*β*,23-dihydroxyolean-28,13*β*-olide from Fraction C are predicted to have the best affinity towards *C. albicans*, with binding energies of -10.9 kcal/mol and -10.1 kcal/mol, respectively (Table 3).

In *S. aureus*, Periplocoside M forms hydrogen bonds with the residues Ala39, Gln174, Asp177, and Phe232 (Figure 5A). These hydrogen bonds are crucial for maintaining the stability of the compound within the active site of TyrRS. The hydrogen bonds facilitate the proper orientation of the molecule, allowing for optimal interactions with the enzyme, which in turn enhances the compound's affinity for the target enzyme.[54] Additionally, hydrophobic interactions with the residues Tyr36, His47, His50, Leu223, and Val224 play a significant role in strengthening the compound's binding to the enzyme's hydrophobic region. Furthermore, 25(S)-ruscogenin also exhibits the ability to bind to the TyrRS enzyme of *S. aureus* through hydrogen interactions with the residues Gly38, Gln174, Asp195, and Val224, which help stabilize the compound within the enzyme's active site (Figure 5B). Hydrophobic interactions with residues His47, Leu223, and Val224 also suggest that the compound is firmly embedded within the enzyme's non-polar pocket. In *E. coli*, saurufuran B forms hydrogen bonds with the residues His298 and Pro303, facilitating stable binding within the active site of the *β*-ketoacyl-ACP synthase enzyme (Figure 6A).

Table 3: Binding energies of compounds from fractions C and E of *I. hippuris*

Sample	Compounds	Binding Energy (kcal/mol)		
		S. aureus	***E. coli***	***C. albicans***
Fraction C	1,2,3,4-tetrahydro-1-oxo-ß-carboline	-8.1	-8.2	-7.4
	11a,12a-Epoxy-23ß,23-dihydroxyolean-28,13ß-olide	-8.0	-6.2	-10.1
	14-Deoxy-11-hydroxy-andrographolide	-8.3	-7.4	-8.1
	25(S)-Ruscogenin	-10.2	-5.9	-8.7
	Abrusoside A	-9.1	-8.1	-10.9
	Arachidonic acid	-6.0	-7.1	-6.5
	Digiprolactone	-6.6	-7.2	-6.6
	Esculentagenic acid	-7.3	-5.7	-7.9
	Leoheterin	-7.0	-6.7	-8.0
	Polyporusterone D	-7.5	-7.4	-9.1
	Saurufuran B	-7.1	-8.5	-7.6
	Siegesbeckic acid	-8.2	-7.4	-8.3
	Sugiol	-7.7	-8.3	-9.4
Fraction E	Siraitic acid E	-9.1	-8.7	-10.7
	Polyporusterone F	-6.6	-6.5	-9.1
	Periplocoside M	-10.2	-8.3	-12.5
Positive Controls	Nystatin	N.A	N.A	-8.4
	Chloramphenicol	-7.3	-8.1	N.A

N.A = not available

These hydrogen bonds are critical for maintaining high binding affinity, thereby enhancing the compound's effectiveness in inhibiting the target enzyme.[55] Additionally, the compound interacts with the residues Pro272, Lys308, Phe390, and Phe392, indicating that saurufuran B is firmly embedded within the enzyme's hydrophobic pocket. Furthermore, Siraitic Acid E exhibits hydrogen interactions with the residues Met204, Asp268, His298, and Gly305 (Figure 6B). The compound also forms hydrophobic interactions with the residues Ala271, Val270, Ala206, Phe392, His333, Cys163, and Phe229, which enhance its affinity for the non-polar regions of the *β*-ketoacyl-ACP synthase enzyme. This binding supports the compound's ability to inhibit enzyme activity, effectively suppressing *E. coli* growth.

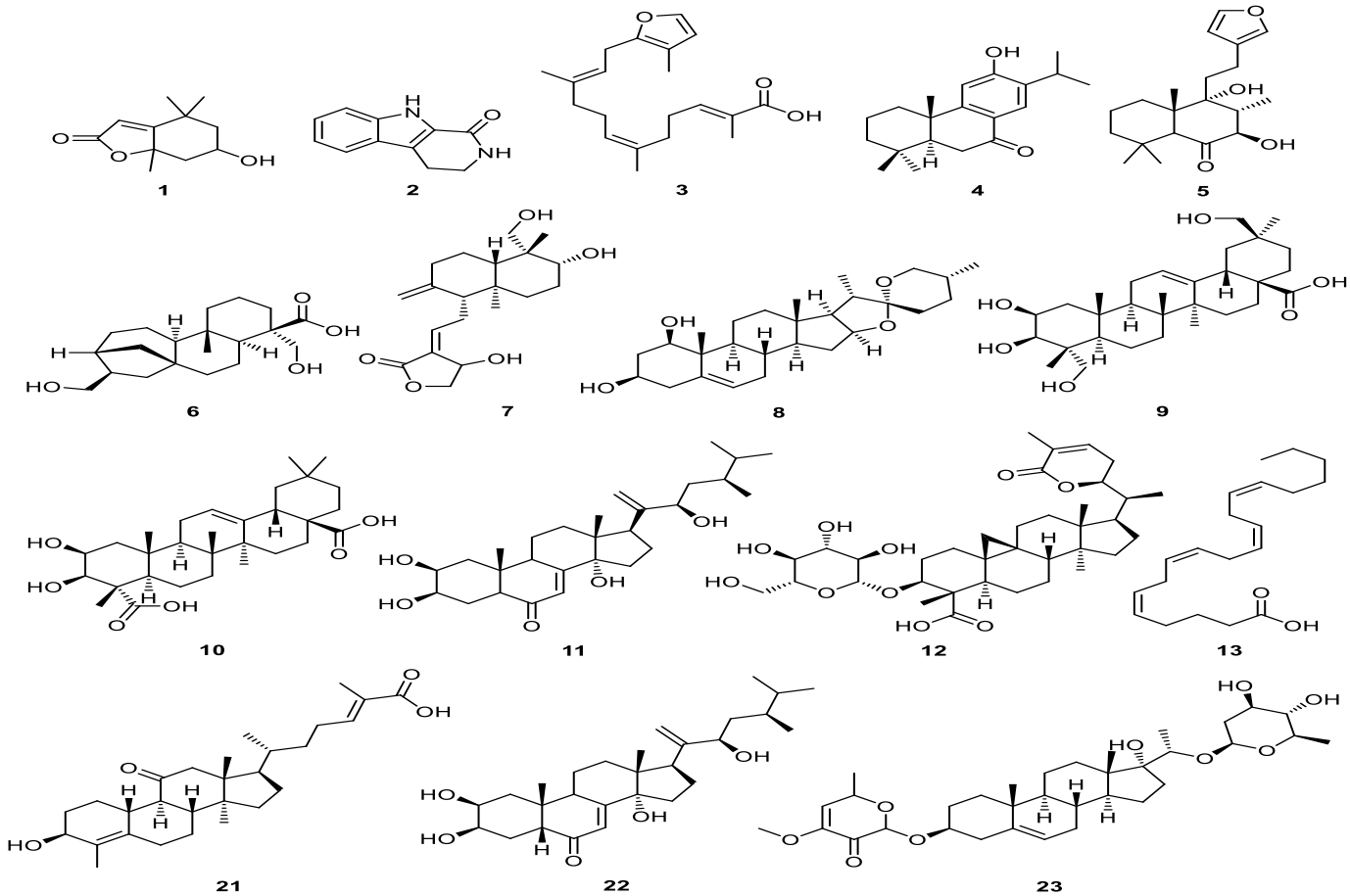

Figure 4: Secondary metabolites structures of Fraction C and E of *I. hippuris*

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

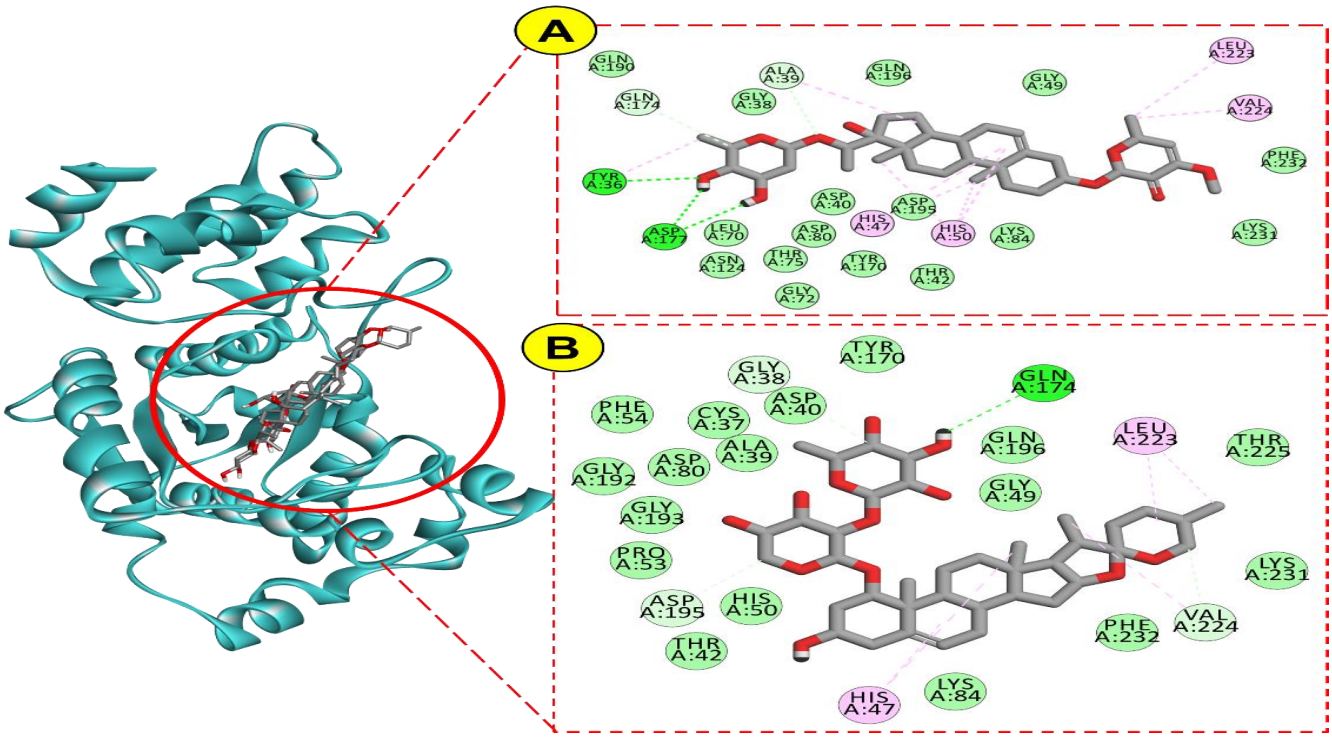

Figure 5: Molecular interactions of (A) periplocoside M and (B) 25(S)-ruscogenin with TyrRS from *S. aureus*.

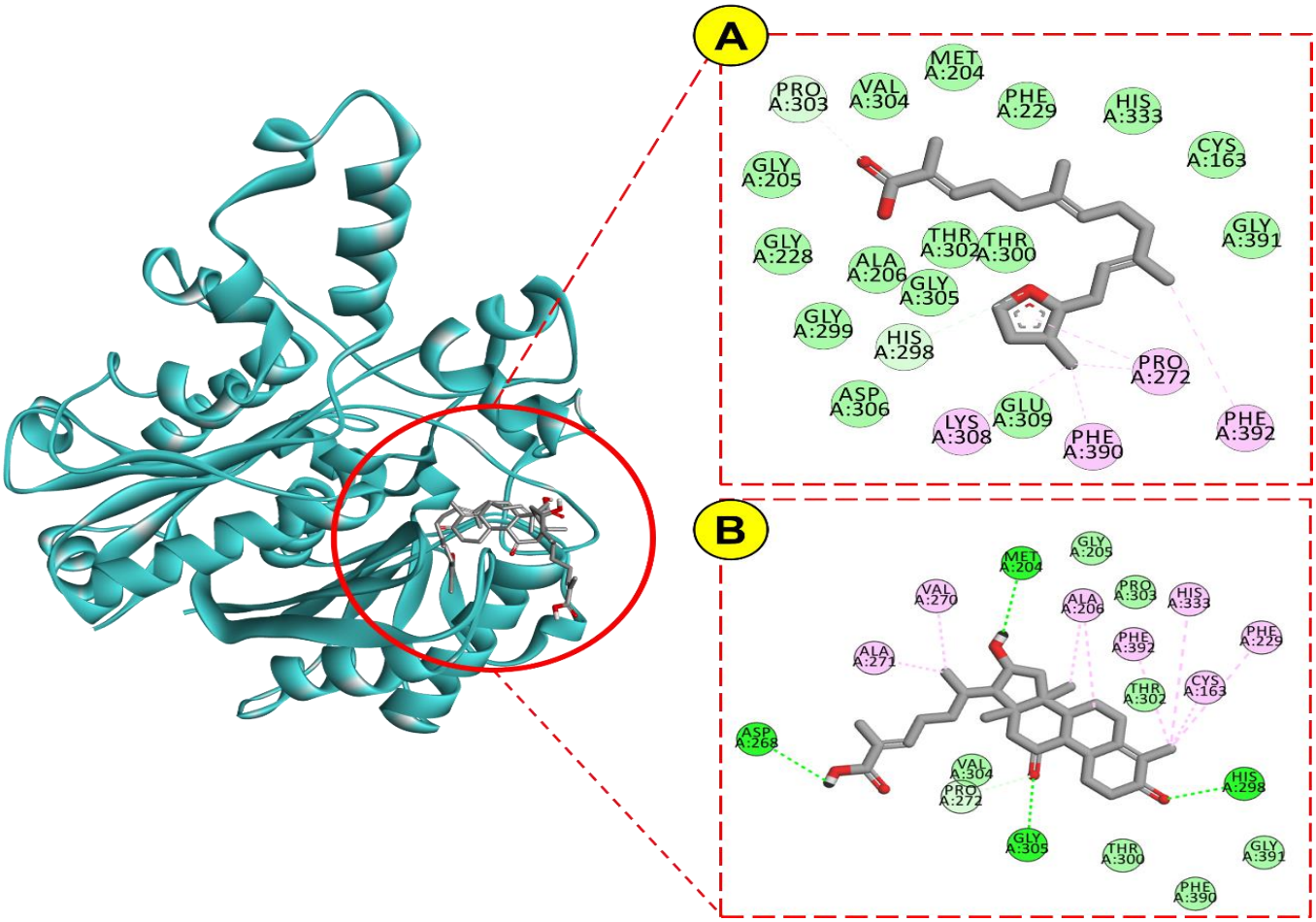

Figure 6: Molecular interactions of (A) saurufuran B and (B) siraitic acid E with *β*-ketoacyl-ACP synthase from *E. coli*.

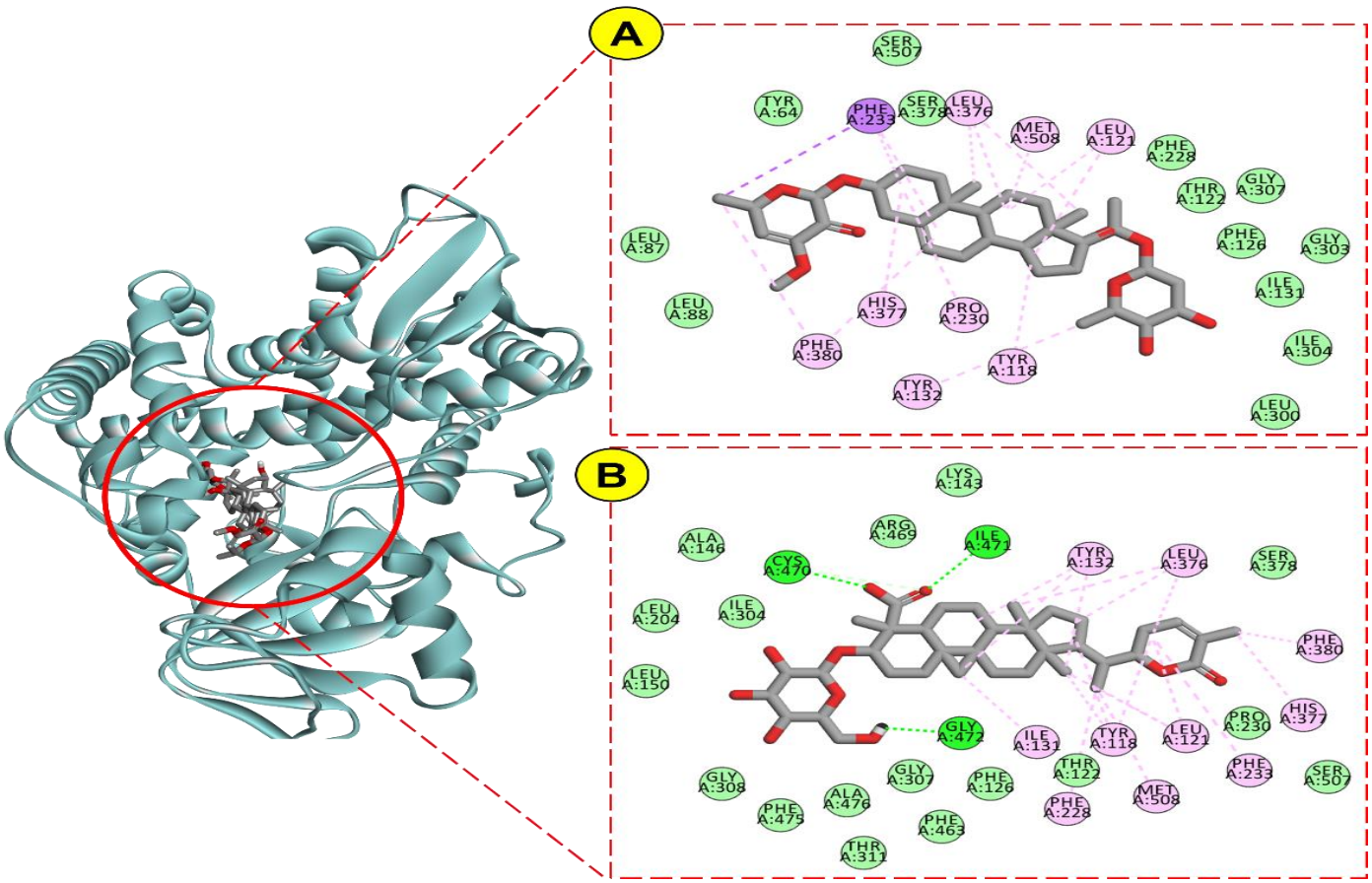

Figure 7: Molecular interactions of (A) periplocoside M and (B) abrusoside A with sterol 14α-demethylase from *C. albicans*.

In *C. albicans*, the compound Periplocoside M from Fraction E did not form hydrogen bonds with the amino acid residues of the target enzyme. However, it demonstrated strong hydrophobic interactions with the residues Phe233, Leu376, Met508, Leu121, Phe380, His377, Pro230, Tyr132, and Tyr118 (Figure 7A). These dominant hydrophobic interactions suggest the compound binds effectively within the enzyme's non-polar pocket.[56] Despite the absence of hydrogen bonds, the strong hydrophobic interactions enable Periplocoside M to remain embedded within the enzyme, potentially inhibiting the activity of sterol 14α-demethylase in *C. albicans*. Additionally, abrusoside A interacts with the enzyme of *C. albicans* through hydrogen bonds with the residues Cys470, Ile471, and Gly472 (Figure 7B), as well as hydrophobic interactions with the residues Tyr118, Ile131, Tyr132, Phe228, Phe233, Leu376, His377, and Phe380, ensuring strong binding within the enzyme's hydrophobic region.

Conclusion

The study highlights the potential of *Isis hippuris* ethyl acetate extract (EAE), particularly Fractions C and E, as potent antimicrobial agents. Fraction E demonstrated significant antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, while Fractions C and E showed potent antifungal activity against *Candida albicans*. *In silico* analysis further supported these findings, revealing that compounds such as periplocoside M, siraitic acid E, abrusoside A, and 11α,12α-epoxy-23β,23-dihydroxyolean-28,13β-olide exhibit strong affinities toward key microbial targets. These findings suggest that *I. hippuris* holds promising potential as a source for developing marine-derived antimicrobial agents in pharmaceutical applications.

Conflict of Interest

Authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgement

We would like to thank The Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia for the research funding with scheme Penelitian Fundamental 2024, with contract Number 17/UN29.20/PG/2024.

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