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Research Article

Antibacterial Activity of *Micrococcus luteus* YSD1 Isolated from Velankanni Water Source, Tamil Nadu

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Abstract

The present study demonstrates the antibacterial activity of marine bacteria isolated from Velankanni Beach, Chennai, India. Five distinct isolates were identified, with *Micrococcus luteus* (YSD1) exhibiting the most promising antibacterial properties. The crude compounds extracted from YSD1 were examined for their antibacterial activity using the agar-well diffusion method against *Proteus mirabilis* ATCC 25933, *Bacillus subtilis* ATCC, *Bacillus cereus* ATCC, and methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300. Gas chromatography-mass spectrometry (GC-MS) analysis revealed 2-pentanone at high intensity, with potential applications in cancer treatment. This research underscores the potential of marine ecosystems as a source of novel antibiotics and the significance of reliable approaches in pharmaceutical discovery. Further research is required to corroborate these findings against a wider range of pathogens and to explore their ameliorative potential and mechanisms of action. The results of this study contribute to the wider field of research by spotlighting the untapped potential of marine ecosystems in bringing novel antibiotics to light, thereby paving the way for future studies to combat antibiotic resistance.

Keywords: Marine; Antibacterial Activity; *Micrococcus luteus*; Agar Diffusion Method; Thin-layer Chromatography (TLC); Gas Chromatography-Mass Spectrometry (GC-MS) Analysis; 2-Pentanone

Introduction

Microorganisms produce antibiotics as secondary metabolites prescribed against bacterial infections in humans and animals [1]. They act as bacteriostatic antibiotics by averting the multiplication of bacteria or bactericidal drugs by killing them. These antibacterial agents target DNA replication, cell walls, cell membranes, folate synthesis, and protein synthesis in bacterial cells. Bacterial pneumonia, urinary tract infections, intra-abdominal infections, chlamydia, gonorrhoea, and meningitis are some infections treated with antibiotics. However, the overuse of antibiotics may lead to antibiotic resistance, which can spawn serious infections and lead to the need for novel therapeutic agents. Thus, exploring antibiotics produced from other sources, such as marine ecosystems, is necessary to address springing illnesses and drug resistant pathogens that pose serious threats to public health.

Oceans cover more than 70% of the Earth's surface and are rich in biodiversity. It is a source of microorganisms that can produce novel antibiotics with enormous potential [2]. This unique habitat harbours microbes that are acclimated to extreme conditions, making them an implicit source of several antibiotics. Maritime microbial communities include viruses, fungi, bacteria, and other microorganisms with significant biotechnological applications. Over the last decade, marine bacteria have emerged as a prominent source of novel medications [3]. These compounds are produced industrially through fermentation processes using large-scale bioreactors under controlled conditions to meet the increasing need for antibiotics globally. Non-toxic and environmentally friendly compounds with renewable and sustainable supply along with bioremediation potential can be derived from marine sources for combating biofouling, cell-cell communication, and microbial growth, thus addressing the importance of "green" chemical compounds [4]. Some marine bacteria, such as *Micrococcus*, *Pseudomonas,* and algicolous marine fungi, have shown potential for combating multidrug-resistant bacteria. Triclosan is a potential antibiotic derived from *Mi-*

crococcus luteus strain associated with sponges [5]. In this study, we identified a few bacteria, including *Micrococcus luteus,* that showed antibacterial activity against bacterial pathogens.

Materials and Methods

Sample collection

Water samples were collected from Velankanni Beach (10.6677654°N, 79.8563603°E) located in Chennai, Tamil Nadu, in sterile bottles and transported to the laboratory. All the bottles were labelled accordingly and then stored at 4°C until further examination [6].

Isolation and purification of bacterial isolates

The collected water sample (100µL) was inoculated onto Zobell Marine Agar plates and incubated for four days at 37°C. Discrete colonies with pigmentation were selected for further analysis. Five morphologically distinct colonies were selected and subcultured to obtain pure cultures. Morphological and biochemical characterisations were performed as per Bergy's Manual of Systemic Bacteriology. The isolates were stored as slants at 4°C for further experiments [7].

Effect of temperature and pH on crude compound production by the isolates

Isolates were cultured in Zobell marine broth with pH ranging from 7.5 to 8.5 and incubated at temperatures of 35°C, 37°C, and 39°C for 24 hours. The ranges of these parameters were selected to ensure efficient cultivation and maximize the yield of crude compounds. A sample of 200µL was added to a 96-well microtiter plate, and the optical densities were recorded every 2 hours for 24 h using an Epoch ELISA reader at 600 nm. The collected data were used to plot a graph using MS Excel software [8].

Extraction of crude compounds from the isolates

The aforementioned isolates were cultured in a 250mL Erlenmeyer flask and incubated at 37°C for five to seven days. The cultures were subjected to centrifugation at 8000 rpm for 15 minutes at 4°C. The pellet containing pigment was washed with distilled water to lyse the cells, after removing the supernatant that did not retain the pigment. The crude compound was extracted by suspending it in 1000µL of Ethanol and placing the tube in a water bath for 60 min. After cooling, the falcon tubes were centrifuged again for 15 min at 5000 rpm until the pellet turned colourless. The resulting supernatant with diffused pigment was filtered through Whatman's No. 1 filter paper (0.22µm pore diameter) to extract the isolate's crude compound [9,10].

Antibacterial efficacy of the crude compound

Bacillus cereus ATCC, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Bacillus subtilis* ATCC, and *Proteus mirabilis* ATCC 25933 were the test microorganisms used in the present study to evaluate antibacterial activity. The stock cultures were subcultured in nutrient broth and incubated at 37°C for 24 hrs before each test. The antibacterial efficacy of the crude compound extracted from the isolates was evaluated against the aforementioned test microorganisms using the agar-well diffusion method. A culture of test microorganisms measuring 100µL was spread on sterile nutrient agar plates. Wells were created in the plates using a sterile well borer. Under sterile conditions, 1000µL of the crude compound was added to the wells and the plates were incubated upright for 24 hours at 37°C. Ampicillin (1000µL) and uninoculated broth (1000µL) were used as positive and negative controls, respectively. After incubation, we assessed the plates and measured the diameter of the inhibition zones in millimetres [11].

GC-MS analysis of the crude compound

This research involved a TLC-mediated bioautography method to analyze the compounds responsible for the antibacterial activity of an isolate. The crude compound of 5µL was applied to TLC plates procured from Merck and developed using different solvent mixtures in a glass chamber. Chloroform/ethanol (2:1 and 3:1), petroleum ether/ethyl acetate (1:9 and 9:1), and acetone/water (5:3) were the solvent mixtures used as the mobile phases. The plates were visualized under UV light, and retention factors were measured. The developed TLC plate was placed on a nutrient agar plate with test bacteria and incubated for 24 h at 37 °C. The zones of inhibition were measured on the plates, and the silica from a similar area on the other developed plates was scraped off for GC-MS analysis using a Shimadzu GC-MS-QP2020 NX instrument from Japan. The carrier gas was helium, and the components were separated at a flow rate of 1 mL per minute. GC-MS analysis determined the components of the unidentified mass spectra by juxtaposing them with recognized components found in the NIST library. Names, structures, and molecular weights of the test material components were accurately identified. Employing both bioautography and GC-MS methods enhances the study by facilitating a thorough evaluation of the crude compound extract [12].

Molecular identification and phylogenetic analysis of YSD1

Considering the evaluated antibacterial activity, one promising isolate, YSD1, was identified using 16S rRNA sequencing. NCBI BLAST was used to analyze the sequence resemblance to the species. A phylogenetic tree was constructed utilising MEGA X software [13].

Results and Discussion

Isolation and characterization of the bacterial isolates

Five bacterial isolates with distinct morphologies were obtained from the water samples collected at Velankanni Beach. They were labelled as YSD1, YSD2, PSD1, WSD1, WSD2 Of these colonies, three (YSD1, YSD2, PSD1) were pigmented. One isolate YSD1 showed bright yellow pigmentation, and the other two, PSD1 and YSD2 formed pink and yellow-pigmented colonies respectively (Figure 1). Hence, they were further studied. In a study conducted by Patel, Nikita., *et al*. (2020), a comparable bacterial isolation method was employed to isolate pigmented bacterial colonies from water samples of the Coast of the Arabian Sea [11]. The authors adopted standard microbiological techniques to isolate a total of nine pigmented bacterial colonies from the samples and characterized them based on morphological and biochemical traits.

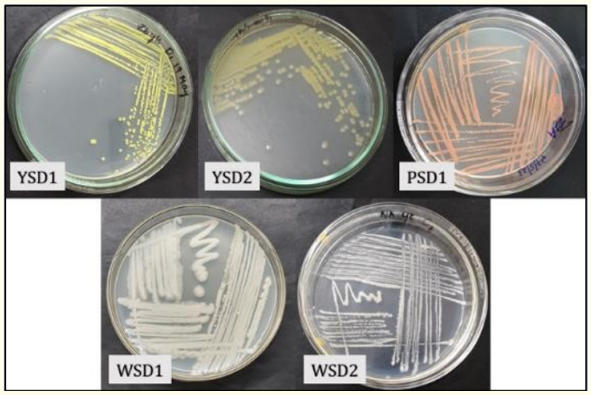

Figure 1: Isolation of marine bacteria: Bacterial colonies were isolated from the marine water sample. Of the 5 colonies obtained, 3 isolates were selected and designated as pigment-producing bacterial isolates.

Morphological characteristics	YSD1	YSD2	PSD1	WSD1	WSD2
COLONY SHAPE	Circular	Circular	Circular	Irregular	Circular
MARGIN	Entire	Entire	Entire	Curled	Entire
PIGMENTATION	Bright yellow	Yellow	Pink	Non-pigmented (White)	Non-pigmented (White)
ELEVATION	Raised	Flat	Raised	Flat	Raised
TEXTURE	Smooth	Smooth	Rough	Smooth	Smooth
OPTICAL PROPERTY	Opaque	Opaque	Opaque	Opaque	Opaque
GRAM NATURE	Positive	Negative	Negative	Positive	Negative
SHAPE	Cocci	Diplobacilli	Bacilli	Bacilli	Bacilli

Table 1: Morphological characteristics of the isolates.

Biochemical tests	YSD1	YSD2	PSD1	WSD1	WSD2
INDOLE TEST	Negative	Positive	Negative	Negative	Negative
METHYL RED TEST	Positive	Negative	Positive	Positive	Negative
VOGES-PROSKAUER TEST	Positive	Positive	Negative	Positive	Positive
CITRATE UTILISATION TEST	Negative	Negative	Negative	Negative	Negative
CATALASE TEST	Positive	Negative	Negative	Negative	Negative
MOTILITY TEST	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile
OXIDASE TEST	Positive	Positive	Negative	Negative	Negative

Table 2: Biochemical characterization of the bacterial isolates.

Effect of pH and temperature on crude compound production

The production of crude compounds by the isolates can be affected by altering the pH and temperature of the medium. Therefore, the selected bacterial isolates (YSD1, YSD2, and PSD1) were cultured in Zobell marine broth at different pH values (7.5, 8.0, and 8.5) and incubated at different temperatures (35 °C, 37 °C and 39 °C). All bacterial isolates exhibited elevated crude compound production at different pH values and temperatures. An increase in crude compound production was *apparent* at 37°C and pH 8.5 for isolate YSD1, 37°C and pH 7.5 for isolate YSD2, and 35°C and pH 8.0 for isolate PSD1 (Figure 2).

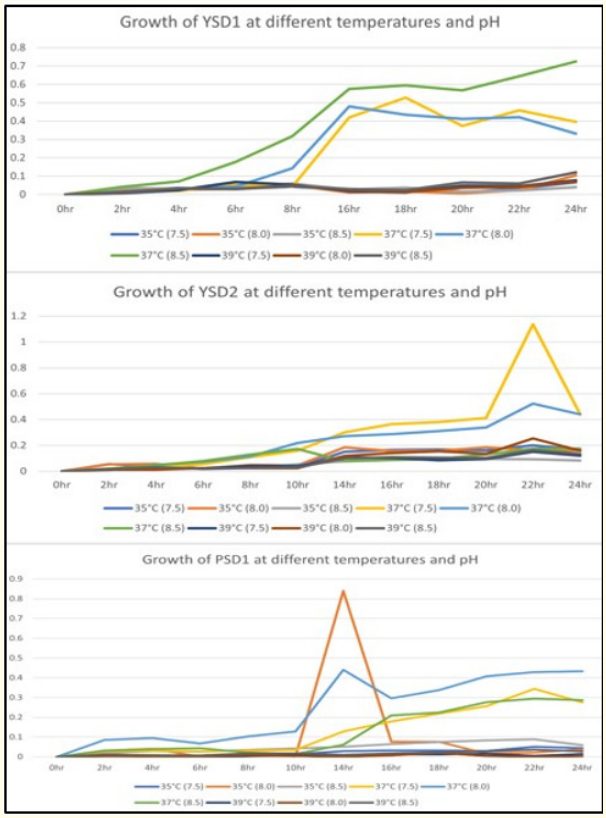

Figure 2: Investigating the effect of pH and temperature on the production of crude compound: Pigment-producing bacterial isolates were cultivated under varying pH and temperature conditions.

Bacterial crude compound extraction

Crude compounds were extracted using solvents, such as ethanol, acetone, chloroform, ethyl acetate, and methanol. However, significant amount of crude compounds could be extracted using ethanol.

Antibacterial activity of crude compounds

The extracted crude compounds were evaluated for their antibacterial efficacy against different test bacteria (Table 3). Of the three pigment-producing bacterial isolates, two isolates, namely YSD1 and YSD2, showed inhibitory activity against the test bacteria, as suggested by the inhibition zones. Among the two isolates, YSD1 exhibited significant antibacterial activity against the four tested microorganisms (Figure 3).

Test organism	YSD1	YSD2	PSD1
Bacillus subtilis	18.5 ± 0.5 mm	13 ± 1 mm	No activity
Bacillus cereus	15 mm	15 ± 1 mm	No activity
Proteus mirabilis	14 mm	No activity	No activity
Methicillin-resistant *Staphylococcus aureus* (MRSA)	13 mm	15 ± 1 mm	No activity

Table 3: Antibacterial efficacy of extracted crude compound against different test bacteria.

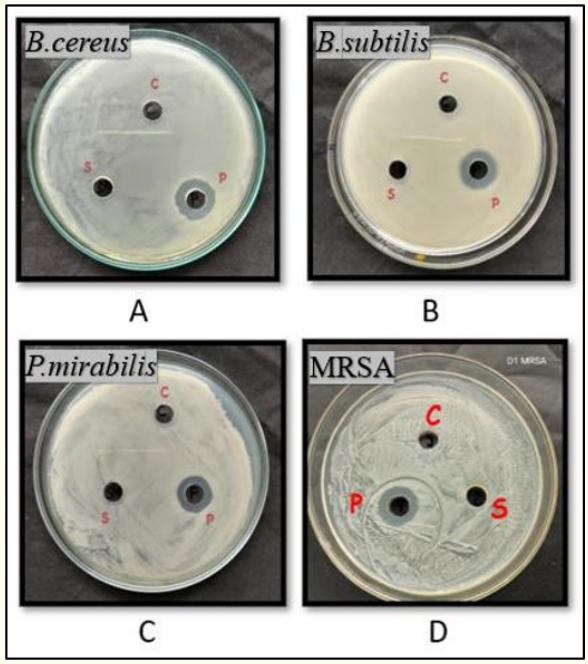

Figure 3: Antibacterial efficacy of the crude compound extracted from YSD1 show inhibitory activity against different pathogens. C-Control; P-Pellet; S-Supernatent.

The antibacterial efficacy of *Micrococcus luteus* YSD1 exhibited varying degrees of inhibition against different test bacteria. The diameter of inhibition zones was 18.5 ± 0.5 mm against *Bacillus subtilis*, 15 mm against *Bacillus cereus*, 14 mm with *Proteus mirabilis*, and 13 mm with MRSA.

Comparing this activity with other studies, *Micrococcus luteus* produces antimicrobial compounds against food-borne pathogens such as *Listeria monocytogenes* and *Salmonella typhimurium* [14]. In addition, a study reported that Bacillus metabolites exhibited inhibition zones of 24 mm against *Staphylococcus aureus*, slightly less than the 25 mm zone observed for *Micrococcus luteus* [15]. In addition, the antimicrobial efficacy of carotenoid pigments produced by *Micrococcus luteus* has been investigated, emphasizing its potential to inhibit the growth, biofilm formation, and virulence of *Proteus mirabilis* [16]. Sharma., *et al*. isolated *Micrococcus* species from a terrestrial source, revealing that the isolate possesses a high potential for combating a diverse range of bacterial pathogens. This strain exhibited an impressive range of antibacterial activity towards harmful microorganisms, such as *Escherichia coli*, *Salmonella typhi*, *Shigella sonnei, Shigella shiga, Shigella dysenteriae*, *Bacillus subtilis*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*. Notably, this strain has demonstrated remarkable adherence properties, competitive exclusion abilities, and antagonism towards *Vibrio harveyi*. In vitro studies have revealed the successful utilization of this strain as a probiotic in *Oncorhynchus mykiss* farming against infections caused by *Aeromonas salmonicida* [20]. Besides these reports, Umadevi and Krishnaveni investigated the antibacterial activity of *Micrococcus luteus* isolated from seawater against *Staphylococcus aureus*, *Klebsiella* species, and *Pseudomonas* [21]. In a study conducted by Syed, Chandini., *et al*. (2017), the diameter of inhibition zones observed against *Proteus mirabilis* was reported to be 11mm [26]. however, in our study, we observed a significantly higher diameter of inhibition zones (14mm) against *Proteus mirabilis*, indicating the potent antibacterial activity of *Micrococcus luteus* YSD1 in our experimental conditions.

These studies collectively emphasize the antibacterial capabilities of the aforementioned bacterium against diverse bacterial strains, highlighting the role of *Micrococcus luteus* in improving fish health through its antimicrobial and probiotic capabilities and supporting its broader application in aquaculture settings.

TLC-mediated bioautography-associated GC-MS analysis

TLC and the separated bioactive fractions analyzed in the crude compound extract of YSD1 were observed using a bioautography assay. The fraction with R_f 0.9 corresponded to the antibacterial activity that was eluted with acetone, which strongly inhibited the growth of *Bacillus subtilis*. GC-MS analysis of this fraction showed 50 peaks. The results revealed the presence of major compounds (Table 4) that were identified and characterized by comparing the mass spectra of the compounds with the library of NIST (Figure 4). The R.Time and peak areas in the analysis provided information regarding the elution time and relative abundance of compounds, respectively. These values are expressed in minutes for retention time and as percentages for peak areas, offering insights into the chromatographic separation and quantification of compounds within the sample. The major components were 2-pentanone, diethyl phthalate, and 4,6dimethyl dodecane. Of these, 2-pentanone exhibited the highest intensity (46.09%). This analytical investigation also indicated caprolactone oxime.

S. NO	Compound	R. time	Molecular Formula	Molecular Weight (g/mol)	Peak (%)	Area
1	2-Pentanone, 4-hydroxy-4-methyl-	2.794	C6H12O2	116	46.09	
2	Dodecane, 4,6-dimethyl	7.345-7.385	C14H30	198.38	5.01	
3	Diethyl Phthalate	10.360-10.405	C24H38O4	390.56	7.36	
4	Caprolactone oxime, (NB)-O-[(diethylborylox y)(ethyl)boryl]-	25.675-25.695	C12H25B2NO2	237	10.31	
5	Hexadecane, 1-iodo	9.331-9.360	C16H33I	396.34	5.62	
6	Heneicosane	9.706-9.735	C21H44	296.57	2.17	
7	Valeric acid, 3,5dihydroxy-2,4-dimethyl-,.delta.-lactone	10.067-10.085	C10H18O4	202.25	1.97	
8	Butanal, 3-hydroxy-	10.605-10.615	C4H8O2	88.11	2.24	
9	Undecane, 3,8-dimethyl-	11.047-11.065	C13H28	184.36	1.80	
10	Tetracosane	11.604-11.640	C24H50	338.65	2.58	

Table 4: Tabulated representation of the major compounds identified through GC-MS analysis.

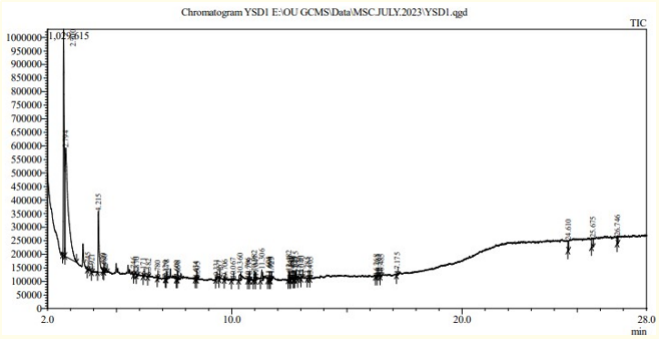

Figure 4: Chromatogram of crude compounds extracted from YSD1.

Interestingly, 2-pentanone is a naturally occurring substance in plants that finds applications as a cleaning solvent in the leather and tanning sectors and as a flavouring element in culinary products. Recent studies have suggested that it may possess therapeutic potential in cancer treatment by reducing prostaglandins and COX-2 synthesis in human colon cancer cells. This indicates that 2-pentanone may be a therapeutic agent in cancer treatment [22]. Diethyl phthalate and 4,6-dimethyl dodecane are widely used in different industries, including cosmetics, plastics, veterinary medicines, and insect repellents [23,24]. Another compound identified in the bioactive fraction, caprolactone oxime, can be utilised for the synthesis of pHsensitive nanoparticles that can be used for drug delivery, especially in cancer therapy. Copolymers with pendant cyclic oximes have been thoroughly studied for potential therapeutic applications in paediatric nanomedicine. Oxime-functionalized iodinated poly (epsilon-caprolactone) shows promise for use in biomedical applications [25].

Overall, a comprehensive analysis of the crude compound extract of YSD1 revealed the presence of several compounds that hold substantial promise for therapeutic applications, beyond their acknowledged antibacterial properties. Specifically, these compounds display significant potential in the cancer treatment and drug delivery domains. These findings advance the field by investigating the potential of marine bacteria as a source of new antibiotics and other therapeutic compounds. They also highlight the significance of exploring diverse and extreme habitats, such as oceanic ecosystems, for drug discovery.

Identification of YSD1 using molecular methods

Isolate YSD1 showed better antibacterial activity among all the selected bacterial isolates. Therefore, molecular identification of this isolate was performed by 16S rRNA sequencing. The results revealed the YSD1 isolate as *Micrococcus luteus*. The phylogenetic analysis of the YSD1 isolate is shown in Figure 5.

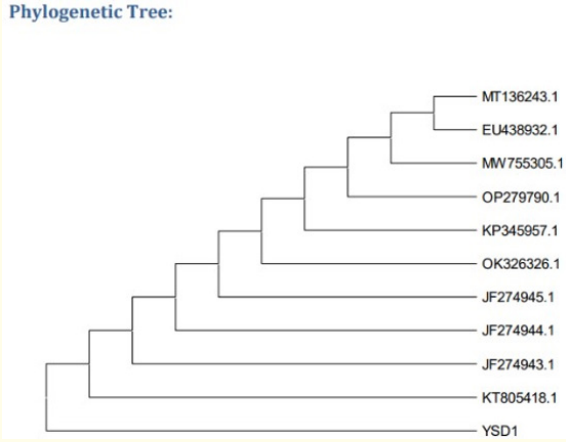

Figure 5: Phylogenetic tree deciphering evolutionary lineages through molecular sequences based on neighbor-joining, suggesting YSD1 isolate as Micrococcus luteus.

Conclusion

To conclude, the study identified five bacterial isolates, namely *Micrococcus luteus* (YSD1), YSD2, PSD1, WSD1, and WSD2, from Velankanni Beach, showing exceptional antibacterial activity and potential for pharmaceutical applications. Among these bacterial isolates, the crude compounds from the YSD1 isolate exhibited promising antibacterial properties. Notably, the compound 2-pentanone, which showed the highest intensity in the GC-MS analysis, has potential applications in cancer therapy. This research has significant implications for the antibiotic discovery field and calls attention to the potential of marine ecosystems as a source of novel antibiotics, which could be pivotal in the fight against increasing antibioticresistant pathogens that pose a serious threat to public health. It also emphasizes the discovery of new pharmaceutical agents by prioritizing sustainable and eco-friendly approaches. However, further validation of these crude compounds against a wider range of pathogens, including fungi, viruses, and other bacteria, is necessary. The effectiveness of the isolate is to be determined

through *in vivo* analysis. Comprehensive characterization, scale-up production, and animal model studies of these compounds are essential to fully investigate their potential therapeutic properties and mechanisms of action for future applications. Such endeavours will enable a deeper understanding of the pharmacokinetics and pharmacodynamics of these compounds and pave the way for their translation into clinical use.

Acknowledgements

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Conflict of Interest

We affirm that there exists no conflict of interest in this matter.

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