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Chemical and biological investigation of the marine bacterium *Rhodococcus* sp. UA13

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Abstract

In this work, a new indole alkaloid, rhodindohyde was isolated from the ethyl acetate fraction of the marine bacterium *Rhodococcus* sp. UA13, previously obtained from the Red Sea sponge *Callyspongia* aff. *implexa*. The structure of the new compound was identified with the aid of 1D and 2D NMR experiments, as well as HR-ESI-MS technique. Rhodindohyde was found to exert weak inhibitory activities (IC_{50} > 100 µg/mL) against both *Staphylococcus aureus* NCTC 8325 and *Trypanosoma brucei brucei* TC221. On the other hand, a limited number of studies have deliberated the anti-inflammatory and pain-relieving potential of marine bacteria and their natural metabolites as yet; therefore, the possible analgesic, anti-inflammatory, and antipyretic properties of the methanol fraction of *Rhodococcus* sp. UA13 were explored using a number of pharmacologically relevant test models. Results showed that the methanol fraction have demonstrated noticeable and statistically significant activities that were also comparable to those of the tested reference drugs; highlighting the helpful role of marine actinomycetes in developing of future therapeutic agents against inflammatory conditions associated with pains and fever.

Key words

Analgesic, Anti-inflammatory, Antipyretic, Marine actinomycetes, Rhodindohyde, *Rhodococcus*.

1. Introduction

Among various inhabitants of deep-sea environments, sponges have been reported to display the most diverse microbial communities, largely represented by actinomycetes [1]. Marine actinomycetes are a wide group of aerobic Gram-positive microorganisms that have been acknowledged as a source of many of currently available medicines as well as of new drug candidates [2]. These bacterial species are prolific producers of novel compounds with a range of biological and pharmacological actions, including among others, antitumor, antioxidant, antimicrobial, and enzyme inhibition [3]. Moreover, secondary metabolites from marine actinomycetes usually show distinct and often complex chemical structures, which help expand the basis for efficient development of further therapeutic agents [2]. Taxonomically, the genus *Rhodococcus* is a member of Actionbacteria, belonging to the order Actinomycetales and the Nocardiaceae family. It comprises an assortment of non-motile, non-sporulating Gram-positive species [4], which have noteworthy metabolic ability to produce ubiquitous antimicrobial compounds like rhodopeptins, aurachins, rhodostreptomycins, lariatins, and rhodozepinone, in addition to peptides, carotenoids, indoles, and some enzymes [4, 5]. Besides, *Rhodococcus* species also enjoy several industrial, biotechnological, and environmental applications [4].

As common global health themes, pain and inflammation have become the focus of extensive scientific research due to their implication in a variety of chronic diseases [6]. Inflammation is a complex biological or pathophysiological response of tissues to injuries brought about by physical, chemical or biological insults, such as pathogens, damaged cells, irritants, toxic chemicals, and tumors [7]. Inflammatory conditions are mediated by several signaling molecules usually produced by macrophages, mast cells, and leukocytes, involving nitric oxide, prostaglandins, cytokines, chemokines, and tumor necrosis factor-α (TNF-α). Such inflammatory molecules also act synergistically to elicit and maintain hyperalgesia via sensitization of nociceptors; hence, most inflammatory disorders are associated with marked pain [8]. In the same way, fever is another secondary impact of inflammation triggered by the increased release of pyrogens, e.g. interleukins, interferon, and TNF-α. These mediators promote the synthesis of prostaglandins E2 from the hypothalamus leading to hyperthermia [9]. Correspondingly, the usefulness of currently available agents to treat inflammation and its associated disorders, like opioids and NSAIDs, has been limited by their severe adverse effects, together with some other economic and potency issues, making the search for other effective natural alternatives an urgent necessity [6]. On account of the aforementioned facts, this work investigates the chemical metabolites and biological potential of the marine sponge-associated bacterium *Rhodococcus* sp. UA13 as a promising source of new therapeutic agents.

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2. Experimental

2.1. General experimental procedures

^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were obtained in CD_3OD on a Bruker Avance III HD 600 instrument and were referenced to the residual protonated signals (δ_H 3.3 and δ_C 49.0) of the solvent. Chemical shift values (δ) were recorded in ppm units, whereas coupling constants (J) in Hz. Heteronuclear correlations were assigned by means of HSQC (optimized for $^{1}J_{HC}$= 145 Hz) and HMBC (optimized for $^{n}J_{HC}$= 8.3 Hz or $^{n}J_{HC}$= 4.0 Hz) pulse sequences. Positive HR-ESI-MS analysis was carried out using a Synapt G2 HDMS QTOF-mass spectrometer (Waters, Germany). Vacuum liquid chromatography (VLC) was performed using silica gel GF_{254} for thin layer chromatography (TLC; El-Nasr Company for Pharmaceuticals and Chemicals, Egypt). HPLC purifications were performed on an Onyx Monolithic semi-preparative RP-18 column (5 µm, 10 × 100 mm; Phenomenex, Germany).

2.2. Chemicals and reagents

Solvents of analytical grade, carboxymethylcellulose (CMC), and sodium chloride were obtained from El-Nasr Company for Pharmaceuticals and Chemicals, Egypt. Carrageenan (Sigma, USA), ibuprofen (Abbott Co., Egypt), indomethacin (El-Nile Company for Pharmaceutical and Chemical Industries, Egypt), and acetyl salicylic acid (The Arab Drug Company, Egypt) were also used in biological assays.

2.3. Sponge collection

Callyspongia aff. implexa (family: Callyspongiidae) was collected in 2006 from the Red Sea (Ras Mohamed, Sinai, Egypt; GPS: 34˚12.904' W; 27˚47.655' N) at a depth of 10 m. The collected sponge was identified by Dr. Michelle Kelly (National Institute of Water and Atmospheric Research, Auckland, New Zealand). A voucher (SAA-12) was kept in Pharmacognosy Department, Faculty of Pharmacy, Suez Canal University, Egypt.

2.4. Isolation of *Rhodococcus* sp. UA13

Isolation of the marine bacterium *Rhodococcus* sp. UA13 was carried out as described by Abdelmohsen et al. [10]. In brief, the collected sponge was cleaned by washing with sterile seawater, cut into small pieces of 1 cm3, and finally homogenized with 10 volumes of sterile seawater. The supernatant was diluted in tenfold series, and then plated out on agar plates containing eight different media [5]. To help isolation of the slow-growing actinobacteria, all media were supplemented with cycloheximide and nystatin (100 and 25 µg/mL, respectively) to inhibit fungal growth. Nalidixic acid (25 µg/mL) was also added to stop the growth of most of the fast-growing Gram-negative bacteria. Distinct colony morphotypes were picked after an incubation period of 6–8 weeks at 30 °C, and re-streaked till visually free from contaminants. *Rhodococcus* sp. UA13 was finally cultivated on ISP2 medium and the isolates were kept in a medium provided with 30% glycerol at −80 °C. Phylogenetic analysis was performed according to Hentschel et al. [11].

2.5. Extraction and isolation

The liquid cultures of *Rhodococcus* sp. UA13 containing ISP2 medium were grown at 30 °C and 150 rpm for 10 days. Cultures were then filtered, followed by extraction of the supernatant with ethyl acetate; the combined extracts were concentrated under vacuum. The crude ethyl acetate extract (10.55 g) was fractionated on a VLC column (6 × 20 cm; 500 g silica gel GF254 for TLC) using gradient elution with petroleum ether, petroleum ether–ethyl acetate (1:1), ethyl acetate, methanol, and n-butanol. Each fraction was concentrated separately under vacuum to provide five fractions (E1–E5). The resulting ethyl acetate fraction (E3; 340 mg) was subjected to semi-preparative HPLC purification using H_2O–CH_3CN (95:5) for 5 min, followed by a linear gradient to 40% CH_3CN within 5 min, and finally with a further isocratic elution of CH_3CN for 20 min at a flow rate of 3 mL/min to give compound (1) (1.2 mg; Rt= 17.3 min). On the other hand, since all fractions were obtained in minor amounts (ranging between 10–400 mg), only the methanol fraction (E4; 8.04 g) was subjected to pharmacological screening tests.

2.6. Evaluation of antibacterial activity

The antibacterial activity of compound (1) was tested against Staphylococcus aureus NCTC 8325 using the standard disc diffusion assay [3, 5]. The optical densities of bacterial cultures in 96-well plates were measured at 550 nm using an ELISA microplate reader in comparison with the control wells without bacteria.

2.7. Evaluation of antitrypanosomal activity

The antitrypanosomal activity of compound (1) against Trypanosoma brucei brucei strain TC221 was evaluated according to the protocol described by Elsayed et al. [3, 5]. The inhibitory activities were measured after 48 and 72 h by light absorption using an MR 700 microplate reader at wavelengths of 550 (test) and 650 (reference) nm.

2.8. Experimental animals

Pharmacological investigations of the methanol fraction of *Rhodococcus* sp. UA13 were conducted on healthy adult male albino mice (30 ± 5 g, each) according to the Institutional Animal Ethical Regulations. The animals were bred under standardized conditions in the pre-clinical animal house, Department of Pharmacology, Faculty of Medicine, Assiut University, Egypt. They were kept in mesh-bottomed stainless steel cages (six per cage) with standard laboratory animal diet and water ad libitum. The animals were left to acclimatize to the environment for at least one week prior to inclusion in the experiments. Mice were handled only at the time of experiments and during cleaning of cages. All conditions were also adjusted to minimize animal suffering.

2.9. Evaluation of analgesic activity

The analgesic activity was determined by the hot plate method [12]. Mice were randomly allocated to three groups (six mice per group). Group 1 was orally administered 300 mg/kg of the methanol fraction suspended in 0.5% CMC solution. Animals of group 2 (positive control) were administered the reference drug ibuprofen (100 mg/kg) in 0.5% CMC solution, p.o., while those of group 3 (negative control) were only administered 0.5% CMC solution orally. A cut off time of 20 sec were applied to avoid tissue damage. The reaction time taken to either lick the hind paw or to jump up when mice were placed on the hot plate (50 ± 1 ºC) was recorded. After each testing, the hot plate was cleaned to remove urine and faeces.

2.10. Evaluation of anti-inflammatory activity

The anti-inflammatory activity was tested by the carrageenan-induced paw edema method [13–15]. Mice were divided into four sets; each of six mice. Inflammation was induced in the paws of mice by a subcutaneous injection of 0.1 mL of 1% carrageenan solution in 0.9% NaCl into the sub-planter tissue of the right hind paw, while the left one was injected by an equal volume of saline solution. One hour before carrageenan injection, the first test group of mice was given the methanol fraction at 300 mg/kg in 0.5% CMC solution orally, whereas the second group was orally administered indomethacin (8 mg/kg) in 0.5% CMC solution as the reference anti-inflammatory drug. Animals of the third group (negative control) were only given the vehicle (0.5% CMC solution, p.o.). The fourth group, on the other hand, included untreated mice and served as the normal control. The paw thickness was measured in mm using Varnier Caliber immediately before injection of carrageenan and then 0.5, 1, 2, 3, 4, and 5 h after injection. The difference between the thicknesses of the two paws was regarded as a measure of edema. The anti-inflammatory efficacy was estimated by comparing the magnitude of paw swelling in pretreated animals with those induced in the control group. The percentages of edema and inhibition of inflammation were calculated as follows [16]:

% Edema= [Right paw thickness – Left paw thickness] × 100 / Right paw thickness

% Inhibition of inflammation= [V0 – Vt] × 100 / V0

Where V0= the average paw thickness of the control group, and Vt= the average paw thickness of the treated group.

2.11. Evaluation of antipyretic activity

The antipyretic activity was evaluated by the yeast-induced pyrexia in mice [15, 17]. Mice were distributed into three groups of six mice. Hyperthermia was induced by subcutaneous injection of 20 mL/kg of 20% w/v aqueous suspension of Brewer's yeast in normal saline 18 h before treatment. The first group was injected intraperitoneally with the methanol fraction at a dose of 300 mg/kg. Animals of the second group (reference group) received an intraperitoneal injection of 100 mg/kg acetyl salicylic acid, whereas the last group served as the negative control and was injected intraperitoneally with 0.5% CMC solution in normal saline. The rectal temperature was taken using a thermometer before and 0.5, 1, 2, 3, 4, and 5 h after administration of either the methanol fraction or aspirin.

2.12. Statistical analysis

Results of the aforementioned pharmacological tests were expressed as mean ± standard deviation (S.D.) One-way analysis of variance (ANOVA) followed by Dunnett's test were used when groups were compared only to the control group. p values less than 0.05, 0.01, and 0.001 were regarded as significant. Graph Pad Prism 5 was employed for statistical calculations (Graph pad Software, San Diego, California, USA).

3. Results and discussion

3.1. Identification of compound (1)

Compound (1) was obtained as a pale yellow powder. It responds to Dragendorff's reagent, suggesting its alkaloidal nature. The molecular formula of (1) was established as $C_{10}H_9NO_2$ based on HR-ESI-MS (found at m/z 175.1480, calcd. 175.0630), requiring seven degrees of unsaturation. Preliminary investigation of the NMR spectral data of compound (1) (Table 1) suggested the presence of a substituted indole structure [3]; represented by three one-proton broad singlets at δH 6.78, 6.59, and 7.31 assignable to H-2, H-4, and H-6, respectively, together with their corresponding carbon resonances at δC 122.4, 109.1, and 109.5 as shown by the HSQC analysis. These assignments were also substantiated via the observed HMBC correlations of H-2 with (C-8, C-9, and C-10), H-4 with (C-3 and C-6), as well as H-6 with (C-4, C-5, and C-7). Moreover, substitution of the indole skeleton at C-3 with a methyl group was indicated by the three-proton singlet at δH 2.56 and its carbon signal at δC 23.0, which was also confirmed through the HMBC connectivities of CH_3-10 with both C-2 and C-9. Likewise, the observed HMBC three-bond correlation between H-6 and C-11 suggested the position of the formyl group (CHO-11, δC 187.8) at C-7, whereas the presence of an additional hydroxyl group at C-5 was deduced from the downfield shift of the latter to 155.2 ppm. Besides, the ^{13}C NMR spectrum of compound (1) displayed 10 carbon resonances attributable to one methyl, three methines, and five quaternary carbons, in addition to one carbonyl carbon of the aldehydic group. Therefore, compound (1) was identified as 5-hydroxy-3-methyl-1H-indole-7-carbaldehyde that was named rhodindohyde (Figure 1).

3.2. Antibacterial and antitrypanosomal activities

Compound (1) showed weak inhibitory potential against both S. aureus NCTC 8325 and T. brucei brucei TC221 with IC50 values exceeding 100 µg/mL.

Table 1: ^{1}H and ^{13}C NMR spectroscopic data of rhodindohyde **(1)** (600 and 150 MHz, CD_3OD).

No.	δ_H	δ_C	HMBC
2	6.78 (1H, br.s)	122.4	8, 9, 10
3	–	110.9	–
4	6.59 (1H, br.s)	109.1	3, 6
5	–	155.2	–
6	7.31 (1H, br.s)	109.5	4, 5, 7, 11
7	–	130.7	–
8	–	137.1	–
9	–	133.9	–
10	2.56 (3H, s)	23.0	2, 9
11	Missed	187.8	–

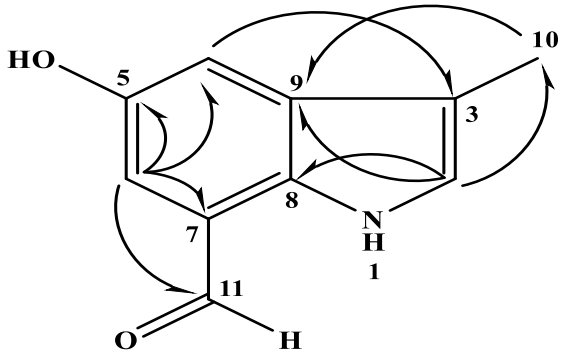

Figure 1: Chemical structure of rhodindohyde **(1)** (significant HMBC correlations are indicated by arrows).

3.3. Analgesic activity

The analgesic properties of the methanol fraction of *Rhodococcus* sp. UA13 were evaluated using the hot plate method. The results presented in Table 2 revealed that both the methanol fraction and the standard drug ibuprofen significantly increased the latency to the reaction against the thermal stimulus from 1 h till the end of the experiment, with their maximum effects were obvious at 4 h. In comparison with the standard drug, the methanol fraction also caused a relatively higher prolongation of the hot plate reaction time at 1 h ($p<0.001$) and its suppression of pain sensation was statistically significant ($p<0.01$) up to 5 h of the experiment. As one of the most common thermal nociception models, the hot plate test is beneficial in clarifying the centrally mediated anti-nociceptive responses [13, 17], and thus the remarkable increase in pain threshold produced by the methanol fraction suggests the involvement of central pathways. It is worth mentioning that a limited number of studies have deliberated the analgesic potential of marine bacteria or their natural compounds. In this context, Ramasamy and Kumar have reported significant anti-nociceptive properties of the total extracts from some seaweed- and ascidian-associated bacterial strains in the hot plate model that were shown to be centrally mediated [18], whereas this is the first report on the analgesic potential of marine actinomycetes, especially *Rhodococcus* species.

3.4. Anti-inflammatory activity

The anti-inflammatory potential of the methanol fraction of *Rhodococcus* sp. UA13 was appraised by the carrageenan-induced paw edema assay in mice. The induction of edema with carrageenan is a well-known biphasic model with substantial involvement of several vascular and inflammatory mediators [13]. The initial or early phase (0–1 h) of edema involves the release of histamine, serotonin, bradykinin, and 5-hydroxytryptamine, whilst higher levels of prostaglandins and inducible cyclooxygenase (COX-2) are common during the second or late phase of swelling (2–5 h), which also enhance the development of further inflammatory reactions [13, 19]. The obtained results (Table 3) revealed that pretreatment of the test groups either with the methanol fraction or indomethacin markedly reduced the carrageenan-induced edema. The anti-inflammatory effects obtained with both treatments were statistically significant at $p<0.01$ after 2 h and prolonged to the end of the experiment at $p<0.001$ in comparison with the non-treated control group that remained the highest edematous one. Besides, the indomethacin-treated group exhibited a relatively better reduction of paw swelling throughout the total experimental time. Nevertheless, the protective effects of the methanol fraction were comparable to those of indomethacin at 4 and 5 h (Table 3), with the highest inhibition of paw swelling (50.65%) produced by the methanol fraction was witnessed at 5 h versus 51.95% for indomethacin. These results demonstrated the noticeable anti-edematous potential of the methanol fraction of *Rhodococcus* sp. UA13, particularly during the late phase of inflammation, which is most likely attributed to inhibition of prostaglandins' release. It is noteworthy that a variety of microbial metabolites with anti-inflammatory properties have been described from some marine actinomycetes, such as saphenic acid, lipomycin, and diazepinomicin (ECO-4601) from Micromonosproa sp. [20, 21]. Cyclomarin A, a cyclic heptapeptide antibiotic from Streptomyces sp., was also shown to possess significant in vitro and in vivo anti-inflammatory actions [22]. Likewise, two anti-inflammatory bicyclic depsipeptides, namely salinamides A and B, were reported from Streptomyces sp. CNB-091 previously isolated from the jellyfish Cassiopeia xamachana [23]. Moreover, Ramasamy and Kumar have demonstrated the potential of total extracts from some seaweed- and ascidian-derived marine bacteria in reducing the carrageenan-induced edema in mice [18]. In the same context, the anti-inflammatory potential of *Rhodococcus* species was considered herein for the first time.

3.5. Antipyretic activity

The effect of the methanol fraction of *Rhodococcus* sp. UA13 on Brewer's yeast-induced pyrexia in mice is depicted in Table

4. Both the methanol fraction and aspirin produced a statistically significant reduction in the rectal temperature of mice from 0.5 h till the end of the experiment. For the methanol fraction-treated group, the maximum decrease in temperature (~1.6 ºC) was recorded after 4 h, while that of aspirin-treated animals (~2.53 ºC) was obvious at 3 h, as compared to the initial corresponding rectal temperatures of each pretreatment group (at zero time). The results generally showed that the antipyretic effect of the methanol fraction was evidently comparable to that of aspirin throughout all the experiment. Yeast-induced pyrexia is a pathogenic fever and possibly includes the production of prostaglandins; therefore, the observed antipyretic actions of the methanol fraction of *Rhodococcus* sp. UA13 might be ascribed to the inhibition of prostaglandin synthesis that is probably mediated by blocking the activity of cyclooxygenase enzyme [24]. Based on literature survey, this is the first report describing the antipyretic potential of marine actinomycetes, and in particular of *Rhodococcus* species

Table 2: Effect of the methanol fraction of *Rhodococcus* sp. UA13 on the hot plate reaction time.

Group no.	Treatment	Hot plate reaction time (s) (Mean ± S.D.)†						
		Pretreatment	0.5 h	1 h	2 h	3 h	4 h	5 h
1	Methanol fraction	43.33 ± 7.37	49.67 ± 5.03	66.67 ± 2.08***	69.00 ± 3.00**	96.00 ± 8.54**	112.33 ± 26.41*	66.67 ± 6.81**
2	Ibuprofen	45.33 ± 6.51	49.33 ± 5.86	59.67 ± 5.03*	81.00 ± 7.21**	108.33 ± 8.33***	162.67 ± 8.02***	86.33 ± 12.06**
3	Negative control	44.33 ± 9.02	44.67 ± 1.53	45.67 ± 3.06	43.67 ± 4.16	49.67 ± 4.16	45.00 ± 10.00	47.00 ± 2.00

†Differences with respect to the control group were evaluated using the student *t*-test (*p<0.05, **p<0.01, and ***p<0.001).

Table 3: Effect of the methanol fraction of *Rhodococcus* sp. UA13 on the carrageenan-induced edema.

Group no.	Treatment	Paw swelling or thickness in mm (Mean ± S.D.)† (% of inhibition of inflammation)						
		Pretreatment	0.5 h	1 h	2 h	3 h	4 h	5 h
1	Methanol fraction	0.73 ± 0.03	0.70 ± 0.00 (6.67%)	0.68 ± 0.03 (9.33%)	0.62 ± 0.03** (19.48%)	0.52 ± 0.03*** (32.47%)	0.40 ± 0.05*** (48.05%)	0.38 ± 0.03*** (50.65%)
2	Indomethacin	0.73 ± 0.03	0.68 ± 0.03 (9.33%)	0.60 ± 0.05* (20.0%)	0.53 ± 0.06** (31.17%)	0.42 ± 0.03*** (45.45%)	0.38 ± 0.03*** (50.65%)	0.37 ± 0.03*** (51.95%)
3	Negative control	0.73 ± 0.03	0.75 ± 0.05	0.75 ± 0.05	0.77 ± 0.03	0.77 ± 0.03	0.77 ± 0.03	0.77 ± 0.03
4	Normal control	0.30 ± 0.00	0.30 ± 0.00	0.30 ± 0.00	0.30 ± 0.00	0.30 ± 0.00	0.30 ± 0.00	0.30 ± 0.00

†Differences with respect to the control group were evaluated using the student *t*-test (*p<0.05, **p<0.01, and ***p<0.001).

Table 4: Effect of the methanol fraction of *Rhodococcus* sp. UA13 on yeast-induced pyrexia.

Group no.	Treatment	Average rectal temperature (˚C) ± S.D.†						
		Pretreatment	0.5 h	1 h	2 h	3 h	4 h	5 h
1	Methanol fraction	39.07 ± 0.47	38.70 ± 0.26*	38.27 ± 0.25**	37.80 ± 0.26***	37.67 ± 0.15***	37.47 ± 0.06***	37.97 ± 0.47**
2	Acetyl salicylic acid	39.63 ± 0.38	38.43 ± 0.15**	37.80 ± 0.20***	37.73 ± 0.25***	37.10 ± 0.10***	37.17 ± 0.15***	37.93 ± 0.55**
3	Negative control	39.47 ± 0.49	39.23 ± 0.06	39.60 ± 0.10	39.70 ± 0.20	39.73 ± 0.15	39.80 ± 0.10	39.87 ± 0.06

4. Conclusion

Chemical investigation of the ethyl acetate fraction from the crude extract of the marine bacterium *Rhodococcus* sp. UA13 has resulted in the isolation of rhodindohyde as a new indole alkaloid. The isolated compound demonstrated weak antibacterial and antitrypanosomal activities against S. aureus NCTC 8325 and T. brucei brucei TC221. The current study also represented the first report on the analgesic, anti-inflammatory, and antipyretic properties of *Rhodococcus* sp. UA13, where the methanol fraction prepared from this marine bacterium was proved to induce substantial reduction of experimentally induced pain, inflammation, and hyperthermia in mice that were also comparable to the effect of standard NSAIDs drugs. Such findings imply the presence of pharmacologically active biomolecules in this fraction, which also strongly recommend their isolation and chemical characterization in the future followed by a detailed biological testing.

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