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Purification and Structural Characterization of an Antimicrobial Compound, Lipoxazolidinone a Produced by a *Lactobacillus Apis* YMP3

Dayanidhi Satish Kumar[1,2] and Palanisamy Venkatachalam[1]*

[1]Department of Microbiology, Sengunthar Arts and Science College (Affiliated to Periyar University), Tiruchengode – 637205, Tamil Nadu, India.
[2]Department of Microbiology, Indira Gandhi College of Arts and Science (Affiliated to Pondicherry University), Kathirkamam – 605009, Puducherry, India.

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Strains of Vibrio cholerae are one among the most causative and serious disease causing human pathogenic agents, its infections are caused mostly by ingesting contaminated water and/or food. According to the recent estimates, between 1.3 and 4.0 million individuals are infected all around the world every year. The lactic acid bacteria are an important class of probiotics microbes have their ability to produce diversified bioactive compounds, hence this study focused on the identification of a promising antimicrobial agent from a Lactobacillus apis YMP3. This strain was cultured on MRS broth and the cell free supernatant was ethyl acetate extracted for the antimicrobial agent. The crude extract was further purified with C18 silica gel column chromatography and structurally characterized by FT-IR, NMR, GC and MS/MS spectrum. The chemistry of the compound was confirmed as Lipoxazolidinone A which has the IUPAC name of (2E)-5-butyl-2-[(E)-4-methyl-2-oxoundec-3-enylidene]-1,3-oxazolidin-4-one. This is the first report of Lipoxazolidinone A produced by a bacterium, L. apis YMP3 which was originally isolated from yoghurt. This finding expands the scope of identifying more promising bioactive compounds from probiotic Lactobacillus sp., further, this systematic procedure for purification of this antimicrobial agent stood as the baseline data for more elaborate therapeutic studies in future.

Keywords: Antimicrobial compound; V. cholerae; Lipoxazolidinone A; Lactobacillus apis; Purification; YMP3.

Antibiotics have historically been derived from natural compounds; therefore, novel scaffolds are typically of interest. Concerns have been raised concerning the potential source of new chemical compound (NCEs) that can address the issue of constantly emerging resistance in the lack of new antibiotics. Till 2002, the vast majority of NCEs approved for use as antibiotics were derived largely from microorganisms[1,2]. Lactobacilli are essential microorganisms renowned for their fermentative activities as well as their nutritional and physiological benefits[3]. Lactobacilli have traditionally been utilized as natural bio-preservatives in food and animal feed viz.,

*Corresponding author E-mail: venmalar.2007@rediffmail.com

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Lactobacillus sp.[4], *L. plantarum*[5] and *Lactobacillus* sp.[6]. Some of the Lactobacilli isolated from cow milk and dairy products are *L. gasseri*, *L. reuteri* and *L. salivarius*[7].

Despite the development and broad use of antibiotics, bacterial infections continue to create a significant threat to human health; for example, increases in morbidity and death due to enteric diseases are a worldwide concern[8, 9, 10, 11]. In developing nations, acute microbial diarrheal illnesses constitute one of the most major public health concerns. Similar to numerous probiotic bacteria, Lactobacilli possesses bactericidal or bacteriostatic characteristics. Lactobacillus species produce substances with direct antimicrobial activity, including bacteriocins, hydrogen peroxide, organic acids and low-molecular-weight compounds. Diarrheal illness patients have the weakest financial resources and the poorest sanitary conditions. Children under the age of five are disproportionately impacted by waterborne microbial illnesses, particularly in Asia and Africa[12].

The quest for new bioactives is an integral part of the fight against by the threat posed by the increase in pathogenic infections. *V. cholerae* has developed drug resistance due to the extensive use of antibiotics to treat diarrhoea[13]. Antibiotics can also disturb gut homeostasis by eliminating normal gut flora. Therefore, alternative therapies that are safe and effective against gut-bacterial infections are required. The aim of this study was to explore the purification and identification of an antimicrobial component from a *Lactobacillus apis* YMP3 isolated from yoghurt purchased in Thanjavur, Tamil Nadu, India, which has been demonstrated to have an antibacterial activity against the growth of human pathogenic *V. cholerae.*

MATERIALS AND METHODS

Microorganism

The production and purification of an antimicrobial compound from a *Lactobacillus apis* YMP3 was performed in this study. This strain was previously published for its isolation from a yoghurt originated from Thanjavur region, Tamil Nadu, India and screened for the production of a promising antimicrobial agent against human pathogenic *V. cholerae* [4]. Further, this strain was also earlier reported for its identification performed by 16S rRNA sequence which was submitted in the NCBI GenBank nucleotide database and OM843103.1 has been provided as its accession number. The maintenance of this strain is carried out in MRS agar slants under 4°C refrigerated conditions.

Culture conditions and extraction of antimicrobial compound

In this study, MRS broth was used for the production of an antimicrobial compound using *L. apis* YMP3 with the basic cultural conditions of pH 6.5 and 35°C. After 72hrs incubation time, the extraction was performed from the cell free broth using an equal volume of ethyl acetate. After 8hrs hold up, the organic phase containing the antimicrobial compound was removed and dried using a rotary vacuum evaporator at 50ºC. The resultant dried extract was dialyzed against a dialysis membrane with phosphate buffer solution at pH 7 for 24hrs with intermediate PBS changes to remove the salt, and the final solution was lyophilized. At each step till the purification, the antimicrobial activity was estimated against the clinically isolated human pathogenic *V. cholerae* using the method as described by Rufino *et al*.[14].

Purification

The dried crude sample was diluted in a 5ml solvent which has the 3:2 ratio of acetonitrile and methanol, and then it was filtered using a 0.2 m syringe filter. Purification was performed using a Reverse Phase (RP)—C_{18} silica gel (230–400 mesh) column at 30°C. The solvent system used in the purification process consisted of acetonitrile (solvent I) and methanol (solvent II), and elutions were done at a flow rate of 0.5 ml/min with a stepwise gradient beginning at a ratio of 50:50, vol/vol (I:II) and ending at a ratio of 100:0, vol/vol (I:II) (A:B). The absorbance of the individual elutions were measured at 210 nm and the antimicrobial activity of the individual fractions was determined[14].

Chemical characterization

Spectral studies are the keys for the detailed chemical characterization of any metabolites and a series of different spectral analysis can interpret and confirm any chemical structures[15, 16]. Among the collected fractions, the fraction showed antimicrobial activity was dried

using a rotary vacuum evaporator at 50ºC and purified dry compound was identified using the following spectral analysis. All the spectra were individually analysed and structurally interpreted for the identification of the purified antimicrobial compound.

Fourier Transform Infrared Spectroscopy (FT-IR)

FTIR was performed to identify the functional groups of the purified antimicrobial compound present in it. About 5mg of the purified compound was mixed with dry potassium bromide (KBr) and the mixture was thoroughly mixed in a mortar and pressed at pressure of 6 bars within 2min to form a KBr thin disc. Then the disc was placed in a sample cup of a diffuse reflectance accessory. Infrared absorption spectrum was recorded on an IR affinity FTIR system (Shimadzu, Japan) at a spectral resolution of $4cm^{-1}$ with an average of 10scans in the wave number range of $400–4000cm^{-1}$.

Nuclear Magnetic Resonance Spectroscopy (NMR)

The purified antimicrobial compound (30mg) was suspended in 0.5ml of deuterated chloroform (CDCl3) solvent. ^{1}H & ^{13C} NMR spectrum of the antimicrobial compound was recorded at 25°C on a Bruker AV600NMR spectrometer (Germany) in which the chemical shifts were expressed in parts per million (ppm) scale downfield from an internal standard of tetra methyl silane (TMS). The spectrum was recorded at 297.9 K at a frequency of 500MHz.

Structural characterization using gas Chromatography and Mass Spectroscopy (GC-MS)

The purified antimicrobial compound was structurally evaluated using GC-MS. The GC-MS was performed on a Thermo Trace GC Ultra coupled with Polaris Q MS and TriPlus auto-sampler using a DB-5 (0.25mm × 30m × 0.22ìm) column in which helium was used as carrier gas. The temperature was set between 50°C to 250°C at a rate of 10°C min^{-1}. The initial temperature was held for 2min and final temperature of 250°C was held for 10min. The GC flow rate was 1ml min^{-1} and the total run time was 32min. MS/MS was performed at scan mode between 0 – 350m/z with an Ion trap EI+. (Jenkins). The fragmentation pattern resulted from the methylated compound was evaluated using NIST database for the structural confirmation of the purified antimicrobial substrate.

RESULT AND DISCUSSION

Extraction of antimicrobial compound

In the current investigation, an antimicrobial compound was produced from a probiotic *L. apis* YMP3 and the cultured broth was extracted for the antimicrobial agent using ethyl acetate [Fig. 1]. The crude extracted was dried which exhibited antibacterial activity of 67.82% against a human pathogenic *V. cholerae*. Similarly, a lactic acid bacterium procured from the samples of cow and buffalo milk showed antimicrobial activity against *Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Mycobacterium smegmatis*, *M. fortuitum* and *Staphylococcus aureus*[6]. Hor and Liong[17] also found that the crude extracts of lactic acid bacterium suppress the biofilm formation by *Staphylococcus aureus*. Owusu-Kwarteng *et al*.[18] reported that the bacterial strains isolated from fermented milk products are commonly regarded as safe for human use which can also tolerate low pH and have the possibility of producing antibacterial compounds.

Purification of antimicrobial compound

The antimicrobial compound was purified with a RP - C_{18} silica gel column chromatography

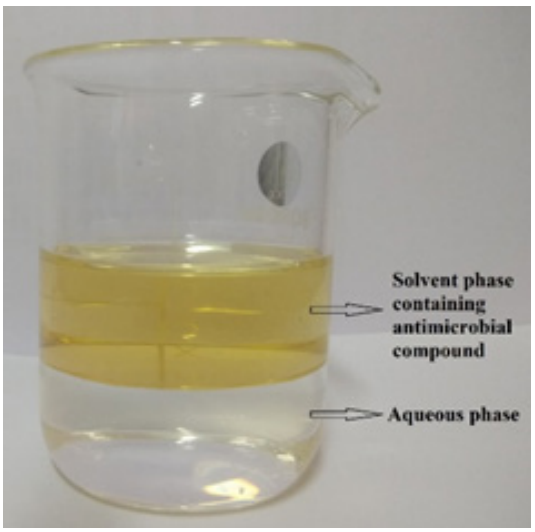

Fig. 1. Ethyl acetate extraction of antibacterial substrate from a *L. apis* YMP3 cultured broth

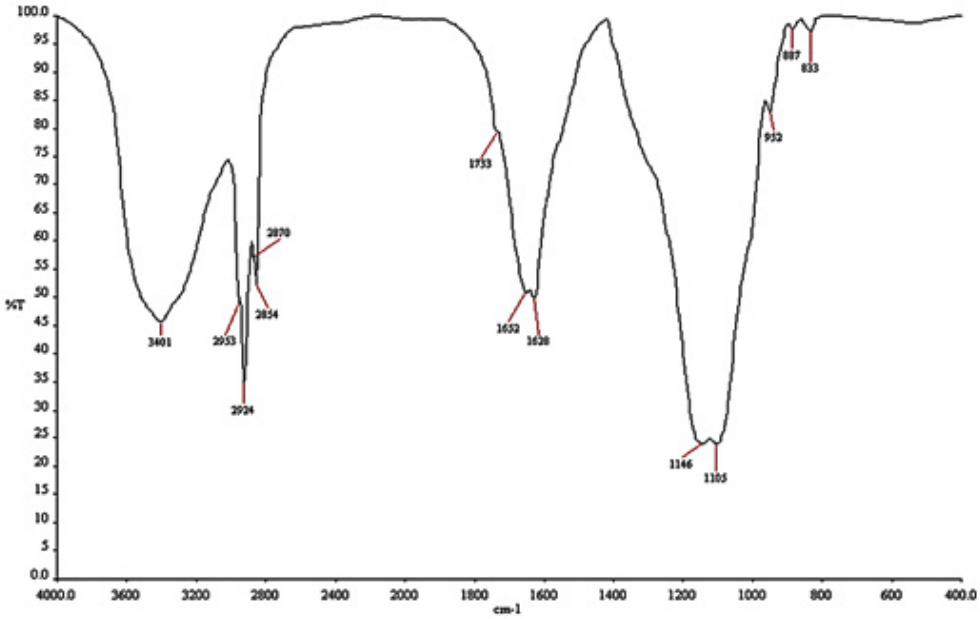

Fig. 2. FT-IR spectrum of the antibacterial substrate from a *L. apis* YMP3

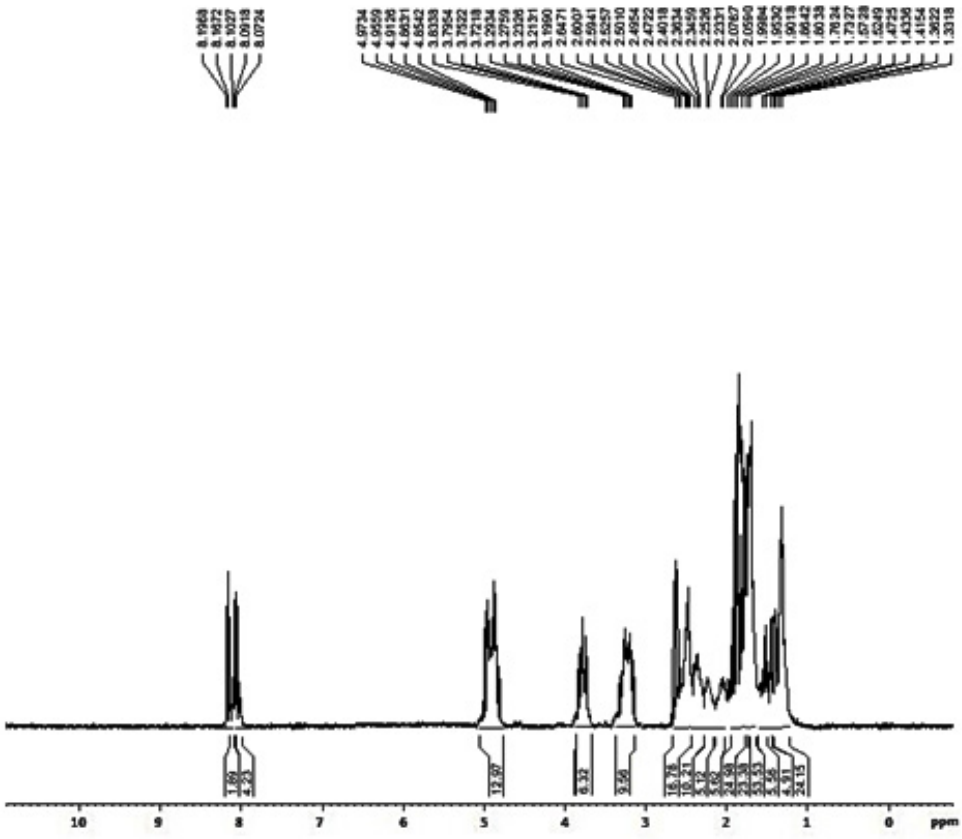

Fig. 3. H^1 NMR spectrum of the antibacterial substrate from a *L. apis* YMP3

at room temperature. Among the collected fifty fractions, fraction representing the ratio of 77:23 acetonitrile and methanol revealed 100% inhibition activity against human pathogenic *V. cholerae*. In the same manner, Macherla[19] also purified a bioactive compound, 4-oxazolidinone antibiotics lipoxazolidinone A, B, and C from a marine *Marinispora* sp. which demonstrated antimicrobial activity against many multidrug-resistant pathogens. Similarly, Arumugam *et al.*[20] isolated a marine *Streptomyces* sp. that was found to be producing antimicrobial metabolites from culture supernatant which was extracted with n-butanol and purified by using silica gel column chromatography. Likewise, an antimicrobial compound purified from a marine *Staphylococcus*

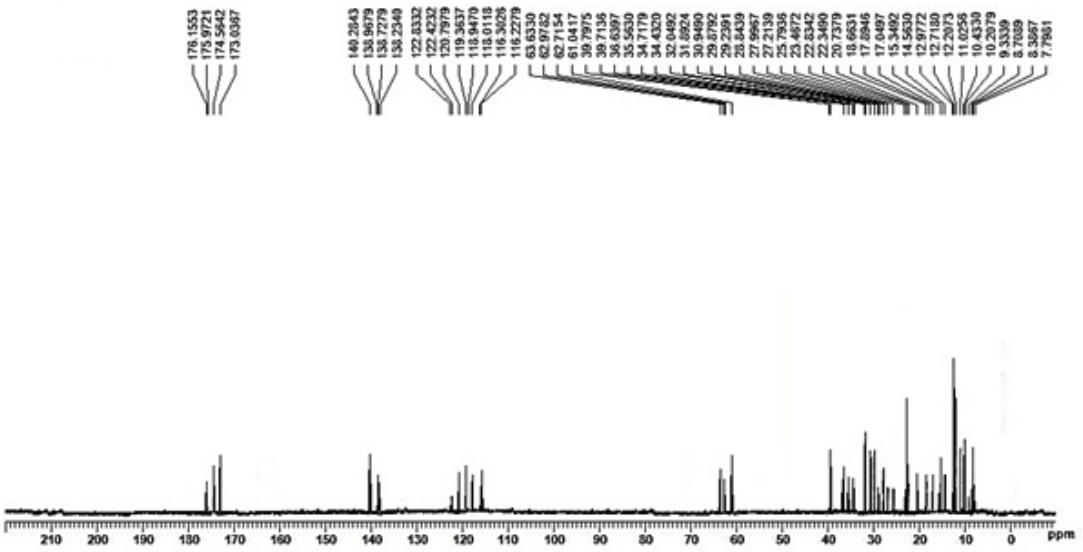

Fig. 4. C^{13} NMR spectrum of the antibacterial substrate from a *L. apis* YMP3

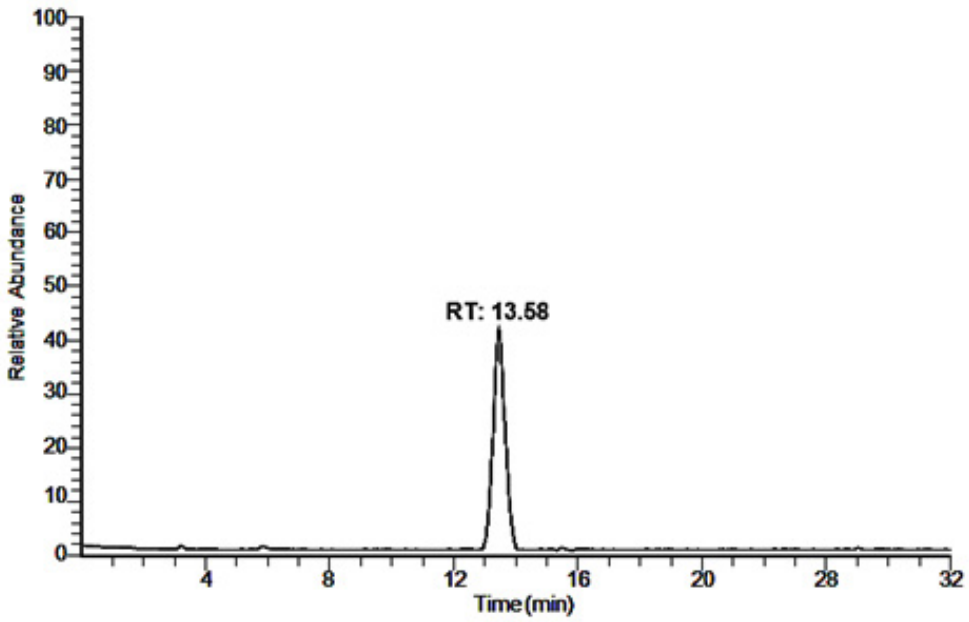

Fig. 5. GC spectrum of the antibacterial substrate from a *L. apis* YMP3

saprophyticus SBPS 15 showed broad spectrum antibacterial activities against clinically isolated human pathogens[21].

FT-IR analysis of antimicrobial compound

The functional groups present in the purified antibacterial compound were predicted using FT-IR analysis [Fig. 2]. Spectral peaks at 2953, 2924, 2870, and 2854cm^{-1} showed the presence of aliphatic alkane, while the peaks at 952, 887, and 833cm^{-1} indicated the presence of aliphatic alkene. Similarly, the aromatic alkane was predicted from the peaks at 1652 and 1628cm^{-1} and the amine (NH) group was evidenced from the peak at 3401cm^{-1}. Ketone (R-CO-R) was found at 1733cm^{-1} and ether (R-O-R) was revealed at 1146 and 1105cm^{-1}. All of the predicted functional groups in the FT-IR spectrum revealed the possible structure of (2E)-5-butyl-2-[(E)-4-

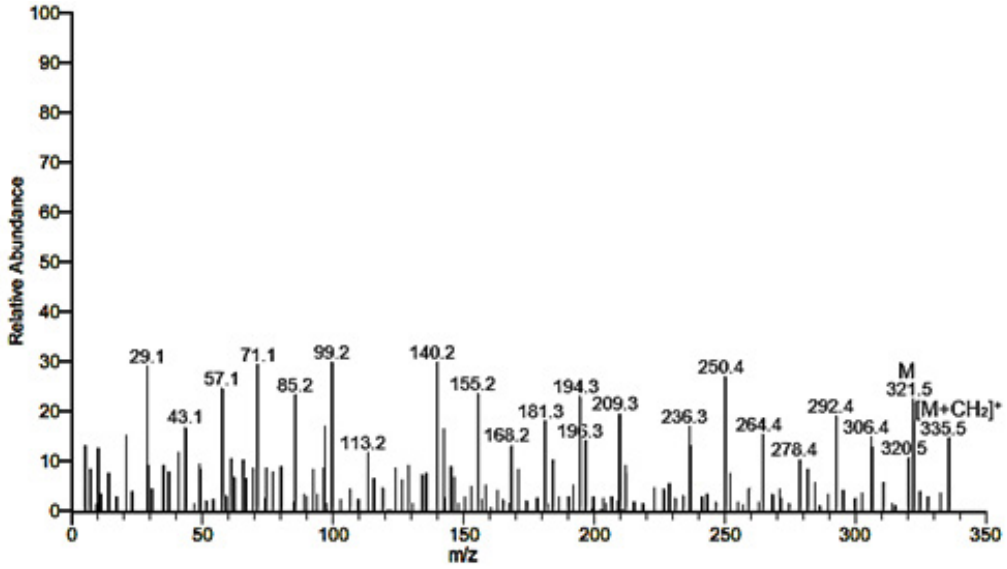

Fig. 6. MS/MS spectrum of the antibacterial substrate from a *L. apis* YMP3

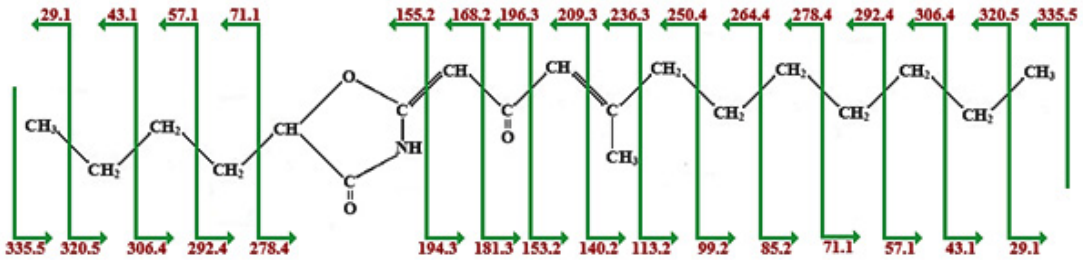

Fig. 7. Fragmentation pattern of antibacterial substrate after methylation process, depicts the methylated (CH_2) molecular weight pattern of the active compound

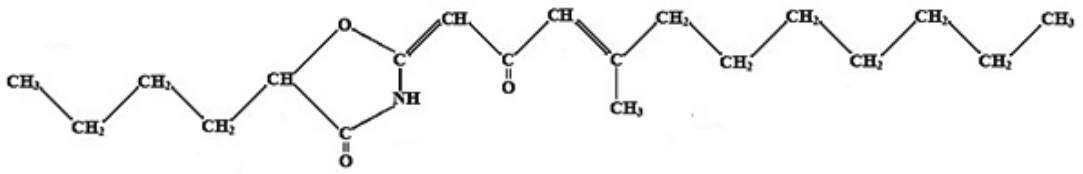

Fig. 8. Predicted chemical structure of the active compound is (2*E*)-5-butyl-2-[(*E*)-4-methyl-2-oxoundec-3-enylidene]-1,3-oxazolidin-4-one

methyl-2-oxoundec-3-enylidene]-1,3-oxazolidin-4-one. Likewise, Aneurinifactin and Cybersan, which were extracted from a marine bacterium, *Aneurinibacillus aneurinilyticus* SBP-11 and marine yeast *Cyberlindnera saturnus* SBPN-27 revealed their structural chemistry using FTIR spectrum[22,23]. Han *et al.*[24] also extracted antimycin B1 and B2 from *Streptomyces lusitanu*s and functional groups were confirmed from FT-IR spectrum.

NMR analysis of antimicrobial compound

^{1}H and ^{13}C NMR spectra of the purified antibacterial compound were illustrated in fig. 3 and 4. The presence of aliphatic alkane hydrogen was observed between ä 1.3318 – 2.0767ppm and aromatic alkane hydrogen was found between ä 8.0724 – 8.1672ppm, similarly, the aliphatic alkene hydrogen was predicted within ä 4.8542 - 4.9734ppm. Further, the hydrogen atoms of amide group evidenced at ä 8.0724 (CO-NH), hydrogen atoms present in ketone group (R-CO-R) and ether group (R-O-R) were revealed between ä 2.2331 – 2.6471ppm and ä 3.1990 – 3.8338ppm chemical shifts, respectively.

In the similar manner, ^{13}C NMR spectrum revealed the carbon atom of the aliphatic alkane carbon and aromatic alkane carbon between ä 7.7981 - 39.7975ppm and ä 138.2340 - 140.2843ppm. Furthermore, the presence of carbon atoms in aliphatic alkene was observed within ä 116.2279 - 122.8332ppm and amide carbon (CO-NH) was evidenced at ä 173.0367. Moreover, the carbon atoms of the significant groups viz., ketone (R-CO-R) was observed between ä 174.5642 - 176.1553ppm and ether (R-O-R) was revealed within ä 61.0417 - 63.6330ppm.

Similar to the FTIR spectrum, NMR spectrum also revealed the possible structure of the purified antimicrobial compound is (2*E*)-5-butyl-2-[(*E*)-4-methyl-2-oxoundec-3-enylidene]-1,3-oxazolidin-4-one. In the same manner, the structure of Pontifactin a bioactive compound produced by a marine *Pontibacter korlensis* strain SBK-47 was described from the NMR spectrum[25] and Staphylosan, a glycolipid bioactive substrate purified from a marine *Staphylococcus saprophyticus* SBPS-15 in which the structural chemistry was evidenced from NMR spectrum[26]. Further, NMR spectrum were used to elucidate the structure of an antimicrobial compound, 4-dimethylaminobenzaldehyde (Ochrosin) from the halophilic *Ochrobactrum sp* BS206[27].

GC-MS analysis of antimicrobial compound

The chemistry of the antibacterial compound was studied in detail using GC-MS analysis. At the retention time of 13.58 min., the sample revealed a single major peak in which the observation of a single peak revealed the purify of the compound [Fig. 5]. Using MS/MS analysis, the molecular weight of the compound is 321.5m/z and the molecular mass of methylated compound was predicted at 335.5m/z [Fig. 6]. As per the NIST database, the purified compound was identified as (2*E*)-5-butyl-2-[(*E*)-4-methyl-2-oxoundec-3-enylidene]-1,3-oxazolidin-4-one as well as the fragmentation pattern of the purified compound as evidenced from the MS/MS analysis is shown in the fig. 7. From the overall spectral evaluations, the structure of the antimicrobial compound is (2*E*)-5-butyl-2-[(*E*)-4-methyl-2-oxoundec-3-enylidene]-1,3-oxazolidin-4-one] which is illustrated in the fig. 8.

The same structure has been identified earlier from an actinobacterium, *Marinispora* sp. NPS008920 which was isolated from the sediments of Cocoa Lagoon, Guam and named as Lipoxazolidinone A[28]. To the best of our knowledge, this is the first report of this structure identified from a bacterium showed promising antibacterial activity against a human pathogenic *V. cholerae*. The same systematic spectral profiling of FTIR, NMR, GC, and MS/MS analysis was used to identify -1,3-oxazolidin-4-one purified from a marine *Paenibacillus macerans* SAM 9[29]. Analogously, Jangir *et al.*[30] also used that GC-MS analysis of the evaluation of volatile organic compounds produced by *Bacillus* sp. such as N, N-Dimethyl, 1,2-benzenedicarboxylic acid and 9-octadecenoic acid which were responsible for controlling the growth of *Fusarium oxysporum f. sp. lycopersici.* Similarly, the antibiotic Lydicamycin congener TPU-0037-A, B, C, and D were separated from *Streptomyces platensis* and their structures were determined using MS/MS analyses[31].

CONCLUSION

This investigation purified an antimicrobial compound from the cell free extract of *L. apis* YMP3. This compound possesses an

effective antimicrobial activity against a clinically isolated human pathogenic *V. cholerae*. The purified compound was structurally identified as Lipoxazolidinone A based on various spectral evaluations. These results serve as the essential information for the purification and antibacterial activity of this compound. Further, this study suggesting the antimicrobial efficiency of purified compound needs to be more investigated for its detailed biological evaluations in future.

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Conflict of Interest

The authors declare that they have no conflict of interest on publication of this article.

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