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# Bioactive compounds isolated from submerged fermentations of the Chilean fungus *Stereum rameale*

**Abstract:** Liquid fermentations of the fungus *Stereum rameale* (N° 2511) yielded extracts with antibacterial activity. The antibacterial activity reached its peak after 216 h of stirring. Bioassay-guided fractionation methods were employed for the isolation of the bioactive metabolites. Three known compounds were identified: MS-3 (**1**), vibrallactone (**2**) and vibrallactone B (**3**). The three compounds showed antibacterial activity as a function of their concentration. Minimal bactericidal concentrations (MBC) of compound **1** against Gram-positive bacteria were as follows: *Bacillus cereus* (50 µg/mL), *Bacillus subtilis* (10 µg/mL) and *Staphylococcus aureus* (100 µg/mL). Compounds **2** and **3** were active only against Gram-negative bacteria. The MBC of compound **2** against *Escherichia coli* was 200 µg/mL. Compound **3** inhibited significantly the growth of *E. coli* and *Pseudomonas aeruginosa*, with MBC values of 50 and 100 µg/mL, respectively.

**Keywords:** antibacterial activity; MIC; MS-3; *Stereum rameale*; vibrallactone.

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## 1 Introduction

The higher fungi, Basidiomycetes, offer an exciting field to obtain new structures with high potential for medical and agricultural applications. Higher fungi have an important advantage as producers of bioactive secondary metabolites, as they release them to liquid media. This ability has allowed the development of a powerful line of research and production, with mycelial fermentations reaching industrial volume and valuable bioactive compounds being isolated with antibacterial, antifungal, nematocidal, phytotoxic, antiviral, insecticidal, cytotoxic, anticancer and other activities [1–3].

Chile is abundant in plant species with a wide variety of symbiotic, saprophytic and parasitic fungi, many of which are still unknown. According to Palfner [4], there are approximately 3000 species of fungi reported from Chile, and around 50% of them are higher fungi. Continuing with our research program on biologically active fungal metabolites, we have discovered an unsuspected reservoir of new and potentially useful molecules produced by Chilean Basidiomycetes [5, 6], and recently we have detected that extracts obtained from *Stereum rameale* (Pers.: Fr.) Burt, [synonym: *Stereum ochraceoflavum* (Schwein.) Ellis] showed a potent inhibitory capacity against pathogenic bacteria.

*Stereum rameale* is predominantly distributed in tropical or subtropical regions and often found associated with woody debris, rotting trunks and sometimes on buried dead wood. The fruiting bodies are thin, elastic and tough when moist, becoming hard and brittle when dry. This crust persists all through the year but releases spores only during autumn [7].

In Chile, the first record of *S. rameale* was published by Guillén et al. [8], who collected the fruiting bodies from branches of *Aetoxicum punctatum*, a Chilean native shrub. They also investigated *S. rameale* as a producer of lignocellulolytic enzymes and its tolerance to metal ions.

In previous investigations, a series of interesting new compounds have been isolated from the *Stereum* genus,

including acetylenic aromatics [9] sesquiterpenoids, such as hirsutanes [10], sterpuranes [11], cadinanes [12], stereumanes [13], isolactaranes [14] and illudalanes [15].

In general, in the scientific literature, reports on secondary metabolites and biological activities of *S. rameale* are quite limited. However, Mantle and Mellows [16] and Mellows et al. [17] reported from *Stereum complicatum* (Fr.) Fr. (also used as synonym of *S. rameale*) two new antibacterial sesquiterpenoids: complicatic acid and hirsutic acid C. Thus, the aims of the current study were to isolate and identify the active compounds produced by *S. rameale*, including submerged cultures, as well as to characterize and quantify their antibacterial effects.

## 2 Materials and methods

### 2.1 General

$^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data ( $\text{CDCl}_3$ ,  $\delta/\text{ppm}$  7.27/77.0 and  $\text{DMSO}-d_6$ ,  $\delta/\text{ppm}$  2.50/39.5) were recorded on a Bruker DRX 500 spectrometer at 500 MHz for  $^1\text{H}$  and 125 MHz for  $^{13}\text{C}$  (Avance III-500 MHz, Germany). Optical rotations were measured in  $\text{CHCl}_3$  at 20 °C on a Perkin-Elmer Model 341 polarimeter (Waltham, MA, USA). Fragmentation  $m/z$  data were recorded on a Waters Q-TOF Micro system mass spectrometer, using  $\text{H}_3\text{PO}_4$  as an internal standard for calibration.

### 2.2 Producing organism and fermentation

Fruiting bodies of *S. rameale* (strain 2511) were collected from the bark of a dead tree in a native forest of *Nothofagus* species (Nothofagaceae), near Ñuble National Reserve, Ñuble Province, Chile. The identification was done on the basis of macro- and micromorphological features, size and color of colonies. Mycelial cultures were derived from spore prints of a fruiting body. The strain 2511 is kept on YMG (yeast extract, malt extract, glucose) agar containing the following (g/L): glucose 4, malt extract 10, yeast extract 4 and agar 20, at pH 5.5. A voucher specimen of the fungus is deposited in the herbarium of the Laboratory of Applied Microbiology and Mycology, Department of Agroindustries, Faculty of Agricultural Engineering, University of Concepción. All culture media were purchased from Merck-Chile and Difco-Chile, Santiago de Chile.

Fermentation on a larger scale was carried out in a 10-L glass bottle (DURAN®) containing 7.5 L of YMG medium with stirring (120 rpm) and aeration (3 L/min) at 20 °C. To prevent foaming, silicone antifoam (Merck, Darmstadt, Germany) was added. The fermentor was inoculated with 100 mL of a well-grown culture in the same medium. Daily samples were withdrawn and assayed for pH, glucose and maltose content, biomass production and antibacterial activity. These aliquots were filtered and then extracted with EtOAc. The combined extracts were dried with  $\text{Na}_2\text{SO}_4$  and concentrated in vacuo (45 °C). Extracts were dissolved in MeOH (100  $\mu\text{g}/\text{mL}$ ) and then used for the antibacterial tests. The fermentation was stopped when the glucose was exhausted and the antibacterial

(*Bacillus cereus*, *Salmonella*) activity of the extract had reached its peak [18].

### 2.3 Isolation of bioactive compounds

The culture was filtered to separate the mycelium from the broth. The mycelium was extracted with methanol (MeOH-extract). The broth culture was extracted with EtOAc (3×7 L) and the organic phase concentrated under reduced pressure (EtOAc-extract). This extract was further applied onto a silica gel column (Merck 60, 0.063–0.2 mm; column 3×30 cm) eluted with *n*-hexane and with a gradient of hexane-EtOAc mixtures up to 100% EtOAc to enhance polarity. Fractions and purified compounds were analyzed by analytical and preparative thin-layer chromatography (TLC) and eluted with mixtures of solvents (hexane-EtOAc, chloroform-MeOH). The separated components were visualized under ultraviolet light (254 and 366 nm), and spraying with 20%  $\text{H}_2\text{SO}_4$  followed by heating. The bioactive bands of the preparative TLC were separately scraped from the air-dried plate and eluted with MeOH. This suspension was filtered and the organic phase evaporated. Active compounds were isolated by bioautography-guided fractionation according to Thines et al. [19]. All solvents were purchased from Merck-Chile, Santiago de Chile.

### 2.4 Bacterial strains

Six bacterial isolates were included in this study: *B. cereus* (LMM-876), *Bacillus subtilis* (LMM-013), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (LMM-213), *Salmonella* sp. (LMM-352) and *Staphylococcus aureus* (LMM-292). All strains, except *E. coli*, had been isolated from different food products. Bacterial strains were grown in Mueller-Hinton Broth at 37 °C.

### 2.5 Antimicrobial assays

Antimicrobial activity was determined in the serial dilution assay or the plate diffusion assay as described by Anke et al. [20].

Total extracts, fractions and isolated compounds **1**, **2** and **3** dissolved in MeOH were assayed in the disc diffusion test at 100  $\mu\text{g}/6$  mm disc. Bacterial cultures were diluted with sterile water to obtain a microbial suspension of  $10^6$ – $10^8$  CFU/mL. Petri plates containing 20 mL of culture medium with 2% (w/v) of molten agar were inoculated with 200  $\mu\text{L}$  of microbial suspension and allowed to solidify in a sterile chamber. After solidification, the discs were placed on the inoculated culture medium. The positive control was penicillin G and streptomycin (streptomycin sulphate) 100  $\mu\text{g}/6$  mm disc. Methanol was used as negative control. The plates were incubated at 37 °C, and the inhibition zones (diameters) around the discs were measured (mm) after 24 h.

Compounds that showed the highest activity against one or more test microorganisms in the primary assay were considered potentially active and selected to determine their minimal inhibitory concentration (MIC) value in serial dilution assays. The MIC of compounds **1**, **2** and **3** was determined for Gram-positive and Gram-negative bacteria, using the serial agar dilution assay in concentrations ranging from 1  $\mu\text{g}/\text{mL}$  to 200  $\mu\text{g}/\text{mL}$ . The tested compounds were

placed in wells, and the methanol was evaporated. Suspensions of bacteria were dispensed at 0.2 mL/well in 96-well microtiter plates. Bacterial suspensions without additions were used as a negative control and streptomycin as a positive control. The MIC values were taken as the lowest concentrations of the compounds in the wells that did not allow visible bacterial growth after 24 h of incubation at 37 °C, as evidenced by the turbidity of the cultures. The contents of the wells in which a MIC had been observed were streaked, using a sterile wire loop, on a sterile agar plate and incubated at 37 °C for 24 h. The lowest concentration of the compounds that did not allow bacterial growth was assigned as the minimal bactericidal concentration (MBC).

3 Results and discussion

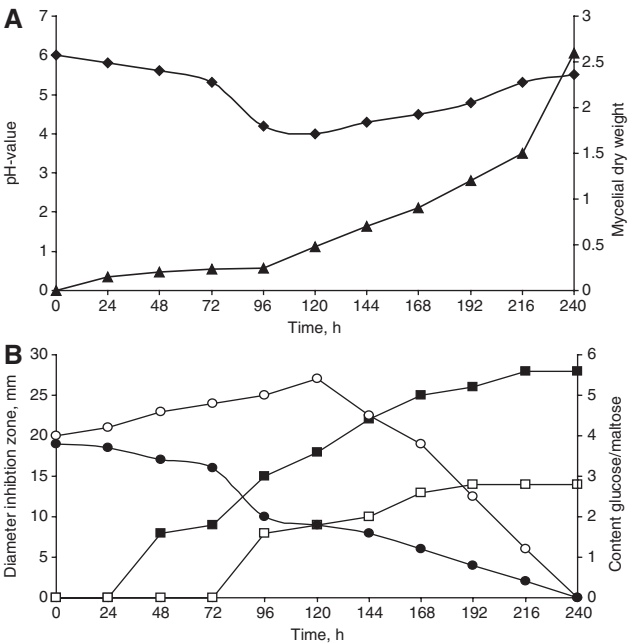
As shown in Figure 1, after 216 h of fermentation, the mycelial biomass had markedly increased (Figure 1A), while the content of maltose decreased after 72 h at a rather continuous rate; glucose increased gradually over the first 120 h and then decreased continually. At 240 h, the medium was depleted of both maltose and glucose, and the fermentation was stopped (Figure 1B).

The antimicrobial activities of the extracts obtained from the daily aliquots increased during this period, against both *B. cereus* and *Salmonella* sp. (Figure 1B). The fermentation was terminated after 240 h. Evaporation of the organic phase yielded 954 mg of EtOAc-extract. Silica gel column chromatography yielded seven fractions.

The crude extract exhibited strong antibacterial activity against the three Gram-positive bacteria, *B. cereus*, *B. subtilis* and *S. aureus*, while it was weak (*E. coli*, *P. aeruginosa*) or negligible (*Salmonella* sp.) against the three Gram-negative bacteria. Mycelial extracts did not contain antibiotic activity.

Table 1 shows the results obtained by the in vitro plate diffusion method. Following the bioassay-guided fractionation, the MeOH-extract and fractions 1, 3, 4, 6 and 7 exhibited a weak antibacterial activity, while the EtOAc-extract and fractions 2 and 5 exhibited distinct antibacterial activities. Elution with *n*-hexane/EtOAc (7:3 and 4:6) yielded fraction 2 (112 mg) and fraction 5 (123 mg), respectively. Fraction 2 was active against *B. cereus*, *B. subtilis* and *S. aureus*, while fraction 5 was active against *E. coli* and *P. aeruginosa*. Both fractions were further purified by preparative TLC. Through a direct bioautographic assay, compounds 1 (21.2 mg), 2 (27.1 mg) and 3 (17.5 mg) were isolated and their structures identified (Figure 2). All NMR data (<sup>1</sup>H-<sup>13</sup>C) were assigned by comparison with previously reported data [21, 22].

Compound 1 was obtained as colorless crystals (soluble in MeOH). The molecular formula was found to be C<sub>21</sub>H<sub>24</sub>O<sub>7</sub>. The HRMS-ESI calculated for the dimer C<sub>42</sub>H<sub>48</sub>O<sub>14</sub> calcd. for [M+H]<sup>+</sup> *m/z* 777.3122. Found: 777.3154. calcd. for [M+Na]<sup>+</sup> *m/z* 799.2942. Found: 799.2968 [M+Na]<sup>+</sup>. From the NMR data, this compound was identified as 3-hydroxy-4, 5-bis (hydroxymethyl)-2-(3'''-methyl-2'''-butenyl)-phenyl 2', 4'-dihydroxy-6'-methyl-benzoate (MS-3). <sup>1</sup>H NMR (500 MHz,

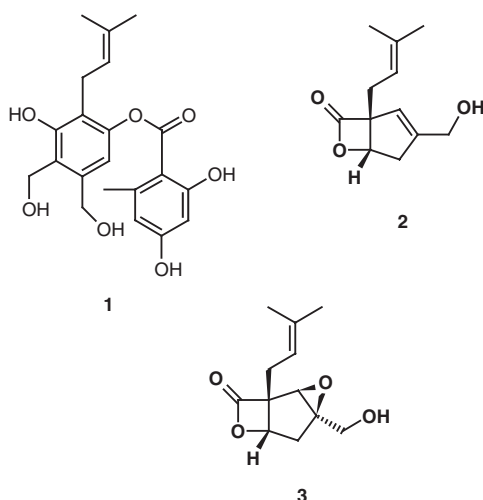


**Figure 1:** Fermentation of *S. rameale* (2511). (A) ♦, pH; ▲, mycelial dry weight (g/L). (B) ■, inhibition zone *B. cereus* (mm); □, inhibition zone *Salmonella* (mm); ○, glucose (g/L); ●, maltose (g/L).

**Table 1:** Antibacterial activities of extracts, fractions and pure compounds isolated from *S. rameale*.

Sample	Bc	Bs	Sa	Ec	Pa	Sal
EtOAc-extract	28	27	14	10	10	–
MeOH-extract	–	8	–	–	–	9
Fr.1	–	–	–	–	–	–
Fr.2	29	28	32	–	–	–
Fr.3	8	8	–	–	–	–
Fr.4	–	9	–	–	–	–
Fr.5	–	–	–	14	10	–
Fr.6	–	–	–	9	–	–
Fr.7	–	–	–	–	–	–
MS-3 (1)	25	25	28	–	–	–
Vibralactone (2)	–	–	–	12	9	–
Vibralactone B (3)	–	–	–	14	13	–
Streptomycin	32	28	30	25	14	22
Penicillin G	30	28	27	25	24	14

Numbers indicate diameter of the inhibition zone (mm). Active=15 or more; moderate=10–15; weak=<10 or diffuse; –, no inhibition zone. Bc, *B. cereus*; Bs, *B. subtilis*; Ec, *E. coli*; Pa, *P. aeruginosa*; Sal, *Salmonella* spp.; Sa, *S. aureus*; EtOAc-extract, ethylacetate extract; MeOH-extract, methanolic extract; Fr., fraction.



**Figure 2:** Compounds isolated from *S. rameale* (2511). **1**, MS-3; **2**, vibrallactone; and **3**, vibrallactone B.

DMSO- $d_6$ )  $\delta$ /ppm: 1.53 (s, 3H, 5''), 1.54 (s, 3H, 4''), 2.38 (s, 3H, 6''), 3.21 (d, 2H,  $J=6.7$  Hz), 4.48 (d, 2H,  $J=5.4$  Hz), 4.68 (d, 2H,  $J=3.4$  Hz), 5.03 (t, 1H,  $J=6.9$  Hz), 5.15 (t, 1H,  $J=5.4$  Hz), 5.69 (t, 1H,  $J=3.4$  Hz), 6.22 (s, 1H), 6.23 (s, 1H), 6.70 (s, 1H), 9.27 (s, 1H), 10.09 (s, 1H), 10.60 (s, 1H).  $^{13}\text{C}$  NMR (125 MHz, DMSO- $d_6$ )  $\delta$ /ppm: 168.4, 161.3, 161.0, 154.8, 148.1, 140.7, 139.1, 130.5, 122.5, 121.6, 120.0, 112.4, 110.2, 107.8, 100.5, 60.5, 56.8, 25.5, 22.9, 21.8, 17.5.

Compound **2** was obtained as colorless oil (soluble in MeOH).  $[\alpha]_D^{20}$ :  $-126.5^\circ$  ( $c=0.10$ ,  $\text{CHCl}_3$ ). Molecular formula:  $\text{C}_{12}\text{H}_{16}\text{O}_3$ . HRMS-ESI calc. for  $[\text{M}+\text{Na}]^+$   $m/z$  231.0997. Found: 231.0917  $[\text{M}+\text{Na}]^+$ . Based on NMR data, compound **2** was identified as vibrallactone.  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$ /ppm: 1.64 (s, 3H), 1.73 (s, 3H), 2.43 (dd, 1H,  $J=15.1$ , 7.4 Hz), 2.62 (dd, 1H,  $J=15.1$ , 7.4 Hz), 2.76 (m, 2H), 4.25 (s, 2H), 4.81 (dd, 1H,  $J=1.3$ , 4.7 Hz), 5.13 (t, 1H,  $J=7.4$  Hz), 5.62 (s, 1H).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$ /ppm: 173.0 (C-7), 146.5 (C-3), 136.0 (C-10), 122.5 (C-2), 117.2 (C-9), 78.5 (C-5), 75.1 (C-1), 61.4 (C-13), 37.3 (C-4), 27.6 (C-8), 25.8 (C-12), 18.0 (C-11).

Compound **3** was obtained as colorless crystals (soluble in MeOH).  $[\alpha]_D^{20}$ :  $-56.5^\circ$  ( $c=0.14$ ,  $\text{CHCl}_3$ ). The molecular formula was found to be  $\text{C}_{12}\text{H}_{16}\text{O}_4$ . HRMS-ESI calc. for  $[\text{M}+\text{Na}]^+$   $m/z$  247.0946. Found: 247.0976  $[\text{M}+\text{Na}]^+$ . From the NMR data, compound **3** was identified as vibrallactone B.  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$ /ppm: 1.66 (s, 3H), 1.74 (s, 3H), 2.09 (dd, 1H,  $J=16.5$ , 6.6 Hz), 2.44 (d, 1H,  $J=16.5$  Hz), 2.51 (dd, 1H,  $J=15.1$ , 7.5 Hz), 2.65 (dd, 1H,  $J=15.1$ , 7.2 Hz), 3.54 (s, 1H), 3.82 (d, 1H,  $J=12.7$  Hz), 3.91 (d, 1H,  $J=12.7$  Hz), 4.81 (d, 1H,  $J=6.5$  Hz), 5.15 (t, 1H,  $J=7.4$  Hz).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$ /ppm: 168.5 (C-7), 137.0 (C-10), 116.2 (C-9), 82.2 (C-5), 77.1 (C-3), 69.0 (C-1), 61.2 (C-2), 60.8 (C-13), 31.7 (C-4), 25.9 (C-8), 25.8 (C-12), 18.0 (C-11).

Compound **1** exhibited the highest antibacterial activity with inhibition zones of 25, 25 and 28 mm against *B. cereus*, *B. subtilis* and *S. aureus*, respectively. This compound showed no antibacterial activity against *E. coli*, *P. aeruginosa* and *Salmonella* sp. Compound **2** was not active against most of the bacteria tested, except *E. coli*, which was moderately inhibited with inhibition zones of 12 mm. Compound **3** had a moderate activity against *E. coli* and *P. aeruginosa*, generating inhibition zones of 14 and 13 mm, respectively.

The MIC and MBC values are shown in Table 2. These results showed that compound **1** exhibited a potent bactericidal effect, and its MBC value was 10  $\mu\text{g/mL}$  against *B. subtilis* and 50  $\mu\text{g/mL}$  against *B. cereus* and *S. aureus*.

Compound **2** showed a MBC value of 200  $\mu\text{g/mL}$  against *E. coli*. Compound **3** inhibited significantly the growth of *E. coli* and *P. aeruginosa*, with an MBC value of 50 and 100  $\mu\text{g/mL}$ , respectively.

In conclusion, the antibacterial activity of *S. rameale* seems to be related mainly to the compounds **1**, **2** and **3**. While compound **1** was able to inhibit only Gram-positive bacteria, compound **2** and **3** were exclusively active against Gram-negative bacteria. Compound **3** differs from **2** in that it has an epoxide group in the pentacyclic ring, which replaces the original double bond.

This difference apparently confers activity against a selective and reduced spectrum of sensitive bacterial strains. We have reported that the presence of an epoxide group in fungal metabolites can decisively affect antimicrobial activity, such as the compounds favolon and favolon B. Hence, in the absence of this group weak or null activity was observed [23].

These molecules have already been reported from several species of the genera *Stereum* and *Boreostereum*. Thus, compound **1** was previously isolated from an unidentified mushroom by Kurasawa et al. [24] and characterized as inhibitor of glyoxalase I and of the proliferation of Yoshida sarcoma cells. Other benzoates related to compound **1** and isolated from *S. hirsutum* exhibited antibacterial, anti-inflammatory and anti-tumor effects

**Table 2:** MIC and MBC of compounds isolated from *S. rameale*.

Bacteria	$(\mu\text{g/mL})$		
	MS-3 (1)	Vibrallactone (2)	Vibrallactone B (3)
<i>B. cereus</i>	10 <sup>a</sup> 50 <sup>b</sup>	nt	nt
<i>B. subtilis</i>	10 <sup>b</sup>	nt	nt
<i>E. coli</i>	nt	100 <sup>a</sup> 200 <sup>b</sup>	20 <sup>a</sup> 50 <sup>b</sup>
<i>P. aeruginosa</i>	nt	–	100 <sup>b</sup>
<i>S. aureus</i>	10 <sup>a</sup> 50 <sup>b</sup>	nt	nt

<sup>a</sup>Bacteriostatic (MIC); <sup>b</sup>bactericidal (MBC); –, no effects up to 200  $\mu\text{g/mL}$ ; nt, not tested.



[25]. Vibrallactone (**2**) and vibrallactone derivatives (B-M) were isolated from cultures of *Boreostereum vibrans* [26–29], of which compound **2** potently inhibited pancreatic lipase with an  $IC_{50}$  value of 0.4 mg/mL [22], while vibrallactones D-F were active against human and mouse  $11\beta$ -HSD1 and  $11\beta$ -HSD2 cell lines [30]. Recently, the unusual fused  $\beta$ -lactone bicyclic system of compound **2** has been established as a potent and selective inhibitor of diverse disease-associated classes of enzymes, such as the caseinolytic peptidase (ClpP1P2) complex in *Listeria monocytogenes* [31] and acyl-protein-thioesterases (APT1, APT2) in HeLa cells [32]. Even though compounds **1–3** have previously been identified, this is the first time that these metabolites and their antibacterial properties are reported from *S. rameale*.

Based on the above information, we might infer that the antibacterial activities exhibited by compounds **2** and **3** inhibit one or more pathways involved in the reproduction and probably the virulence of the Gram-negative bacteria assayed (*E. coli*, *P. aeruginosa*), in which the presence of the  $\beta$ -lactone system and the epoxide group would be key in the observed activity.

Our investigation has revealed that extracts and pure compounds from *S. rameale* have considerable antibacterial activity toward important pathogenic bacteria, making them a good alternative as potential antimicrobials agents. Further in vivo studies are necessary to confirm their safety and efficacy, which may lead to its widespread use in the preservation of raw and processed food.

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