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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2020.813.819

Research Article
Evaluating the Effect of Methyl 5-(Hydroxy-Methyl) Furan-2-Carboxylate on Cytotoxicity and Antibacterial Activity

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Abstract

Background and Objective: Methyl 5-(hydroxymethyl)furan-2-carboxylate (MFC) was isolated as a major compound from *Streptomyces zerumbet* W14. This compound was evaluated the cytotoxicity and antibacterial activities. **Materials and Methods:** Isolation and structure elucidation of the bioactive compound were carried out using silica gel column chromatography and nuclear magnetic resonance. The cytotoxicity and antibacterial activities were reported. **Results:** MFC showed high antibacterial activity against Gram positive bacteria, *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis* and moderate activity against Gram negative bacteria, *Escherichia coli*, *Salmonella Typhi*, *Serratia marcescens* and *Pseudomonas aeruginosa*. This compound showed bacteriolytic effects on the tested microorganisms, causing cell wall and membrane damage by using the Sytox Green assay. It showed moderate cytotoxic activity on both normal cells, LLC-MK2 cells and L929 cells with IC_{50} values of >512.00 and 239.06 µg mL^{-1}, respectively. The strongest cytotoxicity was observed in HeLa and HepG2 cells with IC_{50} values of 64.00 and 102.53 µg mL^{-1}, respectively. **Conclusion:** The findings revealed that MFC affects selective cytotoxicity on cancer cells and Gram positive bacteria at a low concentration. It suggested that MFC has a potential usage as an alternative agents for treatments of some cancers and some bacterial infections.

Key words: Antibacterial activity, bioactive compound, cytotoxicity activity, methyl 5-(hydroxymethyl)furan-2-carboxylate, *Streptomyces zerumbet*, Sytox Green assay

Citation: Thongchai Taechowisan, Tipparat Samsawat, Winyou Puckdee and Waya S. Phutdhawong, 2020. Evaluating the effect of methyl 5-(hydroxy-methyl)furan-2-carboxylate on cytotoxicity and antibacterial activity. Pak. J. Biol. Sci., 23: 813-819.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Many types of bacteria and fungi have been found to be colonized in the internal tissues of plant as endophytes. They are synergistic to their host, some of them would produce bioactive substances to prevent the host plants from attacking phytopathogens and pests, benefiting on plant growth and physiology[1,2]. Endophytic actinomycetes have been reported to produce novel bioactive compounds with wide range of biological activities[3-9]. Most recently, *Streptomyces zerumbet* W14 was isolated from the rhizome tissue of *Zingiber zerumbet* (L.) Smith. Methyl 5-(hydroxymethyl)furan-2-carboxylate (MFC) was isolated as a major compound from its culture products. It has been reported that furan-2-carboxylic acids exhibited cytotoxicity against various cancer cell lines[10] and the furanoid toxin isolated from *Curvularia lunata* and also synthesis showed strong antibacterial activity[11-14]. Due to the structure relationship of furan derivative compounds, the antibacterial and anticancer activities of MFC were investigated. Sytox Green assay provided convenient alternative to traditional methods for assessment of bacterial sensitivity to bactericidal agents. This study also showed the evidence of cellular damages caused by MFC using the Sytox Green assay. The results of this study may contribute the applications of MFC in treatments for bacterial infections and cancers.

MATERIALS AND METHODS

Isolation and cultivation of endophytic actinomycete: The study was carried out at Faculty of Science, Silpakorn University, from July, 2019 to January, 2020. Actinomycete strain W14 was isolated from the rhizome tissue of *Zingiber zerumbet* (L.) Smith by the surface sterilization method as described in the previous studies by Taechowisan *et al.*[15], Coombs and Franco[16] and Taechowisan *et al.*[17]. Identification of the isolate was based on chemotaxonomy, morphological, cultural, physiological and biochemical characteristics and also 16S rDNA sequencing[15,18]. The organism was grown for 14 days on ISP-2 agar at 30°C. The compound isolation was carried out by silica gel 60 column chromatography and the structure elucidation of the purified compound was determined by NMR spectroscopy as previously described[15].

Antimicrobial activity assay: For screening of antibacterial activity against tested bacteria, the paper disk-diffusion method was performed as described in the Clinical Laboratory Standard Institute[19]. The bioautography assay was performed according to the established protocols and procedures[20]. Details of the procedures have been described in a previous publication[15].

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC): Microbroth dilution assays of the crude extract and purified compound were carried out as described in the National Committee for Clinical Laboratory Standards[21]. The assays were performed in 96-well plates. Details of the procedures have been described in a previous publication[15]. The MIC was defined as the lowest concentration of compound that inhibited the visible growth of a microorganism. For the determination of MBC, a portion of culture (10 µL) from each well that showed no visible growth was cultured on nutrient agar and incubated at 37°C for 24 h. The lowest concentration that showed no growth after subculturing was determined as the MBC.

Sytox Green assay: The bacteria strains were grown on nutrient broth (Himedia, India) for the exponential growth phase. Cells were collected by centrifugation (6,000 rpm for 10 min), washed with phosphate-buffered saline (PBS), resuspended in PBS and diluted to be 5×10^7 cells mL^{-1}. Sytox Green assay was carried out in microcentrifuge tubes of 0.5 mL final volumes. Fifty microliter of bacterial suspension was mixed with tested compound. The compound was assayed at the final concentration of 25-400 µg mL^{-1}. Bacterial growth controls replaced the compound with PBS. The tubes were added with 5 µL of Sytox Green solution (Molecular Probes Inc., Eugene, Oreg.) and incubated at 37°C for 1 h. Fifty microliters of samples was placed on glass slides and observed with incident light fluorescence of a Nikon fluorescence microscope (Nikon Fluophot) with Osram HBO 200 W/2 mercury vapour lamp, exciter filter IF 420-490. The photomicrographs were taken with Olympus' cellSens imaging software (version 1.16).

MTT assay for cell viability: The cancer cells (HeLa: human cervical carcinoma and HepG2: hepatocellular carcinoma) and normal cells (LLC-MK2: rhesus monkey kidney epithelial cell lines and L929: murine fibroblast cell lines) were obtained from the Korean Cell Line Bank (Seoul, Korea). These cells were grown in DMEM medium supplement with 10% FBS, penicillin (100 U mL^{-1}) and streptomycin sulfate (100 µg mL^{-1}) at 37°C in a humidified atmosphere of 5% CO_2. Cytotoxicity studies were performed on a 96-well plate. Details of the procedures have been described in a previous publication[17].

RESULTS

Identification of microorganism: The strain W14 obtained from the rhizome tissues of *Zingiber zerumbet* (L.) Smith was determined to be a member of *Streptomyces*, according to the chemotaxonomy, morphological, cultural, physiological and biochemical characteristics and also 16S rDNA sequence analysis, it represented a novel species which we proposed the name *Streptomyces zerumbet* W14.

Isolation and structure elucidation of purified compound: Bioautography of the TLC analysis against *S. aureus* ATCC 25923, *B. cereus* ATCC 7064 and *B. subtilis* ATCC 6633 exhibited that the crude extract of this strain produced at least one compound with Rf values of 0.50 against the tested microorganisms (Fig. 1). Using column chromatography, MFC was isolated as a major compound. Its ^{1}H-NMR and ^{13}C-NMR spectral data were identical with those of furanoid toxin (Table 1). The structure of purified compound was shown in Fig. 2.

Antibacterial activity of purified compound: MFC from *S. zerumbet* W14 was investigated the antibacterial activity by MIC and MBC methods. This compound was effective against *S. aureus* with MIC and MBC values of 1 µg mL^{-1} and 16 µg mL^{-1}, respectively, *B. cereus* ATCC 7064 and *B. subtilis* ATCC 6633, with MIC and MBC values of 4 µg mL^{-1} and 64 µg mL^{-1}, respectively. It was also active against *E. coli* ATCC 10536, *S. Typhi* ATCC 19430, *S. marcescens* ATCC 8100 and *P. aeruginosa* ATCC 27853, with MIC and MBC values of 32-64 and 256-512 µg mL^{-1}, respectively (Table 2).

Bacterial exposed to purified compound showed intense fluorescence after Sytox Green stain (Fig. 3). These findings suggested that this compound exerted the antibacterial effects by damage to bacterial cell walls and membranes.

Assessment of cytotoxicity activity of purified compound: MFC was also investigated for cytotoxicity activity by MTT-assay on the cancer cells and normal cells. The cytotoxicity assay showed that purified compound had

Table 1: Comparison of the spectral data of purified compound and furanoid toxin FT[a]

Numbers	δ_C compound 1	δ_C FT	δ_H compound 1	δ_H FT	HMBC (H→C)	COSY
1	159.4, C	160.3, C	-	-	-	-
2	144.3, C	142.8, C	-	-	-	-
3	119.1, CH	119.0, CH	7.12 (d, 3.3)	7.21 (d, 3.4)	2, 4, 5	4
4	109.7, CH	109.0, CH	6.42 (d, 3.3)	6.44 (d, 3.4)	2, 3, 5	3
5	158.6, C	158.2, C	-	-	-	-
6	57.7, CH_2	56.1, CH_2	4.66 (s)	4.45 (d, 5.8)	4, 5	-
1-OMe	52.2, CH_3	51.9, CH_3	3.88 (s)	3.77 (s)	1	-
6-OH	-	-	-	5.80 (t, 5.8)	-	-

[a]FT: Furanoid toxin from *Curvularia lunata* (data from Liu *et al.*[11]), ^{1}H and ^{13}C-NMR assignments on purified compound [^{1}H (300 MHz), ^{13}C-NMR (75 MHz), $CDCl_3$, J = Hz], furanoid toxin [^{1}H (400 MHz), ^{13}C-NMR (400 MHz), DMSO-d6, J = Hz]

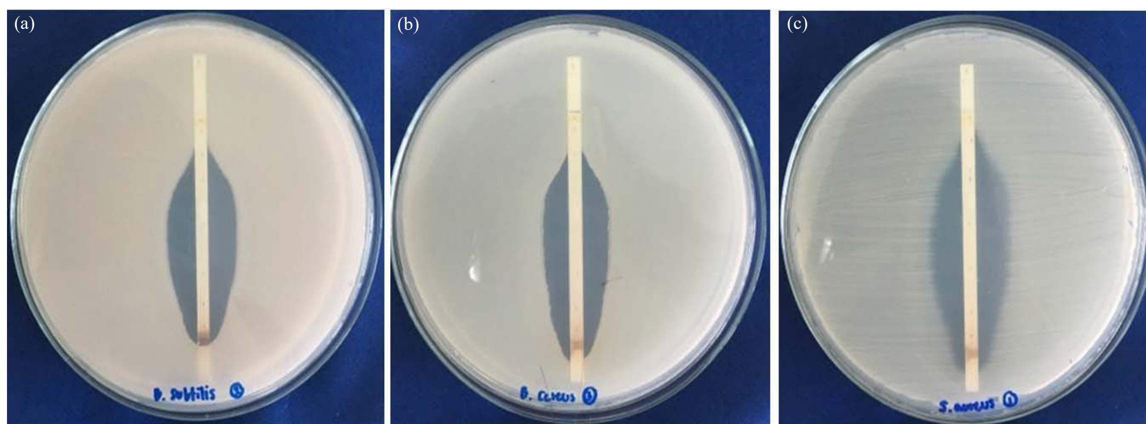

Fig. 1(a-c): Bioautography for antibacterial activity of crude extract of the strain W14 against (a) *Bacillus subtilis* ATCC 6633, (b) *Bacillus cereus* ATCC 7064 and (c) *Staphylococcus aureus* ATCC 25923 on nutrient agar after 24 h of incubation at 37°C

Table 2: Antibacterial activity of purified compound

Bacteria	Concentration ($\mu g\ mL^{-1}$)	
	MIC	MBC
S. aureus ATCC 25923	1.00	16.00
B. cereus ATCC 7064	4.00	64.00
B. subtilis ATCC 6633	4.00	64.00
E. coli ATCC 10536	32.00	256.00
S. Typhi ATCC 19430	32.00	256.00
S. marcescens ATCC 8100	32.00	256.00
P. aeruginosa ATCC 27853	64.00	512.00
MRSA[a]	1.00	64.00
BCG[b]	128.00	128.00

[a]MRSA: Methicillin resistant *S. aureus* strain Sp6 (clinical isolate), [b]BCG: Bacillus Calmette-Guérin (vaccine strain)

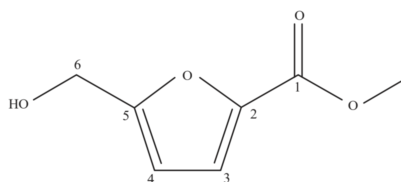

Fig. 2: Chemical structures of methyl 5-(hydroxymethyl) furan-2-carboxylate

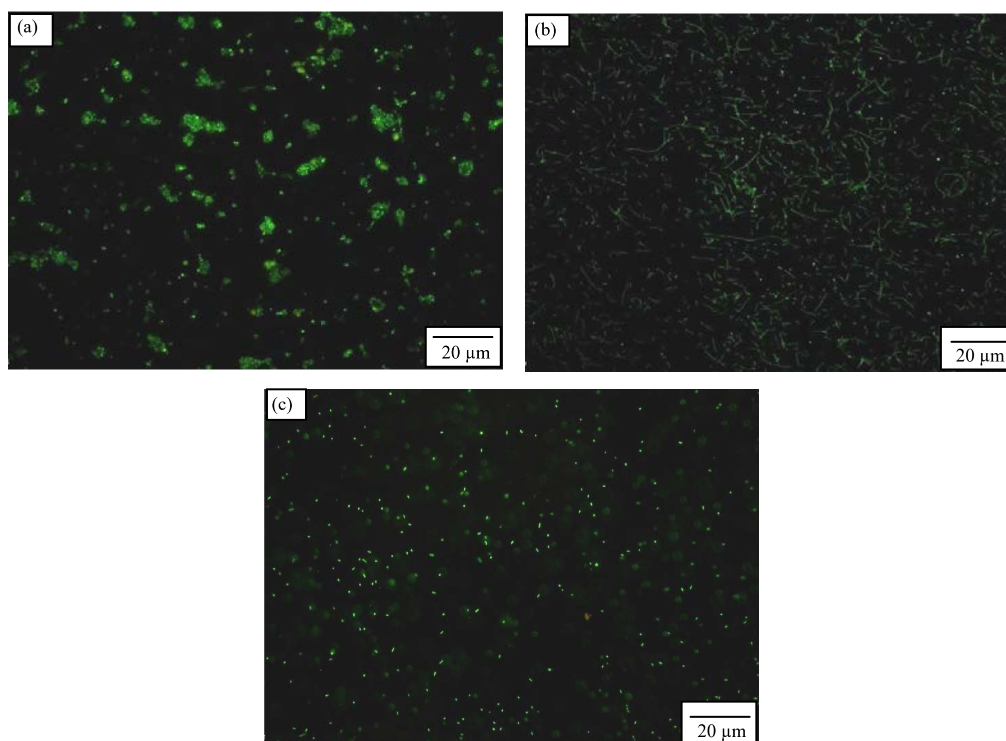

Fig. 3(a-c): Fluorescence microscopy of bacterial cells treated with purified compound by Sytox Green assay, (a) *Staphylococcus aureus* ATCC 25923, (b) *Bacillus subtilis* ATCC 6633 and (c) *Escherichia coli* ATCC 10536

moderate cytotoxic activity on both normal cells, LLC-MK2 cells and L929 cells with IC_{50} values of >512.00 and 239.06 $\mu g\ mL^{-1}$, respectively. The strongest cytotoxicity was observed in HeLa and HepG2 cells with IC_{50} values of 64.00 and 102.53 $\mu g\ mL^{-1}$, respectively (Table 3). When compared with the cancer cell lines, this compound showed less cytotoxicity to the normal cells.

DISCUSSION

In this study, MFC showed antibacterial activity on Gram positive better than Gram negative bacteria. This is due to the composition of bacterial cell walls. Most of the cell wall of Gram-positive bacteria consists of peptidoglycan and other associated molecules. This structure allows hydrophilic molecules to easily penetrate the cells. The cell wall of Gram-negative bacteria is more complex. It is composed of a double layer of phospholipids that is linked to inner membrane by lipopolysaccharides (LPS) which provides Gram-negative bacteria to be more resistant to antibacterial agents. Small hydrophilic solutes are able to pass through the outer membrane via only abundant porin proteins that serve as hydrophilic transmembrane channels and this is one reason that Gram-negative bacteria are relatively resistant to hydrophilic antibiotics and toxic drugs more than Gram-positive bacteria[22,23]. The outer membrane is almost but not totally impermeable to hydrophilic molecules, some of them can slowly cross through porins[24,25]. The mechanisms of action of MFC is similar antimicrobial effects but has different activity against Gram-positive and Gram-negative bacteria. These data confirm that furan derivatives exhibit antibacterial activity against a large number of Gram positive and Gram negative bacteria[26]. It has been observed that the mode of action of this compound is based on the ability to disrupt cell wall and cytoplasmic membrane, leading to lysis and leakage of cells which show intense fluorescence after Sytox Green stain. This stain is a high-affinity nucleic acid stain that does not cross the membranes of live cells and yet easily penetrates cells with compromised plasma membranes. This dead-cell indicator was used for rapid viability assessment after exposure of cells to disinfectant or antibiotics[27-29]. The disturbance of the cell membrance will disturb many vital processes such as energy conversion, nutrient processing, the synthesis of structural macromolecules, the secretion of growth regulators, leakage of cellular components and loss of ions[30,31].

For the determination of IC_{50}, two cancer cells and two normal cells were assessed for the cytotoxicity of this compound. Based on these findings it is concluded that the cancer cells are more sensitive to MFC compound than normal cells and needs to be investigated in depth. According to the previous report that furan-2-carboxylic acid and 5-3-(hydroxymethyl)-4,5-dimethoxyphenyl]-3-methylfuran-2-carboxylic acid exhibited cytotoxicity against various cancer cells: NB4, A549, SHSY5Y, PC3 and MCF7 with low IC_{50} values of 2.5, 1.2, 2.2, 3.6 and 1.9 μm, respectively[10]. The other studies reported that, furan derivative isolated from New Zealand sponge *Hymeniacidon huraki* exhibited cytotoxicity against P-388 with IC_{50} value of 13.4 μg mL^{-1})[32], a novel furan type isolated from *Streptomyces* sp. HS-HY-071 showed cytotoxicity against HCT-116 cells (IC_{50} value of 18.2 μg mL^{-1})[33] and a novel furan-2-yl acetate extracted from *Streptomyces* sp. VITSDK1 showed the IC_{50} values were less than 15 μg mL^{-1} against various tumor cells (HeLa, MCF7, MDA-MB 435S, HT-29, HepG2 and A549 cells), whereas it was >25 μg mL^{-1} against non-tumor cells (NFF, HEK-293 and Vero cells)[34]. Similarly MFC exhibited cytotoxic potential against tested cancer cells under *in vitro* conditions. The molecular mechanism of MFC in inducing cell death on various cancer and normal cell lines will be investigated elsewhere.

CONCLUSION

MFC from *Streptomyces zerumbet* W14 was found to be a potential antibacterial and anticancer activities with weak cytotoxic to normal cell lines. It could be useful for treatments of bacterial infections and cancers. However, the molecular mechanism of this compound needs to be investigated in large set of tumor and normal cell lines. The results of the studies also indicate that the endophytic actinomycetes from the medicinal plants could provide the compounds of beneficial value in biotechnology and biomedicine.

SIGNIFICANCE STATEMENT

This study discovered the MFC from the endophytic *Streptomyces zerumbet* W14. MFC affected on some cancer cells and Gram positive bacteria. This study will help the researcher to uncover the furan derivative compounds as a potential alternative agents for treatments of some cancers and some bacterial infections.

ACKNOWLEDGMENT

The authors are grateful to Ms Chanjira Jaramornburapong and Ms Chanakan Winyakul in the Department of Chemistry, Faculty of Science, Silpakorn University, Thailand, for measuring NMR and MS data, respectively. This work was supported by Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand.

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