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Contributed Paper

Helvolic Acid, A Secondary Metabolite Produced by *Neosartorya spinosa* KKU-1NK1 and Its Biological Activities

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ABSTRACT

Helvolic acid, an antimicrobial metabolite was isolated from the crude ethyl acetate extract of the soil fungus *Neosartorya spinosa* KKU-1NK1. The isolated helvolic acid inhibited growth of Gram positive pathogenic bacteria (Methicillin-resistant *Staphylococcus aureus* DMST 20654, *Staphylococcus aureus* ATCC 25923, *Staphylococcus saprophyticus* ATCC 15305, *Streptococcus pneumonia* DMST 15319, *Enterococus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633) and Gram negative plant pathogenic bacteria (*Ralstonia solanacearum*, *Xanthomonas campestris* pv. *vesicatoria*) with the MIC ranging from 16-32 µg/ml. It also showed an unprecedented antimicrobial activity against *Mycobacterium tuberculosis* H37Ra strain with the MIC ranging from 12.5-25.0 µg/ml. In addition, the result of time kill assay revealed that helvolic acid was bacteriostatic, which inhibited the growth of pathogenic bacteria at 2 fold of MIC. As a result of irregular-shaped cell division was observed by scanning electron microscopy.

Keywords: antibacterial metabolite, antimycobacterium, bacteriostatic, helvolic acid, *Neosartorya* sp.

1. INTRODUCTION

Natural products of major interest in the treatment of infectious diseases and cancers are potentially leading to new sources of innovative therapeutic agents and pharmaceutics [1]. Many groups of fungi are considered to be potential sources of natural products useful in medicine, agriculture, and industry. Fungi present in soil have been well described as an enormous source of biodiversity found in the environment that can be used in the screening of antibiotics production [2]. *Neosartorya* species are the sexual (teleomorphic) state of *Aspergillus* species in section of *Fumigati*. Further, the sexual state of *Neosartorya* species produces ascospores, while in asexual state (*Aspergillus*

species) it produces only conidospores [3]. In previous studies, it was reported that various species of *Neosartorya* could be isolated from forest soil surrounding Pha Nok Kao Silvicultural Station, Khon Kaen Province, Thailand. Among them, the crude mycelial extract of *Neosartorya spinosa* KKU-1NK1 exhibited the strongest inhibition of growth of Gram-positive pathogenic bacteria [4]. In continuation of our research, the main bioactive compound of this fungus was isolated and its structure was elucidated as the known fungal metabolite, helvolic acid.

Helvolic acid is a nordammarane triterpenoid in fusidane antibiotic group which has been reported to be produced by many fungal species including *Aspergillus fumigatus*, *Xylaria* sp., *Metarhizium anisopliae*, and *Cordyceps taii* [5, 6, 7, 8]. So far, helvolic acid has been reported to be active against Gram-positive bacteria [9], shown antifungal activity against plant pathogens [10] and exhibited cytotoxicity against cancer cell lines [8]. In this paper, several bioactivities, including anticancer, antiviral, antimalarial, and antibacterial activities of helvolic acid isolated from a laboratory culture of *N. spinosa* KKU-1NK1 are described. Additionally, antimycobacterial activity against *Mycobacterium tuberculosis*, the causative agent of tuberculosis is also described.

2. MATERIALS AND METHODS

2.1 Isolation and Identification

Neosartorya spinosa KKU-1NK1 was previously isolated from soils collected from forests surrounding Pha Nok Kao Silvicultural Station, Khon Kaen Province in Thailand [4]. The identification of the ascomycetous fungus was carried out initially on the basis of culture and morphological characteristics. Moreover, the identification was also confirmed by molecular technique. For isolation of genomic DNA, strain KKU-1NK1 was purified by culturing on PDA medium for 4 days. Thereafter, genomic DNA of the strain was extracted from fresh mycelial cultures using the method described by Moller *et al.* [11] with some modifications. The internal transcribed spacer (ITS) region of the rRNA gene was amplified using a pair of universal primers, ITS1 (5'-TCCG TAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR reaction was done in a thermal cycler (GENE Q Thermal Cycler model TC24H/b) for an initial denaturation at 94°C for 5 min; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 2 min; a final extension at 72°C for 5 min was done at the end of amplification. PCR products were sequenced externally by First BASE Laboratories Sdn Bhd, Malaysia. The DNA sequence was submitted to GenBank for homology analysis using the BLASTN program. The partial sequence of the ITS1-ITS4 region of *Neosartorya spinosa* KKU-1NK1 was deposited in the DNA Data Bank of Japan (DDBJ) with accession number AB674770.

2.2 Cultivation of Fungus by Large Scale Fermentation

Ascomycetous fungus, *Neosartorya spinosa* KKU-1NK1 was firstly grown on malt extract medium (2% malt extracts, 2% glucose, 0.1% peptone, 1.5% agar). Next five activated fungal mycelial discs (d = 5 mm) from this culture was inoculated into 200 ml malt extract broth medium (2% malt extracts, 2% glucose, 0.1% peptone) contained in 10 of 1 liter Erlenmeyer flasks, followed by cultivation for 28 days at 30°C under static conditions.

2.3 Extraction and Isolation

After fungal growth for 28 days at 30°C

under static conditions, the fungal biomass was filtered and dried at 50°C for 24 h. The dried fungal biomass (74 g) was mashed with mortar and pestle, and then extracted three times by shaking with ethyl acetate (EtOAc, 300 ml each) for 24 h and filtered. The combined EtOAc underwent complete evaporation to yield the EtOAc extract (25 g). The crude ethyl acetate extract was chromatographed on silica gel (4.5 × 48 cm, 230-400 mesh, Merck) and eluted with a gradient solvent system of 30% EtOAc-hexane to 100% of EtOAc. Each fraction (100 ml) was monitored by TLC, fractions with similar TLC pattern were combined to yield 20 fractions (F_1-F_{20}). Fractions F_{14} and F_{15}, which showed activity against *Bacillus subtilis*, *Staphylococcus aureus,* Methicillin-Resistant *Staphylococcus aureus, Staphylococcus saprophyticus, Streptococcus pneumonia*, and *Enterococcus faecalis* concentration of 1 mg/ml per disk, were combined and precipitated with hexane. The solid was filtered out and recrystallized from methanol to yield colorless needles (0.2050 g). The structure of pure compound was elucidated using nuclear magnetic resonance (NMR) spectroscopy and Infrared spectrometer (IR). IR spectra were obtained using a PerkinElmer Spectrum One FT-IR spectrometer (PerkinElmer, Inc., Shelton, CT, USA).^{1}H, ^{13}C and 2D NMR spectra data were recorded on varian mercury plus 400 spectrometer (Varian, Inc., Palo Alto, CA, USA), Chemical shifts were recorded on *d* (ppm) scale using deuterated chloroform ($CDCl_3$) as solvent.

2.4 Biological Assay

Antimicrobial activity was determined using the disk diffusion test of the Nation Committee for Clinical Laboratory standard [12]. In standard disk assays, the purified helvolic acid isolated from strain KKU-1NK1 and dissolved in dimethyl sulfoxide (DMSO) was absorbed onto individual paper discs (6 mm diameter) at a concentration of 1 mg/ml disc^{-1} which were loaded to 20 µl on each disc . Fourteen human pathogens of clinical isolates, including bacteria *Escherichia coli* ATCC25922, *Salmonella typhi* DMST22842, *Shigella sonnei* ATCC11060, *Vibrio cholera* non 01 DMST2873, *Pseudomonas aeruginosa* ATCC27853, *Acinetobacter baumannii* ATCC19606, *Staphylococcus aureus* ATCC25923, Methicillin-Resistant *Staphylococcus aureus* DMST20654, *Bacillus subtilis* ATCC6633, *Enterococcus faecalis* ATCC29212, *Staphylococcus saprophyticus* ATCC15305, *Streptococcus pneumonia* DMST5851, and pathogenic yeast, *Candida albican* TISTR5779, *Cryptococcus neoformans* DMST15319 were used in this study. The antimicrobial agents, tetracycline (A.N.B Laboratories Co., LTD., Thailand), vancomycin (Oxoid) and nystatin (T.O Pharma co., LTD., Thailand) were used as positive controls. The minimum inhibitory concentration (MIC) of the compound was determined by the standard microdilution assay described by Jorgensen *et al.* [13].

Antimycobacterial activity was assessed against *Mycobacterium tuberculosis* (H37Ra strain) using the Green fluorescent protein microplate assay (GFPMA) [14]. The standard drugs, rifampicin, streptomycin, isoniazid, ofloxacin, and ethambutol were employed as positive controls at MIC value of 0.013, 0.625, 0.047, 0.781, and 0.938 µg/ml, respectively.

For the study of antimalarial activity, the parasite *Plasmodium falciparum* (K1, multidrug resistant strain) was evaluated. Quantitative assessment of malarial activity *in vitro* was done based on the microculture radioisotope technique which described by Desjardins *et al.* [15]. Dihydroartemisinin and mefloquine were used as positive controls with IC_{50} of 2.65 nM and 0.0284 µM, respectively.

Antiviral activity was assessed against

Herpes simplex virus type-1 (HSV-1) using cytotoxicity against Vero cells, derived from the kidney of the African green monkey, the green fluorescent protein (GFP)-based assay was evaluated [16]. Ellipticine with IC_{50} of 0.733 µg/ml, and 0.5% DMSO were used as positive and negative controls, respectively.

Cytotoxicity activity against MCF7-breast cancer and KB-Oral cavity cancer were assessed by the colorimetric method, according to Skehan *et al.* [17]. The positive substances were ellipticine for KB-Oral cavity cancer and tamoxifen for MCF7-breast cancer, which showed IC_{50} values of 1.25 and 7.31 µg/ml, respectively.

Antibacterial activity of helvolic acid against the plant pathogenic bacteria *Ralstonia solanacearum*, *Xanthomonas campestris* pv. *vesicatoria* was also tested by disk diffusion test. In addition, antagonistic activity against the plant pathogenic fungi, *Colletotrichum capsisci*, *Alternaria brassicicola* ,*Rhizoctonia solani*, *Fusarium oxysporum* and *Sclertium rofsii* were tested using the dual test plate technique [18]. All of plant pathogenic microorganisms were obtained from Department of Plant Science and Agricultural Resources, Section Plant Pathology, Faculty of Agriculture, Khon Kaen University, Thailand.

2.5 Time Kills Assay

The assay for bacteria kill-rate was modified from a plating technique previously described by Eliopoulos and Moellering [19]. The pathogenic bacterial inoculums were grown with and without the bioactive compound at various minimum inhibitory concentrations of 1 × MIC and 2 × MIC for a period of 24 h at 37°C. Sampling of bacterial cultures was carried out at regular time intervals (0, 2, 4, 6, 8, 12 and 24 h) for determination of CFU/ml by the plate count technique. The tests were controlled by comparison with the culture broth inoculated with tested microorganisms without bioactive metabolite and culture broth incorporated with the antibiotic at concentration of 30 µg/ml and the tested microorganisms. The killing rate was determined by plotting the total number of viable cells as log 10 CFU/mL versus time.

2.6 Scanning Electron Microscopic (SEM) Analysis

The influence of purified helvolic acid from *N. spinosa* KKU1- NK1 for their possible effect on bacterial cells was observed by scanning electron microscope (SEM). The clinical isolates of *B. subtilis*, Methicillin-Resistant *S. aureus* and *S. pneumonia* were cultured in 50 ml of Mueller-Hinton broth medium (MHB) and incubated at 37°C for 18 h. Helvolic acid was dissolved in dimethyl sulfoxide (DMSO) and the culture without helvolic acid mixed with DMSO were used as control. For SEM preparation [20], 100 ml of bacterial cells suspension at concentration of 1×10^{-6} were treated with 50 µl of helvolic acid (4 times of MIC) and then incubated at 37°C for 2 h. They were transferred to nucleopore membrane (whatman, 13 mm) and fixed overnight in 2.5% glutaraldehyde. The fixed samples were washed with phosphate buffer, dehydrated in serial dilution of alcohol up to 100%, allowed to dry, and mounted on stub and gold-coated. The prepared stub was placed on the LEO-1455VPSEM for observations.

3. RESULTS

3.1 Identification of *Neosartorya* sp. KKU-1NK1

After 14 days of incubation at 30°C, colonies morphology of the fungus on Czapek's solution agar showed often growing rapidly with margin entire or irregular, presenting a dissected appearance,

characterized by abundant cleistothecia in colors near cartridge buff to light buff (Figure 1A) [21]. The reverse plate of Czapek's solution agar showed brown shade (Figure 1B) while this fungal specie secreted the violet exudate in potato dextrose broth after an incubation time of 21 days (Figure 1C). Cleistothecia globose (Figure 1F), mostly 200 to 300 µm; asci eight-spored; ascospore with nearly globose spore bodies (Figure 1D) averaging 3.0 to 5.0 µm in diameter with two widely separated (1.0 to 1.5 µm) equatorial crests ranging from 1.0 to 2.0 mm wide and with convex surfaces bearing spine like projections ranging from <0.5 µm to 2.0 µm, were observed. Conidial heads showed characteristic flask-shaped vesicle with uniseriate sterigmata (Figure 1G-H) [24]. SEM observations showed that the ascospores of KKU-1NK1 have convex surfaces with distinct spines (Figure 1E). The fungal isolate KKU-1NK1 were initially identified using morphological characteristics as *Neosartorya spinosa*. In addition, the internal transcribed spacer (ITS) rDNA was further examined. The homology analysis by BLASTN program showed that strain KKU-1NK1 had maximum similarity 99% with *Neosartorya spinosa* (accession no. AB185271.1). Based on these data, the isolate KKU-1NK1 was identified as *Neosartorya spinosa* KKU-1NK1.

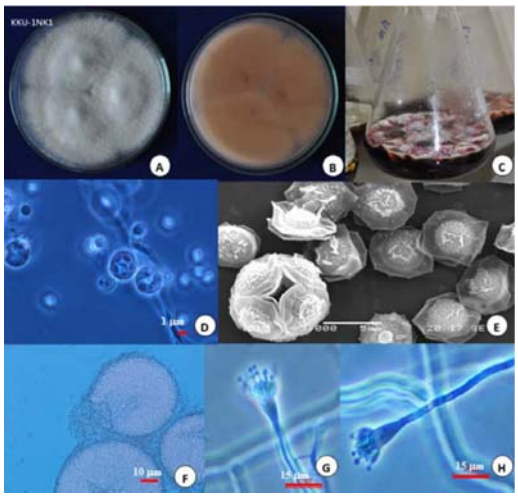

Figure 1. Morphology of *Neosartorya spinosa* KKU-1NK1. (A), Colonies on Czapek's agar (CZA) 14-day-old at 30°C; (B), Reverse plate of colonies on CZA; (C), Fungal growth in Potato dextrose broth 35 days at 30°C; (D), Asci and ascospores; (E), SEM of ascospores; (F), Ascomata (cleistothecium-formed); (G-H), Conidial head and vesicle.

3.2 Structure Elucidation of the Isolated Compound

The pure isolated compound (0.2050 g) was analyzed by spectroscopic methods [IR, 1D and 2D NMR (^{1}H, ^{13}C and COSY, HSQC, HMBC, NOESY) and by comparison with spectral data of the known compound reported in literature [7, 22] as helvolic acid (Figure 2).

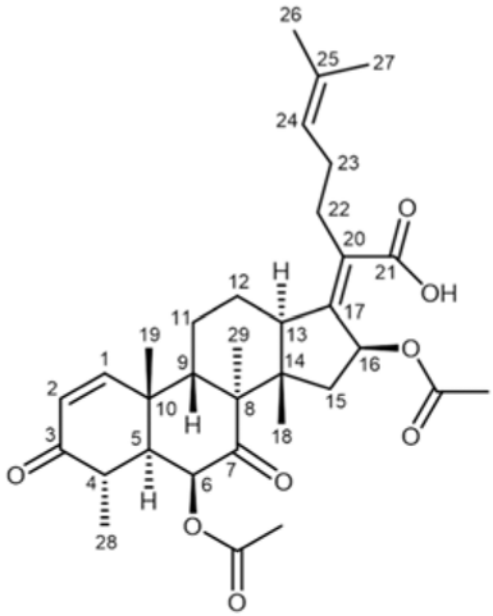

Figure 2. Structure of the isolated helvolic acid.

Helvolic acid; Colourless needles; *melting point* 212°C; IR (KBr) n_{max}: 3230 (OH), 2979 (C-H), 1747 (C=O, OAc), 1714 (C=O, ketone), 1664 (α,β-unsaturated C=O), 1445, 1375, 1250,1210, 1030 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm), 7.31 (1H, d, *J* = 10.0 Hz, H-1), 5.87 (1H, d, *J* = 8.5 Hz, H-16), 5.86 (1H, d, *J* = 10.0 Hz, H-2), 5.22 (1H, brs, H-6), 5.09 (1H, t, *J* = 7.0 Hz, H-24), 2.78 (1H, dq, *J* = 12.5, 7.0 Hz, H-4), 2.60 (1H, dd, *J* = 13.5, 2.5 Hz, H-9), 2.57 (1H, brd, *J* = 11.0, H-13), 2.47 (2H, m, H-22), 2.41 (1H, m, H-12), 2.26 (1H, d, *J* = 12.5 Hz, H-5), 2.23 (1H, dd, *J* = 15.0, 8.5 Hz, H-15), 2.14 (1H, m, H-23), 2.10 (3H, s, 6-OCO*CH*$_3$), 2.09 (1H, m, H-23), 1.97 (1H, m, H-11), 1.93 (3H, s, 16-OCO*CH*$_3$), 1.90 (1H, d, *J* = 15.0 Hz, H-15), 1.81 (1H, dd, *J* = 13.0, 3.5 Hz, H-12), 1.68 (3H, s, H-27), 1.61 (3H, s, H-26), 1.56 (1H, m, H-11), 1.44 (3H, s, H-19), 1.27 (3H, d, *J* = 7.0 Hz, H-28), 1.17 (3H, s, H-29), 0.91 (3H, s, H-18); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm), 157.2 (C-1), 127.8 (C-2), 201.3 (C-3), 40.4 (C-4), 47.2 (C-5), 73.8 (C-6), 168.9 (6-O*CO*CH$_3$), 20.7 (6-OCO*CH*$_3$), 208.7 (C-7), 52.6 (C-8), 41.7 (C-9), 38.1 (C-10), 23.9 (C-11), 25.9 (C-12), 49.4 (C-13), 46.6 (C-14), 40.6 (C-15), 74.2 (C-16), 170.2 (16-O*CO*CH$_3$), 20.4 (16-OCO*CH*$_3$), 147.5 (C-17), 17.9 (C-18), 27.5 (19), 130.4 (C-20), 174.0 (C-21), 28.5(C-22), 28.3 (C-23), 122.8 (C-24), 132.8 (C-25), 17.9 (C-26), 25.7 (C-27), 13.1 (C-28), 18.3 (C-29).

Table 1. Antimicrobial activity of helvolic acid from *N. spinosa* KKU-1NK1 determined by the disk diffusion assay and wells microdilution plate assay.

Microorganisms	Diameter of the inhibition zone (mm), helvolic acid 1.0 mg/ml*	MIC helvolic acid µg/ml
Gram positive bacteria		
Staphylococcus aureus ATCC25923	15.0±0.6	32
Methicillin-Resistant *S. aureus* DMST20654	13.6±1.0	32
Staphylococcus saprophyticus ATCC15305	14.0±1.5	32
Enterococcus faecalis ATCC29212	13.0±0.7	16
Bacillus subtilis ATCC6633	14.6±0.7	16
Streptococcus pneumonia DMST5851	16.3±0.3	16
Gram negative bacteria		
Escherichia coli ATCC25922	(-)	>1000
Acinetobacter baumannii ATCC19606	(-)	>1000
Pseudomonas aeruginosa ATCC27853	(-)	>1000
Vibrio cholera non 01 DMST2873	(-)	>1000
Shigella sonnei ATCC11060	(-)	>1000
Salmonella typhi DMST22842	(-)	>1000
Ralstonia solanacearum	16.0±0.7	32
Xanthomonas campestris pv. *vesicatoria*	16.0±0.7	32
Yeast		
Candida albican TISTR5779	(-)	>1000
Cryptococcus neoformans DMST15319	(-)	>1000

*The values include diameter of paper disk. (-), no growth inhibition

Table 2. Anti-*Mycobacterium tuberculosis* (Anti - TB) H37Ra strain of helvolic acid using the green fluorescent protein microplate assay (GFPMA).

Compounds	Anti-*Mycobacterium tuberculosis* MIC range (μg/ml)
Helvolic acid	12.50-25.0
Rifampicin	0.00156-0.025
Ofloxacin	0.391-1.56
Streptomycin	0.625
Isoniazid	0.0469
Ethambutol	0.469-0.938

3.3 Biological Activities of Helvolic Acid

Helvolic acid was tested for antimicrobial, antimycobacterial, antiviral, antimalarial, cytotoxicity activities against cancer cell lines and anti-plant pathogenic microorganisms. The results showed that helvolic acid exhibited strongly antibacterial activities against the Gram-positive bacteria (*B. subtilis*, *S. aureus,* MRSA*, S. saprophyticus, S. pneumonia*, *E. faecalis*), and plant pathogenic bacteria, *R. solanacearum* and *X. campestris* pv. *vesicatoria*, with MIC values of 16-32 μg/ml as shown in Table 1. Moreover, helvolic acid showed an unprecedented antimycobacterial effect against *M. tuberculosis* (H37Ra strain) with the MIC ranging from12.5-25.0 μg/ml (Table 2). Helvolic acid had no effect to antiviral, antimalarial, cytotoxicity activity against cancer cell line and antifungal agent. However, this study showed that helvolic acid exhibited broad spectrum activity against some pathogenic bacteria: Gram-positive bacteria, acid-fast bacteria, and Gram-negative bacteria.

3.4 Time Kill Assay

With the time kill curves data, the effect of helvolic acid was determined over time on either bacteriostatic or bactericidal activity. The result presented in Figure 3, showed that after 4 h of incubating with the 1 × MICs and 2x MICs, the viable cell count of the tested strains (*B. subtilis*, MRSA*, R. solanacearum*, *X. campestris* pv. *vesicatoria*) reduced till 24 h. There was a significant decrease in bacterial growth at incubating time of 24 h under helvolic acid concentrations of 2 × MICs (32 μg/ml) when compared with untreated bacterial cells. According to the results, helvolic acid showed bacteriostatic action against pathogenic tested strains.

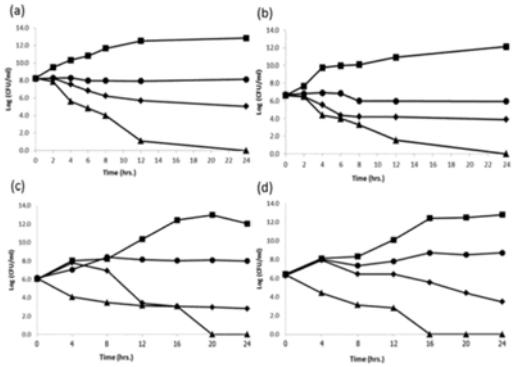

Figure 3. Time kill curves of (a) Methicillin-Resistant *S. aureus* DMST20654, (b) *B. subtilis* ATCC6633, (c) *R. solanacearum*, (d) *X. campestris* pv. *vesicatoria* were treated with helvolic acid at 1xMICs, filled circles and 2xMIC, filled trapezoids. When untreated control, filled squares; Antibiotic positive control, filled triangles.

3.5 SEM Studies of the Effects of Helvolic Acid on Bacterial Cells

The effects of helvolic acid on the cell wall and cell membrane of pathogenic microorganisms were observed by SEM. Gram-positive bacteria including *B. subtilis*, MRSA and *S. pneumonia* presented sensitivity towards helvolic acid which their cell morphology slightly changed after treatment with helvolic acid (Figure 4). The SEM data suggested that helvolic acid had effect on the morphology of *B. subtilis* and

S. pneumonia as a result of the irregular-shaped cell division observed at 4 × MIC of concentration. Furthermore, bacterial cell growth was inhibited as well at 2 × MIC (Figure 4). In contrast, the bacterial cells control treated with DMSO had well defined, intact shapes with smooth surfaces and a lot of cell division (Figure 4).

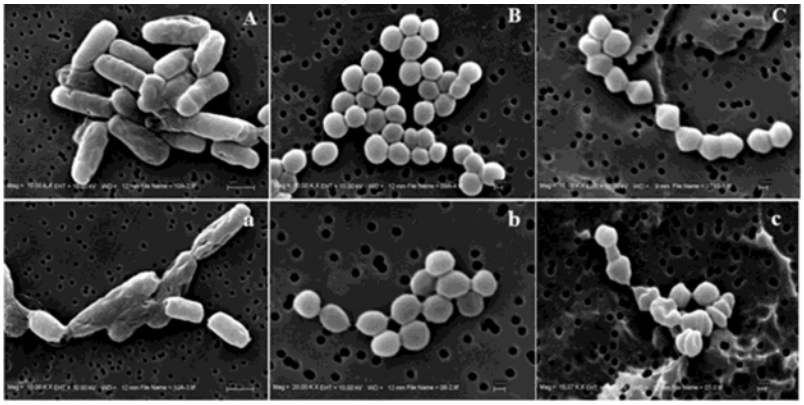

Figure 4. Antibacterial effect of helvolic acid on selected bacterial cells. (A) *B. subtilis*ATCC6633, (B) Methicillin-Resistant *S. aureus* DMST20654, (C) *S. pneumonia* DMST5851. Untreated cells used as control (A, B, C), (a) treated cells of *B. subtilis*ATCC6633, (b) treated cells of Methicillin-Resistant *S. aureus* DMST20654, (c) treated cells of *S. pneumonia* DMST5851as observed by scanning electron microscopy.

4. DISCUSSION

Neosartorya species have a worldwide distribution in soil and have been reported for the production of thermophilic enzymes, antimicrobial proteins and bioactive secondary metabolites [23, 24]. In present study, it has been found that helvolic acid is a major compound in fungal mycelia extracts from *N. spinosa* KKU-1NK1 when cultivated on the submerged culture fermentation. The spectroscopic data of helvolic acid from this fungus was identical to that from *Aspergillus* sp., *Metarhizium anisopliae*, and *Pichia guilliermondii* [6, 7, 22].

Helvolic acid demonstrated stability at various pH levels, was stable to heat and sensitive to *B. subtilis* [25]. It has been previously reported that helvolic acid exhibits antibacterial activities mainly against Gram-positive bacteria, antifungal; *Candida albicans, Tricophyton rubrum, Aspergillus niger* [26] and it has also been shown to inhibit spore germination of *Magnaporthe oryzae* [22]. Likewise, helvolic acid from *N. spinosa* KKU-1NK1 showed strong antimicrobial activities against bacteria tested strains including *B. subtilis*, *S. aureus,* MRSA*, S. saprophyticus, S. pneumonia*, *E. faecalis*, *R. solanacearum*, *X. campestris* pv. *vesicatoria* with MIC range of 16-32 µg/ml. Furthermore, hevolic acid from this fungus exhibited a great inhibitory effect against *M. tuberculosis* (H37Ra strain) at MIC ranging 12.5-25.0 µg/ml. Among the compounds in the fusidane group (steroid-like structural compounds) including cephalosporin P1, fusidic acid and hevolic acid [27], only fusidic acid has been reported for antimycobacterial activity against *Mycobacterium tuberculosis* [28]. Therefore, this is the first report on the activity of hevolic acid isolated from this fungus against *M. tuberculosis* (H37Ra strain).

The action of antimicrobial metabolites can be classified as bactericidal: causing the

death of bacteria, or bacteriostatic: preventing bacterial growth [29]. The time kill curve assay demonstrated that helvolic acid exerted bacteriostatic activity against Gram-positive bacteria of human pathogens (*B. subtilis*, MRSA) and Gram-negative bacteria of plant pathogens (*R. solanacearum*, *X. campestris* pv. *vesicatoria*) which prevented the growth of bacteria during their growth phase. At the initial phase of incubation in time kills assay (t=0 h), the viable bacteria cells remained constant, and gradually increased until 4 h when the compound was added. After 4 h of incubation, the number of viable cells in the treatment of applied helvolic acid at the concentrations of 2 × MIC was significantly decreased until 24 h of incubation when compared with untreated control. As the results of these findings show, helvolic acid might act as bacteriostatic compound. Besides, the SEM microscopy of bacterial cells treated with helvolic acid revealed irregular-shaped cell division, particularly in *B. subtilis* and *S. pneumonia* (Figures 4a-c). This indicated that this compound might not act on bacterial cell surface including cell wall and cell membrane. Martemyanov *et al.* [30] reported that one of compounds belonging to fusidane group, fusidic acid had effected to impairing GTPase of bacterial cells in the step of translation system and ribosome-dependent GTP hydrolysis. Nevertheless, for the fusidane group, only fusidic acid has been studied regarding its mode of action for inhibition of the protein synthesis. Therefore, in order to prove the actual nature of inhibitory activity of bacterial cell growth by hevolic acid, its mode of action needs to be clarified in further study.

5. Conclusion

Helvolic acid isolated from *Neosartorya spinosa* KKU-1NK1 showed broad inhibition activity against Gram-positive bacteria (*B. subtilis*, *S. aureus,* MRSA*, S. saprophyticus, S. pneumonia*, *E. faecalis*), and Gram-negative of plant pathogenic bacteria (*R. solanacearum*, *X. campestris* pv. *vesicatoria*). In addition, the compound was active against acid-fast bacteria (*M. tuberculosis*). It is believed that this is the first report of helvolic acid against *M. tuberculosis* cause by Tuberculosis. Helvolic acid was, however, inactive in antiviral, antimalarial, anticancer and antifungal (plant pathogenic) assay. Time kill assays of this compound against Gram-positive and Gram-negative bacteria revealed that its effects on the growth of bacterial cells were due to bacteriostatic action. The compound had no effect on bacterial cell wall and cell membrane. However, it may inhibit cell division as indicated by SEM observations of the bacterial cell surface.

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