

## Crude extracts of an endophytic fungus attenuate the growth of pathogenic bacteria in aquaculture

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### ABSTRACT

**Background and Objectives:** Endophytic fungi are believed to possess compounds as antibacterial agents. This study was designed to determine *in vivo* antibacterial activity of the crude extracts from *Lasiodiplodia pseudotheobromae* IBRL OS-64 against pathogenic bacteria.

**Materials and Methods:** The qualitative and quantitative screenings were performed using agar plug and disk diffusion antimicrobial tests, respectively. Besides that, the MIC and MBC value of the extracts were determined using broth microdilution assay and morphological changes of the bacterial cells exposed to the extract were observed under Scanning Electron Microscope (SEM).

**Results:** Agar plug diffusion assay revealed that *V. parahaemolyticus* ATCC 17802 and *Exiguobacterium profundum* IBRL MA6 were the most sensitive to the extract with the size of inhibition zones of 11 to  $\leq 20$  mm. The MIC and MBC values of the extract varied depending on the test bacteria. Observation through SEM revealed that the bacterial cells exposed to the extract experienced severe damage such as irregular shape with crumpled and shrunken cells which led to cell death.

**Conclusion:** The data suggest that the crude extracts of *L. pseudotheobromae* IBRL OS-64 exert antibacterial activity against test bacteria and principally affect the cell wall in growing pathogenic bacterial cells.

**Keywords:** Antibacterial agents; Disk diffusion antimicrobial tests; Crude extracts

### INTRODUCTION

Aquaculture is playing a vital role in developing countries, especially for national economic development as well as global food supply (1). However, the emergence of infectious diseases has been triggered by stress factors due to a lack of optimal environmental and nutritional conditions (2) and attributed to the major obstruction to growth in the aquaculture industry that causes severe economic loss (3). Viruses, fungi, bacteria, and parasitic invasions are frequent microorganisms that cause infectious aquacul-

ture diseases. However, diseases of bacterial origin could be considered the most significant problem in the aquaculture industry (4). The genus of *Vibrio* are natural marine inhabitants that play important roles in nutrient cycling as well as the formation of association with zooplankton and intensive aquaculture systems are the best selective medium for bacterial virulence such as traits that enhance infectivity and transmission (5).

*Vibrio* species are Gram-negative bacilli, halophilic bacteria rod-shaped that are closely related to *Enterobacteriaceae*, and they exhibit rapid motility

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with a single polar flagellum and are distributed in surface waters worldwide with estuarine and coastal areas are their natural habitat (6). Luminous vibriosis is a shrimp disease due to bacterial infection caused by *Vibrio* species that leads to major economic losses in the shrimp industry as a result of massive shrimp kills (7). Besides that, vibriosis has resulted in severe economic losses to aquaculture worldwide and affects many farm-raised shrimps, *Artemia*, fishes, and crustaceans. *V. alginolyticus*, *V. anguillarum*, *V. harveyi*, and *V. parahaemolyticus* are the major species causing vibriosis in shrimp aquaculture which leads to high mortality. According to Jones and Oliver (8), consumption of seafood infected by *Vibrio* spp. could cause severe and fulminant systemic infection in humans including nausea, fever, hypotensive septic shock, chills, the formation of lesions on the extremities patients, and septicemia. The vibriosis and its infection of humans are getting worse due to the emergence of multi-drug resistant strains. Multiple antibiotic resistance patterns of vibrio from luminous vibriosis affect cultured tiger shrimp have been reported in Andhra Pradesh, India. They revealed the occurrence of highly virulent strains of vibrio in shrimp culture ponds, which developed resistance to many antibiotics such as chloramphenicol, cephazolin, metronidazole, nitrofurazone, and penicillin G due to the indiscriminate usage of antibiotics. Thus, a new source of antibiotic agents should be explored to overcome this problem.

Endophytic fungi are the most promising source of bioactive compounds that can be used for the treatment of numerous diseases. Endophytic fungi are a diverse group of fungi that are symbiotically associated with plants, and they are ubiquitous and can be found within all known plant species. For instance, Chatterjee (9) reported that an efficient bioactive compound with bactericidal potential exhibited by endophytic fungal strain, *Alternaria alternata* AE1 was previously isolated from leaves of *Azadirachta indica*. Therefore, the study was focused on the endophytic fungus, *L. pseudotheobromae* which was previously isolated from the medicinal herb, *Ocimum sanctum* leaves due to its promising antimicrobial activity. *L. pseudotheobromae* is known as a common fungal pathogen that causes several diseases on a large number of host plants such as stem canker of Chinese hackberry, dieback of Mango (10), and stem-end rot of coconut (11). However, it has been recently known as an endophytic fungus that resides

in various parts of host plants and exhibits several pharmaceutical properties including antibacterial (12) and anticancer activities. In general, the study of antimicrobial activity of *L. pseudotheobromae* is very scarce and thus the present study was designed to investigate the effect of crude extracts of endophytic fungus, *L. pseudotheobromae* IBRL OS-64 against bacteria-related disease in aquaculture.

## MATERIALS AND METHODS

### Endophytic fungus and cultural maintenance.

An endophytic fungus, *L. pseudotheobromae* IBRL OS-64 that was previously isolated from the leaf of *O. sanctum* was provided from the Industrial Biotechnology Research Laboratory (IBRL) Culture Collection, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia (13). The fungal isolate was cultured on potato dextrose agar (PDA) supplemented with powdered host plant (2 g/L) and incubated at 30°C for 7 days. All the cultures were stored at 4°C until further use. To maintain culture purity and viability, subculturing was done once a month.

### Test bacteria and inoculum preparation.

Five pathogenic bacteria in aquaculture including *V. parahaemolyticus* ATCC 17802, *V. alginolyticus* CCB-PB317, *V. owensii* CCB-PG2, *V. azureus* CCB-ST2H16, and *E. profundum* IBRL MA6 were used as test microorganisms in the study and obtained from the IBRL Culture Collection. The bacterial cultures were grown on marine agar, cultivated at 28°C for 24-h and the cultures were stored at 4°C until further use. The bacterial inoculum was prepared by transferring 3-4 single colonies into 5.0 mL of sterile physiological saline and mixing well using a vortex. The inoculum was compared with 0.5 McFarland standards to obtain cell suspensions of approximately  $1 \times 10^8$  CFU/mL (13).

### Fungal cultivation and extraction.

Yeast extract sucrose (YES) broth was prepared by adding yeast extract, 20 g/L; sucrose, 40 g/L; magnesium sulfate, 0.5 g/L into a 2L beaker containing 1000 mL of host plant water extract. Before that, host plant water extract was prepared by boiling 2.0 g fine powdered *O. sanctum* leaves material (host plant) in 1000 mL distilled water and filtered with Muslin cloth, followed by Whatman No. 1 filter paper. Two actively growing

pure mycelial agar plugs were then inoculated into a 250 mL Erlenmeyer flask containing 100 mL of YES broth and incubated at 30°C for 16 days under dark and static conditions. After the cultivation period, the cultures were filtrated using Whatman No. 1 filter paper and the separated fungal biomass and fermentative broth were extracted thrice with an equal volume of ethyl acetate extract and methanol (1:1; v/v). Both extracts were concentrated under reduced pressure to dryness by a rotary evaporator and dried in a fume hood to obtain a dried crude paste (13).

**Agar plug diffusion assay.** Primary screening was performed through the agar plug diffusion assay according to the procedures described by Taufiq and Darah (13). The agar plugs of the endophytic fungus were cut into 10 mm diameter and 4 mm thickness using a cork borer and then placed on the marine agar (MA) that was previously seeded with test bacteria. Prior to that, the endophytic fungus was grown on potato dextrose agar (PDA) supplemented with the host plant and incubated at 30°C for 20 days. The plates were initially kept in the chiller (4°C) overnight to allow diffusion of bioactive compounds into the agar and then incubated at 28°C for 24 hours. PDA supplemented with host plant and impregnated with 30 µg per plug chloramphenicol were set as negative and positive controls, respectively. The diameter of the inhibition zone formed surrounding agar plugs was measured and recorded after the incubation period. The experiment was performed in triplicates.

**Disk diffusion assay.** Disk diffusion assay was performed according to methods described by the Clinical and Laboratory Standards Institute (CLSI) standard M2-A9 (13). The antibiotic discs impregnated with fungal extract (1 mg per disc) were placed on the marine agar (MA) seeded with test bacteria and the plates were incubated at 28°C for 24 hours. One percent of DMSO was included to detect the solvent effects (negative control) whereas chloramphenicol (30 µg per disc) was used as a positive control. After the incubation period, the diameter of inhibition zones surrounding the discs was measured and recorded. The experiment was carried out in triplicate.

**Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).** The MIC and MBC values of the extract were determined by broth microdilution

assay in a sterile 96-wells, U-shaped microtiter plate using procedures described by Clinical and Laboratory Standards Institute (CLSI) standard M26-A. Both assays were performed according to procedures described by Taufiq and Darah (13). The MIC value was determined and recorded as the lowest concentration of the ethyl acetate crude extract that was capable to inhibit the visible growth of test microorganisms after the incubation period. Meanwhile, the lowest concentration that was able to kill up to 99.9% of bacterial cells was reported as the MBC value.

**Time-kill assay.** The efficacy of extract against test bacteria was determined through a time-kill assay. A total of 50 mL of Erlenmeyer flask containing 9.9 mL of marine broth (8.9 mL) and extract (1.0 mL) were prepared with a final concentration of  $\frac{1}{2} \times \text{MIC}$ , MIC, and  $2 \times \text{MIC}$ . A volume of 0.1 mL bacterial inoculums with a concentration of  $1 \times 10^8$  CFU/mL was added, and the flasks were incubated at 28°C in a rotary shaker (150 rpm) for 48 hours. A volume of 1.0 mL of the mixture was withdrawn every 4 h intervals and the colony-forming per unit (CFU) on the spread plate was counted and the result was recorded.

**Brine shrimp lethality assay (BSLA).** Brine shrimp eggs were provided by the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang. An amount of 0.1g brine shrimp eggs (*Artemia salina* cysts) were allowed to hatch in artificial seawater for 48 h at 25°C under constant aeration and illumination (14). Prior to that, stock solutions (1000 mg/mL) were prepared and diluted serially to obtain six final concentrations ranging from 625 to 10000 µg/mL. Ten 48-h old nauplii were transferred into each universal bottle containing serially diluted test solutions with the aid of a Pasteur pipette. The test was carried out in triplicate. For negative control, ten nauplii were transferred into a universal bottle containing artificial seawater without the fungal extract. The number of dead larvae and the percentage of mortality was counted and calculated after the 24 h incubation, respectively. The  $\text{LC}_{50}$  value and the standard error mean were calculated using a non-linear regression curve contained in the Graph pad prism statistical software.

**Scanning electron microscopy (SEM).** The destructive changes of bacterial cells were observed under the SEM. A volume of 9.9 mL of a mixture

(containing 8.9 mL marine broth and 1.0 mL extract with a concentration of MIC) was prepared in 50 mL Erlenmeyer flasks and 0.1 mL of bacterial inoculums ( $1 \times 10^8$  CFU/mL) were then added. The flasks were incubated at 28°C in an orbital shaker (150 rpm) for 48 hours. The SEM samples were prepared according to procedures described by Mares (15) and the prepared samples were then viewed under an SEM (Leica Cambridge, S-360, United Kingdom).

**Statistical analysis.** The triplicate data obtained were subjected to an analysis of a completely randomized design using SPSS software version 12.0 (Chicago, IL, USA), and  $p \leq 0.05$  was considered to indicate statistical significance.

## RESULTS

**Primary screening for antibacterial activity.** The endophytic fungus, *L. pseudotheobromae* IBRL OS-64, was qualitatively screened on agar plug diffusion assay to excess the existing antibacterial activity against pathogenic bacteria in aquaculture (Table 1). The results revealed that the endophytic fungus exhibited antibacterial activity against all test bacteria. *E. profundum* IBRL MA6 was the most susceptible tested bacteria towards the endophyte with the size of inhibition zones of 15 mm. Besides that, the other two bacteria *V. owensii* CCB-PG2 and *V. azureus* CCB-ST2H16 were least susceptible to the fungal endophyte isolate since the inhibition diameter of the inhibition zone was less than 10 mm.

**Quantitative antibacterial activity on disk diffusion assay.** The antibacterial activity of *L. pseudotheobromae* IBRL OS-64 extracts was quantitatively determined using disk diffusion assay and the results are shown in Table 2. Generally, the ethyl acetate extract that was previously extracted from the fermenta-

tive broth (extracellular) showed better antibacterial activity against pathogenic bacteria in aquaculture compared to methanolic extract that was prepared from the fungal biomass (intracellular). The results exhibited that ethyl acetate extract exerted antibacterial activity against all five test bacteria with the size of the inhibition zone ranging between  $9.2 \pm 0.4$  to  $15.4 \pm 0.6$  mm. On the other hand, the methanolic extract exhibited antibacterial activity towards only the two test bacteria (*V. parahaemolyticus* ATCC 17802 and *E. profundum* IBRL MA6) with the diameter of inhibition zone ranging between  $8.2 \pm 0.8$  to  $12.2 \pm 0.6$  mm. The present finding revealed that the antibacterial substances were not only associated with fungal biomass but released extracellularly into the fermentative broth.

**Determination of MIC and MBC.** Table 3 shows MIC and MBC values of ethyl acetate and methanolic extracts of *L. pseudotheobromae* IBRL OS-64 against test bacteria. The results exhibited that the MIC and MBC values of the ethyl acetate extract ranged between 125-1000  $\mu\text{g/mL}$  and 125-8000  $\mu\text{g/mL}$ , respectively. As for methanolic extract, the MIC and MBC values ranged between 250-500  $\mu\text{g/mL}$  and 250-2000  $\mu\text{g/mL}$ , respectively. Ethyl acetate extract exhibited antibacterial activity against all five test bacteria with *E. profundum* IBRL MA6 was the most susceptible to the extract with the same value of MIC and MBC which was 125  $\mu\text{g/mL}$ . Meanwhile, *V. azureus* CCB-ST2H16 was the least susceptible to the extract with the MIC and MBC values of 1000  $\mu\text{g/mL}$  and 8000  $\mu\text{g/mL}$ , respectively. However, the methanolic extract was able to inhibit only two test bacteria. The MIC and MBC values of the methanolic extract towards *V. parahaemolyticus* ATCC 17802 were 500  $\mu\text{g/mL}$  and 2000  $\mu\text{g/mL}$ , respectively. On the other hand, similar MIC and MBC of the extract were observed towards *E. profundum* IBRL MA6 with a value of 250  $\mu\text{g/mL}$ . Again, fermentative broth (ethyl acetate extract)

**Table 1.** Qualitative screening of antibacterial activity of *L. pseudotheobromae* IBRL OS-64 against pathogenic bacteria in aquaculture on agar plug diffusion assay.

Test bacteria	Presence of inhibition zone	Diameter (mm)
<i>Vibrio parahaemolyticus</i> ATCC 17802	++	$12.4 \pm 0.6$
<i>Vibrio alginolyticus</i> CCB-PB317	++	$11.3 \pm 0.2$
<i>Vibrio owensii</i> CCB-PG2	+	$8.4 \pm 0.2$
<i>Vibrio azureus</i> CCB-ST2H16	+	$7.2 \pm 0.4$
<i>Exiguobacterium profundum</i> IBRL MA6	+++	$15.0 \pm 0.4$

**Table 2.** Antibacterial activity of crude extracellular and intracellular extracts of *L. pseudotheobromae* IBRL OS-64 against pathogenic bacteria on disk diffusion assay.

Test bacteria	Diameter of inhibition zone (mm)				Positive control*
	Extracellular extract		Intracellular extract		
	Ethyl acetate	Methanol	Ethyl acetate	Methanol	
<i>Vibrio parahaemolyticus</i>	12.4 ± 0.4	-	-	8.2 ± 0.8	22.6 ± 0.2
<i>Vibrio alginolyticus</i>	10.2 ± 0.2	-	-	-	24.4 ± 0.4
<i>Vibrio owensii</i>	11.4 ± 0.6	-	-	-	21.4 ± 0.4
<i>Vibrio azureus</i>	9.2 ± 0.4	-	-	-	23.2 ± 0.6
<i>Exiguobacterium profundum</i>	15.4 ± 0.6	-	-	12.2 ± 0.6	26.2 ± 0.8

Notes: extract concentration = 1000 µg/ml, positive control = chloramphenicol (30 µg/ml)

**Table 3.** Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the ethyl acetate and methanolic extracts against test bacteria on broth microdilution assay.

Test bacteria	Ethyl acetate extract			Methanolic extract		
	MIC	MBC	Ratio	MIC	MBC	Ratio
	(µg/mL)	(µg/mL)		(µg/mL)	(µg/mL)	
<i>Vibrio parahaemolyticus</i>	250	250	1	500	2000	4
<i>Vibrio alginolyticus</i>	500	1000	2	-	-	-
<i>Vibrio owensii</i>	1000	4000	4	-	-	-
<i>Vibrio azureus</i>	1000	8000	8	-	-	-
<i>Exiguobacterium profundum</i>	125	125	1	250	250	1

showed superior antibacterial activity compared to fungal biomass (methanolic extract). The ethyl acetate extract could be a bactericidal agent against *V. parahaemolyticus* ATCC 17802, *V. alginolyticus* CCB-PB317, *V. owensii* CCB-PG2 since the MBC/MIC ratio was less or equal to four.

**In vitro toxicity.** The best extract from the antibacterial activity against test bacteria has proceeded for the toxicity test. The brine shrimp assay results shown in Fig. 1. revealed that the ethyl acetate extract was virtually non-toxic on shrimps as they exhibited very low toxicity giving LC<sub>50</sub> values greater than 1000 µg/ml after 24 hours. The results obtained also showed that the activity of ethyl acetate extract of endophytic fungus was concentration dependent.

**Time-kill assay.** Time-kill study was performed over a period of 48 h with the *V. parahaemolyticus* ATCC 17802 and *E. profundum* IBRL MA6 being exposed to ½ × MIC (125 µg/mL; 62.5 µg/mL), MIC (250 µg/mL; 125 µg/mL) and 2 × MIC (500 µg/mL; 250 µg/mL) values of ethyl acetate extract of endophytic fungus, *L.*

*pseudotheobromae* IBRL OS-64. Fig. 2. shows a graph of the killing activity of bacteria cells that was plotted between logarithmic numbers of CFU/mL versus time (h). For *V. parahaemolyticus* ATCC 17802, the time-kill assay showed a 93.7% reduction of bacterial cells at ½ × MIC after 32 h of the incubation period. However, the bacterial cells resumed their growth thereafter to obtain 4.12 × 10<sup>7</sup> CFU/mL of viable cell counts. At a higher extract concentration (2 × MIC), the result exhibited that 99.9% reduction was achieved after 28 h of the incubation period, and the reduction was continued until 48 h. For *E. profundum* IBRL MA6, the time-kill study showed 97.8% and 99.9% reduction of bacterial cells at ½ × MIC and 2 × MIC values of extract concentrations, respectively after 24 h of incubation. The results revealed that the effects of the ethyl acetate extract were bacteriostatic and bactericidal against *V. parahaemolyticus* ATCC 17802 at lower (½ × MIC value) and higher (2 × MIC value) extract concentrations, respectively. In contrast, the effect of the fungal extract was bactericidal against Gram-positive bacteria, *E. profundum* IBRL MA6 at sub-MIC (½ × MIC) and higher (2 × MIC) concentrations.

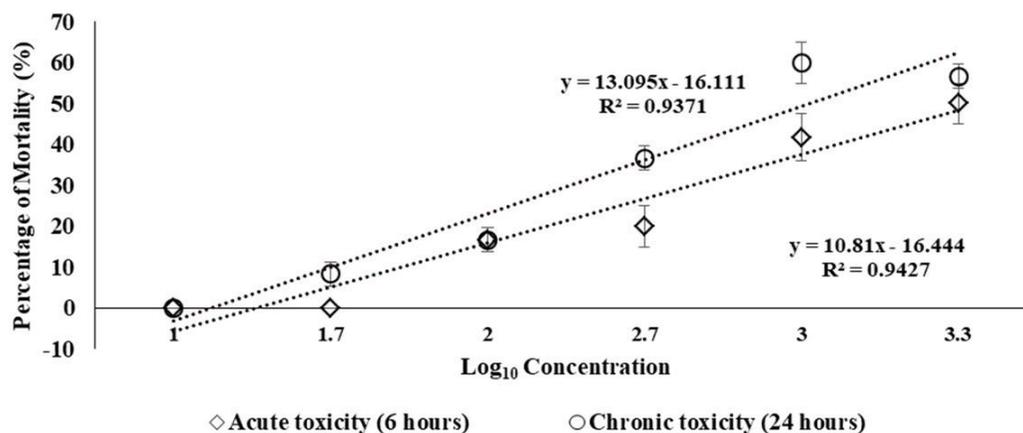


Fig. 1. Toxicity of ethyl acetate extract

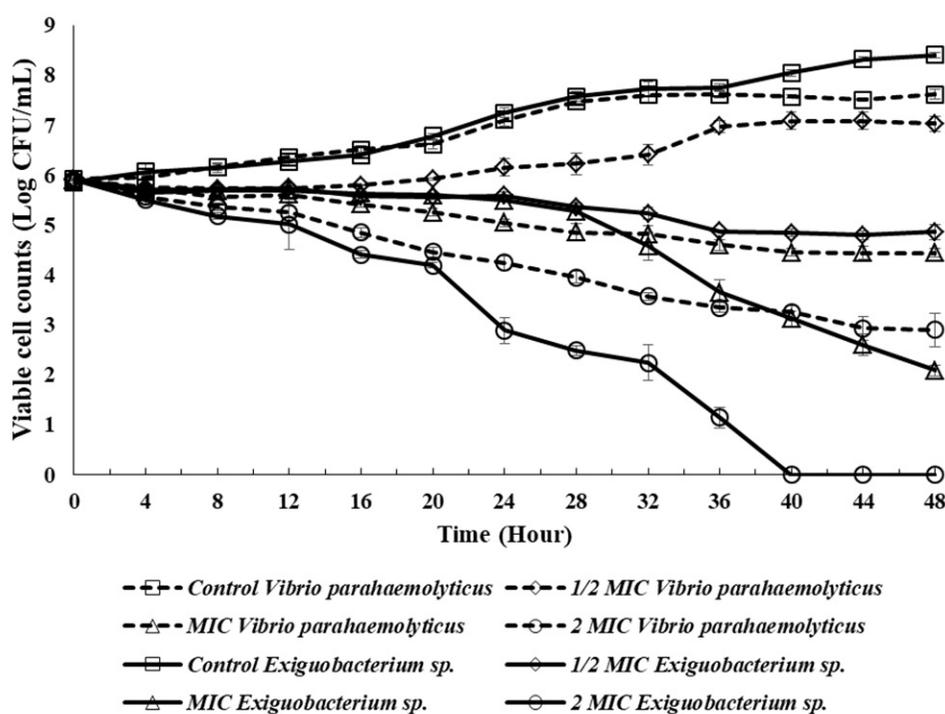


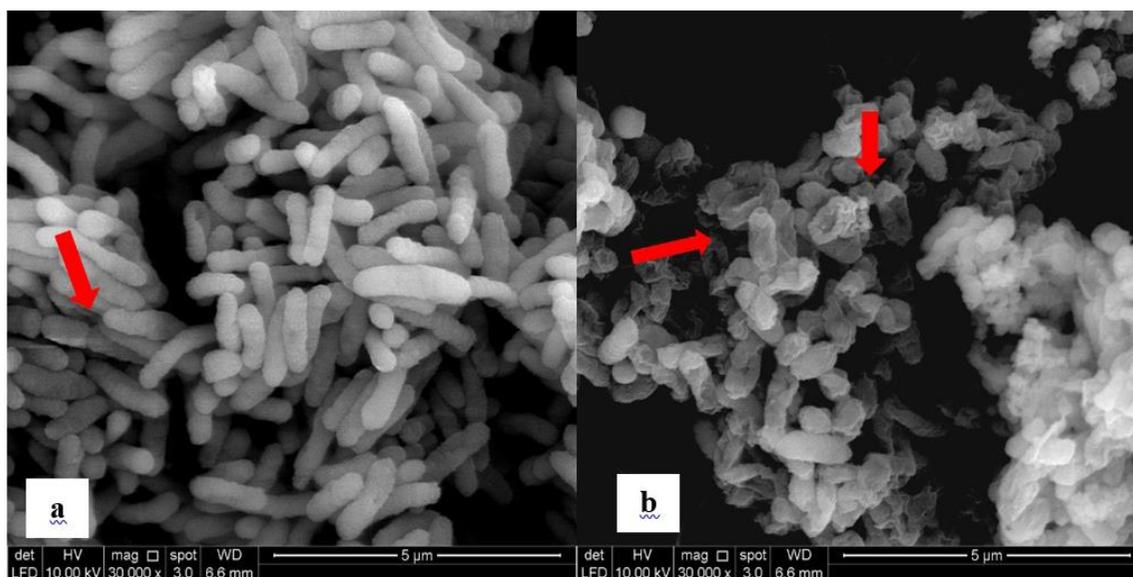
Fig. 2. Time-kill curve of ethyl acetate extract of endophytic fungus, *L. pseudotheobromae* IBRL OS-64 against *V. parahaemolyticus* ATCC 17802 and *E. profundum* IBRL MA6

**Structural degeneration of bacterial cells exposed to the extract.** The effects of the ethyl acetate extract on the exterior part of bacterial cells were investigated through SEM photomicrographs and this has brought conformity to the previous result on the screening part. Generally, the microscopic analysis revealed the severity of morphological deterioration undergone by the extract-treated cells beyond repair. Fig. 3. shows photomicrographs of untreated (control) and ethyl ac-

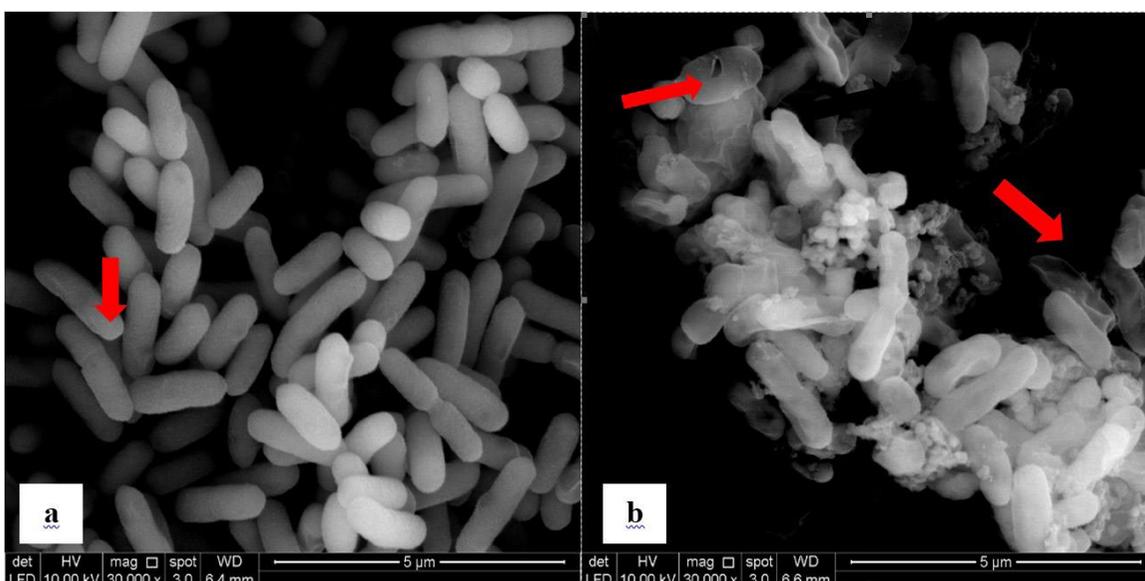
etate extract-treated Gram-negative bacteria, *V. parahaemolyticus* cells. Fig. 3a is the control (untreated cells) that exhibits undamaged cells with the presence of regular Gram-positive bacterial cells and a rough surface with an intact rod shape. Fig. 3b represents the 36 h of exposure to the extract where the cells experienced some morphological changes. In the meantime, irregularities in the cells were observed and almost all individual cells underwent a shrunken phenomenon.

Besides that, there were cavities formed on the outer layer of cells and this phenomenon might be due to the leakage of cells' cytoplasm. Fig. 4 demonstrates the SEM micrograph of untreated and treated *E. profundum* IBRL MA6 cells. Fig. 4a illustrates the control cell without any treatment and it shows a typical normal cell of *E. profundum* with rigid, rod-shaped,

and smooth morphology. Some of the cells were undergone binary fission (indicated by the red arrow), which is a typical replication process of bacteria. However, progressive changes occurred throughout exposure to fungal extract. After 36 h of exposure, the surface of bacterial cells was shrunken and crumpled (Fig. 4b). This phenomenon leads to the formation of



**Fig. 3.** SEM photomicrographs showing the effect of ethyl acetate crude extract of *L. pseudotheobromae* IBRL OS-64 at a concentration of 500 µg/mL on Gram-negative bacteria, *V. parahaemolyticus* ATCC 17802 cells. (a) untreated cell [control] (b) extract-treated cell. Scale bars: 5 µm.



**Fig. 4.** SEM photomicrographs showing the effect of ethyl acetate crude extract *L. pseudotheobromae* IBRL OS-64 at a concentration of 500 µg/mL on Gram-positive bacteria, *E. profundum* IBRL MA6 cells. (a) untreated cell [control] (b) extract-treated cell. Scale bars: 5 µm.

pores that resulted in leakage of the cytoplasm cell. As a result, the bacterial cells loss their cellular materials and some of them lost their rod-shaped and thus, showed indistinctive morphology compared to the control cells.

## DISCUSSION

The emergence of multi-drug resistant strains has urged searching for new bioactive substances with a wide broad of antimicrobial activity and pharmaceutical properties. Thus, an endophytic fungus has been utilized in the present study to screen antibacterial activity against pathogenic bacteria in aquaculture. Endophytic fungi are microorganisms that live within plants without causing any disease symptoms to their hosts (16). Hamzah (17) reported that endophytic fungi help their host plants to adapt to abiotic and biotic stress factors and tolerance to biotic stress by some plants has been associated with the production of fungal natural products. During the long evolutionary process, different relationships have been between fungal endophytes and their hosts through fungus-host interaction recognized as antagonism, continuum mutualism, and neutralism, and this interaction benefit endophytes due to ecological habitat, genetic background, and nutrient level of host plants and in turn, benefits host plants by induced their growth, increased resistance to herbivore or disease and accumulated bioactive substances. Thus, mutual interaction between endophytes and their hosts can affect the formulation of some bioactive compounds that benefit humans (18). Endophytic fungi have been shown to produce a promising source of new natural products and they have been reported to produce several bioactive compounds that can inhibit pathogens. Due to the capability of endophytic fungi to synthesize bioactive compounds with pharmaceutical properties, the recent study was designed to evaluate the antibacterial activity of *L. pseudotheobromae* IBRL OS-64 against pathogenic bacteria in aquaculture.

Primary screening of antibacterial activity of endophytic fungus revealed that the fungal isolate, *L. pseudotheobromae* IBRL OS-64 exerted the highest antibacterial activity against *E. profundum* IBRL MA6 compared to the *Vibrio* sp. This phenomenon might be due to the differences in the cell structure of both bacteria since *E. profundum* IBRL MA6 is a Gram-positive and *Vibrio* sp. is Gram-negative

bacteria. Generally, Gram-negative bacteria consist of complex structures such as an outer membrane layer containing lipopolysaccharide, a thin peptidoglycan layer, and periplasm compared to Gram-positive bacteria. The outer membrane layer is a structure that differentiates between bacteria comprised of lipopolysaccharides, proteins, and phospholipids separating the external environment from the periplasm (19). The outer membrane of Gram-negative bacteria has restricted flow and serves as a selective barrier that prevents antibiotic compounds but allows the transportation of valuable nutrients to the cell. Besides that, the outer membrane is also anchored by membrane proteins known as porins which act as a selective channel that allows the transportation of specific size hydrophilic substances into the periplasm (20). The high resistance of Gram-negative bacteria towards antibiotic agents might be due to the presence of a complex cell envelope that surrounded the peptidoglycan layer even though it is very thin. Furthermore, the special cell envelopes act as a permeability barrier that restricts the flow of outsider molecules into the cell such as hydrophobic, hydrophilic, and charged molecules (21). Besides that, the concentration of extract also plays a vital role in inhibiting bacterial growth. The low concentration of antibiotic agents leads to insufficient execution of the inhibitory action and thus, the bacteria become resistant to the antibiotics (22).

Disk diffusion assay was carried out to determine the antibacterial activity of fermentative broth and fungal biomass extracts. The present study revealed that the fermentative broth extract (extracellular) exerted more antibacterial activity against test bacteria compared to fungal biomass extract (intracellular). The result was in agreement with previous study who claimed that the endophytic fungi usually secreted their secondary metabolites extracellularly into the fermentative broth and seldom kept them intracellularly. Moreover, secondary metabolite compounds secreted by endophytes are meant to protect them from pathogens or predators and therefore, they are more suitable to be secreted extracellularly. The result of the disk diffusion assay revealed that the highest antibacterial observed against *E. profundum* IBRL MA6 (on primary screening) might be due to the synergic effect of different bioactive compounds from extracellular and intracellular since both fermentative broth (ethyl acetate extract) and fungal biomass (methanol extract) exhibited good antibac-

terial activity. Bag and Chattopadhyay (23) evaluated synergistic antimicrobial efficacy by combining essential oils of spices and herbs. They revealed that the combination of cumin and coriander seeds oil was significantly increased antimicrobial activity and this is due to synergistic interactions of both compounds.

Broth microdilution assay was employed to determine the MIC and MBC values of the fungal extract. The results revealed that both fungal extracts (either ethyl acetate or methanolic extracts) exhibited a higher antibacterial effect on Gram-positive bacteria as compared to Gram-negative bacteria. Current results are in agreement with the previous study by Marasini (24) who claimed that MIC values of plant extract against Gram-positive bacteria are significantly low than Gram-negative bacteria. They postulated that this phenomenon may be due to the different nature of cell walls for both types of bacteria and the existence of the efflux system in Gram-negative that facilitates them in enhancing their resistance to antibiotic agents. Besides that, both fungal extracts showed a bactericidal effect against all test bacteria except *V. azureus* CCB-ST2H16 since the MBC/MIC ratio is less or equal to 4. Antimicrobial compounds are considered bactericidal agents when the MBC/MIC ratio is less or equal to 4, whilst bacteriostatic agents if the MBC/MIC ratio is greater than 4. Current findings also revealed a wide range of MIC values, and this indicates different susceptibility levels of test bacteria towards fungal extracts. The variation in susceptibility of test microorganisms might be due to different compositions of the fungal extracts whereby some extracts tend to exhibit selective activity against certain microorganisms and this phenomenon leads to the low MIC value. On the other hand, some of the microorganisms are protected by special cell components including an extra cell wall layer and degrading enzymes that can be less accessible to the fungal extract and this leads to a higher MIC value (25). Besides that, the MIC and MBC values of the extract were similar for *V. parahaemolyticus* ATCC 17802 and *E. profundum* IBRL MA6 indicating the high sensitivity of the test bacteria towards the antibacterial substances in the extract. Olajuyigbe and Afolayan (26) reported equal MIC and MBC values indicating the bactericidal effect of the extract with great therapeutic potential and broad-range spectrum.

A time-kill assay was employed to evaluate bac-

tericidal and bacteriostatic bioactive compounds whereby bactericidal activity is indicated by 99.9% of cell death which is equivalent to a  $3\text{-log}_{10}$  reduction of bacterial cells (27). The present study revealed that the sub-MIC level of extract leads to the regrowth of bacterial cells. Wistrand-Yuen (28) reported that a low level of antibiotic can enrich pre-existing resistant mutants in a bacterial population showing that certain antibiotics could contribute to resistance evolution at a concentration level hundred-folds below the MIC. They also stated that the bacterial cells can still grow at a low concentration of antibiotics since they are capable to generate a different trajectory of evolution with a progressive increase in resistance via accumulation of resistance mutations with individually smaller effects. A biphasic killing curve with a decline in the killing rate of bacterial cells was observed in the present study and this phenomenon might be due to the emergence of persister cells which are less susceptible to antimicrobial drugs (29). In addition, Lewis (30) reported that persistence occurs in a subpopulation of bacterial (approximately < 1%) that are not killed by antibiotics, and thus, the heterogeneous response is repeated when they are exposed to the same antibiotic. According to Pankey and Sabath (31), it is advantageous to have antimicrobial drugs with bacteriostatic action rather than the bactericidal effect in some cases during treatment.

The study of new bioactive substances' toxicity is essential for the antibiotic development process whereby toxicity of the substances can be evaluated by *in vitro* studies towards cell lines, *in vivo* exposure on animals, and accidental exposure studies to a substance (32). The current study revealed that the fungal extract exerted a low toxicity effect on the nauplii may be due to its symbiotic relationship with its host plant. According to Strobel and Daisy (33), endophytes reside in the cell tissue of their host by producing secondary metabolites with low toxicity to not harm or cause death to their host. Furthermore, endophytic fungi could be quiescent or latent pathogens in their host, but their pathogenic behavior will be activated if the host environment is changed (34). Besides, Owen and Hundley (35) postulated that bioactive compounds secreted by endophytes are relatively low toxicity towards higher organisms. According to Meyer's toxicity index, the crude extracts with  $LC_{50} < 1000 \mu\text{g/mL}$  were considered toxic whereas the crude extracts with  $LC_{50} > 1000 \mu\text{g/mL}$  were assumed non-toxic. The results also showed

that the prolonged exposure of brine shrimp to the fungal extract resulted in decreasing LC<sub>50</sub> value. The result was in parallel with the previous study that studied the toxicity of Malaysian red seaweed, *Acanthophora spicifera*. The decreasing LC<sub>50</sub> value with the increase in the exposure time may be due to the tolerance of the tested nauplii towards toxic substances. However, inaccurate results may be obtained if the exposure time was increased beyond 48 hours. This phenomenon occurred due to a food shortage since nauplii can survive without food for up to only 48 hours as they depend on their yolks-sack as their food source (36). The present study also revealed that the fungal extract was not toxic against brine shrimp, and this is very impressive, and the extract may be used in the aquaculture field as an antibacterial agent. Moreover, the extract may be used in the food industry to combat food pathogens. Similarly, previous studies suggested that hexane extract of *Pilea microphylla* can be used to cure pathogenic bacterial infections such as *Bacillus cereus* that cause food poisoning symptoms in humans due to its low toxicity. However, the crude fungal extract should be further characterized and purified to determine the toxicity activity of a single pure compound.

Cell membrane plays a vital role in Gram-positive and Gram-negative bacteria since it is responsible for various essential functions including osmoregulation, transportation, respiration processes, and biosynthesis of peptidoglycans and lipids. Hence, the cell membrane integrity is important to facilitate these functions and any disruption could cause metabolic dysfunction, which leads to cell death beyond repair (37). In the present study, the ultrastructural changes in *V. parahaemolyticus* ATCC 17802 and *E. profundum* IBRL MA6 after being treated with the fungal extract were analyzed by scanning electron microscope and the results revealed the efficiency of the extract since the cell membrane underwent severe damage indicated by cell shrinkage, crumpled, the formation of cavities, and broken cells. The result was in agreement with a previous study that reported that the microbial extracts would affect the cell wall synthesis and the cell membrane permeability. According to Jeyanthi and Velusamy (38), cell damage would promote the penetration of the active compound into the inner cell cytoplasm, which leads to the disruption of its contents. Similar observations reported by previous studies revealed the severe damage observed in the lysed cells of Gram-positive

and Gram-negative bacteria with broken cytoplasmic membrane and leakage of cytoplasmic contents. Another previous study also reported fungal extract exhibited antibacterial activity against *P. mirabilis* by disrupting its normal cell envelope (cell membrane) which then causes cell lysis and ultimate death. Thus, the present study speculated that the active compound(s) from the fungal extract penetrate the bacterial cell membrane and disturbed its integrity resulting in cell shrinkage, the formation of cavities, broken and leakage of the cell contents which leads to cell death beyond repair. However, further study should be done to investigate the mode of action of the fungal extract towards the test bacteria.

## CONCLUSION

With an increasing number of bacteria developing resistance to several commercial antibiotics, extracts from *L. pseudotheobromae* IBRL OS-64, an endophytic fungus holds great promise for novel bioactive compounds with antibacterial properties. The fungal extracts showed significant antibacterial activity against several pathogenic bacteria in aquaculture including *V. parahaemolyticus* and *E. profundum*. The extract was also able to attenuate the bacterial growth and cause severe damage to bacterial cells.

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## REFERENCES

1. Pradeepkiran JA. Aquaculture role in global food security with nutritional value: a review. *Transl Anim Sci* 2019; 3:903-910.
2. Marudhupandi T, Kumar TTA, Prakash S, Balamurugan J, Dhayanithi NB. *Vibrio parahaemolyticus* a causative bacterium for tail rot disease in ornamental fish, *Amphiprion sebae*. *Aquac Rep* 2017; 8: 39-44.
3. Plant KP, Lapatra SE. Advances in fish vaccine delivery. *Dev Comp Immunol* 2011; 35: 1256-1262.
4. Soto-Rodriguez SA, Gomez-Gil B, Lozano-Olvera R,

- Betancourt-Lozano M, Morales-Covarrubias MS. Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in Northwestern Mexico. *Appl Environ Microbiol* 2015; 81: 1689-1699.
5. Goulden EF, Hall MR, Bourne DG, Pereg LL, Hoj L. Pathogenicity and infection cycle of *Vibrio owensii* in larviculture of the ornate spiny lobster (*Panulirus ornatus*). *Appl Environ Microbiol* 2012; 78: 2841-2849.
  6. Scharer K, Savioz S, Cernela N, Saegesser G, Stephan R. Occurrence of *Vibrio* spp. in fish and shellfish Collected from the Swiss market. *J Food Prot* 2011; 74: 1345-1347.
  7. Adam D, Boopathy R. Use of formic acid to control vibriosis in shrimp aquaculture. *Biologia* 2013; 68: 1017-1021.
  8. Jones MK, Oliver JD. *Vibrio vulnificus*: disease and pathogenesis. *Infect Immun* 2009; 77: 1723-1733.
  9. Chatterjee S, Ghosh R, Mandal NC. Production of bioactive compounds with bactericidal and antioxidant potential by endophytic fungus *Alternaria alternata* AE1 isolated from *Azadirachta indica* A. Juss. *PLoS One* 2019; 14(4):e0214744.
  10. Kwon J, Choi O, Kang B, Lee Y, Park J, Kang D, et al. Identification of *Lasiodiplodia pseudotheobromae* causing mango dieback in Korea. *Can J Plant Pathol* 2017; 39: 241-245.
  11. Rosado AWC, Machado AR, Freire FCO, Pereira OL. Phylogeny, identification and pathogenicity of *Lasiodiplodia* associated with postharvest stem-end rot of coconut in Brazil. *Plant Dis* 2016; 100: 561-568.
  12. Wei W, Jiang N, Mei YN, Chu YL, Ge HM, Song YC, et al. An antibacterial metabolite from *Lasiodiplodia pseudotheobromae* F2. *Phytochemistry* 2014; 100: 103-109.
  13. Taufiq MMJ, Darah I. Fungal endophytes isolated from the leaves of a medicinal plant, *Ocimum sanctum* Linn and evaluation of their antimicrobial activities. *Afr J Microbiol Res* 2018; 12: 616-622.
  14. Ogbole OO, Segun PA, Adeniji AJ. *In vitro* cytotoxic activity of medicinal plants from Nigeria ethnomedicine on Rhabdomyosarcoma cancer cell line and HPLC analysis of active extracts. *BMC Complement Altern Med* 2017; 17: 494.
  15. Mares D. Electron microscopy of *Microsporium cookiei* after *in vitro* treatment with protoanemonin: A combined SEM and TEM study. *Mycopathologia* 1989; 108: 37-46.
  16. Dhayanithy G, Subban K, Chelliah J. Diversity and biological activities of endophytic fungi associated with *Catharanthus roseus*. *BMC Microbiol* 2019; 19: 22.
  17. Hamzah TNT, Lee SY, Hidayat A, Terhem R, Faridah-Hanum I, Mohamed R. Diversity and characterization of endophytic fungi isolated from the tropical mangrove species, *Rhizophora mucronata*, and identification of potential antagonists against the soil-borne fungus, *Fusarium Solani*. *Front Microbiol* 2018; 9: 1707.
  18. Jia M, Chen L, Xin HL, Zheng CJ, Rahman K, Han T, et al. A Friendly relationship between endophytic fungi and medicinal plants: a systematic review. *Front Microbiol* 2016; 7: 906.
  19. Beveridge TJ. Structures of Gram-negative cell wall and their derived membrane vesicles. *J Bacteriol* 1999; 181: 4725-4733.
  20. Miller SI, Salama NR. The gram-negative bacterial periplasm: Size matters. *PLoS Biol* 2018; 16(1):e2004935.
  21. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* 2003; 67: 593-656.
  22. Poole CF. Thin-layer chromatography: challenges and opportunities. *J Chromatogr A* 2003; 1000: 963-984.
  23. Bag A, Chattopadhyay RR. Evaluation of synergistic antibacterial and antioxidant efficacy of essential oils of spices and herbs in combination. *PLoS One* 2015; 10(7):e0131321.
  24. Marasini BP, Baral P, Aryal P, Ghimire KR, Neupane S, Dahal N, et al. Evaluation of antibacterial activity of some traditionally used medicinal plants against human pathogenic bacteria. *Biomed Res Int* 2015; 2015: 265425.
  25. Wright GD. Bacterial resistance to antibiotics: enzymatic degradation and modification. *Adv Drug Deliv Rev* 2005; 57: 1451-1470.
  26. Olajuyigbe OO, Afolayan AJ. *In vitro* antibacterial and time-kill evaluation of the *Erythrina caffra* Thunb. extract against bacteria associated with diarrhoea. *ScientificWorldJournal* 2012; 2012:738314.
  27. Balouiri M, Sadiki M, Ibsouda SK. Methods for *in vitro* evaluating antimicrobial activity: A review. *J Pharm Anal* 2016; 6: 71-79.
  28. Wistrand-Yuen E, Knopp M, Hjort K, Koskiniemi S, Berg OG, Andersson DI. Evolution of high-level resistance during low-level antibiotic exposure. *Nat Commun* 2018; 9: 1599.
  29. Nielsen EI, Viberg A, Lowdin E, Cars O, Karisson MO, Sandstrom M. Semimechanistic pharmacokinetic/pharmacodynamic model for assessment of activity of antimicrobial agents for time-kill curve experiments. *Antimicrob Agents Chemother* 2007; 51: 128-136.
  30. Lewis K. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* 2007; 5: 48-56.
  31. Pankey GA, Sabath LD. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clin Infect Dis* 2004; 38: 864-870.
  32. Parasuraman S. Toxicological screening. *J Pharmacol*

- Pharmacother* 2011; 2: 74-79.
33. Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. *Microbiol Mol Biol Rev* 2003; 67: 491-502.
  34. Fesel PH, Zuccaro A. Dissecting endophytic lifestyle along the parasitism/mutualism continuum in Arabidopsis. *Curr Opin Microbiol* 2016; 32: 103-112.
  35. Owen NL, Hundley N. Endophytes: the chemical synthesizers inside plants. *Sci Prog* 2004; 87: 79-99.
  36. Pelka M, Danzl C, Distler W, Petschelt A. A new screening test for toxicity testing of dental materials. *J Dent* 2000; 28: 341-345.
  37. Hartmann M, Berditsch M, Hawecker J, Ardakani MF, Gerthsen D, Ulrich AS. Damage of the bacterial cell envelope by antimicrobial peptides Gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. *Antimicrob Agents Chemother* 2010; 54: 3132-3142.
  38. Jeyanthi V, Velusamy P. Anti-methicillin resistant *Staphylococcus aureus* compound isolation from Halophilic *Bacillus amyloliquefaciens* MHB1 and determination of its mode of action using electron microscope and flow cytometry analysis. *Indian J Microbiol* 2016; 56: 148-157.