Ultrastructural effects of ethyl acetate extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64 on foodborne bacterium

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Abstract

The antibacterial activity of ethyl acetate extract of Lasiodiplodia pseudotheobromae IBRL OS-64 isolated from the leaf of Ocimum sanctum Linn. was studied and tested against foodborne bacterium Yersinia enterocolitica. The extract exhibited favourable antibacterial activity with the zone of inhibition as 20.1±0.6 mm. On the other hand, minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values were 250 µg/mL and 500 µg/mL respectively. The extract exerted bactericidal effect against the test bacterium since the MBC/MIC ratio is less than 4. The time-kill study of the extract against the test bacterium suggested that the extract possessed antibacterial properties at higher concentrations and eradicated the growth of bacterial cells.

The SEM and TEM observations revealed that several abnormalities occurred on the treated cells including irregular shape, cell's leakage, shrunken and crumpled cells which resulted in cell death. Therefore, the present study suggested that the ethyl acetate extract of L. pseudotheobromae IBRL OS-64 could be an effective antibacterial agent to combat foodborne pathogens.

Keywords: *Lasiodiplodia pseudotheobromae*, *Ocimum sanctum*, *Yersinia enterocolitica*, Minimal Inhibition Concentration (MIC), Minimal Bactericidal Concentration (MBC), Time-kill study.

Introduction

Foodborne disease is a worldwide human health problem and most of them are associated with a wide spectrum of pathogenic bacteria. However, foodborne diseases can be also caused by parasites, viruses, or toxins from contaminated food and water. These illnesses occurred as a result of the consumption of contaminated food and it will cause several common gastrointestinal symptoms including fever, nausea, vomiting, diarrhea, abdominal cramps and dehydration[1,14]. Furthermore, the foodborne diseases may result in several serious symptoms such as hepatic, neurologic and renal syndromes which may lead to death[24]. *Yersinia enterocolitica* is a psychotropic zoonotic pathogen with typical rod-shaped gram-negative bacteria belonging to the family of *Enterobacteriaceae* that can cause acute gastroenteritis and more serious diseases to human[19,41]. Human yersiniosis which is primarily acquired via the gastrointestinal tract as a result of ingestion of contaminated food such as raw or inadequately cooked pork is one of illnesses caused by *Y. enterocolitica*[11]. The strain is being frequently isolated from a variety of foods, water, soil and animals and can at refrigeration temperatures[5].

Nowadays, this strain was reported to be resistant to several antibiotics including clindamycin, ampicillin, amoxicillin and tetracycline and the occurrence of virulence strain in pigs and porcine products revealed that the pigs are an important reservoir of *Y. enterocolitica*. Therefore, the increasing of multidrug-resistant strain becomes a public health concern.

Fungal endophytes of medicinal plants contain a tremendous source of antimicrobial agents. For instance, 134 out of 148 isolates (90.5%) of endophytic fungi from *Ocimum sanctum* Linn. were reported to exert an inhibitory effect against at least one test microorganism[36]. Furthermore, endophytic fungi also proved to possess antimicrobial activity against foodborne pathogens. Yin et al[39] reported *Aspergillus terreus* MP15, a fungal endophyte isolated from the leaf of *Swietenia macrophylla* exhibiting significant antibacterial activity towards foodborne bacteria including *Bacillus cereus*, *B. subtilis*, *B. spizizenii* and *Staphylococcus aureus*. Hence, searching for fungal endophytes from medicinal plants is increasing worldwide due to its significant potency as a source of powerful drugs.

Lasiodiplodia pseudotheobromae is a well-known plant pathogen from family Botryosphaeriaceae that frequently infected grapevine, persimmon, woody plants and mango. It can cause several symptoms to the infected plant including fruit rot[26], dieback[18] and cankers[6]. This strain was reported to possess several biological activities including cytotoxic and antibacterial activities[23,37]. However, the information regarding on bioactive compounds and biological activities of *L. pseudotheobromae* is very scarce. Thus, the study was designed to investigate the potential of *L. pseudotheobromae* IBRL OS-64 isolated from the leaf of *Ocimum sanctum* Linn. as an antibacterial agent towards foodborne pathogens.

Material and Methods

Microorganisms and cultural maintenance: The endophytic fungus *Lasiodiplodia pseudotheobromae* IBRL OS-64 was deposited at the Industrial Biotechnology

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Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The fungal culture was grown and maintained on potato dextrose agar (PDA) with the addition of a powdered host plant (2 g/L). The test bacterium *Yersinia enterocolitica* culture was also obtained from the IBRL. The bacterial culture was grown on nutrient agar (NA) and inversely incubated at 37°C for 24 h. All the bacterial and fungal cultures were kept at 4°C until further use. Subculturing was done every month to ensure their purity and viability.

Preparation of bacterial suspension: The bacterial suspension was prepared by picking five single colonies from 24 h old culture and transferred into 5 ml of 0.85% sterile physiological saline (w/v). The turbidity was adjusted using McFarland standards to obtain approximately 1×10^8 CFU/mL of the bacterial suspension.

Culture medium: Yeast extract sucrose (YES) broth was prepared by adding 20 (g/L) yeast extract (Merck, Germany), 40 (g/L) sucrose and 0.5 (g/L) into 1000 mL of host plant water extract. Before that, the host plant water extract was prepared by boiling two grams of host plant powder in 1000 mL distilled water for 30 min and the mixture was filtered with muslin cloth. The pH of the culture medium was calibrated using pH meter to 6.0 and then it was autoclaved at 121ºC for 15 min.

Fermentation and Extraction: Two mycelial plugs of 3 d old fungal culture were transferred into 250 mL Erlenmeyer flasks containing 100 mL YES broth and incubated at 30ºC in the dark for 16 d under static condition. The fungal biomass and fermentative broth were separated using Muslin cloth followed by filtration using filter papers (Whatmann no. 1). The filtered broth was then extracted thrice with an equal volume of ethyl acetate (1:1; v/v). The ethyl acetate extract was collected, concentrated using a rotary evaporator and kept in a fume hood to dryness to obtain ethyl acetate crude paste.

Disk diffusion assay: The antibacterial activity of the extract was determined following the method described by NCCLS[27]. An amount of 20 µL of the fungal extract was impregnated to the sterile antibiotic disc (Whatmann, 6.0 mm diameter) and then placed on the surface of MHA previously seeded with test bacteria. Five percent of ethyl acetate was used as a negative control whilst chloramphenicol (30 µg/mL) was set as a positive control. The plate was then inversely incubated at 37ºC for 24 h. The diameter zone of inhibition formed surrounding the disc was measured and recorded. The experiments were carried out in triplicate.

MIC and MBC Determination: The MIC and MBC values of the extract were determined following the method described by NCCLS[27] in sterile, 96 wells, U-shaped microtiter plates. The plate was loaded with 50 µL Muller Hinton Broth (MHB) and 100 µl of the extract (at different concentrations) was dispensed into each well accordingly. On the other hand, 50 µl of bacterial suspension was added into each of the wells to obtain a final volume of 200 µl with a final bacterial suspension of 5×10^5 CFU/ml. Chloramphenicol (30 µg/mL) and 5% methanol were used as positive and negative control respectively. The plate was then incubated at 37ºC for 24 h. A volume of 20 µL p-iodonitrotetrazolium violet (INT) was loaded to each well after the incubation period as a growth indicator. The lowest dilution of the extract showing no bacterial growth (no changes in broth color) was recorded as the MIC value of the extract. The lowest concentration of the MIC well with no visible of bacterial growth on MHA was regarded as the MBC value of the extract.

Time-kill study: The time-kill study was carried out to investigate the efficacy of the fungal extract towards test bacteria at different concentration levels *viz.* ½ × MIC (125 µg/mL), MIC (250 µg/mL) and 2 × MIC (500 µg/mL). A volume of 0.1 mL of bacterial suspension (as previously explained) was transferred into 4 different 50 mL Erlenmeyer flask containing 18.9 mL Muller Hinton broth (MHB) labeled as A, B, C and D with a final bacterial suspension approximately 5×10^5 CFU/mL. Flasks A, B and C were then added with 1.0 mL of the extract at ½ × MIC, MIC and 2 × MIC levels respectively. Flask D was added with 1.0 mL of 5% methanol (v/v) and set as growth control. The cultures were then homogenized and incubated in an orbital shaking incubator (150 rpm) at 37ºC for 48 h.

Due to the determination of viable cell count, a volume of 0.1 mL of aliquot sample was taken out every 4 h during a time interval of 0 to 48 h. The samples were serially diluted followed by spread plating onto fresh Muller Hinton agar (MHA) and incubated at 37ºC for 24 h. To determine the colony unit per milliliter (CFU/mL), the plates only with the number of colonies ranging from 30 – 300 were counted. A time-kill curve ($\log_{10}$ CFU/mL vs. time) was constructed for each extract concentration and control. In the meantime, the time needed to reduce 50%, 90% 99% and 99.9% of bacterial cell growth was determined according to the equation described by Taufiq and Darah[36].

Scanning Electron Microscopy (SEM): The bacterial sample was prepared according to the method described by Taufiq and Darah[36]. The mixture was then incubated at 37ºC, 150 rpm for 36 h. At every 12 h of the time interval, the mixture was harvested and centrifuged to obtain the bacterial cell pellet. The bacterial pellet then underwent primary fixation, post-fixation and dehydration process. Prior to SEM viewing, the dried bacterial cells were mounted on a specimen stub using conductive tape coated with 5 – 10 nm gold using a sputter coater machine Fison SC-515, UK. The specimen was then viewed under a Scanning electron microscope (Leica Cambridge, S-360, UK).

Transmission Electron Microscopy (TEM): The bacterial samples for TEM were prepared as per SEM procedures. The

bacterial cell pellet was fixed with 1.0 mL McDowell-Trump fixation solution and then the fixed pellet was processed according to the method described by Ibrahim et al[13]. The cell samples on the copper grids were then viewed under a Transmission electron microscope (LIBRA 120 EFTEM, Germany).

Statistical analysis: All the experiments were performed in triplicate (n = 3) and the experimental data were expressed as mean ± standard deviation (SD). The data were analyzed by means of the One Way ANOVA using SPSS 15.0 and Duncan test was used to access the differences between means. The results were considered statistically significant if $p < 0.05$.

Results and Discussion

Table 1 shows the diameter zone of inhibition of the ethyl acetate extract against foodborne bacteria bacterium, *Y. enterocolitica* on disk diffusion assay. As shown in table 1, the extract of *L. pseudotheobromae* IBRL OS-64 exhibited good activity against the test bacteria based on larger inhibition zone produced which was 20.1±0.6 mm. The results from the disc diffusion assay revealed that the extract is potentially effective in suppressing the growth of the test foodborne bacteria. According to Ortez[29], the diameter zone of inhibition on disc diffusion assay can be interpreted as resistant (< 16 mm), intermediate (17 – 20 mm) and susceptible (> 20 mm). Based on the disc diffusion assay interpretation, the test bacteria showed an intermediate susceptibility towards the extract. Disc diffusion assay is a simple method with the ability to rapidly identify active metabolites and is useful in the primary screening for antimicrobial activity[15].

However, the efficacy of this method was influenced by several factors including the diffusion rate of the extract, the potency of the extract, a function of the molecular size and the hydrophilicity of the compounds[16,17]. Besides that, the inoculum size and depth of the agar medium also were reported to influence the efficacy of this assay[22,33]. Thus, the non-standardized in disc diffusion assay especially on bacterial inoculum and rate of diffusion might lead to the false-resistant result and might not represent the actual potency of the extract's antimicrobial activity[31,40].

In the present study, the broth microdilution method was employed to overcome the drawbacks of the agar diffusion test including the inability of some extracts to diffuse into agar[3]. The MIC determination is an alternative assay to evaluate *in vivo* antimicrobial activity of the extract. This assay is more reliable compared to disc diffusion assay as it has the advantage of producing a more defined estimation of the level of resistance in less sensitive strains[4]. Moreover, combination of MIC and MBC would give a piece of important information regarding bactericidal and bacteriostatic properties of the extract[25]. The MIC is defined as the lowest concentration of a drug that will inhibit the visible growth of an organism after overnight incubation whereas MBC is defined as the lowest concentration of antimicrobial agent that will prevent the growth of an organism after subculture on the antibiotic-free solid media[2].

The susceptibility of the test bacteria towards the extract is also presented in table 2. The results demonstrated that the MBC value (500 µg/mL) was significantly two-fold higher than the MIC value (250 µg/mL). The result was in agreement with Lim et al[21]. This observation revealed that the concentration of the extract plays a significant role to kill bacterial cells rather than just inhibiting it. The results also showed that the extract possesses bactericidal effect towards the test bacterium since the MBC/MIC ratio was less than 4. Antimicrobial agents are considered as bacteriostatic when the ratio is MBC/MIC>4 and bactericidal when the ratio is MBC/MIC≤4[10].

Besides that, the extract was found to exhibit a bactericidal effect due to the low MBC/MIC ratio (equivalent to 2) and the MBC was only two-fold higher than MIC. According to Levison[20], the good bacterial drugs should have the MBC values not more four-fold higher than their MIC. Furthermore, the current results revealed that the extract exhibited bactericidal effects when exposed to a higher concentration of extract but bacteriostatic effects at a lower concentration. Thus, the concentration of the extract is believed to have a significant effect on antimicrobial activities.

In drug studies, determination of the time-kill curve is important since the comprehensive pharmacodynamics information of a drug may not be simply gained via MIC assay. Thus, time-kill assays are required to quantitatively evaluate the pharmacodynamic of a bacterial drug by quantifying the bacterial growth reduction at a specified time and drug concentration[32]. The study was performed over a period of 48 h with the *Y. enterocolitica* cells being exposed to different level of extract viz. ½ × MIC (62.5 µg/mL), MIC (125 µg/mL), 2 × MIC (250 µg/mL) and the results as shown in figure 1. For control, the growth curve was observed to be similar to the normal bacterial growth with log (0 – 4h), lag (4 – 28h), stationary (28 – 44h) and death (44 – 48h) phase.

At ½ × MIC value of the extract, the cell growth was significantly reduced after 4 h of exposure time and the growth was stagnant thereafter. However, after 24 h of exposure time, there was a significant increase in bacterial growth indicating that the bacteria resume their growth. A similar growth pattern was also observed at the MIC value of the extract with a longer growth reduction phase. At 2 × MIC value, the extract eradicated the bacterial cell numbers over the exposure time.

The present study revealed that higher extract concentration was significantly suppressed and killed bacterial growth. A similar observation was reported by Darah et al[7] who studied the effect of *Caulerpa sertularioides* extract on foodborne diarrhea-caused bacteria. The time-kill study results for 2 ×

MIC were consistent with the MBC determination. As previously discussed, the MBC of the extract was 500 µg/mL and demonstrated no visible bacterial growth indicating that the MBC of the extract completely killed the bacterial cells (bactericidal effect). A similar phenomenon may also occur in 2 × MIC (500 µg/mL) of the time-kill assay since the concentration of the extract was the same.

In other words, the results from time-kill studies validated the MIC/MBC results. At low extract concentration especially for ½ × MIC and MIC, the re-growth of bacterial cells was observed. The result was consistent with the previous studies in the time-kill kinetics of amikacin, cefoxitin and clarithromycin[9]. This phenomenon may be attributed to two distinct subpopulations with different susceptibility in which the selective amplification of the resistant sub-population takes over the preferential killing of the susceptible sub-population at specified exposure time[35]. On the other hand, the biphasic killing curve was observed in the present study in which the extract was initially suppressed to the bacterial cells but increased thereafter at a specified time. According to Nielsen et al[28], the occurrence may be due to the presence of persister which was less susceptible to antimicrobial drugs. The persister cells arise due to a dormancy state in which cells are metabolically inactive and enable cells to escape the effects of antibiotics. The formation of persister cells is generated by a toxin-antitoxin (TA) system that consists of a stable toxin (protein) that disrupts an essential cellular process and a labile antitoxin (RNA/protein) that prevents toxicity[38].

Besides that, the results showed that the MIC result on broth microdilution was inconsistent with the MIC result in the time-kill study. This may be due to the exposure period of the extract to combat the test bacteria. Indirectly, the results also revealed that the time-kill curve is more reliable and provides more precise pharmacodynamic information of the extract compared to broth microdilution.

Figure 2 depicts the structure of bacterial cells treated with the extract observed under SEM for 0, 12, 24 and 36 h of exposure times. In general, the structural degeneration of *Y. enterocolitica* was correlated with the exposure time whereby the severity of cell damage increased with the increment of time contact of the cell to extract. Figure 2a shows the normal cells of *Y. enterocolitica* with its typical long rod shape, rigid and the rugose cell surfaces. Red arrow indicates that the binary fission of normal bacterial cells occurred during growth and cell replication. After 12 h of exposure to the extract, the bacterial cells were observed to be in irregular shape and shrunk abruptly (Figure 2b). At 24 h of exposure time, the bacterial cell started to lysis indicated by the formation of small cell debris (Figure 2c).

Red arrows show the formation of the bacterial cell small particles as a result of cell lysis. The structures of bacterial cell were lysed and collapsed after 36 h of exposure time to the extract whereby most of the cells lost their rigid long rod (Figure 2d). The red arrows indicate the crumpled cell residues of *Y. enterocolitica* formed and the intact, long rod of the bacterial cells were no longer observed.

According to Taufiq and Darah[36], the crude extract of plants or microbes would affect the cell wall biosynthesis and cell membrane permeability. The SEM micrographs revealed that the severity of bacterial cell damage depends on exposure time to the extract and its concentration. The present study showed the rod shaped bacterial cells loss after 12 h exposure to the extract indicated by the shrinkage of the bacterial cells. On the other hand, the longer exposure time would cause several abnormalities to the bacterial cells including cell surface crumpled and the presence of cell debris.

According to Ibrahim et al[13], the shrinkage of the cell, cell surface crumpled, irregular shape and cleavage of cell envelope may indicate the damage of cell envelope and loss of cell organelles from cell cytoplasm. Generally, *Y. enterocolitica* is a gram-negative bacterium with cell enveloped consisting of the outer cell membrane, thin peptidoglycan layer containing teichoic acid and cytoplasmic membrane[34]. Thus, the cleavages of the cell envelope may be due to result of the action extract on either peptidoglycan layer or cytoplasmic membrane or both. This hypothesis was supported by Pasquina et al[30] who revealed that teichoic acid biosynthesis in peptidoglycan layer is one of the main targets of antibiotics.

Figure 3 shows the TEM micrographs of *Y. enterocolitica* treated with 500 µg/mL of the extract at 0, 12, 24 and 36 h of exposure time. Figure 3a illustrates the bacterial cells treated with extract at 0 h. TEM Micrographs revealed that the bacterial cells at 0 h were in good condition with intact rod-shaped (Figure 3a). The cell organelles were observed to resemble and suspended in the cytoplasm including the nucleus, vacuole and granules. The cytoplasm was enclosed by a cell membrane and a thick cell wall. The cell membrane, peptidoglycan layer and outer membrane can be clearly seen through a thin section of the cell enveloped of *Y. enterocolitica* (black arrows).

Figure 3b shows the structural degeneration of bacterial cells after 12 h exposure to the extract. Results revealed the disintegration of the outer cell membrane led to cell bursting and disruption (red arrows). The vacuole was observed to be smaller compared to the control (black arrow) and this might be due to imbalance osmotic pressure.

Figure 3c depicts the morphology of the bacterial cell after treatment with the extract for 24 h. Results clearly showed the disruption of the outer cell membrane whereby a thin-smear cell wall layer was observed. The vacuole disappeared and the organelles have resembled in uneven structures. After 36 h of exposure time to the extract, the organelles inside the bacterial cell were observed to flow out as indicated by red arrows (Figure 3d). In the meantime, the

bacterial cell wall was observed not in the intact shape and completely collapsed indicated by smeared line along with the cells.

TEM studies were performed to have a clearer view of what is actually happening inside the bacterial cells. The results revealed an unusual morphology of bacterial cells exposed to the extract compared to control. It clearly observed the disintegration of the cell wall and cytoplasmic membrane, swollen of the cells and mass leakage of cytoplasmic organelles. The swollen of the cells may be due to osmoregulation that caused the cells look turgid and compact. The periplasmic cell was very thin.

Similar observations were reported by Hartmann et al[12] in the study of *E. coli* cells. They suggested that an increased influx of water into the cytoplasm increased the turgor pressure and thus leads to cytoplasmic leakage resulting in ultimate cell death. According to Denyer[8], cell leakage may also due to the removal of crucial ions and molecules and the initiation of cell's autolytic mechanism.

Table 1
Diameter of inhibition zone of ethyl acetate extract against *Yersinia enterocolitica* on disk diffusion assay.

Test bacterium	Diameter of inhibition zone (mm ± SD)		
	EA	C	NC
Yersinia enterocolitica	20.1±0.6	28.3±1.2	-

Notes: EA = ethyl acetate extract, C = chloramphenicol (positive control), NC = negative control (5% methanol).

Table 2
Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of ethyl acetate extract against *Yersinia enterocolitica*.

Test bacterium	Extract concentration (mg/mL)		Ratio
	MIC	MBC	MBC/MIC
Yersinia enterocolitica	250	500	2

Table 3
The time to achieve 50, 90, 99 and 99.9% growth reduction in initials inoculum of *Yersinia enterocolitica*

Percentage of reduction (%)	Time (h)			
	Control	½ MIC	MIC	2 MIC
50	NR	8 – 12	4 – 8	4 – 8
90	NR	NR	12 – 16	8 – 12
99	NR	NR	16 – 20	16 – 20
99.9	NR	NR	NR	20 – 24

Key: NR = not reached

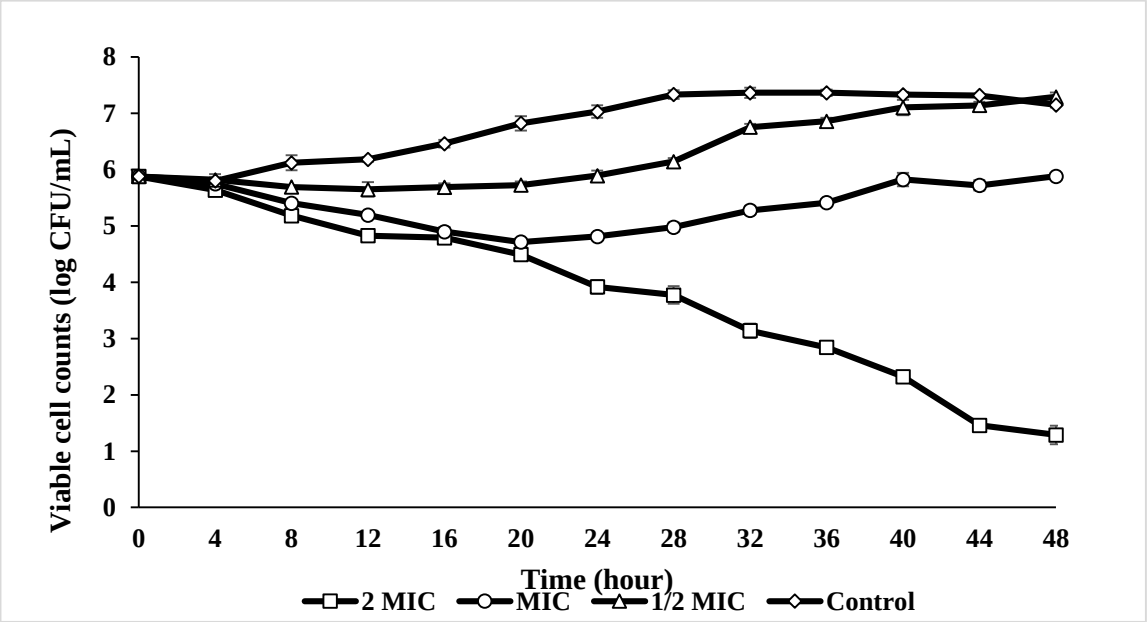

Figure 1: Time-kill curve of ethyl acetate extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64 against *Yersinia enterocolitica* at different extract concentrations.

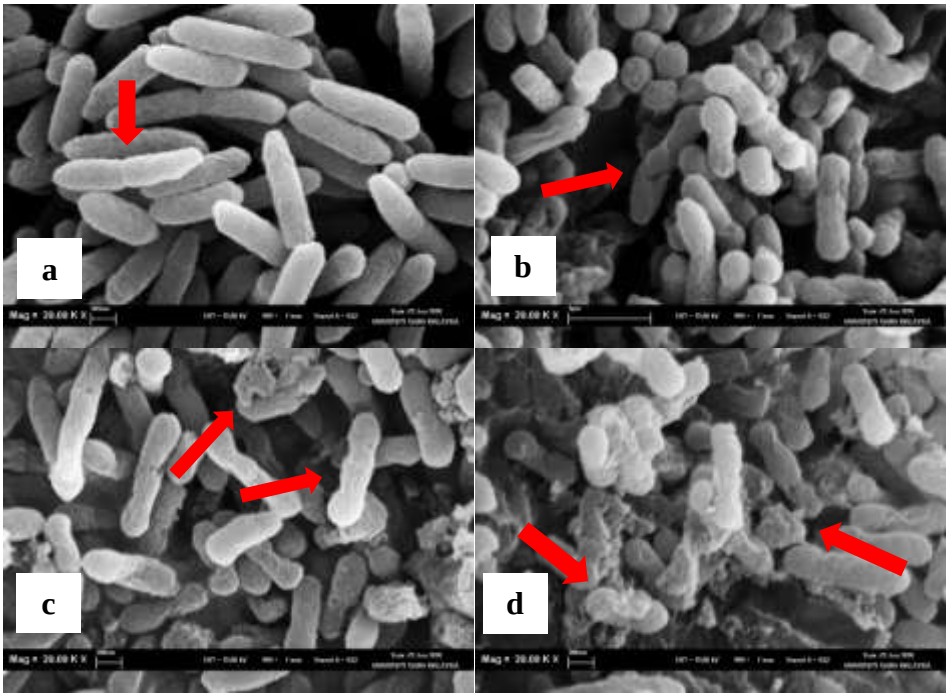

Figure 2: SEM micrographs of *Yersinia enterocolitica* treated with 500 µg/mL of ethyl acetate crude extract at different exposure time. (a) 0 hour [control] (b) 12 hours (c) 24 hours (d) 36 hours. Scale bars: 200nm.

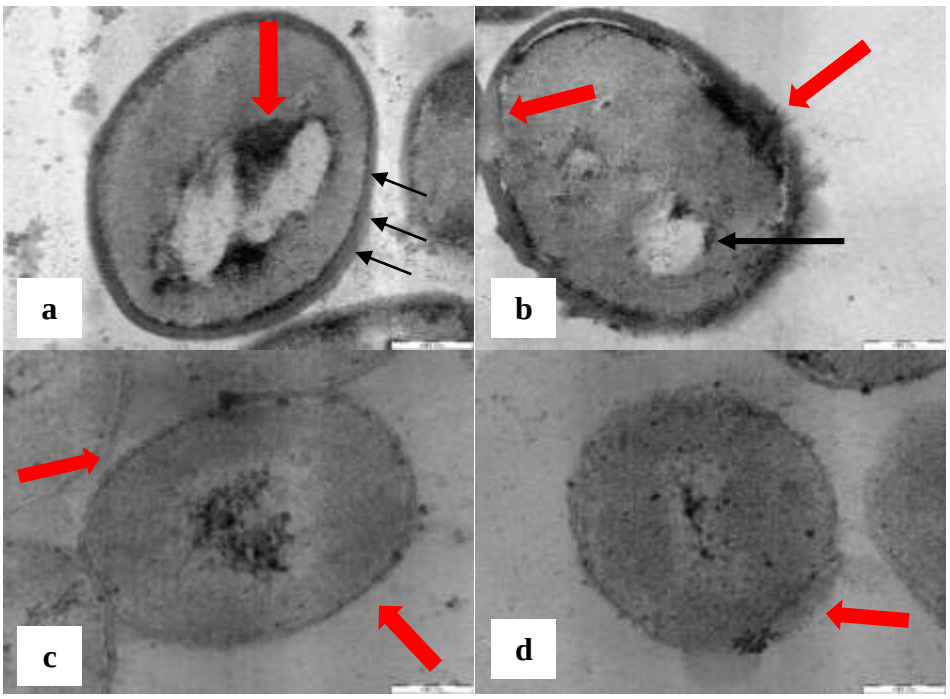

Figure 3: TEM micrographs of *Yersinia enterocolitica* treated with 500 µg/mL of ethyl acetate crude extract at different exposure time. (a) 0 hour [control] (b) 12 hours (c) 24 hours (d) 36 hours. Scale bars: 100nm.

Conclusion

The present investigation confirms the ability of the ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 as an antibacterial drug since it could cause severe disruption of the bacterial cells.

It is important to point out that the extract needs to be further studied to give pure compounds that can be tested for antimicrobial activities.

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