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Growth inhibition of *Escherichia coli* and *Pseudomonas aeruginosa* strains by *Piper betle* Linn. extracts

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Abstract

Piper betle Linn. is a medicinal plant belonging to family Piperaceae. Its leaves are widely applied as a traditional herbal medicine due to their bioactive constituents. In this study, we investigated the antibacterial activity, growth inhibition, bacterial cell morphology effect of *Piper betle* L. extract (PBE) against *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* DMST 37166. PBE at the concentration of 4 mg/ml possessed the widest inhibition zones of 18.50 mm and 22.67 mm against *E. coli* ATCC 25922 and *P. aeruginosa* DMST 37166, respectively. PBE showed the same minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 1 and 2 mg/ml against of *E. coli* ATCC 25922 and *P. aeruginosa* DMST 37166, respectively. The killing kinetics of PBE against bacterial strains were time and dose dependent. Scanning electron microscopy micrographs exhibited morphological alterations of both PBE-treated bacterial strains. These results clearly indicate that the crude extract of *Piper betle* L. has a promising potential to be used as a natural agent for the treatment of infectious diseases caused by *E. coli* and *P. aeruginosa.*

Keywords**:** *antibacterial activity; Escherichia coli; natural agent; Piper betle L.; Pseudomonas aeruginosa.*

1. Introduction

Foodborne illness outbreaks caused by foodborne pathogens have been increasingly a concern in recent years. *Escherichia coli*, a Gram negative bacterium, is one of the common foodborne pathogens. The signs and symptoms of virulent strains of *E. coli* exposure include urinary tract infections, gastroenteritis, meningitis, neonatal etc. (Boulouis et al., 2020). The prevalence of EHEC serotype *E. coli* O157:H7 is found to be 20% of foodborne illnesses globally (Getaneh, Hordofa, Ayana, Tessema, & Regassa, 2021). Similarly, *Pseudomonas aeruginosa*, a Gram negative bacterium commonly found in the hospitals, has become an opportunistic pathogen for humans. This bacterium causes nosocomial infection as well as acute and chronic infection (Bassetti, Vena, Croxatto, Righi, & Guery, 2018). Global foodborne diseases are responsible for 230,000 deaths from 550 million people yearly (Chemoh, Bin-Ismail, & Dueramae, 2021). However, both of these pathogens are increasingly able to tolerant to various classes of antibiotics (Huijbers, Larsson, & Flach, 2020; Khan, Stapleton, Summers, Rice, & Willcox, 2020). Therefore, the development of effective inhibitors in order to overcome drug

resistance in these bacteria has become an urgent necessary.

Piper betle Linn. is a tropical creeper plant and commonly known as betel in English and Phlu in Thai (Dwivedi, & Tripathi, 2014; Phumat, Khongkhunthian, Wanachantararak, & Okonogi, 2018). It is an edible plant belonging to the Piperaceae family and originated in Malaysia (Madhumita, Guha, & Nag, 2019). Betel leaves have been used as a main ingredient in traditional medicines in many Asian countries for treatment of a variety of aliments (Phumat et al., 2018). The extracts of *P. betle* L. leaves have been reported to possess pharmacological effects such as antioxidant, anti-inflammatory, anticancer, anti-allergic, antirheumatic antibacterial, and antifungal activities (Boontha et al., 2020; Murugesan et al., 2020; Sartini, Khaerawati, Kamril, & Febriani, 2020; Sarma et al., 2018). Several bioactive components were found in the extracts such as allylpyrocatechol, chavibetol, 4-chromanol, caryophyllene, eugenol, and hydroxychavicol (Guha & Nandi, 2019; Phumat et al., 2018; Teanpaisan, Kawsud, Pahumunto, & Puripattanavong, 2017). The previous investigations reported that hydroxychavicol found in *P. betle* L. showed antibacterial activity against foodborne pathogens that cause severe infectious diseases including *Staphylococcus aureus*, *Staphylococcus epidermidis, Escherichia coli*, and *Pseudomonas aeruginosa* (Budiman & Aulifa, 2020; Ratridewi et al., 2020; Sedek, Arifin, & Munaim, 2020; Sarma et al., 2018; Gundala, & Aneja, 2014). Other studies showed that 4-chromanol exhibited antibacterial activity against *Streptococcus pyogenes, Streptococcus mutans, Staphylococcus aureus, Proteus vulgaris, Aggregatibacter actinomycetemcomitans, Escherichia coli, Pseudomonas aeruginosa* (Teanpaisan et al., 2017) and disrupted biofilm formation of *Vibrio harveyi* (Srinivasan, Santhakumari, & VeeraRavi, 2017). Interestingly, hydroxychavicol could cause cell death by DNA damage and cell division disruption of *Escherichia coli*. However, the reports of extracellular appearance of bacterial cells inhibited by *P. betle* L. extract are still less.

This study aimed to determine the inhibition potential of *P. betle* L against foodborne pathogens; *E. coli* and *P. aeruginosa* strains. Moreover, this present study investigated the effect of *P. betle* L. extract on cell morphology of these two pathogens using scanning electron microscopy (SEM). The obtained results may help to explain action mechanism of *P. betle* L on extracellular surface of bacterial cells and lead the way to the development of PBE as a natural agent for prevention and treatment of infectious illnesses caused by these foodborne pathogens.

2. Materials and methods

2.1 Plant material and extraction

The leaves of betel (*P. betle* L.) were collected from home-gardens in Udon Thani province, Thailand. The plant was certified by botanist Dr. Sawai Mattapha from Faculty of Science at Udon Thani Rajabhat University. The medicinal plants were extracted in 95% ethanol according to the method of previous study (Sittisart, Piakaew, Chuea-Nongthon, & Dunkhunthod, 2019) with some modifications. Briefly, flesh leaves were washed and dried at room temperature under shade for 5-7 days. The dried leaves were pulverized using an electric grinder. The 100 grams of powder were mixed with 500 ml of 95% ethanol and macerated for 72 h in a shaking incubator (MRC Ltd., Beijing, China). The suspension was filtered through Whatman No. 1 filter paper to remove large debris. The solvent in the extract was removed by rotary evaporation method under vacuum to obtain dried extracts. Then, the dried crude extract was stored at -20°C until used and dissolved in 5% dimethyl sulfoxide (DMSO).

2.2 Antibacterial activity

Antibacterial activity of *P. betle* L. was carried out using agar disc diffusion method (CLSI, 2016). Briefly, frozen cultures of *E. coli* ATCC 25922 and *P. aeruginosa* DMST 37166 were cultivated in Mueller Hinton broth (MHB) (HiMedia Laboratories Pvt. Ltd., Mumbai, India) and incubated at 37°C for 18 h. Then, the bacterial cells were prepared to obtain 10^8 cfu/ml in 0.85% NaCl (Liu, Durham, & Richards, 2000). The bacterial suspension was swabbed evenly on Mueller Hinton agar (MHA) (HiMedia Laboratories Pvt. Ltd., Mumbai, India). Sterilized discs (6 mm diameter) were impregnated with 10 µl of *P. betle* L. extract (PBE) to obtained a final concentration at 1, 2, and 4 mg/ml and dried at 37°C overnight. After the impregnation, the discs were put on inoculated MHA plates and were incubated at 37°C for 18 h. Ceftazidime and DMSO (Sigma-Aldrich, Stockholm, Sweden) were used as controls. The diameters of the inhibition zone were measured and averaged.

2.3 Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The MICs of PBE and ceftazidime were assessed using a modified broth microdilution assay (CLSI, 2016). Shortly, bacterial cultures after 18 h growth were treated in MHB containing different concentrations of PBE and ceftazidime. Bacterial cell suspensions were obtained at a final concentration of 1×10^8 cfu/ml in 0.85% NaCl. The twofold serial dilutions of PBE and ceftazidime in MHB medium at concentrations of 0.5, 1, 2, 4, 8, 16, and 32 mg/ml were prepared in a 96-well polystyrene microtiter plate. A 100 µl of bacterial culture were added into each well and then the microtiter plate was incubated at 37°C overnight. After incubation, MIC was recorded as the lowest concentration of PBE that showed no visible growth of bacterial cultures. For the determination of MBC, a 100 µl of the bacterial culture were spread on MHA plates and incubated at 37°C overnight. MBC value was defined by the lowest concentration of PBE that showed 99.9% or complete inhibition of bacterial inoculums.

2.4 Determination of killing curves

Time killing assay was applied to investigate the antibacterial property of PBE. The assay was carried out according to Siriwong et al. (2015) with some modifications. The bacterial cells at concentration of 5×10^6 cfu/ml in 0.85% NaCl was subjected to MHB containing PBE with the final concentrations ranging from 0.5×MIC to 4×MIC. MHB without antibacterial substance was used as negative control. This made the final concentration of each bacterial suspension per test at 5×10^5 cfu/ml. The samples were incubated at 37°C. After 0.5, 1, 2, 3, 4, 5, 6, 8, and 24 h of incubation, one ml of each test was serially diluted. Then, 0.1 ml of each sample were spread on over dried MHA and were incubated at 37°C for 24 h. Graph was plotted where y-axis was viable counts (log 10 cfu/ml) and x-axis was time. Cell counts at ≥3log10 cfu/ml were considered as bactericidal effect of PBE.

2.5 Scanning electron microscopic study of cell morphology

Scanning electron microscope (SEM) analysis was performed in accordance with some modifications of previous study (Devi, Nisha, Sakthivel, & Pandian, 2010) to observe the alteration in cell surface morphology. Suspensions of bacterial cultures were achieved by inoculating in MHB at 37°C overnight. Then, bacterial cells were added with 1% (v/v) extracts for 3 h at 37°C. Untreated cell controls were also prepared using the same procedure. After incubation, cells were centrifuged at 6,000×g for 15 min at 4°C and washed twice with 0.1 M phosphate buffered saline (PBS). Then, precipitated cells were fixed with 2.5% (v/v) glutaraldehyde in PBS for 2 h at 4°C. The samples were then dehydrated in an ascending series of alcohol concentrations (30%, 50%, 70%, 80%, 90%, and 100%) for 15 min each. The cells were dried and mounted on aluminum stubs and coated with gold under vacuum. The microscopic examination of prepared samples was performed with a scanning electron microscope (SEM) (JEOL JSM-6610LV, Tokyo, Japan).

2.6 Statistical Analysis

All experiments were performed in triplicate. Obtained data were analyzed using one-way analysis of variance (ANOVA) with Tukey's post hoc test (GraphPad Prism 5; San Diego, USA) for determining significant differences between the mean values of multiple groups. $p < 0.05$ was considered to be a significant statistical difference.

3. Results and discussion

3.1 Antibacterial activity

Antibacterial activity of *P. betle* L. extract (PBE) against *E. coli* and *P. aeruginosa* strains was investigated using agar disc diffusion method shown in Table 1. Ethanol was used as extraction solvent to obtain PBE. This is similar to the previous study which demonstrated that the ethanol extract was effective extraction solvent by giving the large inhibition zones (Kaveti, Tan, Sarnnia, & Baig, 2011). Similarly, Ali, Lim, and Wahida (2018) reported that 95% ethanol was considered as the best extraction solvent of phenolic compounds from *Piper betle*. The result exhibited that different concentration of PBE showed a variable degree of bacterial inhibition. PBE at the concentration of 4 mg/ml revealed significant inhibition of both treated *E. coli* ATCC 25922 and *P. aeruginosa* DMST 37166, which gave the largest diameter of inhibition zones of 18.50 mm and 22.67 mm, respectively. This result was in accordance with the previous report of Simanjuntak, Yuniarni, and Prayugo (2016) who found that ethanolic extract of *P. betle* L. at 1 mg/ml possessed obviously antibacterial property against *P. aeruginosa* with the inhibition zone of 20.12 mm. *P. aeruginosa* DMST 37166 was more sensitive to PBE than *E. coli* ATCC 25922 as indicated by a larger diameter of the inhibition zone. However, the previous

finding of Kaveti et al. (2011) showed that the ethanol extract of *P. betle* L. at 50-100 µg/ml had inhibition zones on *E. coli* larger than those observed on *P. aeruginosa*. This indicated that concentration of extract was the main cause of inhibition zone diameters of these two bacteria. Results suggested that PBE possessed inhibition potential against *E. coli* and *P. aeruginosa* strains.

Table 1 Inhibition zone diameters of PBE against *E. coli* ATCC 25922 and *P. aeruginosa* DMST 37166

Extract	Inhibition zone (mm)	
	***E. coli* ATCC 25922**	***P. aeruginosa* DMST 37166**
PBE (1 mg/ml)	12.33±1.53[b]	16.93±0.51[b]
PBE (2 mg/ml)	13.10±0.83[b]	19.10±0.17[b]
PBE (4 mg/ml)	18.50±1.32[a]	22.67±1.04[a]
Ceftazidime (1µg/ml)	18.93±0.60[a]	21.67±1.04[a]

Values with various letters in the same column are significantly different according to Tukey's post hoc test ($p < 0.05$).

3.2 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determination

The results displayed noticeable MIC and MBC values of PBE against both treated *E. coli* and *P. aeruginosa* strains (Table 2). Furthermore, *E. coli* ATCC 25922 and *P. aeruginosa* DMST 37166 treated with PBE exhibited the same MIC and MBC values at 1 and 2 mg /ml, respectively. In addition, the bacterial strains treated with ceftazidime showed MIC and MBC at 0.5 and 1 mg/ml, respectively. Our findings indicated that the potent inhibitory effects of PBE may be associated with the main bioactive components such as anethole, α-copaene, estragole, eugenol, hydroxybenzoic acid, chavibetol, hydroxychavicol, 4-allylpyrocatechol, linalool, chavicol, and caryophyllene (Madhumita et al., 2019; Singh, Chauhan, Agrawal, & Mendiratta, 2019; Phumat et al., 2018; Patel & Mohan, 2017; Harun, Razak, & Musa, 2014) found in *P. betle* L. Recently, it has been reported that hydroxychavicol showed potent antimicrobial activity by inhibiting biofilm formation against *E.coli* (Thamaraikani & Kulandhaivel, 2017). In addition, *P. betle* extract inhibited biofilm development and extracellular polymeric substance extraction effectively caused by *P. aeruginosa* (Siddiqui, Sakinah, Ismail, Matsuura, & Zularisam, 2012).

Table 2 MICs and MBCs of PBE against *E. coli* ATCC 25922 and *P. aeruginosa* DMST 37166

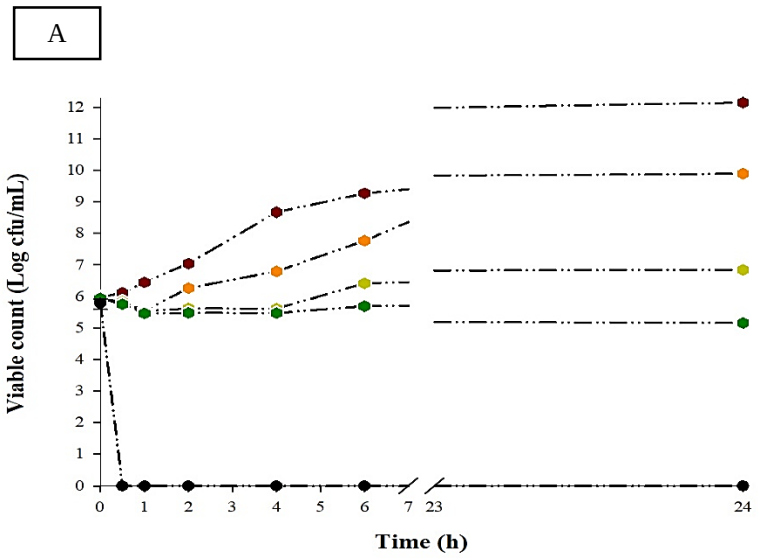
Extract	***E. coli* ATCC 25922**		***P. aeruginosa* DMST 37166**	
	MIC (mg/ml)	**MBC (mg/ml)**	**MIC (mg/ml)**	**MBC (mg/ml)**
PBE	1	2	1	2
Ceftazidime	0.5	1	0.5	1

3.3 Time Killing Assay

The survival rates of *E. coli* ATCC 25922 and *P. aeruginosa* DMST 37166 treated with PBE were determined by time killing assay. The time killing curves were summarized in Figure 1. For both treated bacteria, higher concentration of PBE decreased bacterial cell number more rapidly. Interestingly, *E. coli* ATCC 25922 treated with 2×MIC PBE exhibited the lowest cell counts (1.4×10^5 cfu/ml) after treatment for 24 h (Figure 1A). On the other hand, PBE at 2×MIC reduced cell numbers of *P. aeruginosa* DMST 37166 to around 4.3×10^2 cfu/ml after treatment for 6 h and a complete extermination was observed after treatment for 6 h (Figure 1B). When PBE concentration attained to 4×MIC, the bacterial counts were not found after treatment for 30 min. The results indicated that the growth inhibition of PBE against *P. aeruginosa* was earlier than that of *E. coli*. We assumed that the ions' and nutrients' uptake metabolisms of the treated bacteria might have been deactivated in the presence of PBE as previously shown in *Candida* species (Harun et al., 2014). Furthermore, we also observed that PBE showed a dose- and time-dependent bactericidal effect on the test bacterial strains. Similar finding reported by Phumat et al. (2018) revealed that the killing kinetics of extract from ethyl acetate of *P. betle* against *Streptococcus mutans* and *Streptococcus intermedius* were dose and time dependent. Similarly, Thamaraikani and Kulandhaivel (2017) have reported that killing response against *E. coli* of hydroxychavicol purified from *P. betle* L. extract possessed a time and concentration dependent.

3.4 Observation of bacterial cell morphology by SEM

Scanning electron microscopy (SEM) was employed to observe the morphological changes of *E. coli* ATCC 25922 and *P. aeruginosa* DMST 37166 as shown in Figures 2 and 3, respectively. The untreated cells of *E. coli* ATCC 25922 and *P. aeruginosa* DMST 37166 showed typical morphology with entirely smooth and intact surface which are considered as a normal appearance (Figures 2A and 3A). *E. coli* ATCC 25922 cells treated with 1×MIC of PBE displayed intact surface which was similar to the control (Figure 2B). However, the treated cells of *P. aeruginosa* DMST 37166 with 1×MIC of PBE revealed corrugated surface (Figure 3B). In addition, we found that the cell membrane of *E. coli* ATCC 25922 subjected to 2×MIC of PBE was disrupted and membrane integrity was also completely lost (Figure 2C). Interestingly, the entire cells of *P. aeruginosa* DMST 37166 treated with the same concentration of PBE were fully lysed (Figure 3C). Similar findings were also reported with regard to the effects of *P. betle* L. extract on the morphology of other pathogens. In this regard, the extract contributed to physical alterations and morphological damage of *Candida* cells as well as cell destruction in *Streptococcus mutans* and *Streptococcus intermedius* (Harun et al., 2014). Phumat et al. (2020) confirmed that 4-allylpyrocatechol extracted from *Piper betle* caused bacterial cell membrane destruction of these harmful oral pathogens. This finding suggests that PBE contains bioactive compounds which can suppress the morphological development of the bacterial cells (Fathilah, Yusoff, & Rahim, 2009) and destroy bacterial cells by disrupting cell membrane with its positive charge (Phumat et al., 2018). Eugenol could cause membrane nonspecific permeability and inhibit ions and ATP transports (Nguyen, Nguyen, Nguyen, & Bui, 2020). Recently, Singh, et al. (2018) reported that hydroxychavicol generates oxidative stress inside the *E. coli* cell and further causes the loss of cell viability. It also indicates that hydroxychavicol induces bacterial cell death by generating various reactive oxygen species (ROS) and leading to damage of DNA.



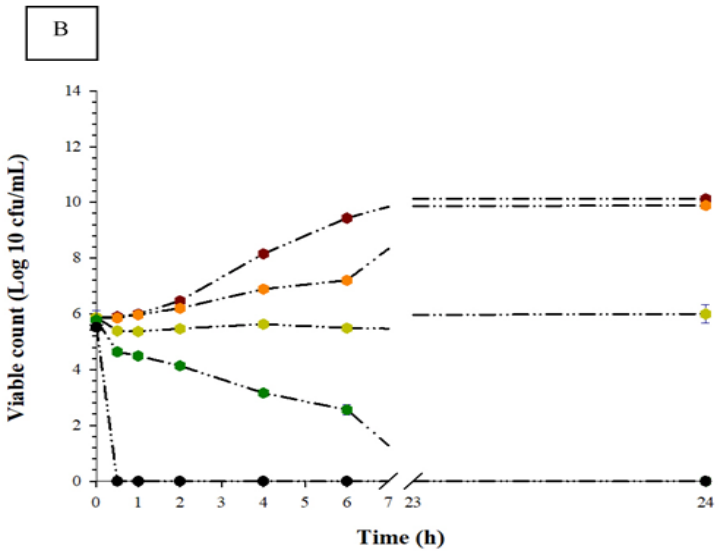


Figure 1 Time killing curve of *E. coli* ATCC 25922 (A) and *P. aeruginosa* DMST 37166 (B) treated with PBE

Control = PBE free; PBE 0.5×MIC = PBE at 0.5 mg/ml;
PBE 1×MIC = PBE at 1 mg/ml; PBE 2×MIC = PBE at 2 mg/ml;
PBE 4×MIC = PBE at 4 mg/ml

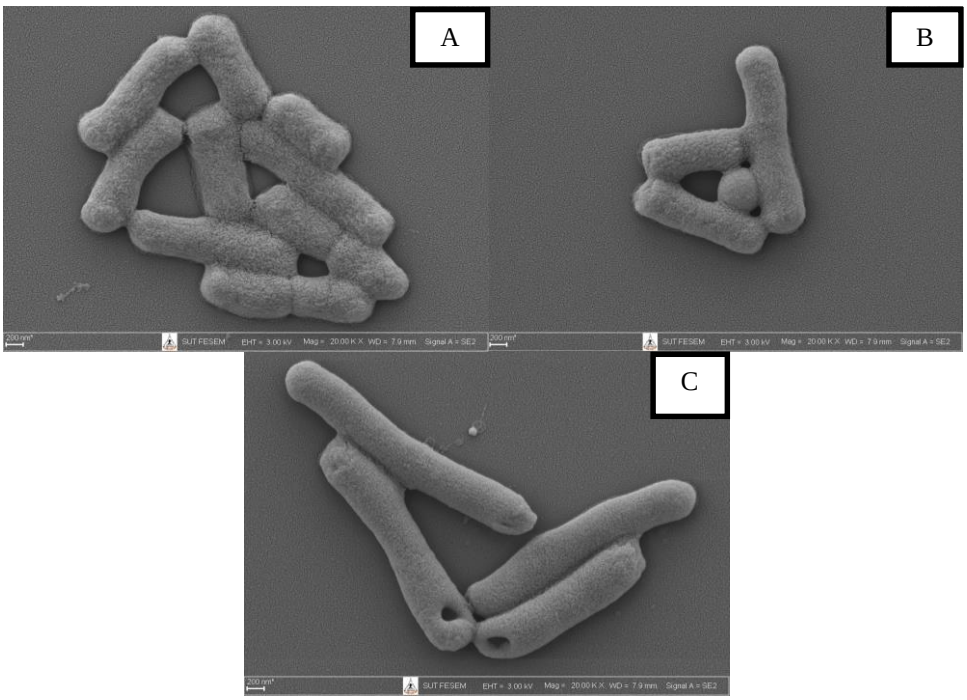


Figure 2 SEM micrographs of untreated (A), and treated *E. coli* ATCC 25922 cells with 1×MIC (B) and 2×MIC (C) of PBE

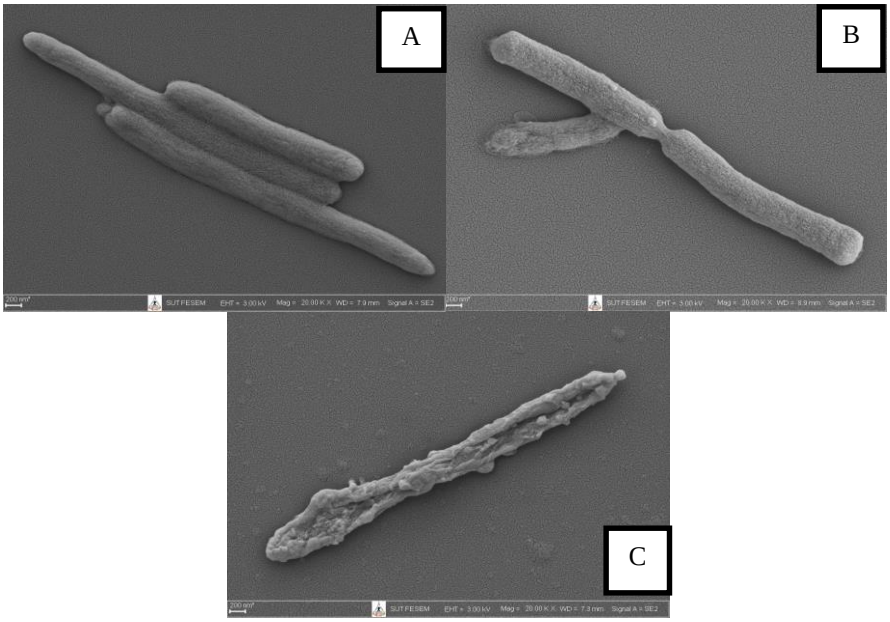


Figure 3 SEM micrographs of untreated (A), and treated *P. aeruginosa* DMST 37166 cells with 1×MIC (B) and 2×MIC (C) of PBE

4. Conclusion

Antibacterial property of the crude extract of *P. betle* L. against *E. coli* and *P. aeruginosa* strains was studied. In addition, SEM was applied to investigate the effect of *P. betle* L. extract on cell morphology of *E. coli* and *P. aeruginosa* strains. The study demonstrates that the ethanolic crude extract of *Piper betle* L. leaves possesses potent antibacterial activity on *E. coli* and *P. aeruginosa*. It was the first report of scanning electron microscopy micrographs of *E. coli* ATCC 25922 and *P. aeruginosa* DMST 37166 cells treated with *Piper betle* L. ethanolic extracts. These findings suggest that *Piper betle* L. has a promising potential to be used as antibacterial agent for therapeutic option to treat infectious diseases caused by *E. coli* and *P. aeruginosa* strains.

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