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Antimicrobial enhancement of red onion crude extract using epsilon-polylysine

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Abstract The antimicrobial activities of red onion extract were investigated by using 50% ethanol solution and combination with epsilon-polylysine against 5 strains of foodborne microorganisms. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidial Concentration (MBC) were determined using Macrobroth dilution method against selected test organism including *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* O157:H7. The MIC of red onion crude extract (ROE) was ranged from 15.00 to 19.00 % (w/v) and the MBC was ranged from 25.00 to 35.00 % (w/v). For epsilon-polylysine (EPL), the MIC was ranged from 0.0150 to 0.0400 % (w/v) and the MBC was ranged from 0.0300 to 0.0650 % (w/v). In addition, the results from the determination of Fractional Inhibitory Concentration Index (FIC_{index}) of the combination between red onion crude extract and epsilon-polylysine using Checkerboard assay against *Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Salmonella* Typhimurium and *Escherichia coli* O157:H7 were 0.50, 0.50, 0.50, 0.50 and 0.75, respectively. These FIC_{index} values indicated the synergistic activity. The study on the efficacy of the combination system were also determined by Time Killing analysis without interfering substances. The result indicated that the antimicrobial activity of combination system was depended on the concentration of red crude extract and epsilon-polylysine. Therefore, synergistic effect of red crude extract and epsilon-polylysine potentially enhances their antimicrobial activities and may be used as an alternative natural antimicrobial material for food preservative.

Keywords: Antimicrobial activity, Epsilon-polylysine, Red onion extract, Isobolograms and synergistic effect

Introduction

Outbreaks of foodborne pathogen disease is a vital facter resulting in food deterioration, shelf-life reduction, and edible value losses (Ye *et al.,* 2013a). The food degradation by microorganism activity during production, storage, and marketing is an important problem in food industries. Recently, the using of chemical presurvatives are limited to use in food products due to residues of harmful chemicals. Therefore, these has been increasing

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interest into utilization of effective natural preservative compounds for improve the safety of food product, which does not affect the nutritional value. Onion is one of the oldest cultivated plants in Allium family which are used for ingredients of many recipes and medical purposes. The onion represented a rich source of antimicrobial active components which has high phenolic and sulphur compounds. The major part of phenolics in onion are flavonoids including mainly quecertin, isorhamnetin tannins and kaempferol (Miean and Mohamed, 2001). In addition, the onion contains organosulfur compounds such as allyl propyl disulfide and diallyl disulfide (Ye *et al.,* 2013a). Onion extract is effective natural antimicrobial against many bacteria species. Sharma *et al.* (2018) have been observed that the red onion extract could inhibit the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus* which mostly due to contains the quecertin compound. In addition, Kabrah *et al.* (2016) have been reported that red onion extract had the antimicrobial effect against *Pseudomonas aeruginosa* and *Klebsiella* Spp., which were depended on concentration of the extract. However, the using only plant extract might not be enough on inhibit the microbial growth, and may be use at the high concentration. Therefore, the binary combination system probably an alternative preservative treatment against microorganisms. Epsilon-polylysine (EPL) is a hydrophilic natural antimicrobial substance which is defined in the generally regarded as safe (GRAS) as an alternative food preservative (Hyldgaard *et al.,* 2014). EPL are water soluble molecules which are nontoxic and biodegradable, and can be apply in many fields of food (Chheda and Vernekar, 2015). The EPL molecules are cationic peptide, surface active component due to their positively charged amino groups which exhibits a broad spectrum of antimicrobial activity (Najjar *et al.,* 2007). The activity of EPL against microbial has been due to its cationic charge adsorb into negative charge of microbial membrane surface, lead to disrupt the cell envelope (Hyldgaard *et al.,* 2014). Therefore, EPL has been used as a preservative material in several food products such as cooked rice, bread, surimi, chilled pork, yogurt, blueberry juice, and other food (Tuersuntuoheti *et al.,* 2019).

In this research, the antimicrobial possiblity effect of the combination system between red onion crude extracts and epsilon-polylysine against foodborn pathogen microorganisms was designed. Therefore, the objective of this study was to investigate the antimicrobial of red onion crude extracts and epsilon-polylysine in combination system against foodborne pathogen, by determined the MIC, MBC, MIC isobolograms, fractional inhibition concentration (FIC_{index}) and Time Killing Analysis.

Materials and methods

Material and tested microorganisms

Fresh red onion was purchased from a wholesale market named Talaad Thai, PathumThani, Thailand. Epsilon-polylysine (ε-polylysine) was purchased from Taster Pro Limited Partnership, Thailand. Phosphatidylcholine and cholesterol were purchased from Sigma-Aldrich, USA. *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* DMST 17303, *Salmonella* Typhimurium ATCC 13311, and *Escherichia coli* O157:H7 DMST 12743were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand. Each microorganism was activated in 50 mL of Tryptic soy broth (TSB; Difco, USA) and incubated at 37°C for 18 h before using.

Preparation of red onion crude extract

Fresh red onions were peeled and cut into small cubes and dried at 60ºC using tray dryer for 10 h. After that, the dried red onion were crushed into powder (80 mesh). For extraction, 25 g of dried red onion powder was extracted with 100 ml of 50% ethanol solution for 24 h which was stirred throughout the extraction period using overhead stirrer (IKA, RW20 digital, Germany). Afterwards, the liquid phase was evaporated to collect crude extracted using rotary evaporator (IKA, RV10, Germany) at 40°C. Finally, the red onion crude extracted were kept in sterile bottle at 4±1ºC.

Determination of the antimicrobial properties of red onion crude extract and epsilon-polylysine by studying the Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial ingredient or agent that is bacteriostatic (prevents the visible growth of bacteria). The MIC of red onion extract (ROE) and epsilon-polylysine (EPL) against the test organisms were determined using the method described in the DVG guidelines (Hunsinger, 2005). Different concentrations of the ROE and EPL were differently prepared by serial dilutions in the Trypticase Soy Broth. Each tube was inoculated with 100 µL of each bacterial strain (1.0×10^6 CFU/mL). Two blank TSB tubes, with and without bacterial inoculation, were used as the growth and sterility controls. The bacteria-containing tubes were incubated at 37ºC for 24 h. After the incubation, the tubes were detected the MICs by observation the visible of growth. The first tube in the series with no visible growth after the incubation period was taken as the MIC.

Determination of the antimicrobial properties of red onion crude extract and epsilon-polylysine by studying the Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) of red onion crude extracts and epsilon-polylysine against the test organisms were determined using a series of steps, undertaken after a MIC test has been completed. One loop full of each tube was transferred on a Trypticase Soy Agar plate. The bacteria-containing plates were incubated at 37ºC for 24 h. After the incubation, the MBC were detected by checking the agar that showed no visible colony growth.

Determination of the Isobolograms of red onion crude extract and epsilon-polylysine combinations

The MIC values from the checkerboard assay were plotted as MIC isobolograms which represented the antimicrobial interactions between ROE and EPL. The concentrations of the ROE and EPL are plotted on the x- and y-axis, and the MICs of each component alone are plotted on the graph which are joined by a dashed line. After that, the mixed MICs of ROE and EPL are plotted and compared with the dashed line (Najjar *et al.,* 2007).

Determination of the Fractional Inhibitory Concentration Index (FIC_{index})

The Fractional Inhibitory Concentration Index were determined using checkerboard assay according to Shang *et al.* (2019) with some modification. The concentrations of ROE and EPL were diluted to 0.00, 0.25-, 0.50-, 1.00- and 1.50- fold serial dilutions of the MICs. The FIC_{index} was calculated with the following equation.

$$FIC_{index} = FIC_{ROE} + FIC_{EPL}$$

Where:

$$FIC_{ROE} = (MIC_{(ROE \text{ in the presence of EPL})})/(MIC_{(ROE)})$$

$$FIC_{EPL} = (MIC_{(EPL \text{ in the presence of ROE})})/(MIC_{(EPL)})$$

Among of the FIC_{index} calculated for all isoeffective combinations, the FIC_{index} of < 1 indicates synergism effects, $= 1$ indicates additive effects, > 1 indicates antagonism effect (van Vuuren *et al.,* 2009).

Determination of the antimicrobial activity by studying the Time Killing Analysis without interfering substances

Time Killing of red onion crude extract and epsilon-polylysine were determined using the method of Appiah *et al.* (2017) with some modification. The 5-strain of microorganisms were stimulated in 50 ml of

Trypticase Soy Broth and were incubated at 37ºC for 18 hr. Afterwards, they were centrifuged at 4000g for 15 min and washed cell pellets with phosphate buffer solution for 2 times. Diluted cell pellet with a phosphate buffer solution to 1.0×10^6 CFU/ml. The final concentration of ROE and EPL solutions were defined to at 0.00, 5.00, 10.00, 15.00 and 20.00 % (w/v) of ROE and 0.00, 0.01, 0.02, 0.03 and 0.04 % (w/v) of EPL. Inoculum size of 1.0×10^6 CFU/mL was added and incubated at 37 ºC. 1.0 mL of the mixture were taken at time intervals of 0_A, 0_B, 30, 60, 120, 180, 240 and 360 min and inoculated aseptically into TSA using Spread Plate Technique and incubated at 37°C for 24 h. (0_A is an initial test time and 0_B is a contact time within 15 sec). Control was performed for the organisms without the antimicrobial agents. The colony forming unit (CFU) of the organisms was determined. The procedure was performed in triplicate (three independent experiments) and a graph of the $\log_{10}$ CFU/mL was plotted against time.

Results

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of red onion crude extract

Figure 1A showed the results of antimicrobial activity of red onion crude extract against the strains of *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmanella* Typhimurium and *Escherichia coli* O157:H7. The ROE inhibited all tested microorganism at different concentration. The lowest MIC value was 15.0 % (w/v), which was found in *Bacillus cereus* and *Listeria monocytogenes*. The hightest was 19.0 %w/v obtained in *Salmanella Typhimurium* and *Escherichia coli* O157:H7. In addition, the lowest concentration of ROE which killed the tested microorganism was presented in MBC values. The MBC of REO against three gram-positive tested (*Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes*) were 30.0 – 35.0 % (w/v) and two gram-negative (*Salmanella* Typhimurium and *Escherichia* coli O157:H7) were 40.0 % (w/v). This result indicated that the ROE expressed the better antimicrobial activity against gram-positive than gram-nagative tested mocroorganisms.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of epsilon-polylysine

The antimicrobial activity of epsilon-polylysine against the strains of *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Escherichia coli* O157:H7 was showed in Figure 1B. The EPL had activity against all tested microorganism with the MIC range from 0.0150 to 0.0400 % (w/v). The lowest MIC value was

found in *Bacillus cereus* which is gram-positve bacteria. The hightest value was found in gram-negative bacteria which is *Escherichia coli* O157:H7. In addition, the EPL were destroyed the tested microorganisms at the low concentration around 0.0300-0.0650 % (w/v) which was showed in MBC values. The lowest MBC value was found in *Bacillus cereus*, and the hightest value was found in *Escherichia coli* O157:H7. This result indicated that the gram-negative tested bacteria also demonstrated resistance to the EPL as well as using ROE.

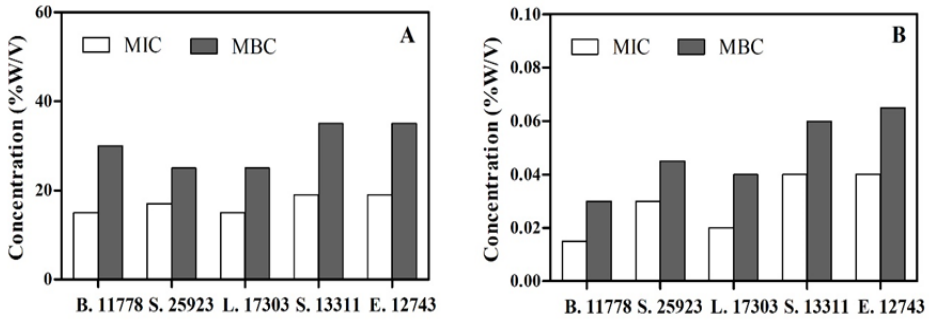

Figure 1. Minimum inhibitory concentrations and Minimum bactericidal concentrations of red onion crude extract (A) and epsilon-polylysine (B) against difference test organisms (B. 11778: *Bacillus cereus*; S. 25923: *Staphylococcus aureus*; L. 17303: *Listeria monocytogenes*; S. 13311: *Salmonella* Typhimurium; E. 12743: *Escherichia coli* O157:H7)

Minimum inhibitory concentration (MIC) isobologram of red onion crude extract and epsilon-polylysine combinations

Figure 2 presented the MIC isobolograms which were demonstrated the interaction of the combinations between red onion crude extract and epsilon-polylysine against foodborn pathogen. On the isobolograms, the synergistic effect was indicated by the mixed MICs fall below the dashed line. The mixed MICs above the line represent the antagonistic effect, and on the dashed line marks as no interaction or additive effect. The synergistic effect was obsevered in the ROE and EPL combinations system against all tested microorganisms including *Bacillus cereus*, *Staphylococcus aureus, Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* O157:H7, which indicated by isobolograms with MIC points falling to reference line (Figure 2A-2D).

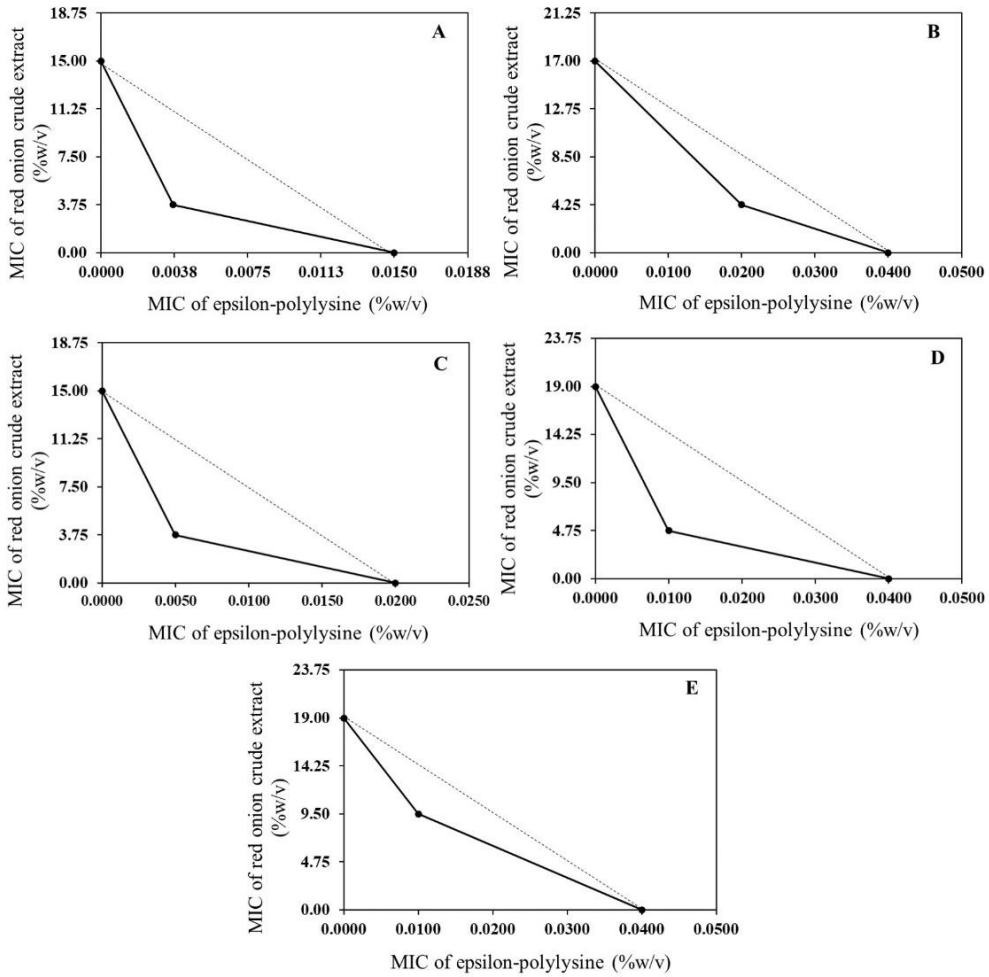

Figure 2. Minimum inhibitory concentration (MIC) isobologram of red onion crude extract and epsilon-polylysine combination against *Bacillus cereus* (A), *Staphylococcus aureus* (B), *Listeria monocytogenes* (C), *Salmonella* Typhimurium (D) and *Escherichia coli* O157:H7 (E)

Fractional Inhibitory Concentration Index (FIC_{index}) of red onion crude extract and epsilon-polylysine combinations

The Fractional Inhibitory Concentration Index (FIC_{index}) is commonly used to explain the effect of antimicrobial combinations. The combinations effect of red onion crude extract and epsilon-polylysine were showed in Table 1 which were correlated with the MIC isobolograms (Figure 2). MICs of ROE and EPL in the combinations system showed antimicrobial potential better than ROE or EPL alone. The MICs of ROE in the combinations system against *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* O157:H7 were 3.75, 4.25, 3.75, 4.75 and 9.50, respectively. The MICs of EPL in the

combinations system against the tested microorganisms were 0.0037, 0.0200, 0.0050, 0.0100 and 0.0100, respectively. The FIC_{index} was calculated which equal to 0.50 in 3 strains of microorganism, including *Bacillus cereus*, *Listeria monocytogenes*, and *Salmonella* Typhimurium, which were indicated the synergistic activity. For *Staphylococcus aureus* and *Escherichia coli* O157:H7, the FIC_{index} were calculated which equal to 0.75, which were also considered to has the synergistic activity. The additive and antagonism effect were no found against microorganisms tested.

Table 1. Fractional inhibitory concentration index of combinations of red onion crude extract and epsilon-polylysine

Bacterial strains	MIC (% w/v) of combinations		FIC		FIC_{index}
	ROE	EPL	ROE	EPL	
B. cereus	3.7500	3.7×10^{-3}	0.25	0.25	0.50
S. aureus	4.2500	2.0×10^{-2}	0.25	0.50	0.75
L. monocytogenes	3.7500	5.0×10^{-3}	0.25	0.25	0.50
S. Typhimurium	4.7500	1.0×10^{-2}	0.25	0.25	0.50
E. coli	9.5000	1.0×10^{-2}	0.50	0.25	0.75

Time Killing Analysis of the combinations of red onion crude extract and epsilon-polylysine without interfering substances

Figure 3 showed the time kill curves of red onion crude extract and epsilon-polylysine combinations system against 5 difference tested microorganisms varied with the concentration and contact time. In control groups of all tested microorganisms, the initial microbial population were around 6.03-6.52 $\log_{10}$ CFU/ml which were remained constant over the period time. Figure 3A showed the reduction cruve of *Bacillus cereus* at time intervals of 0-360 min with difference concentrations. At the present of 5.00 %w/v ROE and 0.01 %w/v EPL combinations, the population was reduced from 6.03 to 1.94 $\log_{10}$ CFU/ml within 360 min. In addition, the tested microorganism was decreased to undetectable level when they contacted with 10.00 %w/v ROE and 0.02% EPL within 360 min. At the present of 20.00 %w/v ROE and 0.04 %w/v EPL, the population of *Bacillus cereus* was significantly decreased in 30 min and were decreased to undetectable level in 60 min. The time kill curve of *Staphylococcus aureus* in Figure 3B was noticeably difference with the *Bacillus cereus*. At the present of 5.00 %w/v ROE and 0.01 %w/v, the population was slightly decreased from 6.34 to 4.56 $\log_{10}$ CFU/ml in 360 min. The population of *Staphylococcus aureus* was reduced to undetectable level at 15.00 % ROE and 0.03% EPL, and 20.00 % ROE and 0.04% EPL combinations in 360 and 120 min, respectively. Figure 3C showed the population of *Listeria monocytogenes*, the population was reduced from 6.52 to 2.85 $\log_{10}$ CFU/ml

in 360 min when using 10.00 %w/v ROE combined with 0.02 %w/v EPL. At the present of 20.00 %w/v ROE and 0.04 %w/v EPL, the *Listeria monocytogenes* was reduced to undetectable level within 60 min which was similar to the killed time of *Salmonella* Typhimurium in Figure 4D. For the time kill curve of *Escherichia coli* O157:H7 in Figure 4E, the reduction trend was similar to the time kill cruve of *Staphylococcus aureus* in Figure 4B.

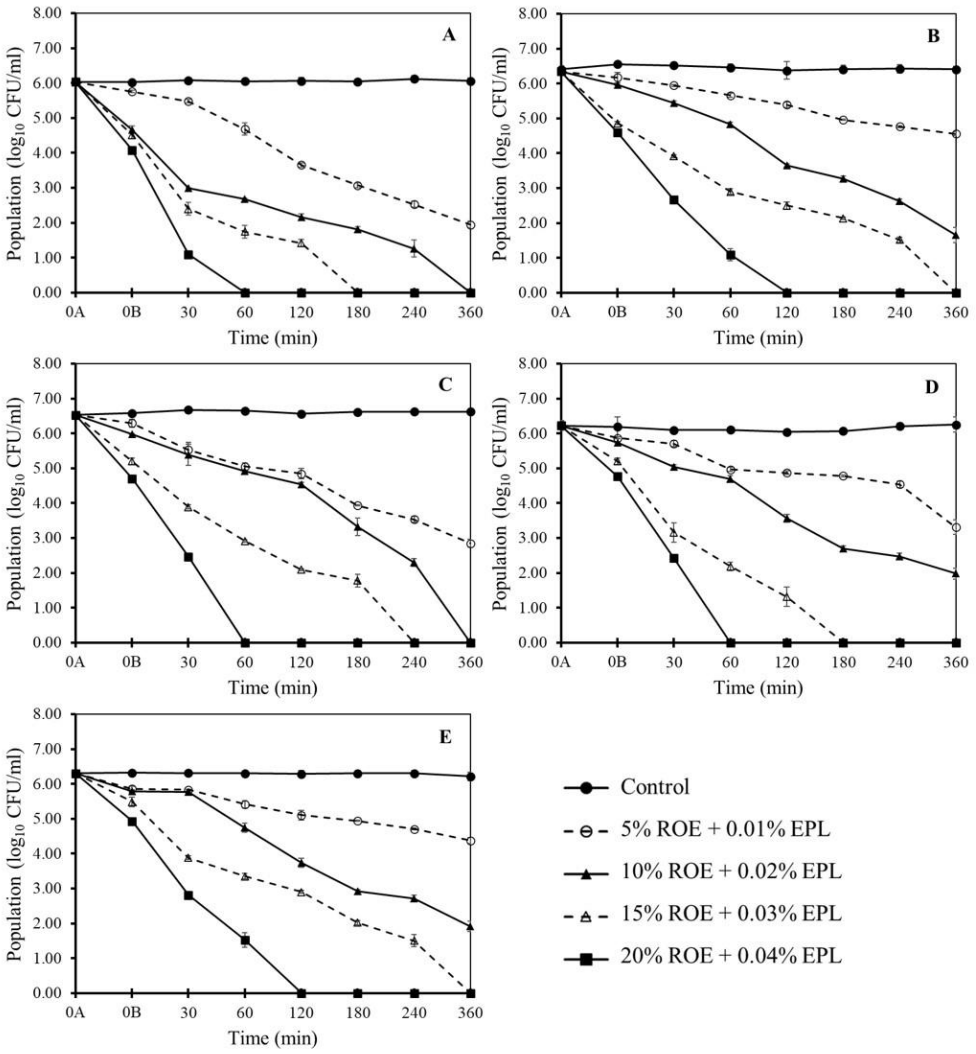

Figure 3. Time-kill curves of *Bacillus cereus* (A), *Staphylococcus aureus* (B), *Listeria monocytogenes* (C), *Salmonella* Typhimurium (D) and *Escherichia coli* O157:H7 (E) exposed to different concentrations of red onion crude extract combined with epsilon-polylysine without interfering substances

Discussion

Red onion extract had antimicrobial activity against microorganisms. The important bioactive compounds of onion are phenolics and flavonoids, mainly quercetin which shows a high antibacterial activity (Santas *et al.,* 2010). Previous reports suggested that the red onion contains higher amounts of total phenolic compounds than white and yellow varieties which are related to the potential of antioxidant activity (Elhassaneen and Sanad, 2009). Comparing among tested 5-strain of microorganisms, three strains gram-negative bacteria were more resistance to red onion crude extract than two strains gram-positive bacteria. This result was supported by a previous study on onion against gram-negative and gram-positive bacteria using a modified Kirby–Bauer disc diffusion method. They found that the red onion extract had the highest zone of inhibition for gram-positive bacteria while the inhibition zone of gram-negative bacteria was found relatively smaller (Sharma *et al.,* 2018). It was informed that phenolic compounds might incorporate into the lipid monolayers of gram-positive microorganism led to increase the membrane fluidity. In addition, phenolic compounds might interrupt the lipid–protein complexes and increase membrane permeability, affecting their physiology and metabolism resulting in cell death (Wang *et al.,* 2018).

In addition, epsilon-polylysine was also exhibited antimicrobial activity against all tested miroorganisms. Ye *et al.* (2013b) have been report that the antimicrobial mechanism of epsilon-polylysine against *Escherichia coli* O157:H7 might be attributed to disturbance on membrane integrity and effects on various gene expressions, such as regulation of oxidative stress and changes in virulence. The mode of action of epsilon-polylysine have been clarify by Zhilei *et al.* (2019) which was studied on metabolite properties of *Staphylococcus aureus*. The result indicated that epsilon-polylysine interrupted the cell integrity, disrupted the cytoplasmic membrane, and induced the structure change of peptidoglycan in cell wall, resulting in cell leakage. These mechanisms were supported by Zhang *et al.* (2018), they demonstrated that the epsilon-polylysine against *Escherichia coli* due to change the permeability and integrity of cell membrane, and change in ultrastructure which investigated by SEM and TEM analysis.

In current study, the combination system of red onion crude extract and epsilon-polylysine showed the synergistic antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* o157:H7. The synergistic activity of red onion crude extract and epsilon-polylysine combinations migh be due to some mechanisms of red onion and epsilon-polylysine have difference mode of action on antimicrobial. Many reserchers have been found the similar phenomenon of antimicrobial combinations. For example, Zahi *et al.* (2017) reported that the D-limonine and epsilon-polylysine

combinations exhibit the synergistic effect against *Escherichia coli* and *Bacillus subtilis*. Najjar *et al.* (2007) have been studied the combination of Nisin A and epsilon-polylysine, the result showed synergistic activity against *Bacillus cereus* and *Listeria monocytogenes*. The mechanism of the nicin and epsilon-polylysine combinations against *Bacillus subtilis* have been explained by Liu *et al.* (2015). They indicated that the bacterial cell treated with the combinations system were markedly damaged which observed by SEM analysis, suggesting that the ¼ MIC of nisin damaged the cell structure, promoting the uptake of epsilon-polylysine into the cell and leaking of the protoplasm through the pores to the exterior of the cell, eventually leading in cell death.

The time killing analysis of all tested microorganisms indicated that the potential of the combination system to killed the microbial was depended on the concentration of red crude extract and epsilon-polylysine. Zhang *et al.* (2018) confirmed that the bacterial cell membrane was significantly damaged when the concentration of epsilon-polylysine was increased. Overall, the antimicrobial activity of red onion crude extract and epsilon-polylysine in combination system was better than the using red onion crude extract or epsilon-polylysine alone. The synergistic effect of the combination system has contributed to the enhancement of the antimicrobial activity. These results indicate that the combination system between red onion crude extract and epsilon-polylysine could be apply into a food product which can be used for preservative materials.

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