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Antimicrobial susceptibility of chili extracts against foodborne pathogens and food related bacteria

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Abstract The comparative evaluation of antimicrobial activities of chili crude extract was inestigated using aqueous, 50 and 95% (v/v) ethanol. Chili crude extract demonstrated inhibitory potential against 25 tested microorganisms, including foodborne pathogens and food-related bacteria. The chili crude extract using 95% (v/v) ethanol solution had the highest efficacy on microbial inhibition. Time-killing analysis were evaluated. The lowest MIC and MBC of 25 bacterial stains was found in *Vibrio cholerae* DMST 9700 at 0.5% and 1.0% respectively. According to the Time killing analysis, the results indicated that the completed destruction phenomenon of bacterial mixture was detected at the concentration of more than 10.0%w/v of chili crude extract.

Keywords: Antimicrobial, Foodborne, Chili extract, Soxhlet, Time killing

Introduction

Recently, food safety has become an important concern to both food industries and consumers. It can be determined that, spoilage and a decrease in quantity and quality might be caused by the presence and growth of contaminated microorganisms. In addition, the emergence of foodborne pathogens has lately turned out to be a major public health concern. Contamination of foodborne pathogens such as *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus* caused around 76 million food poisoning incidences in the United States in 2010. Furthermore, approximately 9.4 million people were infected, resulting in leading causes of death (Scallan *et al.,* 2011). There are several techniques applied for contamination reduction of foodborne pathogens, including thermal processing, cooling and freezing, and even the use of chemical preservatives. However, the mentioned techniques affected the physical characteristics and certain sensory characteristics of food, which presented a direct impact on consumer acceptance (Oh *et al.,* 2017). High quality, nutritious, safe and long shelf-life food products without chemical

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preservative agents are highly preferred by most consumers. The people who require the non-toxic product or natural preservatives has been increasing awareness and reports of illness by synthetic chemicals present in foods.

Many plants are increasingly considered in several fields of research, and many report in Asia (Sinclair, 1998). In Thailand, such plants are common in use for many purposes and are known to possess antibacterial properties (Voravuthikunchai *et al.,* 2002). Natural compounds present in plants and other natural products have been reported to have antibacterial activities and could serve as a source of antibacterial agents in food (Conner and Beuchart, 1984; Zaika, 1988; Dorman and Deans, 2000; Bagamboula *et al.,* 2003 Moreover, their components such as essential oils are known to be antibacterial agents against microorganisms, including Gram-negative bacteria (Helander *et al.,* 1998, Sivropoulou *et al.,* 1996). The information could indicate that they may be applied in controlling pathogens in food (Delaquis and Mazza, 1995; Bowles and Juneja, 1998). The report of Pinilla and Brandelli shows that garlic extracts could inhibit *Listeria monocytogenes*, *Salmonella Enteritidis*, *Escherichia coli*, and *Staphylococcus aureus* (Pinilla and Brandelli, 2016). The use of ethanolic extract from Psidium guajava, Phyllanthus niruri, Ehretia microphylla and Piper betle presented antimicrobial activity on gram-positive bacteria, Methicillin-resistant *Staphylococcus aureus* and Vancomycin-resistant *Enterococcus* sp. Piper betel showed the highest antimicrobial activity with disk diffusion (16–33 mm). The minimum inhibitory concentration (MIC) was 19–156 mg/mL and the minimum bactericidal. concentration (MBC) was 312 mg/mL (Valle *et al.,* 2015).

Chili (*Capsicum annuum* L.) is a widely used species in traditional Thai cuisine. The flavor and pungent power of these vary greatly, as do their capsaicin and capsaicinoid analog contents (Dorantes *et al.,* 2000). There was a report about extracts from *Capsicum annuum* L. that had been investigated for antimicrobial properties. It has been reported with mixed results. Crude extracts from several different *Capsicum annuum* L. varieties could inhibit *Bacillus* sp., *Clostridium* sp., *Pseudomonas* sp., *Listeria* sp., *Salmonella* sp., *Staphylococcus* sp., and *Streptococcus* sp. (Bacon *et al.,* 2017). Organic solvent extracts of capsaicin such as acetone and acetonitrile have antimicrobial properties. It could inhibit *Klebsiella pneumonia* and *Staphylococcus aureus* (Gayathri *et al.,* 2016). Moreover, the n-hexane and chloroform chili extracts could inhibit *Pseudomonas aeruginosa, Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Candida albicans* (Gurnani *et al.,* 2016). However, the extract solvents used in previous studies were toxic. Therefore, nonhazardous extraction solvents should be considered.

This study was undertaken to determine the antibacterial activity of Chili (*Capsicum annuum* L.) to extend shelf-life or promote safety in the food industry by investigating the in vitro antibacterial activity of water,

50% ethanolic and 95% ethanolic extracts. A dilution test for determination of the MIC values was performed according to the method cited by DVG (Deutsche Veterinaermedizinische Gesellschaft; German Veterinary Medicinal Association). Suspension tests were performed modified according to EN 1040 using 25 bacterial strains, which are commonly found in food and can cause either foodborne illness or food spoilage.

Materials and methods

Bacterial strains and culture conditions

Stock cultures of *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Escherichia coli* DMST 703, *Escherichia coli* O157:H7 ATCC 12743, *Enterococcus faecalis* ATCC 2860, *Listeria monocytogenes* DMST 17303, *Listeria monocytogenes* DMST 21164, *Listeria monocytogenes* DMST 23708, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas fluorescens* ATCC 13525, *Staphylococcus aureus* ATCC 25923, *Salmonella* Choeraesuis ATCC 8614, *Salmonella* Enteritidis ATCC 17368, *Salmonella* Paratyphi A ATCC 8486, *Salmonella* Paratyphi B var. Java ATCC 28118, *Salmonella* Typhi ATCC 22842, *Salmonella* Typhimurium ATCC 13311, *Salmonella* Virchow ATCC 32758, *Shigella dysenteriae* ATCC 2137, *Shigella flexneri* ATCC 4423, *Shigella sonnei* ATCC 561, *Vibrio cholerae* DMST 16261, *Vibrio cholerae* DMST 9700, *Vibrio parahaemolyticus* ATCC 17802 and *Yersinia enterocolitica* ATCC 27736 were provided by the Laboratory of the Department of Medicinal Science, Ministry of Public Health, Thailand. All test organisms were maintained in Trypticase Soy Broth (TSB; Difco, USA) with cryoprotectant at -80 ℃. For preparation of the inoculum. After that, each test organism suspension was stroked out on Trypticase Soy Agar (TSA, Difco, USA) and incubated at 37 ℃ for 24 hours. A single colony was transferred to TSA slants. These stock cultures were kept at 4 ℃. For use in experiments, take a loop on test organism was transferred to 50 mL of TSB, and incubated for 18 hours at 37 ℃. This culture was used for antibacterial assay.

Extract preparation

Chili (*Capsicum annuum* L.) was purchased from the local market in PathumThani. Dried samples were washed in hypochlorite solution, sliced and air-dried. Those were ground to powder using a mechanical grinder, and kept separately in plastic bags and dry until use. 60 grams of chili powder were added to the Soxhlet extractor for extraction. 200 mL of 95%, 50% (v/v) ethanol and distilled water were used as extraction solvents. The extraction time was 6 hours. Crude extracts were evaporated by a Rotary

evaporator. The remaining extracts were stored at 4+2 ℃ in a light brown bottle.

Determination of the minimum inhibitory concentrations (MIC)

A broth dilution susceptibility assay was performed using the DVG: Deutsche Veterinärmedizinische Gesellschaft (Hunsinger, 2005) method for the determination. Briefly, all crude chili extracts were dissolved in sterile water of standardized hardness (WSH; Wasser standardisierter Härte, 300 ppm). Then, 2.0 mL of dilution was transferred to sterile tubes containing 2.0 mL of Trypticase Soy Broth (double concentrated), resulting in the desired final concentration. To each tube, 0.1 mL of suspension test organism (equivalent to 6.0 Log_{10} CFU/mL) was added. Tubes were incubated for 72 hours at 37 ℃. Each test was carried out in duplicate and in three repetitions. The lowest concentration of each extract that inhibited visible growth was determined as the Minimum Inhibition concentration (MIC).

Determination of minimum bactericidal concentration (MBC)

Referring to the results of the MIC assay, all tubes showing a complete absence of growth were identified. One loop full of each tube was transferred to the TSA plate. All plates were incubated at 37 ℃ for 24 hours. The complete absence of growth was considered as the Minimum Bactericidal Concentration (MBC).

Visual appearance measurement

The color attributes of chili extract include L*, a*, b* were determined using Colorimeter CX2687 (Colorflex Co., USA).

Time killing analysis

The antimicrobial activity of chili was determined against selected test organisms including *E. coli* O157:H7 ATCC 12743, *S. Enteritidis* ATCC 17368, *S.* Typhi ATCC 22842, *S.* Typhimurium ATCC 13311, and *S. dysenteriae* ATCC 2137. 95% ethanolic extracts demonstrating the highest activity in the dilution test, were chosen and the test method was performed modified according to the "Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics" (EN 1040). 1 mL of a bacterial test suspension containing 108-109 CFU/mL was added to 9.0 mL of the 95% ethanolic extract test concentrations (20.0, 15.0, 10.0, and 5.0 g/100 mL) and kept at room temperature for the chosen contact times. Survivors were monitored at intervals time by taking a

sample, preparing serial dilutions and plating out on TSA. 0A was the determination of the viable counts in the microbial test suspension and 0B was the contact time of 15 seconds. Plates were incubated at 37℃ for 24 hours before counting. The rate of reduction (k) was accordinly calculated to equation 1.

$$N_t = N_0 e^{-kt} \quad (1)$$

N_t = the number of microbes on t
N_0 = the number of initial microbes
k = a constant
t = time

Results

Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) of chili extract

The inhibitory efficiency of chili crude extracts extracted by different method had different potentials for the inhibition of microorganisms. Figure 1 showed a Minimum Inhibitory Concentration of chili extract with different extraction methods. It was observable that the used of 95% (v/v) ethanol as extracted solvent, presented the highest antimicrobial properties compared to others. The MIC of 50% ethanolic extract and aqueous extract was ranged between 3.0% to 41.0% w/v and 2.5% to 41.0% w/v, respectively. However, 95% ethanolic extract demonstrated the MIC of approximately 0.5% to 16% w/v. The Vibrio group was the least resistant to the chili extract. On the other hand, *Y. enterocolitica* was the most durable to extract.

Table 1. L*, a*, b* of chili extract with different solvent

Color parameter	EtOH-95	EtOH-50	Water
L*	0.84	2.36	4.63
a*	0.16	5.20	11.79
b*	0.85	2.68	7.59

Table 1 demonstrates the physical appearance of chili extract. The 95% ethanolic extraction of chili showed a dark color more than 50% ethanolic extract and water extract. Water extract presented high L*, a*, and b*. These results resulted according to the dissolvable of pigment in chili powder. The red pigment was more dissoluble in water than ethanol.

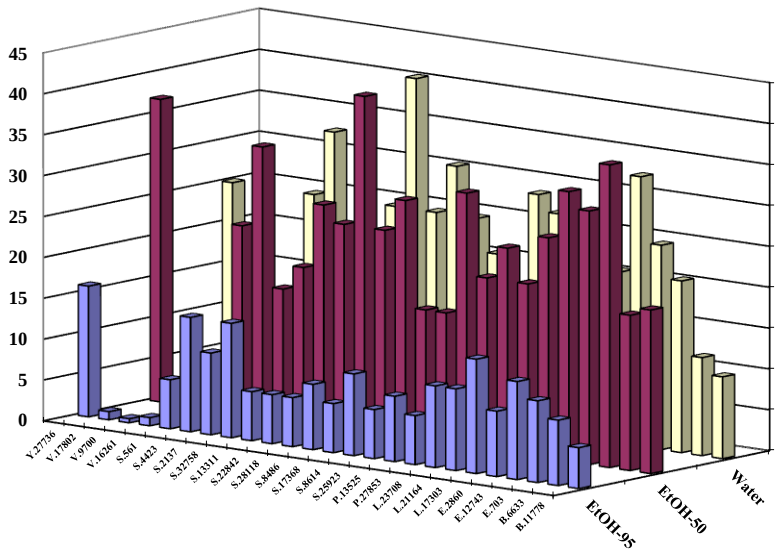

Figure 1. Minimum inhibitory concentration (MIC) of chili extract with different extraction solvent (*B. cereus* ATCC 11778; B.11778, *B. subtilis* ATCC 6633; B.6633, *E. coli* DMST 703; E.703, *E. coli* O157:H7 ATCC 12743; E.12743, *E. Faecalis* ATCC 2860; E.2860, *L. monocytogenes* DMST 17303; L.17303, *L. monocytogenes* DMST 21164, L.21164, *L. monocytogenes* DMST 23708; L.23708, *P. aeruginosa* ATCC 27853; P.27853, *P. fluorescens* ATCC 13525; P.13525, *S. aureus* ATCC 25923; S.25923, *S. Choeraesuis* ATCC 8614; S.8614, *S. Enteritidis* ATCC 17368; S.17368, *S.* Paratyphi A ATCC 8486; S.8486, *S.* Paratyphi B var Java ATCC 28118; S.28118, *S.* Typhi ATCC 22842; S.22842, *S.* Typhimurium ATCC 13311; S.13311, *S.* Virchow ATCC 32758; S.32758, *S. dysenteriae* ATCC 2137; S.2137, *S. flexneri* ATCC 4423; S.4423, *S. sonnei* ATCC 561; S.561, *V. cholerae* DMST 16261; V.16261, *V. cholerae* DMST 9700; V.9700, *V. Parahaemolyticus* ATCC 17802; V.17802, *Y. Enterocolitica* ATCC 27736; Y.27736)

The minimum bactericidal concentrations (MBC) of 95% ethanolic extract were also determined for the entire susceptible organisms and it had ranged from 1% to 25% w/v. MBC of 50% ethanolic extract and aqueous extract were 3.5% to 50% w/v and 3% to 50% w/v, respectively. It represented in (Figure 2.). MBC of all microorganisms was higher than MIC. Chili extract with ethanol 95% still demonstrated the best antimicrobial efficacy.

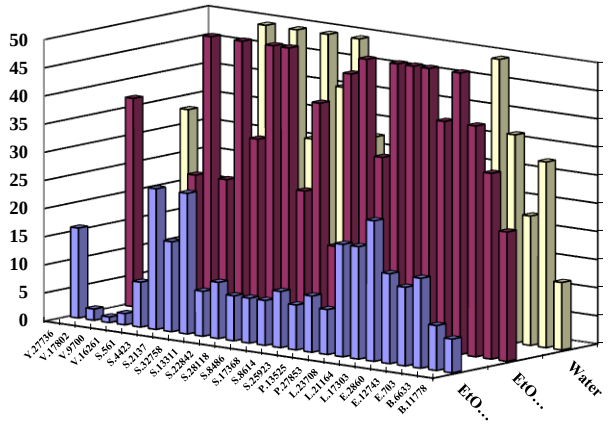

Figure 2. Minimum bactericidal concentration (MBC) of chili extract with different extraction solvent (*B. cereus* ATCC 11778; B.11778, *B. subtilis* ATCC 6633; B.6633, *E. coli* DMST 703; E.703, *E. coli* O157:H7 ATCC 12743; E.12743, *E. Faecalis* ATCC 2860; E.2860, *L. monocytogenes* DMST 17303; L.17303, *L. monocytogenes* DMST 21164, L.21164, *L. monocytogenes* DMST 23708; L.23708, *P. aeruginosa* ATCC 27853; P.27853, *P. fluorescens* ATCC 13525; P.13525, *S. aureus* ATCC 25923; S.25923, *S.* Choeraesuis ATCC 8614; S.8614, *S.* Enteritidis ATCC 17368; S.17368, *S.* Paratyphi A ATCC 8486; S.8486, *S.* Paratyphi B var Java ATCC 28118; S.28118, *S.* Typhi ATCC 22842; S.22842, *S.* Typhimurium ATCC 13311; S.13311, *S.* Virchow ATCC 32758; S.32758, *S. dysenteriae* ATCC 2137; S.2137, *S. flexneri* ATCC 4423; S.4423, *S. sonnei* ATCC 561; S.561, *V. cholerae* DMST 16261; V.16261, *V. cholerae* DMST 9700; V.9700, *V. Parahaemolyticus* ATCC 17802; V.17802, *Y. Enterocolitica* ATCC 27736; Y.27736)

The results demonstrated a wide range of activities of chili extracts against tested organisms. Minimum Inhibition Concentration (MIC) and Minimum Inhibition concentration (MBC) of chili were determined by broth dilution assay cited by DVG. MIC was defined as the lowest concentration that completely inhibited the growth up to 24 hrs. (Hammer *et al.,* 1999; Delaquis *et al.,* 2002). As the same concept, MBC was defined as the lowest concentration at which no growth was observed after incubating 48 hrs. and up to 5 days.

Table 2. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) of chili extract to selected test organism

Test organism	EtOH-95		EtOH-50		Water	
	MIC	MBC	MIC	MBC	MIC	MBC
S. Enteritidis	6	8	29	41	25	33
S. Typhi	6	10	25	50	41	50
S. Typhimurium	6	8	27	33	25	31
S. dysenteriae	10	16	16	25	21	25
E. coli O157:H7	12	14	31	50	25	37

Time killing analysis

The bactericidal effect can be determined by suspension test or time kill analysis (survivor curve plot) whereby the number of viable cells remaining after different contact times with the disinfectants is plotted against time. Ethanolic extract of Chili using 95% ethanol (EtOH-95), demonstrated the highest antimicrobial activity among all extracts and were, therefore, selected for the suspension test. The suspension tests were performed without (EN 1040) interfering substances. The results of suspension tests against selected test organisms are shown in Figures 3.

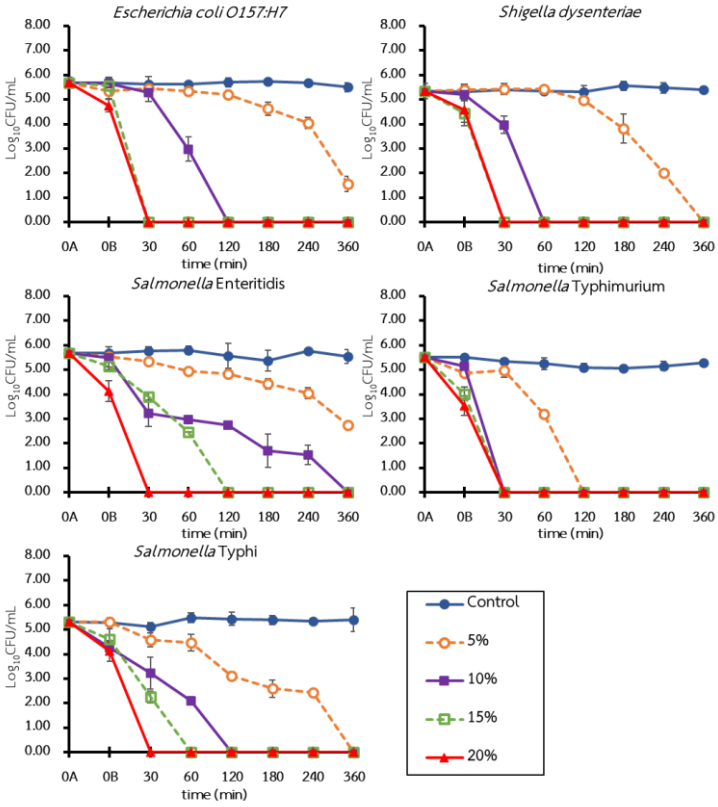

Figure 3. Effects of Chili EtOH-95 extract on the number of test organisms at room temperature without interfering substances according to the EN 1040 method

Figure 3 shows the Log_{10} CFU/mL recorded for each strain at the chosen contact time with the tested concentrations of chili EtOH-95 extract. The extract demonstrated good antibacterial activity against several test organisms. The time kill analysis reveals a time-dependent or a concentration-dependent antimicrobial effect (Balouiri *et al.*, 2016).

The result showed that *E. coli* O157:H7 and *S. dysenteriae* demonstrated time-dependent effectiveness noticeable that population was not reduced before 60 min of contact time. Nevertheless, at high

concentration of chili extract at 10.0%, 15.0% and 20.0% w/v, time-dependent effectiveness was disappeared. The concentrations of chili extract at 20.0 %w/v presented the completely destructive potential against all test microrganism within 30 min. At the concentration of 15.0 %w/v, *E. coli* O157:H7, *S. dysenteriae*, *S.* Enteritidis, *S.* Typhimurium and *S.* Typhi demonstrated the completely destructive potential within 30, 30, 120, 30 and 60 min, respectivly.

Table 3 demonstrated the k-value (ln CFU mL^{-1} min^{-1}) against tested organisms. It was found that the higher the concentration, the greater the increase in k-value could be significantly detected. The k-value of *S.* Typhimurium, *S. dysenteriae* and *E. coli* O157:H7 at the concentration of 15.0% w/v were -0.4245, -0.4112 and -0.437 ln CFU mL^{-1} min^{-1}, respectively. The k-value was not significantly different at 20% w/v. Both concentrations of EtOH-95 chili extract at concentrations of 15% w/v and 20% w/v were enough to destroy all test microorganisms within 30 minutes. On the other hand, *S.* Enteritidis and *S.* Typhi at concentration of 20% w/v, k-value were -0.4383 and -0.4089 ln CFU mL^{-1} min^{-1}, respectively, demonstrated antimicrobial efficiency more than the concentration of 15% w/v (-0.1135 and-0.2103 ln CFU mL^{-1} min^{-1}, respectively).

Table 3. Rate of reduction (k-value) against *E. coli* O157:H7, *S. dysenteriae*, *S.* Enteritidis, *S.* Typhimurium and *S.* Typhi

Test organism	k-value (ln CFU mL^{-1} min^{-1})				
	Concentration of chili extract (%w/v)				
	0.0	5.0	10.0	15.0	20.0
E. coli O157:H7	-0.0006	-0.0209	-0.1045	-0.437	-0.4375
S. dysenteriae	0.0008	-0.0294	-0.1855	-0.4112	-0.4111
S. Enteritidis	-0.001	-0.0178	-0.0421	-0.1135	-0.4384
S. Typhimurium	-0.0031	-0.0996	-0.4238	-0.4245	-0.4248
S. Typhi	0.0007	-0.0332	-0.1089	-0.2103	-0.4089

Discussion

The result of MIC and MBC was noticeable that the use of 95% ethanol as extracted solvent had the most effective inhibiting to all teat organisms because of its polar characteristic. Therefore, the important substances such as capsaicin and dihydrocapsaicin in chili were better leached by ethanol than by polar solvents (Rollyson *et al.,* 2014; Hansen *et al.,* 2001). Chili extract with ethanol 95% still demonstrated the best antimicrobial efficacy. It was commonly acknowledged that if a MIC showed inhibition, plating the bacteria onto agar might result organism become regenerate because the antimicrobial did not cause death.

Comparing all results, the chili extract was showed low antibacterial activity against the tested microorganism. Previous studies on the other plants, water extracts showed antibacterial activity of medicinal plants to be

limited but extract with alcoholic solvent was high, especially associated with essential oils (Hayes and Markovic, 2002; Wilkinson *et al.,* 2003; Burke *et al.,* 2004). Water has limited ability to extract some components from plants such as oil-based components. The results of the actual study indicate that several antibacterial active compounds from chili were oil-based compounds. Alcohol extraction should be used to obtain extracts with higher antibacterial activity.

High MBC-values were often seen when Gram-negative microorganisms were tested. Gram-negative bacteria were endured to external substance (Negi, *et al.,* 2005). It is attributed to the presence of lipopolysaccarides, making them naturally resistant to antibacterial agents, in their outer membrane (Nikaido and Vaara, 1985). On the other hand, Gram-negative showed lower sensitivity against the tested medicinal plants than the Gram-positive bacteria. The reason could be attributed to the differences between their cell wall compositions. Gram-positive bacteria contain an outer peptidoglycone layer, which is an infective permeability barrier (Scherrer and Gerhardt, 1971).

Time killing analysis was in the agreement with the research of Dong and Sun (2021), who observed that the time–killing and the kinetic curve showed that the bactericidal activities of Splys-i were time-dependent. It is widely accepted that higher concentrations of natural material extracts were required in the presence of protein and fat than in distilled water (Farbood *et al.,* 1974). Moreover, the high level of nutrient can protect the bacteria from plant extracts (Tassou *et al.,* 1995). For instance, if the plant extracts are dissolved in the lipid phase, they will be relatively less interaction on bacteria in the aqueous phase (Mejlholm and Dalgaard, 2002). A reaction between carvacrol, a phenolic component of various plant extracts, and proteins has been suggested as a limiting factor in their antimicrobial activity against *B. cereus* in the presence of milk protein (Pol *et al.,* 2001). Similarly, protein interaction has been suggested as a factor reducing the action of clove oil against *S.* Enteritidis in diluted low-fat cheese (Smith-Palmer *et al.,* 2001). Carbohydrates do not appear to protect bacteria from the action of medicinal plant extracts as much as fat and proteins do (Shelef *et al.,* 1984).

From this research, it can be concluded that extracts of chili possess *in vitro* antimicrobial activity against foodborne pathogens and food related bacteria. The appropriate extraction process in order to obtain the maximal antimicrobial activity from both was alcoholic extraction with 95% ethanol. The results supported the healing potency of some traditional plants used in Thai traditional cosine. By suspension test, they may therefore be useful as food additives for controlling the growth of foodborne pathogens and food related bacteria. In ongoing experiments, the antimicrobial activity of these extracts is being tested in model foods. The interaction of the extracts with

the food flavour should be evaluated. These factors may influence the applicability of medicinal plant extracts in certain food products.

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