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Antibacterial Potential of Combined Thai Herb Extracts against Methicillin-Resistant *Staphylococcus aureus*, *Edwardsiella tarda* and *Pseudomonas aeruginosa* in Dried, Seasoned, and Crushed Squid

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Abstract

Food-borne diseases have become a serious public health issue. Due to the occurrence of multi-antibiotic resistant bacteria in seafood-based products, there is a need for novel tools to control the growth of pathogens. In this study, two herb-extract combinations (80 mg/mL), namely lemongrass (*Cymbopogon citratus* (DC.) Stapf) and black pepper (*Piper nigrum* Linn.) extracts, and lemongrass and chili spur pepper (*Capsicum frutescens* Linn.) extracts, were supplemented into dried, seasoned, and crushed squid to investigate their inhibitory activity against pathogenic methicillin-resistant *Staphylococcus aureus* (MRSA) and *Edwardsiella tarda*, and food-spoilage *Pseudomonas aeruginosa*. Combined addition of lemongrass and chili spur pepper extracts resulted in a significant ($p < 0.05$) decrease in MRSA and *E. tarda* numbers, compared to the control during 28-day refrigerated storage. *P. aeruginosa* appeared to be vulnerable to the both herb-extract blends supported by a significant ($p < 0.05$) 1-log reduction in the dried squid during chilled storage. This study suggests that a mixture of lemongrass and chili spur pepper extract had the potential use as natural preservative to reduce the risk of diseases associated with the consumption of contaminated dried seafood products.

Keywords : Chon Buri; Pepper; Chili; Lemongrass; Dried squid; Food safety

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1. Introduction

Seafood is a healthful diet containing a variety of essential nutrients such as proteins, vitamins, minerals, unsaturated fatty acids, and taurine [1]. Demand of seafood-based products in Thailand increases every year with an annual per capita consumption of 33 kg in 2016. Due to the perishable nature of seafood and its improper storage environment, food-borne diseases caused by pathogen contamination have been an important food-safety issue. In Thailand, traditional dried seafood, a popular processed seafood product, is reported to contaminate with several pathogenic bacteria, e.g., *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Edwardsiella tarda*, and *Salmonella* [2]-[4].

Food poisoning caused by enterotoxigenic strains of *S. aureus* has become a public health concern in Thailand. In 2014, 207.52 food poisoning cases per 100,000 populations were reported by Bureau of Epidemiology, Thailand by which *S. aureus* was one of the most frequently isolated pathogenic bacteria identified from 22.2% of the patients [5]. Unfortunately, the misuse of antibiotics in medical treatments and animal farm practices has resulted in the emergence and dissemination of methicillin-resistant *S. aureus* (MRSA) in food chain, thereby harming a food safety. MRSA has been reported to be resistant to common used antimicrobial agents leading to more complicated treatments of patients with severe infections. Aside from causing infections in both healthcare facilities, and communities ranging from mild skin infections to life-threatening diseases, viz. pneumonia, septicemia, endocarditis, and nosocomial infection, MRSA also causes food-poisoning. Human infections caused by food-borne MRSA strains have been documented worldwide. For example, there was the incidence of food poisoning caused by a staphylococcal enterotoxin C producing MRSA following ingestion of roasted pork contaminated by food handlers [6]. Currently, the occurrence of MRSA in fresh seafood, ready-to-eat seafood, and seafood-derived products has been increasingly reported in many countries [7]-[8]. In Thailand, MRSA strains were recently isolated from a multi-recipe of traditional dried seafood products sold in Chon Buri province in our laboratory.

E. tarda, a causative agent of hemorrhagic septicemia in fish, has been isolated from a number of fresh water and marine life. The pathogen can rarely cause infections in human following consumption of contaminated fish or seafood, and accidental ingestion of contaminated water. Most clinical symptoms (approximately 80%) of *E. tarda* infection include gastroenteritis, and other diarrheal syndromes, e.g., dysentery, chronic diarrhea, and enteric fever [9]. *Pseudomonas* sp., in particular *P. aeruginosa*, is one of the most dominant spoilage bacteria frequently isolated from dried ready-to-eat seafood products in Chon Buri province. This bacterium is degenerative to product quality through slime formation, discoloration, and generation of off-flavors and odors due to production of volatile substances, such as alcohols, aldehydes, esters, and sulphur compounds [10]. *P. aeruginosa* is also considered as an opportunistic human pathogen frequently implicated in nosocomial infections, and gastrointestinal syndromes, particularly in immunocompromised individual [10]. Infections caused by *P. aeruginosa* are generally complicated to cure owing to the limited susceptibility to antimicrobial agents and the high frequency of an emergence of antibiotic resistance during remedy [11]. This situation indicates that Thai traditional seafood products pose a serious risk of food-borne diseases. As such, an effective technology based on preservative is required to improve the safety and extend the shelf-life of such products.

Chemical preservatives are commonly applied in several recipes of seafood-based products in Thailand. However, their safety issues have been questioned by consumers in terms of the potentially life-threatening side effects on human health. For example, sulphites are implicated in allergic and asthma reactions in sensitive people, and chronic skin symptoms [12]. Nitrites are toxic at high concentrations and can react with proteins to form carcinogenic nitrosamines, when exposed to certain heats or acid environments. A positive correlation has been reported between increased levels of nitrates in food and increased deaths from Alzheimer's, Parkinson's, and type 2 diabetes [13]. In this sense, alternative practices using natural substances have been drawn much attention. In Thai cuisine, several gastronomical and traditional herbs commonly used as flavoring ingredient, such as ginger, lemongrass, garlic, clove, chili pepper, kaffir lime, cumin, shallot, and galangal, have been claimed to represent antibacterial activity against food-borne pathogens. Until now, a study focused on antimicrobial efficacy of Thai herb extracts/essential oils on pathogenic bacteria has received very little interest in a food-model condition. Therefore, this study aimed to investigate antibacterial activity of two herb extract mixtures (lemongrass-black pepper extract and lemongrass-chili spur pepper extract) against MRSA, *E. tarda*, and *P. aeruginosa* in dried, seasoned, and crushed squid used as a seafood model.

2. Research Methodology

2.1 Herb extraction

In our preliminary study, unsatisfactory inhibitory activity against MRSA, *E. tarda*, and *P. aeruginosa* was observed in individual herb extract of lemongrass (*Cymbopogon citratus* (DC.) Stapf), chili spur pepper (*Capsicum frutescens* Linn.), and black pepper (*Piper nigrum* Linn.). In contrast, their combined extracts (lemongrass and chili spur pepper or lemongrass and black pepper) were *in vitro* effective in inhibiting the tested pathogens. Lemongrass (stems), chili spur pepper (fruits), and black pepper (fruits) were purchased from a local spice store in Chon Buri province, Thailand. Herb preparation and extraction were performed according to P. Soodsawaeng et al. [14] method. After rinsing with running water, all herb materials were shade-dried, and chopped using a table knife. Then, the herbs were dried at 35 °C for 72 h, and ground using an electronic blender. The herb powders were extracted with denatured 95% ethanol at a ratio of 1:10 of material to extractant in a shaking incubator at 120 rpm, 30 °C for 72 h. Supernatants were passed through a Whatman filter membrane No.1 prior to evaporation at 40 °C and 175 mbar using a rotary evaporator (Buchi R-215, Flawil, Switzerland). Ethanolic extracts were diluted with 10% dimethyl sulfoxide to produce stock solution (80 mg/mL) and sterilized using a 0.45 μm syringe filter prior to storage at -20 °C until use.

2.2 Pathogen preparation

Two pathogenic bacteria (MRSA T18 and *E. tarda* DS 002), and one strain of food-spoilage bacteria (*P. aeruginosa* DS001) isolated from dried seafood products sold in Chon Buri, province Thailand in our laboratory were used in this study. The stock cultures were frozen at -80 °C in Trypticase Soy Broth (BD Difco, Sparks, Maryland, USA) containing 20% glycerol. The culture was propagated on Trypticase Soy Agar (TSA; Becton BD) at 35 °C for 24 h with two consecutive transfers to produce active sub-cultures. Then, the bacterial isolate was cultured in a tube containing TSB, and incubated at 35 ºC for 24 h. Cell concentration was adjusted to 10^4 CFU/mL using the McFarland turbidity standard before being used.

2.3 Synergistic study of the mixed herb extracts against pathogenic and spoilage bacteria in dried, seasoned, and crushed squid

Antibacterial potential of the mixed herb extracts against food-borne and food-spoilage bacteria was investigated following N. Butkhot et al. [3] method. Briefly, dried, seasoned, and crushed squid was purchased from a retail store in Chon Buri province, Thailand. The squid samples were cut using a sterile scissors to obtain a 2 x 2 cm piece. Experimental design in this study consisted of three treatments including addition of tested bacterial suspension in the squid samples together with sterile distilled water (control), lemongrass-black pepper supplement, and lemongrass-chili spur pepper supplement. A square piece sample was experimentally inoculated with the suspension of MRSA T18, *E. tarda* DS 002 or *P. aeruginosa* DS001 at concentration of 10^4 CFU/mL. After air-drying in a biosafety cabinet for 15 min, a minute volume (0.1 mL) of either the combined herb supplement (80 mg/mL) or distilled water was introduced onto entire surface of a 4 cm^2 piece of the pathogen-inoculated squid. The squid samples were then air-dried for 15 min, packed in a sterile plastic bag (3 x 5 inches; 1 sample: 1 bag) to prevent cross contamination, and stored in a 4 ºC refrigerator. During 28 days of storage, the squid samples were retrieved at 15-min, 2, 4, 7, 14, 21 and 28 day post-inoculation to monitor change in the pathogen numbers (see below). An additional treatment of squid exposed to sterile distilled water without the addition of pathogen suspension was included in this study to obtain background data of pathogen contamination in dried, seasoned, and crushed squid.

2.4 Enumeration of the tested pathogens

Enumeration of tested pathogenic bacteria was carried out using a spread plate method. The sample (2 g) at each defined interval was mixed with 0.1% (w/v) peptone water (18 mL), and vigorously homogenized using a stomacher for 2 min. A 10-fold dilution was made, and then a 0.1-mL aliquot was spread-plated onto Baird-Parker agar (BD Difco) supplemented with egg yolk tellurite enrichment, Hektoen Enteric Agar (BD Difco), and Pseudomonas Isolation Agar (Himedia, Mumbai, India) for enumeration of MRSA, *E. tarda*, and *P. aeruginosa*, respectively [7], [15]-[16]. All petri dishes were incubated at 35 °C for 48 h. Suspicious colony grown on the media was streaked onto TSA to gain pure culture prior to being characterized by Gram staining, and selected biochemical tests, as described elsewhere [17]. Typical isolates were compared with physical and biochemical characteristics of the original strains experimentally inoculated onto the dried squid. All confirmed typical colonies were counted and calculated as colony forming unit (CFU) per g of sample. All experiments were conducted in triplicate. Reference strains, namely MRSA ATCC 43300, *E. tarda* ATCC 15947, and *P. aeruginosa* ATCC 15442 were used during enumeration and confirmation of the pathogen.

2.5 Data analysis

Data are expressed as mean ± standard deviation (S.D.). The numbers of bacteria were normalized by 10-log transformation, when needed prior to statistical analyses. Data were analyzed using a Two-way ANOVA, and followed by the Tukey's multiple comparison test to identify any difference among treatments. All statistical analyses were conducted at a significant level of $p < 0.05$ using a GraphPad Prism version 7.0 (GraphPad software, San Diego, California, USA).

3. Results

3.1 Antibacterial activity against MRSA

Antistaphylococcal activities of the two herb-extract combinations were significantly ($p < 0.05$) greater than that of the control (**Fig. 1**). The strongest inhibitory activity against MRSA in dried squid was present following addition of the herb extract blend of lemongrass and chili spur pepper due to a significantly ($p < 0.05$) reduced MRSA number, compared to the control during 28-day storage. MRSA number in squid added with lemongrass-chili spur pepper extracts significantly ($p < 0.05$) reduced from 1.43 ± 0.95 x 10^3 CFU/g at beginning of experiment to undetectable level at 28-day storage (**Fig. 1**).

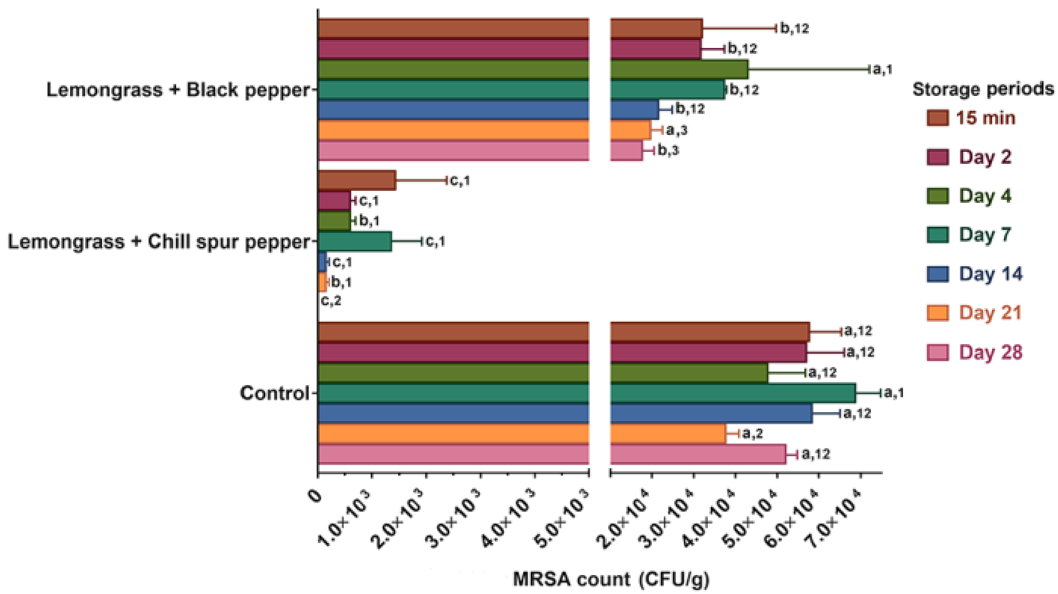

Fig. 1 Antibacterial potential of the combined herb extracts on growth of MRSA in dried squid during 28-day refrigerated storage. MRSA was absent in the squid samples exposed to sterile distilled water without inoculation of the pathogen suspension. Bars with different letters at each sampling period denote significant difference ($p < 0.05$) among treatments. Bars with different numbers within the same treatments denote significant difference ($p < 0.05$) over the time.

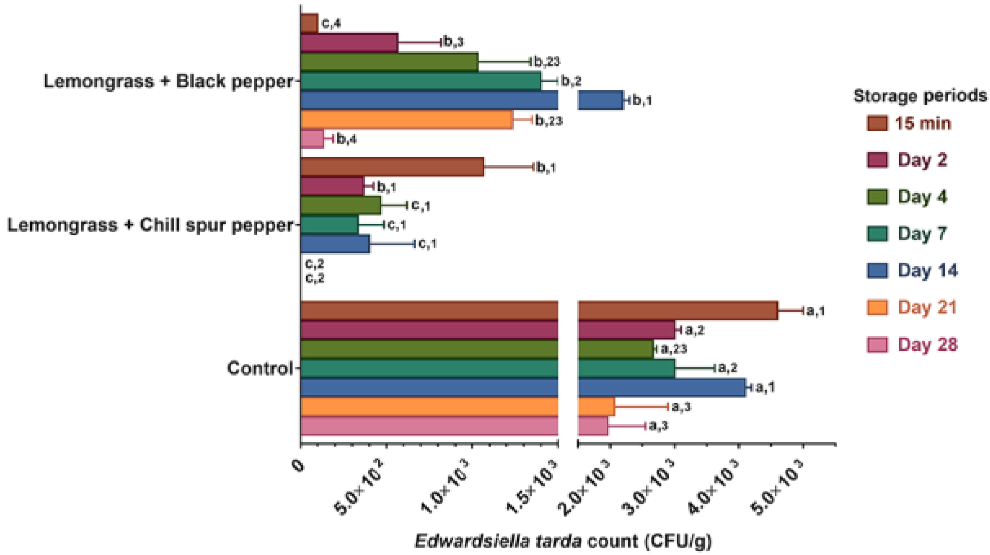

Fig. 2 Change in *E. tarda* in dried squid treated with the mixed herb extracts during 28 days of refrigerated storage. No *E. tarda* was isolated from the squid samples added with sterile distilled water without inoculation of the pathogen suspension. Bars with different letters at each sampling period denote significant difference ($p < 0.05$) among treatments. Bars with different numbers within the same treatments denote significant difference ($p < 0.05$) over the time.

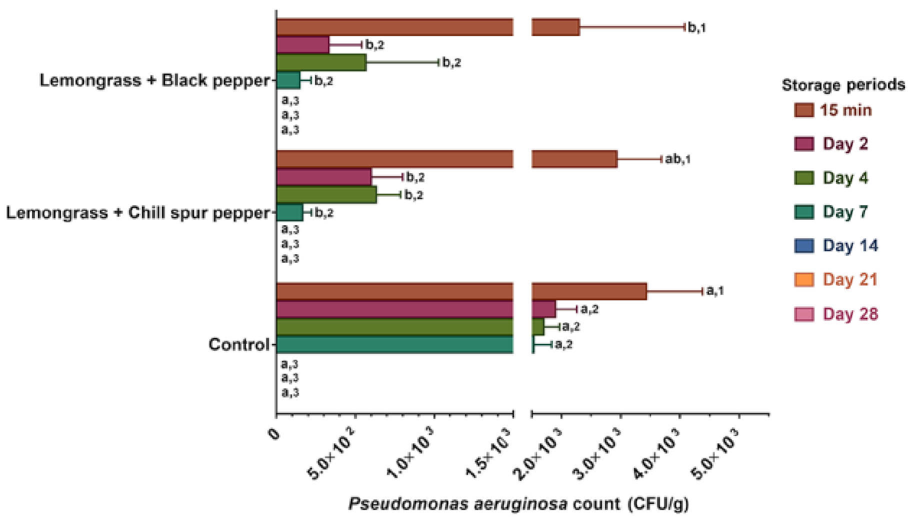

Fig. 3 Antibacterial efficacy of the combined herb extracts on *P. aeruginosa* count in dried squid during 28-day refrigerated storage. *P. aeruginosa* was absent in the squid samples exposed to sterile distilled water without inoculation of the pathogen suspension. Bars with different letters at each sampling period denote significant difference ($p < 0.05$) among treatments. Bars with different numbers within the same treatments denote significant difference ($p < 0.05$) over the time.

3.2 Antibacterial activity against *E. tarda*

Similar to MRSA vulnerability, the combined extracts from lemongrass and chili spur pepper had the strongest bacterial activity in dried squid by which *E. tarda* number significantly ($p < 0.05$) decreased from 1.07 ± 0.28 x 10^3 CFU/g at 15-min post-storage to undetectable level at day 21 of refrigerated storage, compared to those of the control and lemongrass-black pepper added samples (**Fig. 2**).

3.3 Antibacterial activity against *P. aeruginosa*

Number of *P. aeruginosa* in the control was 3.43 ± 0.95 x 10^3 CFU/g at beginning of experiment, and progressively decreased until vanishing at day 14 of storage (**Fig. 3**). Addition of either lemongrass extract in conjunction with chili spur pepper or a mixture of lemongrass and black pepper extracts in the dried squid resulted in a significant ($p < 0.05$) 1-log reduction of *P. aeruginosa* numbers during first 7 days of storage. The absence of *P. aeruginosa* in the control, and the two treated groups was observed at day 14 of storage.

4. Discussion

Growth of pathogenic and spoilage bacteria in dried ready-to-eat seafood products causes a serious harmful effects on consumer health and has highlighted the need of an effective novel technology to control the pathogen proliferation. In this study, the two combined extracts could effectively inhibit the growths of pathogenic MRSA and *E. tarda*, and food-spoilage *P. aeruginosa* in dried squid. However, the strongest inhibitory activity against all tested bacteria was observed in the extracts from lemongrass in combination with chili spur pepper. Despite inhibitory effect of the two herb-extract combinations on food-borne pathogens never reported, chili pepper extract acted synergistically with ginger extract against *E. coli*, *S. aureus*, and *P. aeruginosa* in culture medium [18]. A mixture of lemongrass and turmeric essential oils was effective in retarding growth of mesophilic and psychrophilic bacteria in chilled-stored green mussel (*Perna viridis*) during storage [19]. Antibacterial activities of the two herb-extract blends used in this study are expected perhaps due to active phytochemical constitutes in herb extracts. The inhibitory phenolic components, e.g., cinnamic acid, and *m*-coumaric acid, have been found in chili pepper extract, and contribute to exerting antibacterial action against food-borne pathogens [20]. Citral chemotype: geranial, and neral has been reported to be the major active component in lemongrass essential oil/extract responsible for bactericidal action against multi-strains of methicillin-susceptible *S. aureus* and MRSA [21]-[22] while piperine, terpines, and flavones are the predominant chemicals in black pepper extract and play a role in inhibiting *E. coli* and *S. aureus* [23]. In our preliminary *in vitro* study, degree of bacterial inhibition of individual herb extract: lemongrass, chili spur pepper, and black pepper, was lower than those of the combinations of herb extracts (unpublished data). The results in this study revealed synergistic action among the components in the combined herb extracts that may exert antibacterial activities through binding the bacterial cell surface, penetrating into the cell membrane causing cytoplasmic membrane damage, allowing the efflux of DNA materials, amino acids and ions, changing in the proton motive force, destroying bacterial respiratory metabolism, and ultimately leading to pyknosis and cell death [21], [23]-[24]. Mode of action of the blended herb extracts remains unknown. Therefore, identification of the active compounds present in the two novel combinations should be further performed which may help to explain their synergistic mode of mechanisms.

Our results provided evidence that addition of the extract from lemongrass in conjunction with chili spur pepper was more effective in controlling the growth of MRSA and *E. tarda* than those of a mixture of lemongrass and black pepper extracts. The results were accordant with previous study [25]. A combined essential oil of basil and thyme produced a greater *in vitro* antibacterial activity against *Bacillus cereus*, *S. aureus*, *P. aeruginosa*, and *E. coli* than those of a mixture of basil-parsley, thyme-parsley, basil-lovage, thyme-lovage, and parsley-lovage essential oils. Similarly, bacteriostatic activity of various mixtures among clove, rosemary, cassia bark, and liquorice extracts was studied against four meat spoilage and pathogenic bacteria, e.g., *Listeria monocytogenes*, *E. coli*, *P. fluorescens*, and *Lactobacillus sake*. A combination of rosemary and liquorice extracts represented the best inhibitory potential towards all tested bacteria in culture medium [26]. In a food model study, B. Kong et al. [27] added either the mixed herb extract (honeysuckle, *Scutellaria* and *Forsythia suspense* Thunb) or mixed spice extract (cinnamon, rosemary, and clove oil) as food natural preservative into vacuum-packaged fresh pork. The authors claimed that despite a significantly reduced bacterial count observed in the extract-added pork in comparison with the control, the spice mixture had a stronger antibacterial effect in chilled pork during storage. Such phenomenon may be explained by herb extracts/essential oils generally containing a wide divergence of active constituents whose antibacterial activities are largely dependent on chemical composition, configuration, amount, and interactions (additive, synergistic, and antagonist) among the components [28]. It might be also possible that the minor compounds present in each herb extract may act in a synergistic manner, thereby exhibiting a greater inhibitory activity. G. O. Onawunmi et al. [29] demonstrated that myrcene, a chemical composition in lemongrass essential oil, showed no antibacterial activity, but appeared to enhance the activity, when the presence of citral (geranial, and neral). Likewise, the interaction between two components in tea tree (*Melaleuca alternifolia*) essential oil, 1,8-cineole and terpinene, was reported by C. F. Carson et al. [30]. 1,8-cineole exhibited marginal antibacterial activity inherently, but it was capable of improving the lethal action of terpinene. The authors postulated that 1,8-cineole may permeabilize bacterial membranes and facilitate the entry of other more active components into the cells. These results suggested that chemical components in essential oil/extract blends may act synergistically against target organism and the presence of a small amount of the components could improve antibacterial activity.

This study demonstrated that food-spoilage *P. aeruginosa* seemed to be inhibited by the both herb-extract blends supported by a significantly reduced population during first 7 days of refrigerated storage. In contrast, pathogenic MRSA and *E. tarda* were more vulnerable to blended extracts from lemongrass and chili spur pepper. The variation in sensitivity to antibacterial activity may mainly due to difference in structural organization of lipid bilayer (types, and arrangement of hydrophobic molecule chains) of the membrane and cell wall as well as metabolism machinery among the bacteria [31].

5. Conclusion

This study indicates that herb combination of lemongrass and chili spur pepper extracts (80 mg/mL) was effectively active against MRSA, *E. tarda*, and *P. aeruginosa* in dried, seasoned, and crushed squid. Such synergistic combination of lemongrass and chili spur pepper extracts would have a great implication to reduce the risk of diseases related to pathogenic MRSA and *E. tarda* as well as to improve product quality caused by food-spoilage *P. aeruginosa*. The use of the blended herb extracts as natural preservative provides a more acceptable way to satisfy a current consumer trend of “green”, “natural” or “no chemical added” labels in seafood industry. However, an additional study associated with organoleptic impact of the herb extract mixture should be conducted in the future to ensure sensorial acceptability in food products by consumers.

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