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Evaluation of cloves *(Syzygium aromaticum)* against antibiotics resistant *Vibrio parahaemolyticus* on seafood

[1*]Elexson, N., [2]Rukayadi, Y., [3]Nakaguchi, Y., [3]Nishibuchi, M. and [2]Son, R.

[1]Department of Molecular Biology, Faculty of Resource Science and Technology, University Malaysia Sarawak, 94300, Kota Samarahan, Sarawak, Malaysia
[2]Food Safety Research Center (FOSREC), Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

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Abstract

Vibrio parahaemolyticus is a main foodborne disease in seafood and generally seafood is easily deteriorates in quality of color and flavor. In this study, clove *(Syzygium aromaticum)* extract shows potent antibacterial activity against growth of antibiotics resistant *Vibrio parahaemolyticus* on seafood samples (cockles and shrimps). *Vibrio parahaemolyticus* was artificial contaminates on the samples with 10^6 CFU/ml. The samples were treated with different concentration of cloves extract with 10 mg/ml which are 0.5%, 5% and 10% concentration from methanol food grade extraction in 0 hr, 5 min, 10 min, 15 min, 30 min, 60 min and 120 min. Tab water and deionized water were selected as a negative control. As a result, the amount of 10 % cloves managed to mitigates the number of V. parahaemolyticus on seafood samples in 5 minutes and 15 min on both samples. Therefore, our results signify the fact that cloves can be apply as natural sanitizer which could meet consumer demands for safe and traditionally consumed either raw without any undesirable effect when applied in the seafood system industries.

Introduction

Vibrio parahaemolyticus is a bacterium which causes mild gastroenteritis in humans on consumption of seafood (Oliver and Kaper, 1997). According to Feldhusen (2000), *V. parahaemolyticus* caused about 25% of total foodborne diseases in comparison to other *Vibrio* species. In Japan, a variety of seafood has been traditionally consumed and raw or lightly cooked seafood is highly selected as favourite. This habit of eating seems to provide a justification for many cases of foodborne illness by *V. parahaemolyticus* in the country. Moreover, this Japanese style of cuisine of eating raw and lightly cooked seafood is increasingly popular in Europe, United States even Asian countries and seems to be also adopted in the local cuisines with globalization of food.

Nowadays, microorganisms have become resistance to many antibiotics due to increase use of drugs, and decrease the drug efficiencies. Therefore, it has become necessary to find out new antimicrobial agents. Spices and herbs have been used as food additives since ancient times, both as flavouring agents and as natural food preservatives. They have many functional components such as phytochemicals, phenols, polyphenols, essential oils and micronutrients (Cowan, 1999). The study from Elexson *et al.* (2013) has demonstrates cloves contained the lower Minimum Inhibition Concentration at 19.531 µg/ml to inhibit and killed antibiotics resistant *V. parahaemolyticus* from seafood.

In addition, this was in agreement with the memorandum between Forest Research Institute Malaysia (FRIM) and Malaysia Agriculture Research and Development Institution (MARDI) to boost the herbal industry in Malaysia and to encourage research collaboration and sharing scientific knowledge between these two institutions (Star Online, 2013). Therefore, the objectives of this research is to evaluate the effectiveness of clove *(Syzygium aromaticum)* on seafood samples to mitigate the growth of antibiotic resistant *Vibrio parahaemolyticus*.

Materials and Methods

Collection of seafood samples

Freshly samples (shrimps and cockles) were collected from wet market nearby Serdang, Selangor and transported to the laboratory where they were analyzed within 5 hours upon collection.

*Corresponding author.
Email: *alexsnillian@gmail.com/nelexson@frst.unimas.my*

Extraction of cloves (Syzygium aromaticum)

The dried powder of cloves (100 g) was extracted twice with 200 ml of 100% (v/v) food grade methanol for 48 h at room temperature. Cloves extracts were filtered with Whatman filter paper no. 1 (Whatman International Ltd. Middlesex, England) and concentrated with a rotary vacuum evaporator (Heidolph Instruments, Germany) at 50°C and a speed of 150 RPM, yielding methanol extracts. The yield of the extract was calculated in percentage and the extract was dissolved in sterile deionized water (DIW) to prepare three different concentrations. The method used in this work was according to Rukayadi *et al*. (2009) with slight modification.

Bacterial strains and inoculum preparation.

A reference of *V. parahaemolyticus* ATCC 17802 used in this study was obtained from the American Type Culture Collection (Rockville, MD, USA). As referring to Elexson *et al.* (2013), resistant strain of *V. parahaemolyticus* toward antibiotics was selected for further study. The antibiotics resistant isolates towards bacitracin (10 µg/ml), streptomycin (10 µg/ml), rifampin (2 µg/ml), ampicilin (10 µg/ml), vancomycin (30 µg/ml), penicillin (10 µg/ml), and spectinomycin (25 µg/m) which are commonly used in aquaculture industries.

Preparation of treatment solution by dilution method

Sterile deionized water (DIW) was purchased from Megamal Pharmacy Sdn. Bhd., Penang with the brand name of "Water For Injections B. P." (B. Braun Medical Industries Sdn Bhd, Penang, Malaysia). Methanolic extract of *(Syzygium aromaticum)* was dissolved in 10% of (DMSO) and followed by dilutions to make treatment solutions of 10%, 5% and 0.5% (mg/ml) for the soaking in terms of sanitizing purpose for the seafood samples.

Experimental procedure

From the purchased seafood, damaged and wilted portions were discarded, and the whole pack of seafood was cut into small pieces of 3 x 1 cm and mixed thoroughly to enhance homogeneity of natural background microflora that might present originally in the sample. The homogenate seafood was stored at 4°C and used within 24 hours.

Following treatments, the seafood samples were dried out and treatment under UV light to inhibit the growth of other microorganism for 15 minute. Then, the amount of log 10^6 CFU/ml of antibiotics resistant *Vibrio parahaemolyticus* strain was spiked onto the dried seafood samples. Then, all samples were aseptically removed using a flame-sterilized knife together with a clamp and drained on absorbent paper for drying.

The seafood samples of 10 g each were immersed in 50 ml of filtered tap water and DIW at different concentrations (mg/ml) of 0.5%, 5% and 10% extract solutions at room temperature (23±2°C) for treatment time for 0 minutes, 5 minutes and 10 minutes, 15 minutes, 30 minutes, 1 hour and 2 hour. A sample was prepared without soaking with any solution to be held as the positive control. Microbial analysis was conducted on the antibacterial activity of different concentration of *(Syzygium aromaticum)* extract against gram negative, antibiotics resistant *Vibrio parahaemolytiucs* based on the conventional spread plate method.

This methodology was adapted to the work of Oh *et al.*, (2011). For the detection of the specified pathogenic microorganisms, each 10 g seafood samples was weighed and diluted into the stomacher bag (BAGLIGHT, BagSystem, Interscience, France) with 90 ml of 0.85% saline solution for Vibrio parahaemolyticus. The mixture was homogenized using a stomacher machine (BagMixer 400-P, Interscience, France) for 2 minutes at 250 rpm. After homogenization, the enrichment was serially diluted with 1 ml of the enrichment into 9 ml 0.85 % saline solution to make up 10^{-1} to 10^{-4} dilutions.

Total plate count

The total of 0.02 ml of each dilution was pipette into separate, duplicate, appropriately marked petri dishes with plate count agar. The logarithm numbers of colony forming unit per grams ($\log_{10}$ CFU/g) of samples were calculated by observing and enumerating the presumptive colonies formed after incubation at 37°C for 48 h.

Statistical analysis

Means of bacterial populations ($\log_{10}$ CFU/g) from each treatment were calculated from three replications of each experiment. Data were performed with using Minitab version 16 for Windows. P values of < 0.05 are considered statistically significant different.

Results and Discussions

Nowadays, consumers are starting to incorporate more fish and seafood as regular choices for their menu at home and serve in the menu of many restaurants over the years. The development of strategies to combat bacteria growing is a challenging task as these bacteria are more resistant to classical antimicrobial therapies and they exchange genetic

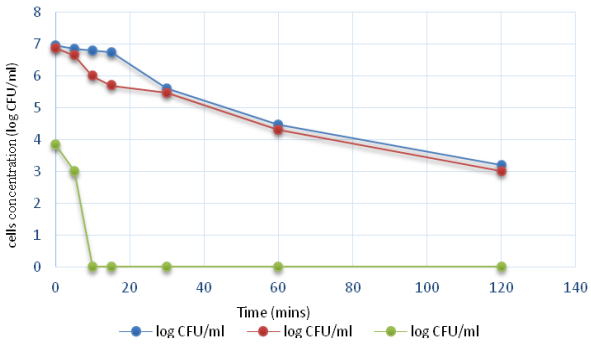

Figure 1. Effect of tab and deionized water on the growth of antibiotics resistant *Vibrio parahaemolyticus*

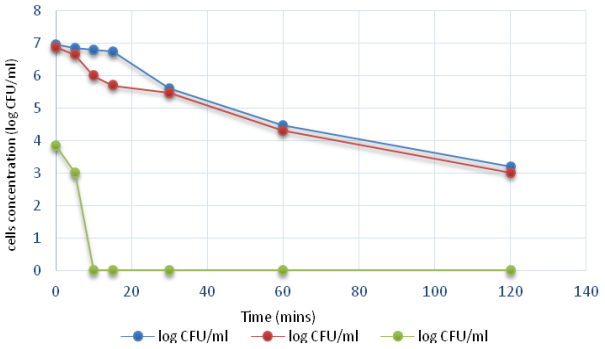

Figure 2. Effects of different concentration of cloves against antibiotics resistant *V. parahaemolyticus* on cockle samples

material more easily

As seen in Figure 1, tab water and deionized water were used as negative control, however, *V. parahaemolyticus* growth is increase in tab water. The presence of chlorine in drinking water has been successfully used to deactivate the harmful microorganisms and is the most widely used method of disinfection in Malaysia today. A study from Lim *et al*. (2008) stated, chlorination is the most important method of disinfection in Malaysia which aims at ensuring an acceptable and safe drinking water quality. Chlorine dioxide (ClO_2) is considered to be a safe and effective disinfectant and is routinely applied for treatment of drinking water and seafood (Wang *et al.*, 2010). Furthermore, Lim *et al*. (2008) stated, the chlorination has virtually eliminated serious waterborne outbreaks such as dysentery, cholera and typhoid fever. However, from this results, it seem *V. parahaemolyticus* resistant toward chlorine based which could raise a concern. Nevertheless, deionized water as negative control showed slow kinetic growth of *V. parahaemolyticus* without treatment. Then, almost all of its mineral ions such as cations like sodium, calcium, iron, and copper and anions such as chloride and sulfate has been removed.

Figure 2 showed the effect of different concentration of cloves on the cockle samples Cockles are common name for a small, edible, saltwater clams and marine mollusk shells. It is a popular ingredient in local foods and as in many areas of Asian countries and they are frequently consumed in a partially-cooked condition. This underlines the need to protect consumer against infection by potential pathogenic strains of bacteria in cockles. In present study, when antibiotics resistant *V. parahaemolyticus* has been treated with cloves, it shown that the concentration of 10% of cloves was effective compared to 5% and 0.5% cloves extract. In 5 min, with the 10% of extracts concentration, a total amount of *V. parahaemolyticus* was reduced to 3.00 log CFU/ml and it killed at 10 min. This mean, the higher the concentration the faster the antimicrobial inhibits the growth of the microorganism.

As seen in 5% of extract, the log CFU/ml was reduce from 6.85 log CFU/ml in 5 min to 3.00 log CFU/ml in 120 min. The 0.5% cloves extracts showed the mitigation of log CFU/ml as well from 7.00 log CFU/ml to 3.20 log CFU in 120 min. *V. parahaemolyticus* revealed the ability to grow at both concentration. Due to it resistance toward antibiotics (Elexson *et al.*, 2013), *V. parahaemolyticus*, rapidly develop resistance to antibacterial agents, this leads to the ineffectiveness of treatment given. Best practices in aquaculture management should be prioritized to avoid entrance of pathogens into the seafood cultivation systems, and antibiotics should only be administered as the last resort.

Figure 3 showed the 10% clove extract able to mitigate the growth of *V. parahaemolyticus* which is from 6.48 log CFU/ml in 5 min to 3.00 log CFU/ml in 15 min. However, *V. parahaemolyticus* was killed at 30 min. There are slow growth of *V. parahaemolyticus* on shrimp when it was treated with 0.5 % and 5% of cloves. As seen, in 5 min period, the amount of 3.726 log CFU/ml treated with 0.5% was reduced to 5.30 log CFU/ml after 120 min while 3.723 log CFU/ml in 5 min was treated with 5% and reduce to 4.00 log CFU/ml after 120 min. Shrimp is the most susceptible seafood which can be contaminated with variety of bacteria especially Vibrio genus (Krupesha, 2000).

On the other hand, shrimps are one of the major aquaculture products of export with importance from the tropics. Eighty percent of the world's farm that raise shrimps are contributed by aquaculture in Asia (Bhaskar *et al.*, 1998). Shrimps contain chitin, which is free amino acid that provides nutrient to encourage the growth of microorganism (Rusell, 2014). Therefore, shrimp is the most susceptible seafood, which can be contaminated with a variety of bacteria, especially marine bacterium, such as *V.*

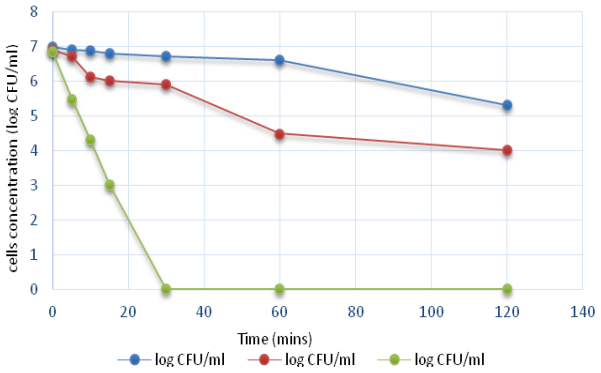

Figure 3. Effects of different concentration of cloves against antibiotics resistant *V. parahaemolyticus* on shrimp samples

parahaemolyticus. The occurrence of *Vibrio* species in tropical shrimp culture environments contribute to the implications of food safety toward economical values since Malaysia is one of the biggest shrimp exporters to the United States (The Star Online, 2013).

The resistance of *V. parahaemolyitcus* could occur is due to the ability to form of biofilm. According to Elexson *et al*. (2013), V. parahaemolyticus can form biofilm which are the formation on biotic surfaces which can cause an implications for the outbreak of disease. The bacteria within a biofilm have been shown to have increased resistance to a variety of stresses, including antimicrobial agents, dehydration, acid conditions, oxidative environments, and UV light (McDougald and Kjelleberg, 2006). Research by Jervis *et al*. (1991), has revealed that the bacterial adherent, expanding in consortium known as biofilms, is present in virtually in almost natural and pathogenic ecosystems.

Sapers (2001) stated that addition of a sanitizing agent to wash water can greatly reduce the population of bacterial cells and thus lower the risk of cross contamination. Various sanitizing agents and methods of application have been employed in attempts to improve the disinfection of microorganisms in vegetables (Sapers and Juneja 2003). Growing concern over the development of resistance to certain therapeutic drugs has led to questions over microorganisms developing resistance to sanitizers. As described by Davidson (1997), the major antimicrobial components in cloves have been known to be terpenes such as eugenol and carvacrol while the antiquity, antiseptic properties of plant have been recognized by Dorman *et al*. (1999). Therefore, it is not a surprise that, clove contained an antimicrobials agent that important in food industries as well.

Hence, from this funding, suggested amount of cloves as natural sanitizer for seafood samples such as cockles and shrimp can be below 10% to treats the antibiotics *V. parahaemolyticus* contamination on samples. This was in agreement with Mahmoud and Louis (1999) stated that the small percentage ranged from 1 to 10% of antimicrobial are suitable used as foods preservation. However, for sanitizer purpose it was propose that the total amount of antimicrobial was commonly used is 62%. Food antimicrobial in this study was not only used to mitigates the growth of *V. parahaemolyticus* growth, which can grow on seafood contact surfaces, processing line, equipment, and facilities, thus prolong shelf life and preserve the quality of seafood. This is especially important for the seafood industry since seafood is a product that gets contaminated easily or spoiled.

As it is generally recognized as safe to be consume, it can be incorporated in any food supply chain involving preharvest of seafood to marketable level in future. From this study, a baseline is provided for other food borne related study on the effects of herbs and spices as natural antimicrobial. The inhibitory factor responsible for the antimicrobial activity can further be identified and used as an alternative to currently used drugs against the pathogenic microbes. Nowadays microbes are increasingly developing resistance against the drugs in used. To combat against these drug resistant microbes, a large library of novel compounds is required. Natural products from plants may give us a solution to this alarming problem.

Conclusion

In this study, cloves showed effective antimicrobial features in treating antibiotics resistant *V. parahaemolyticus* growth in seafood samples with 10 mg/ml and 10% of the cloves extract within 5 min period. The finding can be implemented in our current food safety system and incorporated with the CIPs, GMPs, GHPs, and HACCPs to mitigate the risk of V. parahaemolyticus in future. The future study can also use other herbs and spices against other food borne pathogens studied to prevent any outbreak related to the food industry.

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