

Antibacterial synergistic effect of sea cucumber extract and kelulut honey combination

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Abstract

Natural products remain one of the major sources of new drug molecules today. The combination of sea cucumber extract with stingless bee honey holds great potential for health supplement. However, the specific results of the combination have yet to be determined. In this study, the antibacterial activity of kelulut honey and *Phyllophorus spiculata* sea cucumber extract samples individually and in combination were assessed with disk-diffusion assays and broth dilution method. Most of the samples exhibit small or absence of inhibitory activity using disk-diffusion assay method. Only kelulut honey sample with 100% concentration has a positive antagonism effect against *Escherichia coli* and *Staphylococcus epidermidis*. For the broth dilution method, Gram-positive bacteria were found to be more susceptible as compared to Gram-negative bacteria species, with lower minimum inhibitory concentration (MIC) value of kelulut honey, sea cucumber or combination sample. The growth of gram-positive *S. epidermidis* is inhibited by sea cucumber extract at MIC value of 0.031 mg/mL, kelulut honey at MIC value of 0.063 mg/mL and combination sample at MIC value of 0.063 mg/mL, all of which are of lower MIC value as compared to *E. coli* inhibition. The absorbance at optical density (OD) 600nm wavelength was also measured to monitor the growth of bacteria in solution. From the results, broth dilution proves to be a more effective method to assess the antibacterial activity in the combination samples. Two combinations showed the best antibacterial synergy (OD₆₀₀ value of 0.000) against both Gram-positive and Gram-negative bacteria, which were (1) ½ sea cucumber: ½ kelulut honey combination and (2) ¾ sea cucumber: ¼ kelulut honey combination. This preliminary evidence on their antibacterial synergistic activities is promising and shows possible commercialization potential.

1. Introduction

Antimicrobial susceptibility testing can be used for drug discovery. However, its effectiveness is lost due to increased microbial resistance. Currently, its impact is considerable with treatment failures associated with multidrug-resistant bacteria (Parmanik *et al.*, 2022). For this reason, the discovery of new antibiotics is an exclusively important objective. Natural products have continued to be one of the major sources of new drug molecules today. Interest in natural products as drug indicators is being revived, particularly to address antimicrobial resistance (Atanasov *et al.*, 2021).

solution produced naturally by both honeybees and stingless bees. It is well-known for its high nutritious content and health benefits. *In vitro* and *in vivo* research has revealed its antibacterial activities. Common honey producing bees in Malaysia include *Heterotrigona itama*, *Apis dorsata*, and *Apis mellifera*, which produce Kelulut, Tualang, and Akasia honey, respectively (Mustafa *et al.*, 2018). Kelulut honey is from the stingless bee honey type. Kelulut honey is more diluted compared to other types of honey. Kelulut honey has been claimed to have various pharmacological properties as well as antibacterial properties (Mohd Kamal *et al.*, 2021).

Honey is a natural substance with flavoured viscous

Sea cucumbers have leathery skin and gelatinous

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bodies, shaped like a soft body cucumber. They are one of many marine animals with valuable nutrients and medicinal properties. Due to the presence of bioactive agents, sea cucumber has been linked with therapeutic potential. Several Holothurian species such as *Holothuria atra* and *Holothuria leucospilota* have been reported to exhibit antibacterial activity. A bioactive compound in sea cucumber extract possessing antibacterial activity is saponins (triterpene glycosides) (Pangestuti and Arifin, 2018). The combination of sea cucumber extract with stingless bee honey holds great potential as a health supplement. However, the specific results of the combination have yet to be determined.

In this study, we evaluate the antibacterial activity of sea cucumber extract and kelulut honey generally and in combination. A variety of laboratory methods can be used to evaluate or screen the *in vitro* antimicrobial activity of an extract or a pure compound. The most known and basic methods are the disk-diffusion and broth or agar dilution methods. Therefore, disk-diffusion assays and broth dilution methods were employed to obtain qualitative and quantitative data on the antimicrobial activity of these two natural substances.

2. Materials and methods

2.1 Source of materials

The study investigated the antibacterial test of individual and combination of sea cucumber extract and kelulut honey using disk-diffusion assays and broth dilution method. Both sea cucumber and kelulut honey are obtained from extracted readymade commercial products. The *Phyllophorus spiculata* sea cucumber samples were obtained from Selera Suarasa Foods, Sungai Petani, Kedah, while kelulut honey samples were obtained from Madu Kelulut Asli Rimba, Kahang, Johor. Both natural substances have been extracted and are in a liquid form. All antibacterial agents were tested against two different bacteria which are *Staphylococcus epidermidis* representing Gram-positive bacteria and *Escherichia coli* representing Gram-negative bacteria. Nutrient agar (NA) and nutrient broth (NB) were used as media for these assays.

2.2 Disk-diffusion assay

Sea cucumber extract or kelulut honey individually and in combination were diluted with sterile distilled water into several different concentrations as shown in Table 1. A volume of 100 μ L inoculum (1×10^8 CFU/mL referred to as 0.5 McFarland standard) from each bacterium was spread on NA plates and wells (7 mm diameter) were made in all inoculated plates before each being filled with previously diluted samples. A six mm filter paper disk containing 80 μ L of diluted samples was

used to test the bacteria growth on the inoculated agar medium. Sterile filter paper disk containing distilled water was used as negative control while ampicillin antimicrobial susceptibility disks (10 μ g) were used as positive control. All prepared inoculated plates were incubated at 35 – 37°C for 16 – 24 hrs. The experiments were performed in triplicate. Finally, the diameter of inhibition zones was observed and measured. An inhibition zone with a diameter \geq 10 mm is considered a positive antagonism effect.

Table 1. Antibacterial activity of samples against *E. coli* and *S. epidermidis*

Samples (% v/v)	Inhibition Zone Diameter Measurement (mm)	
	<i>E. coli</i>	<i>S. epidermidis</i>
Sea Cucumber		
100%	0.00	0.00
70%	0.00	6.10
50%	0.00	0.00
25%	0.00	0.00
Kelulut Honey		
100%	12.00	17.00
70%	0.00	0.00
50%	0.00	6.40
25%	0.00	6.40
Sea Cucumber (SC) + Kelulut Honey (KH)		
50% SC + 50% KH	0.00	0.00
70% SC + 30% KH	0.00	6.80
30% SC + 70% KH	0.00	0.00

2.3 Broth dilution method

The MIC was determined by the broth dilution method to determine the power of the antimicrobial activity of the samples. Serial two-fold dilutions of the working concentrations were made in sterilized test tubes using NB. First, a dilution of $\frac{1}{2}$ of 1×10^8 CFU/mL antibacterial agent was prepared in sterile NB. Then, 1 mL of broth medium was prepared for each test tube starting from the 1st to 6th tube. The sterilized macro dilution test tubes were filled with 1 mL of serial two-fold dilution of antimicrobials. After that, 1 mL was serially transferred from the first tube to the 6th tube through each of the tubes along the way. The 7th and 8th tubes were set as a control growth which are negative control (NB and sample) and positive control (containing bacterial suspension and NB, without antimicrobial substance) respectively. Each test and the positive control test tube were inoculated with 100 μ L bacterial suspension. All experiments were performed in triplicate. All test tubes prepared were incubated at 37°C for 18 hrs. On the next day, absorbance was measured at 600 nm (OD₆₀₀) using a UV-visible spectrophotometer (T80 series, Germany). To determine the best combination ratio of sea cucumber: kelulut honey samples for antibacterial activity, another set of experiments was

performed by using pure samples without the addition of NB broth in the mixture (combination ratio can be referred to Table 4). The same procedure described above was used to determine the absorbance at OD₆₀₀.

3. Results and discussion

3.1 Disk-diffusion assays

The antimicrobial results obtained from this study (Table 1) indicate that most of the samples did not exhibit any inhibitory activity and if any, it is relatively small. However, kelulut honey samples with 100% concentration have a positive antagonism effect against *E. coli* and *S. epidermidis*. For kelulut honey samples, all concentrations except for 70% showed an inhibitory effect against *S. epidermidis* at the range of 6 to 7 mm in diameter of the inhibition zone. For combination, only a combination of 70% sea cucumber and 30% kelulut honey showed an inhibitory effect against *S. epidermidis* at 6.80 mm of the inhibition zone. Collectively, sea cucumber had shown no inhibitory effect at all except for *S. epidermidis* bacteria with a diameter inhibition zone at 6.10 mm. In this study, the results obtained showed significant differences as compared to previous published studies. This may be due to the chosen bacteria, whereby Moguel-Salazar *et al.* stated that *E. coli* is one of the most sensitive microorganisms (Moguel-Salazar *et al.*, 2013). Our study used standard laboratory strains of *E. coli* as it is widely used as the standard Gram-negative strain of preliminary assay (Zainol *et al.*, 2013). However, Zainol *et al.* indicated that *E. coli* may have developed resistance to ampicillin antibiotics (Zainol *et al.*, 2013). This situation suggests that Gram-negative *E. coli* is less susceptible to available antibiotics.

Moreover, the antibacterial activity of samples against *S. epidermidis* is higher as compared *E. coli*. This agrees with a previous study that showed the higher susceptibility of Gram-positive bacteria towards *Phyllanthus niruri* extract as compared to Gram-negative bacteria (Ibrahim *et al.*, 2013). This may be due to the differences in the component structure of their bacterial cell walls.

From the result, sea cucumber samples obtained a negative effect on the antibacterial activity except for *S. epidermidis* at 70% concentration. The sea cucumber extract used in this study is from the coelomic fluid of the sea cucumber that has been filtered several times. The sample has also been stored in the refrigerator for two months before use. Thus, the length of time taken may have reduced the antibacterial effect of the sea cucumber extract. The species of the sea cucumber is different from holothuroids species that has commonly

been studied previously. There are not many studies on the antimicrobial effects of *Phyllophorus spiculata*, locally known as tennis-ball sea cucumber or 'gamat bola' by locals.

Phyllophorus spiculata is listed as 'Vulnerable' on the Red List of threatened animals of Singapore. In Singapore, the main threat is habitat loss due to reclamation or human activities along the coast that pollute the water (Lane and VandenSpiegel, 2003). In Pantai Merdeka, Kedah, Malaysia, the company that has been selling the coelomic fluid of *P. spiculata* commercially adopted a sustainable approach of cutting the sea cucumber, collecting the coelomic fluid, and then throwing it back into the sea. This is to ensure it enables the repetitive harvesting of the sea cucumber's coelomic fluid without endangering the species (personal communication, 2024). Published studies have analysed microbial population within the coelomic fluid (Lukman *et al.*, 2014) from *Stichopus chloronotus* and *Holothuria (Mertensiothuria) leucospilota*; as well as cytotoxic potential of coelomic fluid extracted from *Holothuria tubulosa* against breast cancer cell line (Luparello *et al.*, 2019).

Two publications have reported on the anti-microbial activity of stingless bee honey from *T. carbonaria* (Irish *et al.*, 2008; Kimoto-Nira and Amano, 2008). Both used the agar diffusion method with one reporting no antimicrobial activity (Kimoto-Nira and Amano, 2008) whilst the other showed considerable activity (Irish *et al.*, 2008). In the current study, little activity was seen by agar diffusion, which concurs with one of these previous studies. Differences in results, however, may be attributable to variation in the stingless bee honeys tested. The method by which activity was assessed and variations in protocols between different research groups are also likely to be factors. In particular, the method for inoculating the agar, whether it be surface inoculation such as was used in the current study or incorporation of the organisms into the agar plate itself (Irish *et al.*, 2008) may influence results. This suggests that the agar disk-diffusion method does not necessarily generate results that are representative of total antimicrobial activity.

3.2 Broth dilution method

MIC is the lowest concentration of antibiotic being tested that inhibits bacterial growth, producing a sample that has low or no turbidity. Table 2 shows the MIC value of all samples against *E. coli* and *S. epidermidis* while Table 3 shows absorbance at 600 nm (OD₆₀₀) for various dilutions of samples. The higher the OD₆₀₀ value, the higher the degree of light scattered by bacteria in the solution, which indicates the higher bacterial count. Conversely, the lower the OD₆₀₀ value, the lower the

bacterial count.

Table 2. Minimum inhibitory concentration (MIC) of samples.

Samples	MIC (mg/mL)	
	<i>E. coli</i>	<i>S. epidermidis</i>
Sea Cucumber	0.250	0.031
Kelulut Honey	0.125	0.063
Sea Cucumber + Kelulut Honey	0.250	0.063

From the result, the MIC values of samples against *S. epidermidis* are lower than *E. coli*, ranging from 0.031 to 0.125 mg/mL. Higher MIC values were observed for samples against *E. coli*, ranging from 0.125 to 0.250 mg/mL. The lowest MIC value is for sea cucumber samples against *S. epidermidis* with a value of 0.031 mg/mL. The low turbidity or bacterial growth below the detectable limit (absorbance at OD₆₀₀ value of 0.000) was observed for all sea cucumber dilution samples from 0.031 mg/mL to 0.500 mg/mL, except for the 1/64 dilution, with OD₆₀₀ value of 0.206.

Lower MIC values indicate that fewer antibacterial agents are needed to inhibit bacterial growth. Therefore, antibacterial agents with lower MIC scores are more effective antimicrobial agents. This result was comparable with the values obtained in a previous study against *S. aureus* as a Gram-positive bacterium at 37.4 g/mL and for *V. cholerae* as a Gram-negative bacterium at 8.4 g/mL (Moguel-Salazar et al., 2013). However, this is difficult to compare as techniques for finding the value of MIC are different and the bacteria chosen are also different. For kelulut honey, the lowest MIC value obtained to inhibit *E. coli* is 0.125 mg/mL and 0.063 mg/mL against *S. epidermidis*. This is in line with studies in which it stated that Gram-positive bacteria proved to be more susceptible to Malaysian honey than Gram-negative bacterial species (Zainol et al., 2003).

From the result of the two different methods used to determine the antibacterial activity, the broth dilution method is more effective compared to the disk-diffusion assays. However, it is important to note that optical density measurements also have some limitations (Nychas et al., 2003). Specifically, turbidometry detects only the upper part of microbial growth curves and requires calibration to correlate the results with viable counts obtained on media (Skandamis et al., 2001). MIC is easily influenced by bacterial properties, inoculum size, culture medium composition, incubation time and incubation conditions, such as pH, temperature and aeration (Moguel-Salazar et al., 2013).

The OD₆₀₀ of sea cucumber extract and kelulut honey combination at different concentrations was also evaluated. From Table 4, the result of the combination ratio of pure sea cucumber: kelulut honey without any nutrient broth was shown. Two combinations that showed the best antibacterial synergy against both Gram-positive and Gram-negative bacteria are (1) ½ sea cucumber: ½ kelulut honey combination, and (2) ¾ sea cucumber: ¼ kelulut honey combination. The OD₆₀₀ value for these two combinations is the lowest among all concentrations (absorbance of 0.000) indicating the absence of bacterial growth or bacterial growth below the detectable limit. These results are significant as there

Table 4. Absorbance measured at the wavelength of 600 nm (OD₆₀₀) for the various concentrations of sea cucumber and kelulut honey combination.

Concentration of Sea Cucumber + Kelulut Honey (v/v)	Absorbance at 600 nm (OD ₆₀₀)	
	<i>E. coli</i>	<i>S. epidermidis</i>
1/2 + 1/2	0.000	0.000
3/4 + 1/4	0.000	0.000
1/4 + 3/4	0.001	0.028
1/8 + 7/8	0.002	0.049
7/8 + 1/8	0.203	0.424

Table 3. Absorbance measured at the wavelength of 600 nm (OD₆₀₀) for the various samples.

Bacteria serial dilutions (from 1×10 ⁷ CFU/mL stock)	Absorbance measured at 600 nm (OD ₆₀₀)			
	<i>E. coli</i>	Sea Cucumber	Kelulut Honey	Sea Cucumber + Kelulut Honey
1/2		0.000	0.843	0.627
1/4		0.110	1.025	0.709
1/8		0.357	1.501	0.712
1/16		0.469	1.534	0.752
1/32		0.500	0.728	0.887
1/64		1.400	1.767	0.989
<i>S. epidermidis</i>				
1/2		0.000	0.000	0.000
1/4		0.000	0.000	0.000
1/8		0.000	0.270	0.006
1/16		0.000	0.486	0.173
1/32		0.000	0.711	0.643
1/64		0.206	0.624	0.419

is no previous study conducted to evaluate the synergistic combination of sea cucumber and kelulut honey.

4. Conclusion

Sea cucumber and stingless bee honey give a wide range of benefits for human health. Among common honey produced in Malaysia, kelulut honey was chosen as kelulut honey possessed higher nutritional values and antioxidant activities as indicated in published studies. Meanwhile, sea cucumber is one of the marine animals with valuable nutrients and medicinal properties. As there is much evidence from published literature on the medical benefits of kelulut honey and sea cucumber individually but not in combination, the antibacterial activity of both these natural substances was evaluated in this study using disk-diffusion assays and broth dilution method. For disk-diffusion assays, most of the samples exhibited small or no inhibitory activity. Only kelulut honey samples with 100% concentration have a positive antagonism effect against *E. coli* and *S. epidermidis*. For the broth dilution method, Gram-positive bacteria proved to be more susceptible compared to Gram-negative bacteria species, where the MIC value against *S. epidermidis* is lower than the MIC value against *E. coli* for each sea cucumber, kelulut honey and in combination samples. Two combinations showed the best antibacterial synergy (OD₆₀₀ value of 0.000) against both Gram-positive and Gram-negative bacteria, which are (1) ½ sea cucumber: ½ kelulut honey combination and (2) ¾ sea cucumber: ¼ kelulut honey combination. From the result, the broth dilution method is shown to be a more effective method to find the antibacterial activity compared with disk-diffusion assays. This preliminary evidence on their antibacterial synergistic activities is promising and shows possible commercialization potential.

Conflict of interest

The authors declare no conflict of interest.

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