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Antibacterial Activity of Cough Suppressant Functional Drink from Betel Leaves (*Piper sarmentosum*), Lime (*Citrus aurantifolia*) and Honey

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ABSTRACT

In Indonesia, dried betel seating leaves are commonly used as a natural traditional medicine which can cure respiratory tract infection, along with the mixture of honey and lime juice. The objective of this research was to determine the antibacterial activity of betel seating leaves extract, and its combination with lime and honey, towards upper respiratory tract infection-causing bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*), of which one of the main symptoms is cough. The experimental method consists of extraction of betel seating leaves with ethanol for 1, 3, 5 and 7 d and formulation of functional drink by mixing betel seating leaves with lime and honey. Results showed that the extract macerated for 7 d had strong antibacterial activity with MBC of 34.25 ± 1.03 mg/mL and 41.35 ± 1.47 mg/mL against *P. aeruginosa* and *S. aureus*, respectively, and MIC of 8.56 ± 0.31 mg/mL and 10.34 ± 0.36 mg/mL against *P. aeruginosa* and *S. aureus*, respectively. Betel seating leaves extract were formulated with honey and lime to mask its bitter taste. The most preferred formulated drink with the highest antibacterial activity was a mixture of lime-honey (ratio 1:2) and addition of betel seating leaves extract of 3 MBC. This formulation exhibited the strongest inhibition against *P. aeruginosa* and *S. aureus*, with inhibition diameter of about 14.71 ± 0.04 mm and 14.05 ± 0.09 mm, respectively, by causing bacterial cell wall damage.

Keywords: Antibacterial, Cough, Honey, Betel seating leaves, Lime juice.

Introduction

Betel seating plant (*Piper sarmentosum* Roxb. ex Hunter) is commonly found in tropical and subtropical countries,[1] including Indonesia. Betel seating leaves are frequently used as a traditional medicine that can cure several diseases, such as asthma, cough, bone pain, toothache, and other diseases that are caused by bacteria and fungi.[2] The utilization of betel seating leaves as a medicine is related to its bioactive compounds that have antibacterial activity. Methanol or ethanol extract of betel seating leaves have shown antibacterial activity against Gram-positive and Gram-negative bacteria (*Pseudomonas aeruginosa* and *Staphylococcus aureus*),[3,4] which are known as the URTI (Upper Respiratory Tract Infection)-causing bacteria.[5] In Malaysia, roots and leaves of betel seating plant are used as medicine against headache, cough, and asthma, and also as an expectorant, however, betel seating leaves have slightly bitter taste that is unfavourable.[4]

Other than betel seating leaves, a plant that is also commonly used as a traditional medicine is lime (*Citrus aurantifolia* Swingle). Lime is utilized as a traditional medicine to reduce fever, as anti-diarrheal, anti-inflammation and antibacterial agent.[6] Lime juice has been reported to inhibit the growth of bacteria and molds, such as *Escherichia coli, Streptococcus haemolyticus, Staphylococcus aureus, Aspergillus niger* and *Candida albicans*.[7-10] Lime has also been reported to inhibit the growth of cough-causing bacteria, for example *Staphylococcus aureus* and *Streptococcus pyogenes*.[11] The activity is also preserved even if lime is mixed with honey or sweet soy sauce. Antibacterial activity of a mixture of lime and honey is reported to be stable against heat up to a temperature of 100°C.[12] Based on this result, it is also reported that honey has antibacterial activity.[13] Fresh honey bee has inhibition activity up to 50% against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Moreover, honey can also be used as a sweetener that can affect colour, aroma and taste of a drink. The higher the honey concentration, the more intense the colour, aroma and taste.[4] Honey also has antibiotic properties that can heal the dead tissues, wound and ulcer.

To overcome bitter taste from betel seating leaves, lime and honey which possess favorable sour and sweet taste, respectively, were used. This research was focused on formulation of cough-suppressant functional drink and determination of its antibacterial activity against URTI-causing bacteria. Functional drink formulation was done as it is more easily consumed and more preferred compared to drugs.

Materials and Methods

Plant materials

Materials used for functional drink formulation were betel seating leaves (*Piper sarmentosum* Roxb. ex Hunter), lime (*Citrus aurantifolia)* and honey. Betel seating leaves were obtained from Balittro (Balai Penelitian Tanaman Rempah dan Obat), Bogor, Indonesia in September 2019. Lime and honey ("Sari Bunga Alam" Brand) were obtained from Pasar Modern (Modern Market), BSD, Tangerang, Indonesia.

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Organisms and culture media
Staphylococcus aureus ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 (collection of Microbiology Department, Faculty of Medicine, Universitas Indonesia), Nutrient Agar (Merck), Blood Agar Base (Oxoid), defibrinated sheep blood obtained from Microbiology Department, Universitas Indonesia.

Chemicals and reagents
Ethanol, Dragendorff and Mayer reagent, 2 N hydrochloric acid, sodium hydroxide, anhydrous sodium sulfate, dimethylsulfoxide, sulfuric acid, chloroform, anhydrous sodium acetate, boric acid, Folin-Ciocalteau, sodium carbonate, ferric chloride, gallic acid, aluminium chloride, potassium acetate, quercetin, glutaraldehyde 2%, were all products of Merck - Germany, and drinking water (Aqua brand, Indonesia).

Preparation of betel seating leaves extract
Betel seating leaves were washed and cut into smaller pieces with width around 2 cm. The pieces were dried using oven at temperature of 70^{o}C for 5 h.[15,16] Dried betel seating leaves were then size reduced using dry blender and sifted using 35 mesh sifter.
Extraction was performed using maceration method using food grade ethanol.[17] The amount of 15 gram sample was mixed with 300 mL ethanol and macerated at room temperature for 1 d, 3 d, 5 d and 7 d. The result of maceration was filtered using a Buchner vaccuum pump and the filtrate was then evaporated using rotary evaporator at 50^{o}C to obtain betel seating leaves extract.

Formulation of functionald drink
The ingredients used to make cough suppressant drink include; betel seating leaves extract, lime juice and honey. Lime and honey (Sari Bunga Alam brand) were obtained from 'Pasar Modern', Indonesia. First, lime juice and honey were prepared. Lime juice was prepared by cutting lime into two pieces and squeezing them, then filtering using tea strainer. Formulations were prepared from 50 mL of selected betel seating leaves extract (1 MBC, 2 MBC, 3 MBC, and 4 MBC), added to 50 mL of a mixture of lime juice and honey (ratio 1:1, 1:2, 1:3 and 1:4). The ingredients were mixed and heated at 70^{o}C for 30 min over a water bath, and then cooled down to reach room temperature.

MIC and MBC analysis
Analysis of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) was done using agar well diffusion method.[18,19] About 1 mL of bacterial (*S. aureus* or *S. pyogenes*) suspension was inoculated into Petri dish and poured with Nutrient Agar. After solidification, 6 mm diameter wells were made aseptically and 60 µL of extracts with concentration of 100 mg/mL, 200 mg/mL, 300 mg/mL, 400 mg/mL, and 500 mg/mL, were added into the wells. Petri dish was then incubated for 24 h at 37^{o}C. Inhibition zone formed was used to calculate MIC and MBC. MIC was determined by making linear regression between ln extract concentration as the X-axis and square of inhibition diameter (Z^2) as the Y-axis. Intersection of equation curve on X-axis was called Mt value. MIC value is equal to 0.25 Mt and MBC is 4 times MIC.

Brine shrimp lethality test (BSLT)
Extract toxicity was analyzed using BSLT method.[20,21] This method used brine shrimp larvae (*Artemia salina* Leach). Larvae was put in a container and acclimatized for 48 h at room temperature with sufficient oxygen and light supplies. About 20 mg of extract was added into container that consisted of 0 μL Tween 80 and 10 mL of seawater to obtain stock solution (2000 μg/mL). This stock solution was used to make samples with concentrations of 0, 10, 100, 500 and 1000 μg/mL. Each tube was added with 10 living larvae and the amount of living larvae was counted after 24 h. The data obtained were then analyzed to determine LC_{50} (50% Lethal Concentration) value, i.e. the concentration required to kill 50% of the brine shrimp larvae.

Phytochemical analysis
Qualitative phytochemical tests for alkaloids, flavonoids, terpenoids, steroids, saponins and tannins were carried out.[22]
Test for flavonoids: The extract (0.5 mg) was dissolved in 5 mL distilled water then heated. Two (2) mL of the extract was then added with 0.4 mL amyl alcohol containing 37% HCl and 96% ethanol, 0.5 mg magnesium, and 70% alcohol. The presence of red or orange color indicated the presence of flavonoid.
Test for tannins: 2 drops of 1% $FeCl_3$ and H_2SO_4 were added to 2 mL extract. The presence of blackish green precipitate indicated the presence of tannin.
Test for alkaloids: 2 mL extract was added to 0.5 mL of 2% HCl and the solution was divided into two tubes. To the first tube was added 2-3 drops of Dragendorff's reagent, to the second tube was added 2-3 drops of Mayer's reagent. The presence of orange colour in the Dragendorff's reagent tube and yellowish precipitate in the second tube indicate the presence of alkaloids.
Test for saponins: to the extract was added water (1: 1) while shaking for 1 min, formation of foam which persist for 10 min after adding 1 N HCl, is a positive test for saponins.
Test for terpenoids/steroids: 2 mL of extract was dissolved in 0.5 mL of chloroform, and then supplemented with 0.5 mL of acetic acid anhydride. To this mixture was added 1-2 mL of concentrated H_2SO_4 through the tube wall. The formation of a brownish or violet ring on the interface of two solvents indicate the presence of triterpenoids, whereas the formation of a bluish green colour indicate the presence of steroids.

Total phenolic content analysis
Total phenolic content was determined using Folin-Ciocalteau method.[16,23] About 0.3 mL sample was added to 1.5 mL of Folin Ciocalteau solution. To the mixture was added 1.2 mL of Na_2CO_3 (7.5%). The mixture was incubated for 1 h in the dark room and phenolic content was determined colorimetrically using spectrophotometer at wavelength of 750 nm. Standar curve was prepared using gallic acid solution with concentration of 10-100 ppm. Total phenolic content was expressed as mg gallic acid equivalent/L (mg GAE/L sample).

Total flavonoid content analysis
Total flavonoid content was determined using aluminium chloride spectrophotometric method.[16,23] 1 mL of sample was added to 1 mL of aluminium chloride (2%). The mixture was then incubated at room temperature for 30 min after which the absorbance was measured with a spectrophotometer at wavelenght of 415 nm. Standard curve was prepared using quercetin with concentration of 5-50 ppm. Total flavonoid content was expressed as mg quercetin equivalent/L (mg QE/L sample).

Hedonic test[24]
Organoleptic test using hedonic was performed to determine panelist preference towards the betel seating leaves extract functional drink, in terms of aroma, bitter taste, sour taste, aftertaste and overall acceptance. Hedonic test was done using 70 panelists with 7 rating scales, i.e. from 1 means dislike extremely to 7 means like extremely. Panelists were asked to give scores to each attribute without comparing among samples.

Proximate analysis[25]
Proximate analysis were done on betel seating leaves extract functional drink. Parameters determined include; moisture content using oven method, protein content using Kjeldahl method, fat content using Soxhlet extraction method, ash content using dry ashing method and carbohydrate content using by difference method.

Statistical analysis
All data obtained in this research were analysed statistically using SPSS program version 22.0.

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Results and Discussion

Table 1 shows the phytochemical constituents of betel seating leaves extract. The phytochemical tests revealed the presence of saponins, flavonoids, tannins and alkaloids. Saponins, flavonoids, tannins and alkaloids have been reported to have antibacterial properties against both the Gram-negative and Gram-positive bacteria.[26,27] Tannins have antibacterial properties by inhibiting reverse transcriptase and DNA topoisomerase.[28] Tannins can also attack polypeptides of cell wall, resulting in imperfect cell wall. As a result, bacterial cells undergo lysis because of physical and osmotic pressure, and eventually death.[29] On the other hand, flavonoids exhibit antibacterial properties by forming complexes with extracellular proteins and soluble proteins that can damage bacterial cell membrane, followed by lysis.[30-32] Other than inhibiting DNA-RNA synthesis, flavonoids can also inhibit energy metabolism using similar mechanism that inhibits respiration system.[33] Since flavonoids are parts of phenolic group, phenolics have a main target which is cytoplasmic membrane, based on its natural hydrophobic properties.[34] Saponins also have antibacterial properties by reducing surface tension to increase cell wall permeability ond leakage of intracelluler materials.[28] This compound diffuses through susceptible outer membrane or cell wall, then bind the cytoplasmic membrane and disrupt its stability. As a result, cytoplasm leaks and causes cell death.[35]

Antibacterial activity of betel seating leaves extract

The tested cough-causing bacteria were *S. aureus* and *P. aeruginosa*. Antibacterial activity of betel seating leaves extract was measured based on MIC (Minimum Inhibition Concentration) and MBC (Minimum Bactericidal Concentration) values, as can be observed in Table 2. Lower MIC and MBC values showed higher antibacterial properties of a compound, and vice versa. Table 3 shows that betel seating leaves extract showed inhibiton towards *S. aureus* and *P. Aeruginosa.* This result correlated with the phytochemical analysis results which showed that the extract contains saponins, alkaloids, tannins and flavonoid (Table 1). Lower MIC and MBC towards *P. aeruginosa* indicates that betel seating leaves extract might have better inhibition towards *P. aeruginosa* compared to *S. aureus*.

Based on maceration time, it was shown that extracts from 3, 5 and 7 d of maceration time resulted in similar inhibition towards *S. aureus*, but different towards *P. aeruginosa*, in which the highest inhibition against *P. aeruginosa* was the extract from 7 d of maceration time. Based on this result, betel seating leaves extract that were used for the drink formulation was extract from 7 d of maceration.

The linear equations for the correlation between the 7 d extract concentration (X) and inhibition diameter (Y) were Y = 0.0097X + 11.39 (R^2 = 0.99) for *S. aureus* and Y = 0.0098X + 12.21 (R^2 = 0.97) for *P. aeruginosa*. When MBC value for both bacteria are put into the formula, the inhibition diameter would be 11.78 mm for *S.aureus* and 12.54 mm for *P. aeruginosa*. These results are comparable to a previous research that obtained inhibition zone of 11 mm towards *S. aureus* and 12 mm towards *P. aeruginosa.*[36] Based on its inhibition zone, betel seating leaves extract is considered to have a very strong (very active) antibacterial activity, because inhibition zone formed was more than 10 mm.[37] Therefore, betel seating leaves extract could actively inhibit *S. aureus* and *P. aeruginosa*, which are URTI-causing bacteria and could be further applied in URTI or cough-suppressant drink. However, the disadvantage of using betel seating leaves extract was its unwanted bitter taste and mixing with other ingredients should be done to reduce this effect.

Toxicity of betel seating leaves extract

Toxicity of extract was measured based on LC_{50} value using Brine Shrimp Lethality Test (BSLT). LC_{50} value can be categorized into four, i.e. LC_{50} value $\leq$ 30 ppm means strong toxicity, LC_{50} value between 30-100 ppm is categorized as toxic, LC_{50} value between 100-1000 ppm means low toxicity and LC_{50} value > 1000 ppm means non-toxic.[38] The toxicity test result of betel seating leaves extract that was macerated for 7 d was 227.06 ppm. Therefore it can be categorized as low toxicity. This low toxicity level may be attributed to the saponins content of the leaves.[39]

Table 1: Phytochemical compounds of betel seating leaves extract

Phytochemical compound	Result
Saponin	+
Tannin	+
Flavonoids	+
Alkaloids	+
Steroids	-
Triterpenoids	-

Notes: + = detected; - = not detected.

Table 2: MIC and MBC values for *S. aureus* and *P. aeruginosa*

Maceration time	MIC (mg/mL)	
	S. aureus	***P. aeruginosa***
1 day	$23.36 \pm 0{,}76^{a}$	21.99 ± 0.81^{c}
3 days	$10.22 \pm 0{,}23^{b}$	9.97 ± 0.31^{b}
5 days	10.33 ± 0.45^{b}	9.93 ± 0.19^{b}
7 days	10.34 ± 0.36^{b}	8.56 ± 0.31^{a}
Maceration time	**MBC (mg/mL)**	
	S. aureus	***P. aeruginosa***
1 day	93.44 ± 3.05^{b}	87.95 ± 2.65^{c}
3 days	40.88 ± 0.93^{a}	39.891 ± 1.03^{b}
5 days	41.32 ± 1.82^{a}	39.74 ± 0.63^{b}
7 days	41.35 ± 1.47^{a}	34.25 ± 1.03^{a}

Notes: different superscript shows significant difference at $p < 0.05$

Antibacterial activity of functional drink from betel seating leaves extract, lime and honey

Formulation of cough suppressant drink used ratios of 1:1, 1:2, 1:3, 1:4 between lime juice and honey, and betel seating leaves extract with dosage 1× MBC, 2× MBC, 3× MBC and 4× MBC. Antibacterial activity was measured based on its inhibition diameter. Antibacterial activity based on inhibition zone can be categorized as very active (>10 mm), active (7-10 mm), slightly active (6-7 mm) and not active (less than 6 mm).[37] Inhibitory power of cough suppressant drink towards *P. aeruginosa* and *S. aureus* is presented in Figure 1. Figure 1 shows that there are two similar inhibition phenomena on both cough-causing bacteria. The first phenomenon is related to the increase of extract dosage in the drink, in which the higher the extract concentration, the higher the inhibition power towards *P. aeruginosa* and *S. aureus*. The second phenomenon is an increase of inhibition until ratio of 1:2, and then decrease on lime:honey with ratio of 1:3 and 1:4 on both cough-causing bacteria. Inhibition power phenomenon caused by higher concentration of betel seating leaves extract is supported by previous report[3] which stated that the increase of betel seating leaves extract concentration would increase its inhibition towards *P. aeruginosa* and *S. aureus.* The inhibition diameter is affected by flavonoids and other phytochemical component of betel seating leaves extract.[40] It is also influenced by antibacterial properties of lime juice[41] and honey.[13] This is supported by the fact that mixture of lime juice and honey with ratio 1:1 could inhibit cough-causing bacteria,[12] and its antibacterial activity was stable upon heating until 100°C.

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Based on these results, the optimum inhibition power of drink formulated with lime juice, honey and betel seating leaves extract consists of lime and honey with ratio of 1:2 and extract concentration of 4 MBC with inhibition diameter towards *Pseudomonas aeruginosa* of 15.28 ± 0.06 mm and towards *Staphylococcus aureus* of 14.45 ± 0.03 mm. These inhibition diameters are larger compared to inhibition diameters of betel seating leaves extract with concentration of 100 mg/mL and 200 mg/mL which only reached 12 mm and 13 mm for *S. aureus*, respectively, and 13 mm and 14 mm for *P. aeruginosa*, respectively. The addition of lime juice and honey into betel seating leaves extract could increase its antibacterial activity towards *S. aureus* and *P. aeruginosa*. Thus, it has potential for use as a cough suppressant drink.

Total Phenolic and Total Flavonoids Content

Phenolic and flavonoids compounds can act as antibacterial agents.[26,28,31-32] The total phenolic and flavonoids content of cough suppressant is presented in Figure 2. Figure 2A and 2B show the similar phenomenon, i.e. the higher the concentration of betel seating leaves extract, the higher the total phenolic and flavonoids content. On the other hand, the higher the ratio of lime juice and honey, the lower the total phenolic and flavonoids content. The phenolic and flavonoids content in the extract indicates that betel seating leaves contain many phytochemical compounds.[34] Flavonoid content in the extract can be influenced by flavonoids content in betel seating leaves and lime juice nipis.[3,42] Mixture of lime juice and honey with ratio 1:1 and extract concentration of 4 MBC gave the best phenolic and flavonoids content.

Hedonic Characteristics of betel seating leaves functional drink

Addition of extract concentration and mixture of lime juice and honey impacted significantly on aroma, bitter taste, sour taste, aftertaste and overall acceptance (overall preference) of betel seating leaves drink ($p < 0.05$). The results of hedonic test of betel seating leaves drink is shown in Table 3. The addition of honey and extract affected the panelists' preference towards aroma, bitter taste, sour taste, aftertaste and overall acceptance. On the other hand, the addition of betel seating leaves extract affected the panelists' preference towards colour, aroma, bitter taste, sour taste, aftertaste and overall acceptance. In this case, addition of extract until 3 MBC resulted in no significant difference in terms of preference towards bitter taste and aftertaste, compared to addition of extract of 1 MBC. Bitter taste may be attributed to saponins[42] which can give bitter aftertaste.[44] Sour taste can be attributed to the presence of citric acid in the lime juice.[11] Besides, bitter taste can be influenced by phenolic compounds in the extract.[17] The presence of saponins and tannins in *Sapondias mombin* leaves influences the bitterness of leaves.[45] Bitter taste of leaves can be influenced by alkaloids, saponins and tannins.[46] Furthermore, high concentration of the extract resulted in darker colour, more pronounced leaves aroma caused by the presence of volatile compounds and more pronounced bitter taste.[17] The most preferred combination of bitter taste and aftertaste from betel seating leaves, sweet taste from honey and sour taste from lime was the mixture of lime juice and honey with ratio of 1:3, with addition of betel seating leaves extract of 3 MBC.

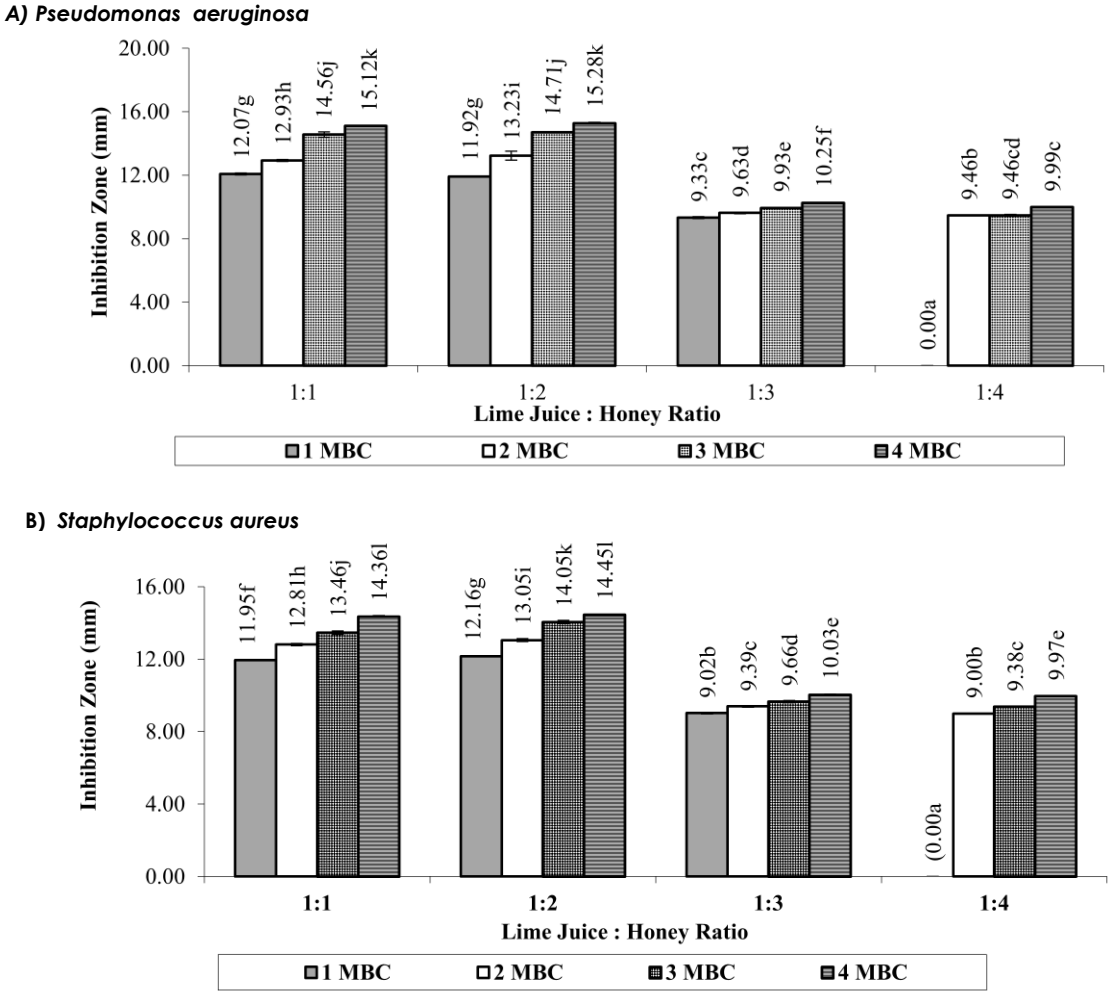

Figure 1: Inhibitory power of functional drink towards *P. aeruginosa* **(A)** and *S. aureus* **(B)**.
Note: Different superscript indicate significant difference at $p < 0.05$.

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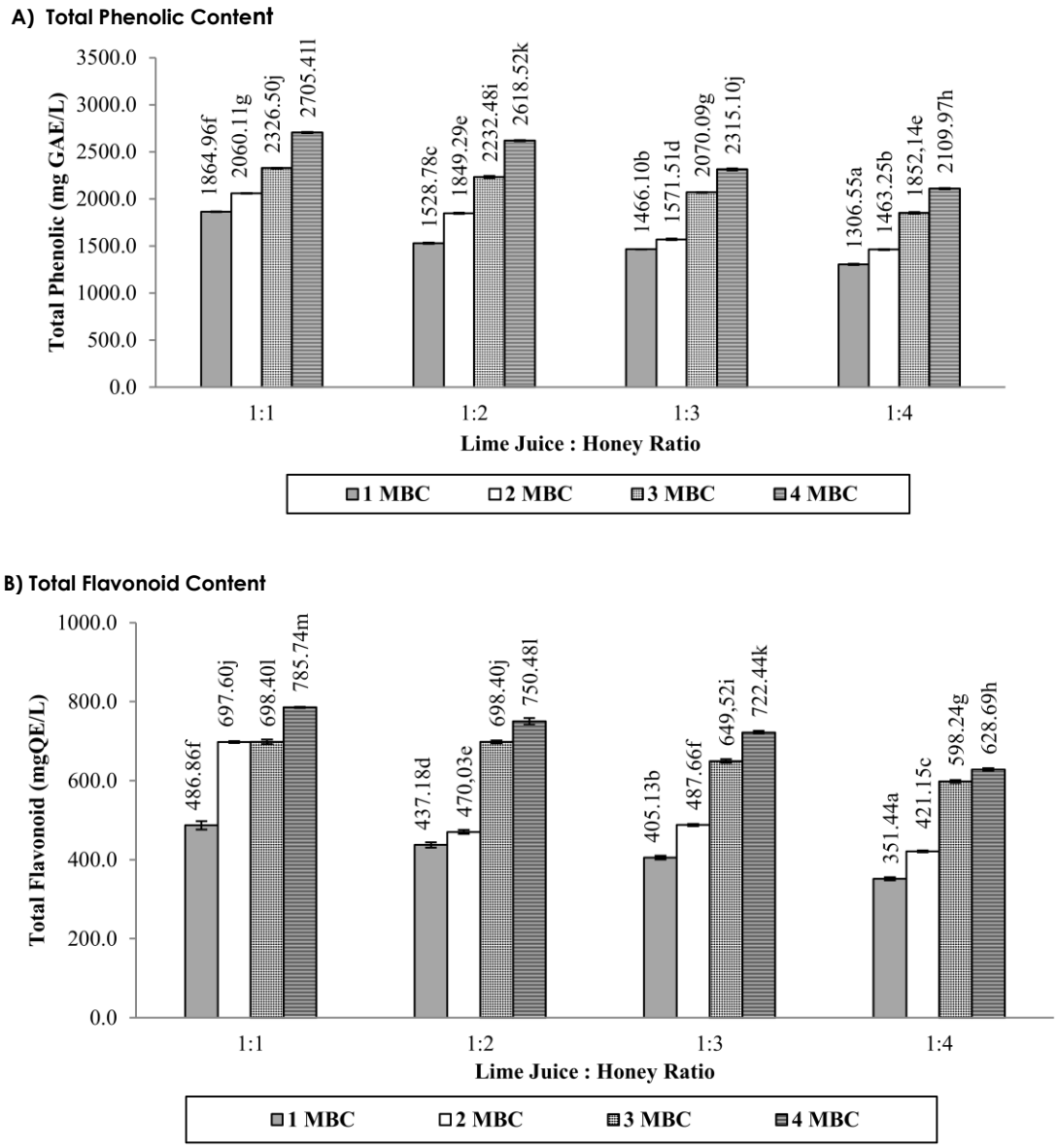

Figure 2: Total phenolic (**A)** and total flavonoids **(B)** contents of the cough-suppressant drink
Note: Different superscript shows significant difference at $p < 0.05$.

Table 3: Results of hedonic test of betel seating leaves drink

Lime : Honey + Extract	Hedonic Attribute				
	Aroma	**Bitter taste**	**Sour taste**	**After taste**	**Overall acceptance**
1:1 + 1MBC	5.2 ± 0.4^{b}	4.6 ± 0.3^{c}	4.1 ± 0.2^{a}	4.6 ± 0.3^{ab}	4.5 ± 0.1^{b}
1:2 + 2MBC	5.2 ± 0.2^{b}	4.4 ± 0.3^{b}	4.2 ± 0.2^{a}	4.7 ± 0.3^{b}	4.6 ± 0.3^{b}
1:3 + 3MBC	5.1 ± 0.1^{ab}	4.7 ± 0.1^{c}	4.4 ± 0.3^{b}	4.8 ± 0.1^{b}	4.8 ± 0.1^{c}
1:4 + 4MBC	4.9 ± 0.2^{a}	4.0 ± 0.2^{a}	4.2 ± 0.2^{a}	4.4 ± 0.4^{a}	4.3 ± 0.3^{a}

Notes: different superscript indicate significant difference at $p < 0.05$. For each attribute, panelists were asked to give score from 1-7, with: 1 = dislike extremely; 2 = dislike; 3 = slightly dislike; 4 = neutral; 5 = slightly like; 6 = like; 7 = like extremely.

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Characteristics of selected functional drink

The highest inhibition diameter from betel seating leaves drink was from treatment using lime juice and honey with ratio of 1:1 and extract concentration of 4 MBC. This treatment was not significantly different from treatment using lime juice and honey (ratio of 1:2) and extract concentration of 4 MBC. The best total phenolic and flavonoids content was observed in the treatment using lime juice and honey with ratio of 1:1 and extract concentration of 4 MBC. However, the organoleptic test showed that the panelists prefer the treatment using extract concentration of 3 MBC with lime juice and honey (ratio 1:3), which was not significantly different from ratio 1:2. Since ratio 1:2 and extract concentration of 4 MBC was slightly disliked by the panelists, the selected formulation of betel seating leaves drink as cough suppressant drink was a mixture of lime and honey (ratio 1:2) and extract concentration of 3 MBC.

Physicochemical characteristics of selected cough suppressant functional drink is shown in Table 4. High concentration of carbohydrate in betel seating leaves drink is related to high concentration of carbohydrate in lime and honey.

The ability of cough suppressant functional drink from combination of betel leaves extract, lime juice and honey in killing cough-causing bacteria can be observed in Figure 3. Figure 3 shows the damage of *P. aeruginosa* and *S. aureus* cells that has been exposed with selected formulation, compared to control. The damage of *P. aeruginosa* and *S. aureus* can be seen from incomplete, shrinked and curved cell walls.

Table 4: Physicochemical characteristics selected cough suppressant functional drink from betel seating leaves

Parameter	Concentration
Moisture (%)	64.43
Ash (%)	0.44
Fat (%)	0.33
Protein (%)	0.50
Carbohydrate (%)	36.84
pH	2.69
Overall acceptance based on hedonic test (score 1-7)	4.86

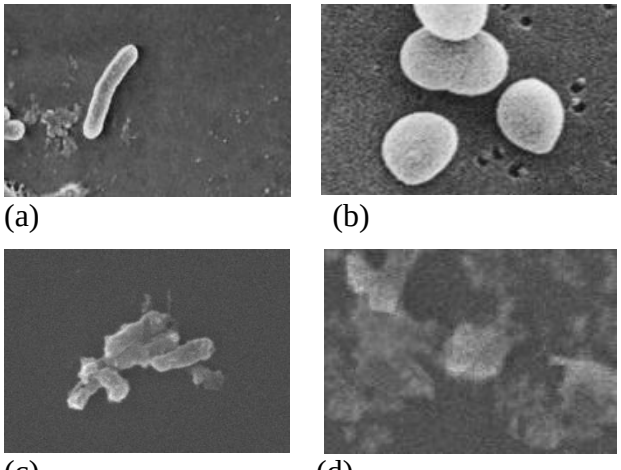

Figure 3: Photomicrographs of normal bacterial cells of *P. aeruginosa* **(a)**, *S. aureus* **(b)**, *P. aeruginosa* that was exposed to drink **(c)** and *S. aureus* that was exposed to drink **(d)** at 10,000x magnification

This damage may have been caused by the presence of saponins, tannins, flavonoids and alkaloids in betel seating leaves extract which have been shown to cause the lysis and death of bacteria. This cell damage may also be related to bacterial cell wall structure. Gram-positive bacteria have simpler cell wall structure compared to Gram-negative bacteria. Gram-negative bacteria have layered structure and higher lipid content (11-12%) which makes them more resistant to environmental changes caused by chemicals. On the other hand, Gram-positive bacterial cell wall consists of 90% peptidoglycan layer and the remaining is made up of teichoic acid.[47,48] Therefore, it is easier for antibacterial compound to disrupt Gram-positive bacterial cell wall.

Conclusion

The ethanol extract of betel seating leaves actively inhibited the growth of *S. aureus* and *P. aeruginosa*, which are URTI-causing bacteria, this makes it suitable for formulation into cough-suppressant functional drink. The MBC values towards *S. aureus* and *P. aeruginosa* were 34.25 ± 1.03 mg/mL and 41.35 ± 1.47 mg/mL, respectively. Addition of lime juice and honey to betel seating leaves functional drink formulation could increase its inhibitory power towards *P. aeruginosa* and *S. aureus*. Formulation of functional drink made from lime juice and honey with ratio 1:2 and betel seating leaves extract of 3 MBC (124.05 mg/mL) inhibited the growth of *P. aeruginosa* and *S. aureus*, with inhibition diameter of 14.71 ± 0.04 mm and 14.05 ± 0.09 mm, respectively. This functional drink was also slightly liked by the panelists.

Conflict of interest

We declare that no conflict of interest with the data contained in this manuscript.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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