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Short communication

Antibacterial Activity of Lemongrass-Scented Betel Herbal Tea

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Abstract

Betel (*Piper betle*) and lemongrass (*Cymbopogon citratus*) are well known medicinal plant that exhibit good antimicrobial properties. This study aims to investigate antibacterial potency of Lemongrass-Scented Betel Tea (LSBT). Four herbal infusions formulations (B-100:L-0, B-95:L-5, B-85:L-15 and B-75:L-25) with different percentage of betel leaves and lemongrass stem powder were prepared. These herbal infusions were tested against four foodborne pathogens, which are *Staphylococcus aureus* (ATCC 29213 and ATCC 33591) (Gram-positive cocci) and *Escherichia coli* (ATCC 25922 and ATCC 35218) (Gram negative bacteria) using Kirby-Bauer disk diffusion method. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays were determined by broth dilution method. B-100:L-0 showed strongest antimicrobial properties with inhibition zones of 28.5 ± 0.71 to 29.0 ± 1.41 mm and 10.0 ± 0.00 mm for *S. aureus* and *E.coli*, respectively. Moreover, B-100:L-0 infusion also showed the lowest MIC (0.63 mg/mL) and MBC (1.25 mg/mL) values for *S. aureus* as well as the lowest MIC (5.0 mg/mL) value for *E.coli*. In conclusion, all four formulations of the herbal tea showed inhibitions against *S. aureus* (ATCC 29213 and ATCC 33591) and *E. coli* (ATCC 25922 and ATCC 35218). However, Gram-negative bacteria were more resistant to antibacterial effects of the herbal tea. It can be suggested that the use of B-100:L-0 would be hepful in the treatment of infections caused by *S. aureus* (ATCC 29213 and ATCC 33591) and *E. coli* (ATCC 25922 and ATCC 35218).

Keywords: *Antibacterial activity; herbal tea; betel leaves; lemongrass stem; foodborne pathogen.*

1. Introduction

In the recent years, the number of food-related illness caused by pathogens such as *Staphylococcus aureus* and *Escherichia coli* is increasing worlwide. Foodborne diseases have been intensified due to the increased use of antibiotics, causing increased resistance to these compounds [1]. Furthermore, antibiotics have been found to be associated with side effects and addiction to the users [2]. The emergence of antibiotic resistance as well as the side effects of conventional treatments has led to an alternative options to overcome this problem, which is traditional medicinal sources [3]. There is wide use of the herbal plants as a source of medicine in many medicinal therapies until the development of synthetic drugs in the nineteenth century [4,5]. Several studies showed that some plants contains good amount of active compounds and nowadays, many drugs in medicine are similar to plant origin substances [6,7,8]. Extracts of plants used in traditional medicine showed antimicrobial effects against human pathogens as they contain antimicrobial compounds that capable of reducing the number of pathogens [9,10]. Therefore, high interest in the development of alternative antimicrobial agent based from plants such as herbal tea has risen.

Generally, the term "tea" refers to beverage steeped from the leaves of *Camella sinensis* while herbal tea or more accurately known as tisanes referred to the infusions or decoction or maceration of any plants other than *Camella sinensis* [11]. The preparation of herbal tea may consist exclusively of one or more herbs. Herbal tea may derive from different parts of plants such as leaf, stalk, bark, root, seed, berry, fruit, and flower [11]. Apart from being the easiest method to earn benefits from plant, herbal tea also gained popularity all over the world due to their antioxidant activity, therapeutic applications, and fragrance that is believed to exert a calming effect to the mind [12]. Herbal tea from leaves has beneficial effects on lifestyle-associated diseases due to their anti-oxidant, anti-microbial, anti-carcinogenic, anti-atherogenic, and chemopreventive activities [13]. Sreeramulu et al. [14] reported that tannins and phenolic compounds extracted from teas managed to inhibit a broad spectrum of bacteria while Yoo et al. [15] found that polyphenols from plants cause functional or structural damage to the cell membrane of bacteria. Moreover, the functional hydroxyl groups and conjugated double bonds in herbal tea extracts may involved in binding to cell wall components of microbial cells, as the microbial cells are negatively affected by the plant derived compounds via various mechanisms of actions that attack the phospholipid bilayer of the cell membrane and disrupt enzyme systems [16].

Piper betle Linn. is the scientific name to betel which belongs to family Piperaceae. The shade loving, semi-woody tropical plant is native to Peninsular Malaysia and largely distributed in tropical and subtropical regions of the world [17,18]. *Piper betle* is a well-known edible plant that is traditionally used for medical purposes. The deep green heart shaped leaves of betel are aromatic, pungent, bitter and sharp in taste ranging from yellowish to dark green colour with glossy upper surface. Betel plant contain a large number of biomolecules which show diverse pharmacological activity such as antioxidant, antifungal, antimicrobial, antiplatelet aggregation, antiulcergenic, antidiabetic, anti-inflammatory, antiproliferative, antiplague, antifertility, cardiotonic, respiratory depressant, antihelminthic, cytoprotective, anti-hyperglycemic, antidermatophytic, antinaceptive, and radioprotective [19, 20, 21, 22]. The major bioactive compounds found in betel leaves are

polyphenols, alkaloids, steroids, saponins, and tannins [23]. Betel leaves were reported to possess many beneficial bioactivities and its extract has great potential to be developed into commercial products [24].

Cymbopogon citratus (DC.) stapf, belongs to the family of Gramineae, is commonly known as lemongrass. Lemongrass is a tall, monocotyledonous, aromatic and coarse perennial grass that grows in tropical and subtropical habitats. The plant is native to Sri Lanka and India and it is now cultivated in tropical regions of Asia, America and Africa [25]. Lemongrass has been a preferred component of many Asian cuisines for centuries because of its excellent aromatic properties. Infusion of lemongrass leaves give an aromatic drink with lemon flavour characteristic [26]. Studies have shown that bioactive constituents of lemon grass are responsible for its wide range of pharmacological and physiological properties [27]. Due to the abundance of citral and essential oil components, *C. citratus* showed antimicrobial activity against a number of microorganisms [28,29]. Lemongrass has been widely reported to have antidiabetic, antimicrobial, antioxidants, and antihelmintic [25, 30, 31].

Plant extracts have been used for many thousands of years in food preservation, alternative medicine and natural therapies. Therefore, it is necessary to investigate those plants scientifically to improve the quality of healthcare. The antimicrobial potentials of an individual betel and lemongrass have been widely reported in literature using different solvent for extraction. However, information about the development of betel and lemongrass as herbal tea is very limited. Moreover, the antibacterial activity of the infusion prepared using the combination of betel leaves and lemongrass stem has yet to be reported. Hence, this study aimed to assess the antimicrobial activity of lemongrass-scented betel herbal tea. In the present study, for different formulation of lemongrass-scented betel herbal tea were tested against four bacteria from different strain, namely *Staphylococcus aureus* (ATCC 29213 and ATCC 33591) and *Escherichia coli* (ATCC 25922 and ATCC 35218). *Staphylococcus aureus* is a facultative anaerobic, Gram-positive coccus and it causes range of illness from minor skin infections like pimples, boils to life threatening diseases such as pneumonia and meningitis. *Escherichia coli* is a Gram negative rod shaped bacterium, its virulent strains can cause gastroenteritis, urinary tract infections and neonatal meningitis. These microorganisms secrete different types of toxins, mainly enterotoxins and exotoxins and cause of diseases in human beings. Therefore, it is interesting to investigate the antimicrobial activity of herbal tea against these food pathogens which can be an alternative option to control bacterial infection or using the herbal infusions as an active extract in food products. The antimicrobial activity was determined by the disk diffusion method, minimum inhibitory concentration and minimum bactericidal concentration.

2. Materials and methods

2.1. Materials

Fresh betel (*Piper betle*) leaves and lemongrass (*Cymbopogon citratus*) stem were procured from a local wet market located in Besut, Terengganu, Malaysia. Gram-positive cocci, *Staphylococcus aureus* (ATCC 29213 and ATCC 33591) and Gram-negative rod, *Escherichia coli* (ATCC 25922 and ATCC 35218) were obtained from Microbiology Laboratory, Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, were subcultured and used as test organisms throughout this study. The culture media (Muller-Hilton agar, Muller-Hilton broth) were purchased from Merck (Darmstadt, Germany). Standard antibiotic ciprofloxacin was purchased from Oxoid (Hampshire, England) while empty discs and filter papers were purchased from Whatman (Maidstone, UK).

2.2. Preparation of betel leaves and lemongrass stem powder

Betel (*Piper betle*) leaves and lemongrass (*Cymbopogon citratus*) stem were washed under running tap water and rinsed with distilled water to remove adhering dirt. The excessive water were removed as they were bolted with tissue papers before being dried in hot air drying oven at 50 ºC for 24 h using a cabinet dryer. The dried matters were ground using a laboratory mill and sieved using an electromagnetic sieve shaker. Coarse powder (0.5 – 1.0 mm) were collected and kept in an airtight bottle and stored in a freezer (- 21 °C) prior to use.

2.3. Preparation formulation of lemongrass-scented betel herbal tea

Four different formulations of Lemongrass-Scented Betel Herbal (LSBH) infusions were prepared from different ratios of betel leaves and lemongrass stem powder by steeping 1 g of LSBH powder in 100 mL boiling water for 10 min and immediately cooled with ice-water bath one the desired time was achieved. The formulation of each infusion is shown in Table 1.

Table 1: Percentage of betel and lemongrass in each formulation[1].

Ingredient	Percentage (%)			
	B-100:L-0	B-95:L-5	B-85:L-15	B-75:L-25
Betel leaves	100	95	85	75
Lemongrass stem	0	5	15	25

[1]B: percentage of betel leaves; L: percentage of lemongrass stem

2.5. Test organisms

Reference strains of Gram-positive cocci, *Staphylococcus aureus* (ATCC 29213 and ATCC 33591) and Gram-negative rod, *Escherichia coli* (ATCC 25922 and ATCC 35218) were used as test organisms.

2.6. Inoculum preparation

The test organisms from nutrient agar slant were transferred into a Muller-Hinton agar (MHA) to obtain a pure colony by incubation at 37 ºC for 24 h. Three to five single colonies were transferred into 5 mL of sterile Muller-Hinton broth and incubated at 37 ºC and left in an incubator shaker (Jeio Tech, Korea) at 250 rpm until it reached the turbidity of 0.5 McFarland standards (equivalent to 10^5 – 10^6 CFU/mL). Density of the suspension was compared to the standard by holding the suspension and McFarland turbidity standard in front of a light against a white background with contrasting black lines.

2.7. Kirby-Bauer's susceptibility test

A antimicrobial activity of the extracts was carried out based on the disc diffusion concept of Kirby-Bauer's susceptibility test [32, 33] with modifications. The agar plates were prepared by pouring approximately 25 mL (4 mm depth) of molten Muller-Hinton agar onto sterile petri plates. A sterile cotton swab was dipped into the adjusted suspension. The surplus suspension was removed by pressing and rotating the swab firmly against the inside of the wall of the tube above the fluid level. The swab was evenly streaked over the surface of the MHA plate in one direction, then the plate was rotated trough an angle of 180 º and streaked again at that direction in order to form a bacteria lawn. The rotations were repeated three times to ensure an even distribution of the inoculums. After the inocula dried (approximately 5 min), sterilized discs (6 mm) were placed on the agar using sterile forceps and were gently pressed down to ensure adherence to agar. For each plate, 3 discs of different formulations were placed in a way that they were no

closer than 24 mm. The discs were impregnated with 20 µL of the infusions (2.5 mg/disc). The plates were left at room temperature for 30 min to allow better absorption. During this time microorganisms will not grow but absorption of extracts would take place. Standard antibiotic disc, ciprofloxacin (5 µg) was used as positive control while sterilized distilled water acted as the negative control. The inoculated plates containing the impregnated discs were incubated in an inverted position at 37 ºC for 24 h in an incubator. Finally, antibacterial capacity of LSBH infusions was determined by measuring the diameter (mm) of the inhibition zone surrounding the discs as bacterial growth inhibition.

2.8. Determination of minimum inhibitory concentration (MIC)

The MIC of the crude extract against *Escherichia coli* and *Staphylococcus aureus* were performed using two folds broth microdilution methods using microtiter plates based on Clinical Laboratory Standard Institute M07-A8 [34] with some modifications. The extract solution with initial concentration of 10 mg/mL was serially diluted with Muller-Hinton broth as 1:2, 1:4, 1:8, 1:16, :1:32, and 1:64 to bring 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.63 mg/mL, 0.31 mg/mL, and 0.16 mg/mL concentrations, respectively. Next, 75 µL of standard suspension of the test organism was added to each well containing 75 µL of LSBH infusion in the dilution series, and mixed. Wells containing broth without infusion were also prepared where one of these well was inoculated with the test organism while the other well was left inoculated and served as a control for media sterility. The microtiter plate was covered with lid and incubated at 37 ºC for 24 h in an incubator. Following incubation, growth inhibition in the tubes was observed. The lowest concentration, at which there was no turbidity (absence of growth), was regarded as MIC value of the extract.

2.9. Determination of minimum bactericidal concentration (MBC)

The MBC is defined as the lowest concentration required for an antibiotic to kill a microorganisms. The MBC were determined by sub-culturing 20 µL of the test dilutions onto fresh Muller-Hinton agar plates and then incubated at 37 ºC for 24 to 48 h until visible growth of colonies were observed. The lowest concentration of the infusion that killed the entire bacterial colony (no growth or fewer than three colonies) on the plates was recorded as MBC value of the extract.

2.10. Statistical analysis

Experimental data were analyzed using the Statistical Package for the Social Sciences for Windows® version 20.0 software (SPSS Inc., Chicago, IL, USA). The reported results in the present study are represented as the mean values of duplicates ± the standard deviation. A one-way analysis of variance (ANOVA) procedure followed by Tukey test was used to determine the significant differences between the mean values at a significance level of $p<0.05$.

3. Results and discussion

3.1. Disc diffusion assay of lemongrass-scented betel herbal tea

The results of zone of inhibition against test organisms of lemongrass-scented betel herbal tea using Kirby-Bauer disc diffusion method (2.5 mg/disc) are presented in Table 2. Results of disk diffusion assay showed that all LSBH tea demonstrate a good antibacterial activity to inhibit *Staphylococcus aureus* (ATCC 29213 and ATCC 33591) and *Escherichia coli* (ATCC 25922 and ATCC 35218). The negative control did not show any antibacterial activity for all organisms tested. The diameter of the zones of inhibition obtained by the herbal infusions in comparison to those imputed by commonly used antibiotic ciprofloxacin (5 µg/disc) used as standard, were found to be significant ($p<0.05$). Among the four formulations, B-100:L-0 showed the largest zone of inhibition zone (28.5 ± 0.71 – 29.0 ± 1.41 mm), followed by B-95:L-5 (27.5 ± 0.71 mm), B-85:L-15 (26.5 ± 0.71 – 27.0 ± 0.00 mm), and B-75:L-25 (25.5 ± 0.71 – 26.0 ± 0.00 mm) for *S. aureus*. In addition, B-100:L-0 also exhibited significantly ($p<0.05$) greater zones of inhibitions compared to standard antibiotic ciprofloxacin (27.5 ± 0.71 – 28.0 ± 0.00 mm) against *S. aureus*. However, the inhibition zone for two strains of *E. coli* were not affected by the four herbal infusion formulations ($p>0.05$). With exception to *E. coli* strains, the antimicrobial activity of LSBH infusions significantly ($p<0.05$) increased as the percentage of betel leaves powder were increased. This result is in agreement with Nouri and Nafchi [35] who reported the antibacterial activity increased as percentage of betel leaves extract increased for sago starch film incorporated with betel leaves extract. In support of the present study, Tan and Chan [36] reported that fresh and processed leaves of *P. betle* are an effective antibacterial agent. Syahidah et al. [37] also reported two compounds; eugenol and hydroxychavicol, acted as main active compounds which responsible for antibacterial activities of *P. betle*. *P. betle* were also reported to possess other active compounds such as allylpyrocatechol, chavibetol, chavibetol acetate and caryophyllene, which are related to its biological activities [38,39,40]. Apart from that, Albert and Ransangan [41] reported that betel leaves showed higher and broader spectrum of antibacterial activities compared to lemongrass stem. In previous studies, Caburian and Osi [42] and Olaiya et al. [43] reported 67.5 mm and 24 mm zones of growth inhibition against *S. aureus* for pure volatile oil of *P. betle* and *C. citratus*, respectively, while Nyarko et al. [44] reported that the aqueous extract of *C. citratus* showed no antimicrobial activity against *S. aureus*.

Table 2: Zone of inhibition against test organisms of lemongrass-scented betel herbal tea[1] using Kirby-Bauer disc diffusion method (2.5 mg/disc).

Microorganism	Diameter of inhibition zone (mm)				
	B-100: L-0	B-95: L-5	B-85: L-15	B-75: L-25	Ciprofixacin
Staphylococcus aureus (Gram positive bacteria)					
ATCC 29213	29.00^{a} ± 1.41	27.50^{ab} ± 0.71	27.00^{ab} ± 0.00	26.00^{b} ± 0.00	28.00^{ab} ± 0.00
ATCC 33591	28.50^{a} ± 0.71	27.50^{ab} ± 0.71	26.50^{ab} ± 0.71	25.50^{b} ± 0.71	27.50^{ab} ± 0.71
Escherichia coli (Gram negative bacteria)					
ATCC 25922	10.00^{b} ± 0.00	10.00^{b} ± 0.00	10.00^{b} ± 0.00	10.00^{b} ± 0.00	36.50^{a} ± 0.71
ATCC 35218	10.00^{b} ± 0.00	10.00^{b} ± 0.00	10.00^{b} ± 0.00	10.00^{b} ± 0.00	34.50^{a} ± 0.71

Data are mean values ± standard deviation (n = 2)
Values in the same row with different superscript lower case letters are significantly different at $p< 0.05$
[1]B: percentage of betel leaves; L: percentage of lemongrass stem

On the other hand, it was observed that *S. aureus* (Gram-positive bacteria) was more susceptible (25.5-29.0 mm) to LSBH infusions compared to *E. coli* (Gram-negative bacteria) (10 mm). As stated by Burt [45], Gram-positive bacteria are slightly more susceptible to the action of antimicrobial than Gram-negative bacteria. This might due to the variation in cell wall structures and complexity of these bacteria [46,47]. According to Arias et al. [48], the different sensitivity between Gram-positive and Gram-negative bacteria could be attributed to cell wall differences as Gram-positive bacteria have thick outer peptidoglycan layer. Gram-negative bacteria have a phospholipidic membrane in their outer layer which carry the structural lipopolysaccharide components, which makes their cell wall strong and impermeable to lipophilic solutes. A number of authors also reported larger inhibition zone for *S. aureus* than *E. coli* such as Nouri and Nafchi [35] for sago starch film incorporated with betel leaves extract, Anibijuwon et al. [49] for lemongrass tea and Hartini et al. [50] for *P. betle* leaves extract.

This may suggest the active compound in betel leaves exert its antibacterial activity by disrupting the formation of peptidoglycan.

3.2. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of lemongrass-scented betel herbal tea

The MIC values of LSBH tea against test organisms using two fold broth dilution methods are shown in Table 3. For *S. aureus*, the lowest MIC value was obtained from B-100:L-0 (0.63 mg/mL), followed by B-95:L-5 (1.25 mg/mL) and equally last, B-85:L-15 and B-75:L-25 (2.5 mg/mL). However, the MIC values for *E. coli* were not significantly ($p>0.05$) affected by the different formulations of betel lemongrass infusions. The MIC value of the herbal infusion against *S. aureus* ranged from 0.63 to 2.50 mg/mL and 5.0 mg/mL for *E. coli*, respectively (Table 3). The trend showed that MIC values of of Gram-positive bacteria were lower than Gram-negative bacteria. Against *S. aureus*, Hoque et al. [51] showed similar MIC value (0.63 mg/mL) for ethanol extract of *P. betle* leaves while Caburian and Osi [42] reported 125 µg/mL as MIC value for pure volatile oil of *P. betle*. On the other hand, Olaiya et al. [43] reported the MIC value of pure volatile oil of *C. citratus* against *S. aureus* is less than 106.25 mg/mL. However, Taukoorah et al. [52] reported aqueous *P. betle* leaves did not inhibit the growth of *E. coli*. It is observed that most plant extracts have activity against both Gram-positive and Gram-negative bacteria [53]. Even so, some plant extract such as *Psidium guajava* leaves extract only exhibit inhibitory activity against Gram-positive bacteria such as *S. aureus* and *Bacillus cereus* but cannot inhibit the growth of *E. coli* and *Salmonella enteritidis* [54].

Table 3: The minimum inhibitory concentration (MIC) values of lemongrass-scented betel herbal tea against test organisms using two fold broth dilution methods.

Microorganism	Concentration of Lemongrass-Scented Betel Herbal Tea[1] (mg/mL)			
	B-100:L-0	B-95:L-5	B-85:L-15	B-75:L-25
Staphylococcus aureus				
ATCC 29213	0.63	1.25	2.50	2.50
ATCC 33591	0.63	1.25	2.50	2.50
Escherichia coli				
ATCC 25922	5.00	5.00	5.00	5.00
ATCC 35218	5.00	5.00	5.00	5.00

Data are mean values ± standard deviation (n = 2)
[1]B: percentage of betel leaves; L: percentage of lemongrass stem

The results of the minimum bactericidal concentration (MBC) values of lemongrass-scented betel herbal tea against test organisms using two fold broth dilution methods are presented in Table 4. As for MBC, B-100:L-0 demonstrated the lowest value (1.25 mg/mL) to inhibit bacteria strain *S. aureus*. This indicates that betel leaves contained higher bactericidal properties than lemongrass to inhibit *S. aureus*. On the other hand, all four formulations of LSBH infusions were not bactericidal for *E. coli*. Mugweru et al. [55] also reported no MBC value for *Senna spectabilis* leaves against *E. coli*. Usually, bioactive compounds from medicinal plants or herbal extracts showed to present more effectively against Gram-positive than Gram-negative bacteria [56]. Olaiya et al.[43] reported 425 mg/mL as MBC value of essential oil of *C. citatus* against *S. aureus*, while Naik et al. [57] reported 0.06% and 0.125% MBC values for of *C. citatus* oil against *S. aureus* and *E. coli*, respectively. Another authors [58] also reported that MBC values for *E. coli* (2.50 mg/mL) is higher than *S. aureus* (1.25 mg/mL) for *C. citratus* extracts.

Table 4: The minimum bactericidal concentration (MBC) values of lemongrass-scented betel herbal tea against test organisms using two fold broth dilution methods.

Microorganism	Concentration of Lemongrass-Scented Betel Herbal Tea[1] (mg/mL)			
	B-100:L-0	B-95:L-5	B-85:L-15	B-75:L-25
Staphylococcus aureus (Gram positive bacteria)				
ATCC 29213	1.25	2.50	2.50	5.00
ATCC 33591	1.25	1.25	5.00	5.00
Escherichia coli (Gram negative bacteria)				
ATCC 25922	-	-	-	-
ATCC 35218	-	-	-	-

Data are mean values ± standard deviation (n = 2)
-: no MBC value
[1]B: percentage of betel leaves; L: percentage of lemongrass stem

4. Conclusion

In conclusion, LSBH tea showed antibacterial activity against *Staphylococcus aureus* (ATCC 29213 and ATCC 33591) and *Escherichia coli* (ATCC 25922 and ATCC 35218). However, *E. coli* were more resistant to inhibitory effects of the herbal tea. The results also could be considered as a new finding since no antibacterial study of herbal infusion made up using betel leaves and lemongrass stem. Furthermore, the data would serve valuable information for further study regarding antibacterial activities of the herbal infusion.

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