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In vitro antibacterial activity of crude medicinal plant extracts against ampicillin+penicillin-resistant *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is the leading cause for foodborne diseases. Extensive use of antibiotics has led to emergence of antibiotic-resistant *S. aureus*. Hence, interest on natural plant-based alternative which limits the use of synthetic chemicals is growing. The present work evaluated the antibacterial capacity of garlic, aloe vera, galangal, pineapple peel, neem, papaya leaf, lemongrass, peppermint, nutmeg and clove separately extracted with hexane, ethanol and water to a final concentration of 10% w/v against ampicillin+penicillin-resistant isolates of *S. aureus in vitro*. Streptomycin was used as a drug control against the resistant isolates; BRS023, BRS068 and DRS072. According to the interpretive standards for inhibition zone diameter provided by the Clinical and Laboratory Standards Institute, isolates BRS068 and DRS072 were considered resistant (≤ 12 mm), and isolate BRS023 was considered intermediate (13-14 mm). Against these isolates, all crude plant extracts exhibited varying degrees of inhibition. However, a coherent trend was observed in the inhibition between resistant and intermediate isolates regardless of plants and solvents used. It was also found that extraction solvent types impacted the resulting antibacterial activity. In terms of positive inhibition, the solvents were ranked in the order of hexane (77%) > water (73%) > ethanol (57%). 10% hexane extract of galangal gave the overall highest inhibition zones (17.8 ± 1.4 mm) closely followed by 10% ethanol extract of nutmeg (16.3 ± 1.1 mm). Further phytochemical analyses of the antibacterial compounds from galangal and nutmeg, and their minimum inhibitory concentration (MIC) are needed. Potential applications of plant-based antibacterial compounds as natural, cost-effective and less-toxic food preservatives against drug-resistant foodborne pathogens should be explored.

Introduction

Staphylococcus aureus is one of the primary causes of foodborne diseases, and commonly found in a variety of foods especially the improperly stored ones (Andreja, 2012). *S. aureus* is a major health concern to the public because of its ability (i) to produce pyrogenic toxic superantigens (i.e., antigens with the capacity to stimulate the proliferation of T-lymphocytes which in turn induces the release of proinflammatory cytokines and finally causing cell death), and (ii) to survive in adverse environmental conditions (Jung *et al*., 2015). An example of the superantigens is the staphylococcal enterotoxin (SE) which is the leading cause for staphylococcal food poisoning (SFP) in humans. SFP is a form of gastroenteritis caused by the intoxication of one or more SEs which are produced by *S. aureus* throughout the logarithmic growth phase on food, and also during the transition from the exponential to the stationary phase (Sihto, Susilo, Tasara *et al*., 2016; Sihto, Tasara, Stephan *et al*., 2016). The clinical symptoms of SFP include the increased saliva secretion, vomiting, abdominal cramping, and diarrhoea with or without blood (Jung *et al*., 2015). Although 23 different SEs have been described thus far (Larkin *et al*., 2009), the International Nomenclature Committee for Staphylococcal Superantigens (INCSS) has proposed that only staphylococcal superantigens capable of inducing emesis (i.e., vomiting) in humans and other primates following oral administration should be designated as SEs, and those which do not should

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instead be designated as SE-like toxins (Vasconcelos and Cunha, 2010). Staphylococcal enterotoxin-A (SEA) to staphylococcal enterotoxin-E (SEE) are the most commonly isolated SEs in SFP outbreaks (Argudín *et al*., 2010). *S. aureus* is a highly versatile pathogen and its infections range from mild to severe to fatal. In humans, *S. aureus* can also inflict illnesses such as rheumatoid arthritis, conjunctivitis and atopic eczema, and toxin-mediated diseases such as Toxic Shock Syndrome and Kawasaki's Syndrome (Vasconcelos and Cunha, 2010).

In the past, penicillin antibiotics were among the first-line medications prescribed in the treatment of staphylococcal and streptococcal infections (Houbraken *et al*., 2011). However, following extensive therapeutic use of the antibiotics, penicillin-resistance has developed in various strains of the pathogens (Jung *et al.*, 2015). In *S. aureus*, the resistance to penicillin is facilitated by penicillinase (also known as β-lactamase) production by the bacterium which cleaves the β-lactam ring within the penicillin structure, rendering it ineffective. In addition, the occurrence of horizontal gene transfer has also resulted in the spread of antibiotic-resistance among the pathogens all the while increasing the needs for alternative antibiotics (Kay *et al*., 2002; Gyles and Boerlin, 2014). Nevertheless, in recent years, considerable interest and attention has already been directed towards researches on plant-based products with efficacious antimicrobial properties against a wide range of foodborne pathogens (Smith-Palmer *et al*., 2004; Bajpai *et al*., 2007) in an effort to combat the pathogenic resistance (Goñi *et al*., 2009) and the side-effects of synthetic chemical food preservatives (Janovska *et al*., 2003). In this regard, plants have been shown to be an excellent biofactory of natural and safe products for the maintenance of human health (Ben Hsouna *et al*., 2014). Traditional medicinal plants and spices such as turmeric (Curcuma longa; Lew *et al*., 2015), king's salad (Cosmos caudatus; Yusoff *et al*., 2015), and lesser galangal (Boesenbergia rotunda; Zainin *et al.*, 2013) have been studied on their antimicrobial effects against foodborne pathogens.

In 2014, a susceptibility test of 10 antibiotics performed on nearly 150 isolates of *S. aureus* isolated from food handlers' hands in Selangor, Malaysia, gave >70% ampicillin-resistant isolates and >50% penicillin-resistant isolates (Tan *et al*., 2014). Streptomycin (aminoglycoside, non-β-lactam) which has different antibiotic mechanism from the aminopenicillin group was not tested. Therefore, in the present work, several of these antibiotic-resistant isolates were further tested against crude extracts of various traditional medicinal plants in Malaysia with streptomycin used as a drug control. The present work was aimed to determine the antibacterial properties of selected medicinal plants extracted with different solvents (i.e., hexane, ethanol, water) against antibiotic-resistant *S. aureus* isolates as measured by the Kirby-Bauer antibiotic testing.

Materials and Methods

Microorganisms

A total of three ampicillin- and penicillin-resistant isolates of the Gram-positive *Staphylococcus aureus* (BRS023, BRS068, DRS072) previously isolated from food-contact surfaces (Tan *et al*., 2014) were used in the present work. These indigenous isolates were used instead of type species because their resistance against commercial antibiotics has been established and documented (Tan *et al*., 2014). All isolates were aseptically cultivated (37°C), and axenically maintained (4°C) on a general growth medium Trypticase Soy agar (TSA; Merck, Germany).

Materials, solvents and reagents

All solvents and reagents were of analytical grade unless otherwise stated. Hexane, ethanol and dimethyl sulphoxide (DMSO) were purchased from Merck (Germany). Water was purified by passing through a Direct-Q UV treatment system with 0.65 μm filter (Millipore, France), and sterilised by autoclaving (Hirayama, Japan) at 121°C for 15 minutes at 15 psi. Streptomycin sulphate was purchased from Fisher (USA). Müeller-Hinton agar and broth for antibiotic susceptibility testing were also purchased from Merck (Germany).

Preparation of plant materials

Plant materials were either obtained commercially or collected from the Department of Crops, University Agriculture Park, Universiti Putra Malaysia. All plant materials were rinsed thoroughly with dH_2O after which they were thinly sliced, and oven-dried (Binder, Germany) at 50 ± 2°C for 72 hours. The dried samples were then pulverised to increase the surface area, followed by sieving to obtain uniform and fine plant powder for optimum solvent extraction. Table 1 lists the scientific names, English names, vernacular names and the plant parts used in the present work.

Solvent-extraction of plant materials

Twenty grams dried powder of each plant material was weighed and separately added to 200 mL of water, ethanol and hexane in Erlenmeyer flasks to a final

Table 1. Scientific, English and vernacular names of the plant materials used

Scientific name	Family	English name	Vernacular name	Plant part
Allium sativum	Amaryllidaceae	Garlic	*Bawang putih*	Bulb
Aloe vera	Xanthorrhoeaceae	Aloe vera	*Lidah buaya*	Leaf
Alpinia galanga	Zingiberaceae	Galangal	*Lengkuas*	Rhizome
Ananas comosus	Bromeliaceae	Pineapple	*Nenas*	Peel
Azadirachta indica	Meliaceae	Neem	*Semambu*	Leaf
Carica papaya	Caricaceae	Papaya	*Betik*	Leaf
Cymbopogon citratus	Poaceae	Lemongrass	*Serai*	Base stem
Mentha piperita	Lamiaceae	Peppermint	*Pudina*	Leaf
Myristica fragrans	Myristicaceae	Nutmeg	*Pala*	Fruit*
Syzygium aromaticum	Myrtaceae	Clove	*Cengkih*	Flower bud

*nutmeg rind / nutmeg flesh

concentration of 10% w/v. The mixtures were then agitated in a rotary shaker (New Brunswick, USA) at 100 rpm and 35°C for 24 hours. The solvent-extracts were then filtered twice (Whatman No. 4, 70 mm Ø; GE Healthcare, UK) into new Erlenmeyer flasks to remove plant debris and sediments. The solvents were evaporated to dryness using a rotary evaporator (Heidolph, Germany) at 80°C for hexane, 70°C for water, and 40°C for ethanol. Following evaporation, 1 g dried crude extracts were collected and dissolved in 2 mL DMSO (1:2 w/v) to obtain stock solutions. The stock solutions were refrigerated at 4°C in the dark until further analysis.

Preparation of antibiotic (streptomycin sulphate; positive control)

Ten milligram streptomycin sulphate was diluted with 1 mL dH_2O to a final concentration of 10 mg/mL stock solution. To prepare 0.5 mg/mL working solution, 50 µL stock solution was added to 950 µL dH_2O in a fresh microtube. The prepared streptomycin sulphate working solution was refrigerated at 4°C in the dark until further use.

Determination of bactericidal activity by Kirby-Bauer antibiotic testing (disc diffusion method)

Axenic culture of *S. aureus* isolates maintained on TSA were aseptically subcultured onto separate Müeller-Hinton broth (MHB) which were prepared fresh and sterilised prior to inoculation. Inoculated flasks were then incubated for 18 hours (exponential phase) at 37°C with 100 rpm agitation. At the end of MHB incubation, fresh Müeller-Hinton agar (MHA) was prepared, sterilised, and kept in the water bath at 50°C to obtain molten agar before being poured onto 90 mm Ø Petri plates (≈5 mm depth or ≈15 mL agar). A 1 mL aliquot of bacterial inoculum from MHB was aseptically and separately transferred onto the molten MHA, and swirled to ensure even distribution of the bacterial inoculum, before being left to solidify. A total of five filter paper discs (Whatman No. 4, 6 mm Ø) were prepared for each replicate of treatment (bacterial isolate x extraction solvent x plant material) on each MHA plate, and impregnated with 20 µL (i) water extract, (ii) ethanol extract, (iii) hexane extract, (iv) DMSO as negative control, and (v) streptomycin sulphate as positive control. The discs were then placed onto the MHA plates in equal-distance of each other, and pressed gently to ensure firm attachment to the agar surface. The MHA plates were then incubated for 18 hours at 37°C (Memmert, Germany) in an inverted position. Following incubation, the diameter of the inhibition zones surrounding the various discs were measured to the nearest whole millimetre (mm).

Statistical analysis

The antibiotic testing (bacterial isolate x extraction solvent x plant material) was performed twice to verify the results. Measurements were then averaged and presented as mean ± SE (standard error). Normal distribution of datasets was checked by the Kolmogorov-Smirnov normality test following which, Analysis of Variance (ANOVA) was applied on normally distributed datasets to analyse the variation between and within group means with 95% confidence interval. $p < 0.05$ was accepted as significantly different. Fisher's Least Significant Difference (LSD) with $\alpha = 0.05$ was applied to compare significance of differences between means of treatments using the Statistical Package for the Social Sciences (SPSS) version 22.0

(IBM Corporation; New York, USA).

Results and Discussion

Figures 1-3 depict the inhibition zones (mm) of antibiotic-resistant *S. aureus* isolates (BRS023, BRS068, DRS072) incubated with plant materials which were extracted with different solvents (hexane, ethanol, water) when compared to the positive control (streptomycin sulphate) using the disc diffusion method. According to the interpretive standards for inhibition zone diameter provided by the Clinical and Laboratory Standards Institute (2014), isolates BRS068 and DRS072 were considered resistant (≤ 12 mm), and isolate BRS023 was considered intermediate (13-14 mm). In terms of positive inhibition (3 isolates n (3 isolates x 10 plants), the solvents were ranked in the order of h 10 plants), the solvents were ranked in the order of hexane (77%) > water (73%) > ethanol (57%). As expected, the negative control (DMSO) did not give any inhibition zone across all isolates tested which confirmed that its application in dissolving the dried crude plant extract neither heighten nor lessen the antimicrobial property of the plant extracts. Therefore, the DMSO data were omitted from the figures.

Against the isolates, the 10 crude plant extracts tested exhibited varying degrees of inhibition. In general, among the plant materials tested, aloe vera, neem, papaya, nutmeg, and peppermint exhibited bacterial inhibition using all three extraction solvents which suggests that biochemically different bioactive compounds (i.e., from polar to non-polar) with antimicrobial properties exist within these plants. However, not all of these inhibitions were statistically higher ($p > 0.05$) than the positive control as evidenced in the Figures. Nevertheless, it is noteworthy that the ethanol extracts of nutmeg gave significantly high inhibition zones against the resistant isolates BRS068 (Figure 2) and DRS072 (Figure 3) when compared to the positive control. For clove, only the extracts from hexane (non-polar) and water (polar) exhibited inhibition zones, with hexane constantly giving higher inhibition zones across all isolates tested. Ethanol extracts of clove however, did not show any inhibition against the isolates. For garlic, only hexane and water extracts exhibited inhibition zones on the resistant isolates (BRS068, DRS072), while only ethanol and water extracts exhibited inhibition zone on the intermediate isolate (BRS023). Similarly for peppermint, extracts from all solvents exhibited inhibition zones on the resistant isolates, but only water exhibited inhibition zone on

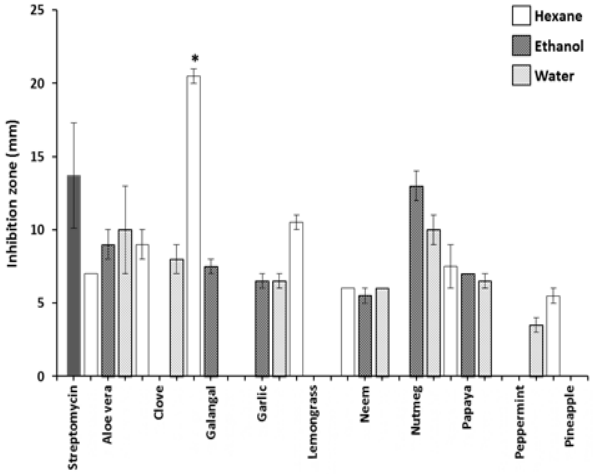

Figure 1. The bactericidal activity of 10% plant extracts extracted with different solvents (hexane, ethanol, water) as compared to commercial antibiotic (streptomycin) against the growth of *S. aureus* BRS023 *in vitro*. Data are means with bars indicating standard error (SE). Asterisks indicate significantly higher inhibition zones of treatments (Fisher's LSD; $\alpha = 0.05$) when compared with positive control (streptomycin)

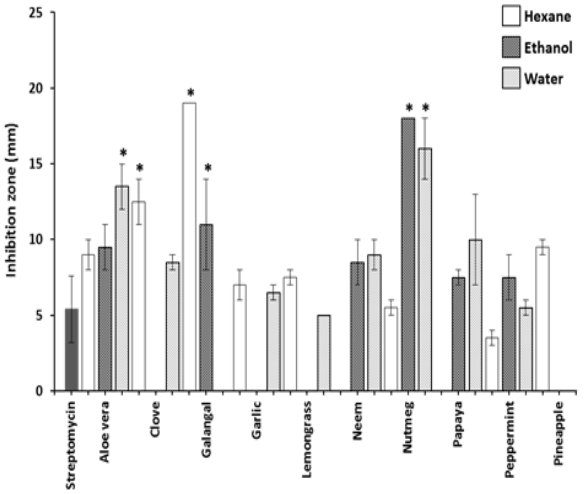

Figure 2. The bactericidal activity of 10% plant extracts extracted with different solvents (hexane, ethanol, water) as compared to commercial antibiotic (streptomycin) against the growth of *S. aureus* BRS068 *in vitro*. Data are means with bars indicating standard error (SE). Asterisks indicate significantly higher inhibition zones of treatments (Fisher's LSD; $\alpha = 0.05$) when compared with positive control (streptomycin).

the intermediate isolate. For galangal, its hexane extracts were the only treatment that significantly ($p < 0.05$) exceeded the positive control across all isolates tested. However, galangal water extracts did not inhibit any of the isolates. In short, a coherent trend was observed in the bacterial inhibition between resistant and intermediate isolates regardless of plants and solvents used. This might indicate that

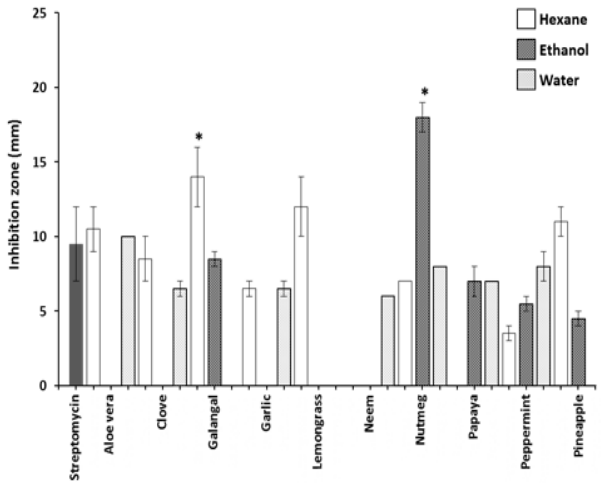

Figure 3. The bactericidal activity of 10% plant extracts extracted with different solvents (hexane, ethanol, water) as compared to commercial antibiotic (streptomycin) against the growth of *S. aureus* DRS072 *in vitro*. Data are means with bars indicating standard error (SE). Asterisks indicate significantly higher inhibition zones of treatments (Fisher's LSD; $\alpha = 0.05$) when compared with positive control (streptomycin).

resistance of pathogens strongly relies on multiple virulence factors, and that different plants extracted with different extraction solvents will yield different bioactive metabolites which in turn can attack and disarm different aspects of pathogenicity.

According to Cunha *et al.* (2006), the virulence factors of *S. aureus* include among others; surface components (capsule, peptidoglycans, teichoic acid, protein A, collagen cell attachment protein); enzymes (lipases, esterases, fatty-acid modifying enzymes, proteases, hyaluronidase, hydrolytic enzymes, deoxyribonucleases, coagulase, catalase, β-lactamase, staphylokinase); and toxins (exfoliative toxins, leukocidins , enterotoxins, haemolysins). In addition, over 30 different extracellular proteins have been characterised from pathogenic *S. aureus* isolates with some being the causative agents in staphylococcal diseases (Lisa, 2004). Regarding the ingestion of staphylococcal enterotoxin (SE) which manifests into staphylococcal food poisoning (SFP), although the exact mechanism is yet to be fully established, one hypothesis has been widely accepted. It is hypothesised that enterotoxin enters the bloodstream via transcytosis which enables interaction with T-lymphocytes → production of cytokines → systemic toxicity → suppression of the adaptive immune response (Balaban and Rasooly, 2000). The virulence factor of *S. aureus* which also includes surface components is what contributes to its resistance to various antimicrobials (Vasconcelos and Cunha, 2010).

Based on the findings of the present work, the resistant and intermediate *S. aureus* isolates exhibited susceptibility towards crude extracts of several plants such as galangal (hexane) and nutmeg (ethanol). The data also demonstrated that the solvent types used in the extraction impacted the resulting antibacterial activity. The data on galangal (hexane) are in agreement with those of Weerakkody *et al.* (2010) who also shown that the hexane extracts of galangal resulted in the highest overall antimicrobial activity against the tested foodborne pathogens (*Listeria monocytogenes, Staphylococcus aureus, Salmonella* Typhimurium, *Escherichia coli*). This could be explained by the fact that hexane, as a non-polar solvent, has a high affinity to react with non-polar compounds, which consequently leads to the extraction of essential oil (Thongson *et al*., 2004). Although the use of galangal essential oil has found its way as anti-oxidant (Mahae and Chaiseri, 2009), aromatherapy (Damayanti *et al*., 2015), anti-inflammatory (Raina *et al*., 2002), its antibacterial application in food preservation is comparatively recent with relatively scarce information as reviewed by Tripathi *et al.* (2013) and Yasurin (2015). Among the widely researched antimicrobial compounds from galangal are zingiberene (Zancan *et al*., 2002; Norajit *et al*., 2007) and galangin (Cushnie and Lamb, 2005, 2006). Even though the bioactive compound in the present work was not characterised, the data obtained provide an idea of the phytochemical nature of the galangal extracts in which the ethanol extracts did not significantly inhibit the tested *S. aureus* isolates when compared to the positive control and hexane extracts, while water extracts did not yield any inhibition at all.

Nutmeg is a widely-used spice and aromatic component in the food, fragrance and flavour industries. The antibacterial property of nutmeg oil or extracts has been demonstrated among others in *Streptococcus* spp. (Pilna *et al*., 2015), *Listeria monocytogenes* (Lin *et al*., 2016), *Helicobacter pylori* (Safavi *et al*., 2015), *Clostridium botulinum* (Cui *et al.*, 2010), *Bacillus subtilis* (Huang *et al*., 2015), and *Campylobacter jejuni* (Smith-Palmer *et al*., 1998). In nutmeg, the antibacterial compounds frequently extracted are myristicin, trimyristin, and myristic acid which have been shown to exhibit good inhibition towards Gram-negative and Gram-positive bacteria (Narasimhan and Dhake, 2006). The antibacterial effect of nutmeg ethanol extract against resistant *S. aureus* isolates in the present work is in accordance to that of Joseph and George (2014) who also showed remarkable activity from ethanol and methanol extracts against *S. aureus* and *Salmonella typhi*.

Conclusion

In conclusion, the present work has demonstrated the ability of different solvents to extract compounds with different antibacterial capacity. The positive effect of 10% galangal hexane extract and 10% nutmeg ethanol extract against the antibiotic-resistant *S. aureus* isolates in vitro has also been shown. Further analyses of the antibacterial compounds from galangal and nutmeg, and their minimum inhibitory concentration (MIC) are warranted. The potential application of the antibacterial compounds from galangal (essential oil) and nutmeg (water-soluble compound) as natural, cost-effective and less-toxic food preservatives should also be explored.

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