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Qualitative Phytochemical Analysis and Antimicrobial Activity of *Illicium Verum* Against Foodborne Pathogens

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ABSTRACT

The occurrence of food-borne diseases caused by pathogenic microorganisms led to a public concern on food safety. Moreover, chemical preservatives that are known to be toxic are widely contained (used) in food (industry) while synthetic antibiotics that are used to treat these diseases might cause side effects to the consumers. Due to the potential human health risks, antimicrobial drugs derived from organic products (i.e. herbs and spices) can be an alternative source for modern medicine. The aim of this study was to evaluate antimicrobial activity of *Illicium verum* (star anise) extract against foodborne pathogens and investigate phytochemical compounds found from the spices. Antimicrobial Susceptibility Testing (AST) by disc diffusion method, determination of Minimum Inhibitory Concentration (MIC) using broth microdilution method and Minimum Bactericidal Concentration (MBC) via sub-cultivation in well suspension onto Tryptic Soy Agar media were performed in this study. The largest diameter zone of inhibition was shown by *S. aureus* and *E. coli* followed by *S. typhimurium* and *B. cereus*. In addition, the phytochemical compounds were screened and results revealed that glycosides, phenols, alkaloids, tannins and terpenoids were found present in the extract. In conclusion, the methanolic extract of *Illicium verum* contained antimicrobial activity against selective food-borne pathogens, hence, considered to be an alternative for food preservative.

Keywords: *Illicium verum*, antimicrobial activity, pathogenic bacteria, food-borne diseases, phytochemical compound

ABSTRAK

Penyakit bawaan makanan yang berlaku disebabkan oleh mikroorganisma patogenik membawa kepada kebimbangan orang ramai terhadap keselamatan makanan. Selain itu, bahan pengawet kimia yang toksik juga banyak digunakan secara meluas di dalam makanan manakala antibiotik sintetik yang digunakan untuk merawat penyakit tersebut boleh menyebabkan kesan sampingan kepada para pengguna. Oleh kerana potensi risiko kesihatan terhadap manusia, ubat-ubatan antimikrobial yang diperolehi daripada produk organik (iaitu herba dan rempah) boleh menjadi sumber alternatif kepada perubatan moden. Tujuan kajian ini adalah untuk menilai aktiviti antimikrobial bagi ekstrak *Illicium verum* (bunga lawang) terhadap patogen bawaan makanan dan mengenalpasti sebatian fitokimia yang didapati daripada rempah tersebut. 'Antimicrobial Susceptibility Testing' (AST) dengan kaedah 'disc diffusion', 'Minimum Inhibitory Concentration' (MIC) menggunakan kaedah 'microdilution' dan 'Minimum Bactericidal Concentration' (MBC) melalui pengkulturan dalam media 'Tryptic Soy Agar' telah dijalankan dalam kajian ini. Zon diameter terbesar telah ditunjukkan oleh *S. aureus* dan *E. coli* diikuti oleh *S. typhimurium* dan *B. cereus*. Di samping itu, sebatian fitokimia daripada ekstrak tersebut telah disaring dan didapati mengandungi glikosida, fenol, alkaloid, tanin dan terpenoid. Kesimpulannya, ekstrak metanol bunga lawang mengandungi aktiviti antimikrob terhadap patogen bawaan makanan yang terpilih, dan berpotensi sebagai alternatif sebagai pengawet makanan.

Kata kunci: *Illicium verum*, aktiviti antimikrobial, bakteria patogenik, penyakit bawaan makanan, sebatian fitokimia

INTRODUCTION

Globally, foodborne outbreak is still an ongoing issue and considered a public health threat that resulted in social and economic problems (Wilcock et al. 2004; Jeyaletchumi et al. 2010; WHO 2015). The method of transmission or infection of foodborne diseases is either due to the ingestion of bacteria, viruses or parasites or consumption of non-infectious agents like toxin and chemicals (Linscott 2011).

Moreover, diarrhoea, the most known symptom of foodborne diseases, had affected 1 in every 10 people each year with African and South-East Asia regions revealed the highest burden of foodborne cases (WHO 2015). In Malaysia alone, approximately 50% of foodborne cases resulted due to unhygienic food handlers (Sharifa Ezat 2013; Abdul-Mutalib et al. 2015). *Salmonella typhi, Staphylococcus aureus, Escherichia coli* and *Clostridium perfringens* are some of the common agents of foodborne outbreaks (Pires et al. 2012). To make matters worse, these pathogens does not emit foul odour or food spoilage characteristics (New et al. 2017) resulting in difficulties to observe or sense that food may not be suitable to be consumed.

Numerous efforts that involved the physical and chemical approaches, had been carried out to control the increasing rate of foodborne pathogen (Sibi et al. 2013) ranging from changing of temperature, pH, osmotic pressure, usage of weak organic acids, hydrogen peroxide and organic biomolecules (Ray 1996; Brull & Coote 1999). Meanwhile, chemical preservatives that includes the usage of monosodium glutamate (MSG), aspartame, saccharin and nitrates are widely used as food additives to produce desirable effects (Chaudhary 2010). However, prolonged consumption of these synthetic materials can cause adverse effects such as childhood hyperactivity (Tuormaa 1994) and other behavioural disorders as well as eczema (Gultekin et al. 2013). Another alarming concern centered on the rising of antibiotic resistance of several pathogens associated with foodborne disease (White et al. 2002; Walsh & Fanning 2008; DeWaal & Grooters 2013). Hence, many had resorted to natural-based approach for food safety, especially spices. Spices had become a very important commodity in each household. However, limited findings available on highlighting the antimicrobial activity of spices (Arora & Kaur, 1999; Ceylan & Fung, 2004; de Souza et al. 2005).

One of the most known spices, star anise is not only a common choice but it also contained health benefits. Star anise or scientifically known as *Illicium verum Hook. f.*, is commonly used star-shaped spice that produced the scent of anise (Parthasarathy *et al*., 2008). Star anise is classified as family *Illiciaceae*, order *Austrobaileyales*, subclass *Magnoliidae*, class *Magnoliopsida* and division *Magnoliophyta* (Wang et al. 2011). Although star anise is commonly grown in Asian countries, its usage is not limited within the region but had yet disseminated worldwide. In China, star anise is one of the essential spices in the five-spice powders used in Chinese cooking and act as flavour enhancer in Chinese stew whilst in Europe countries, the spice was first introduced in the seventeenth century (Wang *et al.*, 2011). From then onwards, the usage of star anise had broaden until it is today – used in confectionary industries and added as flavours to liquors (Parthasarathy *et al*., 2008).

The aim of this study is to investigate whether the methanol extract of *Illicium verum* had the antimicrobial activities to selected foodborne pathogens that commonly caused foodborne diseases. Furthermore, this study would also provide the information regarding the phytochemical constituents or secondary metabolites present in *Illicium verum* that may contribute to its antimicrobial properties. In addition, preliminary phytochemical screening test was also performed in order to qualitatively screen the presence of compounds and associate their presence with antimicrobial activity.

MATERIALS AND METHODS

Sample preparation

One kg of *Illicium verum* (star anise) was bought and grinded into fine powder by a grinding machine or blender. The powdered form of star anise was kept in airtight container and stored in dry place until further use.

Extraction method

The extraction method was carried out based on a study by Vijayakumar et al. (2012) with slight

modification. 250 g of star anise powder was weighed with analytical balance and soaked in 1 L of methanol for 72 hrs with intermittent shaking. Then, the solution was filtered into a Schott bottle by using Whatmann No. 1 filter paper. The filtrate was concentrated under reduced pressure by using rotary vacuum evaporator at 40°C. The crude extract obtained was stored in a sealed, sterile container at 4°C until use.

Bacterial strains and antibiotics

There are four types of bacterial strains that were used, namely *Staphylococcus aureus* (ATCC 43300), *Bacillus cereus* (ATCC 14579), *Escherichia coli* (ATCC 25922) and *Salmonella typhimurium* (ATCC 13311) with antibiotics consisted of Tetracycline (30 µg), Gentamicin (10 µg), Streptomycin (10 µg) and Ampicillin (10 µg), respectively. The bacterial stocks were obtained from Microbiology Laboratory, Centre of Medical Laboratory Technology, Faculty of Health Sciences, UiTM Puncak Alam Campus, Selangor. Each bacterial suspension was prepared by inoculating 5 mL of Tryptic Soy Broth (TSB) with three to five colonies of bacteria obtained from Blood Agar plate prior to incubation at 37°C for 2 to 3 hrs. The turbidity of the suspension was adjusted equivalent to 0.5 MacFarland standards (Cavalieri 2009). Meanwhile, the confirmation tests of these organisms were performed for each organism that include sub-culturing on different agar medium (i.e. Blood Agar, Nutrient Agar, Mueller Hinton Agar).

Antibiotic susceptibility testing (AST) test

The *Illicium verum* crude extract was prepared by transferring 20 µL of 250 mg/mL onto sterile filter paper discs prior to dry at room temperature for 24 hrs. Positive control was carried out using standard antibiotics as mentioned above (Cockerill 2012; Vijayakumar et al. 2012) while for negative control, 10 µL of 10% DMSO was placed onto a filter paper disc. Then, three to five colonies of organism were selected from nutrient agar and suspended into Tryptic Soy Broth (TSB) as stated by Cavalieri (2009). Intepretation of sensitivity or resistance of the inhibition zones was determined by the measurement of diameter. The experiment was performed three times to confirm the reproducible results and the mean±SD (Standard Deviation) for zone of inhibition were determined.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests

Bacterial suspension that only showed sensitivity against *Illicium verum* crude extract was prepared as mentioned by Cavalieri (2009). In order to determine MIC value, 96-microtiter well plate was used by detecting the well with the lowest concentration of extract that completely inhibit the growth of the tested organism. Meanwhile, for the determination of MBC, a loop contained bacterial suspension (Shalayel et al. 2017) that showed no visible growth in MIC well was sub-cultured on Tryptic Soy Agar (TSA) prior to incubation at 37°C for 24 hrs. Both procedures were performed in triplicate to obtain reproducible results.

Phytochemical analysis

The qualitative test of methanol extracts of *Illicium verum* was performed in triplicates according to Deb et al. (2013) and Sibi et al. (2013) with slight modification. The methanol extract of *Illicium verum* were screened for presence of glycosides (using Fehling's test method), phenols (using Ferric Chloride's test method), alkaloids (using Wagner's test method), tannins (using Ferric Chloride's test method) and terpenoids (using Salkowski's test method).

RESULTS AND DISCUSSION

In this study, dried *Illicium verum* was used as most scientists reported that the water content may influence the solubility of subsequent separation, hence, the secondary metabolic plants components need to be stable (Ncube et al. 2008), especially in determining the antimicrobial agent from a plant material. Besides, a better efficacy of extraction can be achieved by increasing the surface area of the plant material by grinding it into powdered-form (Tiwari et al. 2011). Meanwhile, methanol was used during the extraction process as the solvent has been found easier to penetrate into the cellular membrane of plant material and to extract out the intracellular compounds (Jones & Kinghorn 2006; Tiwari et al. 2011). Previous studies had reported that methanol able to exhibit more activity than aqueous extract (Ahmad & Beg, 2001; Nair et al. 2005;) and proven to be more consistent (Parekh et al. 2005). In addition, other

findings of *Illicium verum* also used methanol as solvent in extraction procedure (Shan et al. 2007; Sibi et al. 2013). In addition, positive control consisted of Ampicillin, Gentamicin, Streptomycin and Tetracycline were selected in this study as the aforementioned are considered commercial standards that are commonly used in most studies that exhibit against a wide range of gram-positive and gram-negative bacteria (Halawani 2009; Ahmed et al. 2010; Aburowais et al. 2017).

Subsequently, agar disk diffusion method was used during the preliminary screening of the extract's antimicrobial activity. This technique was chosen as it adopts simple process as well as least costly among other susceptibility methods (Reller et al. 2009; Das et al. 2010). The findings were then evaluated via the presence of inhibition zone and measuring of the diameter (Othman et al. 2011). The extract produced antimicrobial activity against both Gram-positive and Gram-negative bacteria, similarly to a previous study by Shan et al. (2007). However, results revealed that the inhibition zone for *Illicium verum* appeared smaller in comparison to the standard antibiotic. The usage of 100% extract is unable to produce larger zone of inhibition as it may be due to the limitation of disk diffusion method that is considered less sensitive (Manoharan et al. 2003; Valgas et al. 2007) against the selected antibiotics. Hence, higher concentration is required as poor activity at lower concentrations may influence the solubility of the active compounds (Jagtap et al. 2010) while the variation results may also influence by several factors that include climate, environmental condition, test organism and dose (Ncube et al. 2008). Manoharan et al. (2003) had also suggested to further confirmed the findings with MIC tests.

Then, broth microdilution method was employed for the determination of MIC. This technique was preferred due its simplicity and practicality. Furthermore, due to the miniaturization by the use of small, disposable plastic microdilution tray which was microtiter plate has made this method popular and widely accepted by researchers (Jorgensen & Ferraro 2009). Other than that, broth microdilution method has been proven to be more sensitive accurate, appropriate for rapid quantitative determination of antimicrobial activity of plant extract as well as inexpensive and consumes less time than screening agar method (Jorgensen & Ferraro 2009; Klančnik et al. 2010). Meanwhile, for MBC, the test was done via sub-culturing the clear dilution suspension in MIC onto Tryptic Soy Agar (TSA). The methanolic extract of *Illicium verum* produced antimicrobial activity against both Gram-positive and Gram- negative bacteria. According to previous studies, the Gram-negative bacteria have been reported to be more resistance towards plant extract compared to Gram-positive bacteria (Shan et al. 2007; Sibi et al. 2013). However, contradicting to other reports in which *E. coli* also showed same sensitivity as *S. aureus* towards the *Illicium verum* extract. Furthermore, *B.cereus* that belongs to Gram-positive bacteria showed the most sensitivity towards the extract. Meanwhile, the absence of bacterial growth indicated that the tested extract was bactericidal whilst the presence of bacterial growth displayed the bacteriostatic or bacterial-inhibiting at a particular concentration. The results for AST, MIC and MBC are further summarized in Table 1.

In addition, qualitative phytochemical analysis was employed by carrying tests using colour changes and precipitation of the chemicals reagents. Based on our findings in Table 2, all of the tested compounds were present in the *Illicium verum* extract. The results showed similarities with previous reports by Das and Kumar (2013), Harsha et al. (2013) and Sibi et al. (2013). According to Vijayakumar et al. (2012), Das & Kumar (2013) and Harsha et al. (2013), the compounds found in the extract possessed properties that exhibit the antimicrobial activity. These compounds might inhibit the microorganisms in several mechanism of action.

TABLE 1. AST, MIC and MBC results for *Illicium verum* extract against tested bacterial strains

Bacterial Strains	AST – Diameter Zone of Inhibition (Mean±SD; mm)			MIC (%)	MBC (%)
	Methanolic extract (100%)	Positive control	Negative control		
S. aureus (ATCC 43300)	9.00±0.00	21.00±1.00	0	6.25	6.25
B. cereus (ATCC 14579)	8.30±0.58	20.3±0.58	0	0.78	0.78
E. coli (ATCC 25922)	9.00±0.00	14.30±0.58	0	3.13	6.25
S. typhimurium (ATCC 13311)	8.70±0.58	22.70±2.08	0	1.56	3.13

ATCC = American Committee of Clinical Laboratory Standards; AST = Antimicrobial Susceptibility Testing; MIC = Minimum Inhibitory Concentration; MBC = Minimum Bactericidal Concentration; mg = milligram; mL = milliliter; mm = millimeter;
% = Percentage; SD = Standard deviation; ± = plus minus

TABLE 2. Phytochemical analysis of methanolic extract of *Illicium verum*

Phytochemical compounds	Reaction
Alkaloids	+ve
Glycosides	+ve
Phenols	+ve
Tannins	+ve
Terpenoids	+ve

+ = Presence of compound (positive)

CONCLUSION

In summary, *Illicium verum* extract contained the antimicrobial activity against selected foodborne pathogens, hence, a potential substitute for synthetic chemical in food preservative. Further studies are required to fractionate and isolate the active compounds in *Illicium verum* and the study on the evaluation of the possible synergistic action among multiple active compounds present. Moreover, other solvents can also be tested to enhance the antimicrobial effects.

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