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Screening of selected Malaysian plants against several food borne pathogen bacteria

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Abstract: This study was conducted to evaluate antimicrobial properties of ethanolic extracts of the leaves of *Nephelium lappaceum*, *Curcuma longa*, *Cinnamomun cassia*, *Durio zibethinus*, *Vitex trifolia*, *Amaranthus tricolor*, *Syzygium samarangense* and *Manihot esculenta*. Antibacterial properties of the extracts were studied against fifteen strains of different gram positive and gram negative pathogenic bacteria, including *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio para*, and *Escherichia coli* using the agar disk diffusion method. Among the tested extracts, only *Amaranthus tricolor* exhibited specific inhibition of one of the tested bacteria; *Bacillus cereus*. Using the microdilution method, its minimum inhibitory concentration (MIC) value was determined to be 20 mg/mL.

Keywords: Malaysian plants, food borne pathogens, antimicrobial activity

Introduction

In an era of growing populations, the size of food chains increases and the challenge of producing safer food become increasingly tough. The official figures concerning food safety in Malaysia released by the Malaysian Ministry of Health (2009) revealed a sharp increase in reported food poisoning cases from 6930 to 17320 cases between 2006 and 2008. Moreover, in U.S, a country that is reknown for having safer food sources, the Center for Disease Control and Prevention estimated that 76 million people sicken, 300000 are hospitalized and 5000 people die each year between year 1991 and 2000 as a consequence of food poisoning (Olsen *et al*., 2000).

The use of herbs to prevent and cure disease have been rising in recent years (López-Muñoz *et al*., 2006) and its preventive use for the treatment of food borne pathogens is believed to be safer than the inclusion of synthetic antibiotics due to the long history of herbal usage. It is important, however, to consider issues such as the dosage and frequency of consumption of these products (Thomson *et al*., 2001). Moreover, spices and herbs are able to enhance digestion, due to the salivary and gastric secretion induced by the pungent taste and unique aroma. This effect increase the activity of digestive enzymes, bile volume, and bile acids secretion, and also can decrease the time food takes to travel through the gastrointestinal tract thus providing some protection against gastrointestinal disease (Low Dog, 2006).

In India, two herbs, *Vetvier (Vetiveria zizanoides)* and *Tulsi (Ocimum basilicum)*, are commonly used for the treatment of food borne pathogens (Bais *et al*., 2002; Champagnat *et al*., 2008). Chinese use linseed, dandelion, golden thread and skullcap root possibly due to the presence of phenolic compounds (Pérez *et al*., 1994; Lokar *et al*., 1988; Franzblau *et al*., 1986; Yu *et al*., 2004), while ginseng and licorice have been used in Korea and Japan (Chang-Xiao *et al*., 1992; Gupta *et al*., 2008) for these purposes.

In Malaysia and Indonesia several native herbs have been traditionally used to treat food borne pathogens (Lu *et al*., 2006; Arya *et al*., 1989; Soedibyo, 1998; Chan *et al*., 2007; Arora *et al*., 1999; Aziz *et al*., 2009; Ikram *et al*., 2009). However, this use is lack of scientific proof and validation. In the third Herbal Asia Business Dialogue 2009, Kuala Lumpur, the prime goal was to transform Malaysian herbs production into the economy of tomorrow. To achieve that, incorporation of modern facilities to the production of traditional medicine has been widely developed.

The aim of this study was to evaluate the antimicrobial activity of the ethanol extracts of the leaves of *Nephelium lappaceum*, *Curcuma*

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longa, *Cinnamomun cassia*, *Durio zibethinus*, *Vitex trifolia*, *Psidium guajava*, *Amaranthus tricolor, Syzygium samarangense* and *Manihot esculenta* against 5 pathogenic gram positive and negative bacteria: *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio para*, and *Escherichia coli*.

Materials and Methods

Chemicals

Tetracycline 30 µg antibiotics disc was purchased from Oxoid (Cambridge,UK), Nutrient agar, Mueller Hinton agar and broth, dimethylsulfoxide and ethanol were purchased from Merck (Darmstadt, Germany).

Herb selection and extraction

Herbs were selected based on the traditional usage of these plants for the treatment of food borne pathogen disease as listed in Table 1. Nine types of plants were collected from Botanical Farm Putrajaya in Malaysia on December 1, 2009 at 9.00-11.00 am. The herbs were identified by botanist, Mr. Mohammad Ishak. The voucher specimens were stored in Faculty of Food Science and Technology, Universiti Putra Malaysia. Dirt was removed under running water, and air dried for 3 days at constant room temperature 27°C with 70% relative humidity. Plants were then ground to powder using a mechanical grinder. A volume of 70 ml of ethanol was added to 10 g of plant powder, and the mixture was sonicated for 50 min at 30°C and 40 kHz frequency. The extracts were then filtered through filter paper and concentrated under reduced pressure using a rotary evaporator at 40°C for 15 min. Plant extracts were prepared by triplicate. The crude extracts were then kept at -20°C until used for analysis.

Table 1. List of selected plant used for study and related folk indication

No.	Scientific name	Malaysian local name	Part of the plant used	Folk indication
1	*Nephelium lappaceum*	*Daun rambutan*	leaf	Fever, diarrhea
2	*Vitex trifolia*	*Lemuni putih*	leaf	Diarrhea and gastrointestinal affections
3	*Curcuma longa*	*Kunyit*	leaf	Bloating
4	*Cinnamomun verum*	*Kayu manis*	leaf	Diarrhea and headache
5	*Syzygium samarangense*	*Jambu madu*	Shoot	Diarrhea
6	*Manihot esculenta*	*Ubi kayu*	leaf	Belly pain, diarrhea, fever, headache, aches and pains
7	*Psidium guajava*	*Jambu*	leaf	Headache, diarrhea
8	*Amaranthus tricolor*	*Bayam merah*	leaf	Sinus and cold fever, diarrhea
9	*Durio zibethinus*	*Durian*	leaf	Fever, diarrhea

Cited from Anderson *et al*. (1996); Argueta *et al*. (1994); Chooi (2003); Chooi (2004); Soedibyo (1998)

Bacterial strains and inoculum preparation

Three strains of five types of pathogenic bacteria were selected for this study. The Gram positive bacteria were *Bacillus cereus* strains BC205, BC66, BC41, *Listeria monocytogenes* strains LM1, LM3, LM22 and *Staphylococcus aureus* strains SA25, SA2, SA5, while the gram negative bacteria were *Vibrio para* strains VB34, VB36, VB37,and *Escherichia coli* strains EC6, EC8, EC5. Growth media was Nutrient Agar from Merck (Darmstadt, Germany). Each bacteria was activated by transferring a loopful of culture onto the agar plate and after overnight incubation, all cultures were kept at -20°C in 50% glycerol stock to be used no longer than a month later.

Preliminary screening for antimicrobial activity

The disk diffusion agar method was applied, following National Committee for Clinical Standards (NCCLS, 2000). Mueller Hinton agar from Merck (Darmstadt, Germany) was used as the growth media of the pathogenic bacteria. After conformation with 0.5 Mc Farland turbidity, a sterile cotton swab was used to swab the culture evenly on the plate. The nine different extracts obtained were diluted in 10% DMSO to reach a concentration of 40 mg/mL. Sterile Whatman No.1 disk (6 mm) were soaked in the diluted extract for 1 min then dried completely before repeating the procedure until a total of 10 µL of the sample extract was absorbed onto the disk. Each extract was tested in triplicate with DMSO 10% as a negative control, and Tetracycline 30 µg was used as a positive control. Agar plates containing bacteria and extracts were incubated at 35°C for 24 hr. Size of inhibition ring was measured as the difference in the diameter between the growth-free zone and the disc.

Minimum inhibitory concentration

Sterile flat bottom tissue culture plates (96 well) obtained from Jet-Biofil® (Guangzhou, China) were filled with 100 µL Mueller Hinton broth (Darmstadt, Germany), 50 µL of the herbs extract and 50 µL bacterial suspension were inoculated into them. A stock solution of 40 mg/mL of herbs extract was prepared in DMSO 10%. An aliquot of this solution was serially diluted (two-fold) with nutrient broth to obtain a concentration range of 40 to 0.313 mg/mL. Each extract was assayed by triplicate. Controls were prepared using the same method as explained above but without adding the microbial culture. Plates were incubated at 35°C for 24 hr aerobically. A Benchmark Plus microplate spectrophotometer from Bio-Rad Laboratories Inc. (California, US) with Microplate Manager® software was used to read the absorbance

at 600 nm. The lowest concentration of the herbal extract to inhibit bacteria was considered as the MIC value of the tested extract. All experiments were conducted by triplicate for each type of bacteria and the values exhibited are Mean ± SD.

Results and discussions

There was a large variability in the antimicrobial activities displayed by the tested herbs during this screening as shown in Table 2. The classification of the activity as weak, medium, strong and very strong followed that of used by US National Committee for Clinical Laboratory Standards (2000) and Lin *et al*. (1999). About 44 % of herbs showed some degree of inhibition of both gram negative and/ or gram positive bacteria. Among the active herbs, 57% inhibited gram positive bacteria and only 43% inhibited gram negative bacteria. Therefore, this proved that Gram negative bacteria were more resistant than gram positive, a fact explained by the presence of lipopolysaccharide in their outer wall (Russel, 1991).

In most previous research of this type, when an inhibitory effect of pathogenic bacteria growth was detected, the plant extracts responsible for this activity were reported to be capable of inhibiting various types of microorganisms. This result is considered to be a 'false-positive' result since the mechanism of inhibition could well be due to cell toxicity (Cos *et al*., 2006). A plant that is able to inhibit only specific bacteria is the plant which is worthwhile studying. Out of the 9 herbs tested in this study, only *A. tricolor* showed a specific inhibition of one type of bacteria, *B. cereus*. This is the first report on the specific antimicrobial activity of *A. tricolor* ethanolic extract against *B. cereus*. Sharma (1993) reported non- specific antimicrobial activity of this plant extracted with petroleum ether on both gram positive and negative bacteria. The difference with the results obtained in this study is very likely due to difference in polarity of the two solvents used which would result in different types of compounds being extracted. As shown in Figure 1, the MIC values of the *A. tricolor* extracts calculated for all strains of *B. cereus* was 20 mg/mL. A MIC value below 50 mg/ml is considered to correspond to an interesting antibacterial activity which is worth while continuing to study (Arias *et al*., 2004; Motamedi *et al*., 2010; Safary *et al*., 2009; Fabry *et al*., 1998; Valsaraj *et al*., 1997).

A. tricolor belongs to the Amaranthaceae family. Screening of antimicrobial activity on Amaranthaceae family members such as the ethanolic extract of aerial parts and roots of *Blutaparon portulacoides* showed

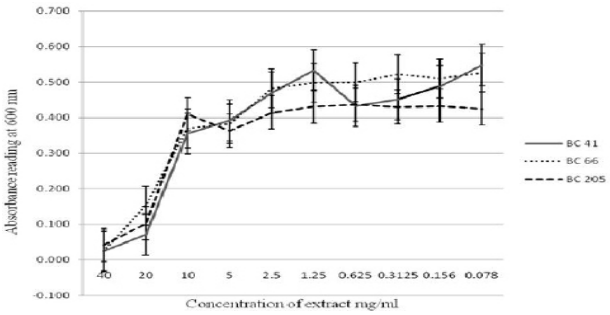

Figure 1. Minimum inhibitory concentration (MIC) of *A. tricolor* toward *B. cereus* strains

specific inhibition towards a gram positive bacteria, *Streptococcus* sp (Salvador *et al*., 2002). Another study on plants from this family revealed that an 80% ethanol extract of leaf and stems of *Achyranthes aspera*, also had a specific inhibition towards two gram positive bacteria, *B. subtilis* and *S. aureus* (Valsaraj *et al*., 1997) while the methanolic and water extract of leaves of *Alternanthera brasiliana*, were reported to present inhibition to both gram positive and gram negative bacteria at 25 mg/ml (Coelho de Souza *et al*., 2004).

Several sterols such as cholesterol, 7,22-ergostadienol,campesterol, stigmasterol, 7,24(28)-ergostadienol, 7-ergostenol, spinasterol, sitosterol, stigmastanol, 7, 25-stigmastadienol, 7-stigmastenol, and 7,24(28)-stigmastadienol have been isolated from *A. tricolor* (Sharma, 1993; Xu *et al*., 1986). Alkanol groups that were found to be present were hexacosanol, and octacosanol while the alkanes nonacosane, and hentriaconetane were also reported (Sharma, 1993). The presence of β-sitosterol and stigmasterol in *A. tricolor* has been related to a strong inhibition against gram-positive bacteria and a moderate activity against gram negative bacteria (Sharma, 1993). Alkanes are the most inert organic compound to give wide spectrum of antibacterial effect (Sharma, 1993). However, no compounds from *A. tricolor* had been reported to have specific inhibition towards *B. cereus*.

The other ethanolic plant extracts tested showed non- specific antibacterial activity. This was the case of *N. lappaceum*, *S. samarangense*, and *V. trifolia* extracts. The ethanolic extract of *N. lappaceum* leaves strongly inhibited one strain of *S. aureus* and *V. para*, and showed medium inhibition towards one strain of *L. monocytogenes*. The other strains of *S. aureus* were weakly inhibited. From the previous literature, only the rind of this plant, extracted with ether, methanol, and water, showed potent activity against several gram positive bacteria including *S. aureus*, *S. epidermis*, and *E. faecalis* and only one gram negative bacteria, *V. cholera*. Phenolic compounds from the peel of this plant were related to

Table 2. Antimicrobial activity of leaf extracts on selected bacterial strains[a]

No	Common name	Gram positive bacteria									Gram negative bacteria					
		Bacillus cereus			*Listeria monocytogenes*			*Staphylococcus aureus*			*Vibrio para*			*Escherichia coli*		
		BC205	BC66	BC41	LM1	LM3	LM22	SA25	SA2	SA5	VP37	VP36	VP34	EC6	EC8	EC5
1	*N. lappaceum*	-	-	-	-	-	++	+++	+	+	+++	-	-	-	-	-
2	*V. trifolia*	+++	+++	-	+++	++	+++	++	++++	++	-	-	-	+++	-	++++
3	*C. longa*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	*C. verum*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	*S. samarangense*	-	-	-	-	-	+	-	-	-	+	-	-	++	++	++
6	*M. esculenta*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	*P. guajava*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	*A. tricolor*	++++	++++	+++	-	-	-	-	-	-	-	-	-	-	-	-
9	*D. zibethinus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	DMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	Te	+++	++++	++++	++++	+++	++++	-	-	-	+++	++++	++++	++++	++++	++++

[a]+: 2-4 mm,weak inhibition; ++: 5-7 mm, medium inhibition; +++:8-10 mm, strong inhibition; ++++:>10 mm, very strong inhibition, excluding the diameter of the disc; -:no activity;
Te: Tetracycline 30µg/disc.

this antibacterial activity (Thitilertdecha *et al*., 2008) and more antibacterial activity was found in the peel than in the seed. However, no antimicrobial study had been conducted on the leaves of this plant.

Ethanolic extract of *S. samarangense* leaves showed medium inhibition towards all strains of *E. coli*, and weak inhibition towards one strain of *L. monocytogenes* and *V. para*. Previous phytochemical studies on the leaves of *S. samarangense* showed the presence of ellagitannins, proanthocyanidins (Nonaka *et al*., 1992), flavanones (Kuo *et al*., 2004), flavonol glycosides (Kuo *et al*., 2004; Nair *et al*., 1999), anthocyanidins (Nonaka *et al*., 1992; Kuo *et al*., 2004), triterpenoids (Srivastaya *et al*., 1995), chalcones (Srivastaya *et al*., 1995; Resurreccion-Magno *et al*., 2005), and volatile terpenoids (Wong *et al*., 1996). Ellagitannin had been reported to have a specific antimicrobial activity against *S. aureus* (Perashar *et al*., 2009). Triterpenoids showed inhibition towards both *B. subtilis* and *E. coli* (Nick *et al*., 1994) while flavonoids exhibited antibacterial activity to both gram negative and gram positive (Cushnie *et al*., 2005; Cook *et al*., 1996).

The ethanolic extract of *V. trifolia* exhibited an inhibitory effect against all tested bacteria of strong to medium strength with the exception of *V. para*. This finding is in accordance with the result reported by Hernández *et al*. (1999) who used hexane, dichloromethane and methanolic extracts of this plant to inhibit a wide range of bacteria such as *S. aureus*, *S. feacalis*, *E. coli*, *Proteus mirabilis*, *Shigella sonei*, *Salmonella typhimurium*, and *Candida albicans*. The acetone extract of this plant showed inhibition towards gram negative and gram positive bacteria (Nyiligira *et al*., 2008). Different groups of compounds had been isolated from this plant, including flavanoids such as casticin, vitexin, artemetin, luteolin (Zeng *et al*., 1996), sterols, such as β-sitosterol (Nair *et al*., 1975), terpenoids, monoterpenes, labdane diterpene and sesquiterpenes. (Nyiligira *et al*., 2008; Hossain *et al*., 2001; Tandon *et al*., 2008; Singh *et al*., 1999) These compounds have all been reported to have antibacterial activity (Cushnie *et al*., 2005).

The ethanolic extract of *P. guajava* and *C. cassia* leaves showed no inhibition towards the tested bacteria. This result coincided with the findings of previous studies which showed no antimicrobial activity of ethanolic *P. guajava* leaf extract towards *S. aureus* and β-Streptococcus (Jaiarj *et al*., 1999). The activity was different when other solvents were used for extraction as reported by Jaiarj (1999), Chang *et al*. (2001) and Wang *et al*. (2009). The water extract from *P. guajava* leaves showed high inhibitory effect towards *S. aureus* (Jaiarj *et al*., 1999). Meanwhile, Chang (2001) and Wang (2009) reported that essential oils from *C. cassia* extracted by ethyl ether exhibited antibacterial activity.

Ethanolic extract of *D. zibethinus*, *M. esculenta*, and *C. longa* leaves exhibited no inhibitory effect on the tested bacteria. No previous report has been found on the antimicrobial activity of these plants.

Conclusions

Some of the plant extracts tested in this study showed inhibition towards both gram negative and/or gram positive bacteria; however the only plant which exhibited a specific inhibition to certain bacteria was considered as the potential candidate for an antimicrobial agent. This study demonstrated that the ethanol extract of *A. tricolor* leaves specifically inhibited all tested strains of *B. cereus* with a MIC value of 20 mg/ml. *B. cerues* is normally found in products containing starch, meat, and vegetable. Some

B. cereus outbreaks have also occurred in "meal-on wheel", where the food has been kept above room temperature for a period of time before being served to consumers. Some cases may originate in stir-fry products since the toxin produced by *B. cereus* is stable to heat (Granum *et al*., 1997).

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