Antibacterial activity of three medicinal Thai plants against *Campylobacter jejuni* and other foodborne pathogens

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Leaves of *Adenanthera pavonina*, *Moringa oleifera*, and *Annona squamosa* are used in traditional Thai medicine to treat dysentery and other diseases. The present study investigated the antibacterial activity of these plants against six species of foodborne pathogen. Methods and solvents employed to extract active constituents were optimised using the disc diffusion assay. Phytochemical analysis of the optimised extracts was performed by thin layer chromatography (TLC). Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined by broth microdilution. *A. pavonina* contained flavonoids, terpenes and tannins, and was the most active extract against *Campylobacter jejuni*, inhibiting growth at 62.5 to 125 µg ml^{-1}. *A. squamosa* extract contained flavonoids, terpenes, tannins and alkaloids, and had the broadest spectrum of antibacterial activity, inhibiting *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus* and *C. jejuni* between 62.5 and 500 µg ml^{-1}. MBCs were just 2- to 4-fold higher than MICs against *C. jejuni* and *B. cereus*, suggesting the extracts are bactericidal against these species. Negligible activity was detected from *Moringa oleifera*. Data presented here shows that *A. pavonina* and *A. squamosa* could potentially be used in modern applications aimed at treatment or prevention of foodborne disease.

Keywords: *Adenanthera pavonina*; *Moringa oleifera*; *Annona squamosa*; antibacterial; foodborne pathogen; *Campylobacter jejuni*

1. Introduction

Foodborne pathogens are an important public health problem, responsible for an estimated 76 million incidents of illness, 325,000 hospitalisations, and 5000 deaths in the United States each year (Centers for Disease Control and Prevention, 2005). In the majority of cases, bacteria are the aetiologic agents. *Campylobacter jejuni* is the most common cause of foodborne infection in the U.S., and a major cause of human gastroenteritis worldwide (Wagenaar, Mevius, & Havelaar, 2006). *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli,* and *Salmonella* spp. are also frequent causes of illness (Ray, 2004). Treatment and prevention of these diseases is becoming increasingly difficult. This is due to various factors including the emergence of antibiotic resistant strains of foodborne pathogen (Andersen et al., 2006), the emergence of resistance to disinfectants (Hegstad et al.,

2010), and rising numbers of people who are susceptible to foodborne infection due to weakened immune systems (Winter et al., 2009).

Plants have a rich history of use in both the treatment and prevention of foodborne disease. Pomegranate, for example, has been used in different cultures to treat diarrhoea and dysentery (Duman, Ozgen, Dayisoylu, Erbil, & Durgac, 2009), and cinnamon was used by the ancient Egyptians to preserve food (Naida, 2000). Antimicrobial plant-derived natural products continue to attract a lot of interest today, and have a wide range of potential uses. Applications currently under investigation include anti-infective drugs (Neamatallah, Yan, Dewar, & Austin, 2005), natural food preservatives (Tajkarimi, Ibrahim, & Cliver, 2010), chill water additives for poultry processing (Dickens, Berrang, & Coxt, 2000) and antimicrobial food packaging (Ravishankar, Zhu, Olsen, McHugh, & Friedman, 2009). It has also been suggested that plant components could be vaporized and used in modified atmosphere packaging for foodstuffs (López, Sánchez, Batlle, & Nerín, 2005).

Thailand is a richly biodiverse country where approximately one third of the population uses medicinal plants (Thailand Public Relations Department, 2009). Leaves from *Adenanthera pavonina*, *Moringa oleifera* and *Annona squamosa* are used in the treatment of various disorders including diarrhoea and dysentery (Table S1). In the present study, the antibacterial activity of these three species was investigated. The first objective of this work was to determine the optimum procedure for extracting antibacterial constituents. The type of solvent used is known to influence the type of compounds extracted, and there is recent evidence to show that the method used is also important (Ndip et al., 2007). The second objective of our study was to identify which classes of phytochemical are present in the optimised extracts, and the final objective was to quantitatively determine the activity of these extracts against *C. jejuni* and other species of foodborne pathogen.

2. Results and discussion

Data presented in Table 1 shows that antibacterial activity of the plants *A. pavonina*, *M. oleifera*, and *A. squamosa* varies significantly ($p<0.05$) when different extraction solvents are used. Water was the most effective solvent for extracting antibacterial constituents from *A. pavonina* and *M. oleifera*. Ethanol was moderately effective by

comparison, and hexane was the least effective. These results indicate that the antibacterial constituents of *A. pavonina* and *M. oleifera* are highly polar. This is potentially advantageous as nonpolar compounds have low aqueous solubility. Compounds with low aqueous solubility suffer from poor bioavailability (Gupta et al., 2005), which can preclude their use as therapeutic drugs. Low aqueous solubility can also limit the use of compounds as food preservatives (Guiotto et al., 2003). For *A. squamosa*, the ethanol extract was most active. Activity was also detected from the hexane extract, but not the aqueous extract. These results suggest that the active constituents of *A. squamosa* have both polar and nonpolar structural moieties.

For *A. pavonina*, the method of extraction was also found to have a statistically significant ($p<0.05$) effect upon antibacterial activity (Table 1). Aqueous and ethanol extracts obtained by ultrasonic assisted extraction were more active than those obtained by the conventional method. It may be that sonication disrupted the integrity of the plant cell walls (Wang & Weller, 2006), facilitating penetration of solvent into the cellular material and release of contents. For *M. oleifera* and *A. squamosa*, conventional extraction produced extracts that were equally active or marginally more active than those obtained using the ultrasonic assisted method (Table 1). This finding shows that, for certain plant species, there are aspects of the conventional method (eg. use of heat, increased extraction time) which facilitate extraction to a similar or even greater degree than sonication. In the case of the *M. oleifera* aqueous extracts, it could be that the bioactive compounds dissolved more readily at 80ºC than room temperature. For the *M. oleifera* ethanol extracts, it may be that dissolution of the bioactive compounds was more readily achieved by 24 h shaking than 30 min sonication. On the basis of the above findings, all subsequent work with *A. pavonina* was performed with water and the ultrasonic assisted method. For *M. oleifera* and *A. squamosa*, extracts were prepared by conventional ethanol extraction and ultrasonic assisted ethanol extraction respectively.

Phytochemical analysis of the optimised *A. pavonina* extract showed that it contains flavonoids, terpenoids, and tannins (Table S2). Very little information is available on the phytochemistry of *A. pavonina*, but there are many reports of compounds in the above classes possessing antibacterial activity, including some recent discoveries with highly potent activity (Saleem et al., 2010). Analysis of optimised *M. oleifera* and *A. squamosa* extracts indicate they contain flavonoids, terpenoids, tannins and also alkaloids (Table S2). A recent study by Verma,

Vijayakumar, Mathela, & Rao (2009) has shown that *M. oleifera* leaves contain the flavonoids kaempferol, quercetin and rutin. These compounds are known to have both direct (Rauha et al., 2000) and synergistic (Arima, Ashida, & Danno, 2002) antibacterial activity. Given our results, kaempferol, quercetin and rutin are almost certainly contributing to the activity of the *M. oleifera* extract. Leaves of *A. squamosa* are reported to contain quercetin-3-*O*-glucoside (Panda & Kar, 2007), but this glycosylated flavonoid is inactive against bacteria (Razavi, Zahri, Zarrini, Nazemiyeh, & Mohammadi, 2009) and unlikely to be contributing to the activity of this extract. All in all, these results show that further in-depth phytochemistry studies are justified for *A. pavonina* and *A. squamosa*. Studies with *M. oleifera* are less likely to yield novel antibacterial compounds.

When MICs were determined, *A. squamosa* was found to have the broadest spectrum of activity (Table 2). Activity was greatest against *L. monocytogenes, B. cereus* and *S. aureus*, with MICs in the range 62.5 to 250 µg ml^{-1}. These values compare favourably with those proposed by Ríos and Recio (2005) for evaluating the antibacterial activity of medicinal plants. The MIC against *C. jejuni* was higher, and no activity was detected against *E. coli* or *Salmonella* serotype Typhimurium. This susceptibility pattern correlates well with those obtained for other medicinal plant extracts. Gram negative bacteria are generally less susceptible to antibacterial agents than Gram positive organisms due to the low permeability of their cell wall (Delcour, 2009).

A. pavonina extract had the most potent inhibitory activity against *C. jejuni* (Table 2), but no activity against any of the other bacteria. This susceptibility pattern, though unusual, is not unprecedented. Friedman, Henika, & Mandrell (2002) report that essential oils from ginger root and other plants are more active against *C. jejuni* than the Gram positive bacterium *L. monocytogenes*. Compared to other bacteria, very few studies have investigated the susceptibility of *C. jejuni* to plant-derived natural products. Data presented here supports the idea that phytochemicals may be particularly suited to the control of infections caused by *C. jejuni*. As to why *C. jejuni* is so susceptible to inhibition by phytochemical compounds, this remains to be determined. Friedman et al. (2002) hypothesise that this may be due to differences between the cell walls of the bacterial species. Conceivably, the plant compounds are targeting structures present in the cell wall of *C. jejuni* that are absent in the other bacteria, or differences in the cell wall are affecting uptake / efflux of the compounds.

It is evident from our results that *A. pavonina* has narrow spectrum antibacterial activity, but this should not preclude the species from further study. In recent years, it has become clear that the use of broad spectrum antibacterial agents, in particular those entering the gastrointestinal tract, can be detrimental to human health (Parkes, Sanderson, & Whelan, 2009).

MICs determined for the *M. oleifera* extract were 1000 µg ml^{-1} or higher, indicating this plant has negligible inhibitory activity against foodborne bacterial pathogens. We cannot conclude from this finding that *M. oleifera* use is unjustified in the treatment of diarrhoea and dysentery. It may simply be that this plant's medicinal properties are attributable to a different type of biological activity. For example, some phytochemicals neutralize bacterial toxins (Choi, Yahiro, Morinaga, Miyazaki, & Noda, 2007), some inhibit virulence factors required for colonization of the host (Xiao et al., 2007), and others have anti-inflammatory activity (González-Segovia et al., 2008). Data presented here indicates that *M. oleifera* is unlikely to contain compounds suitable for protecting food against bacterial growth though.

Results in Table 2 suggest that *A. pavonina* and *A. squamosa* extracts both have bactericidal activity. By definition, bactericidal agents have MBC values within a 2- to 4-fold dilution of the MIC (Prescott, Harley, & Klein, 2005), and this is the case for these two extracts against *C. jejuni* and *B. cereus* respectively. It is not clear why *A. squamosa* extract is merely bacteriostatic against *L. monocytogenes*, *S. aureus* and *C. jejuni*. Sometimes, species-specific bactericidal activity reflects differences in the apoptotic mechanisms of the microorganisms (Stratton, 2005), but this is unlikely here because gentamicin is bactericidal against all four of the species in question (Table 2). An alternative explanation is that the inhibitory activity we have detected from *A. squamosa* is attributable to more than one constituent. If this is the case, then these other compounds may be masking the bactericidal activity of the *A. squamosa* extract. Further work will therefore be performed to fractionate the plant extracts and, if possible, isolate the active compounds.

3. Conclusions

In conclusion, this study shows that leaf extracts of *A. pavonina* and *A. squamosa* possess significant activity against up to four bacterial species including the important foodborne pathogen *C. jejuni*. The constituents responsible for this activity remain to

be identified, but for *A. pavonina* the principal active components are flavonoids, terpenoids or tannins with highly polar structures. For *A. squamosa*, the active components possess both polar and nonpolar structural moieties, and may be flavonoids, terpenoids, tannins or alkaloids. Further investigation is clearly necessary but purified extracts or compounds from these plant species could potentially be useful in the treatment or prevention of foodborne infection.

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Table 1 Efficacy of different methods and solvents for obtaining plant extracts with inhibitory activity against *C. jejuni* ATCC 29428.

Method	Solvent	Zone of inhibition (mm)			
		A. pavonina (5 mg / disc)	*M. oleifera* (5 mg / disc)	*A. squamosa* (5 mg / disc)	Erythromycin (10 µg / disc)
Conventional	Water	52.0±1.7^{b}	17.5±1.1^{a}	-	24.8±1.8
	Ethanol	37.5±2.1^{d}	17.2±1.7^{ab}	13.2±1.6^{a}	26.0±0.9
	Hexane	12.0±1.3^{e}	10.7±1.2^{c}	10.7±0.5^{b}	24.8±1.7
Ultrasonic	Water	54.7±1.8^{a}	17.0±1.3^{ab}	-	24.8±1.2
	Ethanol	42.5±1.5^{c}	14.3±0.8^{b}	12.0±0.9^{ab}	25.0±1.6
	Hexane	13.5±1.8^{e}	11.0±0.9^{c}	11.5±1.1^{ab}	25.2±1.0

Note: Data presented is the mean diameter size of zones of inhibition ±SD (mm) for six replicates. Different superscript letters (a-e) indicate significant differences ($p < 0.05$) between values within the same column as determined by the Scheffe test. -, no activity detected

Table 2 MIC and MBC values of plant extracts obtained using the optimised procedures against various species of foodborne pathogen.

Bacteria	*A. pavonina*		*M. oleifera*		*A. squamosa*		Erythromycin		Gentamicin	
	MIC (µg ml^{-1})	MBC (µg ml^{-1})	MIC (µg ml^{-1})	MBC (µg ml^{-1})	MIC (µg ml^{-1})	MBC (µg ml^{-1})	MIC (µg ml^{-1})	MBC (µg ml^{-1})	MIC (µg ml^{-1})	MBC (µg ml^{-1})
Gram positive										
B. cereus ATCC11778	-	--	-	--	250	500-1000	2	--	0.5	2
L. monocytogenes ATCC19111	-	--	-	--	125-250	--	0.5	--	0.5-1	≥4
S. aureus ATCC 6538	-	--	-	--	62.5-125	--	0.063	--	0.125	0.25
Gram negative										
C. jejuni ATCC 29428	62.5-125	250	1000-2000	4000	250-500	≥2000	2	4	0.5	1
E. coli ATCC 25922	-	--	-	--	-	--	-	--	0.5	2
S. Typhimurium ATCC 13311	-	--	-	--	-	--	-	--	0.5	0.5

-, no inhibitory activity detected at concentrations up to and including 4 mg ml^{-1}; --, no bactericidal activity detected at concentrations up to 8 x MIC

Supplementary material

Antibacterial activity of three medicinal Thai plants against *Campylobacter jejuni* and other foodborne pathogens

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Leaves of *Adenanthera pavonina*, *Moringa oleifera*, and *Annona squamosa* are used in traditional Thai medicine to treat dysentery and other diseases. The present study investigated the antibacterial activity of these plants against six species of foodborne pathogen. Methods and solvents employed to extract active constituents were optimised using the disc diffusion assay. Phytochemical analysis of the optimised extracts was performed by thin layer chromatography (TLC). Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined by broth microdilution. *A. pavonina* contained flavonoids, terpenes and tannins, and was the most active extract against *Campylobacter jejuni*, inhibiting growth at 62.5 to 125 µg ml^{-1}. *A. squamosa* extract contained flavonoids, terpenes, tannins and alkaloids, and had the broadest spectrum of antibacterial activity, inhibiting *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus* and *C. jejuni* between 62.5 and 500 µg ml^{-1}. MBCs were just 2- to 4-fold higher than MICs against *C. jejuni* and *B. cereus*, suggesting the extracts are bactericidal against these species. Negligible activity was detected from *Moringa oleifera*. Data presented here shows that *A. pavonina* and *A. squamosa* could potentially be used in modern applications aimed at treatment or prevention of foodborne disease.

Keywords: *Adenanthera pavonina*; *Moringa oleifera*; *Annona squamosa*; antibacterial; foodborne pathogen; *Campylobacter jejuni*

S1. Experimental

S1.1. Plant material

Leaves from *A. pavonina*, *M. oleifera*, and *A. squamosa* (Table 1) were collected in Maha Sarakham Province in northeast Thailand in September 2009. The identity of these species was confirmed by Ms. Suttira Khumkratok of the Walai Rukhavej Botanical Research Institute (Thailand) and voucher specimens were deposited (Achara 02-10, 01-10, and 03-10). All plant material was rinsed with water, sliced, dried at 50ºC for 24 h, and then ground and sieved with 80 mesh stainless steel sieves.

S1.2. Extractions

Aliquots of the plant powders (6 x 10 g) were extracted using two methods (conventional and ultrasonic assisted) and three solvents [distilled water, 95% (v/v) ethanol, and hexane]. Both methods involved adding an aliquot of plant powder to each solvent [1:10 (w/v)]. For the conventional extraction with water, the suspension was heated at 80ºC in a water-bath for 1 h and then allowed to cool at room

temperature (Ishikawa et al., 2007). Plant tissue was removed by centrifugation (5000 x *g* for 10 min) and filtration of the supernatant. For the conventional ethanol and hexane extractions, the suspensions were shaken at 120 rpm for 24 h (room temperature), centrifuged and filtered, then concentrated using a rotary evaporator at 50ºC (Al-Zubairi et al., 2009). Ultrasonic assisted extractions were performed by immersing the suspensions of powder and solvent in an ultrasonic bath operating at 40 kHz frequency and 40±1ºC for 30 min (Veličković, Nikolova, Ivancheva, Stojanović, & Veljković, 2007). These suspensions were centrifuged, filtered, and evaporated (ethanol and hexane) as before. All samples were freeze-dried (Heto PowerDry PL 3000) and stored in the dark in tightly sealed bottles at 4ºC until required.

S1.3. Bacteria and culture media

All bacteria were from the American Type Culture Collection (ATCC). Prior to testing, *C. jejuni* was cultured on Brucella agar supplemented with 62.5 mg l^{-1} each of ferrous sulfate, sodium metabisulfite and sodium pyruvate. *L. monocytogenes* was cultured on Tryptic soy yeast extract agar, and the remaining bacteria were cultured on Mueller Hinton agar. *C. jejuni* was incubated at 42ºC for 48 h in micro-aerobic conditions (5% O_2, 10% CO_2, and 85% N_2), and the other bacteria were incubated at 37°C for 24 h in aerobic conditions. Inocula were prepared by suspending bacterial colonies in 0.1% (w/v) sterile peptone water and adjusting these to achieve a turbidity equivalent to a 0.5 McFarland standard.

S1.4. Identification of optimum procedures for extracting antibacterial constituents

The efficacy of the different extraction methods and solvents was assessed using the disc diffusion assay (Ratnam & Raju, 2008). In brief, pour plates were prepared containing 20 ml agar seeded with 10^6 CFU ml^{-1} *C. jejuni*. Solutions of 50 mg ml^{-1} plant extract were prepared by dissolving the aqueous and ethanol extracts in distilled water, and the hexane extract in DMSO. Sterile 6 mm discs (Whatman) were impregnated with 100 µl of the plant extract solutions, dried at 40ºC, and added to the agar surface. Negative control discs were prepared with solvent only, and erythromycin discs (10 µg / disc) were used as reference standards. After 48 h incubation at 42ºC, the diameters of the zones of inhibition were measured. Each plant extract was tested in triplicate and each experiment was performed twice.

S1.5. Phytochemical analysis

Phytochemical analysis was performed by ascending thin layer chromatography (silica gel 60 F_{245} aluminum sheets, Merck). Solutions containing 50 mg ml^{-1} of the optimised plant extracts were screened for different classes of secondary metabolite using the standard tests described by Wagner and Bladt (1996).

S1.6. Determination of minimum inhibitory concentrations (MICs)

MICs were determined against *C. jejuni* and the other bacteria using the broth microdilution method (National Committee for Clinical Laboratory Standards [NCCLS], 2000). Assays were performed in 96-well microtitre trays with an inoculum density of $5x10^5$ CFU ml^{-1} in 100 µl of broth. Mueller-Hinton broth supplemented with 5% (v/v) laked sheep blood was used for testing *C. jejuni* and tryptone soy broth was used for *L. monocytogenes*. Mueller Hinton broth was employed for the other bacterial species. Positive control wells were prepared by inoculating broth containing no extract. Negative control wells consisted of an uninoculated dilution series of the plant extract and broth. Erythromycin and gentamicin were used as reference standards. MICs were determined after 48 h incubation at 42ºC for *C. jejuni*, and after 24 h at 37ºC for the other bacteria. Extracts and antibiotics were tested in quadruplicate and each assay was repeated to verify the reproducibility of results.

S1.7. Determination of minimum bactericidal concentrations (MBCs)

MBCs were determined using the microtitre trays from the MIC assay (NCCLS, 1999). The entire volume of liquid (~100 µl) was aspirated from wells with no visible growth, and streaked across the surface of fresh agar plates. After incubation, colonies were counted and the endpoint was determined by identifying the lowest concentration to cause a 99.9% decrease in CFU numbers. Experiments were repeated to verify the reproducibility of results.

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Table S1 Information on the three medicinal plant species investigated in the present study.

Botanical name	Family	Common name	Part used	Traditional medicinal use
Adenanthera pavonina Linn. var. pavonina	Fabaceae	Red sandalwood tree	Leaflet	Treatment of diarrhoea & dysentery (Orwa, Mutua, Kindt, Jamnadass, & Simons, 2009)
Moringa oleifera Lam.	Moringaceae	Horseradish tree	Leaf	Treatment of diarrhoea & dysentery (Saralamp, Chukul, Temsiririrkkul, & Clayton, 1996)
Annona squamosa Linn.	Annonaceae	Sugar apple	Leaf	Treatment of dysentery, ulcers & abscesses (Rueangrangsi & Mangkhlakhup, 2004)

Table S2 Phytochemical analysis of plant extracts obtained using the optimised extraction procedures.

Phytoconstituents	*A. pavonina*	*M. oleifera*	*A. squamosa*
Flavonoids	+	+	+
Cardiac glycosides	-	-	-
Anthraquinone glycosides	-	-	-
Terpenoids	+	+	+
Saponins	-	-	-
Tannins	+	+	+
Alkaloids	-	+	+

+, detected; -, not detected