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Antimicrobial activity of flavonoid extracts from Sabah tea *(Camellia sinensis)* against *Escherichia coli* and *Listeria monocytogenes*

[Aktiviti antimikrob ekstrak flavonoid daripada teh Sabah *(Camellia sinensis)* terhadap *Escherichia coli* dan *Listeria monocytogenes*]

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Key words: flavonoid, catechin, *Escherichia coli* O157:H7, *Listeria monocytogenes*, Sabah tea

Abstract

The antimicrobial activity of tea *(Camellia sinensis)* flavonoids against selected foodborne pathogens, *Escherichia coli* 0157:H7 and *Listeria monocytogenes* was studied. Flavonoid, hydrolysed flavonoid, flavanol and crude catechin were extracted from fresh and dried tea leaf samples. The activities of each extract on both pathogens were tested using paper disc diffusion method. Extracts producing inhibition zone of more than 8.0 mm were further investigated to determine their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Hydrolysed flavonoid of dried samples was the most active extract against *E. coli* O157:H7 and *L. monocytogenes* with inhibition zone of 16.0 ± 1.4 mm and 22.0 ± 1.4 mm respectively. The MIC of hydrolysed flavonoid extract from fresh samples on *E. coli* O157:H7 was 9.7 mg/ml while the MBC was 11.7 mg/ml. *Listeria monocytogenes* was inhibited at a minimum concentration of 5.86 mg/ml by the same extract. Crude catechin from fresh sample was less effective in controlling *L. monocytogenes* with a MIC of 93.8 mg/ml, which was also its MBC. The time required for the reduction of *L. monocytogenes* count by one log cycle was the shortest (1.87 h) in the presence of hydrolysed flavonoid extract at MBC (6.83 mg/ml).

Introduction

In recent years, a number of studies have supported an association between high consumption of plant products and low incidence of certain chronic diseases. This is due to the fact that many of these plants contain naturally occurring compounds that have antimicrobial activity (Sofos et al. 1998; Cowan 1999; Lopez-Malo et al. 2000). The demand by consumers for natural food and food components served as a driving force for the growing interest in natural antimicrobial substances. Among the natural resources, tea has the longest history in the world and the most popular beverage, besides coffee and cocoa (Kim 1996).

The most common types of tea are non-fermented green tea, semifermented oolong tea and fermented black tea of *Camellia sinensis*, with the characteristics of each determined by the manufacturing process. Tea is a dietary source of antioxidant nutrients, such as carotenoids, tocopherols, ascorbic acid and non-nutrient

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phytochemicals generally classified as flavonoids (Wu Christine and Wei 2002). Numerous studies have demonstrated that aqueous extracts of black tea and green tea posses anti-mutagenic, anti-inflammatory, anti-diabetes, anti-bacterial (Hamilton-Miller 1995), anti-tumour (Mitscher et al. 1997) and anti-cariogenic (Wu Christine and Wei 2002) activities in a variety of experimental animal systems.

Green tea is composed of about 30% polyphenols such as flavanols, flavandiols, flavonoids and phenol acids. Polyphenols have been well known to have various excellent biological activities such as anticancer properties (Imai et al. 1997; Ji et al. 1997), inhibition of allergy (Yeo et al. 1995), prevention of gout (An et al. 1996) and inhibition of oxidation. The antimicrobial capacity of tea is effective against various bacteria that cause diarrhoea (e.g. *Staphylococcus aureus, Staphylococcus epidermis, Vibrio cholerae* O1) (Toda et al. 1989) and dental caries *(Streptococcus salivarus, Streptococcus mutans)* (Rasheed and Haider 1998). The mechanism proposed for this antimicrobial activity is that bactericidal catechins primarily act on and damage bacterial membranes (Ikigai et al. 1993).

Although many reports have been published on antimicrobial properties of green tea polyphenols, but knowledge of the effects of Sabah tea flavonoids on the growth of microorganisms related to food, is very limited. Therefore, this study was carried out to investigate the antimicrobial activity of flavonoid extracts of Sabah tea against selected foodborne pathogens such as *Escherichia coli* O157:H7 and *Listeria monocytogenes*.

Materials and methods

Sample preparation

Fresh tea leaves were obtained from Sabah Tea Plantation in Ranau. The harvested samples were kept on ice, transported to the laboratory, and frozen at –40 °C until used. Dried samples were prepared by allowing the tea leaves to expose to sunlight daily (until the leaves were dried) prior to further extraction.

Preparation of test organisms

Strain of *E. coli* 0157:H7 ATCC 35150 was obtained from Veterinary Research Institute Ipoh, whereas *L. monocytogenes* L55 was from Institute for Medical Research, Kuala Lumpur. Cultures were maintained on slant at 4 °C and subcultured monthly to maintain viability. A working culture was prepared by inoculating a loopful of culture into 15 ml of Tryptic Soy Broth (TSB, Merck) and incubated at 37 °C for 18 h. The samples were then centrifuged at 2500 x g for 20 min at 4 °C in a refrigerated centrifuge (Kubota 2100, Japan). Harvested cells were washed twice with sterile saline and the pallet was finally suspended in sterile peptone water by adjusting the optical density in spectrophotometer (Cecil CE 1011, UK) at 640 nm to give a cell density of about 106 CFU/ml.

Preparation of flavonoid extracts

Extraction of tea flavonoid aglycone (hydrolysed flavonoid) was conducted according to the method described by Onyilangha et al. (2003) with slight modification. About 20 g of fresh/dried leaves were cut into tiny pieces and boiled in 50 ml 2 M HCl for 45 min. Hydrolysed leaf extracts were allowed to cool to room temperature. The extracts were then washed trice with equal volumes of ethyl acetate. The pooled ethyl acetate fractions were evaporated to dryness in a fumehood.

For the extraction of flavonoid glycoside, about 100 g of fresh tea leaf was extracted by boiling in methanol (MeOH) for 10 min. The extract was allowed to cool to room temperature, rotary evaporated to dryness, dewaxed with hexane 100%, and the residue was dissolved in water, filtered and washed trice in n-butanol. The combined butanol upper layers were roto-evaporated and the residue was re-dissolved in about 5 ml of distilled water.

Flavanol fraction of tea samples was extracted according to the method of Obanda et al. (1997). Dried tea leaves (20 g) were weighed in duplicate and ground. Aqueous methanol (70%) at 70 °C was added to each flask and placed in a water bath set at 70 °C for 10 min, with mixing after each 5 min. The flasks were then removed from the water bath, allowed to cool for a few minutes and centrifuged at 3000 x g for 10 min. The supernatant was carefully decanted into clean graduated glass tubes and the extraction procedure repeated on the residue. The two volume extracts for each sample were combined and the volume made to 10 ml with aqueous methanol.

The catechins content of dried tea leaves (20 g) were extracted with 200 ml of water at 70 °C for 40 min (Baptista et al. 1999). The extraction was repeated three more times and the combined aqueous extract was filtered and freeze-dried. About 100 mg of green tea powder from the previous extraction was dissolved in 10 ml of hot water (70 °C), and methylxanthines and pigments were extracted with 10 ml of chloroform. Then, 10 ml of ethyl acetate was added to the same volume of aqueous solution for extraction. Similar extractions were repeated three times and the three extracts were combined. Ethyl acetate was evaporated in a vacuum rotary evaporator and the light brown residue, called `crude catechins', was obtained. All extracts were kept in universal bottle wrapped with aluminium foil and stored at 4 °C for further analysis.

Antimicrobial activity

The antimicrobial activity of each tea extracts was measured by a paper disk diffusion method (Kim et al. 1995). Sterile filter paper (diameter 6 mm) was impregnated with 10 μl of various flavonoid extracts and placed in the centre of the Mueller Hinton (MH, Merck) agar plate, on which the test microorganism (10^6 cells/ml) was uniformly inoculated. It was kept in sterile workstation for 30 min to allow it to be absorbed with designated concentration of green tea flavonoids and incubated at 37 °C for 24 h. The diameter of the clear zone shown on plates was measured using calipers and expressed in mm as its antimicrobial activity. Extracts shown inhibitory zone of more than 8.0 mm were further investigated using broth dilution method in MH broth for the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) based on the time kill assays. Extraction solvents without tea extract were used as a negative control in all antimicrobial screening. The MIC is defined as the lowest concentration of extracts that resulted in a significant decrease in inoculum viability (more than 90%) within 24 h, while the MBC referred to the concentration at which more than 99% of the initial inoculum was killed within 24 h (NCCLS 2000).

Results and discussion

Antimicrobial activity using paper disc diffusion

Flavonoid extracts of Sabah tea showed various degree of inhibition against the two foodborne bacterial pathogens in paper disc screening *(Table 1)*. Extracts of fresh tea leaves were much more potent than extracts of dried samples on both bacteria. All flavonoid extracts showed some form of inhibition against both pathogens except flavanol and crude catechin extracts from dry samples, which were inactive against *E. coli* O157:H7. Hydrolysed flavonoid (flavonoid aglycone) from fresh tea samples was the most active against *L. monocytogenes* and *E. coli* O157:H7 with inhibition zone of 16.00 ±1.41 mm and 22.00 ± 1.41 mm respectively. Judged on the inhibition zones, *E. coli* O157:H7 was less sensitive to flavonoid extracts as compared to *L. monocytogenes*.

The active components of tea that are responsible for most biological activities are recognized to be catechins, which includes several isomers, (–)–epigallocatechin

Table 1. Antimicrobial activity of Sabah tea flavonoid extracts against *Escherichia coli* O157:H7 and *Listeria monocytogenes*

Tea leaves	Flavonoid extract	Yield (%)	Inhibition zone diameter[a] (mm)	
			E. coli O157:H7	*L. monocytogenes*
Fresh	Flavonoid glycoside	13.42	++ (12.0 ± 1.4)	++ (18.0 ± 0.0)
	Hydrolysed flavonoid	12.80	++ (16.0 ± 1.4)	++ (22.0 ± 1.4)
	Flavanol	10.51	+ (7.5 ± 0.0)	++ (10.0 ± 1.4)
	Crude catechin	9.35	+ (6.5 ± 0.7)	++ (8.5 ± 0.0)
Dried	Flavonoid glycoside	14.53	+ (6.5 ± 0.7)	++ (12.5 ± 0.7)
	Hydrolysed flavonoid	14.70	+ (7.5 ± 0.7)	++ (15.0 ± 0.0)
	Flavanol	15.60	–	+ (7.5 ± 2.1)
	Crude catechin	16.10	–	+ (6.5 ± 0.4)

[a]Antimicrobial activity: – = Negative; + = Inhibition zone ≤ 8 mm, ++ = Inhibition zone >8 mm; ( ) = Mean values and standard deviations

gallate (EGCg), (–)–epicatechin (EC), (–)–epicatechin gallate (ECg), (–)–epigallocatechin (EGC) and (+)–catechin. Among these, EGCg is the major form (50%) and the most active with regard to a variety of biological activities including antimicrobial properties (Yanagawa et al. 2003). Studies on antimicrobial effects of tea extracts have shown that gram positive bacteria are more sensitive to tea extracts than gram negative ones, and it has been suggested that negatively charges EGCg acts by binding to the positively charged lipids of the membrane causing damage to the lipid bilayer. The low susceptibility to catechin of gram negative bacteria is attributed to the presence of negatively charged lipopolysaccharides (Ikigai et al. 1993).

Toda et al. (1991) found that extracts of tea inhibited and killed *S. aureus, S. epidermis, Salmonella typhi, Salmonella typhimurium, Shigella dysenteriae* and *Vibrio* spp. including *V. cholerae*. However, there is some disagreement precisely over which bacterial species are inhibited by tea. For example, Hara and Ishigami (1989) found that *S. typhimurium* and *Campylobacter jejuni* were resistant, while others reported that the former species was susceptible. Presumably, these differences are due to strain variations, the sources and the infusion strengths of various teas (Hamilton-Miller 1995).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of flavonoid extracts of Sabah tea against *E. coli* O157:H7 and *L. monocytogenes* are shown in *Table 2*. A comparison of bacteriostatic and bactericidal results of the test extracts showed that the MIC values were not the same as the MBC except for flavanol from fresh tea on *E. coli* O157:H7. At the concentration of 27.35 mg/ml, flavonoid glycoside extract showed inhibitory effect against bacterial growth by reducing the numbers of *E. coli* O157:H7 for more than 90%. The MIC of flavonoid aglycone extract from fresh tea against *E. coli* O157:H7 was determined as 9.77 mg/ml while the MBC was 11.72 mg/ml based on the time killed curves. *Listeria monocytogenes* was inhibited at a minimum concentration of 5.86 mg/ml by the same extract, but a higher concentration of 6.83 mg/ml was identified as the MBC. However, extracts of dried samples often seem to have a higher MIC and MBC than extracts of fresh sample indicated that the active compounds found in dried samples were less inhibitory as compared to their fresh counterpart.

The flavonoid content of leaf tissue is very sensitive to environmental conditions such as amount of the light energy and pollutants. Tea leaves also

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (mg/ml) of tea flavonoid extracts against two test organisms *(Escherichia coli* and *Listeria monocytogenes)*

Tea Leaves	Flavonoid extracts	*E. coli* O157:H7		*L. monocytogenes*	
		MIC	MBC	MIC	MBC
Fresh	Flavonoid glycoside	27.35	27.35	7.81	11.72
	Hydrolysed flavonoid	9.77	11.72	5.86	6.83
	Flavanol	–	–	39.06	46.88
	Crude catechin	–	–	93.75	93.75
Dried	Flavonoid glycoside	–	–	19.53	23.44
	Hydrolysed flavonoid	–	–	11.72	11.72

– = Not studied

Table 3. Decimal reduction times (hours) of the test strains *(Escherichia coli* and *Listeria monocytogenes)* in various flavonoid extracts at their respective minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Tea Leaves	Flavonoid extracts	*E. coli* O157:H7		*L. monocytogenes*	
		MIC	MBC	MIC	MBC
Fresh	Flavonoid glycoside	4.99	4.99	7.86	2.75
	Hydrolysed flavonoid	11.3	3.49	17.39	1.87
	Flavanol	–	–	7.87	3.02
	Crude catechin	–	–	4.06	4.06
Dried	Flavonoid glycoside	–	–	8.03	3.44
	Hydrolysed flavonoid	–	–	2.96	2.96

– = Not studied

contain polyphenol oxidase enzymes in separate layers of the leaf. When cell ruptures, catechins may come in contact with the enzymes resulting in the formation of the aflavins and thearubigins, which are relatively less inhibitory against microorganisms (Frei and Higdon 2003). Crude catechins from green tea was less effective in controlling both the tested strains and required as high as 93.75 mg/ml to completely inhibit *L. monocytogenes*. These results are inconsistent with the notion that gallocatechins and their gallates are the main chemical moieties responsible for the antibacterial activity of green tea.

Results of the decimal reduction time of each test strain at their respective MIC and MBC levels were shown in *Table 3*. The time required for the reduction of one log *L. monocytogenes* count was the shortest (1.87 h) in the presence of hydrolysed flavonoid extract at MBC (6.83 mg/ml) as compared to 17.4 h if put at its MIC. It seemed that flavonoid aglycone had the most potent inhibitory/bactericidal activity against *L. monocytogenes,* followed by flavonoid glycoside, flavonol and catechins. The results obtained were similar to that reported by Otshudi et al. (1999) and Rauha et al. (2000) who demonstrated that flavonoid aglycones are more active than their glycosides forms naturally present in plants.

Conclusion

Hydrolysed flavonoid extract from fresh tea leaves showed the strongest antibacterial activity on both gram positive and gram negative bacteria and it is therefore a potential candidate for further study in searching an alternative antimicrobial for food preservation.

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Abstrak

Aktiviti antimikrob flavonoid teh *(Camellia sinensis)* terhadap patogen bawaan makanan terpilih iaitu *Escherichia coli* O157:H7 dan *Listeria monocytogenes* telah dikaji. Flavonoid, flavonoid terhidrolisis, flavanol dan katekin telah diekstrak daripada daun teh segar dan kering. Kaedah peresapan cakera kertas telah digunakan untuk menguji aktiviti setiap ekstrak. Ekstrak yang menghasilkan zon perencatan melebihi 8.0 mm dikaji selanjutnya untuk menentukan kepekatan minimum perencatan (MIC) dan kepekatan minimum bakterisid (MBC). Flavonoid terhidrolisis daripada sampel kering ialah yang paling aktif terhadap *E. coli* O157:H7 dan *L. monocytogenes,* masing-masing dengan zon perencatan 16.0 ± 1.4 mm and 22.0 ± 1.4 mm. MIC bagi ekstrak flavonoid terhidrolisis daripada daun segar terhadap *E. coli* O157:H7 ialah 9.7 mg/ml manakala MBC ialah 11.7 mg/ml sementara *L.monocytogenes* dapat direncat pada kepekatan serendah 5.86 mg/ml ekstrak yang sama. Katekin kasar daripada sampel segar didapati kurang berkesan untuk mengawal *L. monocytogenes* dengan MIC setinggi 93.8 mg/ml yang juga merupakan nilai MBC. Tempoh masa yang diperlukan untuk mengurangkan satu kitaran log kiraan *L. monocytogenes* adalah paling singkat (1.87 jam) dengan kehadiran flavonoid terhidrolisis pada nilai MBC (6.83 mg/ml).

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