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RESEARCH ARTICLE

Oligomeric proanthocyanidin fractions from fresh tea leaves and their antibacterial activity against *Staphylococcus aureus*

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Abstract: Purification of the proanthocyanidin (PA) extract of fresh tea [*Camellia sinensis* (L.) Kuntze] leaves by Sephadex LH-20 column chromatography followed by high-speed counter-current chromatography (HSCCC) using the two phase solvent system hexane-ethyl acetate-methanol-water (1:5:1:5) furnished three PA fractions PA_1- PA_3. The electro-spray ionization mass spectrometry (ESI-MS) of the PA fractions indicated the presence of oligomeric PAs in all three PA fractions. Antibacterial activity against *Staphylococcus aureus* and their minimum inhibitory concentration (MIC) values were determined using the agar well diffusion assay and the agar dilution method, respectively. The antibacterial activity was observed for the selected PA fractions with MIC values in the range 512 − 1024 µg/mL against methicillin-resistant *S. aureus* (MRSA, 12 strains), methicillin-susceptible *S. aureus* (MSSA, 1 strain) and two standard strains of *S. aureus*, NCTC 6571 and ATCC 25923*S.* This is the first report of antibacterial activity displayed by tetrameric A-type PAs in the extracts of fresh tea leaves.

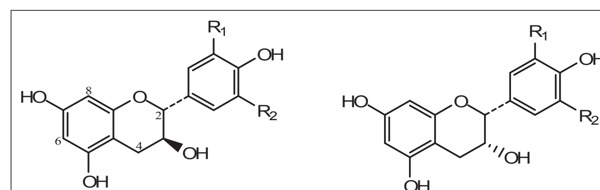
Keywords: Antibacterial, *Camellia sinensis*, oligomeric proanthocyanidins, *Staphylococcus aureus,* tea.

INTRODUCTION

Tea made from the tender leaves of *Camellia sinensis*, var *assamica* is one of the most widely consumed beverages in the world. The health - giving properties of tea are well documented and have been attributed to the wide range of polyphenolic compounds, including flavan-3-ols (catechins), flavanol glycosides and proanthocyanidins (PAs) present in tea leaves.

Dimeric, oligomeric and polymeric PAs, also known as condensed tannins and leucocyanidins are an ubiquitous group of plant phenols, which has attracted a great deal of attention in the fields of nutrition, health and medicine because of their potent antioxidant properties. The term proanthocyanidin derives from the fact that these compounds yield anthocyanidins on heating with aqueous acids. PAs isolated from tea comprise about 2 % of the dry weight of the leaf (Robertson, 1992).

The flavan-3-ols found commonly in PAs are (+)-afzelechin, (+)-catechin and (+)-gallocatechin and their diastereomers (-)-epiafzelechin, (-)-epicatechin and (-)-epigallocatechin (Figure 1). In B-type PAs the inter-flavanol links are C-C bonds between the C4 of one flavanol unit (upper unit) and the C8 or C6 of another (lower unit), while in A-type PAs there are two linkages (C-O and C-C) between two of the flavanol units.



Flavan-3-ol	R_1	R_2	Flavan-3-ol	R_1	R_2
(+)-afzelechin	H	H	(-)-epiafzelechin	H	H
(+)-catechin	H	OH	(-)-epicatechin	H	OH
(+)-gallocatechin	OH	OH	(-)-epigallocatechin	OH	OH

Figure 1: Chemical structures of flavan-3-ols in tea

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B-type PAs have been isolated from many different plant species while A-type PAs have been reported from a few natural sources including cranberry [*Vaccinium macrocarpon* (Aiton)] (Foo *et al*., 2000; Howell *et al*., 2005), cinnamon [*Cinnamomum zeylanicum* (Blume)] (Nonaka *et al*., 1983) and *Pavetta owariensis* (P Beauv.) (Balde *et al*., 1990). Their presence in these plants have been linked with their use in medicinal preparations. The physiological properties of PAs that are beneficial to health depend on the degree of polymerization as well as the nature of the inter-flavan linkage.

The extraction of tea leaves by aqueous 70 − 80 % acetone (Hashimoto *et al*., 1989, Lakenbrink *et al*., 1999) and repeated chromatography have been used for the separation of B-type PAs (Nonaka *et al*., 1984; Kiehne *et al*., 1997; Lakenbrink *et al*., 1999). A dimeric A-type PA, prodelphinidin A-2,3'-*O*-galllate (**1**), along with the other PAs have been isolated from an aqueous 80 % acetone extract of Oolong tea after repeated chromatography (Hashimoto *et al*., 1989). This is the only previous report of the occurrence of A-type PAs in tea.

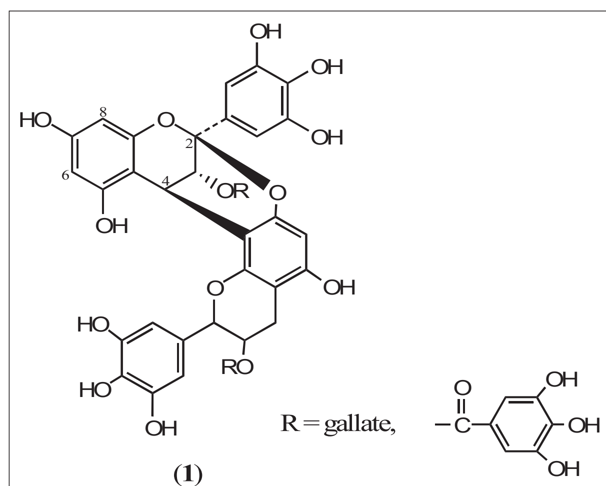

Figure 2: Structure of prodelphinidin A 2,3'-*O*-gallate

Many classes of polyphenolics including PAs from tea, have been separated using high-speed counter-current chromatography (HSCCC), a support-free liquid-liquid chromatographic method (Degenhardt *et al*., 2000; Yanagida *et al*., 2006; Kumar *et al*., 2009).

This paper describes the separation of oligomeric proanthocyanidin fractions from an aqueous acetone extract of tea leaves using Sephadex LH-20 chromatography and HSCCC, and the evaluation of their antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA). The low resolution electrospray ionization-mass spectrometry (LR-ESI-MS) of the PA fractions suggested that these fractions were mixtures of tetrameric PAs (with both A-type and B-type linkages) as well as dimeric PAs. To the best of our knowledge, tetrameric A-type PAs from tea have not been separated before and has been reported by us for the first time (Kumar *et al*., 2013).

METHODS AND MATERIALS

Tender tea shoots were collected from the cultivar TRI 2025 (Tea Research Institute, Talawakelle) and chromatographic fractions were analyzed using commercial TLC plates (Merck Silica Gel $60F_{254}$) and developed with the solvent system ethyl acetate-water-formic acid (90:05:05). A 1 % (w/v) solution of *p*-*N*,*N*-dimethyl aminocinnamldehyde (DMACA) spray reagent in methanolic sulphuric acid (8 mL H_2SO_4 and 100 mL methanol) was used to visualize the spots (Li *et al*., 1996; Rohr *et al*., 2000). The PA containing fractions were located by TLC as purplish blue spots with DMACA reagent.

Extraction of PAs

Cold aqueous 70 % acetone (200 mL) containing sodium ascorbate (0.2 g) was used to extract the freeze-dried tea leaves (100 g), and filtered. The aqueous phase was saturated with sodium chloride (30 g) and the green upper phase was evaporated on a rotavapor under reduced pressure (≤ 30 ^{o}C) to remove acetone, and filtered to remove the precipitated chlorophyll. Distilled water (20 mL) was added into the filtered extract and partitioned with dichloromethane (20 mL × 2) to remove chlorophyll and caffeine. The lower phase was discarded and the upper aqueous phase was partitioned with hexane (100 mL × 2), and the aqueous phase was concentrated and freeze-dried to yield the PA extract as a pale brown powder (2.5 g). The TLC indicated the presence of PAs and some monomeric flavan-3-ols (catechins) in the aqueous acetone extract from tea leaves.

Sephadex LH-20 chromatography

The PA extract (1.5 g in 9 mL of 50 % aqueous methanol) was chromatographed on a Sephadex LH-20 column (Amersham Pharmacia Biotech, Uppsala, Sweden, 20 × 2.3 cm) pre-equilibrated with aqueous 50 % methanol (800 mL), and then eluted with aqueous 50 % methanol (270 mL) to remove the remaining catechins. The eluted fractions were monitored by TLC (Rohr *et al*., 2000):

fractions F_1 (690 mg), F_2 (24 mg), F_3 (83 mg) and F_4 (237 mg). PA containing fractions F_3 (83 mg) and F_4 (237 mg) were eluted using aqueous 70 % acetone. The TLC revealed that fraction F_1 (690 mg) contained monomeric flavan-3-ols while fraction F_2 (24 mg) contained both monomeric flavan-3-ols and PAs. Fractions F_3 (83 mg) and F_4 (237 mg) contained only PAs. The major fraction F_4 was subjected to fractionation by HSCCC.

High–speed counter–current chromatography (HSCCC)

Distilled and degassed (30 min) solvents were used for HSCCC. The HSCCC Model CCC-1000-Pharma-Tech Research Corporation, Baltimore, MD, USA was used for the separation. The preparative coil (volume capacity of 300 mL) had an internal diameter of 2.6 mm, a β-value of 0.8 and a revolution radius of 7.5 cm. The hexane-ethyl acetate-methanol-water system **A** (1:5:1:5) was selected for the separation on the basis of partition coefficient (K_D 1.02), settling time (Ito & Conway, 1996) and stationary phase retention (Shibusawa *et al*., 2000; Kumar *et al*., 2009). The aqueous phase was used as the mobile phase.

The F_4 extract (200 mg in 5 mL of mobile phase) was injected into the HSCCC and fractions (~ 0.5 mL) were collected (elution speed of 2.0 mL min^{-1} and rotational speed of 800 rpm). The effluent from the outlet of the column was monitored continuously with a UV detector and the PA fractions were collected manually.

ESI-MS spectrometry

Low resolution ESI-MS spectra were run by direct injection (detector temperature 225 ^oC) on a thermo electron Finnigan LTQ LC-MS system (Thermoscientific, FL, USA) with ESI system (ionization spray voltage 4.31 V, capillary voltage 2.97 V) at the Harbour Branch Oceanographic Institute at Florida Atlantic University. The compounds were dissolved in MeOH-water (1:1) with a trace of formic acid for positive ion detection while a trace of NH_4OH was added for negative mode detection.

Bacterial isolates and growth conditions

Standard strains of *Staphylococcus aureus* (NCTC 6571 and ATCC 25923) and a total of 12 MRSA and 11 MSSA strains obtained from fresh clinical isolates (collected from the Division of Microbiology, Faculty of Dental Sciences, University of Peradeniya and the Microbiology Laboratory, Teaching Hospital, Peradeniya) were used in the study. These cultures were maintained at –70 °C and the purity of the cultures was ascertained by using standard laboratory techniques. A loopful of the organism was taken from the bacterial colonies grown overnight on blood agar and suspended in sterile distilled water. The turbidity of the bacterial suspension was adjusted using 0.5 MacFarland standard (10^8 CFU/mL).

Antibacterial activity

Susceptibility tests were performed by the Mueller-Hinton-Agar (MHA) well diffusion method (Valgas *et al*., 2007). The MHA plates (90 mm diameter, 20 mL) were inoculated with the bacterial suspension (2.0 mL) by the pouring method. The extra volume was removed with a micropipette and the plates were kept inside an oven at 44 ^oC for 20 min until completely dry. Wells (diameter, 9 mm) were punched in the agar and filled with the extracts (200 µL). The plates were incubated at 37 ^oC for 24 h. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone along the two axes perpendicular to each other. Methicillin (10 µg) for MSSA and gentamicin (5 µg) for MRSA were used as the positive controls, while sterile distilled water was used as the negative control. F_4 (which contained only PAs) was found to be the most active in the agar well diffusion assay and was used for further studies.

Determination of minimum inhibitory concentration (MIC)

The MIC values of the three PA fractions, PA_1-PA_3 obtained from F_4 were determined against two standard *S. aureus* strains (NCTC 6571 and ATCC 25923), 12 MRSA strains and 11 MSSA strains using an agar double dilution (BSAC) method (Andrews, 2001). Each of PA_1-PA_3 (40 mg) was dissolved in sterile distilled water (4.00 mL) to give the stock solutions of 10 mg/mL. A double dilution series was prepared from each stock solution by dispensing the under-mentioned amounts (2048, 1024, 512, 256, 128, 64 µL) into labelled screw-capped bottles with a micropipette to give the final concentrations of PAs (1024, 512, 256, 128, 64, 32 µg/mL). Sterilized cooled molten MHA (20 mL) at 50 ^oC was thoroughly mixed with the previously prepared dilution series in each screw-capped bottle. These were poured into sterile petri dishes (90 mm diameter) and allowed to set on a level surface. Suspenions of NCTC 6571, ATCC 25923, MRSA and MSSA that were adjusted to 0.5 MacFarland standard were spotted onto agar plates (10 µL) using a micropipette. Agar plates were incubated aerobically at 37 ^oC for 24 h.

RESULTS AND DISCUSSION

Separation of PAs using Sephadex LH-20 chromatography

The catechins and PAs present in the lipid free aqueous 70 % acetone extract of freeze dried tea leaves were separated by Sephadex LH-20 chromatography. Fractions F_3-F_4 were collected when the TLC indicated that PAs were being eluted.

Separation of PA fractions by HSCCC

Fraction F_4 having shown considerable antibacterial activity (see below), was subjected to further fractionation using HSCCC. The two phase solvent system hexane-ethyl acetate-methanol-water (1:5:1:5) used for the separation of catechins (Kumar *et al*., 2005) and PAs (Kumar *et al*., 2009) was selected for the separation of the PA extract. TLC (DMACA reagent) was used to monitor, collect and combine the PA containing fractions. Three HSCCC separations of F_4 (200 mg each) yielded the four fractions PA_1 (52 mg), PA_2 (41 mg), PA_3 (41 mg) and PA_4 (26 mg).

The PA fractions separated using HSCCC were studied by TLC and their R_f values were compared with those reported (Rohr *et al*., 2000) for oligomeric PAs, and with the R_f value (0.76) observed for an authentic sample of epigallocatechin gallate. The R_f values observed on TLC suggested the presence of tetramers in PA_1 (R_f 0.26), trimers in PA_2 (R_f 0.35), dimers in PA_3 (R_f 0.6), while PA_4 contained a monomer (R_f 0.76).

ESI-MS mass spectrometry

The stereoisomers of catechin and epicatechin cannot be distinguished during ESI-MS analysis and the notation (epi)catechin has been used in the discussion. Assignments were made based on the molecular mass of the peaks observed.

The presence of both A- and B-type PA tetramers composed of (epi)catechin, (epi)gallocatechin and (epi)afzelechin units, were indicated by the M^+ ions at m/z 1168.6, 1152.8, 1141.7, 1139.9, 1138.7, 1136.8, 1134.8, 1132.7 and 1130.9 observed in the ESI-MS spectrum of PA_1. Possible structures for A- and B-type PA tetramers are shown in Figure 3.

Although the TLC comparison of R_f values suggested that PA_2 was probably a trimeric PA fraction, M^+ ions atttributable to a trimeric PA were not observed in the ESI-MS (positive ionization mode) of PA_2. Peaks due to

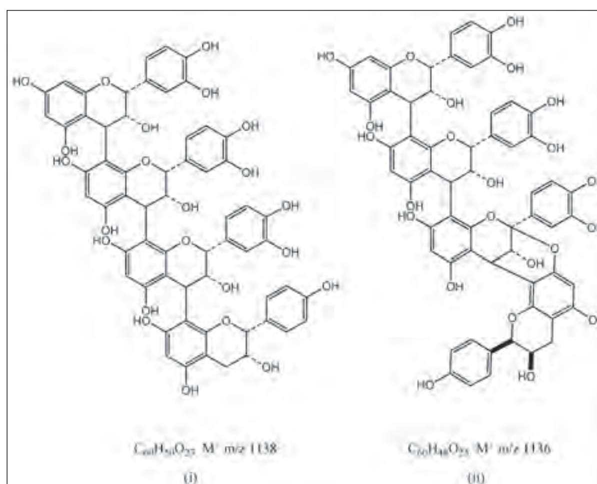

Figure 3: Proanthocyanidin structures: (i) B-type proanthocyanidin tetramer and (ii) A- type proanthocyanidin tetramer

tetramers present in PA_1, as well as sharp intense signals with a narrow signal width were observed at m/z 633.3 and 617.3. It is likely that the fragments at m/z 633.3 and 617.3 had arisen from epigallocatechin 3,5-di-*O*-gallate $[610+Na]^+$ and epicatechin 3,5-di-*O*-gallate $[594+Na]^+$ respectively, which had been isolated and characterised previously from green tea leaves (Nonaka *et al*., 1983; Kiehne *et al*., 1997; Kumar *et al.*, 2009). Therefore PA_1 was a mixture of PA tetramers and the two galloylated catechins above.

The ESI-MS of PA_3 in the negative ionization mode exhibited a cluster of ions at m/z 901.7, 882.1, 881.1 and 878.9. In the absence of an MS/MS system, the authors assigned the peaks at m/z 881.1 and 882.1 to a PA dimer (epi)catechingallate-(epi)catechin gallate, and/or (epi)afzelechin-(epi)gallocatechin gallate based on their previous isolation from a tea extract using HSCCC (Kumar *et al*., 2009).

Antibacterial activity of proanthocyanidin fractions

The results of the current investigation on susceptibility tests clearly indicated the varying levels of activity of fractions F_1-F_4 against standard strains of *S. aureus* (ATCC 25923 and NCTC 6571): F_1 was inactive against both the strains at a concentration of $\leq$ 10 µg/mL; F_2 was inactive against ATCCC 25923 at $\leq$ 10 mg µg/mL but inhibited NCTC 6571 at 6 µg/mL; F_3 inhibited ATCC 25923 and NCTC 6571 at 3 µg/mL and 1 µg/mL, respectively. F_4 was the most active of the four fractions F_1- F_4 and displayed moderate anti-staphylococcal activity against the standard strains of *S. aureus,* and the strains obtained from clinical isolates (Table 1). It is noteworthy that fraction F_1 contained only monomeric flavan-3-ols

Table 1: Diameters[a] (mm) of the inhibition zones for varying concentrations of fraction F_4 against *S. aureus* standard strains and clinical isolates in the agar well diffusion assay

F_4	Strain of *S. aureus*					
Fraction (mg/mL)	ATCC 25923	NCTC 6571	MRSA 2923	MRSA 2855	MSSA 2901	MSSA 2920
1	13	15	17	14	16	14
0.9	13	15	16	13	15	14
0.8	11	15	16	13	14	13
0.7	11	15	15	12	13	13
0.6	11	14	12	11	12	13
0.5	11	14	11	11	12	13
0.4	11	13	NI[b]	NI	11	12
0.3	NI	13	NI	NI	11	12
0.2	NI	13	ND[c]	ND	ND	ND
Water	NI	NI	NI	NI	NI	NI
Positive control [d]	15	15	15	15	15	15

[a] Diameter of the agar well was 9 mm except for positive control (5 mm)
[b] NI = No inhibition; [c] ND = Not determined
[d] Methicillin (10 µg) for MSSA and gentamicin (5 µg) for MRSA

Table 2: Minimum inhibitory concentration (MIC) (µg/mL) of proanthocyanidin fractions PA_1-PA_3 against *S. aureus* reference strains and clinical isolates of MRSA and MSSA

S. aureus strain	PA_1	PA_2	PA_3
NCTC 6571	512	512	512
ATCC 25923	512	512	512
MRSA 0038	1024	1024	512
MRSA 0044	512	512	1024
MRSA 0053	1024	1024	512
MRSA 0056	512	512	512
MRSA 0058	512	512	512
MRSA 0074	512	512	512
MRSA 0151	512	512	512
MRSA 0167	1024	512	512
MRSA 2735	512	1024	512
MRSA 2880	512	1024	1024
MRSA 2919	512	1024	1024
MRSA 3019	1024	1024	512
MSSA 0091	1024	1024	1024
MSSA 0098	1024	1024	512
MSSA 0175	1024	1024	512
MSSA 2815	1024	512	512
MSSA 2848	1024	1024	1024
MSSA 2851	1024	512	512
MSSA 2886	1024	1024	1024
MSSA 2901	512	1024	512
MSSA 2902	512	512	512
MSSA 2920	1024	1024	512
MSSA 2935	1024	1024	512

while fraction F_4 contained oligomeric PAs. This is in agreement with the observation made by Mayer *et al*. (2008), that fractions containing only monomers such as (epi)catechin did not show antibacterial activity except against *Pseudomonas aeruginosa* (ATCCC 27853).

MICs of the three PA fractions obtained after HSCCC of F_4, were determined against a wider range of MRSA and MSSA strains. The values ranged between 512–1024 µg/mL (Table 2) and indicated moderate activity. Of the three fractions, PA_3 appeared to be the most active against *S. aureus*. ESI-MS evidence suggested the dimeric nature of the PAs in this fraction. In this context it is relevant that the biological activity of PAs from grape seed extract is associated with the presence of gallate esters of dimeric and trimeric PAs (Agarwal *et al*., 2007).

The oligomeric PAs that were isolated from fresh tea leaves exhibited moderate activity against MRSA, MSSA, and the standard strains of *S. aureus*, NCTC 6571 and ATCC 25923. The MICs of the PAs that were tested during this study varied between 512 – 1024 µg/mL. However, these results may not accurately reflect the true potency of the PAs because a low rate of diffusion of the potent PAs in the agar medium may have resulted in higher MIC values, due to the fact that the rate of diffusion of PAs in the agar medium may also be influenced by the number of hydroxyl groups present in the polyphenolic molecules (Zheng *et al*., 1996).

CONCLUSION

Oligomeric proanthocyanidins, including those having tetramers with A-type linkages are found in fresh tea leaves and display moderate antibacterial activity against both MRSA and MSSA.

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