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Fractionation of a Methanolic Extract of Tea (*Camellia sinensis* (L) O Kuntze) of Himachal Pradesh, and its Evaluation for Antibacterial Activity

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ABSTRACT

A methanolic extract of made black tea was fractionated using an activated silica gel column. A solvent system with increasing polarity was used (ethyl acetate, ethyl acetate:methanol::80:20, ethyl acetate:methanol::50:50, methanol). Twelve aqueous solutions of the fractions/pooled fractions and aqueous extracts of fresh tea shoots were evaluated for antibacterial activity against 11 selected bacteria. The aqueous extracts of fresh tea shoots, and that of the first fraction eluted with ethyl acetate, were potent and their inhibitory effects were highest when compared with the fractions/pooled fractions eluted later. The antibacterial activity decreased with the order of elution, and the fractions eluted last did not exhibit antibacterial activity. *Serratia marcescens*, *Shigella dysenterae* and *Escherichia coli* (a local isolate) were totally resistant to all the extracts.

Key words: Black tea, antibacterial activity, catechin, methanol

INTRODUCTION

Apart from being one of the most affordable beverages, tea is consumed world-wide for its stimulating effect in association with caffeine. Green and black teas have been reported to contain catechins, a group of polyphenolic compounds with multifunctional activity (Inoue *et al.*, 1996; Wheeler and Wheeler, 2004; Atoui *et al.*, 2005). Green tea differs from black tea because in the former enzymatic activity is arrested by exposing fresh tea shoots to high temperature, whereas in the latter enzymatic activity continues till optimum fermentation is achieved, prior to drying, by means of a blast of hot air. Thus, whereas green tea contains catechins, black tea contains, in addition, theaflavins and thearubigins which are the oxidation products of catechins, responsible for the unique characteristics, *viz.* colour, brightness, strength, 'mouth feel', etc., of black tea liquor (Roberts, 1959; Roberts and Smith, 1963; Biswas *et al.*, 1973). Catechins and their oxidation products have been associated with a number of diverse pharmacotherapeutic properties.

Reviews on the health benefits of tea and its therapeutic value are available in literature (e.g. Stagg and Millin, 1975; Katiyar and Mukhtar, 1996; Dreosti *et al.*, 1997; Dufresne and Farnworth, 2000; Dufresne and Farnworth, 2001). The antibacterial activities of polyphenolics against several bacteria have been reported (Ozkan *et al.*, 2004; Ciraj *et al.*, 2001; Sasaki *et al.*, 2004). However, most studies on the health benefits of tea have been focused on green teas, which are rich in unoxidised flavanols, or the tea catechins, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate.

Tea (*Camellia sinensis* (L) O Kuntze) is grown in the Kangra Valley of Himachal Pradesh, India, on the gentle slopes of the outer Himalayas (elevation: 1290 m above mean sea level, latitude: 32° 13'N, longitude: 76° 56'E). Although references on seasonal variations of theaflavins, thearubugins, quality characteristics and mineral composition are available in literature (Sud and Battacharjee, 1992; Gulati and Ravindranath, 1996; Sud and Baru, 2000), there is hardly any information regarding antibacterial activity of Kangra Valley tea.

The present study was carried out to investigate antibacterial activity of Kangra Valley tea against some selected bacteria.

MATERIALS AND METHODS

Samples of orthodox tea, Tippy Golden Flowery Orange Pekoe (TGFOP-1) grade, were collected from the Palampur Cooperative Tea Factory Ltd., Palampur, Himachal Pradesh. The samples were stored in sealed polyethylene bags, which were only opened at the time of analysis and extraction. Samples of green tea shoots (two leaves and a bud), collected from the surrounding local tea gardens, were employed immediately for extraction.

(a) *Quantitative analysis*

The total polyphenols in green tea shoots, and theaflavins, thearubigins, total colour and brightness in made black tea, were estimated spectrophotometrically by the methods of Swain and Hillis (1959) and Roberts and Smith (1963), respectively. However, the values of total colour were calculated as reported earlier (Sud and Baru, 2000).

(b) Extraction of soluble solids

One hundred grammes of dried and powdered made black tea were immersed in 200 ml of dried methanol (AR) in a flat-bottom flask for 96 h, at a temperature of 27±2°C. The mixture was shaken intermittently, and methanol extract was finally collected in a separating-funnel after filtration though Whatman filter paper. The methanol extract was shaken vigorously for 20 minutes with 25 ml of petroleum ether (40-60°C). The layers were allowed to separate, and the upper petroleum-ether layer was discarded. Washing with petroleum ether was repeated six to eight times till the upper petroleum-ether layer was almost colourless, or faintly yellowish-green. The volume of the lower, dark brown viscous layer was then reduced to about one-third by vacuum distillation on a hot-water bath.

The semi-solid thus obtained was finally vacuum-dried into fluffy, bright brown crusts with a golden lustre, which had a typical tea aroma and was hygroscopic in nature. This dried methanol extract was powdered and employed in further investigations.

(c) *Isolation of fractions*

One gramme of dried methanol extract was thoroughly mixed with 5 g of activated silica gel (60-120 mesh). The silica gel was activated by heating in a hot-air oven at 110±5°C for 1 h. The mixture was vacuum-dried to remove traces of methanol. A glass column, 50 cm long and 2 cm in diameter, was fitted with a stopper and plugged with glass wool near the stopcock. It was packed with 45 g of activated silica gel (60-120 mesh). The mixture of dried methanol extract and activated silica gel was homogeneously spread over the packed silica-gel column. For elution of the column, ethyl acetate, ethyl acetate: methanol (80:20), ethyl acetate: methanol (50:50), methanol, was used, in order of increasing polarity.

A total of 27 fractions (seven eluted with ethyl acetate, nine with ethyl acetate: methanol (80:20), five with ethyl acetate: methanol (50:50), and six with methanol), each 25 to 30 ml, were collected using a column chromatographic technique (Molt and Novotny, 1979). These fractions, except fraction numbers 1, 8, 17, and 22, which had almost identical colours, were pooled into eight solutions to yield a total of 12 solutions. In Table 1 are given the code numbers (1-12) assigned to the fractions/pooled fractions separated from the dried

methanol extract, whose aqueous solutions along with an aqueous solution of caffeine (code number 13), and the aqueous extract of green tea shoots (code number 14), were used in evaluating antibacterial activity.

(d) *Preparation of aqueous solutions*

Ten millilitres of each fraction/pooled fraction was evaporated to dryness on a hot-water bath in a pre-weighed test tube. The dried material was dissolved in 5 ml of sterilized distilled water, and the solution was transferred to a sterilized screw-capped tube and stored in a refrigerator. This for used in carrying out the antibacterial activity studies.

Each of the pre-weighed tubes with left-over insoluble matter was dried again in a hot-air oven and weighed. This allowed a determination of the quantity of solid matter that had passed into aqueous solution.

The dry weights of soluble solids, and the concentrations of the aqueous solutions, are presented in Table 1.

(e) *Antibacterial activity*

Of the 11 bacterial strains used in the present evaluation, eight strains, namely *Escherichia coli* NCTC 10418, *Salmonella typhi*, *Klebsiella pneumonae*, *Serratia marcescens*, *Shigella dysenteriae*, *Acinetobacter* sp., *Staphylococcus aureus* ATCC 25923, and *Psuedomonas aeruginosa* NCTC 10662, were procured from the Department of Microbiology, Indira Gandhi Medical College, Shimla, Himachal Prodesh. Two more, *Staphylococcus albus* and another*Staphylococcus albus*, isolated locally from urine, were obtained from the Department of Microbiology, College of Basic Sciences. A *Proteus* sp. was obtained from the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, C.S.K.H.P. Krishi Vishvavidyalaya, Palampur, Himachal Pradesh.

Pure cultures of these strains were maintained on nutrient-agar slants and stored at 4-8°C. Antibacterial activity was studied using the agar-well technique (Spooner and Sykes, 1972). Fresh culture, grown in peptone water at 37±1°C for 3 h, was spread on nutrient agar plates using sterilized cotton swabs. After 10 minutes, wells of 5 mm diameter were cut out using a sterilized stainless-steel borer. The wells were then filled with 0.1 ml of aqueous solutions of

isolated fraction/pooled fraction. The plates were incubated at 37±1°C for 24 to 48 h. The antibacterial activity was evaluated by measuring the diameter of the zone of inhibition. An aqueous solution of 3 per cent phenol, and sterile phosphate-buffer saline, were used as positive and negative controls, respectively.

RESULTS AND DISCUSSION

Table 2 represents the mean monthly values (April to October) of theaflavins (TF), thearubigins (TR), total colour (TC) and brightness, in liquors of black orthodox tea of the Tippy Golden Flowery Orange Pekoe (TGFOP-1) grade, and the total polyphenols in green tea shoots, along with CV (%) and CD at 5%.

Owing to its weather-related growth-pattern, the plucking of green tea shoots is done from April to October. During the winter months (November to March), the tea bushes experience complete dormancy for about 120 to 150 days.

A perusal of Table 2 indicates that first-flush tea (April-May) was of better quality compared to the rest of the season's teas, as it had higher TF, TC and brightness content. The green tea shoots had a high total polyphenol content during April.

The results reported here are of the studies carried out with Kangra tea samples in April only.

The powdered methanol extract was hygroscopic in nature. On heating in a capillary in a paraffin bath, it started to decompose at 90±2°C, and decomposed completely into a dark-brown mass around 140°C. It was insoluble in acetonitrile, dichloromethane, and diethyl ether; partially soluble in ethyl acetate, isobutyl methyl ketone, and cold water, and completely soluble in hot water and methanol.

Of the 12 fractions/pooled fractions isolated from the powdered methanol extract (Table 1), the aqueous solutions of the fractions with code numbers 6 and 8 to 12, and caffeine in tea (code number 13), did not exhibit antibacterial activity against any of the tested microorganismS (Table 3). A perusal of Table 3 indicates that *Serratia marcescens*, *Shigella dysenterae* and *Escherichia coli* (the local isolate) were resistant to all the solutions as no zones of inhibition were observed. The local isolate of *Escherichia coli* was also found to be resistant to most of the antibiotics routinely used for urine culture sensitivity. However, this strain was sensitive to phenol which was used as a positive control in the present study.

These observations are corroborated by the earlier studies of Simonetti *et al.* (2004) who reported green tea (*Camellia sinensis*) to be inactive against *Candida albicans* and certain strains of *Escherichia coli*. However, the standard strain of *Escherichia coli*, NCTC 10148, and *Proteus* sp., were found to be sensitive to five solutions (1, 2, 4, 5, and 7), and the aqueous green tea shoot extract. These results are in conformity with those of Mahajan *et al.* (1989), who reported *Escherichia coli* NCTC 10148 to be sensitive to black tea and green tea extracts. Of the 12 coded solutions, *Staphylococcus aureus* ATCC 25923 was sensitive to five solutions (1, 2, 3, 4 and 5), whereas *Staphylococcus albus* was sensitive to four (1, 2, 4 and 7).

Sharquie *et al.* (2000) reported strong antibacterial activity of tea liquor against *Staphylococcus aureus* in patients with *impetigo contagiosa*. All these microorganisms, except *Serratia marcescens*, *Shigella dysenterae* and *Escherichia coli* (the local isolate), were sensitive to the aqueous extract of the green tea shoots (code number 14).

The pronounced antibacterial activity of the aqueous extract of green tea shoots, observed in the present studies, could be due to the catechin derivatives present in tea. A preliminary study on tea in Himachal Pradesh showed UV spectral bands that indicated the presence of caffeine, epicatechin gallate and epigallocatechin gallate in aqueous extracts of green tea shoots, and theaflavin-3'-gallate, theaflavin-3-gallate, theaflavin-3, and 3'-digallate in the aqueous solutions of methanolic fractions (code numbers 1 and 2) of made black tea. The steam-dried green tea shoots of the tea cultivar (*Camellia sinensis*), grown in Himachal Pradesh, was reported to contain a high content of epigallocatecin gallate in the range 62.01±4.52 mg/g to 68.89±2.77 mg/g, and epicatechin gallate in the range 14.30±1.15 mg/g to 19.04±0.35 mg/g. (Vasisht *et al.*, 2004) . Tea catechins with a galloyl moiety have been reported to show higher antibacterial activity than those without a galloyl moiety (Sakanaka *et al.* 1989). The pattern of antibacterial activity exhibited by the aqueous solution (code number 1) was comparable to that of the aqueous extract of green tea shoots.

Black tea contains some unoxidized catechin derivatives, which along with theaflavins, could give antibacterial activity to its extracts. Catechins are precursors of theaflavins and thearubigins which are the enzymatic oxidation products present in black tea. During storage of black tea, the initial increase of theaflavins was attributed to the oxidation of catechins that are left unoxidized during processing (Cloughley, 1981; Liyanage *et al.*, 1987). The bactericidal activity of water-ethanol extracts of oolong tea leaves was reported to originate from the synergetic effect of monomeric plyphenols (Sasaki *et al.*, 2004). However, the biological activity, and the chemistry and chemopreventive properties of Himachal Pradesh tea and its polyphenols, are not yet well-defined.

Table 1. Dry weights (g) recovered from various fractions on evaporation of solvents, concentrations (g L^{-1}) of aqueous solutions and their assigned code numbers.

Fraction/Pooled fraction	Dry weight	Concentration	Assigned code
(1) 1	20.81	37.62	1
(2) 2, 3, 4	1.62	2.64	2
(3) 5, 6, 7	0.51	0.65	3
(4) 8	2.00	2.97	4
(5) 9, 10, 11, 12	3.23	6.07	5
(6) 13, 14, 15, 16	0.24	0.21	6
(7) 17	6.55	11.65	7
(8) 18, 19	3.71	7.04	8
(9) 20, 21	1.62	0.35	9
(10) 22	1.05	0.18	10
(11) 23, 24, 25	0.90	0.18	11
(12) 26, 27	0.20	0.40	12
(13) Caffeine	–	1.43	13
(14) Aqueous fresh tea shoots' extract		ND*	14

* Not detected

Table 2. Mean monthly variations in theaflavins, thearubigins, total colour and brightness in tea liquors (TGFOP-1 grade), and in total polyphenols in green tea-shoots from Himachal Pradesh.

	Theaflavins	Thearubigins	Total colour	Brightness	
Month	Theaflavin	Thearubigin	Total Colour	Brightness	Total Polyphenols
	(μ mol g^{-1})	(mg g^{-1})	(l g^{-1} cm^{-1})	(per cent)	(per cent)
April	2.840	52.604	1.303	19.368	19.97
May	2.796	54.180	1.066	16.053	16.81
June	2.624	50.23	1.472	13.526	16.98
July	2.448	50.320	1.604	11.459	18.86
August	2.475	50.920	1.580	12.349	18.89
September	2.446	55.898	1.605	14.489	19.00
October	2.904	52.860	1.544	16.396	17.82
Mean	2.647	52.430	1.459	14.805	18.33
CV (%)	27.720	7.230	8.530	8.840	4.32
CD (5%)	0.587	0.302	0.102	1.062	0.54

Table 3. Antibacterial activity of 0.1 ml aliquots of aqueous solutions of various fractions/ pooled fractions of dried methanol extract, caffeine and green tea-shoot extracts.

Organism	Assigned code													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Status of zones of inhibition*													
Staphylococcus albus	+++	++	-	++	-	-	+	-	-	-	-	-	-	++++
Escherichia coli NCTC 10418	++	++	-	+	+	-	+	-	-	-	-	-	-	+++
Proteus sp	++	++	-	+	+	-	+	-	-	-	-	-	-	+++
Salmonella typhi	+++	-	-	-	-	-	-	-	-	-	-	-	-	+++
Klebsiella pneumonae	+++	-	-	-	-	-	-	-	-	-	-	-	-	+++
Serratia marcescens	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Shigella dysenteriae	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	++	++	-	-	-	-	-	-	-	-	-	-	-	++
Staphylococcus aureus ATCC 25923	++	++	+	+	+	-	-	-	-	-	-	-	-	++
Psuedomonas aeruginosa NCTC 10662	++	++	-	-	-	-	-	-	-	-	-	-	-	++
Escherichia coli (local isolate)	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*Zones of Inhibition: (-) no zone (+) 7-10 mm (++) 10-15 mm (+++) 15-20 mm (++++) 20-25 mm

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