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In vitro antibacterial activity of Sri Lankan orthodox black tea (*Camellia sinensis* L.) belonging to different agro-climatic elevations

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ABSTRACT

Objective: To investigate the antibacterial properties of three grades of orthodox Sri Lankan black tea belonging to the three agro-climatic elevations.

Methods: Methanolic extracts of orange pekoe (OP), broken orange pekoe fannings (BOPF) and Dust No. 1 belonging to three agro-climatic elevations (low, mid and high grown) were made and tested *in vitro* (concentration: 300 µg/disc) against Gram-positive pathogenic bacteria, *Staphylococcus aureus* (ATCC 25923) (*S. aureus*) and *Bacillus cereus* (ATCC 11778) (*B. cereus*), and two Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 9027) (*P. aeruginosa*) and *Escherichia coli* (ATCC 35218) (*E. coli*), using agar disc diffusion assay. Gentamycin (10 µg/disc) was used as the positive control and methanol as the negative control. Minimum inhibitory concentration (MIC) values were evaluated, using micro dilution method.

Results: None of the tea extracts exerted an antibacterial action against *P. aeruginosa* and *E. coli*. In contrast mild to moderate antibacterial activity was exerted against *S. aureus* and *B. cereus*. Further gentamycin exhibited strong antibacterial activity against all the four bacterial species. Further low MIC values were evident for tea samples against the two Gram-positive bacteria. The order of anti-bacterial activity for tea extracts was Dust No. 1 > BOPF > OP.

Conclusions: It is concluded that Sri Lankan orthodox black tea belonging to Dust No. 1, BOPF, and OP possess *in vitro* antibacterial activity against *S. aureus* and *B. cereus* but not against Gram-positive bacteria *P. aeruginosa* and *E. coli*.

1. Introduction

Tea is the most widely drunk beverage besides water[1]. It is estimated that 3.5 billion cups are consumed daily at present[1] with a per capita consumption of 2.52–3.16 kg/person per year[2]. Tea is manufactured from freshly harvested top most immature leaves and unopened buds of an evergreen plant, *Camellia sinensis* (L.) O. Kuntz (*C. sinensis*) (Family: Theaceae) without any additives or preservations in over 25 countries including Sri Lanka[1]. Based on the manufacturing technique there are three main types of tea: black, green, or oolong[1]. Black tea accounts for 78% of

world tea production and about 80% of global tea consumption[1]. Currently, Sri Lanka is the second main black tea exporter and the main orthodox black tea exporter[3]. In Sri Lanka, black tea is manufactured by two mechanisms, orthodox (93%) and crush-tear-curl (7%)[3].

Several studies have shown that black tea has a wide range of beneficial physiological and pharmacological effects[1], including antibacterial activity both *in vivo*[4,5] and *in vitro*[5-12]. In most of these investigations, the origin of the tea sample, the grade of the tea sample, the nature of the tea sample (blend or factory fresh), manufacturing technique or agro-climatic elevation are not specified[5,8,10,11,13]. This is a big limitation since pharmacotherapeutic potential is shown to vary with many factors including country of origin, agro-climatic elevation, processing technique, rainfall, genetic make-up of the plant, method, particle size and grade of tea[14-17]. In this regard, giving these important aforementioned details[14-17], we have shown that orthodox black tea has antioxidant[18-20], anti-clotting[21,22], thrombolytic[23,24],

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anti-hyaluronidase[25], anti-elastase[26], anti-tyrosinase[27], sun screening[28], membrane stabilizing, anti-glycation and cross-link braking[29], cholesterol micellization inhibitory[30], anti-amylase[31], anti-rheumatoid arthritic[32] activities *in vitro* and diuretic[33,34], anti-diarrhoeal[35], anti-inflammatory[36], gastro protective[37], gastric ulcer healing[38], aphrodisiac[39], anti-pyretic[40], anti-nociceptive[41], anxiolytic[42], hepatoprotective[43], hypoglycaemic[44,45], anti-hyperglycaemic[45], and anti-diabetic[44,45] activities *in vivo*.

However, as of now, studies on antibacterial activity of Sri Lankan black tea, especially orthodox black tea are extremely scarce[10-12]. Moreover, unfortunately, these anti-bacterial studies have been reported in abstract form and do not appear to have been followed up. Hence, the aim of this study was to investigate the antibacterial effects of three grades of Sri Lankan black tea manufactured by orthodox technique, namely, Dust No. 1, broken orange pekoe fannings (BOPF) and orange pekoe (OP) belonging to the three agro-climatic elevations [low grown: below 600 m above mean sea level (AMSL), mid grown: between 600–1 200 m AMSL, and high grown: above 1 200 m AMSL][1,13]. This was assessed with methanolic extracts using agar disc diffusion bioassay.

2. Materials and methods

2.1. Sample collection

Black tea samples used in this study (Dust No. 1, BOPF and OP) were manufactured using orthodox rotarvane technique with the top most immature leaves and unopened buds of *C. sinensis* plant harvested during August 2015 in three tea factories belonging to three agro-climatic elevations: Mattakalle tea factory, Tallawakelle [1 382 m AMSL; high grown, latitude 6°55'59" N, longitude 80°39'00" E], Kirimatiya tea factory, Gampola [600 m AMSL; mid grown, latitude 7°9'36" N, longitude 80°34'12" E] and Kottawally, Galle [0 m AMSL; low grown, latitude 6°2'24" N, longitude 80°13'12" E], Sri Lanka. Tea samples were packed in triplicate laminated aluminum foil bags and stored at –20 °C until use.

2.2. Sieve analysis

The composition of “true to size particles” defined for each grade of tea samples was determined in triplicate using a sieve shaker with standard set of sieves (shaking time: 10 min and shaking speed: 50 vibrations/min) as described by Samaraweera *et al.*[46].

2.3. Organoleptic profile analysis

Typical characters (leaf characteristics, infused leaf characteristics, and liquor characteristics) of each grade of tea samples belonging to the three agro-climatic elevations were organoleptically evaluated by professional tea tasters attached to Sri Lanka Tea Board, Colpitty, Sri Lanka.

2.4. Preparation of methanolic tea extracts

Five grams of each grade of tea belonging to the three agro-climatic elevations were separately extracted into 100 mL of 100% methanol, continuously for 5 days with sonication at room temperature (30–32 °C) ($n = 9$). The extracts were filtered through

Whatman No. 1 filter paper and the filtrates were evaporated to dryness under reduced pressure in a rotary evaporator.

2.5. *In vitro* antibacterial assay

Each of the prepared methanolic extracts of tea samples ($n = 9$) were evaluated for antibacterial activity against two Gram-positive pathogenic bacteria, *Staphylococcus aureus* (ATCC 25923) (*S. aureus*) and *Bacillus cereus* (ATCC 11778) (*B. cereus*), and two Gram-negative pathogenic bacteria, *Pseudomonas aeruginosa* (ATCC 9027) (*P. aeruginosa*) and *Escherichia coli* (ATCC 35218) (*E. coli*) using standard agar disc diffusion assay as described by Clinical and Laboratory Standard Institute[47]. Briefly, inoculums of different pathogenic bacteria were evenly seeded using a sterile cotton wool onto Petri dishes (diameter: 90 mm) containing preset Mueller–Hinton agar. Sterilized blank discs (diameter: 90 mm) were loaded with different methanolic extracts of tea samples (concentration: 300 µg/disc) using a micropipette and were firmly placed onto the inoculated agar plates. Gentamycin (10 µg/disc) was used as the positive control and methanol was used as the negative control. These Petri dishes were then incubated aerobically at 37 °C for 24 h. At the end of the incubation period, the diameter of the transparent inhibition zone around each disc was measured (in mm) using a pair of vernier calipers. All experiments were conducted in triplicate. A tea sample was considered to have an effective antibacterial action if the inhibition growth zone around the disc was equal or greater than 8 mm.

2.6. Evaluation of minimum inhibitory concentration (MIC)

Methanolic tea extracts that exhibited an effective antibacterial action against *S. aureus* and *B. cereus* were subjected to the determination of MIC using broth micro-dilution method with slight modifications and using Mueller–Hinton broth as the medium (NCCLS 2002) as described by Clinical and Laboratory Standard Institute[47]. Bacterial culture concentration inoculated was 0.5 McFarland standards ($\sim 1.0 \times 10^8$ CFU/mL). The initial concentration of methanolic tea extracts used was 256 µg/mL, which was serially diluted. Polymyxin B and rifamycin were used as positive controls for *B. cereus* and *S. aureus* respectively (concentration series used: 2.0–0.004 µg/mL). MIC was considered as the lowest concentration of the tea extracts or positive control which inhibited > 90% bacterial growth when absorbance was determined by a DTX plate reader at 600 nm.

2.7. Statistical analysis

Data were expressed as mean inhibition zone diameter \pm SEM. Statistical comparisons, where possible, were made using non-parametric Kruskal–Wallis test. Significance was set at $P < 0.05$.

3. Results

3.1. Sieve analysis

Sieve analysis showed that more than 80% of tea particles were in the size range specified for each grade: Dust No. 1, 300–500 µm; BOPF, 500–800 µm; and OP, 2000–4000 µm.

3.2. Organoleptic profile

Organoleptic evaluation by professional tea tasters revealed that their leaf characteristics, infusion leaf characteristics and liquor characteristics can be accepted as typical to each grade and their agro-climatic elevations.

3.3. Assessment of *in vitro* antibacterial activity

As shown in Table 1, none of the black tea extracts (Dust No. 1, BOPF, and OP) belonging to the three agro-climatic elevations had any antibacterial activity (in terms of the diameter of zone of inhibition) against *E. coli* and *P. aeruginosa* (Gram-negative). In contrast, all the tea extracts showed mild to moderate antibacterial activity against Gram-positive bacteria, *S. aureus* [(7.0–10.8) mm] and *B. cereus* [(7.6–11.3) mm]. Against *S. aureus*, the highest antibacterial activity was exhibited by high grown Dust No. 1 [(10.8 ± 0.3) mm] followed by high grown BOPF [(10.5 ± 1.3) mm] and low grown Dust No. 1 [(9.6 ± 0.1) mm], whilst the lowest antibacterial activity was shown by mid grown OP [(7.0 ± 0.2) mm]. Positive control drug, gentamycin displayed significant ($P < 0.05$) and a remarkable antibacterial activity [(16.1 ± 1.0) mm].

With respect to *B. cereus*, the highest antibacterial activity was evident with high grown Dust No. 1 [(11.3 ± 0.8) mm] followed by mid grown Dust No. 1 [(10.8 ± 0.5) mm] and mid grown OP [(10.3 ± 1.6) mm]. Of all black tea extracts, the least antibacterial activity was exhibited by low grown BOPF [(7.6 ± 0.7) mm]. Conversely, positive control drug, gentamycin exhibited significant ($P < 0.05$) and outstanding antibacterial activity [(18.6 ± 0.8) mm] whilst negative control agent, methanol had no antibacterial activity.

Table 1

In vitro antibacterial activity of different grades of Sri Lankan black tea belonging to the three agro-climatic elevations against *S. aureus*, *B. cereus*, *P. aeruginosa* and *E. coli*.

Black tea grade	Antibacterial activity (mm)				
	<i>S. aureus</i>	<i>B. cereus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>	
High grown	OP	8.0 ± 0.2	9.0 ± 0.3	–	–
	BOPF	10.5 ± 1.3	9.0 ± 0.6	–	–
	Dust No. 1	10.8 ± 0.3	11.3 ± 0.8	–	–
Mid grown	OP	7.0 ± 0.2	10.3 ± 1.6	–	–
	BOPF	9.0 ± 0.4	9.1 ± 0.1	–	–
	Dust No. 1	9.4 ± 0.2	10.8 ± 0.5	–	–
Low grown	OP	7.3 ± 0.4	8.2 ± 0.9	–	–
	BOPF	8.1 ± 0.2	7.6 ± 0.7	–	–
	Dust No. 1	9.6 ± 0.1	8.8 ± 0.5	–	–
Gentamycin (positive control)		16.1 ± 1.0	18.6 ± 0.8	14.6 ± 2.1	15.4 ± 1.5
Methanol (negative control)		–	–	–	–

–: No activity. Data are expressed as mean ± SEM.

MIC values of different black tea extracts, belonging to the three agro-climatic elevations against *S. aureus* and *B. cereus* are depicted in Table 2. The lowest MIC value of 64 µg/mL against *S. aureus* was shown by high grown Dust No. 1, high grown BOPF and low grown Dust No. 1, whilst intermediate MIC value of 128 µg/mL was exhibited by mid grown Dust No. 1, mid grown BOPF and high grown OP. On the other hand, the highest MIC value of 256 µg/mL was shown by low grown BOPF. The positive control drug rifamycin displayed an extremely low MIC value of 0.125 µg/mL.

With respect to *B. cereus* lowest MIC value (16 µg/mL) was evident with high grown Dust No. 1 whilst intermediate MIC value of 32 µg/mL was shown by high grown OP and BOPF and mid grown OP and Dust No. 1. The highest MIC value of 64 µg/mL was observed with mid grown BOPF and low grown OP, BOPF and Dust No. 1. Positive control drug, polymyxin B had a very low MIC value of 2 µg/mL.

Table 2

MIC values of different grades of Sri Lankan black tea belonging to the three agro-climatic elevations against *S. aureus* and *B. cereus*.

Black tea grade	MIC (µg/mL)		
	<i>S. aureus</i>	<i>B. cereus</i>	
High grown	OP	128	32
	BOPF	64	32
	Dust No. 1	64	16
Mid grown	OP	–	32
	BOPF	128	64
	Dust No. 1	128	32
Low grown	OP	–	64
	BOPF	256	64
	Dust No. 1	64	64
Positive control		0.125	2
Negative control (methanol)		–	–

–: No activity. Positive control: Polymyxin B for *B. cereus* and rifamycin for *S. aureus*.

4. Discussion

Limited numbers of studies, some of which are superficial and fragmentary have shown antibacterial properties of black tea against a range of bacteria at strengths/concentrations to those found in a standard cup of tea. Further, most of these studies have many limitations that were described in the introduction section[5,8,9,11,12].

Taking into account these points, this study evaluated *in vitro* antibacterial properties of three grades of unblended Sri Lankan black tea (BOPF, OP, and Dust No. 1) manufactured by orthodox rotarvane technique, belonging to the three agro-climatic elevations (high, mid, and low grown). Furthermore, the tea samples tested were typical and representative to the grade agro-climatic elevations (in terms of sieve analysis, appearance and organoleptic properties). The antibacterial testing was done using a methanolic extract which is considered as the best in the evaluation of plant extracts *in vitro*[7,8]. Four commonly used pathogenic bacterial species, two Gram-positive (*S. aureus* and *B. cereus*) and two Gram-negative (*P. aeruginosa* and *E. coli*) were used.

The results convincingly showed, under our experimental conditions, irrespective of the grade and agro-climatic elevation, none of the tea extract tested was effective against Gram-negative bacteria, *P. aeruginosa* and *E. coli*. Nevertheless it is desirable to extend these studies against other Gram-negative pathogenic bacteria. This is a novel finding for orthodox Sri Lankan black tea. However, others have shown antibacterial activity against *E. coli*[8,48] and *P. aeruginosa*[7]. These differences may be due to variations of strains of bacteria, the source of tea, the manner in which *C. sinensis* leaves were processed following harvest, grade of tea, concentration/strength, solvent used for extraction, and the extraction time employed.

In contrast, all the tea extracts tested showed mild to moderate

antibacterial activity against two species of Gram-positive bacteria, *S. aureus* and *B. cereus* with low MIC values 64–128 µg/mL for *S. aureus* and 16–32 µg/mL for *B. cereus*. Further, overall, grade wise the order of potency of ranking for antibacterial against *S. aureus* was Dust No. 1 > BOPF > OP whilst for *B. cereus* it was Dust No. 1 > OP > BOPF. Agro-climatic elevation wise rank order of potency against *S. aureus* was high grown > low grown > mid grown for Dust No. 1 and OP and high grown > mid grown > low grown for BOPF. Conversely, a different trend of potency ranking with respect to agro-climatic elevations against *B. aureus* was mid grown > high grown > low grown for BOPF and OP and high grown > mid grown > low grown for Dust No. 1. These trends show that severity of the antibacterial action differs according to the grade and agro-climatic elevation. Interestingly, this is the first study, globally, to show such differences in potency of antibacterial activity in any grade of black tea (garden fresh or blended) manufactured either with orthodox or crush-tear-curl technique, although others have reported antibacterial activity of black tea against a range of Gram-positive bacteria where the important information are not indicated[5,6-10]. However, in agreement with the results of this study, we have shown previously that potencies of some bioactivities of Sri Lankan orthodox black tea vary with the grade and agro-climatic elevation, including diuretic activity[33,34], blood glucose regulatory activity[44,45], blood anti-clotting activity[21,22], thrombolytic activity[23,24], anti-oxidative activity[18-20], and anti-rheumatoid activity[32]. Thus, the results of this study together with our previous studies[18-24] suggest that in order to get maximum benefit from a particular bioactivity of Sri Lankan black tea, the grade and agro-climatic elevation should be carefully selected.

Several studies have shown that antibacterial activity of black tea is due to polyphenols (tannins), catechins, theaflavins and thearubigins[1,5,6,8]. We have shown that all the three grades used here have these phytoconstituents but in varying proportions[19, 20,45]. Thus, the antibacterial action of these teas can be attributed to these phytoconstituents and the differences in the potencies can be ascribed to the differences on their phytoconstitutional profile. Lack of any antibacterial activity against Gram-negative bacteria in this study may be a general phenomenon seen with antibacterial agents; in general Gram-negative are less susceptible to plant extracts including tea extracts and antibiotics[5-7,9,10] as their outer membrane of lipoprotein and lipopolysaccharides is able to regulate the access of antibacterial agents into underlying structures[6]. Currently, it is not possible to pinpoint the exact mechanism of action/s of the black tea induced antibacterial action in this study, but may be attributed to inhibition of extracellular microbial enzymes, deprivation of substrates required for microbial growth, and inhibition of phosphorelation as claimed for tea tannings[6].

Based on the results of this study, it is concluded that Sri Lankan orthodox BOPF, Dust No. 1 and OP grades of black tea possess selective antibacterial activity against Gram-positive bacterial species and may have the potential to be used as a safe supplementary beverage during antibacterial therapy.

Conflict of interest statement

We declare that we have no conflict of interest.

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