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Research Article

Comparative Analysis of the Antibacterial Activity of Sri Lankan Black Tea from Different Geographical Areas

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Abstract

Tea is one of the most ancient and popular therapeutic beverages consumed by people all over the world. It is made from the leaves and buds of the plant "*Camellia sinensis*". Tea is cultivated in more than thirty countries around the world as a plantation crop. In the present study, tea infusions from twenty black tea samples belonging to low (Dust (I), BOPF, BOPI, OPI, Pekoe), mid (Dust (I), Dust, BOPF Local, BOPI, BOP, OPI, Pekoe) and up (Dust, BOPF, BOP, FBOP, FBOPI, OPA, OPI, Pekoe) country of Sri Lanka were tested by microdilution assay for antibacterial activities against *Escherichia coli*, Methicillin-resistant *S. aureus* (MRSA) and *M. smegmatis* (MS). The results were statistically analyzed using ANOVA. None of the tea infusions showed antibacterial activity against gram-negative bacterium *E. coli* even at the highest concentration of tea infusion. All the tea samples had antibacterial activity against gram-positive bacteria *M. smegmatis* and *S. aureus*. *S. aureus* was more sensitive to all the tested tea samples than *M. smegmatis*. Samples with the highest and the lowest antibacterial activity against *M. smegmatis* were: low-country Dust (I) (MIC- 1.24 mg/ml) and mid-country BOPF (MIC- 2.78 mg/ml) respectively, while the highest and the lowest antibacterial activity against *S. aureus* were; mid-country BOP (MIC- 0.38 mg/ml) and low-country BOPF (MIC- 1.72 mg/ml) respectively. Agro-climatic elevation of the tea sample affected antibacterial activity of black tea. Based on the results of this study, it is concluded that Sri Lankan black tea possesses selective antibacterial activity against Gram-positive bacterial species and Sri Lankan black tea may have the potential to be used as a safe supplementary beverage during antibacterial therapy.

Keywords: *Black Tea; Microdilution Assay; Upcountry; Mid-Country; Low Country*

Introduction

Tea produced from dried leaves of *Camellia sinesis* [1] is the most widely consumed and the cheapest [2,3] nonalcoholic beverage in the world, after water [1,4]. Medicinal properties of tea such as antibacterial activities against many pathogens such as *Staphylococcus aureus, Vibrio parahemolyticus, Clostridium perfringens, Bacillus cereus, Pleisomonas shigelloides,* etc. antioxidant and anticancer activities have been widely explored [2,5-12]. Human studies suggest that tea may contribute to a reduction in the risk of cardiovascular disease and some forms of cancer, as well as to the promotion of oral health [1]. Depending on the treatment of the harvested leaf teas are classified into black (fermented), green (non-fermented), and oolong (semi-fermented) teas [1,2,13-15]. The chemical composition, flavour, aroma, quality characteristics of different types of tea varies widely [16,17].

The chemical composition of tea has been widely studied where about one-third is contributed by polyphenols on a dry weight basis [16,18-21]. Due to the oxidation of phenolic compounds during the fermentation process, black tea contains multimeric polyphenols thearubigins, and theaflavins [2,15,21,22].

The tea industry is the main source of agricultural foreign exchange in Sri Lanka [23]. Ceylon tea stands for the cleanest and best quality tea as Sri Lanka is the first country in the world to be awarded the ozone-friendly status and it has the lowest pesticide residue of all the teas in the world [24]. Sri Lanka exports approximately 95% of its production [24] and has been able to maintain its global export share at 15% in 2008 [23]. The tea sector contributed 1.2% of GDP (Tea growth rates by economic activity for 2018 Q1 - 10.6%) in 2018 [23]. The product range that is exported by Sri Lanka includes black tea, green tea, organic tea, instant tea, flavored tea, and ready-to-drink (RTD) tea products [25].

Infectious diseases have become a crisis as a major cause of human and animal mortality and morbidity [26] which accounts for one-third of human deaths worldwide [27]. Even though several new antibiotics have been produced in the last three decades, bacterial infections have become a global problem, due to the rapid development of multi-drug resistance [28-31] limited antimicrobial spectrum, and adverse effects of available antimicrobial agents including hypersensitivity, immune-suppression and allergic reactions [32,33]. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs that are utilized as therapeutic agents, and prolonged treatment may cause toxic conditions [26].

The use of herbs in the treatment of diseases of man and animals is gaining popularity at present and has also been practiced in ancient times [34,35]. Antimicrobials of plant origin have enormous therapeutic potential as they are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials [7-10,36]. Studies have been conducted on the possibility of using plant extracts including tea extracts in treating diseases caused by microbial strains resistant to antibiotics [30,37-39]. Although Sri Lanka produces black tea from three different geographical areas, comparative analysis of the antibacterial activity of Sri Lankan black tea of different geographical areas in the country has not been carried out so far.

Objective of the Study

The objective of the present study was to test the antibacterial activity of Sri Lankan black tea infusions from three different geographical areas. As a gram-negative bacterium, *E. coli* was used. *S. aureus* and *M. smegmatis* were used as gram-positive bacteria. These bacteria cause infectious diseases in humans. As a model organism for *Mycobacterium tuberculosis, M. smegmatis* was used. Antibacterial activity was tested using microdilution antibacterial assay.

Materials and Methods

Tea samples

Twenty black tea samples of (Dust, Dust (I), Broken Orange Pekoe Fanning's (BOPF), Broken Orange Pekoe (BOP), Broken Orange Pekoe I (BOPI), Flowery Broken Orange Pekoe (FBOP), Flowery Broken Orange Pekoe I (FBOPI), Orange Pekoe A (OPA), Orange Pekoe I (OPI) and Pekoe) belonging to different geographical areas; up-St. Coombs estate, Thalawakelle; above 1,200m, mid- St Joachim estate, Ratnapura; 600 - 1200m and low, Deniyaya tea estate; between sea level and 600m obtained from Tea Research Institute, Thalawakelle, Sri Lanka. Tea infusions were freshly prepared, double concentration by adding 2g of dry tea sample into 100 ml of boiled water, filtered then cooled to room temperature and used for the assay. The dry matter content of tea infusions has measured using the oven method (at 110 ± 5°C temperatures for constant mass) [40]. Triplicates of infusion were prepared for each sample.

Bacterial strains

For antibacterial assays, Gram (+) MRSA, Gram (+) acid-fast *M. Smegmatis,* and Gram (-) *E. coli* were selected. *S. aureus* was grown under aerobic conditions with constant agitation at 37°C for 18 hrs in Luria broth (LB) [25g of LB in 1L of distilled water]. Autoclaved at 121°C and 15 psi above atmospheric pressure for 15 mins to obtain an inoculum containing cultures in the exponential growth phase

to obtain approximately 2 x 10^8 CFU/ml absorbance at a wavelength of 600 nm (OD_{600}). *E. coli* was also grown at 37°C in LB broth under the same conditions. *M. smegmatis* was grown at 37°C in Middlebrook broth [Sterilized Middlebrook 7H9 broth + 0.5% glycerol and 10% OADC enrichment] for 36 hrs with constant agitation. Bacterial cells were collected by centrifugation at 5000 rpm for 10 min and re-suspended in a phosphate buffer. These cultures were serially diluted (dilution factor - 10^{-1}) in the same buffer to obtain 10^6 CFU/ml of bacteria and used for the antibacterial assay.

Antibacterial assays using microdilution

The minimum inhibitory concentration (MIC) of each tea brew was determined using the broth microdilution colorimetric assay at the botany, university of Ruhuna [41]. The main modification from the reference method was the addition of a resazurin indicator after incubation of the plates instead of adding it before incubation [41].

The microtiter plates containing eight rows and twelve columns, with 96 wells were prepared for the assay under aseptic conditions. An amount of 100 µl of tea brews (2g of tea/100 ml boiled water) was pipetted into all wells in the first row. To all wells in other rows, 50 µl of sterilized distilled water was added. Serial dilutions were performed in the microtiter plate from the first to last row in each column (in serially descending concentrations) using a micropipette to make 50 µl of the tea brew in each well. The excess volume in the last row was discarded.

Forty microliters of 2.5x strength LB broth were added to each well to ensure that the final concentration of nutrient broth was single strength. The bacterial suspension was added to each well to achieve a concentration of 5×10^5 cfu/ml in the well. Then the final tea infusion concentration of each well in the first raw was 2g/200ml. Amoxicillin (5×10^{-4} mg/ml in the first cell in the column and two-fold serial dilutions until 3.906×10^{-6} mg/ml) and distilled water was used as positive and negative controls respectively in each plate. As a sterility control, one tea brew was used without bacteria. Sealed plates were incubated for 18 hours at 37°C and then 0.25 mg/ml resazurin solution as a bacterial growth indicator was added to all microplate wells. The plates were incubated further for 30 minutes. The colour change was then observed visually. Any colour changes from purple/blue to pink/colourless were recorded as positive. The lowest tea concentration (using the dry matter content of tea each infusion) at which colour change occurred was taken as the MIC value which was the MIC for the test material and bacterial strain (Figure 1).

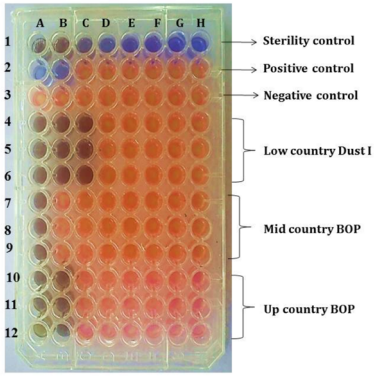

Figure 1: *A microtiter plate used for microdilution resazurin assay, after 24h incubation of bacteria. (Pink colour indicates growth and blue colour means inhibition of growth; the test organism was S. aureus. 1,2,3,4,5,6,7,8,9- tea brew + broth + resazurin indicator + bacteria in two-fold serial dilution along the columns from top to bottom. 10- Control (without tea brew)-bacteria + broth + resazurin indicator. 11-Positive control; Amoxicillin (5×10-4 mg/ml in sterilized distilled water) + broth + resazurin indicator + bacteria in serial dilution. 12- sterility control (no bacteria) - tested tea brew in serial dilution + broth + resazurin indicator).*

Statistical analysis

Determination of MIC values was carried out with nine replicates and the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using the SPSS software. The differences between means of each category of black tea were analyzed separately by the ANOVA test and then Duncan's multiple range tests ($p < 0.05$) to identify the best samples of each category.

Results

Antibacterial activity

None of the tea infusions showed antibacterial activity against *E. coli* even with the highest concentration (2g tea powder in 100 ml boiled water/2) of tea infusion. Results of the broth microdilution assay of black tea samples against *M. smegmatis* had antibacterial activity for all the samples. Samples with the highest and the lowest antibacterial activity against *M. smegmatis* were low country Dust (I) (MIC - 1.24 mg/ ml) and mid-country BOPF (MIC - 2.78 mg/ml) respectively (Table 1). According to the results of the microdilution assay, all the black tea samples tested had antibacterial activity against *S. aureus*. Samples with the highest and the lowest antibacterial activity against *S. aureus* were mid-country BOP (MIC - 0.38 mg/ ml) and low-country BOPF (MIC - 1.72 mg/ml) respectively (Table 1). All tea samples showed strong antibacterial activity against *S. aureus* than *M. smegmatis*.

Tea sub category	Grading	Sample	Broth microdilution MIC values (mg/ml)	
			M. Smegmatis	**MRSA**
Low country (LB)	Dust	Dust (I)	1.24 ± 0.64^{g}	0.70 ± 0.28^{defg}
	Fanning's	BOPF	1.95 ± 0.32^{bcdefg}	1.72 ± 0.49^{a}
	Broken leaf	BOPI	2.05 ± 0.72^{abcdef}	1.03 ± 0.36^{bcd}
	Whole leaf	OPI	2.24 ± 0.63^{abcde}	0.82 ± 0.28^{cdef}
		Pekoe	1.93 ± 0.66^{bcdefg}	0.96 ± 0.33^{cde}
Mid country (MB)	Dust	Dust (I)	1.64 ± 0.85^{efg}	0.86 ± 0.24^{cdef}
		Dust	1.68 ± 0.48^{defg}	0.63 ± 0.18^{efg}
	Fanning's	BOPF Local	2.78 ± 0.89^{a}	0.94 ± 0.33^{cde}
	Broken leaf	BOPI	2.48 ± 0.58^{abc}	0.85 ± 0.29^{cdef}
		BOP	1.43 ± 0.40^{fg}	0.38 ± 0.09^{g}
	Whole leaf	OPI	2.04 ± 0.70^{bcdef}	0.78 ± 0.36^{def}
		Pekoe	1.73 ± 0.80^{cdefg}	0.65 ± 0.18^{efg}
Up country (UB)	Dust	Dust	2.65 ± 0.75^{ab}	1.15 ± 0.39^{bc}
	Fanning's	BOPF	1.92 ± 0.68^{bcdefg}	0.56 ± 0.18^{fg}
	Broken leaf	BOP	2.45 ± 0.83^{abcd}	1.33 ± 0.47^{b}
		FBOP	2.33 ± 0.75^{abcde}	0.83 ± 0.24^{cdef}
		FBOPI	2.00 ± 0.47^{bcdef}	0.94 ± 0.27^{cde}
	Whole leaf	OPA	1.42 ± 0.24^{fg}	0.75 ± 0.00^{def}
		OPI	2.13 ± 0.35^{abcdef}	0.94 ± 0.27^{cde}
		Pekoe	2.00 ± 0.47^{bcdef}	0.88 ± 0.28^{cdef}

Table 1: *MIC values of broth microdilution assays of black tea grades against M. smegmatis and S. aureus. Data are average MIC value of each tea sample ± SD. n= 9. Different letters indicate significant differences ($p \leq 0.05$) between different tea samples. Bold letters are for the best tea samples in each column.*

Discussion and Conclusion

Tea is the most popular beverage in the world and it is consumed frequently by many people all over the world. Turkey, which is maintained the position at the first place among the main Sri Lankan tea export destinations has the largest per capita consumption of tea in the world, is 3.14 kgs per person per year [42,43]. It has been identified as a health-promoting beverage by many researchers and tea can enhance the health of consumers [5,22,23,44,45]. Several studies have shown that tea has a wide range of beneficial physiological and pharmacological effects [23,46] including antibacterial activity both *in vivo* [47-49] and *in vitro* [23,47,49-56]. Although differences among the activities of black and green tea are included in some studies [2,7,47,57] in most of these, the origin, the grade, manufacturing technique, or agro-climatic elevation is not specified [49,53,55]. Only a few studies on the antibacterial activities of Sri Lankan black tea from different geographical areas against different microorganisms have been carried out before [58]. This is a major limitation since the pharmaco-therapeutic potential is shown to vary with many factors, including country of origin, agro-climatic elevation, processing technique, rainfall, the genetic make-up of the plant, particle size, and grade of tea [59-63].

Therefore, this study evaluated *in vitro* antibacterial properties of twenty Sri Lankan black teas manufactured by the orthodox production method, belonging to the three agro-climatic elevations (high, mid, and low grown). All tea infusions were used for the preliminary *in vitro* antibacterial assays to find an activity against *M. smegmatis*, methicillin-resistant *S. aureus* (MRSA), and *E. coli* strains.

The broth microdilution assay is a simple, rapid, efficient, reliable, sensitive, safe and cost-effective method of determining MIC values of the samples against microorganisms.

E. coli was resistant to all tea samples according to the results. A previous study also found that methanol extracts of black tea samples of Sri Lanka did not show any antibacterial activity against gram-negative bacteria, *P. aeruginosa* and *E. coli* [58]. However, some researchers have reported the antibacterial activity of tea against *E. coli* [2,53,64]. These differences may be due to variations of strains of bacteria, the source of tea, how *C. sinensis* leaves were processed following harvest, grade of tea, concentration/strength, the solvent used for extraction, and the extraction time employed.

According to the results of the present study, almost all the tea samples had mild to moderate antibacterial activity against two strains of gram-positive bacteria, *M. smegmatis* and *S. aureus*. In agreement with this study, [58] also have found that the methanol extracts of black tea samples of low, mid, and upcountry of Sri Lanka possess antibacterial activities against gram-positive bacteria, *S. aureus* and *Bacillus cereus* [65] have found that Sri Lankan black tea could inhibit seven strains of *Helicobacter pylori*.

Results of the present study are in agreement with previous studies [5,6,58,66] showing that Gram-positive bacteria were more susceptible to the tea extracts than gram-negative bacteria. High resistance of Gram-negative bacterium, *E. coli* may be due to the presence of thick cell walls composed of lipoprotein and lipopolysaccharides in their outer membranes [58,66]. According to the results of previous studies, Gram-negative bacteria are less susceptible to plant extracts including tea extracts and antibiotics [49,51,52,54,55,58].

Both of the gram-positive bacteria used in the study have shown sensitivity to tea infusions at different levels. *S. aureus* was more sensitive to all the tea samples than *M. smegmatis*. Previous findings have identified that *S. aureus* is more sensitive to tea extracts than *B. cereus* and *E. coli* [66]. According to other findings *S. aureus* was more sensitive to the extracts of *Indigofera lupatana* and *Passiflora foetida* than *Klebsiella pneumonia, P. aeruginosa, Proteus mirabilis, Salmonella typhimurium* and *Salmonella typhi* [67,68]. Also, the literature indicates that terpenoids and phenolic compounds show most of the antibacterial activities, and *S. aureus* is specifically more susceptible to phenolic compounds [69,70]. Hence, the high phenolic content of tea may be the reason for the high antibacterial activity against *S. aureus*. Antibacterial activity was higher against *S. aureus* than *M. smegmatis* for almost all the tea samples tested here.

Grade wise the order of potency of ranking for antibacterial activity against *M. smegmatis* was low country- Dust (I) > Pekoe > BOPF > BOPI > OPI, mid country- BOP > Dust (I) > Dust > Pekoe > OPI > BOPI > BOPF, up country- OPA > BOPF > FBOPI = Pekoe > OPI > FBOP > BOP > Dust. Whilst for *S. aureus* it was low country- Dust (I) > OPI > Pekoe > BOPI > BOPF, mid country - BOP > Dust > Pekoe > OPI > BOPI > Dust (I) > BOPF, up country- BOPF > OPA > FBOP > Pekoe > FBOPI = OPI > Dust > BOP. Both bacteria showed a more or less similar pattern to different tea grades. As an example low country Dust (I) and mid country BOP grade had highest antibacterial activity against both bacteria from their geographical regions. Although OPA, FBOPI, OPI are identified as higher grades of black tea their antibacterial activity was moderate compared to lower grade dust and dust (I).

Agro-climatic elevation wise, the rank order of potency against *M. smegmatis* was upcountry > low country > mid-country for BOPF; mid country > upcountry > low country for OPI; low country > mid country for BOPI; mid country > upcountry for Dust and BOP; low country > mid-country for Dust (I) and mid country > low country > upcountry for Pekoe. As a summary of these results of tea from different agro-climatic regions had different patterns of highest to low antibacterial activity according to tea grades. For example, according to the BOPF grade, upcountry was the best, but mid-country was the best according to OPI grade.

Agro-climatic elevation wise rank order of potency against *S. aureus* was low country > mid country for Dust (I); mid country > low country > upcountry for OPI; mid country > up country > low country for Pekoe; mid country > upcountry for Dust; upcountry > mid country > low country for BOPF; mid country > low country for BOPI and BOP. The same trend of potency ranking for agro-climatic elevations against both bacteria was mid country > low country for Dust and mid country > upcountry for the BOP. These patterns were also similar to the results against *M. smegmatis*. Tea from different agro-climatic regions showed different patterns of high to low antibacterial activity according to tea grades.

These trends show that the effectiveness of the antibacterial activity differs according to the grade and agro-climatic elevation. Although many researchers have reported the antibacterial activity of black tea against a range of Gram-positive bacteria [49,51-53,55] differences in potency of antibacterial activity with tea grade or agro-climatic elevation of black tea had been shown in a few studies only [58]. Several studies have shown that the antibacterial activity of black tea is due to polyphenols (tannins), catechins, theaflavins, and thearubigins [46,49,51,53]. Previous studies have shown that Sri Lankan black tea has these Phyto-constituents but in varying proportions in different grades [71-73].

Based on the results of this study, it could be concluded that Sri Lankan black tea possesses selective antibacterial activity against Gram-positive bacterial species and black tea may have the potential to be used as a safe supplementary beverage during antibacterial therapy. *S. aureus* was more sensitive to the tested tea samples than *M. smegmatis* and *E. coli*. Agro-climatic elevations of tea samples affect antibacterial activity of black tea. According to the results of this study, mid-country tea samples showed better antibacterial activities than up-country and low country samples respectively. Different black tea grades had different antibacterial activity against different bacteria. Tea grades with low particle sizes (Dust/BOP/BOPF) showed better antibacterial activities than the samples with bigger particle sizes (Pekoe/OPI) may be due to the high extraction efficiency of antibacterial-rich compounds.

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