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Research Article

INHIBITORY ACTIVITY OF FRESH GREEN TEA AND BLACK TEA EXTRACTS (*CAMELLIA SINENSIS*) ON INTESTINAL PATHOGENS ISOLATED FROM DIARRHEAL SAMPLE

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ABSTRACT

Medicinal plants have been the major resources for therapeutic agents for remedy of many diseases. In the present investigation, antimicrobial activity of aqueous and methanol extracts of green and black tea (*Camellia sinensis*) against intestinal pathogens was studied by well diffusion method and minimum inhibitory concentration. About twenty six intestinal strains which include *E.coli*, *Salmonella* spp., *Shigella* spp. and *Vibrio* spp. were isolated from diarrheal samples. Results showed that methanol extracts of both green tea and black tea revealed higher inhibitory activity on intestinal bacteria than aqueous extracts. At the concentration of 12.0 mg/ml 8/12 (66%) of *Salmonella* spp., 06/08 (75%) of *Shigella* spp. and 02/04 (50%) of *Vibrio* spp. were sensitive to green tea aqueous extracts. All the isolates (100%) were sensitive to the green tea methanol extracts concentration of 3.0, 6.0, 12, 24 and 48 mg/ml and exhibited zone size ranging from 1.0-37 mm. The MIC of green tea for *E.coli* was found to be 3.0mg/ml, for *Salmonella* spp. was 6.0 mg/ml for aqueous and 12.0 mg/ml for methanol extract. For *Shigella* spp and *Vibrio* spp. it was 12.0 and 6.0mg/ml respectively. Our findings revealed that methanol extracts of green tea exhibited strong antibacterial activity on intestinal pathogens. Thus the extracts of tea leaves can be used as an alternative drug against intestinal pathogens without having any side effects. Green tea and black tea extracts are safe and can be a supplement for other systems of medicines for the treatment of intestinal diseases caused by bacteria.

Keywords: Green tea, black tea, extracts, diarrheal pathogens, MIC

INTRODUCTION

Tea is one of the most popular drink in the world. Tea is produced from the plant *Camellia sinensis* grows best in tropical and subtropical regions[1]. It has been valued for its medicinal properties. Moreover, it has received more attention with regards to health benefits[2]. The beneficial effects of the tea have been attributed to the strong antioxidant activity due to the phenoloic compounds. Tea has wide range of antioxidant, anti-inflammatory, anti- cariogenic and antibacterial activity against many pathogens[3]. Tea components are able to inhibit growth of *Vibrio cholerae*, *Streptococcus pneumonia* and *Shigella dysenteriae*[4,5]. The difference in the activity of tea with the microbes related with the type of fermentation of tea production. Green tea is made from mature unfermented leaves; black tea from fully fermented leaves[6]. Health benefits of green tea are well studied are: as anti inflammatory, an antioxidant, anti carcinogenic, in cardiovascular health, oral health and as a antimicrobial. Studies have shown that green tea have antimicrobial activity against a variety of both gram positive and gram negative bacteria such as *E.coli*, *Salmonella* sp. *Staphylococcus aureus*, *Enterococcus* sp. and some fungi.

The clinical important components of green tea is catechins are 30-40%of the water soluble solids which consists of four major catechin derivatives such as Epicatechins (EC), epigallocatechin (EGC), epicatechingallate (ECG) and epigallocatehingallate (EGCG)[7]. The main constituents of tea catechin, polyphenol content have been reported to inhibit pathogenic bacteria such as *Helicobacter pylori*, *S.aureus*, *Salmonella* sp, *Shigella* sp.[8]. These properties made tea as a good alternate antimicrobial agent.

There are several reports available on the antibacterial property of tea especially on intestinal pathogen[9].The Japanese and others have carried out systemic experiments by using tea extracts and kill various species of intestinal pathogens such as Salmonella, Shigella and Vibrio . Regular consumption of green tea killed *S.aureus*, *Vibr*o spp. *Bacillus cereus* [10]. Green tea contain 30-40% of water extractable polyphenol and black tea contain 3-10% the combined use of tea and antibiotic could be useful in eliminating and controlling of multidrug resistant bacteria associated with enteric disorders. In addition, tea can be cultivated in high elevated regions, generally safe, nontoxic and cheap.

In the present study, we have investigated the inhibitory activity of the extracts of green tea and black tea on intestinal pathogens isolated from diarrheal samples. This study may explore the possibilities of using *C. sinensis* extracts for the control of intestinal pathogens. The photochemical constituent's presents in tea leaves are highly sensitive to oxidation process.

MATERIALS AND METHODS

Collection of Plant samples

The fresh tea leaves (*Camellia sinensis*) used in this study was collected from the field of Valparai tea plantation, Coimbatore,

Tamil Nadu, India, (for green tea preparation). Commercial black tea powder was obtained (black tea preparation) from Super market from Coimbatore. The fresh tea plant leaf sample was shade dried at room temperature (28º C) for three days, made into small pieces. Both dried form of fresh leaf and commercial black tea powder grind into powder by using mortar and pestle.

Preparation of Aqueous and Methanol extracts of green tea and black tea

Extract from fresh leaf of tea plant was done by following standard method [11]**.** For water extraction about 10 g of powdered fresh green tea leaves (green tea) and black tea powder was weighed and soaked in 100 ml of distilled water separately and kept for overnight in shaker (130 r/min at 37º C). The extracts were filtered by using Whatman No.1 filter paper to remove the residues. The filtrate were then concentrated by using rotary evaporator and stored in bottles and refrigerated at 4º C until use.

Methanol extraction was done similarly where about 10 g of powdered fresh green tea leaves and black tea powder was mixed with 70 % methanol. The extracts were prepared by maceration of the plant material with the solvents and kept in a shaker. Then the extract was filtered using Whatman No. 1 filer paper and concentrated and dried powder stored separately in airtight labelled bottles at 4 C.

Isolation and Characterization of intestinal pathogens from diarrheal sample

A total of 26 diarrheal samples were collected from different age group of Patients who were admitted in Govt. Hospital, Erode, Tamil Nadu during the period of November, 2016 – April, 2017. After getting consent from the Patients, the samples were collected, kept in plastic container which contained transport medium and brought to the laboratory immediately by using ice box.

The diarrheal sample was streaked on different selective media agar plates (Hi-media, Pvt. Ltd, Mumbai, India). Mac Conkey agar was used for the isolation of *Escherichia coli,* Salmonella – Shigella Agar for the detection of *Salmonella* spp. Hektoin enteric agar for *Shigella* spp. and TCBS agar (Thiosulphate Citrate Bile salts and Sucrose) for the isolation of *Vibrio* spp. The inoculated plates were incubated aerobically at 37º C for 24 - 48 hours to observe the prevalence of intestinal pathogens. After incubation period the colonies which exhibited characteristics growth appearances for *E. coli*, *Salmonella* spp., *Shigella* spp., *Vibrio* spp. were selected and characterized by various biochemical tests. Intestinal pathogens were identified by using Bergey's Manual of Systematic Bacteriology[12].

Phytochemical analysis of plant extracts[13]

Dried extracts of fresh leaf (green tea) and commercial tea powder (black tea) was taken (about 2 g) and mixed with distilled water. The solution obtained was then subjected to analyze the presence of saponin, alkaloid, phenolics, flavonoid, terpenoid, tannin and proteins.

Antibacterial assay of green tea and black tea extracts on intestinal pathogens

Inhibitory activity of black tea and green tea extracts on intestinal pathogens was determined by standard agar diffusion method and Broth microdilution method.

Agar disc diffusion method

Antibacterial activity of extracts against intestinal pathogen was performed by using agar well diffusion method[14]. The intestinal bacteria were allowed to grown in Mueller Hinton broth (Hi-Media Laboratories Ltd., India) for 18-24 hours on rotary shaker. The concentration of test organisms was adjusted to 10^7 CFU/ml to obtain suitable turbidity. Then Mueller Hinton agar plates were swabbed with 0.1 ml of 18-24 hrs of bacterial culture. After inoculation, plates were kept as such for 5 minutes. A sterile Whatman No. 1 (5mm diameter in size) was impregnated with various concentrations of black tea and green tea extracts were prepared separately (1.5, 3, 6, 12, 24 and 48 mg/ml) and placed above the agar media and incubated at 37 ºC for 24-48 hrs. After incubation the diameter of the zone of inhibition was measured. All experiments were performed in triplicates mean and standard deviation (SD) was calculated and recorded. Filter paper discs containing extracts which did not produce zone of inhibition were considered as negative/resistance to that specific concentration. Commercially available antibiotic disc namely Chloramphenicol (30mcg) was used as a control in this study.

Determination of minimum inhibitory concentration (MIC) of green tea and black tea extracts against intestinal pathogens (Broth Microdilution method)

Broth microdilution method[15] was adopted to determine the minimal inhibitory concentration (MIC) of green tea and black tea extracts against intestinal pathogens. Bacterial cells were harvested by centrifugation (10,000 g, 10 min, 4º C). The pellets were washed with phosphate buffer saline (0.1 M, pH 7.2) for three times then suspended in same buffer and stored. Test organisms were allowed to grown in nutrient broth for 24 hrs at 37ºC on rotary shaker. The concentration of test organisms was adjusted to 10^7 CFU/ml.

Two fold dilutions of extracts were prepared in the appropriate broth culture media from stock solution. About 200 µl of diluted of extracts were dispensed in 96 well culture plates. One hundred microliters of each bacterial suspension was added to each well and incubated at 37º C for 48 hrs. The absorbance was measured at 595 nm. The highest dilution of the green tea and black tea extracts at which no growth was observed was identified as its MIC.

Statistical analysis

All experiments were performed in triplicate. Results of each analysis were expressed as Mean ± Standard deviation.

RESULTS AND DISCUSSION

The present study was undertaken to evaluate and compare different extracts of green tea and black tea on intestinal pathogens isolated from diarrheal samples. The antimicrobial activity of the extracts was carried out by standard procedures such as agar diffusion and microdilution method.

Table 1: Phytochemical analysis of black tea and green tea extracts of *Camellia sinensis*

S.No.	Phytochemical compound	Black tea extracts	Green tea extracts
1	Alkaloids	+	+
2	Flavonoids	+	+
3	Saponins	+	+
4	Tannins	+	+
5	Phenols	+	+
6	Proteins	+	+

+ Presence of phytochemical compound, - Absence of phytochemical compound

Table 2: Cultural characterization of intestinal pathogens on selective agar media

S. No.	Selective media	Appearance of growth	Gram staining
1	MacConkey's Agar (*Escherichia coli*)	Lactose fermenting bright red colour, smooth and raised colonies	Gram negative rod
2	Salmonella-Shigella Agar (*Salmonella* spp)	Opaque translucent colonies	Gram negative rod
3	Hektoein Enteric Agar (*Shigella* spp)	Green colour colonies	Gram negative rod
4	Thiosulphate Citrate Bile salts Sucrose (TCBS) agar (*Vibrio cholerae*)	Yellow colour shiny colonies	Gram positive curve shaped

Table 3: Biochemical Characterization of intestinal pathogens isolated from diarrheal samples

Tests	*Escherichia coli*	*Salmonella* spp.	*Shigella* spp.	*Vibrio* spp.
Motility	+	+	+	+
Catalase	+	+	+	+
Oxidase	-	-	-	-
Urease	-	-	-	-
Indole	+	+	-	-
Methyl red	+	+	+	+
Voges Proskauer	-	+	+	+
Citrate	-	+	+	+
TSI	+	+	+	+
Mannitol	+	+	+	+
Sucrose	+	+	+	+
Lactose	+	+	+	+
Dextrose	+	+	+	+
Gelatinase	+	+	+	+
Hemolysis on blood ager	+	+	+	+

+ Positive result, - Negative result

Table 4: Antimicrobial activity of Aqueous extracts of green tea and Black tea on intestinal pathogens

Intestinal Bacteria	Green Tea extracts -Aqueous (mg/ml) (mm)						Black Tea extracts -Aqueous (mg/ml) (mm)						
	1.5	3.0	6.0	12	24	48	1.5	3.0	6.0	12	24	48	Chlormphenical (30mcg)
Escherichia coli (n=22)	R	3.6± 2.14	5.2± 1.30	17.4± 2.35	20.9± 1.14	32.0± 1.80	R	R	7.0± 2.35	17.4± 1.10	20.7± 1.43	25.6± 1.80	19
Salmonella spp. (n=12)	R	R	3.2± 1.84	12.6± 1.62	17.1± 1.32	25.6± 1.10	R	R	R	10.6± 1.21	11.1± 1.52	20.6± 1.10	16
Shigella spp. (n= 08)	R	R	R	9.6± 1.12	15.6± 1.64	24.1± 1.35	R	R	R	8.1± 1.22	12.6± 1.51	21.1± 1.35	18
Vibrio spp. (n= 04)	R	R	3.0± 2.14	10.0± 1.34	14.5± 1.22	23.6± 2.14	R	R	R	9.0± 1.13	12.5± 1.57	17.6± 2.14	12

Values are mean diameter of zone of inhibition of 3 experiments in millimetre ±SD. R= Resistant; n= number of isolates. Chloramphenicol (30mcg) was used as reference antibiotic compound, n = number of isolates

Table 5: Antimicrobial activity of Methanolic extracts of green tea and Black tea on intestinal pathogens

Intestinal Bacteria	Green Tea extracts -Methanol (mg/ml) (mm)						Black Tea extracts -Methanol (mg/ml) (mm)						
	1.5	3.0	6.0	12	24	48	1.5	3.0	6.0	12	24	48	Chloamphenical (30mcg)
Escherichia coli (n=22)	R	5.2± 1.14	8.6± 1.22	19.4± 2.22	24.7± 1.44	36.8± 1.20	R	R	8.2± 1.35	15.1± 1.40	20.3± 1.13	28.2± 1.12	19
Salmonella spp. (n=12)	R	2.3± 1.42	6.2± 1.32	13.7± 1.42	20.2± 1.62	28.4± 1.18	R	R	R	10.5± 1.30	15.4± 1.32	19.6± 1.20	16
Shigella spp. (n= 08)	R	2.0± 1.05	5.6± 1.11	10.4± 1.82	22.3± 1.24	26.2± 1.14	R	R	R	14.1± 1.31	15.6± 1.11	19.4± 1.12	18
Vibrio spp. (n= 04)	R	1.6± 1.12	4.8± 1.24	12.2± 1.64	10.2± 1.12	30.1± 2.24	R	R	R	15.0± 1.33	17.5± 1.34	24.2± 1.34	12

Values are mean diameter of zone of inhibition of 3 experiments in millimetre ±SD. R= Resistant; n= number of isolates. Chloramphenicol (30mcg) was used as reference antibiotic compound, n = number of isolates

Table 6: Minimum inhibitory Concentration (MIC) of green tea and black tea extracts on intestinal pathogens

Type of Extracts (mg/ml)	Extraction type	Intestinal Pathogens			
		***Escherichia coli* (n=22)**	***Salmonella* spp. (n=12)**	***Shigella* spp.(n= 08)**	***Vibrio* spp. (n= 04)**
Green Tea	Aqueous	3.0±0.2	6.0±0.8	12.0±0.2	6.0±0.4
	Methanol	3.0±0.5	12.0±0.4	12.0±0.5	6.0±0.5
Black Tea	Aqueous	6.0±0.7	12.0±0.6	12.0±0.1	12.0±0.8
	Methanol	6.0±0.1	24.0±0.3	12.0±0.1	12.0±0.4
Chloramphenicol		30[a]	-	-	-

Values are mean MICs of GTE ± SD. [a] MICs of Chloramphenicol is the mean of 3 experiments (mg/ml)

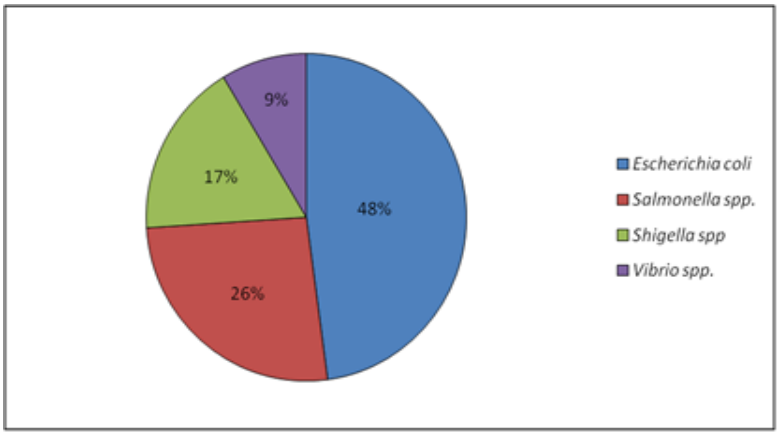

Figure 1: Percentage detection of intestinal pathogens from diarrheal samples

Table 1 shows Phytochemical analysis of black tea and green tea extracts. The present study revealed that both extracts of tea showed the presence of alkaloid, flavonoid, saponin, tannin and phenol. These results were found to be similar with previous studies[16]. The presence of phytochemical substances in tea generally plays a vital role in plant defence mechanism[17, 18].

About 26 numbers of diarrheal samples were collected from patients and observed for the prevalence of intestinal pathogens. Isolation of intestinal pathogens from diarrheal samples was carried out by swabbing them onto selective agar media plates. After incubation the characteristic growth appearance of bacteria were observed and selected for further characterization studies. The percentage of occurrence of intestinal pathogens in diarrheal sample is shown in Figure 1. Among the samples analysed, 84.6% showed the presence of *E.coli*, followed by *Salmonella* spp., (46%), *Shigella* spp., (30.7%) and *Vibrio* spp. (15.3%).

Table 2 showed the characteristic appearance of intestinal pathogens on selective agar media which confirmed the presence of *E.coli*, *Salmonella* spp. *Shigella* spp. and *Vibrio* spp. About 46 numbers of bacterial strains were isolated from diarrheal samples. The bacterial isolates were characterized and identified by studying various biochemical properties. Based on the specific growth on selective agar media and biochemical reactions, the bacterial isolates were confirmed as *E.coli*, *Salmonella* spp. *Shigella* spp. and *Vibrio* spp. All the intestinal pathogens showed beta hemolysis on blood agar media indicates the pathogenicity nature of the isolates (Table 3).

In the present study intestinal bacteria were recovered from more than half of the samples with acute gastroenteritis. Our results are in agreement with the findings of previous studies [19,20] . This might be due to the environmental and personal hygiene factors. The presence of *E. coli* in most of the samples confirmed this as a common causative agent of bacterial diarrhoea and agreement to earlier reports[21]. Salmonella was the second cause of diarrhoea in our study and contradictory to the

earlier reports[22] of who revealed the presence of Shigella spp. in the sample as maximum occurrence. The need for the prevention and treatment of Salmonellosis and Shigellosis is important[23]. Hygiene education should be given for diarrheal disease susceptible groups like school children's, young, old generation and monitoring is also still required. Most of the diarrheal diseases are preventable in nature so that need an efficient surveillance system for monitoring purpose and infection control should be regular in practice[24].

The assessment of the antibacterial activity of tea extracts in this study was based on the measurement of diameter of zone of inhibition produced around the wells. In this study ethanol and aqueous extracts of green tea and black tea was tested on intestinal bacteria isolated from diarrheal samples. The results are given in table 4 and 5.

The results obtained for the aqueous extracts of green tea and black tea showed significant control and antibacterial activity against all the four intestinal bacteria tested (Table 5). Green tea extract exhibited greater inhibitory activity than black tea. All the isolates of *E.coli* (100%) were sensitive to the green tea aqueous extracts at the concentration of 3.0, 6.0, 12, 24 and 48 mg/ml and exhibited zone of inhibition ranging from 4.0-32 mm. *Shigella* spp showed sensitive to the extract concentration of 12, 24 and 48 mg/ml. They exhibited zone of inhibition ranging from10-24 mm. At 3.0 mg/ml concentration 20/22 (90%) of *E.coli* were sensitive to green tea aqueous extract. At the concentration of 12.0 mg/ml 8/12 (66%) of *Salmonella* spp., 06/08 (75%) of *Shigella* spp. and 02/04 (50%) of *Vibrio* spp. were sensitive to green tea aqueous extracts. When compared with aqueous extracts of black tea, *E.coli* showed sensitive to the methanol concentration from 6.0 mg/ml. The zone of inhibition ranged from 7-25 mm. All the other three isolates were resistance to the concentration upto 6.0mg/ml. They were started showing sensitive to the concentration from 12.0 mg/ml and produced zone ranging from 8-21 mm.

Methanol extracts of both green tea and black tea results showed higher activity than aqueous extracts. The antibacterial study for both the extracts revealed that green tea methanol extracts showed promising results in inhibiting all the intestinal isolates effectively. All the isolates (100%) were sensitive to the green tea methanol extracts concentration of 3.0, 6.0, 12, 24 and 48 mg/ml and exhibited zone of inhibition ranging from 1.0-37 mm. The sensitivity observed with methanol extract was higher than the aqueous extracts. All the isolates of *E.coli* 22/22 (100%) were sensitive to the green tea methanol and exhibited zone of inhibition ranging from 5-37 mm. The sensitivity observed was higher than aqueous extracts. Previous reports revealed that the methanol extracts of tea showed higher activity against both Gram positive and Gram negative bacteria[25]. Our study revealed that the organic solvents had greater potential in extracting bioactive components from the plant extracts than water. Results of comparison of antibacterial activity of extracts of different tea against standard pathogens revealed that the methanol extracts of green tea exhibited higher antimicrobial activity[26].

The MIC results for both extracts of green tea and black tea are shown in table 7. The MIC of green tea extracts for *E.coli* was found to be 3.0mg/ml, for *Salmonella* spp. was 6.0 mg/ml for aqueous and 12.0 mg/ml for methanol extract. For *Shigella* spp and *Vibrio* spp. it was 12.0 and 6.0mg/ml respectively. When compared with black tea extracts, the MIC was higher than green tea extracts. For *E.coli* it was 6.0 mg/ml in both aqueous and methanol extracts. Similarly, *Shigella* spp. and *Salmonella* spp. showed 12.0 mg/ml as their MIC. When compared with the standard antibiotic used in this study the extracts showed significant antimicrobial activity. Study of MIC for Gram negative, Gram positive bacteria and reported that *Bacillus subtilis* required least concentration of either methanolic or aqueous extract to inhibit the growth [27]. Our results were correlated with the previous reports) who have reported that the methanol extracts showed higher activity in terms of IC_{50} values [28].

Our results showed that methanol extracts of both green tea and black tea revealed higher inhibitory activity on intestinal bacteria than aqueous extracts. Green tea extracts proved to be the effective antimicrobial agent when compared with black tea[11]. These observations may be correlated with the presence of green tea catechin and polyphenol compounds. These may be responsible for antibacterial activity [29]. The results of the study showed that the leaves of *Camellia sinensis* indicate the presence of potent antimicrobial activity, which confirms its use as antimicrobial agent. The leaves of *Camellia sinensis* contain the phytochemical substances which are responsible for this activity and plays duel benefits as medicinal values and food value. Thus the extracts of tea leaves can be used as an alternative drug against intestinal pathogens without having any side effects[30]. The phytochemical constituents of green tea extracts were able to prevent the adherence of microbes with the intestinal walls and interfere with the cell division thus inhibiting growth and multiplication of microbes[31]. The green tea extract may affect the purine and pyrimidine synthesis in bacteria[32]. In addition to these reports are available that both green and black tea produced larger zone of inhibition (400 µg / ml) which supports our study[33]. The daily consumption of green tea can able to kill Many Gram positive, Gram negative bacteria and many other harmful bacteria[34].

CONCLUSION

In conclusion, hygienic education is needed for the population and an efficient monitoring system has to be set up for the control. Our study revealed that the extracts of green tea and black tea were rich in antibacterial constituents. Moreover, multiple drug resistant strains are in the rise thus become difficult in treatment. Green tea and black tea extracts are safe and can be a supplement for other systems of medicines for the treatment of intestinal diseases caused by bacteria. Further research is required for the bioavailability of the active components before it can be used as a therapeutic agent.

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