

Original Research Article

<https://doi.org/10.20546/ijcmas.2017.610.037>

Assessment of Metal Chelating, Bioautography and Spot Screening by TLC of Green Tea (*Camellia sinensis*)

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ABSTRACT

Keywords

Green tea, Metal Chelating ions, Antibacterial assay, Antioxidant activity, Phytochemical screening, *Camellia sinensis*.

Article Info

Accepted:
04 September 2017
Available Online:
10 October 2017

Tea is receiving increased interest from scientists due to its biological properties and the presence of bioactive substances like polyphenols which give nutraceutical values to well brew cup of tea. The study was carried out as a prerequisite to evaluate the therapeutic potential of green tea. The antioxidant potential of Kangra tea in terms of metal ions chelation was determined. Ferrous ions chelation potential of tea powders (in terms of $IC_{50} (Fe^{2+})$ values) was in between the $IC_{50} (Fe^{2+})$ values of EDTA and citric acid. The $IC_{50} (Fe^{2+})$ exhibited a correlation with TC and flavanols. A comparative TLC (Thin layer chromatography) bioautograph study between aqueous solutions of standard catechins (0.5 mg mL^{-1}) and tea powder (1 mg mL^{-1}) indicated that EGCG was one of the active components present in tea solution which might be responsible for antibacterial activity of Kangra tea. Growth inhibition of bacterial pathogens (*B. cereus* and *S. aureus*) by aqueous solution of tea powder was found to be influenced by the addition of ferrous ions which suggest that the antibacterial activity of tea catechins could be due to their ability to chelate ferrous ions, indicating that this activity is bacteriostatic rather than bactericidal in nature.

Introduction

For more than 1000 years green tea has been used in China and Japan as a medical herb and a healthful beverage. The traditional Chinese medicine has recommended this plant for headaches, body aches and pains, digestion, depression, detoxification, as an energizer and, in general, to prolong life. Tea is made from young shoots and leaves of the plant *Camellia sinensis* (Willson and Clifford 1992; Cabrera *et al.*, 2006; Sharma *et al.*, 2007).

Reasons for the vast use of medicinal plants are high cost of allopathic drugs and their side

effects (Marwat *et al.*, 2008). Majority of the human beings died mostly due to infectious bacterial diseases in the developing countries (Nathan 2004). The synthetic antibiotics that are being used also became ineffective because the pathogens got resistance against these antibiotics (Walsh and Amyes, 2004).

Various strategies have been developed during the past decade e.g., biological screening, isolation as well as clinical trials for a variety of plants to explore the secret of their therapeutic actions. Now a day, bioactive plant extracts are considered as a

promising source of biological friendly antibacterial agents (Koehan and Carter 2005).

Fresh tea leaves contain caffeine, tea polyphenols, tea polysaccharides, and necessary nutrients, such as protein, amino acids, lipids, and vitamins. Generally, four chemical components – free amino acids, total tea polyphenols, soluble sugars, and caffeine – are considered indicators of tea quality (Yamamoto *et al.*, 1997; Bashir *et al.*, 2014; Gogoi and Barua 2017). It is of the interest to note that the polyphenols found in fresh green tea shoots are different from other polyphenols of plant origin as most of them are found only in leaves of tea plant. Tea catechins have been used as antioxidant compounds in many food matrices such as meats, poultries, fishes and vegetable oils (Yilmaz 2006). Since tea has an antimicrobial activity against a large spectrum of pathogenic bacteria (Chou *et al.*, 1999; Gramza *et al.*, 2005; Friedman *et al.*, 2006; Freidman 2007). Its low pH value, of approximately 4.2, makes tea compatible with many food products in term of acidity.

Epidemiological studies during the recent years have suggested that green tea reduces risks of several chronic diseases including cancer and cardiovascular disorders by increasing plasma antioxidant capacity in humans (Nakagawa *et al.*, 1999; Duthie *et al.*, 2000). In the present study, we have investigated the bioactivity of green tea, cultivated at Wah tea estate, Rajpura, HP.

Materials and Methods

Preparation of tea powders

Tea powders were prepared by lyophilizing aqueous extracts of dried green tea with the help of Edwards EF₄ Modulyo freeze dryer and Heto Power Dry LL 3000 freeze dryer. The powders so obtained were immediately

transferred into glass tubes fitted with air-tight stoppers which were stored in vacuum desiccator.

Estimation of total catechins

Total catechins were always estimated in freshly prepared tea extracts by the method of Sun *et al.*, (1998).

Thin-layer chromatography

Thin layer chromatography was done on glass plate coated with silica gel G by the method of Stahl (1969). Standard solutions were prepared (0.5 mg mL⁻¹). (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, (-)-epicatechin gallate and (-)-epigallocatechin were procured from (Sigma, USA). Solvent system was used: Chloroform: Ethyl acetate: Acetic Acid:: 25:75:0.5 (By volume)

Metal ions chelation assay

The binding potency of metal ions by tea catechins was evaluated using method of Tang *et al.*, (2002). The technique for the evaluation of metal ions chelation by the tea catechins a method was standardized using standard chelating agents namely EDTA (1 mM) and citric acid (25 mM). The value of slope and y-intercept were evaluated from a plot (Figures 1) between % chelating activity and concentration of EDTA and Citric Acid.

IC₅₀ (Fe²⁺) value (the amount of metal chelator necessary to decrease the initial ferrous ions concentration by 50%) for EDTA was evaluated using the following equation:

$$IC_{50} (Fe^{2+}) \text{ Value} = (50 - y\text{-intercept}) / \text{slope}$$

Evaluation of tea samples

The metal ions chelation activity of tea catechins was evaluated in aqueous solutions of tea powders by following protocol:

The sample solution have pale yellow color and sample volumes used for evaluating metal ions chelating activity were quite large, it was noted that sample form blue color with ferrous ions.

To correct this occurrence, sample blanks containing the appropriate dilution of each volume of sample with 2 mM FeCl₂.7H₂O solution (0.1 mL) were used. The absorbance of each sample blank was subtracted from the absorbance of sample, respectively. The final absorbance so obtained was used for further calculations.

Antibacterial activity

Growth inhibitory activity of tea powder of fresh green tea shoots was evaluated against selected pathogenic bacteria i.e. *Bacillus cereus* (MTCC-1272) and *Staphylococcus aureus* (MTCC-96) procured from the Institute of Microbial Technology (IMTECH), Chandigarh.

The bacterial cultures were maintained on freshly prepared nutrient agar (NA) medium and were stored at 4±0.5°C for further use.

Agar-well diffusion method for antibacterial activity

Antibacterial activities of tea powder prepared from green tea shoots were evaluated by the method of Toda *et al.*, (1989a). The antibacterial activity was always determined in freshly prepared extracts/solutions.

Bioautography

A TLC bioautographic method as described by Kline and Golab (1965) was used to detect active antibacterial components present in tea powder. For this 50 µL of aqueous solution of tea powder (1 mg mL⁻¹) was applied on silica gel G TLC plate which was developed by

using chloroform: ethyl acetate: acetic acid (25:75:0.5) as the solvent system and then dried for complete removal of solvent. Then TLC plate was placed in the Petri dish and 0.1 mL of inoculum of *Bacillus cereus* (MTCC-1272) grown in nutrient broth was added to agar medium and this medium was distributed over the entire TLC plate.

The plate was incubated at 37°C for 24 h and the inhibition zone was visualized with 0.1% of triphenyl tetrazolium chloride solution. Inhibition zone was observed as clear area against a red colored background.

Influence of metal ions on inhibitory properties of aqueous solution of tea powder

Influence of metal ions on inhibitory properties of aqueous solution of tea powder was evaluated using agar-well diffusion method as described by West *et al.*, (2001). The growth inhibition of aqueous solution of tea powder was determined against *B. cereus* and *S. aureus* using agar-well diffusion method as described above. Zones of inhibition were observed, then 0.1 mL of ferrous sulphate solution was added to one well whereas sterilized double distilled water was added to other well as a control. Plate was kept at 4°C for 1 h to allow diffusion of the solution into the medium. The plate was then incubated at 37°C for 24 h then appearance of growth was observed in inhibition zone.

Results and Discussion

Mean total catechins (TC) content of fresh green tea shoots varied significantly in the range of 143.242 to 120.930 g kg⁻¹. Tea powders obtained by lyophilizing aqueous extracts of green tea shoot samples. The mean TC content in tea powders varied significantly in the range 271.392 to 140.831 g kg⁻¹.

Thin layer chromatography of tea powder

Thin layer chromatographs (TLC) of standard flavan-3-ols [(+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG) and (-)-epicatechin gallate (ECG)] and tea powder were presented in Plate I.

Metal ions chelation

The mean $IC_{50(Fe^{2+})}$ values corresponding to chelation of Fe^{2+} ions by EDTA and citric acid (versatile metal chelators) were estimated to be $1.589 \mu\text{g mL}^{-1}$ and $1455.895 \mu\text{g mL}^{-1}$, respectively (Table 1).

In table 3 is given the mean monthly $IC_{50(Fe^{2+})}$ values (inhibitory concentration necessary to reduce the metal ions concentration by 50 per cent) of aqueous solutions (concentration: 5 mg mL^{-1}) of tea powders obtained by lyophilizing aqueous extracts of samples of fresh green tea shoots along with CD (at 5%) and per cent CV.

A perusal of table 3 indicates that the mean monthly $IC_{50(Fe^{2+})}$ values varied significantly in the range: 1243.002 to $724.953 \mu\text{g mL}^{-1}$ for tea powders. The tea powders obtained by lyophilizing aqueous extracts of fresh green tea shoots harvested during August had lower mean monthly $IC_{50(Fe^{2+})}$ value. Although these values are comparable with the $IC_{50(Fe^{2+})}$ value estimated for citric acid but, far higher in comparison to EDTA.

The metal ions chelating potential in terms of $IC_{50(Fe^{2+})}$ values of tea powders exhibited a significant negative correlation with TC, EGCG, ECG and EGCG+ECG (Table 4).

Antibacterial activity

In order to evaluate green tea for its biological activity, antibacterial potential of aqueous extracts of samples of fresh green tea shoots

were studied against standard bacterial pathogens i.e. *Bacillus cereus* (MTCC-1272) and *Staphylococcus aureus* (MTCC-96)

Sensitivity of selected bacterial pathogens for standard catechins

Before evaluating the tea samples for antibacterial activity, the individual catechins i.e. C, EC, EGC, EGCG and ECG were tested for their antibacterial effect on the selected pathogens (Plate II). Minimum inhibitory concentrations (MIC) of EGC, EGCG and ECG for *B. cereus* were 375, 50 and $250 \mu\text{g mL}^{-1}$, respectively, whereas for *S. aureus* the MIC of EGC, EGCG and ECG were 500, 250 and $375 \mu\text{g mL}^{-1}$, respectively table 5 indicates that the MIC of aqueous extracts of samples of fresh green tea shoots varies significantly in the range 724.953 to $1243.002 \mu\text{g mL}^{-1}$

Bioautography

In Plate IV the TLC bioautographic result of aqueous solution of tea powder against *B. cereus* is given. The component that corresponded to the zone of inhibition against a red background was at the bottom of the TLC plate where the tea solution was spotted

Influence of metal ions on inhibition properties of tea catechins

Growth inhibition of the aqueous solution of tea powders on *B. cereus* and *S. aureus* was influenced by the addition of ferrous ions (Plate V).

Health benefits of tea drinking have been attributed to tea catechins. The present investigations: "Assessment of metal chelating, bioautography and spot screening of TLC of green tea (*Camellia Sinensis*)" were carried out to elucidated biological activity of green tea.

Table.1 Protocol of metal ions chelating activity

Tube no.	Vol. of stock sample solution (mL)	Vol. of double distilled water (mL)	Vol. of FeCl ₂ (mL)	Vol. of ferrozine (mL)
T ₁ (Control)	0.000	2.700	0.100	0.200
T ₂	0.240	2.460	0.100	0.200
T ₃	0.360	2.340	0.100	0.200
T ₄	0.480	2.220	0.100	0.200
T ₅	0.600	2.100	0.100	0.200
T ₆	0.720	1.980	0.100	0.200
T ₇	0.840	1.860	0.100	0.200

Table.2 Mean total catechins (g kg⁻¹) contents in tea powders prepared from Fresh green tea shoots

Months	Fresh green shoots	Total catechins
April	120.930 ^e	140.831 ^f
May	137.730 ^b	197.627 ^d
June	136.242 ^b	226.024 ^c
July	132.429 ^c	249.689 ^b
August	142.644^a	271.392^a
September	135.609 ^b	251.041 ^b
October	127.097 ^d	185.190 ^e
Mean	132.954	217.399
CD (5%)	4.86	10.75
CV (%)	2	2.82

Rankings – means within each column followed by the same letter are not significantly different at $p < 0.05$.

Table.3 Mean IC₅₀(Fe²⁺) values (µg mL⁻¹) of tea powders from fresh green tea shoots (FGTS)

Months	FGTS
Mean IC ₅₀ (Fe ²⁺) values	
April	1243.002 ^a
May	1160.127 ^b
June	884.435 ^d
July	971.934 ^c
August	724.953^e
September	890.731 ^d
October	985.467 ^c
Mean	980.093
CD (5%)	47.66
CV (%)	2.78

Rankings – means within each column followed by the same letter are not significantly different at $p < 0.05$.

Table.4 Correlation coefficient among TP, TC, C, EC, EGC, EGCG and ECG and mean IC₅₀_(Fe²⁺) values of tea powders from fresh green tea shoots (FGTS)

	FGTS
Mean IC _{50(Fe²⁺)} values	
TP	-0.77 ^a
TC	-0.87 ^a
C	NS
EC	NS
EGC	NS
EGCG	-0.92 ^a
ECG	-0.80 ^a
EGCG+ECG	-0.88 ^a
TP	-0.77 ^a
TC	-0.87 ^a

^a – Significant at $p < 0.05$; NS – Not significant.

TP - total polyphenols; TC - total catechins; C – catechin; EC – epicatechin; EGC- epigallocatechin; EGCG - epigallocatechin gallate and ECG - epicatechin gallate.

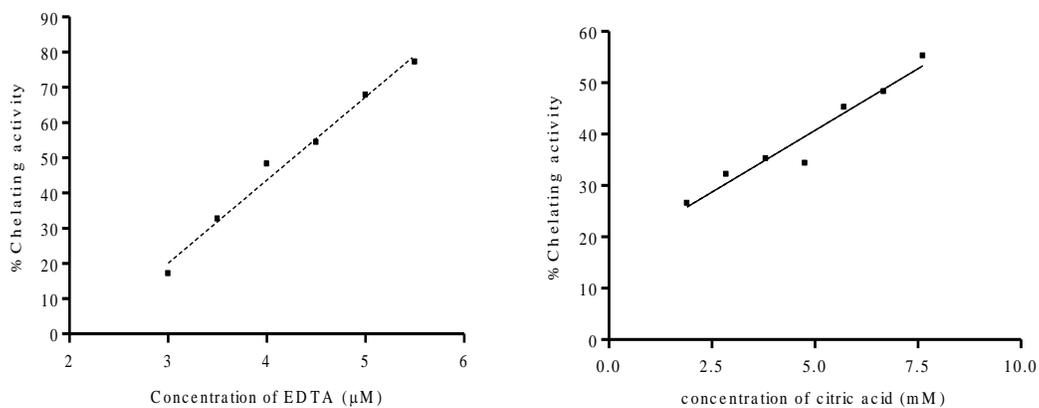
Table.5 Mean minimum inhibitory concentrations (mg mL⁻¹) of aqueous extracts of samples of Fresh green tea shoots against selected bacterial pathogens

	Pathogens	
Months	<i>B. cereus</i>	<i>S. aureus</i>
	Mean minimum inhibitory concentrations*	
April	1.541 ^f	2.042 ^b
May	1.595 ^e	1.595 ^e
June	1.770 ^c	1.770 ^c
July	2.025 ^b	2.025 ^b
August	1.073^g	1.541^f
September	1.723 ^d	1.723 ^d
October	2.253 ^a	2.253 ^a
Mean	1.712	1.850
CD (5%)	0.036	0.036
CV (%)	1.20	1.20

Rankings – means within each column followed by the same letter are not significantly different at $p < 0.05$; NZ – No inhibition zone detected.

*The minimum inhibitory concentrations are based on total catechins content (mg mL⁻¹) of aqueous extracts.

Fig.1 A- Graph between EDTA and per cent chelating activity; B- Graph between citric acid and per cent chelating activity



A

B

A -Slope = 23.56; y-intercept = (-) 50.62; $r^2 = 0.9857$; B- Slope = 0.02290; y-intercept =16.66; $r^2 = 0.9424$

Plate.1 Thin layer chromatogram of tea powders containing significant amounts of flavanols along with standard catechins mixture Solvent system: Chloroform: Ethyl Acetate: Acetic Acid:: 75:25:0.5

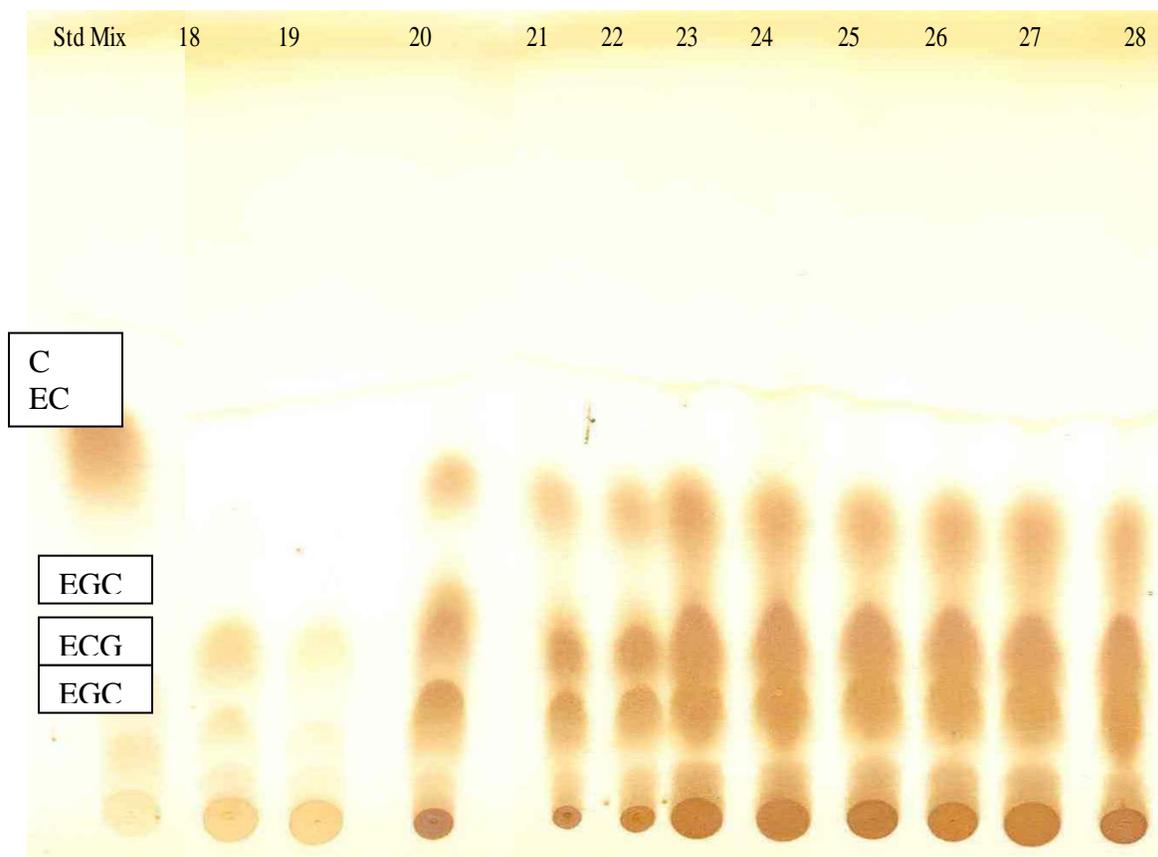
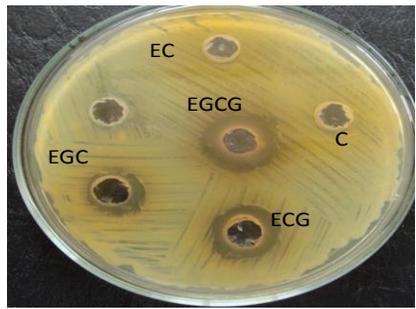
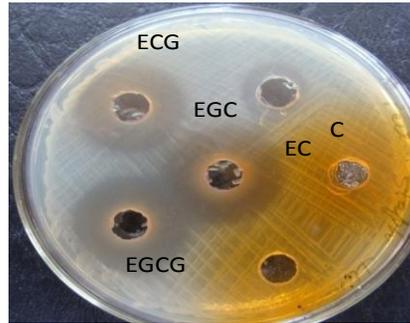


Plate.2 Sensitivity of *B. cereus* and *S. aureus* against standard catechin and its derivatives

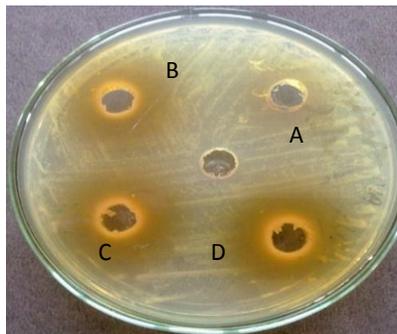


Staphylococcus aureus (MTCC-96)

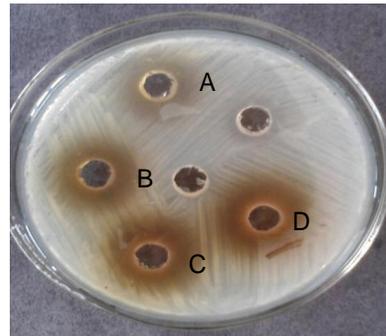


Bacillus cereus (MTCC-1272) L

Plate.3 Sensitivity of *B. cereus* and *S. aureus* for aqueous extracts of fresh green tea shoots

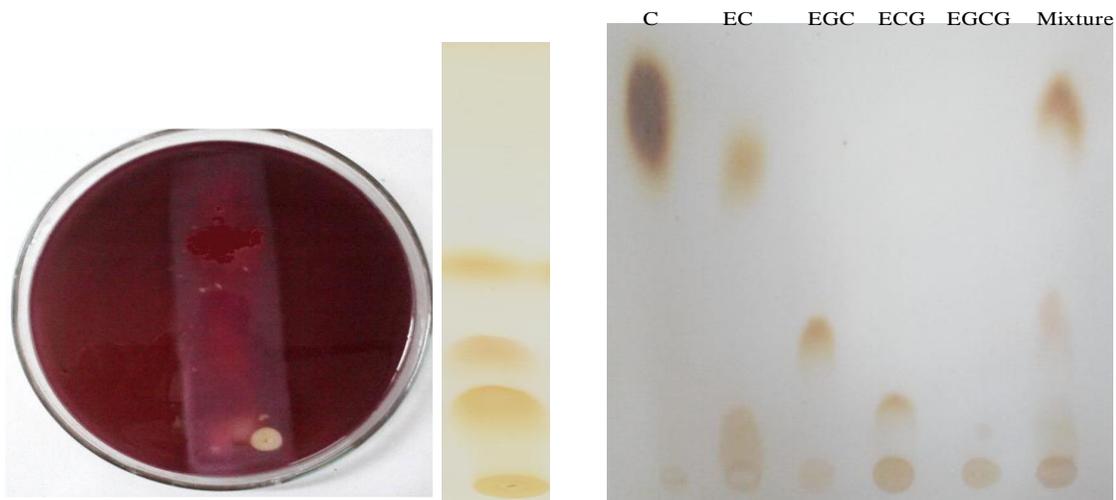


Staphylococcus aureus (MTCC-96)



Bacillus cereus (MTCC-1272) L

Plate.4 TLC bioautography of aqueous solution of tea powder of green tea shoots against *B. cereus* along with TLC of tea solution and standard catechins. Solvent system: Chloroform: Ethyl Acetate: Acetic Acid:: 75:25:0.5

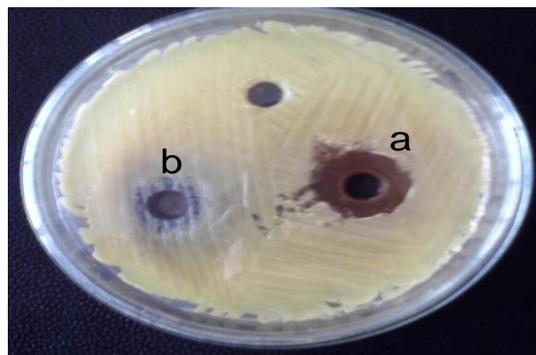
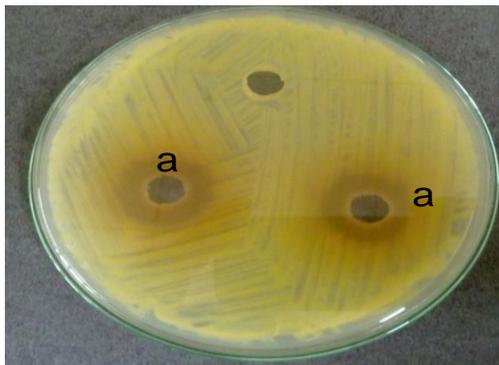


TLC bioautograph of tea solution

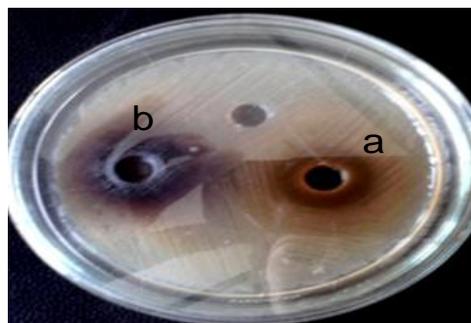
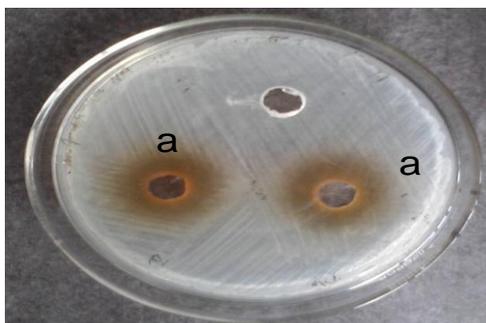
TLC of tea solution

TLC of standard catechins

Plate.5 Growth inhibition of *B. cereus* and *S. aureus* by aqueous solution of tea powder (a) in the absence, and (b) showing restoration of growth in the presence of ferrous ions



Staphylococcus aureus (MTCC-96)



Bacillus cereus (MTCC-1272)

Total catechin content

Significant mean variations in the levels of TC in tea powders were almost similar to those of in fresh green tea shoots (Table 2). Misra *et al.*, (2008) reported that the fresh tea shoots were extremely rich in phenolic compounds which can constitute up to 300 mg/g of dry leaves.

Thin layer chromatography of tea powder

The chromatogram indicates that the tea powders contain all the five flavanols of interest. Bashir *et al.*, (2014) reported that spot screening of TLC-developed plates indicated that the presence of active biological compounds such as flavonoids, phenols, alkaloids and glycosides.

Metal ions chelation

The ability of flavanols to bind cations of metals having variable valency will restrict the metal ions from undergoing oxidation which in turn terminate the generation of free electrons/radicals in biological system. Such a reaction may contribute, indirectly, to the antioxidant potential of flavanols.

The metal chelation potential of the tea powders was better than that of citric acid. This could be due to the presence of flavanols in tea powders. Tea catechins have been reported to exhibit significantly ($p < 0.05$) weaker chelating activity ranging from EGCG (38%), EC (30%), ECG (19%) on Fe^{2+} ions compared to citric acid (54%) or EDTA (100%) at a concentration of 400 ppm (Tang *et al.*, 2002).

From significant negative correlation with TC, EGCG, ECG and EGCG+ECG, it is reasonable to infer that the flavanols with gallate moiety (EGCG and ECG) might be responsible for the metal ions chelation activity among tea catechins. Compounds with gallate groups in their molecules, gallate of catechins and their polymers have been reported to exhibit metal ions chelating activity (Miller *et al.*, 1996).

Antibacterial activity

Sensitivity of selected bacterial pathogens for standard catechins

The lowest mean MIC among the standard catechins was observed with EGCG against *B. cereus*. The selected bacterial pathogens were resistant against catechin and epicatechin. These results are in agreement with Taguri *et al.*, (2004) who reported higher antibacterial effect of EGCG against all bacterial groups: *Staphylococcus aureus* (20 strains), *Salmonella* (26 strains), *E. coli* (23 strains) and genus *Vibrio* (27 strains) as compared to EGC suggesting that this could probably be due to the presence of galloyl group in EGCG which increases its antibacterial activity.

Table 5 indicates that the aqueous extracts of samples of fresh green tea shoots harvested during the month of August had lower MIC and hence more potent antibacterial as compared to rest of the months (Plate III). However, Chou *et al.*, (1999) reported that the extract of oolong tea prepared from samples of tea shoots harvested in summer flush seasons exhibited the strongest antimicrobial activity followed by those prepared from samples of spring, winter and fall. The high antibacterial activity of samples August could be due to the presence of high levels of total catechins in the sample as evident from the results given in table 2. These results corroborate with the earlier studies carried by

Chou and Lin (1987) and Yam *et al.*, (1997) who fractionated tea extract and reported that the fractions rich in EGC, EGCG and ECG exhibit strong antimicrobial activity.

Bioautography

A comparison with standard catechins TLC chromatogram illustrate that EGCG was present at bottom and hence, EGCG was the active component of tea sample which probably responsible for the antibacterial activity of Kangra tea. These results are in agreement with Freidman *et al.*, (2006) who reported that gallate of catechins i.e. (-)-gallo catechin-3-gallate, (-)-epigallocatechin-3-gallate, (-)-catechin-3-gallate, (-)-epicatechin-3-gallate, theaflavin-3,3'-digallate, theaflavin-3'-gallate and theaflavin-3-gallate showed antimicrobial activities at nanomolar levels. Bashir *et al.*, (2014) reported that TLC bioautography of methanol, ethanol and DMSO extracts of *Camellia* varieties indicated the significant inhibition of *S. aureus*, *S. epidermidis* and *S. marcescens*, respectively. Similar results were recorded by spot screening of TLC-developed plates against all clinical bacterial pathogens. The zone of inhibition was recorded in range from 10.00±0.0 mm to 28.00±0.00 mm.

Influence of metal ions on inhibition properties of tea catechins

It was evident from the plate that addition of ferrous ions restored the growth of organism around the well indicating that the antibacterial activity of tea catechins is probably due to their ability to chelate Fe²⁺ ions and making them non-available for microbial growth. These observations are in accordance with the earlier studies of West *et al.*, (2001). These results also suggest that the growth inhibition by tea catechins is of bacteriostatic in nature. Earlier studies by Kono *et al.*, (1994) suggested that EGCG has

a concentration dependant bactericidal activity i.e. at concentration of 1 to 2 times of MIC, the number of bacteria decreased to 1/10th of original load, suggesting bacteriostatic activity, but if concentration was increased to 6 fold greater than MIC, then EGCG showed bactericidal activity. However, Freidman *et al.*, (2006) reported bactericidal activities of the aqueous extract of green tea catechins.

The ferrous ions chelation and antibacterial potency of green tea were directly proportional to total catechins contents of tea in general and (–)-epigallocatechin gallate and (–)-epicatechin gallate contents in particular. The antibacterial effect of green tea was bacteriostatic instead of bactericidal; however, more work is required to be carried out to establish this property. The flavanols with galloyl moiety exhibit potential biological activity.

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How to cite this article:

Arti Ghabru and Sud, R.G. 2017. Assessment of Metal Chelating, Bioautography and Spot Screening by TLC of Green Tea (*Camellia sinensis*). *Int.J.Curr.Microbiol.App.Sci*. 6(10): 302-313. doi: <https://doi.org/10.20546/ijcmas.2017.610.037>