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Bioassay-Guided Isolation and Identification of Antibacterial and Antifungal Component from Methanolic Extract of Green Tea Leaves (*Camellia sinensis*)

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Abstract: The antibacterial activity of methanolic extract of green tea leaves (*Camellia sinensis*; Family: Theaceae) was studied on four different bacteria namely *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* and two fungi of *Aspergillus* species. Antibacterial activity was assigned by measuring the Zone of Inhibition (ZOI) as well as Minimum Inhibitory Concentration (MIC) at four different concentrations of the methanolic extract (10, 25, 50 and 100 mg mL^{-1}) and erythromycin (10 mg mL^{-1}) which was taken as the antibiotic control. Preliminary screening was done to find out the phytochemical components present in the extract. Thin Layer Chromatography (TLC) was performed to separate and isolate the bioactive components present in the extract. Further bioassay-guided fractionation of the methanolic extract with the help of column chromatography was performed using a stepwise gradient with Chloroform: Methanol against the bacteria. The fraction which gave us the best result was further taken for the HPTLC for the identification of the bioactive compound which was identified as catechin. Zone of inhibition was observed in most of the organism. Alcoholic extract of the leaves of *Camellia sinensis* was found to be most effective against *Bacillus cereus*.

Key words: Green tea leaves (*Camellia sinensis*), antibacterial and antifungal activity, TLC, HPTLC, Catechin

INTRODUCTION

Green tea consists of the rapidly dried, freshly picked leaves of *Camellia sinensis*, a plant of the Theaceae family. It was originated in China and has become widespread in the West, where black tea is traditionally consumed. Green tea is one of the most popular beverages consumed all over the world. The plant was originally discovered and grown in Southeast Asia 1000 of years ago (Horiba *et al*., 1991). Green tea has various added advantages over black tea because it undergoes minimal oxidation during the processing, which further prevents various bioactive components from being oxidized. This tea is beneficial to human beings in various ways. The tea extracts shows wide range of activities such as antibacterial, antiviral, antioxidative, antimutagenic and anticarcinogenic (Toda *et al*., 1989a; Kuroda and Hara, 1999; Nakane and Ono, 1989; Akiyama *et al*., 2001; Mabata *et al*., 2006).

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It also contains large number of bioactive components such as methylxanthines, theobromine and theophylline. Green tea contains several vitamin B and C. Other green tea ingredients include 6 to 8% of minerals such as aluminium, fluoride and manganese. Green tea also contains organic acids such as gallic acid and quinic acid and 10 to 15% of carbohydrate and small amount of volatile compounds. Among all these, epigallocatechin gallate is believed to be the most active agent (Cheng *et al*., 1998; Stapleton *et al*., 2007). It is believed that a cup of green tea contains up to 200 mg of catechins, whose biological activity has been attributed to it's antioxidisng activity. Efficiency of green tea extract in oral hygiene and against some intestinal bacteria has been known for centuries and this gave researchers a clue that the beverages might be involved in antibacterial activity (Hara and Ishigami, 1989; Ahn *et al*., 1990). Polyphenols and flavonoids from different other plant extract have also be proven to have potent antioxidising activity (Okpuzor *et al*., 2009). Another important effect is on anti-ageing factor, where it is found to lower blood sugar, chelate iron and control the production of nitric oxide which combinedly protects health and delay ageing (Vinson and Dabbagh, 1998). A recent study refers to lower rate of heart disease and cancer in Asia despite high rate of smoking cigratte correlating it with consumption of green tea daily and has been name as Asian Paradox where it was also found that regular intake of green tea provides high level of polyphenols and other antioxidants which is beneficial to body against various toxicity (Sumpio *et al*., 2002).

All the four bacteria taken for the present study namely *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus* and *Pseudomonas aeruginosa* produces different type of toxin mainly enterotoxin and exotoxin and causes various wild type of diseases in human beings. The fungi *Aspergillus parasiticus* and *Aspergillus flavus* are responsible for causing mycotoxicosis in humans. The present research was carried out to evaluate the efficacy of alcoholic extracts of green tea leaves on four different bacteria and two different fungal species and to isolate and identify the bioactive component responsible for the activity.

MATERIALS AND METHODS

Chemicals

All chemicals used in the present study were of analytical grade procured from Hi Media Laboratories Pvt. Ltd., Mumbai, Thomas Baker (Chemicals) Ltd., Mumbai, Qualigens Fine Chemicals, Mumbai and S.D. Fine chemicals Pvt. Ltd., Mumbai, India. The study was started from 1st Dec., 2008 and was ended in 31st March, 2009.

Microorganisms

Staphylococcus aureus (MTCC No. 737), *Escherichia coli* (MTCC No. 448), *Bacillus cereus* (MTCC No. 135), *Pseudomonas aeruginosa* (MTCC No. 424), *Aspergillus parasiticus* (MTCC No. 8189) and *Aspergillus flavus* (MTCC No. 1883) was obtained from Institute of Microbial Technology, Chandigarh, India. Cells were grown and stored at 37°C on agar slant.

Collection and Identification of Plant

The plant material viz., leaves of Chinese green tea (*Camellia sinensis*) was collected from local market of Anand state, Gujarat, India in the month of November, 2008. It was identified and authenticated from Department of Botany, Gujarat University, Ahmedabad, Gujarat, India.

Preparation of Green Tea Leaves (*Camellia sinensis*) 50% Methanolic Extract

Green tea (*Camellia sinensis*) leaves were allowed to dry in shade for 2 weeks. Twenty five gram gm of dry powder was subjected to soxhelet extraction with 250 mL 50%

methanol as solvent. Soxhelation process was allowed to carry out for 3 h, 8 cycles, temperature was maintained at 50°C. During the experiment known amount of dried extract was re-dissolved in water and was used. The following concentration of the tea extract made was 10, 25, 50 and 100 mg mL^{-1} (WHO Protocol CG-06, 1983).

Preliminary Screening of Secondary Metabolites

The condensed extracts were used for preliminary screening for phytochemicals such as alkaloids (Dragendorff's test), flavonoids (Shinoda test), catechins (Phloroglucinol test), tannins ($FeCl_3$ Test), saponins (Foam test), sterols (Salkowski test), glycosides (Molisch test) and phenols (Folin's test) (Daniel, 1991).

Separation of Secondary Metabolites by Thin Layer Chromatography (TLC)

The chromatoplate spotted with extract was developed in solvent system comprising of N-Butanol: Ethyl acetate: 2 M HCL (1:1:1) (v/v). The developed plates were air-dried and observed under long wave UV light. Later on was sprayed with the colouring reagent (1.5% alcoholic Ferric chloride solution). Spots were obtained which was compared to the standards. Rf value was calculated for each of the spot (Harborne, 1973).

Bioassay Guided Fraction of Methanolic Extract

Bioassay guided fraction of methanolic extract was performed to separate various components from the mixture (scrapped matter of TLC) that exhibited antibacterial activity. The fraction was applied on the top of a silica gel and the components was eluted (5 mL) using a stepwise gradient starting with Chloroform : Methanol (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1). Each time the fraction was collected and evaporated to dryness and once again resuspended in the water. Phytochemical analysis of all the evaporated solvent was conducted further. Each fraction was subjected for antimicrobial activity (*E. coli*), where zone of inhibition was noted. The fraction which gave us the best result was further taken for the HPTLC to check the presence of the bioactive compound in the extract.

Separation and Identification of Bioactive Component by High Performance Liquid Chromatography (HPTLC)

Densitometric HPTLC (Modle-Camag/Switzerland, pressure-60-90 psi, preferably N_2, Column-C-18, CAT Software) was used for determination of bioactive components in the 50% methanolic extract of the green tea leaves. Sample was loaded on to the silica gel plate with the help of a sample injector. The components were separated on C-18 plates, with toluene-ethylacetate-methanol-formic acid 3.8:3:0.4:0.8 (v/v) as mobile phase, which was saturated in an automated development chamber at 75% humidity for 1 h. The sample was allowed to run along with the solvent and then the spots are identified under UV light. The catechins were detected under UV light at 280 nm. The catechin of the plant material was detected on the basis of the amount of (+)-catechin (standard). The method was validated for precision and repeatability.

Determination of Antimicrobial Activity

Zone of Inhibition

The four different concentration of leaf extracts were tested for antibacterial activity using agar well diffusion method and paper disc diffusion method, standardize by National Committee for Clinical Laboratory Standards (2002). The microorganisms were inoculated in the conical flask containing 100 mL of nutrient broth. These conical flasks were incubated at

37°C for 24 h. Media was prepared using nutrient agar, which was poured on petri plates allowed to solidify and then inoculated with the test organisms. Wells were prepared with the help of sterile cup borer of 1 mm diameter and were filled with the plant extract. A bacteria control and antibiotic control (erythromycin-10 mg mL^{-1}) was prepared for the comparative study. The plates were incubated from 37-48°C depending on the bacterial species. Antibacterial activity was assigned by measuring the Zone of Inhibition (ZOI) around the well.

Minimum Inhibitory Concentration

The determination of the Minimum Inhibitory Concentration (MIC) by the extract of the plant at the different dose levels (10, 25, 50 and 100 mg mL^{-1}) was also performed. It was carried out by the method as described as earlier (National Committee for Clinical Laboratory Standards, 2002). The microorganisms were inoculated in the conical flask containing 100 mL of nutrient broth. Then approximately 10 mL of the seeded suspension was taken in different test tubes. The 0.2 mL varying concentrations of the plant extract was introduced in the test tubes containing 10 mL broth and standard bacterial cells. Further the test tubes were incubated for 24 h at 37°C. Controls were set up with the test organism only and one with the antibiotic erythromycin. The minimum inhibitory concentration was taken as the tube with the least concentration of the extract with no visible growth was taken as the minimum bactericidal concentration.

Statistical Test

For each parameter at least 6 replicates were done. Results are expressed as Means±SEM. The results obtained were statistically analyzed by Student's t-test.

RESULTS

Table 1 and 2 show the antibacterial activity of varying concentration of alcoholic extract of *Camellia sinensis* by agar well as well as paper disc diffusion method on different bacterial and fungal species (*E. coli*, *B. cereus*, *P. aeruginosa*, *S. aureus*, *A. flavus* and *A. parasiticus*). Fifty percent methanolic extract of leaves *Camellia sinensis* showed greater antibacterial activity against *Bacillus cereus* and followed by *Pseudomonas aeruginosa*. As the concentration of the extract was increased (10, 25, 50 and 100 mg mL^{-1}) the zone of inhibition was found to increase in the significant manner ($p>0.001$). No activity was seen in case of both the fungal species. In some cases the highest concentration (100 mg mL^{-1}) of extract was found to be much more effective than that of the antibiotic.

Table 3 shows the Minimum Inhibitory Concentrations (MIC) of the 50% methanolic extract of *Camellia sinensis* against *E. coli* and *B. cereus*. The MIC was 100 mg mL^{-1} for *E. coli* and 10 mg mL^{-1} for *B. cereus*.

Table 1: Zone of inhibition of bacterial growth on culture media by varying concentration of 50% methanolic extract of *Camellia sinensis* by agar well diffusion methods

		10	25	50	100
Conc. of extract	Antibiotic	---------- (mg mL^{-1}) ----------			
E. coli	12.66±0.36	3.33±0.55[a]	5.66±1.38[a]	9.5±0.48[a]	10.00±0.96[a]
B. cereus	23.00±0.36	13.00±0.36[a]	16.33±0.55[a]	20.00±0.36[a]	23.66±0.76[a]
P. aeruginosa	23.66±0.55	11.55±0.54[a]	13.66±0.55[a]	19.66±0.55[a]	22.83±0.73[a]
S. aureus	10.66±0.55	3.33±0.55[a]	6.33±0.76[a]	9.16±0.27[a]	11.83±0.45[a]
A. flavns	Nil	Nil	Nil	Nil	Nil
A. parasiticns	Nil	Nil	Nil	Nil	Nil

Values are Mean±SEM; n = 6. Significant at the level:As compared to antibiotic control: [a]p<.001 (Student's t-test)

Table 2: Zone of inhibition of bacterial growth on culture media by varying concentration of 50% methanolic extract of *Camellia sinensis* by paper disc diffusion method

Conc. of extract	Antibiotic	10	25	50	100
		---------- (mg mL^{-1}) ----------			
E. coli	21.33±0.55	3.16±0.45[a]	6.16±0.45[a]	11.16±0.38[a]	15.00±0.73[a]
B. cereus	25.86±0.67	13.83±0.45[a]	16.66±0.76[a]	18.43±0.51[a]	25.66±0.76[a]
P. aeruginosa	21.33±0.55	11.00±0.73[a]	16.33±0.55[a]	20.16±0.38[a]	22.50±0.18[a]
S. aureus	14.33±0.55	3.66±0.55[a]	5.91±0.34[a]	8.38±0.35[a]	12.00±0.73[a]
A. flavns	Nil	Nil	Nil	Nil	Nil
A. parasiticns	Nil	Nil	Nil	Nil	Nil

Values are Mean±SEM; n = 6. Significant at the level: As compared to antibiotic control: [a]p<0.001 (Student's t-test)

Table 3: Minimum Inhibitory Concentrations (MIC) of the 50% methanolic extract of *Camellia sineusis* against *E.coli* and *B. cereus*

Conc. of extract	Volume of extract (mL)	Volume of nutrient broth (mL)	Volume of culture suspension (mL)	Growth	
				E.coli	*B. cereus*
Antibiotic (positive control)	2	10	0.1	----	----
d. H20 (negative control)	2	10	0.1	++	++
10 mg mL^{-1}	2	10	0.1	++	-- --
25 mg mL^{-1}	2	10	0.1	++	-- --
50 mg mL^{-1}	2	10	0.1	++	-- --
100 mg mL^{-1}	2	10	0.1	-- --	-- --

Minimum inhibitory concentration (*E. coli*)-100 mg mL^{-1}. Minimum inhibitory concentration (*B. cereus*) - 10 mg mL^{-1}. ++: Normal turbidity; ----: Complete inhibition (min. inhibitory conc)

Table 4: Preliminary screening of secondary metabolites of the 50% methanolic extract of *Camellia sinensis*

Secondary metabolite	Name of the test	Methanolic extract
Alkaloids	Dragendorff's test	++
Flavonoids	Shinoda test	+++
Catechins	Phloroglucinol test	++++
Tannins	$FeCl_3$ test	++
Saponins	Foam test	+
Sterols	Salkowski test	++
Glycosides	Molisch test	+
Phenols	Phenol test	++

+: Low concentration; ++: Moderate concentration; +++: High concentration; ++++: Very high concentration

Table 5: Rf values (in cm) of the spots obtained in TLC by the 50% methanolic extract of *Camellia sinensis*

Spots	Rf values of alcoholic extract	Proposed bioactive components
1	0.210±0.008	Queretin (flavanol aglycone)
2	0.240±0.002	Equisporol (flavonol aglycone)
3	0.480±0.002	Kaempferitin (flavanol glycosides)
4	0.680±0.004	Catechin (flavone aglycone)
5	0.830±0.002	Quercitrin (flavanol glycosides)
6	0.917±0.001	Morin (flavonol aglycone)

Table 4 shows the preliminary screening of secondary metabolites present in the 50% methanolic extract of *Camellia sinensis*. It was found that the extract was having high concentration of catechin followed by flavonoids. Other than these alkaloids, tannins, saponins, sterols and phenols were found to be present in the extract.

Table 5 and Fig. 1 explains the retention factor (Rf) in cm, obtained in TLC of the crude methanolic extract of *Camellia sinensis*. Queretin, equisporol , kaempferitin, epicatechin, quercitrin and morin were detected in the crude extract. The spot 4 (epicatechin) obtained in TLC was scrapped and subjected to bioassay-guided fractionation with the help of column chromatography using a stepwise gradient with Chloroform : Methanol mixture (1:9 to 9:1).

Table 6 shows the screening of secondary metabolites of the fractions obtained from column chromatography of the extract of *Camellia sinensis*. In all the fractions high

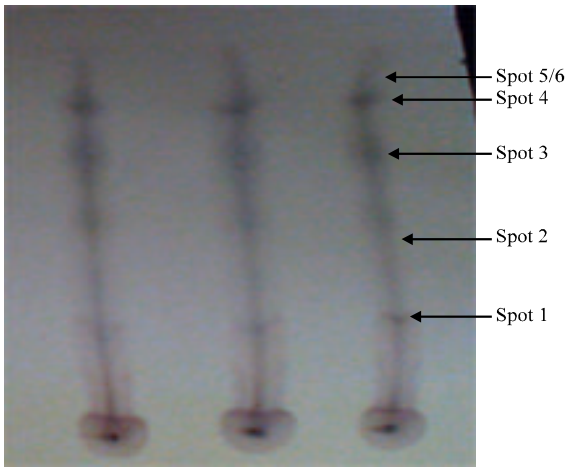

Fig. 1: TLC of the 50% methanolic extract of *Camellia sinensis*

Table 6: Screening of secondary metabolites of the fractions obtained from column chromatography of the 50% methanolic extract of *Camellia sinensis* for different bioactive components

$CHCl_3$:MeOH gradient	Alkaloid	Flavonoid	Catechin	Tannin	Saponin	Sterol	Glycosides	Phenols
1:9	+	+	++	-	-	-	+	-
2:8	+	+	+++	-	-	-	+	-
3:7	-	+	+++	-	-	-	++	+
4:6	-	++	++	-	+	-	++	-
5:5	+	++	+++	-	-	-	++	+
6:4	+	++	++++	+	+	+	+++	+
7:3	+	+++	++++	+	+	+	+++	++
8:2	+	++++	++++	++	-	++	++++	++
9:1	++	++++	++++	++	++	++	++++	++

+: Low concentration; ++: Moderate concentration; +++: High concentration; ++++: Very high concentration; -: Negligible concentration

Table 7: Bioassay guided antimicrobial activity of *E. coli* by paper disc diffusion method of the 50% methanolic extract of *Camellia sinensis*

$CHCl_3$:MeOH gradient	Methanolic extract
1:9	7.16±0.27
2:8	7.00±0.32
3:7	8.60±0.19
4:6	9.83±0.20
5:5	11.50±0.18
6:4	13.30±0.23
7:3	15.40±0.19
8:2	17.56±0.18
9:1	23.40±0.19

concentration of catechins were found to be present. As the concentration of chloroform was increased, more and more separation of the bioactive components was achieved. Table 7 shows the bioassay guided antimicrobial activity of *E. coli* by paper disc diffusion method of the methanolic extract of *Camellia sinensis*. It was observed that the antimicrobial activity of the extract increased with the increase in the concentration of chloroform.

Finally, the fraction which gave us the best result was further taken for the HPTLC for the identification of the bioactive compound which was identified as catechin (Fig. 2a, b and 3).

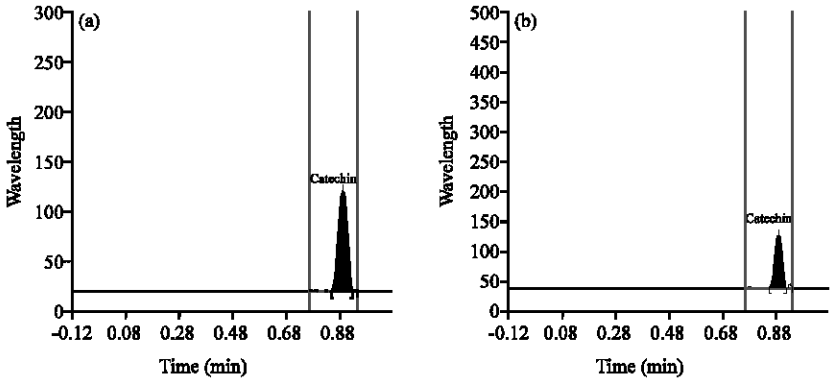

Fig. 2: HPTLC of the 50% methanolic extract of *Camellia sinensis* (a) Std. Catechin and (b) Crude

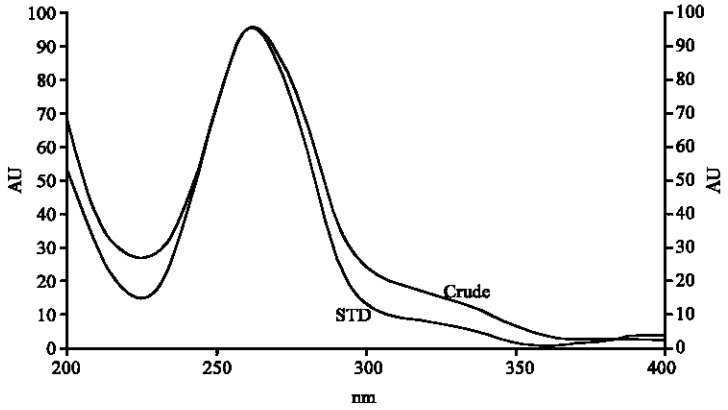

Fig. 3: Linearity between the standard catechin and catechin from crude extract of *Camellia sinensis*

DISCUSSION

According to World Health report of Infectious diseases 2000, overcoming antibiotic resistance is the major issue of WHO for the next millennium. Hence the last decade witnessed an increase in the investigation of plants as a source of human disease management. In the present experiment 50% methanolic extract of *Camellia sinensis* leaves (Green tea) showed broad spectrum of antibacterial activity and catechin was found as the bioactive natural product in it, which may serve as leas for the development of new pharmaceuticas that address hither unmet therapeutic needs. Such screening of various natural organic compounds and identifying bioactive components is the need of the hour, because successful prediction of the lead component and drug like properties at the onset of drug discovery will pay off later in drug development. In conclusion, the present extract of *Camellia sinensis* showed anti-microbial activity of broad spectrum. The anti-microbial activities are due to the presence of identified catechins. Tea extracts have been shown to possess inhibitory effect against an array of pathogenic micro-organisms (Shetty *et al*., 1994; Toda *et al*., 1989b; John and Mukundan, 1979).

The effects of catechins on membrane fluidity were studied by a fluorescence polarization method using liposomes prepared with dipalmitoyl phosphatidylcholine and dioleoylphosphatidylcholine to assess their pharmacological mechanism at μmol L^{-1} levels found in human body fluids after clinical application. All eight catechins tested, ranging from 1 to 1,000 μmol L^{-1}, significantly reduced membrane fluidity in both hydrophilic and hydrophobic regions of lipid bilayers. Catechin gallate esters were superior in fluidity reduction to the corresponding nonesters. Catechin shows antibacterial activity particularly affecting the membrane fluidity in both, hydrophilic and hydrophobic regions of lipid bilayers of the microorganism. The antibacterial activities of catechins were predominantly related to the gallic acid moiety and the hydroxyl group member (Hironori, 1999; Nance *et al*., 2006). The mode of action of catechin involves including rapid leakage of small molecules entrapped in case of intraliposomal space and aggregation of the liposomes have been reported earlier. Catechins also show antibacterial activity by inhibiting the action of DNA polymerases (Jane, 2001). The antibacterial activity of the extract is also may be because of flavonoids. Early report suggests when flavonoid in combination with morin and rutin, were evaluated, based on the minimum inhibition concentration (MIC) in a liquid culture, by using *Salmonella enteritidis* and *Bacillus cereus* as the test bacteria it shows positive result against the bacteria (Marderosian, 1999). Morin was also detected in the present extract. Hence, we can conclude that the antimicrobial activity of the extract is mainly because of the presence catechins and flavonoids in it.

The two methods (ZOI and MIC) used to test the antibacterial activity of the leaf extract proved to be excellent, but the paper disc diffusion method tend to show wider zones of inhibition than the agar-well diffusion method.

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