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EXTRACTION OF FLAVONOIDS FROM GREEN TEA AND ITS *IN VITRO* EFFICACY AGAINST SOME SELECTED MICROBES

ARNAB DEB[1], RAJESH P JAYASWAL[1] AND PRANAY PUNJ PANKAJ[2*]

[1]Lovely Faculty of Applied Medical Sciences, Lovely Professional University, Punjab, India-144402;
[2]Department of Zoology, Nagaland University, Lumami, Nagaland, India-798627

ABSTRACT

Green tea (*Camellia sinensis*) consists of various bioactive components which is useful in new kind of formulation to treat the human diseases. Micro-organisms became resistant to varieties of antibiotics so natural herbal remedy is needed to combat the microbial infections. In the present study, polyphenols such as MQK (myricetin, quercetin, kaempferol) and EGCG (epigallocatechingallate) and C (catechin) were extracted from green tea (*Camellia sinensis*) leaves and tested against test bacteria and fungi. Different concentrations of these polyphenols were examined for antibacterial activities by disc diffusion method. Antimicrobial assay showed significant zone of inhibition against *S. aureus* (Gram positive bacteria), *P. vulgaris* (Gram negative bacteria), *Cryptococcus sp*.(fungus) and *Penicilium sp.* (fungus). Catechin showed broad spectrum antibacterial and antifungal activities. Minimum inhibitory concentration (MIC) of catechin was 3.12 $mgdl^{-1}$ against *S. aureus*, *P. vulgaris* and *Cryptococcus sp*. whereas at 6.25 $mgdl^{-1}$ inhibited *Penicilium sp.* MQK and EGCG were especially effective against *Cryptococcus sp* and *Penicilium sp*.which gave MIC value of 6.25 $mgdl^{-1}$. EGCG at same concentration was also effective against *P. vulgaris.* This study suggests the therapeutic use of green tea against bacterial as well as fungal infections.

KEYWORDS: Green tea, Epigallocatechingallate, Catechin, Antibacterial activity, Antifungal activity, Disc diffusion method.

DR. PRANAY PUNJ PANKAJ
Department(s) and institution(s): Department of Zoology, Nagaland University, Lumami, Nagaland, India-798627

INTRODUCTION

Green tea (*Camellia sinensis*) is the most popular energy drink worldwide. *Camellia sinensis* (CS) has been accepted as an important healthy beverage since ancient times but recently, a great attention has been given among scientific community because of its antioxidant properties.[1] CS leaves have nutritional value due to the presence of amino acids, vitamins (C, E and K), polysaccharides, caffeine and other chemical constituents including flavonoids which are important to human health. Flavonoids usually act on broad spectrum of microbes such as bacteria, fungus and some parasites as well. CS contain bioactive polyphenol compounds that has numerous medicinal value such as antimicrobial[2], antifungal[3], anticancer[4], imunomodulatory[5], anti-HIV effects[6,7], enhancing the activity of insulin[8], protecting cardiovascular[9] and genital diseases[10] and stopping cerebral ischemic damage.[11] Previous studies suggests that CS consumption imparts immunity against intestinal disorders, improve microflora in the intestine, protect the cell membranes from oxidative damages, prevent dental caries due to presence of fluorine, treat against peritonitis in rat model with *Moringa oleifera*[12], burn excess fats[13], normalize blood pressure and prevent coronary heart diseases[14]. Tea also possesses germicidal and germ static activities against various Gram-positive and Gram-negative human pathogenic bacteria such as *Vibrio cholera*, *Salmonella sp., Clostridium sp.*[15] CS leaves are highly rich in polyphenolic compounds (myricetin, kaempferol and quercetin) and Catechins such as Epicatechins (EC), Epigallocatechin (EGC), Epicatechingallate (ECG), Epigallocatechingallate (EGCG), Catechin (C) and Gallo Catechin (GC). Their concentrations are influenced by season, climate, age of the tea leaf and horticultural area and processing technique used.[16, 17] EGC has been shown anti-microbial effects on *E. coli* which suggests that ingesting GT could have beneficial to UTI (Urinary Tract Infection) patient.[18] Synergistic activities of green tea (GT) with commercial antibiotic chloramphenicol have displayed potent action against enteropathogens.[19] Similarly, chloramphenicol and tea extract in combination has been recognized to inhibit the growth of *S. dysenteriae*, *A. fumigatus* and *C. albicans* more effectively. In similar studies, tea extract showed synergistic anti-microbial activity along with other antibiotics like gentamycin, methicillin and nalidixic acid against test strains.[20] Tea extract has also showed synergistic activity with other antibiotics like gentamycin, methicillin and nalidixic acid against test strains. Growth inhibition of *S. typhimurium* 1402/84, *S. typhi* Ty2a, S. *dysenteriae*, *Y. enterocolitica* C770, and *E. coli* (EPEC P2 1265) have already been documented.[21] Generally, antibiotics control the microbial infection by killing or by managing the multiplication rate of the microbes but almost all microbes retain resistant power against the antibiotic drugs and become complete resistant after prolonged treatment of the antibiotic drug so there is a great surge for the formulation of new kind of antimicrobial drug of herbal origin to combat with microbial infection and prevalence of multiple drug resistance (MDR).[22] It is one of the best liked energy drink worldwide and has no side effect if taken in moderate amount. In the present endeavor, it has been attempted to extract some of an important flavonoids such as myricetin, querectin, kaempferol, epigllocatechingallate and catechins from *Camellia sinensis* and investigated *in vitro* efficacy against some human pathogens

METHODS

Procurement of GT leaves

Camellia sinensis leaves in dried form were brought from Darjeeling and West Bengal territory of India during February' 2014. Tea leaves were dried under shade at 40°C. The leaves were crushed to form powder using mortar & pestles.

1. Extraction of various flavonoids from GT

Flavonoids specially Myricetin, Querectin and Kaempferol (MQK), Epigallocatechingallate (EGCG) and Catechin were extracted from GT for controlled studies.

2. Preparation of powder form of MQK, EGCG and C extract

Extract solutions of MQK, EGCG and C were sprayed in vacuum chamber at high temperature (higher than the boiling point of solvent and lower than the melting point of the solute) to obtain powder form of each extract.

2.1 Chemicals required

All the chemicals used for extraction and *in vitro* analysis of flavonoids were of analytical grade, availed from Hi-media Pvt. Ltd. India.

2.2 Extraction of Myricetin, Querectin and Kaempferol (MQK)

One gram of GT powder was mixed with 40 ml of 60% aqueous ethanol and 5 ml of 6M HCl. The solution was refluxed at 95°C for 2 hours and then filtered using filter paper (0.45 µm). Spectroscopic analysis was done using UV-Visual Spectrophotometer for their purity and then preserved for further analysis.

2.3 Extraction of Epigallocatechingallate (EGCG)

20 gm of GT leaves were homogenized using 200 ml of distilled water at room temperature and then filtrate was obtained under reduced pressure. The filtrate was preserved for further analysis.

2.4 Extraction of Catechin*(C)*

5 gm of GT leaves were transferred to 500 ml round bottom flask containing 250 ml of distilled water which was fitted with reflux condenser, thermometer and a glass stopper. The extraction was performed by stirring the mixture at specified time intervals from 5 min to 240 min. The extract was filtered through 0.45 µm membrane filter, after that filtrate was washed with 0.90 ml of chloroform for at least three times followed by 0.90 ml of ethyl acetate for three times. Ethyl acetate phase was dried by evaporation process. Finally the content was dissolved in 0.30 ml of methanol. The mixture was then preserved for further analysis.

3. *Preparation of paper disc containing various flavonoids*

Powder form of MQK, EGCG and C were diluted with ethanol in different concentrations ranging from 1.56 mg/dl to 100mg/dl. Sterilized paper disk was soaked in each dilution for overnight at 4°C; after that each disc was dried and stored for further studies.

4. *Determination of antimicrobial activities*

Following test organisms such as *S. aureus* (Gram positive bacteria), *P. vulgaris* (Gram negative bacteria), *Cryptococcus sp.* (fungus) and *Penicilium sp.* (fungus) were taken to evaluate antimicrobial activity of MQK, EGCG and C. Control antibiotics used were Erythromycin (30 mg/dl), Amikacin (30 mg/dl) and Fluconazole (10 mg/dl), which are generally considered to be highly active against Gram positive bacteria, Gram negative bacteria and fungi respectively. Various antibiotics and culture media used in this study were acquired from Hi-media Pvt. Ltd. India.

5.1 Antibiotic susceptibility testing (AST) by Kirby Bauer method

S. aureus, *P. vulgaris*, *Cryptococcus sp*and *Penicilium sp.* were inoculated separately in 4-5 ml broth and incubated for 2-8 hours at 37°C until the turbidity of the suspension reaches at 0.5% McFarland standards. Sterile nutrients agar plates were inoculated with 100 µl of fresh bacteria and (Potato Dextrose Agar) PDA plates with fungi. The culture suspension was evenly spread on entire surface of the plate. The prepared paper discs containing different extract and control antibiotics discs were placed on the agar plates by using a sterile forceps. The discs were pressed firmly against the agar surfaces and then incubated overnight at specified temperature. Growth pattern and zone of inhibition (ZOI) around each disc were measured and recorded.

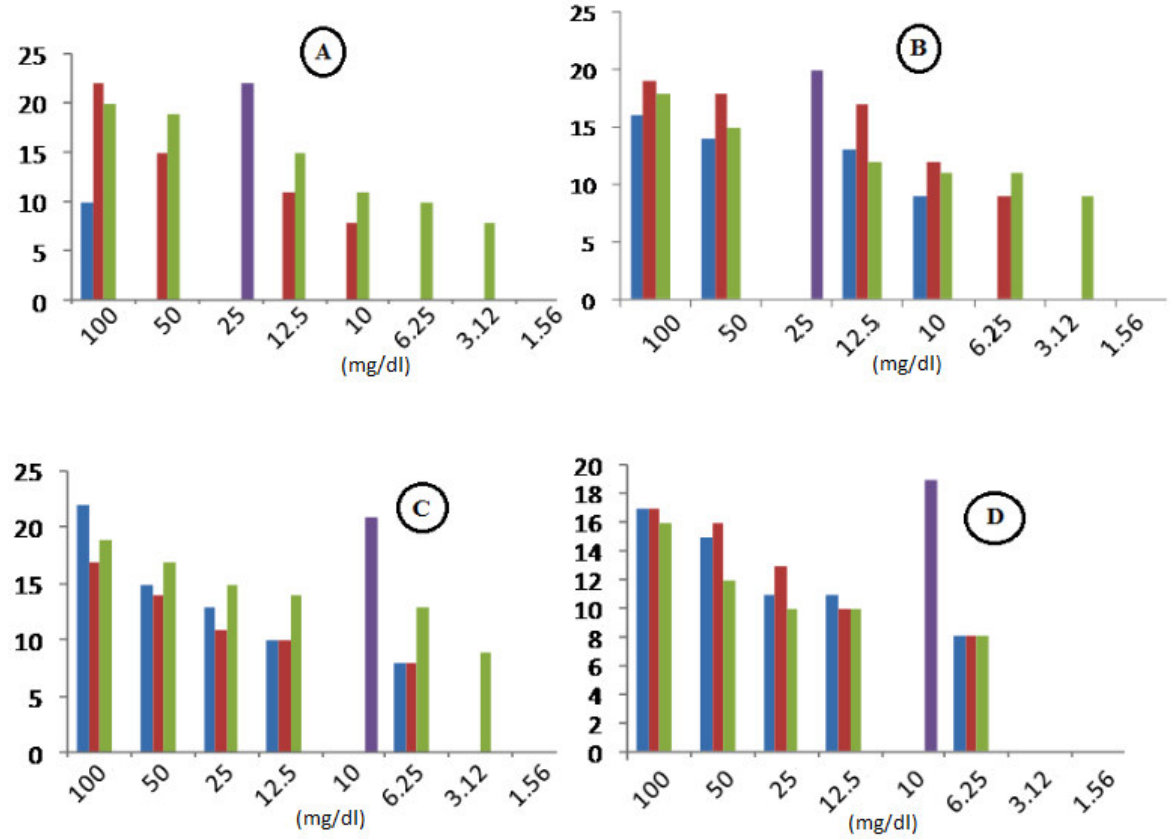
RESULTS

Natural products have been recognized with increasing interest these days by clinical pharmacologists due to their antimicrobial, antifungal, antiviral, antiprotozoal and antihelminthic effects. GT is one of the most popular natural products having been documented with many health benefits and medicinal value. The present investigations include the extraction of flavonoids from GT leaves to carry out *in vitro* efficacy against some human pathogen (Table 1-2). The extracts were partially purified using chemical treatment and filtration. In this work, antibacterial and antifungal activity of GT flavonoids against two selected bacterial pathogens (*S. aureus* and *P. vulgaris*) and two selected fungal pathogens (*Cryptococcus sp.* and *Penicilium sp.*) were investigated *in vitro*. A correlation of antimicrobial activities between herbal individual flavonoids and antimicrobial drugs were compared (Fig 1A-1D). Antibacterial activity against Gram positive and Gram negative bacteria were shown by all the extracts. Quercetin, kaempferol and myricetin produced low activity against *S. aureus*. It produced zone of inhibition at 100 mg/dl concentration but it show much better antimicrobial activity against Gram negative bacteria and fungus. Zone of inhibition were found at concentration ranging between 100 mg/dl to 12.5 mg/dl against the Gram negative bacteria and 100 mg/dl to 6.25 mg/dl against fungus (Table 1-2). EGCG and catechin have shown antibacterial activity against all bacteria and fungus. Zone of inhibition were recorded by EGCG at concentration from 100 mg/dl to 12.5 mg/dl against Gram positive bacteria and from 100 mg/dl to 6.25 mg/dl against Gram negative bacteria as well as fungus by both EGCG and catechin (Fig*1A-1D).*

Table 1
Comparison of molecular weight, molecular formula, melting point and boiling point of various ingredients of CS

Bioactive components of CS	Molecular Weight	Melting Point	Boiling Point (at 760 mmHg)	Molecular Formula
Epicatechin (EC)	290.27	240 °C	~630.4 °C	$C_{15}H_{14}O_6$
Epigallocatechin (EGC)	306.27	>200 °C	685.63 °C	$C_{15}H_{14}O_7$
Epicatechingallate (ECG)	442.38	255 °C	861.67 °C	$C_{22}H_{18}O_{10}$
EpigallocatechinGallate (EGCG)	458.37	212 °C	909.11 °C	$C_{22}H_{18}O_{11}$
Catechin (C)	290.27	200 °C	630.38 °C	$C_{15}H_{14}O_6$
Gallocatechin (GC)	306.27	189 °C	909.1 °C	$C_{15}H_{14}O_7$
Myricetin	318.24	300 °C	747.63 °C	$C_{15}H_{10}O_8$
Kaempferol	286.24	276-278 °C	582.08 °C	$C_{15}H_{10}O_6$
Quercetin	338.27	>300 °C	642.4 °C	$C_{15}H_{10}O_7$

Figure 1
Zone of inhibition against extract and known antimicrobial drugs



Zone of inhibition (mm) are expressed in Y-axis whereas concentration (mg/dl) of extract and antimicrobial drug are represented in X axis. (A): Test microorganism-S. aureus against Extract and Erythromycin. (B): Test microorganism- P. vulgaris against Extract and Amikacin. (C): Test microorganism- Cryptococcus sp. against Extract and Fluconazole. (D): Test microorganism-Penicilium sp. against Extract and Fluconazole.

Table 2
Antimicrobial activity of Myricetin, Querectin and Kaempferol (MKQ), Epigallocatechingallate (EGCG) and Catechin (C) extracted from Camellia sinensis against specific Gram positive bacteria, Gram negative bacteria and fungi.

Test Microorganisms	MIC ($mgdl^{-1}$)			Zone of inhibition (mm) at different concentration ($mgdl^{-1}$) of MQK (M), EGCG(E) and Catechin (C)																			Activity against known antibiotics		
	M	E	C	100			50			25			12.5			6.25			3.12			1.56			Antibiotics used	ZOI (mm)	MIC ($mgdl^{-1}$)
				M	E	C	M	E	C	M	E	22	30	E	C	M	E	C	M	E	C	M	E	C			
S. aureus	100	12.5	3.12	10	22	20	R	15	19	R	11	20	30	08	11	R	R	10	R	R	08	R	R	R	Erythromycin	22	30
P. vulgaris	12.5	6.25	3.12	16	19	18	14	18	15	13	17	21	10	12	11	R	09	11	R	R	09	R	R	R	Amikacin	20	30
Cryptococcus sp.	6.25	6.25	3.12	22	17	19	15	14	17	13	11	19	10	10	14	08	08	13	R	R	09	R	R	R	Fluconazole	21	10
Penicilium sp.	6.25	6.25	6.25	17	17	16	15	16	12	11	13	10	11	10	10	08	08	08	R	R	R	R	R	R	Fluconazole	19	10

M - Myricetin, Querectin and Kaempferol , E - Epigallocatechingallate and C - Catechin

DISCUSSION

The results suggested that consumption of GT can cure a patient suffering from bacterial infections caused by *S. aureus* and *P. vulgaris* as well as fungal infections due to *Cryptococcus sp*. and *Penicilium sp*. Most of the research results have already claimed that GT has powerful antimicrobial property against *V. cholera*, *Salmonella sp.*, *Clostridium sp.*[15], *E. coli*[18], *L. monocytogenes*[23], *S. dysenteriae*, *A. fumigatus*, *C. albicans*, *S. typhimurium* 1402/84, *S. typhi* Ty2a, S. *dysenteriae*, *Y. enterocolitica* C770 and *E. coli* (EPEC P2 1265)[21], *C. albicans*[3] at pH 7 and *H. pylori*[24]. Such findings proved that apart from nutritional (beverage) value, GT has also potential to protect from several infectious diseases. It is hoped that considering the therapeutic use of GT can help from various side effects due to consumption of antibacterial, antifungal and parasitic drugs[3]. The research work will further motivate other researchers to carry out determining the antimicrobial capabilities of GT against all other infectious diseases. For this purpose, the present study will serve as benchmark for all curious researchers to study, assess and compare the results so that GT can be incorporated as antimicrobial compounds in the future.

CONCLUSION

In the present study, *Camellia sinensis* leaves were extracted and partially purified to get flavonoids such as quercetin, kaempferol and myricetin; EGCG & catechin in enough concentration to carry out the experiments. The flavonoids were reported for its antimicrobial activity. Among these flavonoids catechin has shown maximum antimicrobial activity than epigallocatechingallate and then followed by the crude extract of quercetin, kaempferol and myricetin. The study suggested that in order to control the pathogenic infection and manage the problem of drug resistance, green tea can be used as a therapeutic agent with no side effect if consumed moderately.

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