



Antibacterial activities of green tea crude extracts and synergistic effects of epigallocatechingallate (EGCG) with gentamicin against MDR pathogens



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ABSTRACT

Plant extracts and their purified compounds were examined for synergistic antimicrobial activity using selected multi-drug resistant (MDR) pathogens. The study aims to investigate the antibacterial activity of green tea (*Camellia sinensis*) and its purified compound epigallocatechingallate (EGCG). The synergistic relation of the compound with antibiotic was detected against selected potential Gram positive and Gram negative pathogens. *Staphylococcus aureus* and *Escherichia coli* were used as test pathogens which were resistant to different groups of antibiotics. After collection of fresh green tea leaves, samples were washed and air dried. EGCG is one of the bioactive compounds and was separated from tea plant. Antibacterial activity of EGCG and crude extracts of green tea were done by microdilution method (minimum inhibitory concentration and minimum bactericidal concentration). The synergistic effect of EGCG and gentamicin was determined. MIC value of green tea extract was found at 125 µg/mL in case of MDR *E. coli*, MDR *S. aureus* and their reference strains and MBC at 500 µg/mL against *S. aureus*. No MBC value was found against *E. coli*. EGCG showed better activity on Gram positive pathogen compared to that of Gram negative. MBC value of this compound was 1250 µg/mL for *E. coli* where 625 µg/mL for *S. aureus*. Strong synergistic relation (FICI 0.325) was found against pathogens in the combination of EGCG with gentamicin. The purified EGCG compound of green tea has great synergistic effect against MDR pathogens. More investigation is needed to know the inhibitory effect of these plant extracts and their components.

1. Introduction

Infectious diseases are the third most significant reason of mortality around the world. The burden of infectious diseases is high in developing countries. This is because of the emergence of multi-drug resistant pathogens due to poor health-care facilities, and over-the-counter availability and misuse of antimicrobial agents (Vincent et al., 2009).

World Health Organization (WHO) has urged the search for new antimicrobial compounds and natural bioactive compounds which can be a good candidate in this perspective. Despite the fact that many new drugs and technologies have been developed to combat the infectious diseases, these have continued to be global health challenges. The use of conventional antimicrobial agents against these infections is always

associated with problems such as the development of multiple drug resistance and adverse side effects. In some cases, the use of synergistic antibiotic drug combination with bioactive compounds is the only option for the treatment of multi-drug resistant (MDR) bacteria (Bag and Chattopadhyay, 2015).

The discovery and development of medicinal plants as drugs, especially from China, India and some African countries has proven effective in the treatment of multi-drug resistant patterns among clinical and environmental isolates. The primary benefits of using plant-derived medicines are that they are safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment (Ojo et al., 2013). In fact, scientific evidence supports the hypothesis that several plants are composed of biologically active chemical entities and several

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drugs in modern day medicine are actually analogues of plant origin substances (Cowan, 1999). It was thought that some of the plant extracts may display good inhibitory effects against pathogenic bacteria and therefore, may support in the development of antimicrobials supplements. For example, green tea extract from the leaves of *Camellia sinensis* (green tea) has been shown to have a wide range of antimicrobial activity due to the presence of high content catechin specially epigallocatechingallate, EGCG (Song and Seong, 2007).

Green tea contains relatively large amounts of polyphenols, mainly catechins and their derivatives, considered to exert a protective effect against cancer and cardiovascular diseases (Gramza et al., 2005). The combined use of tea and antibiotics may be effective in inhibiting emerging drug-resistance pathogens especially among enteropathogens. Previous works (Toda et al., 1989) showed that moderate daily consumption of green tea killed *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Clostridium perfringens*, *Bacillus cereus*, *Pleisomonas shigelloides*, etc. However, there is limited knowledge about the synergistic effect of the green tea crude extract and its purified products with commercially used antibiotics against MDR bacteria.

In this study, we aimed to determine the antibacterial activity of green tea extract and EGCG against pathogenic bacteria. Then we also want to investigate the synergistic effects of purified green tea bioactive compound EGCG with antibiotic gentamicin.

2. Materials and methods

2.1. Isolation, identification and antibacterial sensitivity test of pathogens

Bacteria were isolated from the clinical samples collected from Dhaka Shishu Hospital, Bangladesh. Clinical isolates were shown to be MDR than reference strain. After isolation, pathogens were identified by standard biochemical methods (Cappuccino and Sherman, 1996). Biochemical tests such as oxidase, catalase, citrate, Kligler iron agar (KIA), indole, MR and VP were performed to identify the isolates. The antibiotic resistance was determined by standard Kirby-Bauer disk diffusion method. These specific bacterial isolates were selected because they are responsible for causing serious infections in human and resistant to a wide variety of antibiotics (Clinical and Institute, 2009).

2.2. Sampling of green tea and crude extract preparation

The fresh, healthy and mature leaves of different tea plants were collected from tea garden. Collected samples were washed, air dried and ground to fine powder.

Ten grams (10 g) of the dried powdered plant samples were soaked in 100 mL of hot distilled water and other organic solvents (e. g. n-hexane, chloroform, ethanol, methanol) in different beakers (1:10 w/v) and left undisturbed for 48–72 h. Then the solution was filtered using sterile filter papers (Whatman No.1 filter paper). The filtrates were evaporated on water bath up to a semisolid mass and air dried (Gumgumjee et al., 2012). They were weighed and stored in sterile labeled containers and kept in the refrigerator at 4 °C until further for use.

For the preparation of dilution of crude extracts of the plant for antibacterial assay, the extracts were reconstituted using distilled water for aqueous extract and DMSO for the organic extracts.

2.3. Preparation and separation of epigallocatechin from green tea plant

After collecting the plant materials, it was washed 2–3 times with tap water and finally with distilled water. Initially, catechins compounds from green tea were extracted by centrifuging with distilled water at 50 °C at 300 rpm for 4 hour. A 5g amount of dry grounded green tea leaves were weighed and placed in a 500 mL triangle flask with 150 mL distilled water. Then, the extract was filtered and concentrated to 30 mL with a rotary evaporator.

For separation, the extract was partitioned with an equal volume of chloroform to eliminate impurities. Catechins were extracted from the water layer with an equal volume of ethyl acetate. To isolate epigallocatechingallate (EGCG) from green tea, a C18 reversed-phase preparative column (250*22 mm) packed with packings (15 μm) with the mobile phase of 0.1% acetic acid in water/acetonitrile, 87/13 vol. % was used. The injection volume and the flow rate of mobile phase were 15 μL/min and 1 mL/min, respectively. The effluent was collected from the column outlet and concentrated to 1 mL for HPLC-analysis. The injection volume was 20 μL and the experimental conditions of mobile phase composition and flow rate were equal to those in the preparative column. In preparative HPLC, the composition of mobile phase was changed in order to improve resolution by adjusting retention times of EGCG.

From 5g of dry grounded green tea leaves stock of EGCG was prepared with 0.01g EGCG and 1mL Distilled water. Then working solution was prepared with 1mL EGCG and 1mL DW. To recheck and confirm our data, we repeated the experiments with commercially purchased EGCG from Sigma- Aldric, USA. We are now working on the synergistic effects of other catechins with gentamicin (data not shown).

2.4. Antibacterial activity of green tea plant crude extracts, EGCG and antibiotics

Antibacterial activity of EGCG, crude extracts of green tea and antibiotic (Gentamicin) were evaluated by microdilution method (MIC and MBC) (Bag and Chattopadhyay, 2015). Gentamicin was chosen because, it is a potent antibiotic and being used for the treatment of patients with a wide range of infections caused by Gram positive and Gram negative bacteria. Besides antimicrobial resistance against this antibiotic has become a common problem. One hundred μL of nutrient broth was dispensed into each well of the 96-well plate. A 200 μL from the stock solution of test extracts was added into the first row of the plate. Then, twofold serial dilutions were performed. Bacterial culture in nutrient broth was prepared and density was compared with 0.5 McFarland solution. One hundred μL of the bacterial suspension was added to each well. A negative control was prepared with only media and a positive control was prepared with the bacterial culture and media. Then the test plates were incubated at 37 °C for 18 h. The lowest concentration at which no growth or turbidity was seen which indicates the minimum inhibitory concentration (MIC). To determine minimum bactericidal concentration, the MIC and at least two concentrated dilutions were given in nutrient agar plate (10 or 7 μL). After incubation, the minimum concentration at which no visible growth found was recorded as the MBC.

2.5. Synergistic effect of purified compound EGCG with antibiotics

After determining the MIC of individual extracts and antibiotic, their synergistic effect were determined by microdilution checkerboard method (Rand et al., 1993; Bag and Chattopadhyay, 2015). All these experiments were done in triplicate.

Antimicrobial activity of purified compound in the presence of different antimicrobial agent, such as gentamicin was determined. Purified compound of green tea, EGCG was tested against multi-drug resistant *E. coli* and *S. aureus*. Concentrations of EGCG extracts ranged from 4.75 μg/mL to 2500 μg/mL. The antimicrobial activity of the extract and antibiotic combination was interpreted as one of the following categories: Synergy; indifferent; additive effect; or antagonism. The fractional inhibitory concentration (FIC) of EGCG extract or gentamicin was calculated as the MIC of the EGCG/gentamicin in combination, divided by the MIC of the EGCG/gentamicin alone. Then the fractional inhibitory concentration index (ΣFIC) would be FIC index = FIC of EGCG extract + FIC of Gentamycin. The FICI results were interpreted as follows: < 0.5 synergy; 0.5 to 1 additive effect; 1–2 indifferent or no effect; and >2 antagonism (Ahmad et al., 2014). Fifty μL of nutrient broth was added in

all wells. One Hundred μL of the EGCG extracts (0.005 g/mL) was added to each wells of column 1 and 100 μL of stock solution of the antibiotic (0.001 g/mL) of gentamycin was added to each wells of row A. Then serially dilution was done of these two agents. Bacterial suspension was added to each well except negative control. Plates were incubated at 37 $^{\circ}\text{C}$ for 18 h. FIC was determined from the lowest concentration of antibiotic and purified compound in combination in which no visible growth of the test organisms was seen.

3. Results

3.1. Antibacterial activity of *Camellia sinensis* (green tea) crude extract

Antibacterial activity of *Camellia sinensis* (green tea) crude extract was determined against multi-drug resistant pathogens. In this study multi-drug resistant *E. coli* and *S. aureus* and their reference strains were used for screening antibacterial activity of green tea. MDR *E. coli* and *S. aureus* were resistant to ceftaxitin, ciprofloxacin, chloramphenicol, clindamycin, oxacillin, tetracycline, gentamicin, ceftazidime and vancomycin.

Green tea crude extract showed minimum inhibitory concentration at 125 $\mu\text{g}/\text{mL}$ for *E. coli* ATCC25922, multi-drug resistant *E. coli*, *S. aureus* ATCC25923, multi-drug resistant *S. aureus*. Green tea crude extract showed minimum bactericidal concentration at 500 $\mu\text{g}/\text{mL}$ against *S. aureus* ATCC25923, multi-drug resistant *S. aureus* where no MBC result was found against *E. coli* ATCC25922, multi-drug resistant *E. coli* (Table 1).

3.2. Antibacterial activity of purified EGCG compound and antibiotic

EGCG showed better antimicrobial activity on MDR *S. aureus* than MDR *E. coli* (Fig. 1). This purified compound showed minimum inhibition at 1250 $\mu\text{g}/\text{mL}$ for MDR *E. coli* and 625 $\mu\text{g}/\text{mL}$ for MDR *S. aureus* (Table 2).

3.3. Synergistic effect of purified EGCG compound with gentamicin

A synergistic effect was found between gentamicin and EGCG extract of green tea in checkerboard method. In the combination of this purified compound with gentamicin, a synergistic effect was observed against MDR *S. aureus* and MDR *E. coli* (FICI 0.325, Table 3).

4. Discussion

Plants and plants derived purified compounds have been used to control infectious diseases. In this study, we used *Camellia sinensis* (green tea) crude extract and purified compound to detect their antibacterial activity. The antibacterial activity was found against multi-drug resistant *E. coli*, *S. aureus* and their reference strains. These pathogen were resistant to different antibiotics, was previously proved. In 2014, the World Health Organization (WHO) warned that the antibiotic resistance crisis is becoming dire (Michael et al., 2014).

Tea is a tree from Theaceae family where *Camelliasinensis* is one of the species of green tea. Fresh leaves of different tea plants were washed and air dried. Tea leaves have been reported to be the only food product containing epigallocatechingallate (EGCG) (Chu and Juneja, 1997). For

Antibacterial Effect of EGCG

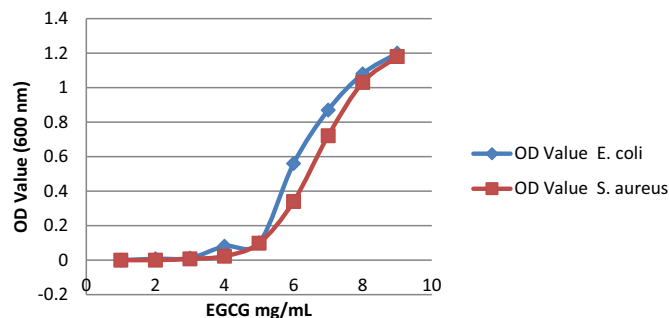


Fig. 1. MIC of EGCG against MDR *E. coli* and MDR *S. aureus*.

Table 2

Antibacterial effect of EGCG and antibiotic.

Bacteria	EGCG ($\mu\text{g}/\text{mL}$)		Antibiotic ($\mu\text{g}/\text{mL}$)	
	MIC	MBC	Gentamicin MIC	MBC
MDR <i>E. coli</i>	1250	2500	32	32
MDR <i>S. aureus</i>	625	-	32	32

different type of cancers such as liver, prostate, stomach, esophagus, colon, pancreas, bladder, skin, lung, and breast EGCG has been reported to be chemopreventive (Lang et al., 2009). EGCG also has chemopreventive effect in carcinogenesis caused by UV light, chemical agents and genetic aberrations (Lambert and Yang, 2003). So it suggests that EGCG could be potentially useful for the prevention or treatment of cancer, though this has to be established formally (Lang et al., 2009).

Green tea crude extract was prepared from the dried leaves. Catechins were also extracted and epigallocatechin was separated from green tea plants. Green tea and the purified compound have shown good inhibitory and bactericidal effect on the MDR pathogens. In our study green tea inhibits the drug resistant pathogen at 125 $\mu\text{g}/\text{mL}$. This was the minimum concentration at which green tea extract inhibited the pathogen. In another study, the antibacterial effects of tea polyphenols (TPP) extracted from Korean green tea (*Camellia sinensis*) have been found against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) (Archana and Abraham, 2011). In present study, green tea extract showed MBC at 500 $\mu\text{g}/\text{mL}$ against MDR *S. aureus* but no MBC was found against MDR *E. coli*. So, we can tell that green tea extract has shown better bactericidal effect on Gram positive pathogen than Gram negative pathogen. Previous studies have reported that catechins from green tea are active against Gram positive bacteria (Yam et al., 1997) but no effect was seen to be antimicrobial against Gram negative bacteria. This may be due to their different cell wall components.

Swiercz et al. (1999) claimed that tea component EGCG showed strong inhibitory properties (Swiercz et al., 1999). In our study, the MIC value of this purified compound EGCG was 1250 $\mu\text{g}/\text{mL}$ for MDR *E. coli* and 625 $\mu\text{g}/\text{mL}$ for MDR *S. aureus*. Thus EGCG showed higher antibacterial activity against *S. aureus* compared to *E. coli*. This purified compound had antimicrobial activity and this was may be due to production

Table 1

Antibacterial effect of *Camellia sinensis* (green tea) crude extract ($\mu\text{g}/\text{mL}$).

Greentea	500	250	125	62.5	31.25	15.62	7.81	3.90	1.95	0.97	+	-
	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	+	+	+	+	+	+	+	+	a
B	-	-	-	+	+	+	+	+	+	+	+	b
C	-	-	-	+	+	+	+	+	+	+	+	c
D	-	-	-	+	+	+	+	+	+	+	+	d

a = *E. coli* reference, b = *E. coli* resistance, c = *S. aureus* reference, d = *S. aureus* resistance.

Table 3
Synergistic effect of EGCG in combination with gentamycin against MDR *S. aureus* and MDR *E. coli*.

Pathogen	MIC of EGCG ($\mu\text{g/mL}$)		MIC of Gentamicin ($\mu\text{g/mL}$)		FICI	Outcome
	Alone	Combination	Alone	Combination		
<i>E. coli</i>	1250	156.25	32	6.4	0.325	Synergistic
<i>S. aureus</i>	625	78.125	32	6.4	0.325	Synergistic

of reactive oxygen species and hydrogen peroxide which are generated from catechins, EGCG. Matsunaga et al. (2002) have reported that the tea catechin, EGCG has a potential immunomodulatory as well as antimicrobial activity (Matsunaga et al., 2002). Yoda et al. (2004) indicated that the structure of the bacterial cell wall and different affinities of EGCG with various cell wall components are responsible for different susceptibilities of *Staphylococcus* and Gram-negative rods to EGCG (Yoda et al., 2004).

The combination of EGCG purified compound and antibiotic (gentamicin) was also used to detect any antimicrobial activity has present or not against MDR pathogen. Bazzaz et al., (2016), found significant decrease in MIC value when EGCG combines with gentamicin which complies with the findings of our study. Partial synergy between EGCG and gentamicin had been determined (Bazzaz et al., 2016) where strong synergistic relationship found in this study. In another study additive or indifferent effects were observed in combinations of EGCG with gentamicin (Hu, 2002) which also not similar with the finding of this study. EGCG induces disruption of the cell wall which increases the permeability of cell membrane to antibiotics, might be the main factor for the additive effects (Hu, 2002). The purity of EGCG could play a significant role behind the synergistic, partial or additive results from the combined use of EGCG with antibiotics. More investigation is needed.

By Fractional Inhibitory Concentration Index (FICI) method, a synergistic relation was found between the purified compound of *Camellia sinensis* (Green tea) and gentamycin. Strong synergistic effect (FICI 0.325) was observed in the combination of EGCG and gentamicin against both MDR *E. coli* and MDR *S. aureus*. This strong result was found because of green tea catechins, EGCG which can cause cell membrane disruption and prevent DNA super coiling eventually leading to bacterial destruction (Namita et al., 2012). Hence, from this study, it could be concluded that green tea crude extracts and their purified compound could play a significant role in the treatment of MDR pathogens.

5. Conclusions

Green tea and the purified EGCG compound showed strong inhibitory effect against Gram positive and Gram negative MDR pathogens. The MIC and MBC of green tea was 125 $\mu\text{g/mL}$ and 500 $\mu\text{g/mL}$ respectively. MIC of EGCG when used alone, found 1250 $\mu\text{g/mL}$ against MDR *E. coli* and 625 $\mu\text{g/mL}$ against MDR *S. aureus*. MIC value decreased significantly when EGCG used in combination with the antibiotic gentamicin and it was found 156.25 $\mu\text{g/mL}$ for *E. coli* where 78.125 for *S. aureus*. A strong synergistic result (FICI 0.325) was found in combination of the EGCG extract with gentamicin. In future, more research is needed so that the combined use of green tea extract and antibiotics can be used to control drug resistant pathogens.

Declarations

Author contribution statement

Md. Anowar Khasru Parvez, Karabi Saha: Conceived and designed the experiments.

Juairia Rahman, Rahath Ara Munmun, Md. Atikur Rahman, Shuvra Kanti Dey, Mohammad Hossain Shariare, Sohridul Islam, Md. Shahedur Rahman: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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