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Research Article

Antibacterial activity of green tea (*Camellia sinensis*) leaf extract against Metallo-β-lactamase producing uropathogens

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Abstract

Increasing incidences of antibiotic resistant pathogens has created a demand to explore alternative treatment approaches. One such approach involves evaluating plant derived compounds for their activity against drug resistant pathogens. This study aims at examining the effect of green tea leaf extracts (GTE) on Metallo-β-lactamase (MBL) producing gram negative uropathogens. An ethanolic extract of GTE was prepared using soxhlet apparatus and its effect was studied on seven gram negative MBL producing uropathogens with respect to its Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). In addition, the synergistic activity of ampicillin (a β-lactam drug) with GTE was carried out by agar dilution method to analyze the reduction in MBC of ampicillin in the presence of sub-lethal concentrations of GTE. The MIC of GTE for the MBL producing uropathogens was found to be similar to that of ampicillin. However, in presence of sub lethal concentrations of GTE, the MIC of ampicillin was reduced for most cultures by a factor of 10. Liquid Chromatography Mass Spectrometry (LC-MS) analysis of GTE identified epicatechin gallate to be the most abundant polyphenol in the extract, the least abundant being epigallocatechin; both of which are known to be antibacterial in nature. The study thus provides promising results in combating MBL producing pathogens.

Keywords: Green Tea, MBL, Gram-negative, Synergy, Ampicillin, Polyphenol, LC-MS.

Introduction

The severity of antibiotic resistance among pathogens has been compounded by the frequent emergence of resistance to newer antibiotics, the localization of antibiotic resistance genes on bacterial plasmids which permits their transfer to different bacterial species and the spread of resistant bacteria beyond the patients in the hospital to individuals in the community (Weiner et al., 1999).

To combat the challenge posed to β-lactam antibiotics by the hydrolytic β-lactamase enzymes, more stable β-lactams such as cephalosporins, carbapenems, and monobactams were introduced in the 1980s (Philippon et al., 2002). Unfortunately, even these drugs are rapidly becoming ineffective owing to the rise in carbapenem resistance (Toye et al., 2009). Resistance to carbapenem antibiotics due to carbapenemase production is more severe because it recognizes and hydrolyses most of the β-lactams, in addition to being resilient to inhibition by most commercially available β-lactamase inhibitors (Nordmann and Poirel, 2002). Among this category are Metallo-β-lactamases (MBLs), which degrade all β-lactam drugs, including the last-resort carbapenems without being hindered by conventional β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam). In addition, they are known to exhibit a host of mechanisms that mediate resistance against other classes of drugs (aminoglycosides and macrolides) too (Walsh et al., 2005).

One such enzyme that has gained particular notoriety and caused equivalent alarm worldwide is the New Delhi Metallo-β-lactamase-1 (NDM-1). The first NDM-1 isolates characterized in 2009 were found to resist all antibiotics tested except colistin, a polymyxin E antibiotic. *bla*$_{NDM-1}$, was found to be a part of a large 180-kb genetic element that also contained genes for

broad-spectrum β-lactamases, enzymes that inactivate erythromycin, ciprofloxacin, rifampicin, and chloramphenicol, as well as growth promoters to drive the expression of the genes on mobile element (Yong et al., 2009). Plasmids carrying bla_{NDM-1} are also reported to carry a number of other genes which confer resistance to most aminoglycosides, macrolides and sulfamethoxazole (Kumarasamy et al., 2010). Given their ability to inactivate such a large number of antimicrobial agents, an increase in the prevalence of NDM-1-containing bacteria can severely compromise the ability to treat hospital- or community-acquired infections (Fritsche et al., 2005).

Urinary Tract Infections (UTIs), in addition to being among the most common community-acquired infections, are also the most common nosocomially-acquired infections (Pechere et al., 1998). Uropathogens have been known to possess an enormous repertoire of various antibiotic-resistance mechanisms, the reason being; administering drugs without carrying out any preceding isolation or sensitivity tests, coupled with a large section of the population being impoverished and illiterate. The lack of completion of the prescribed drug-course and the use of leftover drugs also adds to the problem (Akram et al., 2007). With hot and humid climate prevalent in Mumbai for most of the year, the incidences of UTIs and consequently the number of antibiotic-resistant microorganisms that cause UTIs has witnessed a gradual rise (Aruna and Tariq, 2012).

Green tea is a natural substance traditionally consumed as a beverage in Asia (Kim et al., 2008) and is prepared by steaming young leaves of the tea plant *Camellia sinensis*. This practice inactivates indigenous oxidizing enzymes, thereby maintaining in the leaves a level of polyphenols that is much higher than those found in Black Tea or Oolong Tea (Adak and Gabar, 2011). Green tea polyphenols, collectively known as catechins, comprise 30% -40 % of the extractable solids of dried green tea leaves. They include Epicatechin (EC), Epicatechin gallate (ECG), Epigallocatechin (EGC), and Epigallocatechin gallate (EGCG), all of which have demonstrable bactericidal activity against various gram positive and gram negative bacteria (Ikigai et al., 1993; Blanco et al., 2005). Studies performed on the biological activity of GTE and tea polyphenols have implicated catechins to be the main components, possessing anti-microbial, anti-carcinogenic, antioxidant, and chemo-preventive properties (Van et al., 2003; Banerjee et al., 2005; Mabe et al., 1999; Alschuler, 1998).

This study aims at examining the effect of GTE on the growth of MBL producing uropathogens, and identifying a putative cure for pathogens with limited treatment options.

Materials and Methods

Collection of gram-negative MBL producing uropathogens:

A total of seven gram-negative uropathogens were used in the study that was characterized in a previous study carried out in our laboratory (Tariq and Aruna, 2015). The pathogens were referred as M1-M7. It included 3 *Escherichia coli* (M1, M2 and M4), 2 *Klebsiella pneumoniae* (M5 and M6), 1 *Pseudomonas aeruginosa* (M3) and 1 *Citrobacter amalonaticus* (M7). Among these, M2, M3, M6 and M7 showed the presence of NDM-1 gene. M1 and M4 showed the presence of IMP gene and M5 showed the presence of VIM-1 gene. All three genes were harbored on the extra-chromosomal plasmid DNA of the uropathogens.

Preparation of a hot ethanolic crude extract of green tea leaves:

The ethanolic GTE was prepared by using commercially available green tea leaves. The bioactive components from green tea leaves were extracted using 200ml of 70% ethanol in a soxhlet apparatus and the setup was maintained at 30°C for 6h, following which the extract was placed in a heating oven maintained at 40°C until a dry mass was obtained. The solid residue left behind was stored in a dark container at 4°C (Harbone, 1994).

Sterility of green tea extract:

A loopful of the GTE was streaked onto a sterile Nutrient agar and Sabouraud's agar plates (Sule and Agbabiaka, 2008). These media were incubated at room temperature for 48h, following which they were examined for any bacterial or fungal contaminations respectively.

Determination of Minimum Inhibitory Concentration of the Green Tea Extract:

The MIC of GTE was determined by the broth dilution method. One gram of the dried GTE was completely dissolved in 5ml of 70% ethanol to produce a standard stock solution of 200 mg/ml. Using the standard stock, a working stock of 20mg/ml GTE was prepared by adding 10 ml GTE stock in 90 ml of sterile Luria-Bertani (LB) broth. The MIC of green tea extracts

were determined by using a concentration range of 2 to 20 mg/ml with an interval of 2mg/ml.

Turbidity control tubes were set up for each concentration in addition to the positive and negative controls. All the tubes were incubated at 37°C for 24h. The experiment was performed in triplicates. MIC was defined as the lowest concentration of GTE that inhibited (i.e showed no turbidity) the growth of pathogens.

Determinations of Minimum Bactericidal Concentration of the Green Tea-Extract:

The MBC of the GTE for MBL producing uropathogens was determined using the agar dilution method (CLSI, 2006). Varying volumes of GTE working stock were added to molten sterile LB agar and poured onto sterile Petri dishes to obtain a series of LB agar plates with different concentrations of GTE ranging from 1mg/ml to 20mg/ml with an interval of 1 mg/ml. A control plate was also set up without GTE. The test pathogens were spot inoculated on these plates using a sterile toothpick and incubated at 37°C/24h. The experiment was carried out in triplicates. The MBC was defined as the lowest concentration of GTE that completely killed the culture.

Minimum Inhibitory Concentration of ampicillin:

The MIC of ampicillin was also determined by the broth dilution method, using concentrations ranging between 1-10mg/ml of ampicillin with an interval of 1 mg/ml. The experiment was performed in triplicates.

Determination of the synergistic action by agar dilution method:

The agar dilution method was similarly used to determine the synergistic activity between GTE and ampicillin. It was performed by incorporating sub-lethal (½ MBC and ¾ MBC) concentrations of GTE into a molten NA butt which was cooled to around 40°C along with 100-500 µg/ml of ampicillin with an interval of 100µg/ml (CLSI, 2006).

Liquid Chromatography Mass Spectrometry of the Green Tea-Extract:

Liquid Chromatography Mass Spectrometry (LC-MS) of the GTE was carried out at SAIF, IIT Bombay. The powdered GTE dissolved in 70% ethanol was analyzed using a HiP sampler connected to a MS Q-TOF (Agilent Technologies G6550A). A Zorbax SB C-18 Rapid Resolution HD column with the dimensions 2.1mm x 100mm x 1.8µm was employed along with the solvents 0.1% Formic Acid in Distilled Water (Solvent A) and 100% acetonitrile (Solvent B) used as the mobile phases. At a flow rate of 0.3 ml/min, the following gradient program was applied: B 5% (2 min), B 90% (20 min), B 90% (25 min), B 5% (30 min). The eluted fractions were scanned over a range of 50 to 2000(m/z) in both positive and negative modes and identified using the database available with IIT Bombay.

Results

Sterility checking of GTE:

The ethanol extract of green tea leaves was found to be sterile as evidenced by the absence of any kind of bacterial or fungal growth on nutrient agar and sabouraud's agar plate after 48h incubation at room temperature.

Determinations of Minimum Inhibitory Concentration of the Green Tea-Extract and ampicillin:

The results obtained from assays carried out to study the effect of ampicillin and GTE are shown in Table 1. The MIC of ampicillin obtained using the broth-dilution method was found to exhibit a wide variation among the isolates. An MIC of ampicillin ranging from 3 to >10mg/ml was observed among the test uropathogens. In contrast, the MIC of the GTE was found to be remarkably uniform (i.e between 11 and 12 mg/ml) for the uropathogens tested.

Table 1: Minimum Inhibitory concentrations of ampicillin and GTE

Isolate no.	Organism	MIC of ampicillin	MIC of GTE
M1	*E. coli*	4 mg/ml	11 mg/ml
M2	*E. coli*	>10 mg/ml	11 mg/ml
M3	*P. aeruginosa*	>10 mg/ml	12 mg/ml
M4	*E. coli*	7 mg/ml	11 mg/ml
M5	*K. pneumoniae*	8 mg/ml	11 mg/ml
M6	*K. pneumoniae*	>10 mg/ml	12 mg/ml
M7	*C. amalonaticus*	3 mg/ml	12 mg/ml

Determinations of Minimum Bactericidal Concentration of the Green Tea-Extract and ampicillin:

Table 2 shows the results obtained from assays carried out to study the MBC of ampicillin and GTE. The MBC of ampicillin was found to be >10mg/ml for all the tested uropathogens. Also, the MBC of GTE showed a uniform range of 12-13 mg/ml against the test isolates.

Table 2: Minimum Inhibitory Concentrations of ampicillin and GTE individually and synergistically.

Isolate no.	MBC of ampicillin	MBC of GTE	MBC of ampicillin in presence of	
			½ MBC of GTE	¾ MBC of GTE
M1	>10 mg/ml	13	300 µg/ml	200 µg/ml
M2		12	400 µg/ml	200 µg/ml
M3		12	300 µg/ml	200 µg/ml
M4		12	500 µg/ml	300 µg/ml
M5		13	400 µg/ml	200 µg/ml
M6		12	300 µg/ml	200 µg/ml
M7		13	500 µg/ml	300 µg/ml

Determinations of synergistic activity between GTE and ampicillin:

The results obtained for synergy between ampicillin and GTE is shown in Table 2. The MBC of ampicillin was found to drop sharply in the presence of sublethhal concentrations of GTE. In presence of ½ MBC concentration of GTE, the MBC of ampicillin was found to drop between 300 and 500 µg/ml from >10mg/ml. Further decrease in MBC of ampicillin to 200-300 µg/ml was observed in presence of ¾ MBC of GTE.

Liquid Chromatography –Mass Spectrometry of the Green Tea-Extract:

Analysis of the ethanolic extract of green tea by LC-MS revealed the presence of multiple compounds, principally the polyphenolic catechins to which most of the antimicrobial activity of green tea is attributed. The principal compounds identified in positive ion scanning-mode and their retention times are stated in Table 3. The most abundant among the catechins found in the green tea extract was ECG, followed by EGCG, catechin and EGC.

Table 3: Principal components of Green Tea Extract identified by LC-MS

Retention time (min)	*m/z*	Abundance (ppm)	Compound*
0.842	307.0807	1.62	EGC
3.23	307.0802	3.56	EGC
4.591	307.08	3.62	EGC
4.889	307.0799	4.1	EGC
5.801	291.085	4.51	C
5.809	459.0895	5.77	EGCG
5.91	291.0851	4.09	C
6.034	459.0895	5.7	EGCG
7.001	443.0945	6.27	ECG
7.086	443.0942	6.78	ECG

*(EGC- Epigallocatechin, C- Catechin, EGCG- Epigallocatechin gallate, ECG- Epicatechin gallate)

Discussion

The current study was carried out to examine the propensity of the bioactive compounds in green tea to inhibit the growth of MBL producing uropathogens.

Plant extracts have long been used in assays that study bacterial inhibition (Cox et al., 2000; Caccioni et al., 2000; Tassou et al., 2000; Pednekar et al., 2012). Most of the research conducted on green tea focuses on their

anticancer (Azam et al., 2004), antioxidant (Zhao et al., 1989), anti-inflammatory (Hofbauer et al., 1999) and anti-obesity properties (Ashida et al., 2004; Bose et al., 2008). However, very few studies have been carried out to examine the effect of plant extracts on pathogenic, drug resistant bacteria (Hu et al., 2001; Tiwari et al., 2005). This study is, to the best of our knowledge, the first that deals with the effect of green tea extracts on MBL producing gram negative pathogens.

The MIC (11-12mg/ml) and MBC (12-13mg/ml) of GTE was fairly constant for all the seven pathogens tested. On the basis of this observation, green tea extracts do not seem to possess any specific antibacterial activity by themselves and are seen to be as adept at killing MBL producing uropathogens as ampicillin, a commonly used β-lactam antibiotic, in the treatment of infectious diseases including urinary tract infections. However, the concentration of ampicillin required to inhibit the growth of MBL producing bacteria was drastically reduced when used in combination with GTE. More than 99% reduction was observed in the MBC of ampicillin in the presence of GTE. The most significant finding of this study is the synergy between GTE and ampicillin in killing the extremely resistant MBL producing uropathogens at a mere 300-400µg/ml of ampicillin. This observation expands the potential that green tea is effective only against gram positive pathogens as shown by other researchers previously (Hu et al., 2001; Neyestani et al., 2005; Radji et al., 2013). Owing to its broad spectrum activity, low production cost and comparatively negligible side effects, ampicillin still remains an important antibiotic that can be used for common infections (Jancel and Dudas, 2002). Although resistance by β-lactamase producing pathogens makes it obsolete, this study demonstrates that in combination with GTE, ampicillin does not only inhibit the growth of these pathogens but they are bactericidal. This may open up the doors for formulating preparations comprising a β-lactam drug along with green tea polyphenols to fight infections caused by MBL producing pathogens.

The exact mode of action of green tea extracts on pathogenic bacteria specifically, leaving the eukaryotic cells unharmed, is not yet understood completely but susceptibility of bacterial strains to the tea extract has been shown to be related to differences in cell wall components (Ikigai et al., 1993). A possible mechanism proposed, discusses the anti-adhesive properties of green tea polyphenols that prevents the attachment of pathogenic bacteria on the host cell membrane (Lee et al., 2009). Epigallocatechin gallate, which is a type of proanthocyanidin from green tea has also been reported to interact with the outer membrane of bacterial cell and prevent their adhesion to mammalian epithelial cells (HEp-2) (Sharma et al., 2012; Janecki and Kolodziej, 2010). Catechins also cause partitioning in the lipid bilayer membrane resulting in loss of cell structure and function and finally the cell death (Ikigai et al., 1993; Kumar et al., 2012). A study done by Stapleton et al. (2006) has shown the capacity of catechins to reduce β-lactam resistance by penetration of the green tea polyphenols into membrane bilayers of the pathogenic bacteria. In another study done by Miller (1995), it is observed that, in addition to facilitating a reduction in the β-lactam resistance in *S. aureus*, galloylated catechins promote cell wall thickening and cell aggregation. Other studies show reduction in slime production and inhibition of biofilm formation in *S.aureus* cells in presence of green tea extracts (Blanco et al., 2005; Paul et al., 2007)

Green tea polyphenols, also called catechins, make up for about 30% -40% of the total extractable solids of the plant. Most of the antimicrobial activity of green tea is attributed to these catechins, mainly to EGCG. Since flavonoids are non-volatile and require derivatization prior to analysis using GC-MS (Murakami et al., 2006), LC-MS was the preferred method of identification of the green-tea catechins. LC-MS revealed ECG to be the most abundant of the catechins (13.05 ppm) in the green-tea extract, followed by EGCG (11.47 ppm). EGC was the least abundant of the catechins. This finding is in complete accordance to that identified in other studies conducted on green-tea polyphenols using liquid chromatography methods (Murakami et al., 2006). The absence of EC may be explained by its epimerization at the 2-position to catechin due to exposure to high temperatures (Cheong et al., 2005).

This study serves to showcase the potential that green tea polyphenols serve towards combating the menace of multi-drug resistance, and lays the foundation for further studies to elucidate the interaction between green tea polyphenols and β- lactam drugs as well as their combined effect on gram negative MBL producing pathogens.

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