



Research Article

Antibacterial Effect of Green Tea Extract Against Multi Drug Resistant *Escherichia coli* Isolated from Urine Sample of Patients Visiting Tertiary Care Hospital of Eastern Nepal

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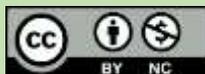
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Abstract

Urinary tract infections (UTIs) caused by drug resistant (DR) Uropathogenic *Escherichia coli* have become a significant worldwide public health problem. Green tea (*Camellia sinensis*), has been reported to have antimicrobial activities against various pathogenic bacteria. The main aim of our study was to estimate the antibacterial effect of green tea extract against drug resistant Uropathogenic *E. coli* isolated from urine samples of patients visiting in tertiary care hospital from eastern Nepal. During the study 360 urine samples were collected from UTI suspected patients visiting a tertiary care hospital of Biratnagar. Urine samples were cultured by using semi-quantitative culture technique and bacteria was identified by standard microbiological procedure. Antibiotic susceptibility testing was done by Kirby-Bauer Disk Diffusion method according to NCCLS (2011) guidelines. The antibacterial effect of green tea extract was performed by preparing the 95% ethanol extract in Soxhlet apparatus which was dispensed in DMSO solution and sterilized by membrane filtration. Antibacterial activity of Green Tea Extract against MDR Uropathogenic *Escherichia coli* was performed by making different concentration of green tea. The overall prevalence of *E. coli* was 27.22% in study population whereas the prevalence of MDR *E. coli* was 21.08%. All the isolated *E. coli* exhibited 100% sensitivity towards Nitrofurantoin and it was still a drug of choice for the treatment of Urinary tract infection caused by *E. coli*. The green tea extracts exhibited effective antibacterial activity against MDR *E. coli*. The MIC of Green Tea Extract was found to be 600µg/ml for 24 MDR isolates and 1000µg/ml for remaining 11 isolates. Based on the present study it is concluded that Green Tea extracts have great potential as an antimicrobial agent against *E. coli*.

Keywords: *E. coli*; Green tea extract; MDR; Phytochemical analysis; UTI

Introduction

Urinary tract infection (UTI) is a serious health problem affecting millions of people each year. It is the most important cause of mortality and morbidity in the world affecting all age groups across the life span (Karki et al., 2004). UTI is the second most common infectious presentation in community practice caused by Uropathogenic *Escherichia coli* (UPEC), one of the

members of the extra-intestinal pathogenic *E. coli* (ExPEC) (Zorc et al., 2005). These strains harbor a variety of virulence factors that allow them to establish an infection, including adhesins, toxins, host defense avoidance mechanisms and multiple ions acquisition systems (Robinson and Le, 2016).

The most common cause of UTI is Gram negative bacteria that belong to the family Enterobacteriaceae, members includes *E. coli*, *Klebsiella*, *Enterobacter* and *Proteus* (Karki et al., 2004; Iroha et al., 2009). In particular, the Extended-Spectrum Beta-Lactamase (ESBL)-producing *Escherichia coli* are emerging worldwide (Cowan 1999; Lee et al., 2005; Ahmad and Aqil, 2007). Drug resistance to the antimicrobial agents is recognized as a major global public health problem, infectious diseases are for approximately one-half of all cases of death in different beings (Ullah et al., 2009).

Early detection and eradication of bacteriuria is very important for prevention of recurrence and complication e.g. chronic pyelonephritis, chronic renal failure etc. (Pradhan and Pradhan, 2017). Today, antimicrobial drugs remain the front line therapy for conquering bacterial infection (Forbes et al., 2016). However, the emergence of drug resistance can significantly affect the course and outcomes of infections, both in the community and in the hospital setting (Knobler et al., 2003). Today the search for other antimicrobial agents to combat infection remains the top most priority. In this regard the medicinal plants have been studied to possess antimicrobial agents against the pathogens (Noormandi and Dabaghzadeh, 2015).

Green tea is an infusion of the leaves of the *Camellia sinensis* plant, a member of the Theaceae family (Brown, 1999). Green tea (*C. sinensis*), has been reported to have antimicrobial activities against various pathogenic bacteria (Suzuki et al., 2012). As the issue of antimicrobial resistance continues to grow, there is a renewed interest in deriving antimicrobial products from natural compounds, particularly extracts from plant material (Kaufmann and Christen, 2002). Therefore, the main aim of our study was to determine the antibacterial effect of green tea extract against drug resistant Uropathogenic *E. coli* isolated from urine sample of patient visiting tertiary care hospital of Eastern Nepal.

Materials and Methods

Study Design

The prospective lab based cross-sectional study of bacterial Uropathogens was conducted among patients suspected of UTI attending tertiary care hospital of Biratnagar, Morang, Nepal. During this study, 360 midstream urine specimens were collected from clinically suspected patients of UTI and processed at bacteriology laboratory of microbiology department of Central Campus of Technology and Tertiary care hospital of Biratnagar. The patient's age ranged above 16 years were included in the study. The consents of the patients were obtained and the history of all the patients including age, gender and symptoms was recorded in the data collection form from the requisition form obtained along with the midstream urine for culture. This research work was conducted from November 2017 to April 2018.

Urine Sample Collection and Evaluation

The patients attending at tertiary care hospital of Biratnagar with clinical features of UTI were given a clean, dry sterile and leak proof container and requested for 5 to 10 ml midstream urine sample and examined immediately. Before proceeding with any testing, the urine specimens were evaluated in terms of their acceptability. A properly labeled specimen contained patient's full name, date of collection. Single urine specimen was collected from each patient.

Urine Sample Processing and Culture

About 10 ml of urine sample was taken in a clean sterile centrifuge tube and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and the sediment was then examined by wet mount preparation. WBCs in excess of 10^4 cells/ml (>10 cells/ml) of urine indicated significant pyuria.

Semi-quantitative culture technique was used to culture urine specimens and to detect the presence of significant bacteriuria by standard methods. An inoculating loop of standard dimension was used to take up approximately fixed ($\pm 10\%$ error is accepted) and known volume (0.001 ml) of mixed uncentrifuged urine to inoculate on the surface of Cystine lactose electrode deficient (CLED) agar (HiMedia, India), Nutrient agar (NA) (HiMedia, India), Eosin-methylene blue agar (EMB) (HiMedia, India), 5% Blood agar (BA) (HiMedia, India) and MacConkey Agar (MA) (HiMedia, India). A loopful of sample was touched on the surface of the culture plate, from which the inoculum was spread across the entire plate. Urine specimen was thoroughly mixed to ensure uniform suspension of bacteria before inoculating the agar plates. The inoculated plates were aerobically incubated overnight at 37°C for 24 hours. Identification of significant isolates was done by conventional microbiological procedure as described by (Cheesbrough 2006). Isolates other than *E. coli* was not included in the study.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testing of the isolates towards the various antimicrobial discs; Amikacin (AK 10mcg), Amoxicillin (AMX 10mcg), Cefixime (CFM 30mcg), Cefotaxime (CTX 30mcg), Ceftazidime (CAZ 30mcg), Ceftriaxone (CTR 30mcg), Cefpodoxime (CPD 30mcg), Ciprofloxacin (CIP 30mcg), Cotrimoxazole (COT 25mcg), Gentamicin (GEN 10mcg), Nalidixic acid (NA 30mcg), Nitrofurantoin (NIT 100mcg), Norfloxacin (NX 10mcg), Ofloxacin (OF 5mcg) and Tetracycline (TET 10mcg) was done by modified Kirby-Bauer discs diffusion technique as recommended by NCCLS 2011. The *E. coli* isolates that showed resistance to at least three or more antibiotic classes were identified as MDR *E. coli*.

Extraction

The Green tea sample was collected from Himalayan tea garden located at Jhapa district, Eastern Nepal from the altitude of 233 ft. from sea level. The plant species was

verified from herbarium collection of the Department of Botany, Post Graduate Campus, Biratnagar, Nepal. The tea sample was washed and dried in the sun and fine powder was made with the help of electric blender. The 25 gm grounded sample was placed in a thimble (HiMedia, India) with 100 ml 95% ethanol. Ethanolic solvent extract of tea was obtained by Soxhlet extraction process. The concentrate (semisolid paste) was then allowed to dry at room temperature under aseptic conditions. The dried extract was dissolved in 5% DMSO to make stock solution of concentration 1600 µg/ml and it was filtrated through 0.45 µm membrane filter paper (Whatman, USA) (Archana and Abraham, 2011; Kumar et al., 2012).

Phytochemical Screening

The aqueous extracts of tea were screened for the presence of secondary metabolites such as alkaloids, saponin, phenolics, tannins, anthraquinones, cardenolides, terpenes, flavonoids and cardiac glycosides as described by Trease and Evans (1989) and Harborne (1998).

Antibacterial Evaluation

Agar well diffusion assay was the key process used to evaluate the antibacterial potential of plant extracts. Mueller Hinton Agar (MHA) (HiMedia, India) media was seeded with 100 µl inoculum of bacterial strain (inoculum size was adjusted so as to deliver a final inoculum of approximately 0.5 McFarland standards. Wells of 8 mm diameter was cut into solidified agar media using a sterilized cup-borer. Different concentrations of green tea extracts (200, 400, 600, 800, 1000, 1200, 1400 and 1600 µg/ml) was prepared. 1 ml of each extract was poured in the respective well and the plates were incubated at 37°C overnight. After 24 hour's incubation the zone of clearance around each well after the incubation period confirmed the antimicrobial activity of extract. Control strain of *E. coli* (ATCC 25922) was used for the standardization of the Kirby-Bauer test and also for correct interpretation of zone of diameter. The experiment

was performed in triplicate and antibacterial activity of each extract was expressed in terms of the mean of diameter of zone of inhibition in mm, produced by each extract at the end of incubation period. The MIC was determined as the lowest drug concentration that inhibited growth of bacteria in 24 hours incubation.

Data Analysis

Data entry, checking and validation were done. All data were entered in MS Excel 2010 and finally analyzed by SPSS software version 16.0. The p-value <0.05 was established statistically significant.

Results

Bacterial Growth Pattern in UTI Patients

Total 360 mid-stream urine samples were collected from patients attending hospital. Among them 166 (46.11%) *Escherichia coli*, 98 (27.22%) non-*Escherichia coli* were isolated whereas 96 (26.67%) mid-stream urine samples showed no growth in Cysteine Lysine Electrode Deficient Agar (CLED). Among them, 35 (21.08%) were MDR *Escherichia coli*.

Age and Sex Wise Pattern of *E. Coli* Isolates in UTI Patients

Among 166 isolated Uropathogenic *Escherichia coli*, age (30-40) showed highest number of bacteria isolates. Female had more (62.65%) *E. coli* isolate than male (38.75%). Results are shown in Fig. 1.

Antimicrobial Susceptibility Pattern of Uropathogenic *E. coli*

All the isolated *E. coli* showed complete 100% sensitivity to Nitrofurantoin (NIT 100mcg) whereas; the MDR *E. coli* didn't show resistance to Gentamicin (GEN 10mcg), Amikacin (AK 10mcg) and Nitrofurantoin (NIT 100mcg) (Table 1).

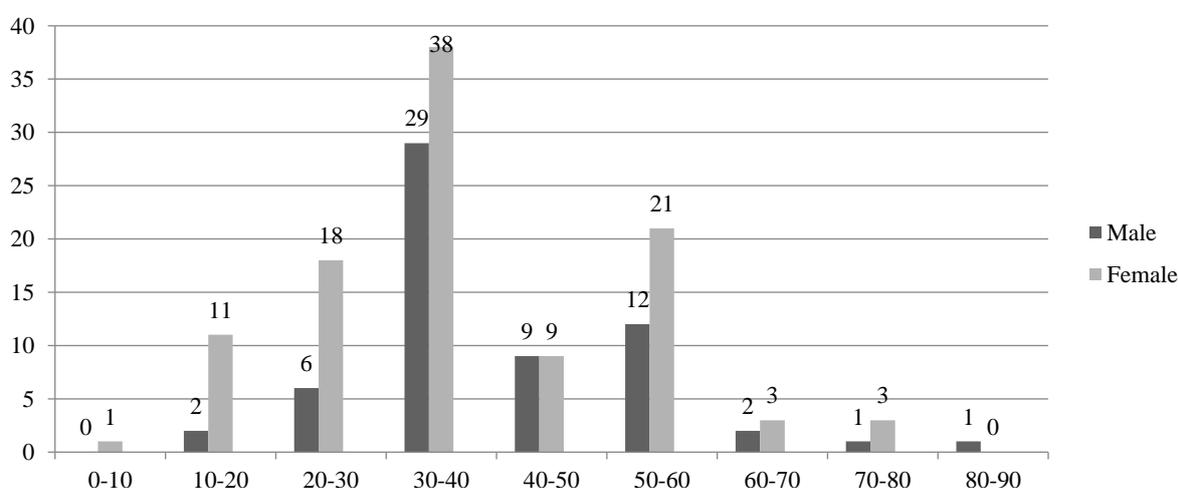


Fig. 1: Age and sex wise pattern of *Escherichia coli* isolates in UTI patients

Table 1: Antimicrobial susceptibility pattern of Uropathogenic *E. coli*

Antibiotics	Resistance (%)	Sensitive (%)	P-value
Amikacin (AK 10mcg)	13 (7.83%)	153 (92.17%)	0.00
Amoxicillin (AMX 10mcg)	162 (97.59%)	4 (2.41%)	0.00
Cefixime (CFM 30mcg)	60 (36.14%)	106 (63.86%)	0.00
Cefotaxime (CTX 30mcg)	39 (23.49%)	127 (76.51%)	0.00
Ceftazidime (CAZ 30mcg)	39 (23.49%)	127 (76.51%)	0.00
Ceftriaxone (CTR 30mcg)	37 (22.29%)	129 (77.71%)	0.00
Cefpodoxime (CPD 30mcg)	35(21.08%)	131 (78.92%)	0.00
Ciprofloxacin (CIP 30mcg)	63 (37.95%)	103 (62.05%)	0.00
Cotrimoxazole (COT 25mcg)	61 (36.75%)	105 (63.25%)	0.00
Gentamicin (GEN 10mcg)	10 (6.02%)	156 (93.98%)	0.00
Nalidixic acid (NA 30mcg)	58 (34.93%)	108 (65.07%)	0.00
Nitrofurantoin (NIT 100mcg)	0	166 (100%)	-
Norfloxacin (NX 10mcg)	58 (34.93%)	108 (65.07%)	0.00
Ofloxacin (OF 5mcg)	60 (36.14%)	106 (63.86%)	0.00
Tetracycline (TET 10mcg)	52 (31.32%)	114 (68.68%)	0.00

Phytochemical Analysis of Green Tea Extract

Qualitative analysis of green tea extract showed the presence of Alkaloids, Flavonoids, Saponins, Terpenes, Anthraquinones, Cardenolides and Cardiac glycosides.

Antibacterial Activity of Green Tea Extract Against MDR Uropathogenic *E. coli*

Antibacterial activity of green tea extract against 35 MDR Uropathogenic *E. coli* was performed by making different concentration of green tea extract as shown in Table 2. The MIC of Green Tea Extract was found to be 600µg/ml for 24 isolates and 1000 µg/ml for 11 isolates.

Table 2: Antibacterial activity of green tea extracts against MDR Uropathogenic *Escherichia coli*

Concentration of Green Tea extract (µg/ml)	For n=24	For n=11
	Formation of Clear Zone (mm)	Formation of Clear Zone (mm)
200	-	-
400	-	-
600	6	-
800	8	-
1000	10	11
1200	10	12
1400	10	12
1600	12	14
Negative control	-	-

Discussions

UTI can occur in any populations and age groups however, infection is most common in women in reproductive age (Karki *et al.*, 2004). A number of *E. coli* isolates have been isolated from urine specimens of patients with UTI that are resistant to common antimicrobial agents (Chomarat 2000; Lee *et al.*, 2005; Ahmad and Aqil, 2007). The past two decades have also witnessed a significant increase in clinically important resistance in a variety of bacterial species as well as the emergence of significant pathogens of intrinsically resistant strains previously considered to be of low pathogenicity (Smith *et al.*, 2002). These challenges have been receiving growing interest to find alternative antimicrobial agents from plant extracts that need to be developed and used to control multidrug-resistant bacteria (Cushnie and Lamb, 2005; Song and Seong, 2007; Reygaert and Jusufi, 2013). *Camellia sinensis* (green tea) is one of the most popular beverages in the world, and has been reported to have antimicrobial effects against various pathogenic bacteria (Taylor *et al.*, 2005; Cushnie and Lamb, 2005; Lee *et al.*, 2005).

In this study, female population had more prevalence (62.65%) of uropathogenic *E. coli* isolate than male (38.75%). In similar study of the age group analysis showed that the female patients in the range of 20-30 years had highest prevalence rate (27.8%) and then the least was found in age group more than 80 years, this might be due to reason that female in the reproductive age groups has a high prevalence rate of UTI and similarly the incidence of symptomatic UTI is high in sexually active young women (Dash *et al.*, 2013). The uropathogens found in this study are similar to uropathogens identified in other studies conducted in different parts of the World (Farajnia *et al.*,

2009). The similarities and differences in the type and distribution of uropathogens may result from different environmental conditions and host factors, and practices such as healthcare and education programmed, socioeconomic standards and hygiene practices in each country (Amin *et al.*, 2009).

In this study antibacterial activity of Green Tea Extract against MDR Uropathogenic *Escherichia coli* was performed by making different concentration of green tea extract as shown in Table 2. Concentration of 200, 400µg/ml showed clear zone for 24 isolates whereas concentration of 200, 400, 600, 800µg/ml and control showed no clear zone in 11 isolates. The MIC of green tea extract was found to be 600µg/ml for 24 MDR *E. coli* and 1000µg/ml for remaining 11 isolates.

It is predicted that one half of all women will experience a UTI in their lifetime, and one in three women will receive antimicrobial therapy for UTI (Foxman 2003). UTI is the most common bacterial infection causing illness in females mostly in the developing countries like Nepal due to illiteracy, unhygienic conditions and lack of proper toilet facilities (Joshi *et al.*, 2016).

Increasing pattern of resistance of urinary tract pathogens against common antibiotics in Nepal have also been reported by other researchers (Sharma *et al.*, 2011; Singh *et al.*, 2013). It is observed that ampicillin, cephalixin, ciprofloxacin and cefixime were poorly effective against Uropathogenic *E. coli*. In our study only 13% of the isolates were found susceptible to all the antibiotics tested. All the isolated *E. coli* exhibited 100% sensitivity towards Nitrofurantoin drug and it was still a drug of choice for the treatment of Urinary tract infection caused by *E. coli*. Cephalosporin, the commonly prescribed antibiotic as empirical therapy in pediatric and adults, resistance to this group of antibiotics was found high. However, compared to previous reports from Nepal, we observed a considerable increase in resistance against penicillin, aminoglycosides, quinolones and ceftriaxone.

In qualitative analysis of Green Tea Extract the substances like Alkaloids, Flavonoids, Phenolic compounds, saponins, terpenes, cardenolides and cardiac glycosides were found and anthraquinones was not found. The presence of the secondary metabolites (alkaloids, terpenes, saponins, flavonoids, cardiac glycosides, cardenolides, anthraquinones and phenols) in tea might partly enhance the antimicrobial activity.

The highest antimicrobial activity of tea is due to presence of catechins and polyphenols which damage the bacterial cell membrane (Cho *et al.*, 2007). Studies on the antibacterial activity have shown that green tea inhibits the growth of *E. coli* by its polyphenolic components also known as catechins. Catechins have been reported to possess strong antioxidant activity and are widely accepted

as important antioxidants, which eliminate free radicals that suppress the bacterial growth (Seeram *et al.*, 2006).

Conclusions

As drug resistance among pathogens is an evolving process. The routine surveillance and monitoring studies should be conducted to help physicians to provide most effective empirical treatments. The present study reveals the medical importance of green tea extract as an alternative antimicrobial to control drug resistant bacteria which are becoming a threat to human health and economic burden worldwide. This research concludes with the evidence of pharmacological significance of green tea to treat disease caused by MDR *E. coli*. However, further research is needed to isolate the secondary metabolites from the extracts and determine its therapeutic uses.

Ethical Approval

This study was carried out as a part of thesis of Master of Science (M.Sc.) Microbiology. Approval was obtained from Department of Microbiology of Central Campus of Technology and Department of Microbiology of Tertiary care Hospital of Biratnagar. Informed consent was obtained from the patients before carrying out the research.

Author's Contribution

Kabita Giri designed the concept, performed laboratory work, analyzed and interpreted the data. Bijay Kumar Shrestha participated in data acquisition, data interpretation and critical revision of the manuscript for intellectual contents and drafting the manuscript. Jenish Shakya participated in data analysis and Manuscript drafting. Shiv Nandan Sah participated in analysis and interpretation of data. Hemanta Khanal participated in data analysis and acquisition. All the authors contributed for final approval of the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest with present publication.

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