ISSN 2321 – 7359 EISSN 2321 – 7367

discovery

Medical Science

The International Weekly Journal for Medicine

Effect of trio agent, Green tea extract, EDTA and antibiotics on *Escherichia coli* urinary tract isolates

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Publication History
Received: 28 January 2014
Accepted: 16 March 2014
Published: 26 March 2014

Citation
Neha Sabharwal, Tiwari RP. Effect of trio agent, Green tea extract, EDTA and antibiotics on *Escherichia coli* urinary tract isolates. *Medical Science*, 2014, 6(22), 83-90

ABSTRACT

This study describes the effects of Green tea *(Camellia sinensis)* and chelating agent (EDTA) against seventeen clinical *E.coli* UTI isolates using combination of GTE (Green tea extract) and EDTA. SIC (Sub-inhibitory concentration), GTE (1.03 mg/ml), SIC EDTA (1mM) resulted in reversion of five MDR (Multiple drug resistant) strains out of seventeen strains used in the study. These agents used at respective SIC (GTE, EDTA) showed synergistic activity against the isolates (Checkerboard titration) and the death rate kinetics revealed that the Trio agent (SIC GTE, SIC EDTA, SIC Nitrofurantoin) reduced the time required for bactericidal activity against the strains. SIC GTE also reduced bacterial adherence to uroepithelial cells upto 50%. Addition of GTE resulted in reduced expression of both cell bound and cell free hemolysin activity. Biofilm formation was also found to be lower in strain grown in presence of SIC GTE. Finally SIC GTE enhanced the phagocytic uptake and killing by mouse intraperitoneal macrophages and killing of test strain as compared to the strain grown in absence of GTE (control).These results are indicative of multifunctional effects of GTE on the virulence traits of uropathogenic *E.coli in vitro* and its efficacy against MDR strains offers possibilities for a novel anti UTI therapy.

Keywords: Urinary Tract Infections, *Escherichia coli*, EDTA, Sub-inhibitory concentration, Green tea

1. INTRODUCTION

Phytochemicals have been used since times immemorial. Efficacy of phytochemicals and combinations of new molecules to fight MDR strains is studied through the past decade. Consistent and indiscriminate use of antibiotics has lead to drug resistance and reemergence of infectious diseases and transmission of traits within same or different species of bacteria. Hence, there is global resurgence in use of phytochemicals to fight and keep pace with drug

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Comparison: This study emphasizes on the problem of emerging multi-drug resistance especially with reference to pathogen *Escherichia coli* and its growing pathogenicity in urinary tract infections. The study suggests an approach using green tea, EDTA and antibiotics to lower the virulence potential of uroisolates of *E.coli*.
Content: The literature for this work has been collected from research articles and books.

resistance emergence in microorganisms. Developing new molecules or combinations has been explored for its antibacterial activity. Much information has been added in the last decade on efficacy of phytochemicals including tea against pathogens to fight MDR emergence.

Urinary tract infection (UTI) has been an extremely common medical problem (Azzarone et al., 2007) and 80% UTI are caused by *E. coli*. UTI's are ranked second only to respiratory infections (Foxman, 2002). Uropathogenic *E.coli* (UPEC) expresses number of specific virulence factors. Tea's antibacterial and bactericidal properties are well documented against various bacterial strains; *Staphylococcus aureus, Staphylococcus epidermidis, Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis, Shigella flexneri, Shigella dysenteriae,* and *Vibrio spp* (Horiba et al., 1991; Okubo et al., 1998; Sakanaka et al., 1989; Toda et al., 1989). Bacteria can resist the action of antibiotics in many ways, notably, presence of enzymes that inactivates the antibiotic, modification of the target of the antibiotic, reduced uptake of the antibiotic and active efflux of the antibiotic (Higgins et al., 2001). Green tea is rich in source of catechins, particularly epigallocatechin gallate (EGCG) (Takahashi et al., 1995). Its increased antimicrobial activity has been related to impairment of barrier function in microorganisms and a depletion of thiol groups (Simonetti et al., 2004). Interestingly, green tea can be complimented in daily routine with EDTA (chelating agent & FDA approved food additive). Thus, the present study describes the potential of Green tea extract against UPEC in presence of EDTA and antibiotics alone and in combination.

2. OBJECTIVE OF THE STUDY

The objective of this study was to attempt to possibly revert the MDR *E.coli* strains of urinary tract to sensitive by using trio agent, Green tea, EDTA and antibiotics under their SICs.

3. MATERIAL AND METHODS

3.1. Bacterial strains

E.coli ATCC 25922, a standard strain of *E.coli* and seventeen *E.coli* clinical UTI strains were used in this study. Clinical strains were isolated (named 1, 2, 4, 9, 12, 15 -18, 22-24, 26, 27, 30-32) in the clinical laboratory, Department of Microbiology, Panjab University, Chandigarh. Each strain was checked for purity and characterized prior to use in the study. The cultures were preserved by stabbing on to Le Minor's medium, maintained at 4^{o}C and were also maintained in 50% glycerol and preserved at -20 ^{o}C.

3.2. Green Tea

Kangra Jwala Green Gold Tea, purchased from Cooperative Tea Factories Federation Ltd., Palampur, Kangra valley, H.P. India. , Darjeeling Green Tea, Vrindavan Green Tea, Lipton Darjeeling Green Tea purchased from general merchandise shop, Chandigarh, India. The chemicals used in the study were procured from standard firms in India.

3.3. Experimental animals

Female LACA mice, 12-20 week old, weighing 25-30g were procured from Central Animal House, Panjab University, Chandigarh. Animals were kept in clean polypropylene cages and fed on standard antibiotic free diet (Hindustan Lever, India). The study had prior approval from the animal ethics committee, Panjab University, Chandigarh. Mice were only used for isolating peritoneal macrophages.

3.4. Green Tea Extracts (GTE)

Other than aqueous extract, organic solvent extracts from different tea varieties were prepared by a modified method (Harbone, 1994). Methanol, ethanol, acetone and acetone: water: acetic acid in 70:29.8:0.2 was used for preparation using soxhlet extraction chamber and the refluxing was continued for 5-6 runs. Extracts thus accumulated were concentrated to one-third volume. They were centrifuged (10,000 rpm for 15 minutes) to remove coarse deposit and stored at 4^{o} C for further use.

3.5. Effect of EDTA and GTE on growth

To sterile nutrient broth (3ml/tube), different concentrations of EDTA and GTE were combined. Antibacterial activity of EDTA and GTE was determined using Checker board dilutions prior adding 10µl young test inoculum. Contents were mixed thoroughly and tubes were incubated for 24 hours at 37^{o} C. Next day tubes were examined visually for growth. Growth inhibition in tubes containing higher concentrations of GTE was determined by plating a loopful of inoculum onto nutrient agar plates. Results were expressed in terms of Fractional inhibitory concentration index (FIC Index) which is equal to the sum of FIC's of each agent.

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FIC of agent A = $\frac{\text{MIC of A in combination}}{\text{MIC of A alone}}$ FIC of agent B = $\frac{\text{MIC of B in combination}}{\text{MIC of B alone}}$

SICs
Sub-inhibitory concentration is a concentration or dosage less than an inhibitory level.

FIC index = ∑ FIC = FIC of A + FIC of B
Synergism = ∑ FIC is ≤ 0.5
Antagonism=∑ FIC is >0.5 and ≤ 4
Indifference = ∑ FIC is > 4

3.6. Antibiotic sensitivity pattern

The method as described was followed (Kirby and Bauer, 1966). Octodisc OD 51 [*Octodisc OD 51 : Cefuroxime, Augmentin, Ceftriaxone, Ceftazidime : 30 µg ; Gentamicin : 10 µg; Nitrofurantoin : 200 µg; Cotrimoxazole : 25 µg; Ciprofloxacin : 5 µg] was put in the centre of each plate swabbed with young culture. The plates were incubated at 37° C for 18-24 hours. Next day the plates were checked for the zone of clearance around individual antibiotics. Zone of inhibition was measured for test strain.

UTI
Urinary tract infections refer to an infection urinary tract caused by the invasion of microorganisms. The pathogen migrated from urethra (causing urethritis) to bladder (causing cystitis) and spread to the upper urinary tract and bacteria may get seeded into the kidney (causing pyelonephritis).

3.7. Death rate kinetics

The test strain bacterial cell suspension (BCS) was exposed to sub inhibitory concentrations (SIC) of inhibitory agents (GTE, Nitrofurantoin and EDTA). 10µl of young culture was added to sterile nutrient broth tubes (3ml/tube) followed by SIC's of nitrofurantoin (12.5µg/ml and 50µg/ml), GTE (1.03 mg/ml) and EDTA (1mM) alone and in combination. From each tube, aliquots were withdrawn aseptically immediately at 0 hour and then at intervals of 2, 4, 6, 8, 24 hours. The aliquots were serially 10 fold diluted in normal saline and spread plated on nutrient agar plates and incubated at 37° C for 24 hours for determining viable bacterial count. Log cfu/ml was calculated and graph plotted against time.

3.8. Adherence to Uroepithelial cells

The following method was followed (Svanborg and Godaly, 1997). BCS was made in normal saline (10^8 cfu/ml) and UEC suspension was prepared from pooled urine from healthy females. The percentage viability was determined by dye exclusion method using 0.01% trypan blue. For Adhesion assay, 1 ml each of BCS grown in nutrient broth and in presence of SIC GTE, was mixed with UEC suspension, mixed gently and kept at 37° C for 1 hour in water bath shaker. At the end of incubation period, tube contents were centrifuged at 5000 rpm for 10 min. The supernatant was decanted off carefully and pellet washed twice with PBS to remove non adherent bacteria. The deposit was used to prepare smear on clean glass slides and stained with Giemsa stain. Average number of bacteria adhering per UEC was calculated by counting bacteria adhering to 30 UEC.

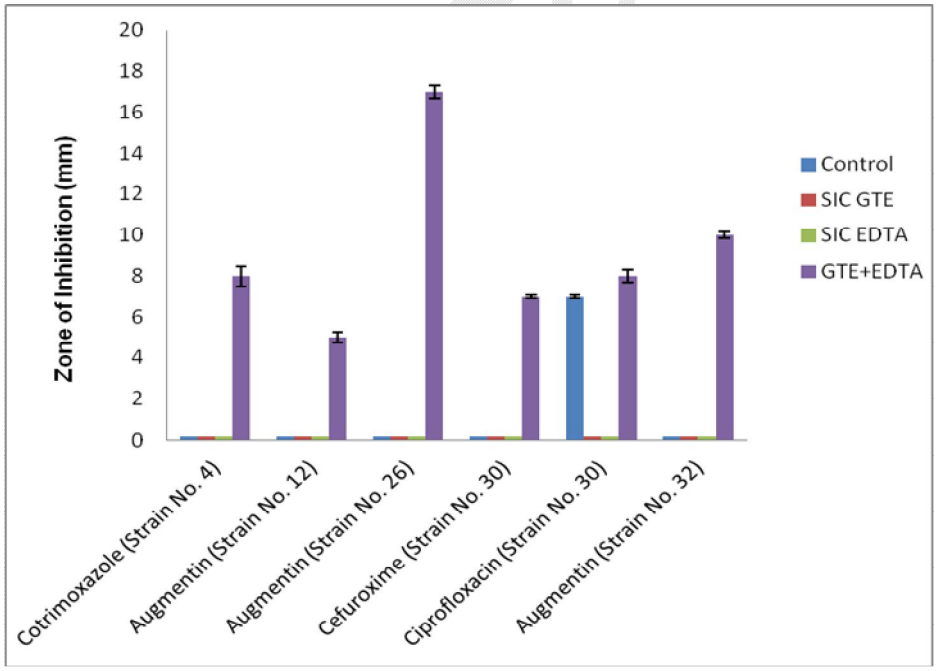

Figure 1
Synergistic inhibition of five MDR *E.coli* clinical UTI isolates by Trio agent

3.9. Effect of GTE on hemolysin production

The following method was followed (Tiwari et al., 2002). 300 µl of CCFS or BCS) was mixed with six ml sRBC suspension (1% v/v). Calcium chloride (10 mM) was added to each tube. Tubes were kept in the incubator at 37° C for three hours with occasional shaking. At the end of incubation period, tube contents were centrifuged at 3000 rpm for 15 min. The supernatant was collected in clean, dry test tubes. The blank was processed alike test except that it contained an equivalent amount of sterile nutrient broth or PBS in lieu of CCFS and bacterial suspension respectively. The hemolytic activity was determined by measuring absorbance of the supernatant against blank at 545 nm and expressed as mg of hemoglobin released.

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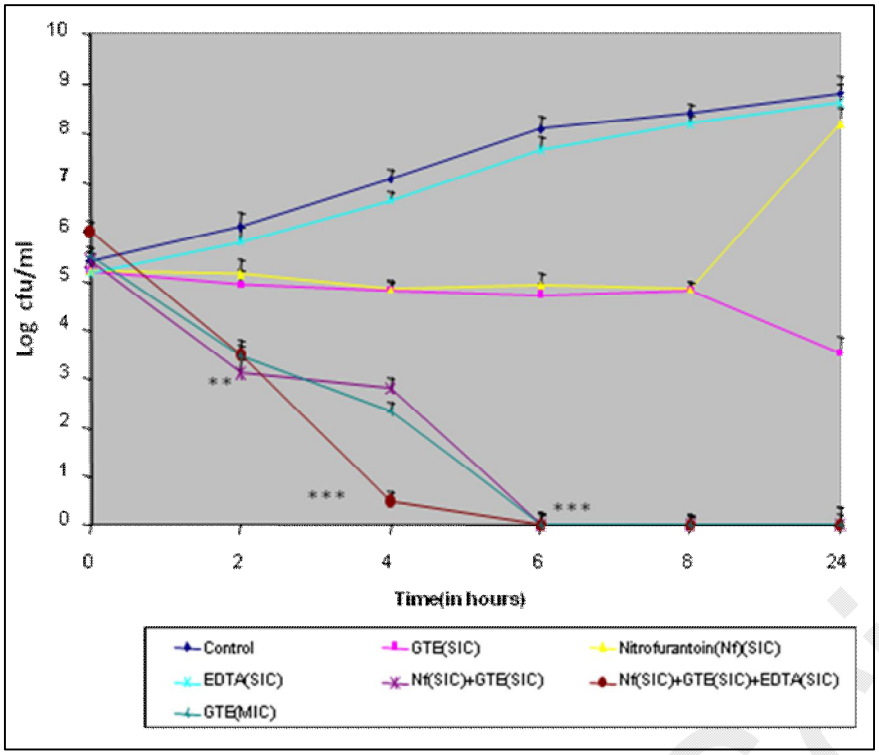

Figure 2a

Death rate kinetics of E.coli ATCC 25922 at SIC Nitrofurantoin(12.5µg/ml), GTE(1.03mg/ml), EDTA (1mM) , Nitrofurantoin plus GTE and Nitrofurantoin plus GTE plus EDTA in nutrient broth at 37°C

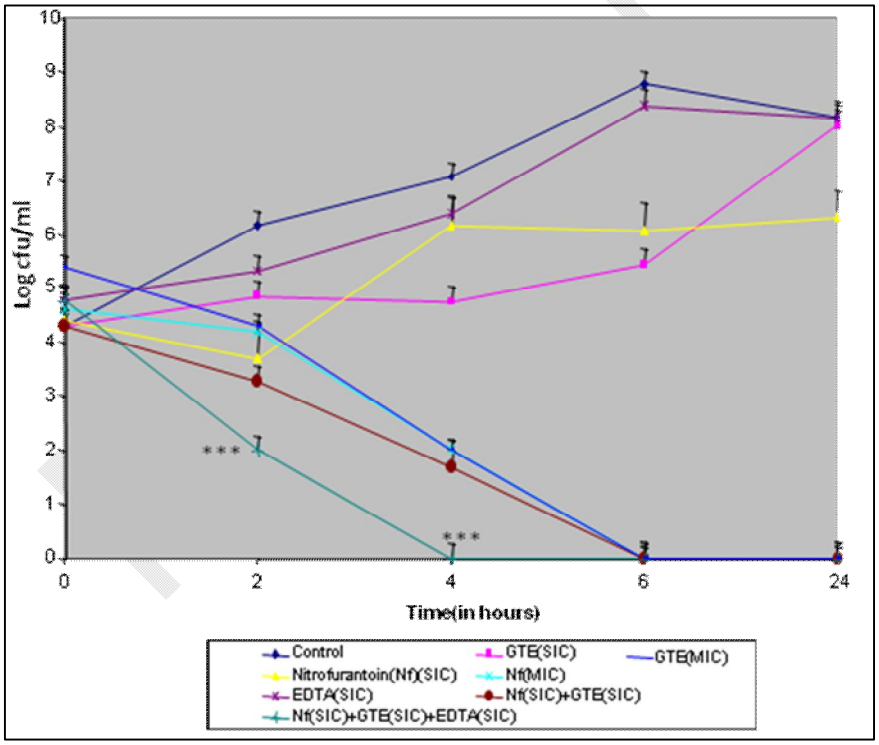

Figure 2b

Death rate kinetics of E.coli clinical UTI isolate 2 at SIC Nitrofurantoin(50µg/ml), GTE(1.03mg/ml), EDTA (1mM) , Nitrofurantoin plus GTE and Nitrofurantoin plus GTE plus EDTA in nutrient broth at 37°C

3.10. Effect of GTE on biofilm formation

The following method was followed (Mathur et al., 2006). 100 µl of test bacterial cell suspension was added to each well of the microtiter plate containing 100 µl of nutrient broth and 100 µl of nutrient broth supplemented with SIC GTE. Plate was kept at 37° C for 16-18 hours for 12 days. Spent culture and planktonic cells were poured off and the wells were washed thrice with 250 µl of PBS (0.02 M, pH 7.2) Adherent bacterial cells were stained with 250 µl of crystal violet (0.1 %) and excess stain was rinsed off with sterile distilled water. Dye bound to adherent cells was resolubilized in 200 µl of 90 % ethanol for ten minutes. Optical density was measured at 490 nm in ELISA reader.

3.11. Effect of GTE on phagocytic activity

A single step phagocytic uptake and killing method was followed (Hampton and Winterbourn, 1999). BCS prepared from cells grown in absence and presence of SIC GTE was mixed with macrophage suspension and kept at 37°C. At the end of assay, the contents of three tubes, control, pellet (for intracellular bacteria) and supernatant (for extracellular bacteria) were serially ten fold diluted and spread plated.

3.12. Statistical Analysis

Mean values and standard deviation were calculated. Differences were analysed by Student's t-test employing Graph Pad software. A value of $p<0.01$ was considered to be statistically significant.

4. RESULTS

Amongst all tea extracts, the antibacterial activity of methanol tea extract was the highest and was in this order: Darjeeling (2.06 mg) > Kangra Jwala (3.06 mg) > Lipton Darjeeling (3.87 mg) > Vrindavan (4.93 mg). Darjeeling tea methanol extract showing minimal inhibitory concentration was selected for use in further study.

4.1. EDTA and GTE effect on growth

SIC of GTE, EDTA and combination inhibited the growth of *E.coli* but it could grow when either agent was used alone (EDTA 1mM and GTE 0.25 mg/ml). The results showed synergy. FIC of GTE was 0.247 and FIC of EDTA was 0.062. FIC for combination was calculated to be 0.309 which showed synergism.

4.2. Effect on sensitivity pattern

Out of seventeen strains, combination of SIC EDTA (1mM) and SIC Darjeeling tea extract (1.03 mg/ml)

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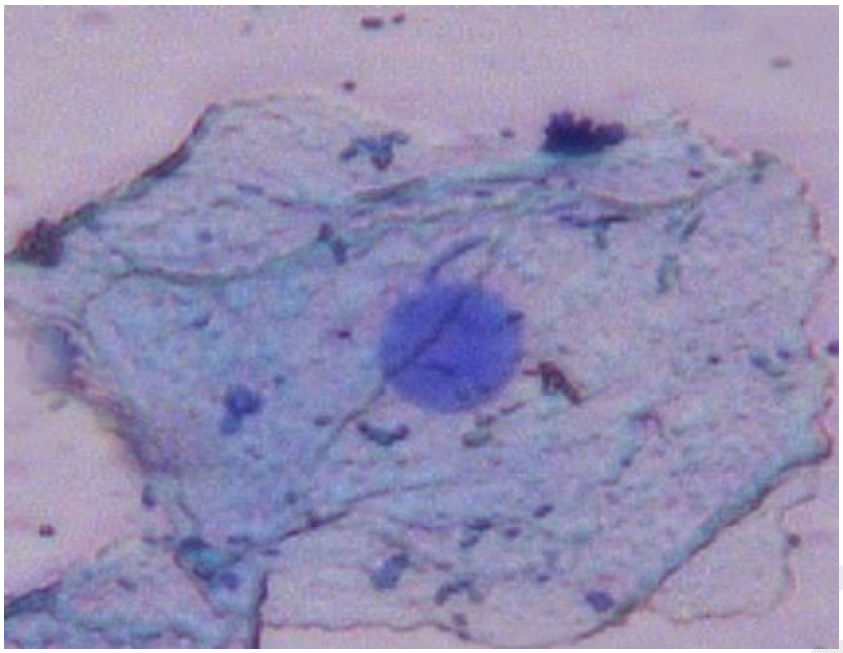

Figure 3a

Adherence of E.coli clinical UTI isolate 2 cultured in nutrient broth at pH 7.0 to human uroepithelial cells

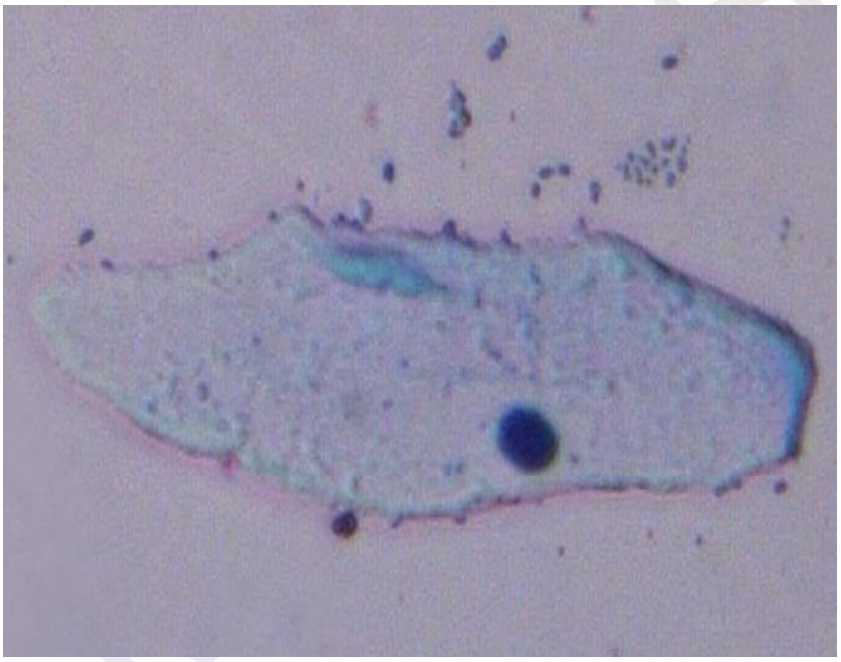

Figure 3b

Adherence of E.coli clinical UTI isolate 2 cultured in nutrient broth containing SIC Darjeeling methanol GTE(1.03mg/ml) at pH 7.0 to human uroepithelial cells

showed synergistic inhibition of five *E.coli* clinical UTI isolates (4, 12, 26, 30, 32). These strains exhibited susceptibility to antibiotics (Cotrimoxazole, Augmentin and Cefuroxime) in presence of SIC EDTA, to which these were found to be resistant on nutrient agar plates. These showed growth on nutrient agar supplemented with SIC GTE or SIC EDTA (Figure 1).

4.3. Death rate kinetics

Viable counts at SIC EDTA and SIC GTE were almost similar to control (Figure 2a). Complete inhibition was observed in *E.coli* UTI isolate 2 on supplementing with SIC EDTA to SIC GTE and SIC Nitrofurantoin (Figure 2b). These results confirm the earlier observation on the antibiogram of *E.coli* UTI isolate 2 whereby addition of SIC GTE and SIC EDTA caused an increase in the zone of inhibition to Nitrofurantoin. The results were found statistically significant. ($p<0.001$).

4.4. Effect of SIC GTE on adherence of *E.coli* clinical UTI isolate to human uroepithelial cells (UECs)

The pictorial representation depicting the adherence of *E.coli* UTI isolate 2 to human UECs, cultured in absence and presence SIC GTE (1.03 mg/ml) have been shown in Figure 3a and 3b. The number of *E.coli* adhering to 30 UEC's was 240 as compared to 130 in test (tea extract treated). Apparently, reduced bacterial adherence by 50% in test *E.coli* was statistically significant ($p<0.01$).

4.5. Effect of SIC GTE on hemolysin production

Hemolysin is an important virulence trait in *E.coli* causing extra intestinal infection. Hemolysin activity showed decrease in hemolysin production in presence of SIC GTE in nine clinical isolates (1, 4, 9, 16, 17, 18, 22, 23, 26) as well as in calcium chloride induced cell bound and cell free hemolysin production in strains 18, 23, 27 (Figure 4).

4.6. Effect of SIC GTE on biofilm formation

Two strains *E.coli* ATCC 25922 and *E.coli* clinical UTI 2 were compared for biofilm formation potential in presence and absence of SIC GTE. Growth in *E.coli* UTI 2 started on the second day and upon addition of SIC GTE, the biofilm disintegrates by sixth day, is reformed and again showed a dip on nineth day as compared to control. However, an overall decrease in biofilm formation under GTE was evident in both clinical isolate and the standard strain. Differences were found statistically significant ($p<0.01$) (Figure 5).

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Table 1
Effect of SIC GTE on phagocytic uptake and killing of *E.coli* clinical UTI isolate 2 *in-vitro* by mouse intra-peritoneal macrophages

Culture	Time (in min)	Supernatant	uptake	% uptake	Pellet	killed	% killing
NB	0	3.0×10^7	-	-	-	-	-
	30	1.1×10^7	1.9×10^7	63.0	1.25×10^7	0.65×10^7	34.2
	60	1.85×10^7	1.15×10^7	38.3	1.0×10^7	0.15×10^7	13.0
	90	1.9×10^7	1.1×10^7	36.6	1.0×10^7	0.1×10^7	9.0
NB+T.E	0	3.0×10^7	-	-	-	-	-
	30	3.9×10^6	2.61×10^7	87.0	5.0×10^6	2.11×10^7	80.8
	60	5.0×10^6	2.5×10^7	83.3	6.0×10^6	1.9×10^7	76.0
	90	1.35×10^7	1.65×10^7	55.0	1.1×10^7	0.55×10^7	33.3

Hemolysin
Hemolysins are certain proteins and lipids that cause lysis of red blood cells by damaging their cell membrane

Phagocytosis
Phagocytosis forms the first line of host defense mechanism against invading pathogens and involves a process of removing pathogens and cell debris by the reticulo-endothelial system of the body (macrophages).

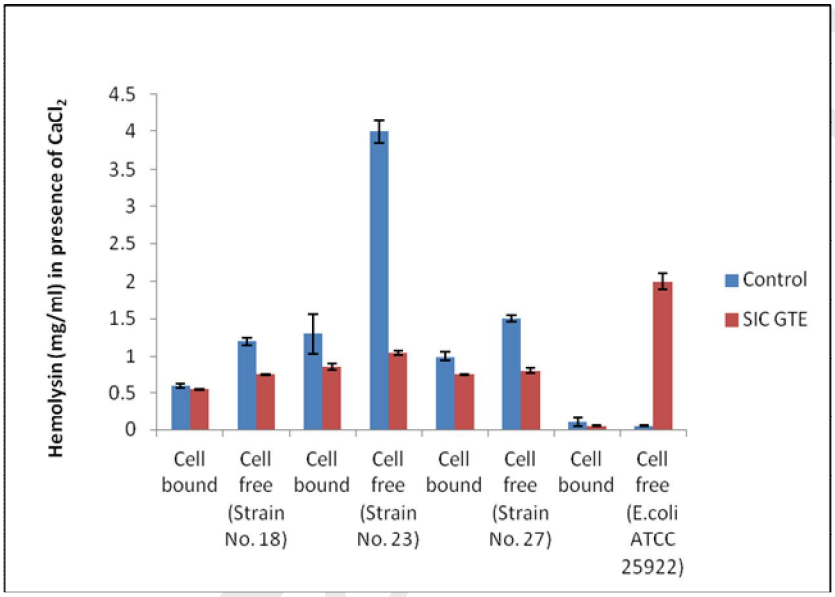

Figure 4
Effect of SIC GTE on calcium chloride (10mM) induced hemolysin production by *E.coli* uroisolates

Uroepithelial cells
Urothelium or uroepithelium (or "transitional epithelium")is the epithelium that lines much of the urinary tract. It is a layer of tissue that lines the renal pelvis, the ureters, the bladder, and parts of the urethra.

4.7. Effect of SIC GTE on phagocytosis

At 90 minutes, net phagocytic uptake was 55% in case of test strain (SIC GTE) and 36.6% in control. Phagocytic killing of bacterial strain grown in presence of SIC GTE after 90 minutes was 33.3% as compared to 9% in control. Alike the phagocytic uptake, the phagocytic killing was also faster for strain grown in presence of tea (Table 1).

5. DISCUSSION

Emerging drug resistant strains have renewed the interest in the natural products. Alternative antibacterial agents are examined alone or in combination for imparting effective treatment to patients. Usage of tea extract in combination with antibiotics routinely used for the treatment of urinary tract infections showed synergistic activity with nitrofurantoin. Cephalosporin and ceftriaxone had differential antibacterial property against drug resistant strains. Synergistic activity of the combination as an antibacterial agent was also found effective at much lower concentration MIC of each agent. The accentuation in antibacterial activity of some of these antibiotics may be related to differing mode of action of GTE and antibiotics and role of EDTA in permeabilisation and as chelating agent (binds Ca^{2+} and Fe^{3+}). EGCg are known to bind to peptidoglycan and induce its precipitation and interfere with cell wall functions (Shimamura et al., 2007). *E.coli* UTI clinical isolates exhibited an increased susceptibility to cotrimoxazole, augmentin and cefuroxime in presence of SIC EDTA (1 mM) and SIC GTE. Test strains were resistant to these drugs. The death rate kinetics showed enhanced bactericidal activity with antibiotic, EDTA along with GTE. Condensed tannins to P-fimbriae in *E.coli* inhibited its binding to uroepithelial cells (Howell et al., 1998). Hemolysin production is characteristic of most stains found associated with urinary tract infections (Felmlee et al., 1985; Hacker et al., 1983). Results showed that at SIC GTE there is interference to calcium chloride stimulatory effect on cell bound and cell free hemolysin activity of isolates. Biofilm formation and phagocytic killing were affected by GTE. Tea catechins incorporate readily into lipid bilayer (Ikigai et al., 1993; Yam et al., 1997) and has been found to stimulate nonspecific component of immune response *in vitro* (Matsunaga et al., 2002).

6. CONCLUSION

It is clear from the results that GTE can be effective as an antimicrobial agent against urinary tract isolates *in vitro*. The trio agent EDTA, GTE and Nitrofurantoin each at SIC was found to be bactericidal. GTE also compromised the

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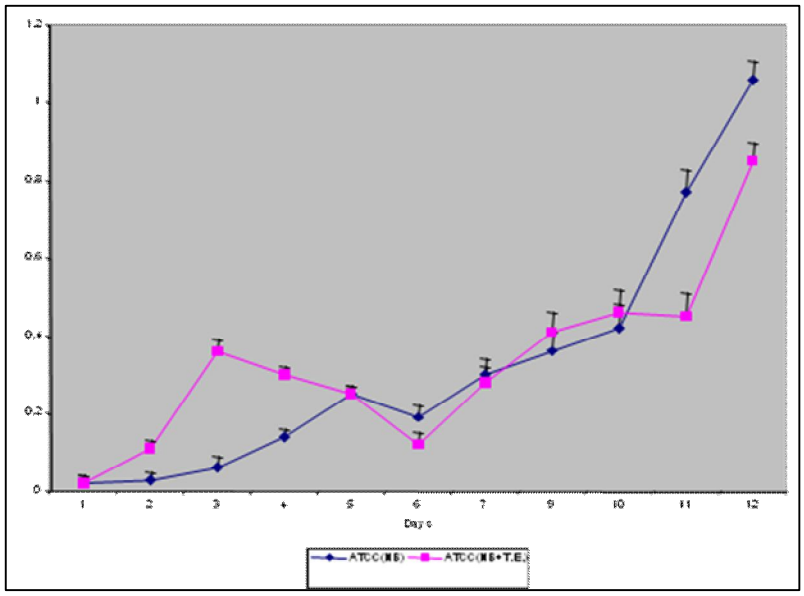

Figure 5a

Effect of SIC Darjeeling tea extract on biofilm formation of *E.coli* ATCC 25922

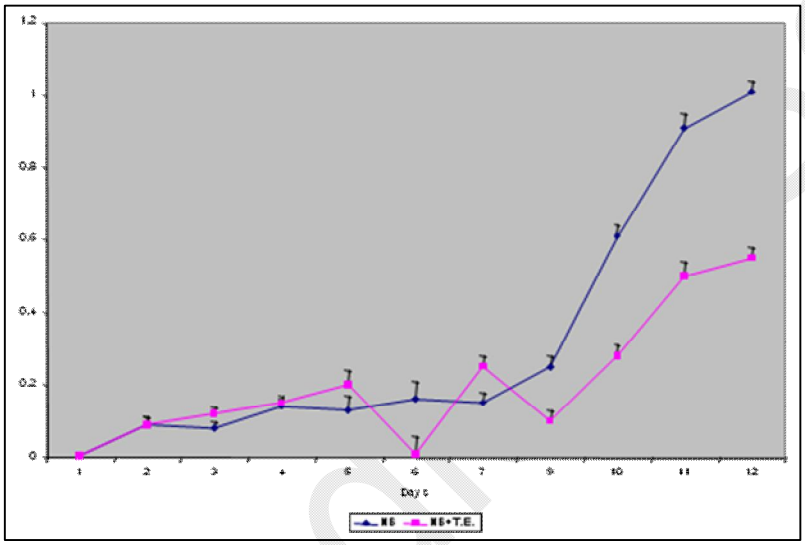

Figure 5b

Effect of SIC Darjeeling tea extract on biofilm formation of *E.coli* clinical UTI isolate 2

organism's ability in expression of virulence traits at SIC GTE. Evidently the tea extract exhibited multifunctional effects as antibacterial agent affecting bacterial virulence and offers possibilities for a novel anti UTI therapy. Further *in vivo* work may provide an insight.

SUMMARY OF RESEARCH

The study suggested a trio agent, green tea, EDTA and antibiotics, under their SICs to lower the virulence factors of uroisolates of *E.coli*. It was very effective to revert back the MDR strains to sensitive, which is the need of hour.

FUTURE ISSUES

In vivo studies would validate this trio agent's efficacy in animal model. This is a very important therapeutic way to tackle the problem of MDR strains in routine hospital settings. Further, clinical trials may lead this agent's way to general public.

DISCLOSURE STATEMENT

There is no financial support for this research work from any funding agency.

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