Original Research Article **Bio Chemistry**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

ANTIBACTERIAL SYNERGISM OF SELECTED COMPOUNDS AGAINST MDR UTI PATHOGENIC BACTERIA.

[1*]ARCHANA MOON, [2]PRANJALI GAJBHIYE, [3]MONALI JARIYA, [4]DEEBA KHAN, [5]AMIT TAKSANDE

University Department of Biochemistry, RTM Nagpur University, LIT Premises, Amravati road, Nagpur, Maharashtra, India.

ABSTRACT

UTI is an infection of the urinary tract, caused by microbes including fungi, viruses, and bacteria. Bacteria are the most common cause of UTIs. The bacterium *Escherichia coli (E. coli)* causes the vast majority of UTIs. Urinary tract infections are the second most common type of infection in the body; accounting for about 8.1 million visits to health care providers each year. Women are especially prone to UTIs for anatomical reasons. For women, the lifetime risk of having a UTI is greater than 50 percent. Antibiotic resistant is a seriously growing problem in medicine. Limited treatment options are available for treatment of infections caused by MDR bacterial strains. Hence, there is an urgent need to search for novel, cost effective, potent therapeutic antibacterial agents. In the current study, we focused on investigation of antibacterial activity of selected phytochemicals against multidrug resistant bacterial isolates. The synergistic effect of antibacterials was checked to ensure drugs working together -where one drug increases the other's effectiveness.

KEYWORDS: *Urinary Tract infection (UTI); Multidrug Resistant (MDR); antibiotics; antibacterial activity; MIC.*

ARCHANA MOON*

University Department of Biochemistry, RTM Nagpur University, LIT Premises, Amravati road, Nagpur, Maharashtra, India.

Corresponding Author

Received on: 01-07-2017
Revised and Accepted on: 30-08-2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.4.b135-141

INTRODUCTION

A urinary tract infection (UTI) is the most common hospital-associated infection, and can lead to death in critically ill patients. It may affect the lower or upper or both UTI. The National Kidney & Urologic Diseases Information Clearing house (NKUDIC) reports that UTIs account for over 8 million visits to the clinicians annually.[1] The bacteria causing UTI includes *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Lactobacillus*, *Enterococcus* and members of the family of *Enterobacteriaceae*, and the fungus *Candida* (yeast). *E. coli* is the cause of 80–85% of urinary tract infections. The usual treatment for a urinary tract infection (UTI) consists of antibiotics. Lower UTIs can be treated with oral antibiotics. Upper UTIs require intravenous antibiotic. In uncomplicated cases, urinary tract infections are easily treated with a short course of antibiotics, although resistance to many of these antibiotics is increasing. In complicated cases, a longer course or intravenous antibiotics may be needed, and if symptoms have not improved in two or three days, further diagnostic testing is needed. The choice of antibiotic depends on the bacteria that are causing the infection, the severity of symptoms, the possibility of complications and the ability of the patient to take medicine by mouth. There are several types of antibiotics available that are used to treat UTI.[2] Oral antibiotics such as Trimethoprim/ Sulfamethoxazole (TMP/SMX), nitrofurantoin, or fosfomycin are typically first line. Cephalosporins, amoxicillin/clavulanic acid, or a fluoroquinolone may also be used.[3] Although a wide variety of antibiotics are available to treat UTI, but the most frequently adviced medication include the antifolates. Trimethoprim either alone or in combination with sulfamethoxazole known as co-trimoxazole forms the most prominent member of this group. Trimethoprim is the derivative of 2, 4-diaminopyrimidine and is referred to as the potent inhibitor of bacterial dihydrofolate reductase. The combination of trimethoprim with sulfonamides, such as sulfamethoxazole has extended the effect of trimethoprim on UTI bacteria. Trimethoprim and sulfamethoxazole have a greater effect when given together than when given separately, because they inhibit successive steps in the folate synthesis pathway. They are given in a 1:5 ratio in tablet formulations, so that when they enter the body the concentration in the blood and tissues is roughly 1:20, the exact ratio required for a peak synergistic effect between the two.[4] One of the component of co-trimoxazole, sulfamethoxazole (SMX) induces its therapeutic effect by competing with p-aminobenzoic acid (PABA) in the biosynthesis of dihydrofolate by inhibiting the enzyme Dihydropteroate synthetase (DHPS). While the other component which is trimethoprim (TMP) serves as a competitive inhibitor of Dihydrofolate reductase (DHFR), thereby inhibiting the de novo synthesis of tetrahydrofolate, the biologically active form of folate. Over the years, the continued use of various antibacterial/antimicrobial agents has led to the development of resistance in microorganisms. Antibiotic resistance is a seriously growing problem in medicine. Limited treatment options are available for treatment of infections caused by MDR bacterial strains. Hence, there is an urgent need to search for novel, cost effective, potent therapeutic antibacterial agents. The current study includes isolation and identification of UTI pathogens and investigation of antibacterial activity of different compounds against MDR uropathogenic bacteria and their synergistic effect to check drug effectiveness.

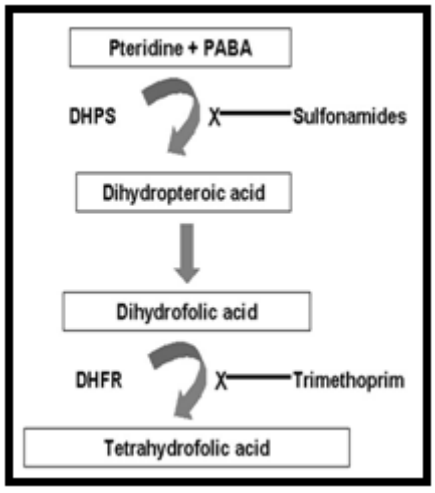

Figure 1
Folate Biosynthesis

MATERIALS AND METHOD

Collection and processing of Urine samples

100 Urine samples from the patients of age group of 45±5 years were collected in a sterile container from pathology laboratories in Nagpur, Maharashtra, India. The collected samples were diluted in proportion of 1:100 using sterile distilled water and stored at 4° C till further use.

Bacterial Isolation

The bacterial colonies were isolated by streaking diluted urine sample on a differential medium UTI agar plates

(Himedia, SM1353) and then further on Eosin Methylene blue (EMB) agar Plates (Himedia, M317), Enterrococcus confirmatory Broth (Himedia, M394). Later isolated single colony was picked and inoculated in 5 ml sterile Luria-Bertani (LB) broth for further use.

Bacterial inoculation

A single bacterial colony was inoculated into 5ml of sterile nutrient broth and incubated at 37^0C for 16-18 h till the turbidity matched with 0.5 Mc Farland's Nephelometer Standard. This culture was then used for antibiotic susceptibility testing.[5]

MDR (Multiple drug resistance) assay

20 antibiotics as mentioned in table 1 were utilized to check the multidrug resistivity (MDR) of the isolated bacteria. The antibiotics discs were purchased from Himedia, Mumbai. The agar Disc diffusion method (Kirby-Bauer) was employed for antibiotic susceptibility assay.[6] *Escherichia coli* ATCC 25922 was used as control as mentioned in table 3. The diameter of zone of inhibition around the disc was measured (Himedia antibiotic scale: PW096) to determine the sensitivity towards particular antibiotics. The MDR assay was done in triplicates. 2% methanol and 0.2% DMSO were also used as control.

Table 1
Antibiotics and Antifolates used for Antibiotic Sensitivity Assay

Sr no.	Name of Antibiotics	Disk Concentration	Sr no.	Name of Antibiotics	Disk Concentration
1.	Ampicillin	10 µg	11.	Gentamicin	10µg
2.	Cefazolin	30µg	12.	Cefixime	5µg
3.	Nalidixic Acid	30µg	13.	Amikacin	30µg
4.	Norfloxacin	10µg	14	Colistin	10µg
5.	Ciprofloxacin	5µg	15.	Netillin	30µg
6.	Cotrimoxazole	25µg	16.	Ceftriaxone	10µg
7.	Levofloxacin	5µg	17.	Cephotaxime	30µg
8.	Nitrofurantoin	300µg	18.	Furazolidone	50µg
9.	Augmentin	30µg	19.	Amoxycillin	10µg
10.	Cefuroxime	30µg	20.	Vancomycin	30µg
21.		Antifolates			
21. a)	Trimethoprim	5µg	21. b)	Sulphamethoxazole	20µg

Determination of antibacterial activity and MIC (Minimum Inhibitory Concentration) of phytochemicals

Selected compounds as mentioned in table 2 were employed to check the antibacterial activity against the isolated bacteria. Well diffusion method by Kirby–Bauer was employed for this test.[6] The diameter of zone of inhibition around the wells was measured using an antibiotic scale. Based on the results of the antibacterial activity, the MIC was ascertained to check the lowest concentration of a phytochemical that prevents visible growth of a bacterium.

Table 2
Phytochemicals concentration used to check their antibacterial activity.

Sr no.	Phytochemicals	Concentration used
1.	Hippuric acid	2, 5, 10, 12, 14, 16, 18, 20, 30, 40 mg/ml.
2.	Chlorogenic acid	1, 2, 5, 10, 12, 18, 20, and 40 mg/ml.
3.	Gallic acid	1, 2, 5, 10, 12, 14, 16, 18 mg/ml.

Synergistic effect of phytochemicals

Concentration based combination of Chlorogenic acid with Hippuric acid and Gallic acid with Chlorogenic acid and Hippuric acid were made, to perform antibacterial activity by well diffusion method to verify the synergistic antibacterial effects.

- Various concentration dependent combination of Chlorogenic acid (2, 12, 20 mg/ml) with Hippuric acid (5, 15, 20 mg/ml) against *E. faecalis* isolates and Chlorogenic acid (2, 12, 20 mg/ml) with Hippuric acid (5, 14, 20 mg/ml) against *E. coli* isolates were tested.
- Combination of Chlorogenic acid (2, 12, 20 mg/ml) with Gallic acid (2, 10, 16 mg/ml) against *E. coli* and Gallic acid (5, 14, 18 mg/ml) against *E. faecalis* isolates and Combination of Hippuric acid (5, 15, 20 mg/ml) with Gallic acid (2, 10, 16 mg/ml) against *E. coli* and Gallic acid (5, 14, 18 mg/ml) against *E. faecalis* isolates were also studied.

RESULTAND DISCUSSION

Bacterial isolation

The diluted urine samples were streaked on UTI agar. The bacterial mixed population from diluted Urine samples was observed on HiCrome UTI agar (Figure 2).

Figure 2a
Diluted Urine on UTI agar

Figure 2b
E. coli

Figure 2d
Metallic green sheen on EMB Agar confirming E.coli

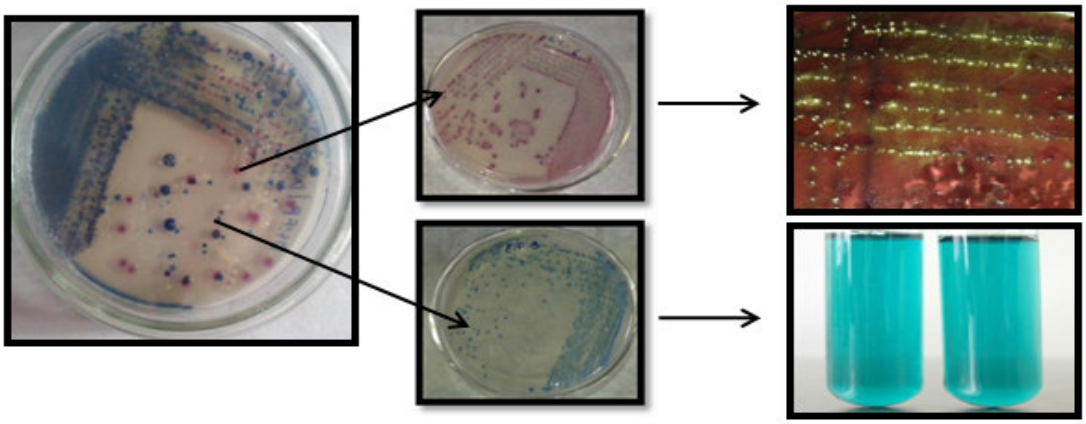

Figure 2c
E. faecalis

Figure 2d
Turbidity on Enterococcus confirmatory broth confirming

Figure 2
Isolation of Bacteria

It was found that out of 60 urine samples collected, 15 *E. coli*, 10 *E. faecalis*, were isolated.

MDR (Multiple drug resistance) assay

Antibiotic sensitivity assay was performed against 15 *E. coli* and 10 *E. faecalis* isolates to study their resistance pattern. The sensitivity results of all the clinical isolates of *E. coli* and *E. faecalis* towards the selected 20 antibiotics was performed. The antibiotic resistance pattern showed that the clinical isolates are multi drug resistant pathogenic UTI causative bacteria as mentioned in table 4&5.

Table 3
Antibiotic sensitivity of control microorganisms(zone of inhibition in mm diameter)

S.No.	Control microorganisms	Antibiotics														
		A	Am	C	E	P	K	T	Cp	Cf	Co	Gf	Nx	Of	Pf	Sc
1.	*Escherichia coli* ATCC 25922	18	22	24	19	17	19	22	15	33	24	29	32	32	30	32

Zone in triplicates

A=Ampicillin, T=Tetracycline, Nx=Norfloxacin, Am=Amoxycillin, Cp=Cephalexin, Of=Oflaxacin, C=chloramphenicol, Cf=Ciprofloxacin, Pf=Pefloxacin, E=Erythromycin, Co=Cotrimoxazole, Sc=Sparfloxacin, P=Penicillin-G, Gf=Gatilfloxacin, S=streptomycin, K=Kanamycin.

Table 4
Antibiotic sensitivity pattern of UTI E. coli (Zone in mm diameter)

Name of Antibiotics	Disk Conc.	*E. Coli* ATCC 25922	EC 1	EC 2	EC 3	EC 4	EC 5	EC 6	EC 7	EC 8	EC 9	EC 10	EC 11	EC 12	EC 13	EC 14	EC 15
Ampicillin	10 µg	15-22	14	R	15	R	27	20	15	15	20	R	R	R	R	R	R
Cefazolin	30µg	21-27	24	21	23	17	31	28	23	24	24	R	R	R	R	R	R
Nalidixic Acid	30µg	22-28	R	R	18	R	16	22	21	25	7	R	R	R	R	25	R
Norfloxacin	10µg	28-35	7	12	20	R	24	27	32	29	23	17	17	16	23	25	R
Ciprofloxacin	5µg	30-40	7	17	25	R	28	35	27	38	30	R	R	R	R	R	R
Cotrimoxazole	25µg	23-29	R	20	26	R	26	25	23	39	R	12	R	R	R	23	R
Levofloxacin	5µg	29-37	10	19	20	R	28	24	28	36	24	R	R	R	R	R	R
Nitrofuran-toin	300µg	17-23	16	18	16	17	17	70	16	16	20	24	21	20	21	22	20
Augmentin	30µg	15-28	29	32	31	R	R	R	R	R	16	R	R	R	10	17	R
Cefuroxime	30µg	20-26	28	20	30	28	R	R	R	R	28	R	24	30	32	24	R
Gentamicin	10µg	19-26	26	21	13	15	R	R	R	R	15	19	16	14	27	20	R
Cefixime	5µg	23-27	28	30	27	31	R	R	R	R	24	R	23	27	34	R	R
Amikacin	30µg	19-26	27	27	16	28	R	R	R	R	30	24	29	22	21	15	R
Colistin	10µg	11-15	18	17	16	18	R	R	R	R	16	15	16	15	19	14	17
Netillin	30µg	22-30	27	23	23	25	R	R	R	R	25	30	26	22	28	19	R
Ceftriaxone	10µg	29-35	31	40	31	32	R	R	R	R	27	R	31	25	32	R	R
Cephotaxime	30µg	30-37	30	24	33	30	R	R	R	R	29	R	32	27	25	22	R
Furazolidone	50µg	20-25	21	21	19	21	R	R	R	R	22	21	22	24	21	21	16
Amoxycillin	10µg	19-25	13	28	35	R	R	R	R	R	R	24	R	R	R	21	R

Zone in triplicates ***R - Resistant strains***

Table 5
Antibiotic sensitivity pattern of UTI E. faecalis (Zone in mm diameter)

Name of antibiotics	Disk conc.	*E. Faecalis* ATCC 29212	EF1	EF2	EF3	EF4	EF5	EF6	EF7	EF8	EF9	EF10
Cefpodoxime	10 µg	-	16	R	20	12	R	16	R	R	24	17
Chloramphenicol	30 µg	25-32	29	29	26	35	R	13	R	R	26	25
Vancomycine	30 µg	-	R	R	R	R	R	R	R	R	R	R
Streptomycin	10 µg	-	11	R	15	18	R	10	R	R	18	10
Rifampicin	5 µg	-	24	18	24	12	20	28	R	R	21	18
Levofloxacin	5 µg	25-32	24	24	27	25	30	16	R	R	27	24
Ceftriaxone	30 µg	-	20	22	27	20	R	20	R	R	31	20
Clindammycin	2 µg	-	R	15	10	13	R	18	R	R	R	10
Augmentin	30 µg	-	22	27	31	24	R	27	32	30	30	25
Amikacin	30 µg	-	10	15	15	16	R	10	R	R	21	10
Cefixime	5 µg	-	16	R	22	R	R	11	20	10	25	12
Tetracycline	30 µg	-	10	14	R	38	R	12	R	R	23	11
Nitrofurantoin	300 µg	19-23	20	22	20	22	23	20	23	24	19	21
Nalidixic acid	30 µg	-	R	R	R	R	R	R	R	R	R	R
Cefuroxime	30 µg	-	R	22	25	R	27	R	28	20	28	R
Co-trimoxazole	25 µg	26-34	22	27	21	30	20	18	24	28	25	21
Norfloxacin	10 µg	16-22	16	16	18	16	17	9	R	16	R	21
Gentamicin	10 µg	16-23	R	15	14	R	18	R	15	17	13	R
Ciprofloxacin	5 µg	19-25	21	R	R	24	R	10	R	R	R	20
Cefazolin	30 µg	-	19	R	R	19	R	24	R	R	R	19
Ampicillin	10 µg	27-33	20	R	R	28	R	33	R	R	R	31

Zone in triplicates ***R - Resistant strains***

Antifolates

Since, antibiotic resistance was seen towards the commonly prescribed antibiotics, it was thought worthwhile to also check antibacterial activity of the antifolate drugs i.e. Trimethoprim and Sulfamethoxazole against isolated 15 *E. coli* and 10 *E. faecalis*.

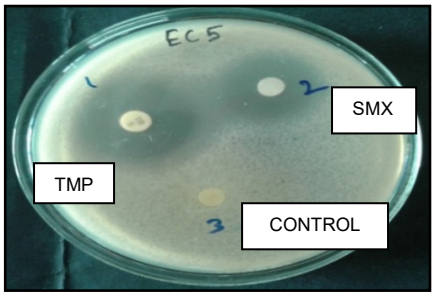

Figure 3
Antibacterial assay of antifolates against isolates

Table 6
Sensitivity and Resistant patterns towards DHFR and DHPS inhibitors for E. coli isolates

Antibiotics	*E.coli* Isolates														
	EC1	EC2	EC3	EC4	EC5	EC6	EC7	EC8	EC9	EC10	EC11	EC12	EC13	EC14	EC15
TMP (5µg)	R	22 (s)	23 (s)	R	25 (s)	28 (s)	R	24 (s)	R	R	R	R	R	R	R
SMX (20µg)	17 (s)	13 (I)	16 (s)	R	24 (s)	16 (s)	R	20 (s)	R	19 (s)	R	R	R	13 (I)	R

Zone of inhibition in millimetre diameter, Zone in triplicates
R - Resistant Strains, S- Sensitive, I- Intermediate

E. coli 1, 4, 7, 9, 10, 11, 12, 13, 14, 15 isolates were found to be resistant to Trimethoprim and *E. coli* 4, 7, 9, 11, 12, 13, 15 isolates were resistant to Sulfamethoxazole and *E. coli* 2, 3, 5, 6, 8 isolates were found to be sensitive to both Trimethoprim and Sulfamethoxazole as mentioned in table 6.

Table 7
Sensitivity and Resistant patterns towards DHFR and DHPS inhibitors for E. faecalis isolates

Antibiotics	*E.faecalis* Isolates									
	EF 1	EF 2	EF 3	EF 4	EF 5	EF 6	EF 7	EF 8	EF 9	EF 10
TMP (5µg)	27	33	22	34	23	17	23	31	23	25
SMX (20µg)	R	R	R	R	R	R	R	R	R	R

Zone of inhibition in millimeter diameter, Zone in triplicates
R - Resistant Strains

All *E. faecalis* isolates were found to be sensitive against Trimethoprim, while all the 10 isolates of *E. faecalis* were found to be resistant against Sulfamethoxazole as mentioned in table 7. TMP (Trimethoprim): Resistant <_10 mm, Intermediate 11-15 mm, Susceptible >_16 mm SMX (Sulfamethoxazole): Resistant <_10 mm, Intermediate 11-16 mm, Susceptible >_17 mm TMP/SMX: Resistant <_10 mm, Intermediate 11-17 mm, Susceptible >_18 mm

Determination of antibacterial activity and MIC of selected phytochemicals and standard antibiotics against E. coli and E. faecalis isolates.

Table 8
Antibacterial activity and MIC of phytochemicals.

Sr no.	Pytochemicals	Antibacterial activity(mg/ml)		MIC(mg/ml)	
		E.C	*E.F*	*E.C*	*E.F*
1	Hippuric acid	5	5	2.2	2.2
2	Chlorogenic acid	5	5	3-5	2.2
3	Gallic acid	1	5	2.2	2.6

Result of Antibacterial activity and MIC of phytochemicals is mentioned in table 8.

Combination of phytochemicals

RESULT

Chlorogenic acid showed the best inhibitory effect against *E. coli* and *E. faecalis* at a concentration of 20 mg/ml and Gallic acid and Hippuric acid showed the best inhibitory against *E. coli* and *E. faecalis* at a concentration of 15 mg/ml and 20 mg/ml for synergistic effect. There was a synergistic effect when antibacterial effect of phytochemical in triple combination including Hippuric acid, Chlorogenic acid and Gallic acid was compared to its isolated effect for both microorganisms.

DISCUSSION AND CONCULSION

Antibacterial activity of selected compounds viz; Chlorogenic acid, Hippuric acid, Gallic acid was performed against 15 *E. coli* and 10 *E. faecalis* UTI clinical isolates. Chlorogenic acid, Hippuric acid, Gallic acid, showed a resistant pattern from 1-5 mg/ml concentration and sensitivity at 10 – 40 mg/ml concentration against *E. coli* and *E. faecalis*. Green colour zone of Chlorogenic acid was observed due to the condensation reaction of two molecules of Chlorogenic acid ester with one molecule of primary amino compound under aeration in alkaline solution. Aeration of Chlorogenic acid in alkali produce only browning. Greening developed with the addition of amino acid such as glycine and alanine. Reduction of green pigment by ascorbic acid gave a yellow product, which readily turns green. The green pigment was assumed to be an oxidized quinone product. From lower MIC values of the compounds, it is conclude that these lead to an inhibitory action on MDR bacteria. Chlorogenic acid, Hippuric acid, Gallic acid are most effective antibacterial agents. MIC Scores are important to confirm resistance of the microorganisms. The synergistic activity was studied for the selected compounds. It was observed that the zone of inhibition did not synergistically increase. This may be attributed to the antibacterial activity of the individual compounds. Hence, they do not show enhanced synergistic antibacterial activity. Though, these compounds after appropriate ADMET studies can be utilized as potentiators of antibiotics/drugs to treat MDR UTI. It was concluded that the tested compounds show significant antibacterial activity *in vitro* against *E. coli* and *E. faecalis* isolates which are resistance to antibiotics i.e. Cotrimoxazole, as it is a combination drug of Trimethoprim and Sulfamethoxazole which are the inhibitors of folate pathway having enzyme DHFR and DHPS. The future perspective lies in studies at molecular level of the folate pathway enzymes.

CONFLICT OF INTEREST

Conflict of interest declared none.

REFERENCES

1. Urinary Tract Infections in Adults. (n.d.). National Kidney and Urologic Diseases Information Clearinghouse. Retrieved May 11, 2012 [cited 2012 May 11]. Available from https://www.niddk.nih.gov/health-information/kidney-disease
2. Grigoryan L, Trautner BW, Gupta K. Diagnosis and management of urinary tract infections in the outpatient setting: a review. jama. 2014 Oct 22;312(16):1677–84.
3. Zalmanovici TA, Green H, Paul M, Yaphe J, Leibovici L. Antimicrobial agents for treating uncomplicated urinary tract infection in women. Zalmanovici Trestioreanu, Anca "Cochrane Database of Systematic Reviews", Cochrane Database Syst Rev. 2010;10(10).
4. Hitchings G.H. Mechanism of action of trimethoprim sulfamethoxazole. J Infect Dis. 1973 Nov 1;128:Suppl-3:S433-6.
5. Bollela VR *et al.* McFarland nephelometer as a simple method to estimate the sensitivity of the polymerase chain reaction using Mycobacterium tuberculosis as a research tool. Braz J Med Biol Res, 1999 Sep;32(9):1073-6.
6. Bauer AW *et al.* Antibiotic susceptibility testing by a standardized single disc method. Am J Clin Pathol. 1966;45(4):493-6.
7. Yabuta G, Koizumi Y, Namiki K, Hida M, Namiki M. Structure of green pigment formed by the reaction of caffeic acid esters (or chlorogenic acid) with a primary amino compound. Bioscience, biotechnology, and biochemistry. 2001;65(10):2121-30.
8. Susan M. Schappert, M.A., and Elizabeth A. Rechtsteiner, M.S. Ambulatory medical care utilization estimates for 2006. National health statistics reports; no 8. Hyattsville, MD: National Center for Health Statistics; Aug 6. 2008.
9. Griebling TL. Urinary tract infection in women. In: Litwin MS, Saigal CS, eds. Urologic Diseases in America. Department of Health and Human Services, Public Health Service, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases. Washington, D.C.: GPO; 2007.07–5512: 587–619.
10. Mohammad Rahbar and Kambiz Diba. *In vitro* activity of cranberry extract against etiological agents of urinary tract infections. Afr. J. Pharm. Pharmacol. 2010May;4(5):286-8.
11. Donald Kaye. Antibacterial Activity of Human Urine. J. Clin. Invest. 1968Oct; 47(10):2374-90.
12. Borges A, Ferreira C, Saavedra MJ, Simões M. Antibacterial Activity and Mode of Action of Ferulic and Gallic Acids against Pathogenic Bacteria. Microb Drug Resist. 2013 Aug;19(4):256-65.
13. Alfreid W. Bauer, *et al.* Single Disk Antibiotic-Sensitivity Testing of Staphylococci; An analysis of technique and results. AMA Arch Intern Med.1959;103(2):208-16.

Reviewers of this article

Dr V.G. Meshram

Professor, Dept. of Biochemistry, RTM Nagpur University, Nagpur.

Prof. Srawan Kumar G.Y

Associate Professor,Nalanda Institute of Pharmaceutical Sciences, Sattenapalli, Guntur, Andrapradesh, India.

Prof.Dr.K.Suriaprabha

Asst. Editor , International Journal of Pharma and Bio sciences.

Prof.P.Muthuprasanna

Managing Editor , International Journal of Pharma and Bio sciences.

We sincerely thank the above reviewers for peer reviewing the manuscript