Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2018; 10(10): 243-248

ISSN- 0975 1556

Research Article

Antimicrobial Efficacy of Traditional Medicinal Plant Extracts Against the Antibiotic Resistant Isolates from Drinking Water Sources

Jadhav A V[1], Agnihori S N[1], Sawant H[1], Bansode S[1], Bankar A[2], Panicker S G[1*]

[1]*Department of Microbiology, Dr. D.Y. Patil Arts, Commerce and Science College, Pimpri, Pune, Maharashtra, India*
[2]*Department of Microbiology, MES-Garware College, Pune, Maharashtra, India*

Available Online:25th October, 2018

ABSTRACT

Water is the second most essential factor for different life forms after oxygen. Most of the people obtain their drinking water from surface and underground sources. However both surface and ground water source could be contaminated by biological and chemical pollutants. In the rural area, majority of people directly use natural water sources like river water, bore well, lake water etc. for drinking purpose. In the present study, 12 different drinking water samples were collected from different villages of Pune, Maharashtra, India. The samples were analyzed bacteriologically and physico-chemically. The MPN positive samples were analyzed for antibiotic resistance by polydisc method. 27 bacterial isolates were reported to be resistant to antibiotic Imipenem (10mcg). Antimicrobial activity of Tulsi and Neem leaf extracts showed a great potential against these antibiotic resistant isolates. Thus, from the obtained results, it can be concluded that these plants can be used as natural therapeutic agents that may serve as lead for the development of new pharmaceuticals addressing the major therapeutic needs.

KEYWORDS: Drinking water, Antimicrobial activity, bacteriological, MPN, Antibiotic resistant.

INTRODUCTION

Water is one of the most important bacterial habitats on earth and known to be a major way of dissemination of microorganisms in nature. The water-bodies are recognized as a significant reservoir of antibiotic resistant bacteria (ARB). The development of multidrug resistant (MDR) bacteria in water supply systems is found to be a serious threat to the public health[1]. The wastewater treatment plants are one of the major sources for continuous release of a variety of antibiotics into aquatic environment. Therefore, the load of ARBs in the aquatic environment has been increasing continuously. This serious issue is observed in developing countries like India due to the poor sanitization practices. The easy availability of antibiotics sometimes without a medical prescription has increased their widespread misuses[2]. Apart from this, large quantities of antibiotics are being used in the clinical settings, agriculture, food industry and aquaculture etc.[3] The global demand of antibiotics has been increased by 40% in the last decade[4]. Therefore, due to heavy usage of antibiotics results into increasing numbers of ARBs. The continuous development of ARBs in an aquatic environment may cause a serious public health issue. Sewage is the most common source of drinking water pollution due to the leakage of water supplying pipelines and thus alters the physiochemical properties of drinking water. In addition to this, the sewage water also spreads several human pathogens via drinking water system[5]. However, it was noticed that many other water sources are also found to be highly contaminated. It is reported that 37.7 million Indians are affected due to waterborne diseases and 1.5 million children are died due to diarrhea, annually. Therefore, there is a great need of an extensive water treatment before it is consumed[6]. The microbiological safety of drinking water is of prime importance to protect public health. The protection of drinking water is generally considered as the primary strategy to make it safe for drinking purpose.

From several years, it is well known that the herbs are commonly used in the preparation of many drugs that are used for the treatment of several human diseases. Thus, the anti-microbial property of these plants made them eligible as most safe and the efficient therapeutic agents against several bacterial pathogens. The plant of Neem has been used in the preparation of the traditional medicine as a source of many therapeutic agents in the Indian culture and grows well in the tropical countries. It is well known that this plant contains several bio-active molecules, having multiple medicinal properties[7]. Similarly, Tulsi leaves also exhibited insecticidal and anti-bacterial activities and are employed in treatment of several diseases on the basis of the traditional experience[8]. Previously, it was also reported

**Author for Correspondence: suneetapanciker@gmail.com*

Table 1: Antibiotic Resistance of different isolates

Isolates	Antibiotic	Imipenem (10mcg)	Meropenem (10mcg)	Ciprofloxacin (5mcg)	Tobramycim (10mcg)	Moxifloxacin (5mcg)	Ofloxacin (5mcg)	Sparfloxacin (5mcg)	Levofloxacin (5mcg)
DS I	DS I 1	R	S	S	S	S	S	S	S
	DS I 2	R	S	S	S	S	S	S	S
	DS I 3	R	S	S	S	S	S	S	S
DS II	DS II 1	S	S	S	S	S	S	S	S
	DS II 2	R	R	S	S	S	S	S	S
	DS II 3	S	S	S	S	S	S	S	S
	DS II 4	R	S	S	S	S	S	S	S
DS III	DS III 1	R	S	S	S	S	S	S	S
	DS III 2	S	S	S	S	S	S	S	S
	DS III 3	S	S	S	S	S	S	S	S
DS IV	DS IV 1	R	S	S	S	S	S	S	S
	DS IV 2	R	S	S	S	S	S	S	S
	DS IV 3	S	S	S	S	S	S	S	S
	DS IV 4	R	S	S	S	S	S	S	S
DS V	DS V 1	S	S	S	S	S	S	S	S
	DS V 2	S	S	S	S	S	S	S	S
	DS V 3	S	S	R	S	S	S	R	S
DS IV	DS VI 1	S	S	S	S	S	S	S	S
	DS VI 2	R	S	S	S	S	S	S	S
	DS VI 3	R	S	S	S	S	S	S	S
	DS VI 4	R	S	S	S	S	S	S	S
SS I	SS I 1,2,3	S	S	S	S	S	S	S	S
	SS II 1	R	S	S	S	S	S	S	S
	SS II 2	R	S	S	S	S	S	S	S
SS II	SS II 3,4	S	S	S	S	S	S	S	S
SS III	SS III 1,2	S	S	S	S	S	S	S	S
SS IV	SS IV 1,2,3,4	S	S	S	S	S	S	S	S
SS V	SS V 1	R	S	S	S	S	S	S	R
	SS V 2	S	S	S	S	S	S	S	S
SS IV	SS VI 1,3,4	S	S	S	S	S	S	S	S
	SS VI 2	R	S	S	S	S	S	S	S

that the Tulsi leaves (*Ocimum sanctum*) and Neem leaves (*Azadirachta indica*) have been used in purification of contaminated drinking water. Thus, the use of such plants for contaminated water treatment could be a safer, cost effective and an eco-friendly process[9]. The side effects of antibiotics and quick development of bacterial resistance are the major problems. Therefore, several bioactive compounds of different plants species can be an alternative to these antibiotics[10]. In the present study, anti-microbial activities of plant leaves extracts of Tulsi and Neem were tested against the antibiotic resistant microorganisms isolated from different drinking water sources of the villages in Pune district, Maharashtra, India.

MATERIALS AND METHODS

Drinking water Sample collection

12 samples water samples were collected from different drinking water sources (surface water and borewell water) of Dehugaon and Sudumbare villages of Pune, India. The water samples were collected in sterile screw capped sterile glass bottles. The bottles were labeled properly with details of source, date and time of collection. The bottles were carried in icebox and brought to the laboratory and stored in the refrigerator until further analysis.

Evaluation of bacterial load

Enumeration of bacterial colonies was performed by using spread plate technique. The water samples were serially diluted with sterile distilled water. The different dilutions (0.1 ml) were spread on sterile MacConkeys agar plates and incubated at 37ºC. The bacterial colonies formed on sterile MacConkeys agar plates were counted. The obtained colonies were also tested for antibiotic resistance study.

Antibiotic resistance study

40 isolated colonies were tested against eight different antibiotics on Mueller Hinton Agar by Kirby-Bauer disk

Table 2: Antibiotic Resistant organisms identified from Bergey's Manual

Sr. No.	Sample & Isolate No.	Genus Name
1	DSI isolate 1	*Salmonella*
2	DSI isolate 2	*Escherichia*
3	DSI isolate 3	*Escherichia*
4	DSII isolate 2	*Hafnia*
5	DSII isolate 4	*Cedecea*
6	DSIII isolate 1	*Cedecea*
7	DSIV isolate 1	*Erwinia*
8	DSIV isolate 2	*Citrobacter*
9	DSI V isolate 4	*Vibrio*
10	DSV isolate 3	*Vibrio*
11	DSVI isolate 2,3,4	*Unidentified*
12	SSII isolate 1	*Escherichia*
13	SSII isolate 2	*Vibrio*
14	SSV isolate1	*Cedecea*
15	SSVI isolate 2	*Edwardsiella*

diffusion method reported earlier. [11] Briefly, overnight grown bacterial cultures in broth were spread separately on sterile Mueller Hinton agar plates and antibiotic impregnated octadisk was placed on it. All plates were incubated overnight at 37^0C and observed for a clear zone of growth inhibition. The obtained results were interpreted using a reference table. The bacterial isolates that showed resistance for two or more antibiotics (multidrug resistant) were further characterized and identified. Antibiotics of different classes used in this study were: Imipenem(10 mcg), Meropenem (10 mcg), Ciprofloxacin (5 mcg), Tobramycin (10 mcg), Moxifloxacin (10 mcg), Ofloxacin (5 mcg), Ofloxacin (5 mcg) and Levofloxacin (5 mcg).

Characterization of antibiotic resistant isolates

The antibiotic resistant bacterial isolates were characterized morphologically and biochemically. The colonial morphology, Gram staining, spore staining, IMViC test, Catalase test, starch and casein hydrolysis were carried out to characterize the obtained isolates. Bergey's manual of systematic bacteriology and the manual of identification of medical bacteria were used for partial identification of antibiotic resistant isolates.

Plant material collection

Fresh leaves of *O. sanctum* (Tulsi) and *A. indica* (Neem) were collected from the local area of Pune, Maharashtra, India. The collected plant leaves were washed with the sterile distilled water thoroughly, air dried and then homogenized to make a fine powder and stored in airtight bottles for further use.

Preparation of plant leaves extracts

Aqueous extraction

The dried powder (5 g) of Neem or Tulasi leaves was added separately into 200 ml of distilled water and boiled on slow heat for 2 h. After boiling, the extract was filtered through a muslin cloth and centrifuged at 5000 rpm for 10 minutes. The obtained supernatant was collected and concentrated by boiling to make one-fourth of the original volume[11].

Solvent extraction

The dried powder (5 g) of Neem or Tulasi leaves was added separately into 50 ml of methanol in a conical flask. The mixture was kept on rotary shaker for 24 h. The obtained solution was then filtered through a muslin cloth and centrifuged at 5000 rpm for 15 minutes. The methanol was evaporated and the leaves extract was re-suspended into 50 % of DMSO to achieve the concentration of 100 mg/ml[12].

Anti-bacterial activity of plant extracts

The anti-bacterial activity of different plant species (aqueous and solvent extract) was evaluated by agar well diffusion method. The test organisms were grown overnight in the nutrient broth. The cell suspension of 0.1ml was inoculated into the molten Mueller Hinton agar media and after proper homogenization it was poured into 100 mm Petri dishes. The wells were prepared in the seeded plates with the help of a cork-borer (8 mm). Then 0.1ml of the plant leaves extracts was added into each well separately and allowed to diffuse at 4^0C for 20 min. The plates were then incubated at 37°C for 24 h. The anti-microbial activity of the plant leaves extracts was determined by measuring the diameter of zone inhibition[13].

RESULTS

Isolation of antibiotic resistant bacteria from different water sources

Altogether, six Dehugaon samples showed presence of 22 different colony forming units and that of Sudumbre showed 18. The water samples collected from Dehugaon region were labeled as DS I-VI and the samples from Sudumbre were labeled as SS I-VI. Each of these isolate was tested for its antibiotic resistance against eight different antibiotics by Kirby-Bauer disk diffusion method. Zone of inhibition thus obtained can be seen in the table 1. It was seen that forty percent isolates were resistant to the Imipenem antibiotic. Isolates such as DS II 2, DS V 3 and SS V 1 were resistant to two antibiotics of the eight from the octadisk. DS II 2 was found to be resistant to Imipenem and Meropenem, DS V 3 showed resistance to Ciprofloxacin and Sparfloxacin while SS V 1 exhibited resistance to Imipenem and Levofloxacin.

Characterization of antibiotic resistant isolates

The Bergey's manual of systematic bacteriology and manual of identification of medical bacteria have been referred for the partial identification of bacterial isolates and is shown in Table 2. All the identified isolates belonged to *Enterobacteriaceae* family reflecting that drinking water source was fecally contaminated.

Antibacterial activity of plant leaves extracts

The antibacterial activity of different plant species was evaluated by agar well diffusion method for both aqueous and solvent leaves extracts. The aqueous extract of Neem and Tulsi showed no antibacterial activity against the bacterial isolates. However, the concentrated organic extracts of Neem and Tulsi leaves extracts showed a prominent antibacterial activity against almost all the resistant isolates. It was observed that when the leaves extracts were 1:1 diluted with DMSO, the extracts found to be only active against the isolates, *Escherichia, Erwinia*

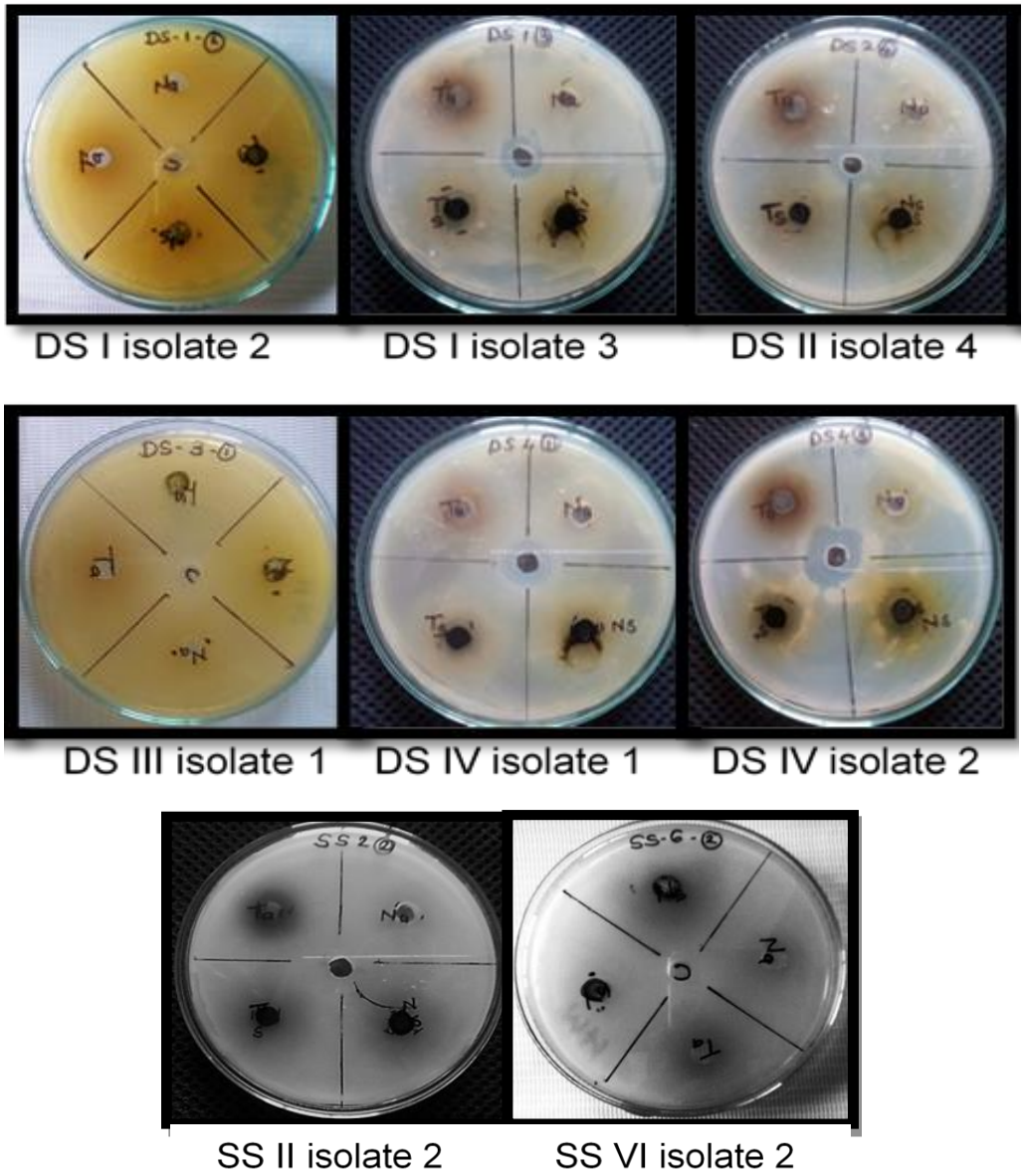

Fig 1: Antibacterial activity of herbal extracts against Resistant Isolates

and Citrobacter. The antimicrobial activity of the extracts is shown in the representative figure 1 and the diameter of the zone of inhibition measured is presented in table 3.

DISCUSSION

Forty different isolates were obtained from 12 different drinking water sources. The antibiotic resistance of different isolates was tested against eight broad spectrum antibiotics having activity against both Gram-positive and Gram-negative bacteria. It was observed that almost 40% isolates were found to be resistant to the Imipenem antibiotic. The isolates DS II 2, DS V 3 and SS V 1 were resistant to two different antibiotics from the octadisk. The antibiotic, imipenem is a particularly active against *Pseudomonas* and *Enterococcus* species. The Gram negative bacteria are found to be resistant to imipenem to varying degrees was reported earlier[14]. Important clinical infections like urinary tract infection are caused due to *Enterococcus*. The meropenem is similar to Imipenem in its action and spectrum. However, the antibiotic meropenem is more active against *Enterobacteriaceae* and less active against the Gram positive bacteria. It is administered to adults with complicated UTI. The identified antibiotic resistant bacteria from the drinking water sources in the present study belong to *Enterobacteriaceae* family which is well known to cause urinary tract infection or gastrointestinal diseases. The presence of co-resistance and MDR signifies that there might be continuous heavy usage of antibiotics in the community. The non-human use of highly-important antibiotics also contributes to the drug resistance against a range of antibiotics[15,16]. The relatively cheap and commonly prescribed drugs commonly favor a high co-resistance is reported previously[17,18]. In rural communities, the high level of bacterial contamination is reported in drinking water sources due to poor sanitation practices and thus results into lack of safe-water supply for domestic purposes[19]. Studies illustrate that surface water

Table 3: Effect of Herbal extracts on antibiotic resistant organisms

Sr. No.	Isolated Resistant Bacteria (Genus)	Zone of Inhibition (mm)				
		Tulsi (Aqueous extract) 1:1 diluted	Neem (Aqueous extract) 1:1 diluted	Tulsi (Methanol extract) 1:1 diluted	Neem (Methanol extract) 1:1 diluted	Tulsi (Methanol extract) Concentrated
1	DS I 2 (*Escherichia*)	0	0	0	0	12
2	DS I 3 (*Escherichia*)	0	0	14	15	17
3	DS II 4 (*Cedecea*)	0	0	0	0	15
4	DSIII 1 (*Cedecea*)	0	0	0	0	0
5	DS IV 1 (*Erwinia*)	0	0	11	10	16
6	DSIV 2 (*Citrobacter*)	0	0	18	16	22
7	SSII 2 (*Vibrio*)	0	0	0	0	0
8	SSVI 2 (*Edwardiella*)	0	0	0	0	0

contamination occurs mainly from livestock operations and human sewage and that decreasing livestock access to surface water reduced the fecal coliforms levels by an average of 94%[20]. Recent study also revealed that six imipenem resistant and 29 meropenem resistant isolates were reported in a village from Ujjain district of Madhya Pradesh, India[21].

To the best of our knowledge, this study is the first of its type for this region and highlights the need for drinking water source monitoring in the community for the presence of fecal contamination along with resistance profiles of the isolates. However, various studies around the world assessing different water sources have revealed total coliform contamination rates 0-100%[22,23,24].

With the widespread development of drug resistance of bacterial pathogens against currently available modern antibiotics, the medical science is now making efforts to discover novel antibiotics. The traditional medicinal plant such as Neem (*A. indica* L.) and Tulsi (*O. sanctum* L.) have been proved to be a better alternative to the antibiotics. In the present study an effort has been made to access the susceptibilities from aqueous and organic extracts of Neem (leaves) and Tulsi (leaves) against the antibiotic resistant bacterial species isolated from different drinking water sources. It was noticed that *Vibrio* and *Escherichia* were found to be more susceptible followed by *Erwinia*and *Citrobacter*. Previous studies have showed that extracts of *A. indica* were found effective against *Escherichia coli* and *Streptococcus faecalis* with fairly high degree of sensitivity (IZ=18-33 mm) to methanol extracts[25]. A study by group[26] revealed that ethanolic extract of Neem bark was more significantly active at 250 µg concentrations than the Neem leaves at all the studied concentrations. *E. coli* was highly susceptible to ethanol extract of Tulsi leaves at 250 µg concentration. Most bacterial strains were resistant at lowest concentrations (5 µg) of various extracts of both plants. The results obtained in present study are in good agreement with the previous reports. In the present study, it is clearly revealed that the organic leaves extracts was more effective against all the tested bacterial isolates from drinking water sources. However, it was also noticed that aqueous leaves extracts did not show anti-bacterial activity at the lower concentrations.

CONCLUSION

The results of the study indicate the poor sanitary conditions of the sampling sites and unhygienic practices of people leading to the presence of organisms belonging to the *Enterobacteriaceae* family. Furthermore, it also indicates the overuse and / misuse of antibiotics as antibiotic resistant bacteria were isolated from the drinking water samples. The study suggests the additional measures (hygienic and sanitary practices) are required by the individual to avoid post contamination of drinking water. From the above studies, it can also be concluded that the traditional plants are new sources of antimicrobials that can establish a scientific base for the use of modern medicine.

ACKNOWLEDGMENT

The authors thank the management of Dr. D. Y. Patil Arts Commerce and Science College, Pimpri, Pune, India, for supporting and providing the opportunity to carry out the research.

REFERENCES

1. IvoneVaz-Moreira, Olga C. Nunes, C_elia M. Manaia. Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human microbiome, FEMS Microbiol Rev 2014; 38: 761–18.
2. Nain VK, Khurana GS, Singh S, Vashitha A, Sangeeta A, Singh N, Aggarwal A, Arora I, Khan G, Thareja K, Gupta R, Jain and Diwan P. Antibiotic Resistance Pattern in Bacterial Isolates Obtained from Different Water Samples of Delhi Region,DU Journal of Undergraduate Research and Innovation 2015; 1(3): 219-227.
3. Van Boeckel TP., Brower C., Gilbert M. Grenfell BT., Levin SA., Robinson TP., Teillant A., Laxminarayan R**.** Global trends in antimicrobial use in food animals, Proc. Natl. Acad. Sci. USA, 2015; 112: 5649–5654.
4. The Review on Antimicrobial Resistance, chaired by Jim O'Neill. Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations. Dec 2014. http://www.jpiamr.eu/wp-content/uploads/2014/12/AMR-Review-Paper-Tackling-a-crisis-for-the-health-and-wealth-of-nations_1-2.pdf

5. Khurana I and Sen R. report. 2009. WaterAid's vision is of a world where everyone has access to safe water and sanitation.
6. Naidoo S and Olaniran. A. Treated Wastewater Effluent as a Source of Microbial Pollution of Surface Water Resources, Int. J. Environ. Res. Public Health 2014; 11: 249-270.
7. Maragatharavlli S., Brinda S., Kaviyaarshi NS. andGangwar SR.. Antimicrobial activity in leaf extract of neem (*Azadirachtaindica*Linn.), Int. J. Sci. Nature 2012; 3(1): 110-113.
8. Nanasombat S., Lohasupthawee, P. Antibacterial activity of crude ethanolic extracts and essential oils of spices against Salmonellae and other enterobacteria, KMITL Sci.Tech. J. 2005; 5(3): 527-538.
9. Bhattacharjee T., Gidde MR., Bipinraj NK.. Disinfection of Drinking Water in Rural Area Using Natural Herbs,International Journal of Engineering Research and Development, 2013; 5(10): 07-10.
10. Fernando Baquero, Jose´ -Luis Martı´nez, and Rafael Canto´n., Antibiotics and antibiotic resistance in water environments, Current Opinion in Biotechnology 2008; 19: 260–265.
11. Ada Zwetlana, Nandini M, KusumaDorcas. Antimicrobial activity of medicinal plant extracts on gram negative bacteria, Journal of Medicinal Plants 2014; 2(5): 51-54.
12. Chang-Geun Kang, Dae-Sik Hah, Chung-Hui Kim, Young-Hwan Kim, Euikyung Kim1 and Jong-Shu Kim. Evaluation of Antimicrobial Activity of the Methanol Extracts from 8 Traditional Medicinal Plants. Toxicol. Res 2011; 27(1):31-36.
13. Balouiri M, Sadiki M, Ibnsouda SK.. Methods for in vitro evaluating antimicrobial activity: A review, Journal of Pharmaceutical Analysis 2016; 6: 71–79.
14. Mohammed ESH, Fakhr AE, Mohammed ESH, Johery SAE, andHassanein WAG. Spread of TEM, VIM, SHV, and CTX-M β-Lactamases in Imipenem-Resistant Gram-Negative Bacilli Isolated from Egyptian Hospitals, International Journal of Microbiology, 2016; ID 8382605: 1- 15.
15. Van Boeckel TP., Gandra S., Ashok A., Caudron Q., Grenfell BT., Levin SA., Laxminarayan R. Global antibiotic consumption 2000 to 2010: An analysis of national pharmaceutical sales data, Lancet Infect. Dis. 2014; 14: 742–750.
16. Hammerum AM., Heuer OE. Human health hazards from antimicrobial-resistant Escherichia coli of animal origin, Clin. Infect. Dis. 2009; 48: 916–921.
17. Abo-State MA.,Mahdy HM., Ezzat SM., Abd El Shakour EH., El-Bahnasawy MA. Antimicrobial resistance profiles of Enterobacteriaceae isolated from Rosetta branch of River Nile, Egypt. Appl. Sci. J. 2012; 19: 1234–1243.
18. Sivri N., Sandalli C., Ozgumus OB., Colakoglu F., Dogan D. Antibiotic Resistance Profiles of Enteric Bacteria Isolated from Kucukcekmece Lagoon (Istanbul–Turkey). Turk, J. Fish. Aquat. Sci. 2012; 12: 699–707.
19. Ichor T., Umeh EU., Duru EE. Microbial Contamination of Surface Water Sources in Rural Areas of Guma Local Government Area of Benue State, Nigeria, Med. Sci. Public Health 2014; 2: 43–51.
20. Hagedorn C., Robinson S.L., Filtz J.R., Grubbs S.M., Angier T.A., Reneau R.B., Jr. Determining sources of fecal pollution in a rural Virginia watershed with antibiotic resistance patterns in fecal *Streptococci*. Appl. Environ. Microbiol. 1999; 65: 5522–5531.
21. Purohit MR, Chandran S, Shah H, Diwan V, Tamhankar AJ and Lundborg CS. Antibiotic Resistance in an Indian Rural Community: A 'One-Health' Observational Study on Commensal Coliform from Humans, Animals, and Water Int. J. Environ. Res. Public Health 2017; 14: 386.
22. Almasi, AAsadi, F., Sharafi, K., Atafar, Z., Mohamadi, A. Efficacy evaluation of drinking water distribution network existence in microbial quality desirability and its chlorination status in small communities - Case study: Kermanshah province villages, J Health in The Field 2013; 1(2): 17-21, (in Persian).
23. Mukhopadhyay C., Vishwanath S., EshwaraVK., and Shankaranarayana SA. Afrin Sagir microbial quality of well water from rural and urban households in Karnataka, India: Across-sectional study, J Infect Public Health 2012; 5: 257—262.
24. AIOtaibi ELS. Bacteriological assessment of urban water sources in KhamisMushait Governorate, southwestern Saudi Arabia, International Journal of Health Geography 2009; 8: 16.
25. Koona S. and Budida S. Antibacterial Potential of the Extracts of the Leaves of Azadirachta indica Linn., Not Sci Biol, 2011; 3(1): 65-69.
26. Rathod GP, Kotecha BM, Sharma R, AminH, Prajapati PK. *In vitro* Antibacterial study of two commonly used medicinal plants in Ayurveda: Neem (*Azadirachtaindica*L.) and Tulsi (*Ocimum sanctum* L.), International Journal of Pharmaceutical & Biological Archives 2012; 3(3): 582-586.