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Phytochemical analysis of some herbal extracts and assessed their antimicrobial effect on the bacterial isolates from urinary tract infected patients

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ABSTRACT

Urinary tract infection is one of the most common infections among women. The diseases is responsible for many visits to physicians and isolating of antibiotic resistance bacteria from infected women encouraging the scientist to isolates bacteria and evaluate the effect of herbal plants on the isolates. For this purpose 100 urine samples were collected from women who admitted in Iranpour hospital, Omideye. The samples were cultivated on Blood agar, MacConkey as well as Eosin methylene blue agar. Then they were identified using biochemical tests and verified using Api 20E. The WBC of the positive culture was analyzed and antibiotic susceptibility of the isolates was checked. Then effect of 3 medicinal plants, including: *Myrtus communis*, *Achillea millefolium* and *Citrullus colocynthis* were evaluated on the islaets. Furthermore, phytochemical screening of the medicinal plants was evaluated and MIC for the best plant was checked. Finally, identification of phytochemical compounds was performed using HPLC. Out of all, 9 isolates were detected and all of them were belonging to Enterobactereacae family. Most of the isolates were resistant to cephalothin and the most effective drug was ciprofloxcacin. Among the water, ethanolic and acetonic extract the most effective were ethanol extract of *Myrtus communis*. The extracts showed different compound including: tannins, glycosides, saponins, alkaloids, flavonoids, quinone and phenoloic compounds with antibacterial capability. In addition presence of phenolic compound verified using HPLC.

1. Introduction

Urinary tract infection defined as a typical type of bacterial infection which could affect the lower part or upper parts of the urinary tract. The disease will increase among female population due to the anatomy of their urethra and certain behavioral factors, including: delay in micturition, sexual activity and the use of diaphragms and spermicides (Nicolle, 2008; Salvatore et al., 2011). Indeed infection in women most often results from personal or pre urethral bacteria that enter the urethra and ascend into the bladder, which is may depend on sexual activity or consuming of the catheter during surgery (Litza and Brill, 2010). UTIs have been well-studied in Sweden and other parts of Europe. The studies have shown that one in 5 adult women experience a UTI at some point, confirming that it is an remarkably common worldwide problem (Naber *et al.*, 2008).

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Furthermore, Anderson and his colleague believed that the incidence of disease will increase during August and they have explained the hot and humid condition during this month is the main factors (Anderson, 1983). The most incorporated bacteria are: *Escherichia coli, Staphylococcus saprophyticus*, *Proteus*, *Klebsiella*, *Pseudomonas*, *Enterobacter* and *Citrobacter* (Lane and Takhar, 2011). According to isolated bacteria from infected women in different parts of world, several antibiotics have been suggested by clinician while nitrofurantoin, trimethoprim/sulfamethoxazol, cephalosporin and fluroquinolons are most effective (Salvatore *et al.*, 2011; Zalmanovici *et al.*, 2010). However, in case of reducing prevalence of antibiotic resistance, the drugs should not be prescribed extremely. In addition presence of different herbal medicine may act as specific role for remedy (Doughari *et al.*, 2009; Doughari, 2012). Natural plants with secondary metabolites may use as a drug source with antibacterial and antifungal activity. Therefore, the present study tried to evaluate the effect of water, ethanolic and acetonic extract of 3 native plants of Khozestan province on isolated bacteria from women with UTI.

2. Materials and Methods

2.1. Sample collections

A total hundred urine samples were collected from hospitalized women at an Iranpour hospital in Omidiyeh city, Iran. The samples were collected in sterile bottles and taken to laboratory within one hour. All collected samples were taken to the laboratory within one hour and subjected to microbiological analysis.

2.2. Isolation and identification of the isolates

The samples were cultivated on blood agar, Eosine methylen blue and MacConkey agar and incubated at 37°C for 18-24 hrs. The isolates were physiological identified using biochemical tests including: gram stain, oxidase, catalase, nitrate reduction, and TSI tests. Then phenotypic identification of the isolates was verified by Api kits 20E (Aiyegoro *et al.*, 2007) and finally, WBC counts were carried out on all positive Urinary tract infection samples (Cosgrove and Avdic, 2013).

2.3. Antibiotic susceptibility of the isolates

Antibiotic susceptibility of the isolates was done by disk diffusion method and the results were interpreted by antibiotic guideline 2013-2014 (Cosgroveand Avdic., 2013). The Antibiotics used were: ciprofloxacin (CP 5mcg), trimethoprim/sulfamethoxazol (SXT 1.25, 23.75 mcg), gentamycine (GM 10 mcg), ceftriaxon (CRO 30 mcg), cephalothine (CF 30 mcg) and nitofurantoin (FM 300 mcg).

2.4. Plant extracts

In the present study, *Myrtus communis*, *Achillea millefolium* and *Citrullus colocynthis* were obtained from retail shops and extracted by hot water, acetone and ethanol (70%). Twenty gram of each plant powder was added in 100 ml of hot water, acetone and ethanol (70%) and the suspensions were kept at room temperature for 24 hrs. Then the suspensions were filtered by filter paper (woman 10) and the filtrates were kept in refrigerators (Handa, 2008).

2.5. Antimicrobial effect of plant extracts on the isolates

The isolates were fully cultivated on Muller Hinton agars and the wells were made in the each plate agar using sterile Borer. Then, 100 µl of each plant extract was added into the well and the plates were incubated at 37°C for 18-24hrs. After this time, the inhibition zone for each plant extract was measured and recorded (Bahador and Baserisalehi, 2011). The test was done in three replicates and the results were analyased using ANOVA test.

2.6. Determination of minimal inhibitory concentrations of the plant extracts

Minimal inhibitory concentrations of the plant extracts against the isolates were determined after cultivation of *Klebsiella oxytoca* (sensitive isolates to extracts) onto Muller Hinton agar. Then, the wells were made in the agar and 100 µl of the plant extract with different dilutions (1^{-2}- 1^{-124}) were added into the wells. The plates were incubated at 37°C for 18-24 hrs and the lowest concentration exhibited inhibition zone considered as Minimal Inhibitory Concentration of each plant extract. The test was done in three replicate.

2.7. Determination of phytochemical compounds of plant extracts

Presence of different compounds including: tannins, glycosides, saponins, alkaloids, flavonoids, quinone and phenoloic compounds were checked according to the protocols.

Detection of tannin and glycoside in the present study was carried out by adding 2 ml ferric chloride and sodium hydroxyl separately into 4 ml diluted (1^{-2}) plant extracts (Sabri et al., 2012; Solomon et al., 2013). Observation of dark green and yellow colors indicated as existence of tannin and glycoside respectively. In addition, Occurrence of Saponine in the plant extracts was evaluated by adding the few volume of distilled water to 1 ml of aqueous extract then observation of foam after shaking the solution vigorously for 15 mins recorded as positive results (Solomon *et al.,* 2013).

Alkaloid compounds in the ethanol plant extracts were detected using Wagner's reagent. 0.5 ml of Wagner's reagent added into 2 ml ethanol plant extracts. Then brown color precipitation considered as presence of Alkaloid compounds in the extracts (Solomon et al., 2013).

In addition, flavonoid, quinone and phenolic compounds in the extracts were detected using Chloric acid, magnesium and ferric chloride. Observation of dark red and yellow colors of the extract after adding 0.5 ml Chloric acid and 0.5 gram magnesium indicated as the existence of flavonoid and quinine compounds respectively. Green color after adding 0.5 ferric chloride (5%) indicated as the existence of phenolic compounds (Prabha et al., 2011; Solomon et al., 2013).

2.8. HPLC analysis

Identification of phytochemical compounds was performed using High-Performance Liquid Chromatography (Knauer UV detector, Germany) equipped with a Diode array detector and An Aqua RP-C18 column (250 mm × 4,6 mm, 5 µm particle size) and an Aqua C18 precolumn were used at ambient temperature. All samples were centrifuged at 5000 rpm for 10 min before injection into the column with an injection volume of 20 µl and at a flow rate of 0.5 ml/min. Chromatographic analysis was carried out at 25°C using simultaneous monitoring of extracts performed at 348 nm. The solvent system was: solvent A, 1% acetic acid and solvent B, was mixture of acetonitrile, methanol and acetic acid (6-2-6) and detection was carried out at 348 nm (Barboni et al., 2010).

3. Results

3.1. Isolation and phenotypic identification of isolated bacteria

Totally nine bacteria were isolated from all the samples. All of the isolates were belonging to Enterobactereacae family. The isolates were *Kluyvera* spp., *Klebsiella pneumonia*, *Entrobacter aerogenes*, *Kelebsiella oxytoca* and *Escherichia coli.* Frequency of occurrence of *Klebsiella pneumonia* followed by *Escherichia coli* was more. The results obtained from WBC count indicated that the number of WBC was high in all the samples, in addition the number of WBC in *Escherichia coli* infection was high and in *Kluyvera* spp. infection was low (Fig.1).

3.2. Antibiotic susceptibility of the isolates

The results obtained from susceptibility of the isolates to antibiotics indicated that most of the isolates were resistant to cephalothin. A more effective antibiotic was ciprofloxacin and less effective antibiotic was cephalothin (Table 1).

3.3. The antibacterial effect of plant extracts

Of all, ethanol and acetone extracts relatively showed more antibacterial effects. *Klebsiella* species were more sensitive and *E.coli* isolates were more resistant to the extracts. Ethanol extract of *Myrtus communis* relatively showed a more antimicrobial effect (Table 2, Fig. 2). The results obtained from determination of minimal inhibitory concentration of the plant extracts illustrated that lowest MIC value was obtained for ethanolic plant extracts (Figs. 3).

3.4. Chemical analysis of the plant extracts

Our finding showed the presence of Tannin, saponine, glycosides, flavonoides, Alkaloides, quinione and phenolic compounds in all of the plant extracts.However, saponine was absence in chemical structure of *Citrullus colocynthis*.

The results obtained from HPLC of ethanolic plant extracts verified the presence of phenolic compounds as well as galic and elagic acids. Therefore, it can be interpreted that probably phenolic compounds are special compounds for inducing the antibacterial effect (Figure 5).

3.5. Statistical analysis

The results obtained from statistical analysis using ANOVA test ($p < 0.05$) indicated that there is significant difference between the isolates except *Klebciella oxytoca*. As shown in Figure 4 identical alphabetical characters showed no significant differences among the extracts on the isolated bacteria.

4. Discussion

Urinary tract infections (UTI) are common infection worldwide and the pattern of antimicrobial resistance of the infectious agents are varied. In the present study we conducted to isolate and identify the infectious agents from urine samples of the hospitalized patients and assessed their antibiotic susceptibilities. In addition, the response of the isolates against the antimicrobial effects of drug plant extracts viz., *Myrtus communis*, *Achillea millefolium* and *Citrullus colocynthis* were tested.

The results obtained showed detection of gram negative bacteria including: *Kluyvera* spp., *Klebsiella pneumoniae, Entrobacter aerogenes, Kelebsiella oxytoca* and *Escherichia coli* as the main agents of UTI. Out of all, *Klebsiella pneumonia* follow by *Escherichia coli* was detected in high frequency. However, our finding concerning the isolation of UTI agents was parallel with several reports (Bokaeian et al., 2013; Dhanalakshmi and Selve, 2013; Bokaeian et al., 2014; Shahba et al., 2014; Vivek et al., 2014), but isolation of *Kluyvera* spp. as UTI agent might be considered as first report in this geographical area.

The results obtained from WBC counts of the urine samples and their correlation to the infectious agent indicated that the number of WBC relatively was more in the urine samples whenever, the infectious agent was *Escherichia coli* and it was less when the infectious agent was *Kluyvera* spp. Although, detection of *Escherichia coli* and their relation to the high number of WBC was predictable, the existence of *Kluyvera* spp. and their relation to the low number of WBC wasn't expectable.

However, the isolates showed different responses against antibiotics, but ethanolic plant extracts relatively showed more antibacterial effects. Of all plant extracts, Ethanol extract of *Myrtus communis* relatively showed more antimicrobial effect.

Although, reports concerning to the responses of UTI agents isolated from the patients were varied, but the antibacterial effect of *Achillea millefolium* against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Micrococcus loteus* was reported earlier (Hasson, 2011). Furthermore, Dhanalakshmi and their colleagues (2013) reported the antibacterial effect of ethanolic extract of *Tribulus terrestris, Punica granatum* and *Cinnamomum verum* on UTI agents. Besides Rigi and their colleagues in 2014 reported the antimicrobial effect of ethanolic extract of *Myrtus communis* on *Staphylococcus aureus (*Rigi et al., 2013). On the other hand, Chemical analysis of the plant extracts verified the presence of different compounds viz., tannin, glycosides, flavonoids, Alkaloids, quinine, phenolic compounds, galic and elagic acids in all the plant extracts. It must be noted that our experiment was qualitative and therefore without quantative experiment recognition of the main antibacterial compounds is difficult however, probably phenolic compounds are the main compound for inducing the antibacterial effect.

In general, our finding verified the presence of different members of Enterobacteriaceae family in the urine samples of the patient suffering from UTI. In addition, some of them were resistant to the antibiotics used as the first line treatment of urinary tract infections.

Although, ethanolic plant extract such as *Achillea millefolium* extract also had antimicrobial effect against the UTI agents, hence, investigation on the usage of drug plant extracts are suggested for introducing a new remedy for treatment of Urinary tract infections and reduction of frequency of occurrence of antibiotic resistant bacteria.

Table 1. Antibiotic susceptibility of the isolates (mm)

Isolates / Antibiotics	GM10	CF 30	CP 5	CRO 30	FM 300	SXT
Kluyvera spp.	13±0.81 INT	12±0.81 R	21.33±0.94 S	21±0.81 S	18±0.81 INT	22.6±0.47 S
K. pneumoniae	10.3±0.47 R	6 R	15.6±0.47 INT	7±0.81 R	9.3±0.47 R	14.3±0.47 INT
K. pneumoniae	9±0.81 R	6 R	14±0.81 INT	6 R	9.3±0.47 R	6 R
Entrobacter	13.6±0.47 INT	13±0.81 R	24±2.1 S	21±0.81 S	19±0.81 INT	22.3±0.94 S
K. pneumoniae	14.6±0.94 INT	10±0.81 R	18.6±0.47 INT	21.6±2.1 S	21.6±1.6 R	11.6±0.47 R
E coli	13.6±0.47 INT	6 R	26.6±0.47 S	6.3±0.47 R	20.3±0.94 S	6.3±0.47 R
K. oxytoca	15±1.41 S	14/6±1.24 INT	21±0.81 S	22.6±1.24 S	14.3±1.69 INT	21±1.41 S
K. pneumoniae	13.6±0.47 INT	14.3±0.47 INT	20.3±0.47 S	21.3±1.24 S	14.6±0.47 INT	20.3±1.2 S
E coli	7.3±0.47 R	6 R	21.3±0.47 S	6 R	17.3±0.47 S	6 R

GM10: Gentamycin 10mcg-CF30: Cephalothin 30mcg –CP5: Ciprofloxacin 5mcg – CRO30:Ceftriaxon 30mcg – FM300: Nitrofurantoin 300mcg – SXT: Trimethoprim/Sulfamethoxazol 1/25-23/75 mcg

Table 2. Effect of different plant extracts on the isolates

Isolates / Plant extract	Water extract			Ethanol extract			Acetone extract		
	M	**A**	**C**	**M**	**A**	**C**	**M**	**A**	**C**
Kluyvera spp	17.6±0.94	6	6	20.6±1.6	11.6±2.4	6	13.3±2.05	6	6
K.pneumonia	24±0.81	19±0.81	17±0.81	31±1.41	26.3±0.47	26.6±0.47	19.6±0.47	6	6
K.pneumonia	18±1.41	6	7.3±0.94	19.3±0.47	21.6±9.6	21.3±3.85	20.6±0.47	6	6.6±0.94
Entrobacter	17.3±0.47	6	6	19.6±1.24	6	6.6±0.94	11.6±0.24	6.6±0.94	6
K.pneumonia	19.6±1.69	8±1.63	6	26±6.48	17.6±8.73	13±4.96	17±1.63	6	6
E coli	18±0.81	7.3±0.94	6	21.6±0.47	6.6±0.94	6	11.6±1.24	6	6
K.oxytoca	18.3±0.47	6	8.6±2.4	44.3±1.69	32.2±2.05	36.6±3.09	20.3±0.47	6	6
K.pneumonia	19.3±0.47	6	6	38.3±7.4	30.3±3.85	31.6±4.18	19.6±0.47	6	6
E coli	17.3±2.62	7.3±1.88	6	19.3±0.47	6	6	19±0.81	6	6

M:*Myrtus communis* ,A: *Achillea millefolium* ,C: *Citrullus colocynth*

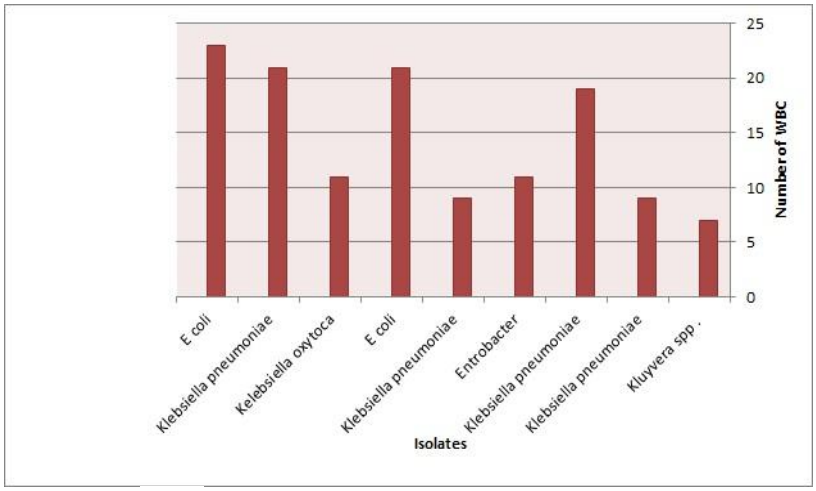

Fig 1. Number of WBC from infected women with UTI

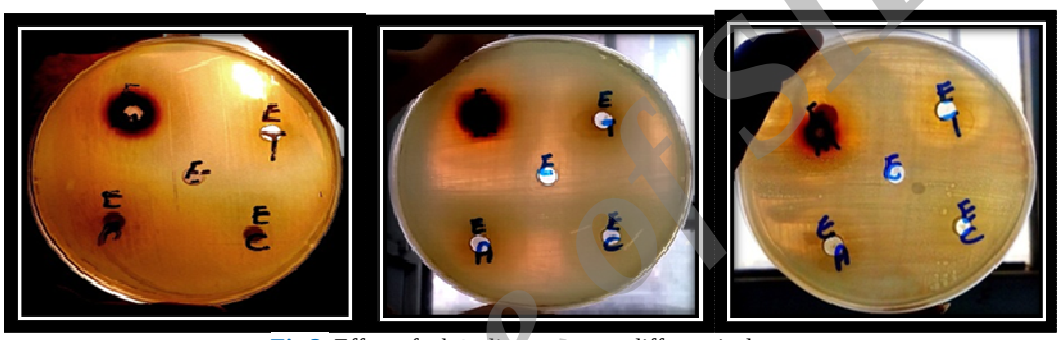

Fig 2. Effect of ethanolic extracts on different isolates

Fig 3. MIC of ethanol extract

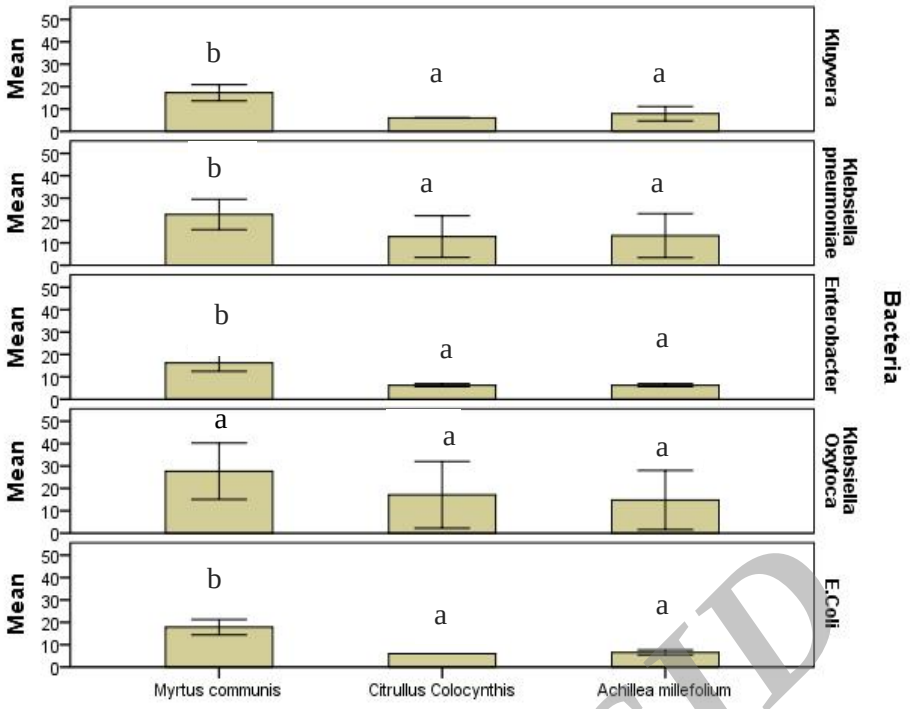

Fig 4. Antimicrobial effect of herbal plants on the isoaltes
*Identical alphabetical characters showed no significant differences

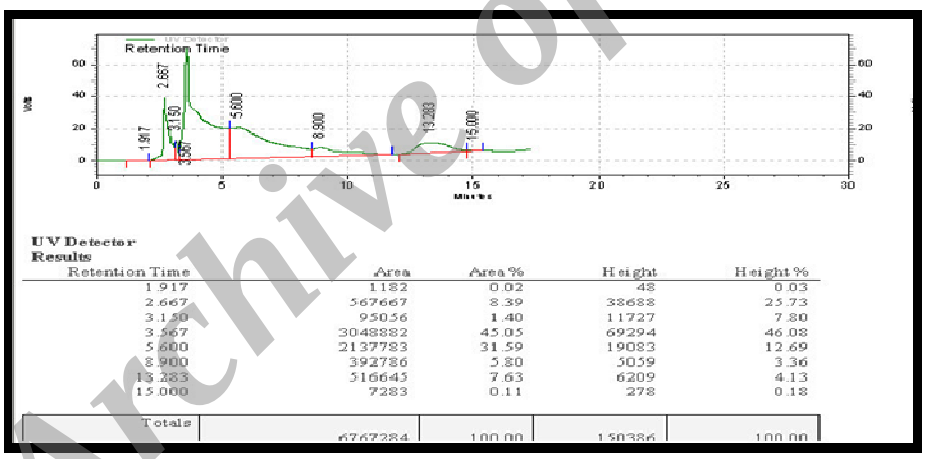

Figure 4. HPLC analysis of Myrtuscommunisextracts

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Aiyegoro, O.A., Igbinosa, O.O., Ogunmwonyi, I.N., Odjadjare, E.E., Igbinosa, O.E., okoh, A.I., 2007. Incidence of urinary tract infections (UTI) among children and adolescents in Ile-Ife, Nigeria. African Journal of Microbiology Research. 013-019

Anderson, J.E., 1983. Seasonality of symptomatic bacterial urinary infections in women. J Epidemiol Community Health. 37(4): 286-90.

Bahador, N., Baserisalehi, M., 2011. The effect of Quercas castaneifolia extract on pathogenic enteric bacteria. Anaerobe. 17: 358-360.

Barboni, T., Cannac, M., Massi, L., Perez-Ramirez, Y., Chiaramonti, N., 2010. Variability of Polyphenol compounds in Myrtuscommunis L. berries from Corsica. Molecules.15:7849-7860.

Bokaeian, M., Shiri, Y., Solouki, M., Saeidi, S., Javadian, F., 2013. In vitro Antibacterial activity of Myrtuscommunis L. against Morganella Morgana. International Research Journal of Applied and Basic Sciences. 6(9):1297-1299.

Bokaeian, M., Gholipour, N., Naruei, T., Saeidi, S., 2014. In vitro evaluation of some Iranian plants against Staphylococcus aureus isolation of UTI. International Journal of Advanced Biological Research. 21:440-445.

Cosgrove, S., Avdic, E., 2013. Antibiotic guidelines 2013-2014: treatment recommendations for adult inpatients.the Johns Hopkins Medicine.102.

Dhanalakshmi, J., Selve, S., 2013. Antibacterial activity of medicinal plants usedagainst urinary tract infection causing pathogens. International Journal of Research in Sciences.1: 01-07.

Doughari, J.H., Human, I.S., Bennade, S., Ndakidemi, P.A., 2009. Phytochemicals as chemotherapeutic agents and antioxidants: Possible solution to the control of antibiotic resistant verocytotoxin producing bacteria. Journal of Medicinal Plants Research. 3(11): 839-848.

Doughari, J.H., 2012. Phytochemicals: extractionmethods, basic structures and mode of action as potential chemotherapeutic agents.Phytochemicals – A Global Prespective of their Role in Nutrition and Health from Dr Vnketshwer Rao Handa, S.S., 2008. An overview of extraction techniques for medicinal and aromatic plants.International Centre for Science and High Technology.p:22.

Hasson, R.N., 2011. Antibacterial activity of water and alcoholic crude extract of flower achillea millefolium .Raf Journal Science. 22:11-20.

Lane, D.R., Takhar, S.S., 2011. Diagnosis and management of urinary tract infection and pyelonephritis. Emergency Medicine Clinics of North America. 29(3)539-552.

Litza, J.A., Brill, J.R., 2010. Urinary tract infections. Prim Care. 37(3): 491-507.

Naber, K.G., Schito, G., Botto, H., Palou, J., Mazzei, T., 2008. Surveillance study in Europe and Brazil on clinical aspectsand Antimicrobial Resistance Epidemiology in Females with Cystitis (ARESC): implications for empiric therapy. EurUrol. 54(5):1164-75.

Nicolle, L.E., 2008. Uncomplicated urinary tract infection in adults in cluding Uncomplicated pyelonephritis .UrolClinice North America .35:1-12.

Prabha, K., Karar, P.K., Hemalatha, S., Ponnudurai, K., 2011. A comparative Preliminary Phytochemical screening on the leaves, stem and roots of three Viburnum linn species. Der Pharmacia Sinica. 2(3):81-93.

Rigi, M., Shafeghat, M., Saeidi, S., 2013. Antibacterial activity of citrullus colocynthis extract against some human pathogenic bacteria causing urinary tract infection. International Research Journal of Applied and Basic Sciences. 5(6): 727-729.

Sabri, F.Z., Belarbi, M., Sabri, S., Alsayadi, M.M.S., 2012. Phytochemical screening and identification of some compounds from Mallow.Journal Nat Prod Plant Resour. 2(4):512-516.

Salvatore, S., Cattoni, E., Siesta, G., Serati, M., sorice, P., Torella, M., 2011. urinary tract infections in women. European Journal of Obstetrics, gynecology and reproductive Biology . 156(2): 131-136.

Shahba, S., Bokaeian, M., Mozafari-Sabet, N.A., Saeidipour-Parizi, A., Bameri, Z., Nikbin, M., 2014. Antibacterial effect of Teocrium polium on the bacteria causing urinary tract infection .Zahedan Journal of Research in Medical Sciences. 16(3):44-49.

Solomon, C.U., Arukwe, U.I., Onuoha, I., 2013. Preliminary phytochemical screening of different solvent extracts of stem bark and roots of dennetia tripetala g baker .Asian Journal of Plant Science and Research. 3(3):10-13.

Vivek, M.N., Manasa, M., Pallavi, S., Sachidanandaswamy, H., Ravikumar, T.N., Prashithkekuda, T.R., 2014. Antibacterial activity of cashew apple juice against antibiotic resistant urinary tract pathogens. World Journal of Pharmaceutical Sciences. 2(1):79-82.

Zalmanovici, A., Green, H., paul, M., Yaphe, J., Leibovici, L., 2010. Antimicrobial agents for treating uncomplicated urinary tract infection in women .Cochrane Database of Systematic Reviews. 10: CD007182.