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Bio-control of clinical bacterial isolates associated with urinary tract infection using wild medicinal plant extract

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ABSTRACT

The goals of treatment for UTIs are to relieve symptoms, eliminate the infection, prevent recurrence and prevent serious complications such as kidney damage and sepsis results due to improper treatment. Exploring unexplored aspect of wild plants for developing antibacterial drugs as novel attempt further investigation. Study aims to screen eleven wild medicinal plants possessing antibacterial activity against the clinical bacterial isolates from urinary tract infection patients. The methanolic and ethanolic plant extract were analyzed by well diffusion assay and phytochemical characterization of the active ingredient were determined possessing antibacterial activity. Relative percent occurrence of E. coli was found to be maximum, followed by Klebsiella, Pseudomonas and Staphylococcus respectively. All the test pathogens were effectively controlled by the methanolic extracts of Acacia nilotica, Tagetus erectus, Thevatia peruviana, Thuja occidentalis and the ethanolic extracts of Acacia nilotica, Tagetus erectus, Murraya koenigii, Lawsonia inermis due to the higher levels of alkaloids, tannins and phenols. Plants possessing phenol, tannin and alkaloids show antibacterial activity. Screening of wild plants can be a novel approach for potential lead molecules and treatment of UTI. Determining exact component in these extract possessing antibacterial activity can be recommended for the alternative therapy for drug designing.

Keywords: anti-bacterial activity, clinical bacterial isolates, medicinal plant extract, urinary tract infection.

INTRODUCTION

Urinary tract infections (UTIs) are some of the most common types of infections in humans with an estimated 34 percent of adults aged 20 or older reported as having had at least one occurrence of a UTI or cystitis. Specifically, over 50% of women and over 13% of men will have a UTI at least once in their lifetime. [1]

Urinary tract infections (UTI) in female is one of the most difficult challenges for the physicians, affecting about 25% of women with a history of isolated urinary tract infection.[2] When bacterial virulence increases or host defense mechanisms decrease, bacterial inoculation, colonization, and infection of the urinary tract occurs. Treatment of recurrent urinary tract infection requires understanding of the pathogenesis of UTI and the role of host and bacterial factors.[3] The urinary tract is normally sterile; bacteria that generally ascend from the peri-anal area reservoir may cause UTIs.

Bacteria in the urinary tract may remain asymptomatic or cause irritative symptoms such as frequency and urgency.[4] The most common uropathogenic gram negative bacteria are *Escherichia coli* and *Klebsiella pneumoniae*.[5]

UTI diagnosis is a multistep process which includes the determination of the concentration of pathogens, and the identification of the responsible bacteria, as well as their susceptibility to various antibiotics, the so called antibiogram.[6] Enteric bacteria (in particular, *Escherichia coli*) have been and remain the most frequent cause of UTI, although there is some evidence that the percentage of UTIs caused by *E. coli* is decreasing. The percentage of UTIs caused by *E. coli*, *Proteus* species, and *Pseudomonas* species decreased, whereas the percentage of UTIs caused by yeasts, group B streptococci, and *Klebsiella pneumoniae* increased.[7,8]

Uncomplicated UTIs typically occur in the healthy adult non-pregnant woman, while complicated UTIs (cUTIs) may occur in all sexes and age groups and are frequently associated with either structural or functional urinary tract abnormalities. Examples include foreign bodies such as calculi (stones), indwelling catheters or other drainage devices, obstruction, immunosuppression, renal failure, renal transplantation and pregnancy.[9]

Different changes in the causative agents of UTI, with a decrease in the percentage of UTIs caused by *Enterobacter* species, but with an increase in the percentage of UTIs caused by *Acinetobacter* species and *Pseudomonas aeruginosa*.[10]

The present study aims at evaluating the antibacterial activity and phytochemical characterization of plant extract (methanolic and ethanolic) of medicinal plant against the clinically isolated bacteria from UTI's samples.

MATERIALS AND METHODS

Plant Material:
Eleven plants were used for the analysis of their antibacterial activity *Cucurma longa* (Haldi), *Thuja occidentalis* (Morpankhi), *Murraya koenigii* (Meetha Neem), *Lawsonia inermis* (Mehndi), *Acacia nilotica (Babool)*, *Tagetus erectus* (Gainda), *Thevatia peruviana* (Kaner), *Riccinus communis* (Arandi), *Catherenthus reseus* (Sadabahar), *Tinospora cordifolia* (Neemgiloya) and *Jatropha curcas* (Ratanjot). Fresh leaves were collected washed thoroughly 2-3 times with running tap water and once with sterile distilled water, air dried at room temperature on a sterile blotter and used for preparation of extracts.[11]

Solvent extraction:
The dried powdered leaves were subjected to methanolic and ethanolic extraction by Soxhlet method. Plant extract were prepared by 15 grams fine powder of leaves was filled in the thimble and extracted successively with methanol for 48 hours at 55^{o}C. All the solvent extracts were concentrated using rotary flash evaporator under reduced pressure. The extracts were preserved in airtight brown bottle until further use. [13,31]

Phytochemical Screening of Plant Extract
All the plant extract were subjected for phytochemical screening by quantitative analysis of alkaloids, flavonoids, saponins, phenols and tannins.

Alkaloid determination
5 g of the sample was weighed into a 250 ml beaker and 200 ml 20% acetic acid in ethanol was added and covered to stand for 4 h. this was filtered and the extract was concentrated using a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitation was collected by filtration and weighed. [15,33]

Flavanoid determination
10 g of the sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatmann filter paper No.1. The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed. [13]

Saponin determination

20 g of plant sample was dispersed in 200 ml of 20% ethanol. The suspension was heated over a hot waterbath for 4 h with continuous stirring at about 55ºC. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90ºC. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of normal butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the sample were dried in the oven into a constant weight. The saponin content was calculated in percentage. [16]

Determination of total phenol content

The total phenolic content of the *M. oleifera* flower extract was determined by using Folin-Ciocalteu reagent following a slightly modified method of Ainsworth. [17] Gallic acid was used as a reference standard for plotting calibration curve. A volume of 0.5 mL of the plant extract (100 µg/mL) was mixed with 2 mL of the Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and were neutralized with 4 mL of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 30 min with intermittent shaking for color development. The absorbance of the resulting blue color was measured at 765 nm using double beam UV-VIS spectrophotometer (UV-VIS Systronics 119). The total phenolic contents were determined from the linear equation of a standard curve prepared with gallic acid. The content of total phenolic compounds expressed as mg/g gallic acid equivalent (GAE) of dry extract.

Determination of tannins

500 mg of the sample was weighed into 100 ml bottle; 50 ml of distilled water was added and shaken for 1 h in a shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtrate was pipette out into a tube and mixed with 3 ml of 0.1 M $FeCl_3$ in 0.1 N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured in a spectrophotometer at 725 nm wavelength within 10 min. A blank sample was prepared and read at the same wavelength. A standard was prepared using tannin acid to get 100 ppm and measured. [18]

Test Bacteria

Urine cultures were collected from Dr. B. Lal Clinical Laboratory, Jaipur. All the samples were subjected to culture on blood agar and Mac conkey agar. Four species of clinically isolated bacteria from the urine culture of UTI patients as *E.coli, Pseudomonas, Kleibsella, Staphylococcus* were used as test bacteria for antibacterial activity assay.

Antibacterial activity by herbal formulations:

The plant materials extracts were tested for antimicrobial activity by the well diffusion method.[19] This method depends on the diffusion of the various extracts from a cavity through the solidified agar layer of Petri dish to an extract such that growth of the added microorganism is prevented entirely in circular area or zone around the cavity containing the extracts.[20] On the Muller Hinton agar plates, C (positive control, streptomycin) (5mg/well (w/v), S (Sample) and R (Reference, solvent) should be marked. The wells were punched with the help of 8 mm corkborer. Bacterial cultures should be swabbed on the plates and in the wells 100µl of the samples were loaded respectively. The plates were incubated at 37 ± 2^{o} C for seven days and triplicates were maintained for each treatment.[21] The zone of inhibition of mycelial growth was determined by antibiotic zone scale (Hi-media). [12,22] Samples showing activity index >1 represent significant control of pathogens.

$$\text{Activity index} = \frac{\text{Zone of inhibition of sample}}{\text{Zone of inhibition of reference}}$$

RESULTS AND DISCUSSION

Urine samples of 50 patients susceptible for UTI were collected. All the samples were subjected to Routine Examination and Culture. Out of 50 samples, 45% were females and 55% were males. Among the samples, 77.8 % females and 63.6 % males were confirmed to be infected with UTI. During routine examination count of Pus cells,

Table 1: Phytochemical Characterization of methanolic and ethanolic Plant Extract

S.No.	Plant Extracts	Solvent	Alkaloid (mg/gm)	Saponins (mg/gm)	Flavanoid (mg/gm)	Tannin (mg/gm)	Phenol (mg/gm)
1.	*Curcuma longa (Haldi)*	Methanolic	0.09	0.12	0.12	0	1.017
		Ethanolic	0.06	0.18	0.06	0.03	1.092
2.	*Thuja occidentalis (Morpankhi)*	Methanolic	0.45	**1.08**	0.36	0.21	0.753
		Ethanolic	2.07	**0.63**	0.15	0.03	0.834
3.	*Murraya koenigii (Meetha Neem)*	Methanolic	0.51	0.72	0.15	0.51	**1.422**
		Ethanolic	0.09	0.24	0.27	0.3	1.356
4.	*Lawsonia inermis (Mehendi)*	Methanolic	0.09	0.51	0.15	0.6	**1.437**
		Ethanolic	0.18	0.75	0.33	0.24	1.332
5.	*Acacia nilotica (Babool)*	Methanolic	0.03	0.51	0.36	1.14	0.636
		Ethanolic	2.22	0.24	0.06	0.63	0.702
6.	*Tagetus erecta (Gainda)*	Methanolic	2.94	0.75	0.09	**2.13**	0.594
		Ethanolic	2.64	0.6	0.03	2.04	0.558
7.	*Thevatia peruviana (Kaner)*	Methanolic	3.81	0.87	0.21	1.14	0.6
		Ethanolic	3	0.9	0.3	1.05	0.801
8.	*Riccinus communis (Arandi)*	Methanolic	0.51	**2.04**	**2.16**	0.87	1.395
		Ethanolic	0.3	1.74	2.37	0.9	**1.47**
9.	*Catharanthus roseus (Sadabahar)*	Methanolic	0.54	**2.04**	0.42	**2.13**	0.798
		Ethanolic	0.6	1.95	0.57	2.28	0.906
10.	*Tinospora cordifolia (Neem- giloye)*	Methanolic	1.08	0.48	**1.11**	**1.74**	1.029
		Ethanolic	0.36	0.42	1.62	1.95	0.9
11.	*Jatropha curcas (Ratanjot)*	Methanolic	0.39	0.27	**1.53**	**1.92**	0.702
		Ethanolic	1.2	0.6	1.29	1.38	0.639

Epithelial cells and WBCs were observed. Upon urine culture, the incidence of *E. coli* (40%) was highest followed by *Klebsiella* (25%), *Pseudomonas* (20%), *Staphylococcus* (10%). (Figure 1) They were subjected to further study and antibacterial activity by various plants. Urinary-tract pathogens such as *S. saprophyticus, Pseudomonas* species, or *Enterococci*.[23] *Klebsiella, Staphylococci, Enterobacter, Proteus, Pseudomonas,* and *Enterococci* species are more often isolated from patients, whereas there is a greater preponderance of *E. coli* in an outpatient population.[24] Symptoms may disappear, but the infectious bacteria can make its way up to the kidney and cause damage and even death. Only a urinalysis can reveal whether all offending bacteria are out of the entire urinary tract. [25] Routine screening of bacteremia is not recommended in diabetic patients, the administration of antibiotic does not prevent the further symptomatic episodes. [26]

Plant extracts, both Methanolic and Ethanolic were subjected to Phytochemical Characterization as Alkaloid, Saponin, Flavanoid, Tannin and Phenol. All the test pathogens were effectively controlled by the methanolic extracts of *Acacia nilotica* (0.03 1.14, 0.636 mg/g), *Tagetus erectus* (2.94, 2.13, 0.594 mg/g), *Thevatia peruviana* (3.81, 1.14, 0.6), *Thuja occidentalis* (0.45, 0.21, 0.753 mg/g) and the ethanolic extracts of *Acacia nilotica* (2.22, 0.63, 0.702), *Tagetus erectus* (2.64, 2.04, 0.558 mg/g), *Murraya koenigii* (0.09, 0.3, 1.356 mg/g), *Lawsonia inermis* (0.18, 0.24, 1.332 mg/g) due to the higher levels of alkaloids tannins and phenols respectively. (Table 1) Whereas, botanicals that can be effective at the first sign of an infection and for short-term prophylaxis include berberine and uva ursi. Estriol cream and vitamins A and C have also been shown to prevent UTIs, while potassium salts can alkalinize the urine and reduce dysuria.[29] In a similar study, methanolic plants extracts of *Catherenthus reseus, Riccinus communis, Tagetus erectus, Acacia nilotica, Lawsonia inermis* and *Thuja occidentalis* were found to be significantly controlling the test fungi. Data revealed that plants possessing higher phenol, tannin and saponin show antifungal activity.[34]

Antibacterial activity against plant extracts was performed by well diffusion method and following conclusions were made Methanolic extract of *Tagetus erecta* (activity index 2.8) and *Thevatia peruviana* (activity index 2.2) was highly active against *Pseudomonas;* extracts of *Acacia nilotica, Tagetus erecta* (activity index 3.46, 2.2 respectively) significantly control *E. coli; Klebsiella* controlled by *Acacia nilotica* (activity index 3.58) and *Thuja occidentalis* (activity index 1.88)*; whereas, Staphylococcus* was significantly controlled by *Acacia nilotica, Tagetus erecta* (activity index 3.4 and 2.1) with respect to the control. The methanolic extracts of *Catharanthus roseus, Tinospora cordifolia* and *Jatropha curcas* did not exhibited any activity against clinical isolates from UTI. (Table 2; Figure 2)

Table 2: Antibacterial activity of methanolic and ethanolic plant extracts against clinical isolates from UTI

S.No.	Plant	Solvent	Well	*Pseudomonas*	*E. coli*	*Klebsiella*	*Staphyloccocus*
				Zone of Inhibition (in mm)			
1.	***Curcuma longa***	Methanol	S	1.34	1.76	1.46	1.84
			C	3.38	3.08	3.44	3.22
			R	1	1	1	1
			Activity Index	**1.34**	**1.76**	**1.46**	**1.84**
		Ethanol	S	2.1	1.98	1.6	2.04
			C	3.38	3.16	3.26	3.26
			R	1.52	2.06	1.2	1.14
			Activity Index	**1.38**	**0.96**	**1.33**	**1.79**
2.	***Thuja occidentalis***	Methanol	S	1.88	1.78	2	1.84
			C	1.74	2.22	4.96	2.38
			R	1	1	1	1
			Activity Index	**1.88**	**1.78**	**2**	1.84
		Ethanol	S	1.68	2	1.58	2.12
			C	3.66	3.6	2.72	3.1
			R	1.24	1.48	1.22	1.5
			Activity Index	**1.35**	**1.35**	**1.3**	**1.41**
3.	***Murraya koenigii***	Methanol	S	2.06	1.44	1	1.48
			C	3.28	2.62	2.96	2.38
			R	1	1	1	1
			Activity Index	**2.06**	**1.44**	**1**	**1.48**
		Ethanol	S	2.32	2.16	2.42	2.72
			C	3.38	2.92	3.18	3
			R	1.88	1	1.58	1.98
			Activity Index	**1.23**	**2.16**	**1.33**	**1.37**
4.	***Lawsonia inermis***	Methanol	S	1,76	2.22	1.88	1.38
			C	2.06	3.02	2.9	1.68
			R	1	1.18	1	1
			Activity Index	**1.76**	**1.88**	**1.88**	**1.38**
		Ethanol	S	2.44	2.96	2.64	1.98
			C	3.16	3.8	3.72	2.78
			R	1.26	2.94	1.44	1.OO
			Activity Index	**1.94**	**1.01**	**1.83**	**1.98**
5.	***Acacia nilotica***	Methanol	S	2.16	3.46	3.58	3.4
			C	1.92	2.66	2.92	2.72
			R	1.28	1	1	1
			Activity Index	**1.68**	**3.46**	**3.58**	**3.4**
		Ethanol	S	1.92	2.88	2.16	2.66
			C	4.06	3.04	3.08	3.16
			R	1.66	1	1	1
			Activity Index	**1.16**	**2.88**	**2.16**	**2.66**
6.	***Tagetus erecta***	Methanol	S	40	32	14	32
			C	14	14	15	15
			R	17	17	18	17
			Activity Index	**2.8**	**2.2**	**0.9**	**2.1**
		Ethanol	S	39	34	17	35
			C	15	15	14	14
			R	18	18	17	17
			Activity Index	**2.6**	**2.2**	**1.2**	**2.5**
7.	***Thevatia peruviana***	Methanol	S	31	24	24	17
			C	14	14	14	14
			R	16	17	17	16
			Activity Index	**2.2**	**1.71**	**1.71**	**1.2**
		Ethanol	S	30	23	25	16
			C	13	14	14	13
			R	17	17	17	16
			Activity Index	**2.3**	**1.6**	**1.7**	**1.2**
8.	***Riccinus communis***	Methanol	S	19	24	22	21
			C	14	15	15	14
			R	32	33	32	33
			Activity Index	**1.3**	**1.6**	**1.4**	**1.5**
		Ethanol	S	14	14	14	14
			C	12	14	12	14

Note:

			R	35	38	13	14
			Activity Index	**1.1**	**1**	**1.1**	**1**
9.	***Catherenthus roseus***	Methanol	S	28	30	30	28
			C	0	0	0	0
			R	28	30	28	30
			Activity Index	**0**	**0**	**0**	**0**
		Ethanol	S	15	11	20	27
			C	0	0	0	0
			R	28	30	28	28
			Activity Index	**0**	**0**	**0**	**0**
10.	***Tinospora cordifolia***	Methanol	S	0	0	0	0
			C	0	14	0	0
			R	35	13	14	11
			Activity Index	**0**	**0**	**0**	**0**
		Ethanol	S	15	13	13	15
			C	0	0	0	0
			R	25	23	25	23
			Activity Index	**0**	**0**	**0**	**0**
11.	***Jatropha curcas***	Methanol	S	0	0	0	0
			C	0	0	0	0
			R	15	15	13	13
			Activity Index	**0**	**0**	**0**	**0**
		Ethanol	S	14	11	14	11
			C	9	10	10	9
			R	28	30	30	28
			Activity Index	**1.5**	**1.1**	**1.4**	**1.2**

Sample: 5 mg (w/v) test methanolic plant extract (50 µl loaded per well)
Control Positive Control itraconazole 5 mg (v/v) per well
Reference: Solvent used for solvent extraction (methanol) 50 µl loaded per well
Diameter of well: 8 mm

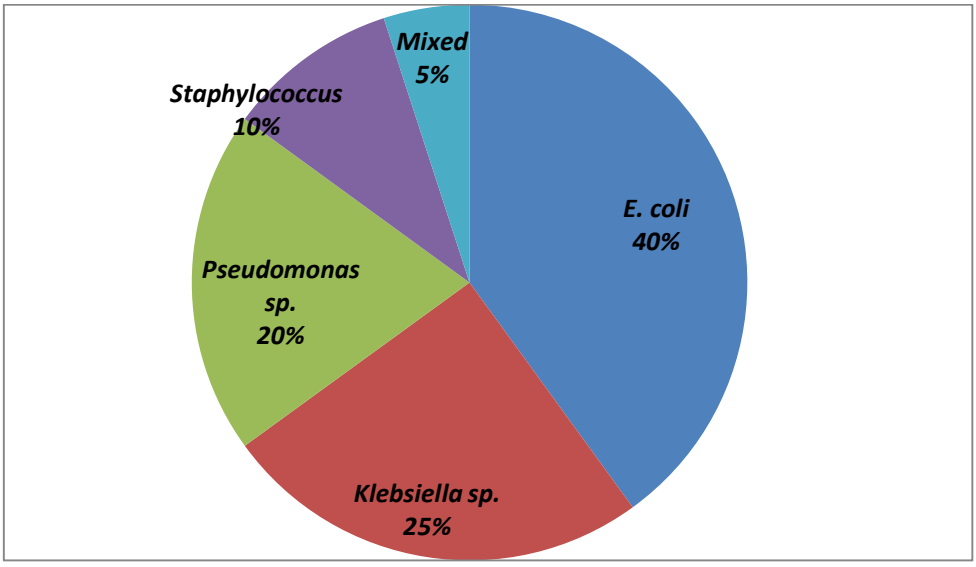

Figure 1 Pie-chart showing the Relative Percent Occurrence (RPO) of the clinical isolates with urinary tract infection

Ethanolic extract of *Acacia nilotica* active against *E. coli*, *Klebsiella* and *Staphylcoccus* (activity index 2.88, 2.16, 2.66); *Tagetus erecta* significantly controlled *Pseudomanas*, *E. coli*, *Klebsiella* (activity index 2.6, 2.2, 2.5); *Thevatia peruviana* controlled *Pseudomanas* (activity index 2.3), *Murraya koenigii* by *E. coli* (activity index 2.16); *Lawsonia inermis* (activity index 1.83).

On the other hand the ethanolic extracts of *Catharanthus roseus* and *Tinospora cordifolia* was inactive against clinically isolated cultures. (Table 2; Figure 2) In a study using Cinnamon it is proven to completely suppress causes of *E. coli* and *Candida albicans*, UTI causing bacteria and fungus, respectively.[27] An ethnomedicinal

survey among the traditional healers of various ethnic groups and in several regions of the country to obtain information on medicinal plants used to treat UTIs. Thirty-one species were reported by traditional healers as being used for UTIs, including leucorrhea, frequent or infrequent urination and cloudy urination and burning sensations during urination.[28] Modern analytical spectroscopies of high intrinsic dimensionality can provide rapid accurate microbial characterization techniques, but only when combined with appropriate chemometrics. [30]

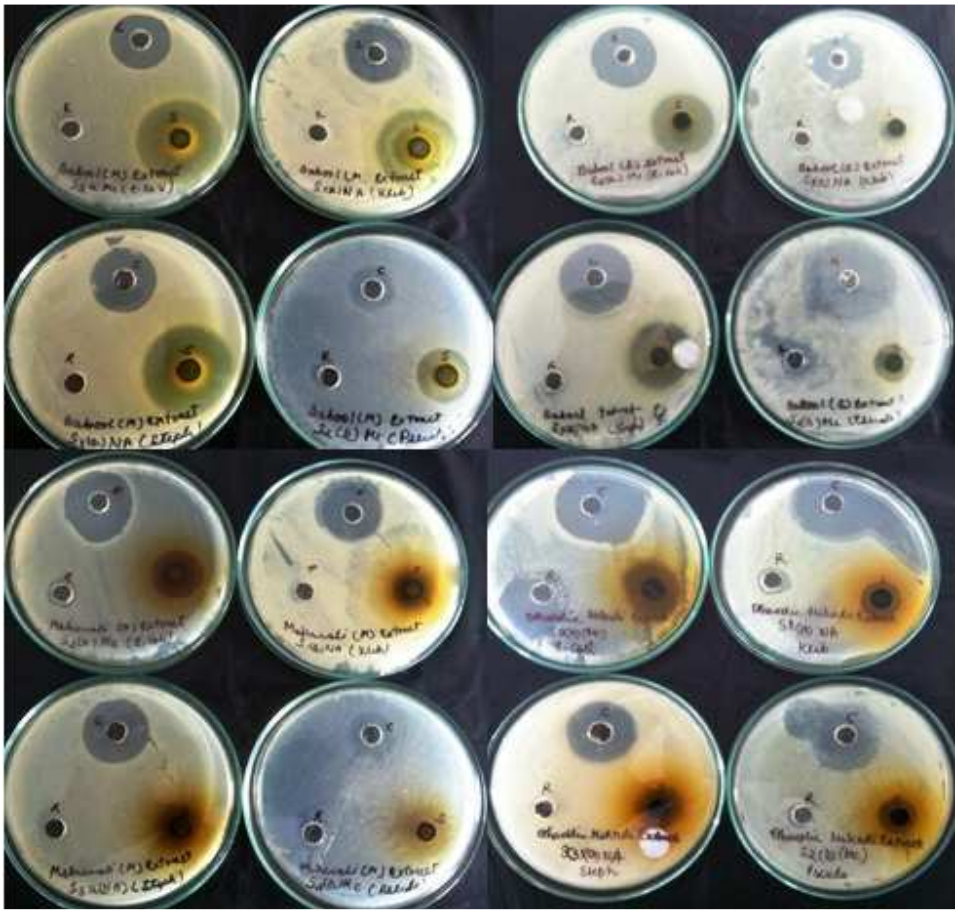

Figure 2: Antibacterial activity of the plant extracts (methanolic and ethanolic) against clinically isolates test bacteria through well diffusion assay

The relative percent occurrence of *E. coli* was maximum followed by *Klebsiella*, *Pseudomonas* and *Staphylococcus* respectively. All the test pathogens were effectively controlled by the methanolic extracts of *Acacia nilotica*, *Tagetus erectus*, *Thevatia peruviana*, *Thuja occidentalis* and the ethanolic extracts of *Acacia nilotica*, *Tagetus erectus*, *Murraya koenigii*, *Lawsonia inermis* due to the higher levels of alkaloids tannins and phenols.

CONCLUSION

The management of UTI infections needs personal hygiene, awareness of infection, proper diagnosis and medication. At present there are a large number of drugs available commercially. With increasing incidence of bacterial infection, microbial resistance to the existing drugs, cost and side effects, there is a need for an antibacterial drug that can overcome all these limitations. Out of eleven plants *Acacia nilotica, Tagetus erectus* and *Thevatia peruviana* remains to be an unexhausted source of bioactive compounds and a boon to the medical field. It was interpreted that the plants possessing higher amounts of phenol, tannin and alkaloids shows effective antibacterial activity against the test bacteria. Screening of plants of wild nature can be a novel approach for obtaining potential lead molecules for clinical trials and later treatment of urinary tract infection as compared to the standard drugs.

We know that primary and secondary metabolites are responsible for antibacterial activity on the basis of review. Now, the future perspectives of the present project is to find out the exact component in these extract responsible for antibacterial activity that can be recommended for the alternative therapy and herbal formulations in reference to the chemical formulations. New treatments can be proposed against UTI and it can also be used in for drug designing.

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