doi.org/10.36721/PJPS.2022.35.2.REG.479-486.1

Datura innoxia antimicrobial activities against *E. coli* isolated from infections of type 2 diabetic patients

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Abstract: The current study was designed to explore the antimicrobial activity from plant sources in order to inhibit the microorganisms responsible for infections. 137 samples were collected from hospitals of district Attock. Agar disc diffusion was used to check the resistance patterns. *Datura innoxia* activity was checked against strains. Integron (class 1) presence was detected through PCR based study. Ethanolic and metabolic extracts were effective against *E. coli* strains. Ethanolic leaf extract showed more activity while chloroform and methanolic extracts were least activities. FTIR analysis of the data showed that plant showed antimicrobial activity due to presence of different compounds including alcoholic, aromatic, carboxlic acid, alkane as main compounds. Results of biofilm detection showed that 69% strains were able to develop strong biofilm. Report of (class 1) integron was really significant because it was reported first time from this area. 16S r DNA also confirmed the results. The *Datura innoxia* extracts activity against *E. coli* was also really significant. This will really help to find out the reason behind prolonged stay of diabetic patients in hospitals and plant activity against strains will really help to explore potent herbal agents against pathogenic strains.

Keywords: Type 2 diabetes, infections, *E. coli*, class 1 integron, *Datura innoxia.*

INTRODUCTION

Different plant species have great antibacterial properties (Khatak *et al*., 2010). *Datura innoxia* showed activity against many bacterial isolates (Eftekhar *et al*., 2005). The genus *Datura innoxia* which is also known as Jimson weed distributed throughout the world, there are 14 species of this genus. *Datura innoxia* is one of them. *Datura innoxia* is well known to be used in medicines as narcotic and treatment for ulcers, wounds, fever, and toothache. Anti-bacterial activities of *Datura innoxia* have been well established. Activities of *Datura innoxia* has been reported against *E.coli*, *Salmonella*, *Klebsiella*, *Staphylococcus*. *Datura innoxia* is well known for its uses in Chinese and Indian medical systems (Lindow *et al*., 2003). Type2 diabetes is a group of complex metabolic disease associated with prolonged underline conditions (Wild *et al*., 2000). Type 2 diabetes patients suffer a lot due to the complications. Different studies have stated that hyperglycemia is the leading cause of diabetes complications. Different bacterial pathogens are responsible for diabetic infections, *Pseudomonus*, *Staphylococcus*, *Bacillus* and *Klebsiella* (Martinez and Jose, 2009). These bacteria are responsible for resistance against antibiotics. Biofilms are special surface linked structures, composed of polysaccharides and proteins (Lindow, 2003). Biofilms may develop up to 80% of the microbe's population on plants surfaces (Mie *et al*., 2014). It has been reported that 99% bacteria exists as biofilms. Keeping in view the disease status of *E. coli* prevalence, this study was focused to evaluate the reasons for prolonged stay of diabetic patients in hospitals. *Datura innoxia* extracts activity was also checked.

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MATERIALS AND METHODS

Patient's samples were collected from hospitals of district Attock, after approval by institutional board of advanced research. All of these strains were taken after confirming the willingness of patients.

Selection of patients

Diabetic patients, diagnosed with UTIs and hospitalized were involved in the study. Patients having no disease or pre diabetes and without UTIs, were excluded from the study. Patients of type 1 diabetes were also excluded.

Study design

137 bacterial strains were isolated from diabetes type 2 patients after informed written consent from patients. All the patients were divided in three groups based on age.

1. <30 years
2. (30-50) years of age
3. (51-70) years of age

The samples were transferred to the microbiology laboratory for further processing.

Isolation and purification of bacteria

Spreading of bacteria was done in order to obtain growth of samples. Streaking was done in order to obtain single purified colony, by using sterile inoculating loop a single colony was picked from previously grown colonies and the picked colony was streaked on freshly prepared nutrient agar media. The plates were incubated at 37^{o}C overnight.

Identification of bacteria

Bacterial strains were identified by using Gram's staining, microscopy and standard biochemical methods. After purification, various confirmatory tests were performed on these bacterial isolates. In order to confirm the genus of the bacteria, tests that includes Gram staining, microscopy, MSA, MacCokney agar, catalase, oxidase, blood agar, cled agar, simmon citrate, starch hydrolysis, urease, VP and methyl Red and indole tests were performed.

Antibiotic disk diffusion

In this study total 15 antibiotics which were commonly prescribed by the physicians to the UTI patients were used. Levofloxacin, Ciprofloxacin, Piperacillin with Tazobactam, Penicillin, Fosfomycin, Ceftriaxone, Ampicillin, with Clavulanic acid, Gentamycin, Trimethoprim, Ceftriaxone, Cefoxitin, Nitofurantoin and Ciprofloxacin are affective or sensitive against UTI pathogens. 5µl of bacterial culture was poured on agar plate with the help of micropipette and spreaded with sterilized glass rod. Antibiotic discs were fixed on this MH Agar media. Plates were incubated at 37°C for 24 hours. Diameters were measured with the help of scale after incubation (Johnson *et al*., 2013). The largest diameter of zone of inhibition against antibiotics was considered as susceptible according to (CLSI, 2019). The smaller diameter of zone and absence of any zone against antibiotics disc showed that the bacterial strains were resistant to these antibiotics.

Collection of plant

Leaves of *Datura innoxia* were collected from different parts of district Attock. Plant samples were randomly collected. The collected plants were brought to microbiology, Department of Biosciences for further processing.

Extracts preparation

Extracts were prepared by cutting the sterilized leaves into small pieces and then these leaves were macerated and 20 gm leaves were added in 200ml of solvents. After that extracts were dried in a beaker and under heating drying oven at temperature less than boiling points of solvents i.e.60^{o}C for ethanol, chloroform and 80°C for methanol extracts. Dry weight of extracts was determined excluding the weight of beaker containing it passed through bacterial filter of size 0.45µm and stored until further processing.

Screening of the plant extracts

Antimicrobial potential of methanolic, chloroform and ethanolic leaves extracts of the selected plant was tested on these bacterial isolates.

Procedure for disc diffusion method

By disc diffusion method the susceptibility of plant extract against different bacterial strains was checked. The 5µl bacterial culture was poured in the center of agar plate with micropipette. The inoculum was spread evenly over the surface of Muller Hinton Agar plate by rotating the plate in four directions. Filter paper disc was soaked with 20µl of extract and allowed to dry (Nascimento *et al*., 2000) and were placed firmly on the surface of inoculated M H Agar plates. After incubation period the diameter were checked through scale.

FTIR related study of extracts

It is normally used to identify certain types of chemicals found in different materials. The intensity of light was traced in the spectra. Dried form of all solvent extracts was used for interpretation. The ratio was selected at 100/10 mg of the powder at 397 KBr pellets. All of the samples were placed in spectroscope with a range of 400 to 4000cm 1; it was fine-tuned on 4 cm.

Detection of Biofilm

Biofilm production by the bacteria was detected by three phenotypic methodologies which include the CRA, TM and TCPM. Biofilm production was graded into three categories strong, moderate and weak. Strong and moderate results were interpreted as positive and non/weak were interpreted as negative results.

Congo red Agar Method

This contains brain heart infusion (BHI) broth with the addition of sucrose, congo red and agar. This solution of congo red was then autoclaved at 121^{o}C for 15 minutes.

Tube Method

10ml of the media in each glass tube was added and incubated in shaking incubator at 37^{o}C for 24 hours. After that tube were emptied and wash with phosphate buffer saline and left to air dry. Crystal violet (0.1%) was used to stain the dried tubes for 15 minutes. Trypticase soy broth with 1% glucose and 10ml of the media in each glass tube and incubated in shaking incubator at37^{o}C for 24 hours. After that tube was emptied and washes with phosphate buffer saline and left to air dry. Crystal violet (0.1%) was used to stain the dried tubes for 15 minutes.

Tissue Culture Plate Method

This method is more reliable for evaluation of biofilm. A loop full freshly cultured isolates was added in10ml of trypticase soy broth with 1mg glucose. The inoculate broth was kept in the incubator for 24 hours at 37^{o}C. The suspension was diluted 1:100 with fresh medium, separated well of a polystyrene tissue culture plate, of 96 flat bottom wells. These wells were filled with 200ul of the bacterial suspension.

PCR amplification of 16S rDNA

Bacterial samples were freshly grown on agar plates. For DNA extraction, inoculum was prepared by growing loop full of bacterial culture in 1ml MHB media at 37°C for 24 hours.

Fig. 1: Extracts of *Datura innoxia.*

PCR optimization of 16S rDNA

Initially denatured at 94°C for 10 minutes and then for 35 cycles of denaturation for 30 seconds, annealed at 52°C for 40 sec (primers annealing) and initial elongation at 71°C for 1 minute, later on extended at 72°C for 10 minutes.

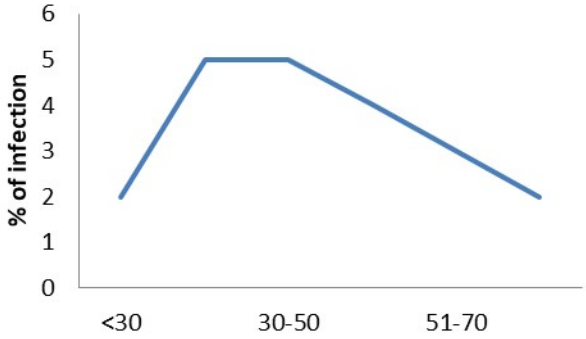

Fig. 2: Infection rate among different age groups

DNA extraction

Bacterial cultures were taken from overnight growth. The centrifugation of bacterial cultures was done at 6000 rpm for around 10 minutes. The upper layer was discarded and lower layer was mixed in 300µl nuclease free water and Placed in water bath for 10 minutes at 100°C and centrifugation was done for 5 minutes at 13000 rpm. Finally 5µl of upper layer was used for PCR reaction.

STATISTICAL ANALYSIS

All the data was analyzed by using SPSS (21). Mean ±SD was calculated. Comparison of means was done in order to find out the significance against antibiotics. Multivariate analysis of different variables was carried out. One way Anova was also carried out for plants extracts with the significance level of ($p<0.05$).

RESULTS

Demographic statistics

Out of 137 urine samples, percentage of UTIs were 76 (63%) among females and 61(37%) in case of males. UTIs were common in (age group 30-50) 70 (51.09%). This is due to the anatomical predispositions of females. This is similar to a previous study by (Adriotimi *et al*., 2006) in which rate of UTIs was 10.2% in females. UTI risk increases with age, poor diet, hygiene standards, and various complications of immune system. UTI development increases with age but in current study as shown in figure 2, 51.09% patients of age group (30-50) were affected as shown in figure 2, this was really significant as it was against the findings of (kaleem *et al*., 2019) in which infection rate was higher in patients of age group (50-70). General linear model multivariate analysis of demographic data shows that different factors including low income, gender, smoking, and lack of diabetes self-management are crucial in diabetic infections prevalence.

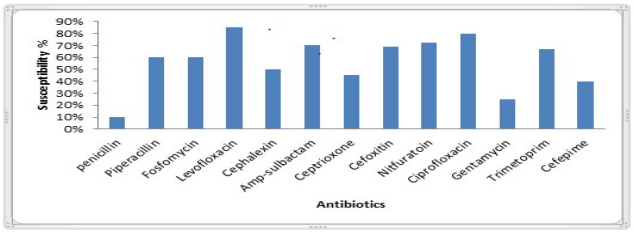

Fig. 3: Susceptibility patterns of antibiotics.

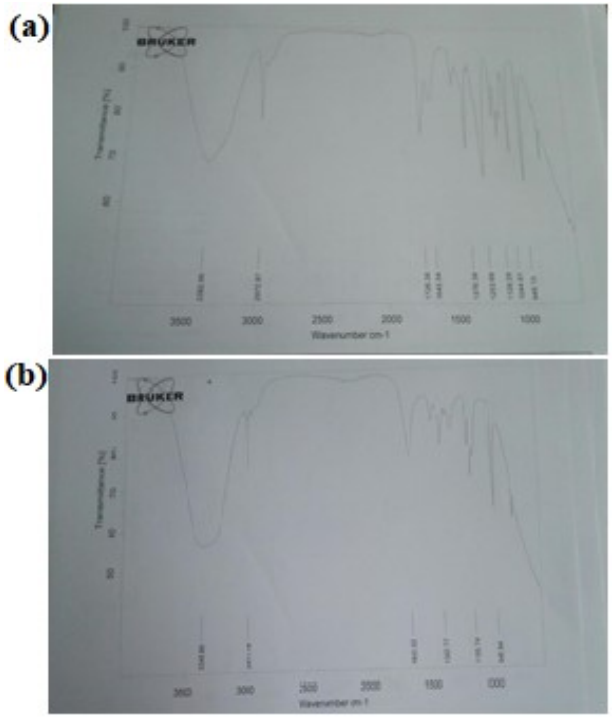

Fig. 4: (a) Ethanolic extracts (b) Methanolic extract.

Table 1: Primers for class 1 integron and 16S rDNA

Sr. No.	Primer	Oligonucleotide designation	Amplicon size (bp)	GC (%)	References
1	16S F1	(5'-GCG GAC GGG TGA GTA ATG-3')	1050	52	(Wang, 2007)
2	16S F2	(5'-GCT GGC AAA TAA GGA AAA-3')	1050	52	(Wang, 2007)
3	Int 1 F	GCCACTGCGCCGTTACCACC	898	51	(Kern, 2002)
4	Int 1 R	GGCCGAGCAGATCCTGCACG	898	51	(Kern, 2002)

Table 2: Multivariate analysis of the demographic variables

Variables	B	S.E	WALD	P value
Gender Males Females	1.304	0.305	6.543	0.003
Marital status Married Un married	-2.422	.785	9.516	0.002
Income 10-20K/pkr 21-40k/pkr	1.767	.834	4.483	0.002
Smoking 10/day 20 or more/day	2.477	.450	30.433	0.000
Weight Normal Overweight	3.076	1.339	5.227	0.002
Prescription Followed Not followed	1.606	.715	5.050	0.002

General linear model multivariate analysis. Significance level, $p < 0.05$.

Table 3: Methods showing percentage of biofilm formation

	Congo Red Agar		Tube Method	
Category.	*No.*	*%age*	*No.*	*%age*
Strong	32	23.33%	54	39.41%
Intermediate	22	16.05%	31	22.62%
Weak	07	5.10%	24	17.51%
Negative	76	55.47%	28	20.43%
	Tissue Culture Plate Method			
Category	*No.*		*%age*	
Strong	95		69.3%	
Intermediate	15		10.9%	
Weak	23		16.78%	
Negative	04		2.91%	

Table 4: One way Anova of plants extracts

Extracts	Mean	SD	p value
Ethanol	9.04	5.72	<0.002
Methanol	8.74	5.59	0.005
Chloroform	6.47	3.68	0.009

SD= standard deviation, p= significance <0.05

Disc diffusion test

Results of disc diffusion assay showed that 90% of strains were resistant against penicillin as shown in figure 3, while 75% were resistant against gentamycin. 60% of bacterial strains were resistant against cefepime. 55% were resistant against ceftriaxone. 50% were resistant against cephalexin. 85% strains were susceptible against levofloxacin and 80% were susceptible against ciprofloxacin while 70% were susceptible against amp-sulbactam (fig. 3).

Table 5: Functional groups of ethanolic extracts

Compound group	Frequency	Compound class
O-H	3392-3550	Alcohol
O-H	3200-3500	Alcohol
N-H	2965-3000	Amine salt
C-H	2913000	Alkane
C-H	1655-2000	Aromatic
C-H	1450	Alkane
O-H	1438-1500	Carboxlic acid
S=O	1380-1415	Sulphate
O-H	1406-1500	Carboxlic acid
C-F flouro	1000-1400	Flouro compound
S=O	1315-1343	sulfone
C=H	1013-1500	Flouro compound
C-H	800	Disubstituted
C=C	665-730	Alkane

Detection of biofilm

For detection of biofilm three methods were used, the most advanced method was tissue culture plate method according to which 69.3% strains developed strong biofilm as indicated in table 3, while in tube method 54 (39.41%) samples developed strong biofilm and in congo red method 32 (23.33%) strains developed strong biofilm.

These strains were able to develop biofilm. In tissue culture plate method only 15 strains showed intermediate biofilm production while in other two methods 31 (22.62%) and 22 (16.05%) strains showed intermediate level production of biofilm. This also showed the difference between these methods. All of these three methods are considered for biofilm detection but tissue culture method is more reliable method.

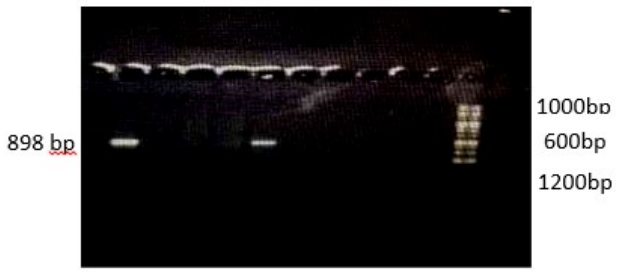

Fig. 5: Class 1integron

Screening of Plant Extracts

Antimicrobial properties of Methanolic, Ethanolic and Chloroform leaves extract of *Datura innoxia* were checked against selected bacterial strains. The maximum activity was showed by the ethanol leaf extract of *Datura innoxia* plant i.e. 20 mm and the minimum zone of inhibition 7 mm by methanolic extract. The mean of ethanolic extract was 9.04 while methanol and chloroform extracts means were 8.74 and 6.47mm respectively. While using one way anova the ethanolic extract activity was statistically significant as compared to methanol and chloroform extracts, p value was <0.02 in case of ethanolic extract (table 4). This was similar to the results of previous study which described that activity of ethanolic extract was 17mm and methanolic extract activity was 15mm against resistant bacteria, Ethanolic extract activity was significant because of the fact that *Datura innoxia contains* flavonoids and ethanol is more potent to extract flavonoids from plant and can kill bacteria by dissolving their cell membrane (Kushik, 2008).

FTIR analysis

The FTIR of ethanolic and methanolic extracts was performed as shown in table 5 anfd 6, in order to find out different functional groups in extracts. Maximum activity was showed by ethanolic extract with 14 peaks while in case of methanolic extracts 11 peaks were observed. Functional groups were determined by the peaks observed after the analysis. These functional groups were recognized according to the procedure explained by (Sasidharan *et al*., 2011).

Table 5 shows that majority of compounds present in the ehanolic extract, these compounds are responsible for antimicrobial activities of plant. The highly active compounds with strong peaks were observed in the range of 3300-3500 while lowest frequency was observed in 600-70 range.

The methanolic extract FTIR analysis showed 11 peaks alcoholic group was most active with strong peak while least peak was observed by alkane group. Presence of phenolic compounds showed that this plant has the antimicrobial potential to kill resistant microbes.

Detection of 16S rDNA

The 16S (rDNA) was used in order to identify the bacteria (Dam *et al*., 2013). The slow evolution rates enabled that universal primer to amplify genes. The PCR results were visualized by gel electrophoresis. Result from these

Table 6: Functional groups of methanolic extracts

Compound group	Frequency	Compound class
O-H	3382-3500	Alcohol
N-H	2972-3000	Amine salt
C-O	1720-1900	Aldehyde
C-C	1645-1728	Alkane
C-H	1450	Methyl group
O-H	1300-1380	Phenol
S=O	1335-1372	Sulphonate
C-O	1200-1276	Alkyl aryl ether
C-O	1124-1205	Tertiay alkcohol
C-H	1020-1250	Amine
C-C	930	Alkane

Compounds identified at different frequencies along with functional groups.

experiments showed that intact 16S rDNA was very rarely detected in a total RNA background at hybridization temperatures above ambient temperature.

Prevalence of integron cassette

The PCR results confirmed class 1 integron presence according to the size, i.e. (898 bp). The current results show the presence of class 1 integron as indicated in figure 5, as a major virulence agent in *E. coli*. The trend of occurrence of the class 1 integron among our isolates was 75 (54.74%) out of 137 isolates.

DISCUSSION

Type 2 diabetes is a lethal disease (Defronzo *et al*., 2009). Different bacterial species have been responsible for causing urinary tract infections (UTIs) (Adriotimi, 2006). With the use of antibiotics bacteria have gained resistance. In current study bacteria were isolated from type 2 diabetic patients. These bacteria were able to form biofilms. Most of these strains were resistant against large number of antibiotics. Large numbers of bacteria are able to form biofilms (Japoni *et al*., 2008). *E.coli*, *Klebsiella pneumonae*, *Pseudomonas aeruginosa*, *S. epidermidus*, *S. aureus* are able to form biofilms. In present study *Datura innoxia* was screened for presence of various compounds. Ethanolic and methanolic extracts were effective against *E.coli* strains. Ethanolic leaf extract showed more activity while chloroform and methanolic extracts were least active (Johnson *et al*, 2013). In a study by *(*Shama *et al, 2014)* methanolic extract of *Datura* was most active against isolates while in current study ethanolic extract was more effective against bacterial pathogens. Leaf extract of *Datura innoxia* showed highest activity against *E.coli, S. aureus, Klebsiella pneumonae* (Kumar *et al*., 2015). Antimicrobial activities of *Datura* against different isolates suggested that the inhibition patterns depend upon which part of the plant was used (Koushik *et al*., 2008) Leaf extract was more effective than stem and root extracts. It is reported that mixture of secondary products including tannins, alkaloids, steroids, and many other components have antimicrobial properties. *Datura* showed antimibacterial activity against three microbes including *Bacillus*, *E. coli*, *amyloliquefactions* and *P.aeruginosa.* Presence of diabetic cystopathy and macro vascular issues of kidneys plays a major role in development of urinary tract infections (UTIs). *E.coli* is normally leading cause of UTIs. (Johnson *et al*., 2011) evaluated the activity of leaves extract of *Datura stramonium*, *Calotropis against* variety of pathogens. In current study activity of ethanolic, chloroform and methanolic extracts were checked against *E. coli* strains. In a similar study by (Gulzar *et al*., 2015) aqueous extract of *Datura* was most effective against bacterial isolates. In a study by (Taye *et al*., 2011) three plants extracts were tested against bacterial pathogens and extracts inhibited the growth of pathogenic strains. Antibacterial activity of aqueous and other extracts from different parts of *plant* was carried out. Gram negative bacteria such as E. coli, *P.aeruginosa, S. typhi, Vibrio sp, while the Gram positive: B. subtilis, S. aureus*, *Bacillus cereus*, *B. subtilis* were checked (Gulzar *et al*., 2015). It was confirmed that leaf extracts have more activity against microbes. In comparison with chloroform, methanolic extract was more active, while chloroform showed activity against *Candida specie* (Eftekhar *et al*., 2005). It was estimated that extracts concentrations were required in larger amount in order to stop bacterial growth. Antimicrobial agents may hinder the formation of peptidoglycan on bacterial cell. Antimicrobial activity of *D. stramonium* ethanolic leaf extract against *E. coli, K. pneumonia*. 13 methanolic and 13 acetone extracts showed antibacterial activity against *Klebseilla pneumonia* was checked the results confirmed the potency of the plants uses in medicine. In addition, these results provide a good base for selection of a plant. Genetic diversity among plants genotypes reduces vulnerability of a plant against attack of insects/disease (Bibi *et al*., 2011).

The current study findings support the use of plants because of their antimicrobial properties. There are many other types of bacteria including *Klebsiella pneumonae, Enterococci, S. aureus*. Different studies have shown that *S. aureus, E. coli, Pseudomonas aeruginosa* were

common among diabetic infected patients (Japoni *et al*., 2008). Out of 137 strains class 1 integron was present in 75 (54.74%) strains. FTIR is used for identification of chemical groups found in mixture plant extract mixture. In a previous study by (Khusboo *et al*., 2016) FTIR analysis detected alkenes, alkanes, alkynes, amides, carboxylic group, aromatic, aliphatic amines and halide groups. Current study shows some additional compounds along with all these compounds including tertiary alcoholic and alkyl aryl groups. (Taye *et al*., 2011) find out integrons in 64% and 66.5% of *E. coli* isolated from infections. Integron class 1was present in 22.05%, 33.34% and 6.25% of *E. coli*, respectively (Hendrik *et al*., 2005) reported integrons in 6.25% of *E. coli* of UTI patients. *E. coli* in current study was resistant against many of antibiotics. *Pseudomonas aeruginosa* was resistant against majority of antibiotics but in current study *E. coli* was resistant against antibiotics. In current study *E. coli* was involved in UTIs. In a similar study *Staphylococcus* strains were resistant against majority of antibiotics except impenem, rifampicin, acefoxitin the sensitivity rate of strains against antibiotics was of 78%, 69% and 65% respectively. The role of empirical therapy in type 2 diabetes is higher than previously considered in Pakistan. There is a dire need to develop new protocols at community level. Those patients suffering from co morbidities require quick medical response.

CONCLUSION

This was really first study based on type 2 diabetes in Attock district to find out the reasons of diabetic patients prolonged stay in hospitals due to diabetes related complications. Detection of class 1 integron was significant to find out the prevalence rate in patients. *Datura innoxia* is a local herb it is commonly used to treat different ailments, its therapeutic affect was detected, and this may be helpful for future studies to investigate its antimicrobial activities further.

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