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Full Length Research Paper

Screening of natural extracts for their antibacterial activity against different enteric pathogens isolated from soil, water and rotten fruit samples

Shama Nazir and Zakia Latif*

Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore-54590, Pakistan.

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Different bacterial strains were isolated from soil, water and rotten fruit samples, and biochemically characterized as *Shigella, Pseudomonas, Escherichia coli, Klebsiella* and *Proteus.* Unhygienic environment (for example, contaminated food, water and air) are the most common habitats of these pathogens. Mostly, all are involved in endemic breakouts causing urinary tract and gastro-intestinal infections. The antibacterial activity of four concentrations of natural crude extracts of medicinal plants namely *Allium sativum* (garlic), *Nigella sativa* (black cumin), *Trigonella foenum-graecum* (fenugreek), *Ficus carica* (fig), *Azadirachta indica* (Indian lilac) and honey was determined against a total of ten isolated organisms by well plate method. Test organisms were found to be more sensitive to aqueous extract of bulb of *A. sativum,* seeds of *Nigella sativa* and honey. The antibacterial activity of crude extract was comparable with ciprofloxacin and ceftriaxone, commonly used antibiotics for the treatment of infections in adults and children, respectively. It was observed from the studies that most of the test strains were resistant to the antibiotics used especially against ceftriaxone. Due to the emergence of drug resistant microorganisms, it is a need to search out more effective antimicrobial agents to cure the disease. Our studies suggest that aqueous crude extract of medicinally important plants and honey could be the alternative of the antibiotic to cure the diseases.

Key words: Ceftriaxone, Ciprofloxacin, *Nigella sativa*, *Shigella*, *Proteus*.

INTRODUCTION

Each year millions of people get sick due to the contaminated food, air and water. Pathogenic bacteria are the most common cause of illness. Worldwide, around 1 billion people lack access to clean water and 2.5 billion have no access to basic sanitation. Common edibles available at local market are not properly cooked and kept in unhygienic conditions are major source of community acquired infections. Most of the people living in undeveloped areas live a life worst than that of animals. Their continued uncivilized existence is because Of their lack of awareness. Problems related to gastrointestinal tract and urinary tract infections mostly prevail among masses are due to the microorganisms belonging to class gammaproteobacteria including *Shigella*, *Pseudomonas*, *Escherichia coli*, *Klebsiella* and *Proteus*.

Due to the resistance emerging in organisms against antimicrobial drugs, it is an immediate need to develop alternate way such as search out novel and new antimicrobial drugs more active against pathogens with high resistance to cater the problem. Medicinal plants are a source of great economic value all over the world. Since pre-historic times, man has used various parts of different plants against common ailments prevailing in the society with varying degree of success. The knowledge of drugs has developed along with the evolution of scientific and social progress. Drugs which are extracted from plants are very effective, easily available and less expensive and they rarely have side effects associated

*Corresponding author. E-mail: zakia.mmg@pu.edu.pk or umna123@yahoo.com.

Abbreviations: MR, Methyl red; **VP,** Voges–Proskauer; **TSI,** triple sugar iron.

with them.

Crude extracts of plants may be used for initial screening for the potential antibacterial and antifungal compounds from plants. Antimicrobial activities of medicinal plants have also been reported. Over the last 40 years, there have been a lot of investigations on natural remedies like crude extracts of medicinal plants as source of new microbicidal activity (Sharma et al., 1977; Elnima et al., 1983; Farbman et al., 1993; Molan, 1999; Adebolu, 2005; Pritee et al., 2007; Salman et al., 2009; Joshi et al., 2011). Therefore, in the present study, natural aqueous crude extract of garlic, black cumin (kalvanjii), fenugreek (methi), Indian lilac, (neem), fig (anjeer) and pure honey were screened out against bacterial isolates involved in gastrointestinal problems. From our finding, we concluded that black cumin seeds, garlic and honey could be the alternatives of antibiotics to cure the diseases of gastrointestinal tracts.

MATERIALS AND METHODS

Isolation of bacterial strains

Samples of uncooked chicken meat, dry milk powder, polluted water, standing rain water, uncooked beef, sewerage mud, rotten potato, canal water, beef, sewerage water, poultry waste and rotten cheese were collected aseptically in sterile bottles and stored in the laboratory at 4°C till further use.

One gram of each sample was emulsified into 10 ml selenite broth and incubated at 37°C on continuous shaking at 150 rpm. After incubation, test tubes were examined for growth of bacteria. The overnight cultures were spreaded into Petri plates containing MacConkey's agar as selective and differential medium. Sub culturing on xylose lysine deoxycholate agar (XLD) was performed to obtain pure bacterial cultures.

Characterization of bacterial isolates

All bacterial isolates were biochemically characterized. Biochemical tests including catalase test, oxidase test, triple sugar iron test, indole test, methyl red test, voges-proskauer test, citrate utilization test, urease test and motility test were performed for the characterization bacterial strains by following the methods described by Cappuccino and Shermann (2002).

Collection of samples

Natural honey from Gilgit (Northern region of Pakistan), garlic (*Allium sativum*), black cumin *(Nigella sativa*), fenugreek (*Trigonella foenum-graecum*), fig (*Ficus carica*) and Indian lilac (*Azadirachta indica*) was purchased from local supermarket.

Preparation of natural crude extracts

All extracts were prepared with protocol followed by Durairaj et al. (2009) with few modifications. Samples except honey were weighed (10 g), and washed thoroughly with tape water. Samples were surface sterilized with absolute ethanol for 5 min. After washing thoroughly with autoclaved distilled water three times to remove extra ethanol, the samples were air dried in a laminar air flow chamber. The sterilized samples were homogenized aseptically using a sterile mortar and pestle in 10 ml autoclaved distilled water. The mixture was centrifuged at 10,000 rpm for 10 min and supernatant was filtered through Whatman filter No. 4. This extract was considered as 100% concentration which was further diluted to 25, 50 and 75% with appropriate volumes of sterile distilled water. The extract was preserved aseptically at 4°C until further use.

Antibacterial activity assay

The antimicrobial activity of all the crude was performed by well plate assay as described by Hannan et al. (2009) and Akujobi and Njok (2010). All bacterial strains were grown overnight in Luria Bertani (LB) broth (Cappuccino and Shermann, 2002). About 100 µl of each culture set at optical density 0.1 at 600 nm were spread on LB agar plates separately and left at room temperature for about 20 min to embed the microorganism before making wells. Each plate was labelled for the respective organism inoculated, and then punched to make four wells of 6 mm diameter with the help of a sterile cork borer. Different concentrations of prepared extracts were pipetted out (50 µl) into the wells in assay plate. Plates were incubated in an upright position overnight at 37°C. Inhibition zones were observed and the diameter was measured in mm using scale and mean were recorded.

Antibiotic sensitivity test

Four concentrations (2.5, 5.0, 7.5 and 10.0 µg/µl) of ciprofloxacin and ceftriaxone antibiotics (manufactured by Bayer and Roche Company, respectively) were prepared in sterilized distilled water. 100 µl of each concentration was used for all isolated bacterial isolates as with natural crude extract and observations were recorded by measuring the inhibition zone area in mm (Durairaj et al., 2009) after 24 h incubation at 37°C.

RESULTS

Isolation and characterization of bacterial isolates

10 morphologically different bacterial colonies were selected from plates inoculated with different samples on MacConkey agar medium and biochemically characterized as *Shigella* sp., *Pseudomonas* sp., *E. coli* sp., *Klebsiella* sp. *and Proteus* sp. (Tables 1 and 2).

Antibacterial activity by well plate method

Effect of honey

Among different test organisms, *E. coli* (isolate M1) showed maximum inhibition zone of 26 mm diameter; although it showed comparatively less inhibition by antibiotic ciprofloxacin and no inhibition by ceftriaxone. So, honey has more antibacterial potency for M1 isolate of *E. coli* (Figure 1) as compared to antibiotics used. Growth inhibition potency of honey shown in Figure 5 clearly demonstrates that two isolates of *E. coli* were sensitive that is, inhibited by honey. Isolates of *Pseudomonas* sp., *Shigella* sp. (isolate C2); *Proteus* sp.

Table 1. Response of bacterial isolates to ten different biochemical tests.

S/N	Source	Isolate	MR test	VP test	Insole test	Citrate test	Urease test	TSI test	Motility test	Oxidase test	Catalase test	Lactose test
1	Polluted water	P1	-	+	-	+	-	y/r/-/-	-	-	+	+
2	Rotten potato	R1	+	-	+	-	-	y/y/+/=	+	-	+	-
3	Sewerage water	S1	-	-	-	+	-	r/r/-/-	+	+	+	-
4	Sewerage water	S2	+	-	+	-	+	y/y/+/-	+	-	+	+
5	Sewerage mud	M1	+	-	+	-	-	y/y/+/-	+	-	+	+
6	Canal water	C1	-	-	-	-	-	r/y/-/-	-	-	-	-
7	Canal water	C2	-	-	-	-	-	r/y/-/-	-	-	+	-
8	Rotten potato	R2	-	-	-	-	+	y/y/+/+	+	-	+	-
9	Poultry waste	L1	+	-	+	-	-	y/y/+/-	+	-	+	+
10	Polluted water	P2	+	-	+	+	+	/y/y/+/+	+	-	+	-

+, Positive result; -, negative slant/butt/gas/H_2S; y/r, yellow slant/ red butt; y/y, yellow slant/yellow butt; r/r, red slant/red butt; r/y, red slant/red butt; +/-, gas production/no H_2S; -/-, no gas/no H_2S; +/+, gas production/H_2S production.

Table 2. Characterization of bacterial isolates by biochemical tests.

S/N	Isolate	Bacterial isolate
1	P1	*Klebsiella* sp.
2	R1	*Proteus* sp.
3	S1	*Pseudomonas* sp.
4	S2	*Escherichia coli*
5	M1	*E. coli*
6	C1	*Shigella* sp.
7	C2	*Shigella* sp.
8	R2	*Proteus* sp.
9	L1	*E. coli*
10	P2	*Proteus* sp.

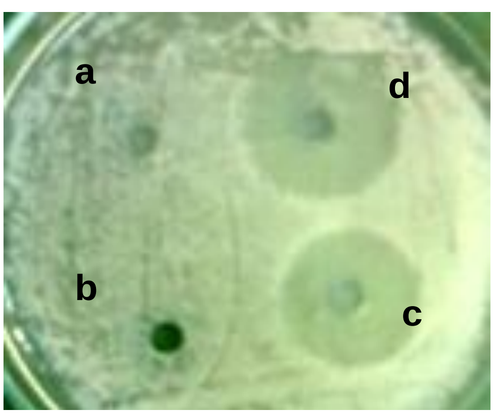

Figure 1. *E. coli* (isolate M1) zone of inhibition by ceftriaxone (a) 5 µg/ml, (b) 10 µg/ml; honey; (c) 50% and (d) 100%.

(isolates R2 and P2) showed no inhibition with crude honey. One isolate of *Shigella* and *Proteus* showed significant inhibition zone (22 mm). *Klebsiella* (P1), *Proteus* (isolate R1) and *Shigella* (isolate C1) were inhibited by honey and showed inhibition zone up to 19 mm in diameter (Figure 5).

Effect of garlic (A. sativum) extract

Maximum diameter of inhibition zone of 29 mm was observed in *Proteus* (isolate R2) in Figure 2 but was totally resistant to antibiotics ciprofloxacin and ceftriaxone (Figure 5). Other bacterial isolates responded differently against garlic extract as well as antibiotics. No activity was observed against *Pseudomonas* (S1) and *Shigella* sp. (C1) whereas antibacterial activity of the extract against other isolated test organisms varied exhibiting inhibition zone area from 14 to 19 mm (Figure 5).

Effect of black cumin (N. sativa) seeds extract

Crude extract from black cumin seeds (common name Kalvanjii) was employed to observe the inhibition of selected strains. Among the isolates, *Proteus* sp. (R2) gave maximum size of the zone of inhibition 31 mm (Figure 3). Extract of black cumin seeds showed no

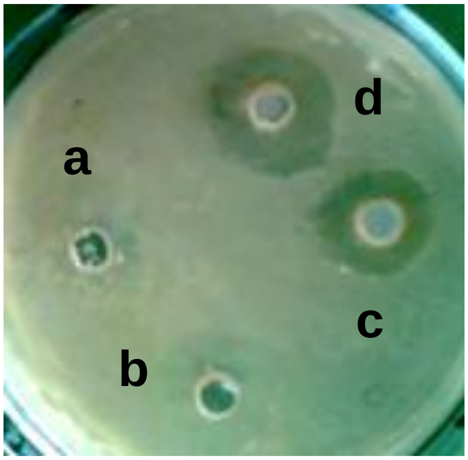

Figure 2. *Proteus* sp. (isolate R2), zone of inhibition by ciprofloxacin: (a) 2.5 µg/ml; (b) 5 µg/ml; garlic extract; (c) 50% and (d) 100%.

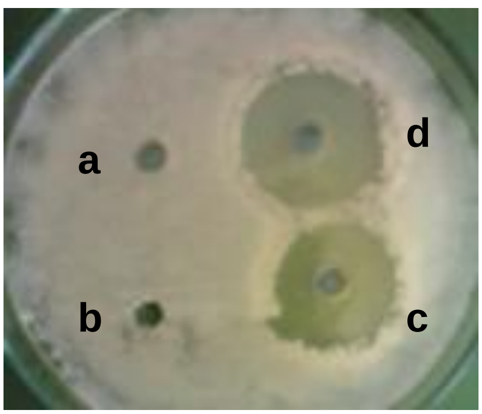

Figure 3: *Proteus* sp. (isolate R2) zone of inhibition formed by ceftriaxone a) 7.5µg/ml, b) 10µg/ml; black cumin seeds c) 50%, d) 100%

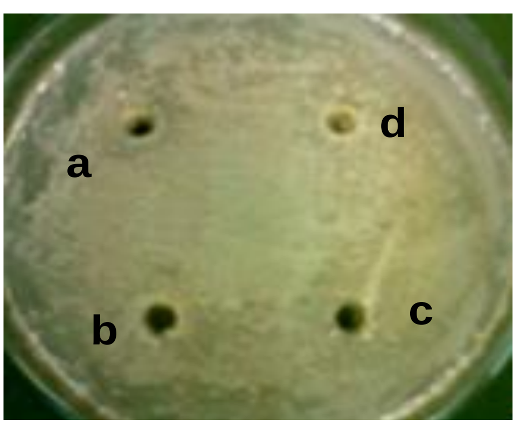

Figure 4. *Klebsiella* sp. (no zone of inhibition by ciprofloxacin: (a) 2.5 µg/ml; (b) 5 µg/ml; (c) 7.5 µg/ml; and (d) 10 µg/ml.

inhibitory effect on *Shigella* (C2) mean completely resistant to the extract of black cumin seeds. All the other test isolates showed moderate inhibition and the zone size ranges from 8 to 22 mm (Figure 5).

Effect of fenugreek (T. foenum-graecum) extract

Crude extract of fenugreek (common name methi) inhibited the growth of only *Proteus* species, the inhibition zone ranging with from 20 to 26.3 mm in diameter. All other bacterial isolates did not show any inhibition by the extract at any concentration.

Effect of Indian lilac (A. indica) leaves extract

Indian lilac leaves (Neem) extract inhibited growth of *Proteus* sp. (R2) at maximum concentration. The measured zone area was only 14 mm, significantly having no importance. *Klebsiella* sp., *E. coli* (isolates S2 and L2) and *Shigella* sp. (isolates C1and C2) showed no inhibition by the extract (Figure 5).

Effect of fig (F. carica) extract

The extract of fig (Anjeer) is not significantly important as having least antibacterial activity. One isolate of each, *Pseudomonas* (S1), *Shigella* (C1) and *Proteus* (R2) showed inhibition but zone area observed was only 12 mm. Other isolates were resistant to the extract of Anjeer (Figure 5).

Antibiotic sensitivity testing

Increase in size of inhibition zone was observed by increasing the concentration of antibiotics as in case of natural crude extracts. Among all bacterial isolated *Pseudomonas* (S1), *Proteus* (P2), all isolates of *E. coli* (L1, M1 and S2) and *Shigella* (C1 and C2) were sensitive to ciprofloxacin, and showed inhibition zone ranging from 21 to 31 mm in diameter. Proteus (R1 and R2, Figure 2) and *Klebsiella* (P1, Figure 4) were totally resistant to ciprofloxacin and showed no inhibition at any concentration. It was observed that ceftriaxone has less potency of bacterial inhibition than Ciprofloxacin although both are broad spectrum antibiotics. Isolates of E. coli (M1 and S2, Figure 1), *Proteus* (R1 and R2, Figure 4), and Shigella (C1 and C2) showed complete resistance against ceftriaxone (Figure 5). Only 40% bacterial isolates showed sensitivity against Ceftriaxone whereas 70% was observed in case of Ciprofloxacin.

Statistical analysis

Kolmogorv-Smirnov test (normality test) was used before

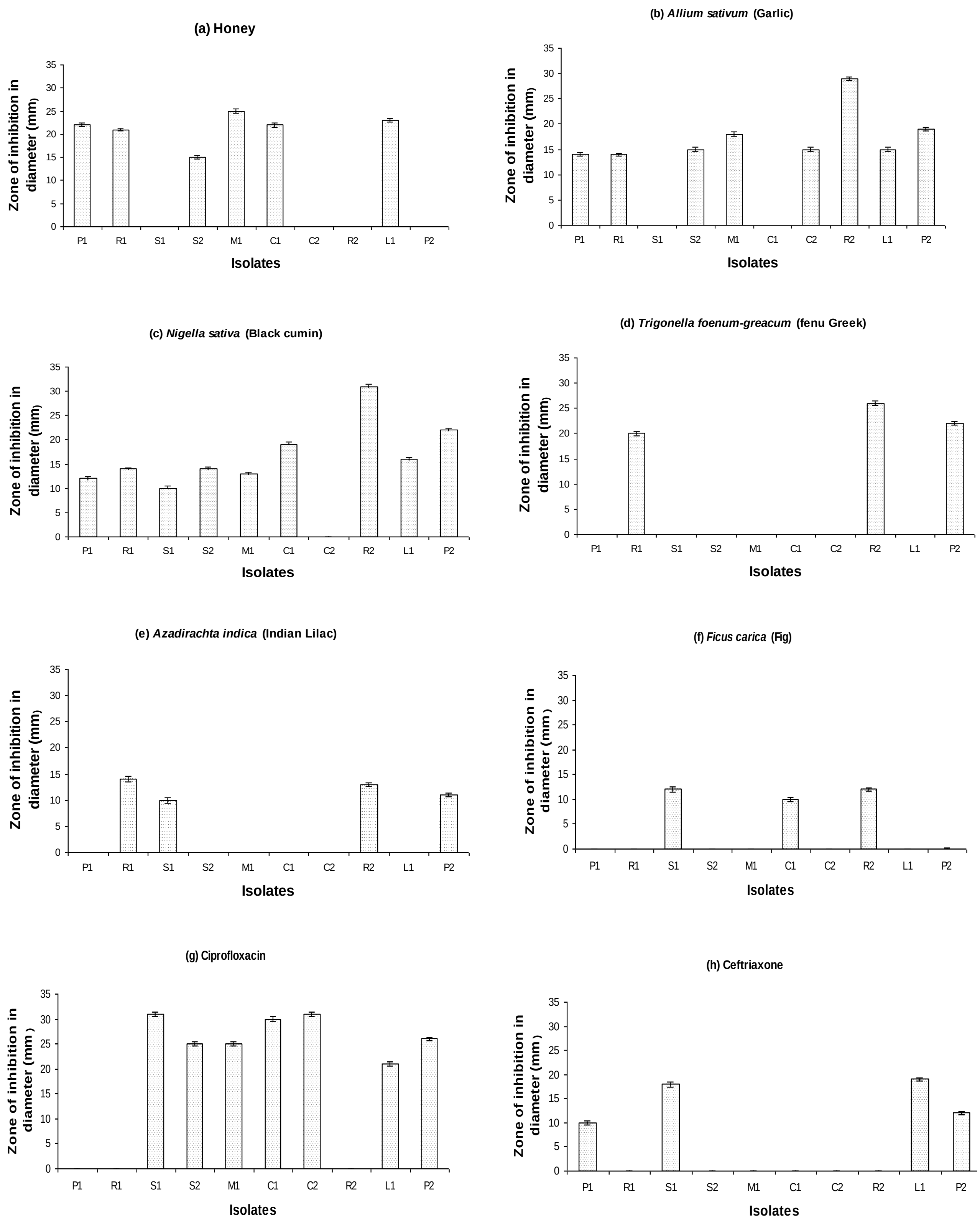

Figure 5. Antibacterial effect of different natural extracts and antibiotics on different isolates of bacteria by well plate method (inhibition zone in mm); P1, *Klebsiella* sp.; R1, R2, P2*, Proteus* sp.; S1, *Pseudomonas* sp.; S2, *Escherichia coli*; M1, *Escherichia coli*; C1, *Shigella* sp.; C2, *Shigella* sp.; L1, *Escherichia coli*. P1, *Klebsiella* sp.; R1, *Proteus* sp.; S1, *Pseudomonas* sp.; S2, *Escherichia coli*; M1, *Escherichia coli*; C1, *Shigella* sp.; C2,*Shigella* sp.; R2*, Proteus* sp.; L1*, Escherichia coli*; P2*, Proteus* sp.

the application of any statistical analysis to check the normality of the data. Analysis of variance (ANOVA) was carried out to determine the significance of selected natural extracts and antibiotics commonly used against enteric pathogens. In order to find which of natural was most active in inhibition of bacterial strain, Tukey's test was used. Overall, all extracts showed different activity against selected bacterial isolates (for pathogens, d.f = 9,160; F = 454.90; P = 0.00 and for extract d.f = 7,160' F = 2624.36; P = 0.00). Although, according to Tukey's test, honey, garlic and black cumin had similar antimicrobial activity as ciprofloxacin against all isolated bacterial strains. Similarly fenugreek, Indian lilac and fig have similar inhibition effect on all bacterial strains as ceftriaxone. To check the similarity in activity of extracts and antibiotics on isolated bacteria was applied using Minitab 13.

DISCUSSION

Since pre-historic times, man has used different plants against common illness prevailing in the society with varying degree of success. The knowledge of drugs has developed along with the evolution of scientific and social progress. Drugs which are extracted from plants are very effective, easily available and less expensive and they rarely have side effects associated with them. The up growing resistance of microorganisms to the convectional antimicrobial agents is a source of great concern to clinical microbiologists. Bacteria evolve some changes in their genome with time, as a result, a large number of bacterial species particularly *Shigella* and *E. coli* (WHO, 2009) have become resistant to the antibacterial drugs due to extensive use and often create a problem in treatment of infectious diseases. Thus it is the need of the hour to develop alternate ways to cater this problem. Natural substances are known to act synergistically with antibiotics, and resistance has not been reported against them. There are some advantages of using antimicrobial compounds of medicinal plants such as often fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature (Vermani et al., 2002).

As a result of the present scenario, efforts are being made to develop antimicrobial agents from local sources for better chemotherapeutic effects. Crude extracts of six naturally occurring substances including honey, garlic, black cumin (kalvanji), Indian lilac (neem), fenugreek (methi) and fig (anjeer) have been used to determine antimicrobial susceptibility. The assay against 10 different strains isolated from water, soil and rotten food etc. was done by well plate method. The present study clearly showed that the plant extracts inhibited bacterial growth but their effectiveness varied *in vitro* studies. All strains were biochemically characterized. Most of these strains belong to the class gammaproteobacteria. They are mostly involved in gastrointestinal diseases and urinary tract infections (Nicolle, 2003; Jeremy, 2006). Such infections are mostly endemic and are very common in Pakistan.

Among the six extracts; honey, garlic and black cumin showed maximum inhibition of the organisms. Black cumin was active against all organisms except one. Present study is also in agreement with Morsi (2000) who reported that black cumin extracts possess antibacterial activity against a wide broad range of microbes, especially antibiotic resistant bacteria. Preliminary clinical trials have reported its therapeutic use for the treatment of variety of ailments and conditions that include diarrhea, asthma, hypertension, diabetes, inflammation, cough, bronchitis, headache, eczema, fever, dizziness, influenza and dental caries (Ali and Blunden, 2003). Its seeds are also a source of Ca, Fe, Zn, Mg, K, Se and Na, required only in small amount by the body. It is one of the important medicines of Tibbe Nabawi (Prophetic Medicine) according to the following Hadiths: Prophet said, 'This black cumin is healing for all diseases except As-Sam.' asked, 'what is As-Sam?' He said, 'Death.' "(Sahih al Bukharia). The antimicrobial activity of fig, fenugreek and Indian lilac was not very prominent but were observed to be active against 3 to 4 organisms. Their antimicrobial activity can be analyzed further by changing some parameter.

One interesting observation was recorded by *Klebsiela* (P1), showing no growth inhibition by any of the antibiotic (Figure 5) but was inhibited in the presence of honey, garlic or black cumin. Overall conclusion is that *Klebsiella* has evolved resistance against antibiotics but not against the natural extracts. Similarly *Proteus* sp. *(*R1*)* and *(*R2*)* were not inhibited by Ciprofloxacin but inhibited by other natural extracts. *Klebsiela* sp., *Pseudomonas* sp., *E. coli (*S2*)*, *E. coli (*M1*)*, *Shigella* sp. *(*C1*)*, and *Shigella* sp. *(*C2*)* also did not show any inhibition on exposure to antibiotic Ceftriaxone. The inability of antibiotics to inhibit the growth of any of these organisms may be as a result of misuse and abuse of this drug.

Conclusion

This antibacterial study of the plant extracts demonstrated that folk medicine can be as effective as modern medicine to conflict pathogenic microorganisms. Very little work has been done on the biological activity and application of natural compounds as medicine. Extensive investigation is needed to exploit their therapeutic utility to combat diseases. Modern drugs can be developed after extensive investigation of their bioactivity, mechanism of action, pharmacotherapeutics and toxicity after proper standardization and clinical trials.

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