

SCREENING OF ANTIBACTERIAL ACTIVITY OF FOUR MEDICINAL PLANTS OF BALOCHISTAN-PAKISTAN

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Abstract

The Balochistan province possesses rich medicinal herbal resources, but has not been evaluated scientifically. Different plants of province or its parts are generally used to treat different ailments of animals as well as human being. In the present study, Crude Methanol Extracts (CME) of four plants of Balochistan (*Berberis baluchistanica*, *Seriphidium quettense*, *Iphionia aucheri*, *Ferula costata*) have been tested for a wide array of antimicrobial activity against three gram positive bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*) and four gram negative bacteria (*Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*). Zones of inhibition of CMEs were determined by disc diffusion method and MIC was tested by agar dilution method and agar well diffusion method. All the plant extracts were found to be effective against all the tested bacteria. *Berberis baluchistanica* was the most effective one showing higher zones of inhibition and lowest MIC values whereas *Ferula costata* performed comparatively least activity. *Salmonella typhimurium* and *Klebsiella pneumoniae* exhibited resistance with the highest rate. These and other medicinal plants of the province have got great potential to be used in research area for the production of different goods in the future.

Introduction

Indigenous knowledge of herbal medicine is a big source of the modern knowledge. Today, thousands of plants, traditionally used as medicines, are being explored for their antimicrobial activities and chemical components (Evans, 1997). Even there are thousands of species of plants which have to be explored in the future (Aberoumand *et al.*, 2010). New and effective antimicrobial agents with broad-spectrum activities from natural sources are now become the demand of the day because of the development of resistance of pathogens against antibiotics caused by the indiscriminate use of modern antibiotics. The American Society of Microbiology has recommended the development of novel antimicrobial agents because of the emergence of multi drug resistant bacterial pathogens (Anon., 1995).

Antibacterial activity of different extracts is being sorted out throughout the world. Ushimaru *et al.*, (2007) reported that CME of *Caryophyllus aromaticus* exhibited excellent antimicrobial activity for *Staphylococcus aureus* and the other tested strains of bacteria (*Escherichia coli*, *Salmonella typhimurium* and *Enterococcus* sp.). Methanolic extract of *Terminalia chebula* leaf and aqueous extract of *T. bellerica* fruit also showed excellent activity against *Staphylococcus aureus* and *Escherichia coli*. Methanol leaf extracts of *Acacia nilotica*, *Sida cordifolia*, *Tinospora cordifolia*, *Withania somnifera* and *Ziziphus mauritiana* were examined against some bacterial species, by disc diffusion method, which displayed significant antimicrobial activity (Mahesh & Satish, 2008). Nascimento *et al.*, (2000) evaluated the antimicrobial activity of ethanolic extracts of ten plants. Out of these ten observed plants, *Caryophyllus aromaticus* and *Syzygium joabolanum* were proved to be the most active. The antibacterial activity of different extracts of some medicinal plants was examined, by agar-well diffusion method and reported that Chloroform and ethanol extracts of *Ventilago madraspatana* had shown broad-spectrum activity against most of the bacteria except *S. aureus*, *E. coli* and *Vibrio cholerae* (Basu *et al.*, 2005). Twenty out of 30 Indian

folklore medicinal plants used by tribal healers to treat infections showed activity against one or more species of bacteria used in an assay by Samy and Ignacimuthu (2000). In the same way out of 66 ethanolic plant extracts, evaluated against nine different bacteria, 39 showed broad-spectrum activity against six or more bacteria (Aqil and Ahmad, 2007). Three Moroccan plants used as traditional medicine, exhibited significant activity against *Streptococcus pneumoniae* (Warda *et al.*, 2009). The hexane, chloroform, ethyl acetate and methanol extracts from the leaves of *Acalypha indica* showed minimum inhibitory concentrations (MIC) between 0.156 to 2.5 mg/ml against the tested Gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Streptococcus faecalis*) and were active only against *Pseudomonas aeruginosa* amongst the tested Gram-negative bacteria (Govindarajan *et al.*, 2008). Antimicrobial activity of CME and its different fractions of *Impatiens bicolor* Royle were determined against 6 bacterial species but neither the CME nor any of its fractions showed any antibacterial activity (Nisar *et al.*, 2010). Naz *et al.*, (2010) studied the antibacterial activity of curcuminoids and essential oil of *Curcuma longa* by agar well diffusion method. Zones of inhibition were observed for both curcuminoids and essential oil. Antibacterial activity of aqueous infusion, decoction and essential oil of *Cinnamomum cassia* (Cinnamon bark) by standard disk diffusion method were investigated against 178 bacterial strains. Oil of Cinnamon bark exhibited 99.4% antibacterial effect, aqueous decoction 70.2% and aqueous infusion exhibited 52.2% effect (Chaudary and Tariq, 2006).

Seriphidium quettense (Podlech) Ling, Vern. Speredh Tarkha (Pashto), a species of the complex *Artemisia maritima* Burk. (Asteraceae). This plant is endemic to Pakistan (Balochistan) and commonly grazed by sheep and goats. It is traditionally used for various digestive problems (Anon., 2003 and Durrani *et al.*, 2003). Six organic solvent extracts of *Artemisia nilagirica* were used against 15 bacterial strains. The methanol and hexane extracts exhibited the highest activity (Ahameethunisa and Hopper, 2010). The aqueous and ethyl acetate

extracts of *Artemisia herba-alba* had a weak or no antibacterial activity against the tested bacteria (Seddik *et al.*, 2010). Ethanol extract of *A. sieversiana* had maximum zone of inhibition against *E. coli* whereas chloroform extract of *A. sieversiana* exhibited maximum zone of inhibition against *B. subtilis* (Nisha *et al.*, 2010). Antibacterial activities of ethyl acetate, methanol, chloroform and acetone extracts of whole plant of *Artemisia absinthium* were studied. The ethyl acetate and chloroform extracts showed activities against some of the test bacteria (Erdogrul, 2002).

Berberis baluchistanica Ahrendt, Vern. Zralga (Pashto) is a shrub which belongs to the family Berberidaceae. This plant is endemic to Pakistan (Balochistan). Decoction of roots is used for the remedy coughing, infection and internal injury of human being and livestock (Ghafoor, 2002). Two new alkaloids (*pakistanine* and *pakistanamine*) and a new phenolic bisbenzylisoquinoline alkaloid (+)-baluchistine have been isolated from *B. baluchistanica* (Shama *et al.*, 1973 and Miana *et al.*, 1979). In an antibacterial study, different extracts of the roots of *Berberis tinctoria* have exhibited broad spectrum antibacterial activity (Sasikumar *et al.*, 2007). The hydroalcoholic extracts of four species of *Berberis* (*B. aristata*, *B. asiatica*, *B. chitria* and *B. lycium*) were examined against eleven bacterial strains (Singh and Srivastava, 2007). All the species were found to be active against most of the tested bacteria. Crude extracts of *Berberis aetnensis* have also shown antibacterial activity mainly against Gram-positive bacteria (Musumeci *et al.*, 2003). Shahid *et al.*, (2009) reported that aqueous and alcoholic extract of *Berberis aristata* DC roots showed wide antibacterial activity against Gram-positive bacteria and against limited tested Gram-negative bacteria.

Iphiona aucheri (Boiss.) Anderb. (Balochi: Kolbur) belongs to the family Asteraceae. *Iphiona aucheri* is distributed in Pakistan, Iran and Oman. In Pakistan, it has been reported that it is inhabitant of Mekran, Khuzdar, Lasbela, Chaghi and Loralai districts ((Qaiser and Abid, 2003). This plant species has been reported to be responsible for camel poisoning in United Arab Emirates (Roeder *et al.*, 1994). They isolated a number of substances from the aerial parts of *Iphiona aucheri* in which atractyloside and carbixyatractyloside were identified as the toxic principle of the plant. Atractyloside was known to be a fatal form of herbal poisoning (Stewart *et al.*, 2000).

Ferula costata Kor. ex Nasir belongs to the family Apiaceae. It has tall stem and long leaves. Leaves are tripinnate with small oblong segments. Flower pedicelate, fruit elliptic to oblong. It is distributed in Pakistan and Afghanistan. In Pakistan, its distribution is restricted to Balochistan and Khyber Pukhtunkhwa. The local community claims that different species of *Ferula* are used for endoparasites of animals and tooth-ache in humans.

In the present study, the antibacterial activity of the Crude Methanol Extract (CME) of *Berberis baluchistanica* (roots), *Seriphidium quettense* (aerial parts), *Iphiona aucheri* (aerial parts) and *Ferula costata* (aerial parts) was tested against three gram positive and four gram negative bacteria to evaluate the potential use of these plants as antimicrobial agents. This study proved that the local community could provide the knowledge and be used as a guide to helping out to improve new and more efficient products against ever-changing pathogens.

Materials and Methods

Plant materials: Aerial parts of *Seriphidium quettense* (Asteraceae), *Iphiona aucheri* and *Ferula costata* and roots of *Berberis baluchistanica* were collected from districts Quetta, Nushki, Killa Saifullah and Ziarat, respectively. Bark of the *B. baluchistanica* roots was removed. The collected materials were air dried and ground to powder. Plants were identified by a qualified plant taxonomist and voucher specimens were deposited in the herbarium of Botany Department University of Balochistan, Quetta Pakistan.

Extract preparation: The powdered material of each plant was soaked in methanol and left for one week. The solvent was filtered through fine cloth. This process was repeated twice for 24 hours. The filtrate was condensed by rotary evaporator. The semi solid extract was refrigerated till use.

Test microorganism: The following test microorganisms (bacteria) were got from a local private diagnostic laboratory and cultured on blood agar media:

1. **Gram positive bacteria:** *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*.
2. **Gram negative bacteria:** *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*.

Antibacterial assay: Antibacterial activity was determined by Disc Diffusion Method (Nostro *et al.*, 2000). Minimum Inhibitory Concentration (MIC) was determined by Agar Dilution Method (Nostro *et al.*, 2000) and Agar Well Diffusion Method (Basu *et al.*, 2005).

Preparation of 0.5 McFarland standard: The required standard was prepared by adding 0.5 ml. of 0.048 M BaC₁₂ (1.17% w/v BaC₁₂·2H₂O) to 99.5 ml. of 0.18 M H₂SO₄ (1% w/v) while stirring constantly (Andrews, 2004).

Disc diffusion method: An inoculum of each test organism was prepared in 5 ml normal saline solution. The bacterial suspension was compared to the 0.5 McFarland standard. The dried Mueller-Hinton agar plates were inoculated evenly by sterile cotton swabs. 200 mgml⁻¹ (w/v) solutions of CME were prepared in dimethylsulfoxide (DMSO). Paper discs of 6 mm diameter were soaked in 20% CME solutions and placed on the inoculated agar plates. Streptomycin and Penicillin were used as positive control while DMSO was used as negative control. The plates were incubated at 37°C for 24 hours. Each extract was used in triplicate.

MIC determination by agar dilution method: Stock solution of 500 mgml⁻¹ concentration of each extract was prepared in DMSO. Six twofold dilutions were made. Mixed 1 ml of each dilution in 20 ml melted Mueller-Hinton agar and poured in a Petri dish. 1-2 µl of bacterial suspension (compared to 0.5 McFarland standard) was deposited on the solidified agar in the form of spots. The plates were incubated for 24 hrs at 37°C. Petri plates of each extract dilution was made in triplicate. As 1 ml of dilution was mixed with 20 ml M-H agar, the concentration of extract was calculated accordingly.

MIC determination by agar well diffusion method:

Mixed 1 ml of bacterial suspension, compared with 0.5 McFarland standard, with 20 ml sterile M-H agar at 45°C and poured into Petri dishes. Wells of 4mm diameter were made in the solidified M-H agar. Two-fold serial dilution of 200 mg ml⁻¹ CME in DMSO (w/v) was introduced in the wells. The plates were incubated at 37°C for 24 hours. Plates were made in triplicate.

Statistical analysis: Standard Deviation of the data was analyzed by MS-Excel.

Results and Discussion

Antibacterial activity of Crude Methanol Extracts (CME) of four plant of Balochistan, *Berberis baluchistanica* (roots), *Seriphidium quettense* (aerial parts), *Iphiona aucheri* (aerial parts) and *Ferula costata* (aerial parts), traditionally used as medicine, were tested against *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The first three are Gram-positive and last four are Gram-negative bacteria.

The result of Disc Diffusion Test (DDT) showed that all the plant extracts were active against all the test bacteria to variable extent. The antibiotic Streptomycin

was active against all the test bacteria while Penicillin was active only against Gram positive bacteria. Similar results of Penicillin activity were reported by Basu *et al.*, (2005). DMSO did not show any activity.

Berberis baluchistanica exhibited the best activity amongst all the studied plants. Its highest activity was against *P. aeruginosa* (19.33mm) and lowest was against *S. typhimurium* (10.67mm) (Table 1, Fig. 1). The MIC value of *B. baluchistanica* for different bacteria ranged from 0.87 to 6.25 mg ml⁻¹ in agar dilution assay (Table 2) and it was 1.3 to 6.25 mg ml⁻¹ in agar well diffusion test (Table 3). The lowest MIC was against *P. aeruginosa* while highest was against *K. pneumoniae*. Results of both the MIC methods (Agar Dilution Method and agar Well Diffusion Method) were almost similar to each other. Other species of *Berberis* have also exhibited excellent antibacterial activities. The methanol extract of *B. tinctoria* root has also shown antibacterial activity against *E. coli*, *Salmonella sp.*, *S. typhi*, *K. pneumoniae* and *Pseudomonas aeruginosa* (Sasikumar *et al.*, 2007). Hydroalcoholic extracts of stem and roots of *B. aristata*, *B. asiatica*, *B. chitria* and *B. lyceum* exhibited significant activity against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. pneumoniae* and some other bacteria. However none of above four *Berberis* species showed any activity against *S. typhimurium* except *B. lycium*.

Table 1. Antibacterial activity of CME (20% in DMSO) of medicinal plants against bacterial species tested by disc diffusion assay.

Plant name	Zone of inhibition (mm)						
	Sa	Spn	Ec	Spy	St	Kp	Pa
<i>Berberis baluchistanica</i>	17.33 (±1.52)	18.00 (±1.52)	12.33 (±0.58)	19.00 (±1.00)	10.67 (±1.04)	12.67 (±0.76)	19.33 (±0.76)
<i>Seriphidium quettense</i>	17.67 (±1.52)	15.00 (±1.52)	12.33 (±1.04)	14.66 (±0.58)	9.67 (±0.29)	10.67 (±1.04)	16.67 (±0.29)
<i>Iphiona aucheri</i>	15.5 (±0.50)	12.50 (±1.32)	13.17 (±1.26)	13.67 (±0.58)	8.83 (±0.76)	9.33 (±0.76)	14.00 (±1.00)
<i>Ferula costata</i>	10.5 (±0.87)	8.83 (±0.29)	9.83 (±0.29)	9.33 (±0.58)	9.50 (±0.50)	9.17 (±0.29)	9.33 (±0.29)
DMSO	NA	NA	NA	NA	NA	NA	NA
Streptomycin	22.00 (±0.50)	19.00 (±1.00)	19.16 (±0.29)	20.83 (±0.76)	15.66 (±0.58)	18.50 (±0.58)	23.33 (±0.58)
Penicillin	14.66 (±0.58)	15.00 (±0.50)	NA	17.83 (±0.29)	NA	NA	NA

Sa; *Staphylococcus aureus*, Spn; *Streptococcus pneumoniae*, Ec; *Escherichia coli*, Spy; *Streptococcus pyogenes*, St; *Salmonella typhi*, Kp; *Klebsiella pneumoniae*, Pa; *Pseudomonas aeruginosa*, NA ; No activity

Table 2. MIC of CME of plants against seven bacterial species (by Agar Dilution Method).

Plant name	MIC values (mgml ⁻¹)						
	Sa	Spn	Ec	Spy	St	Kp	Pa
<i>Berberis baluchistanica</i>	3.12 (±0.0)	1.56 (±0.0)	3.12 (±0.0)	1.56 (±0.0)	4.16 (±1.81)	6.25 (±0.0)	0.87 (±0.0)
<i>Seriphidium quettense</i>	2.08 (±0.90)	3.12 (±0.0)	4.16 (±1.81)	3.12 (±0.0)	4.16 (±1.81)	6.25 (±0.0)	6.25 (±0.0)
<i>Iphiona aucheri</i>	3.12 (±0.0)	6.25 (±0.0)	3.12 (±0.0)	3.12 (±0.0)	8.33 (±3.61)	3.12 (±0.0)	6.25 (±0.0)
<i>Ferula costata</i>	6.25 (±0.0)	12.5 (±0.0)	12.5 (±0.0)	12.5 (±0.0)	6.25 (±0.0)	6.25 (±0.0)	12.5 (±0.0)

Sa; *Staphylococcus aureus*, Spn; *Streptococcus pneumoniae*, Ec; *Escherichia coli*, Spy; *Streptococcus pyogenes*, St; *Salmonella typhi*, Kp; *Klebsiella pneumoniae*, Pa; *Pseudomonas aeruginosa*

Table 3. MIC of CME of plants against seven bacterial species (Agar Well Diffusion Method).

Plant name	MIC Values (mgml ⁻¹)						
	Sa	Spn	Ec	Spy	St	Kp	Pa
<i>Berberis baluchistanica</i>	2.6 (±0.90)	1.56 (±0.0)	2.6 (±0.90)	1.3 (±0.45)	2.6 (±0.90)	6.25 (±0.0)	1.56 (±0.0)
<i>Seriphidium quettense</i>	1.56 (±0.0)	3.12 (±0.0)	3.12 (±0.0)	3.12 (±0.0)	2.6 (±0.90)	5.21 (±1.81)	6.25 (±0.0)
<i>Iphiona aucheri</i>	2.6 (±0.90)	5.21 (±1.81)	2.6 (±0.90)	3.12 (±0.0)	5.21 (±1.81)	2.6 (±0.90)	6.25 (±0.0)
<i>Ferula costata</i>	5.21 (±1.81)	8.33 (±3.61)	10.42 (±3.61)	12.5 (±0.0)	6.25 (±0.0)	4.16 (±1.81)	10.42 (±3.61)

Sa; *Staphylococcus aureus*, Spn; *Streptococcus pneumoniae*, Ec; *Escherichia coli*, Spy; *Streptococcus pyogenes*, St; *Salmonella typhi*, Kp; *Klebsiella pneumoniae*, Pa; *Pseudomonas aeruginosa*

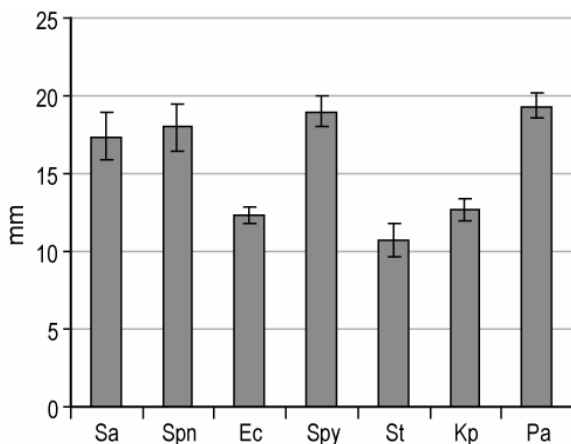


Fig. 1. Antibacterial activity of *Berberis baluchistanica* by Disc Diffusion Method.

Sa; *Staphylococcus aureus*, Spn; *Streptococcus pneumoniae*, Ec; *Escherichia coli*, Spy; *Streptococcus pyogenes*, St; *Salmonella typhi*, Kp; *Klebsiella pneumoniae*, Pa; *Pseudomonas aeruginosa*.

Seriphidium quettense also showed significant antibacterial activities. As shown in Table 1 and Fig. 2, the best activity in the DDT was against *S. aureus* (17.67mm) while the poorest was against *E. coli* (12.33mm). Its minimum MIC values were against *S. aureus* and against *K. pneumoniae* and *P. aeruginosa*, respectively (Tables 2&3). Benli *et al.*, (2007) reported that methanol extract of *Artemisia dracunculus* showed better activity than its chloroform and acetone extracts. Its methanol extract was effective against two strains of *E. coli* and three other bacterial species but was ineffective in case of *S. pyogenes* and *S. aureus*. In another study, the methanol and chloroform extracts showed activity against the highest number of bacteria (Ahameethunisa and Hopper, 2010). In this study, *K. pneumoniae* and *S. typhi* proved to be the most resistant species. Seddik (2010) reported that ethyl acetate and chloroform extracts of *Artemisia herba-alba* Asso., showed excellent activity against bacteria. These extract contained high quantity of phenolic compounds.

Antibacterial activities of *Iphiona* species have not been tested yet. However, it has been reported that it contains atractyloside which is toxic to many organisms (Stewart *et al.*, 2000). In the present study it proved to be quite efficient against all the tested bacteria. In the DDT, its

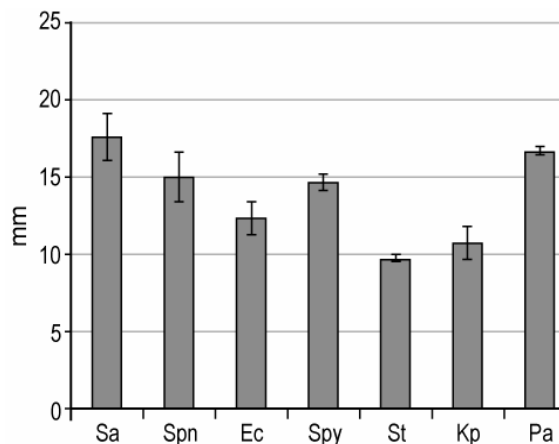


Fig. 2. Antibacterial activity of *Serephidium quettense* by Disc Diffusion Method.

Sa; *Staphylococcus aureus*, Spn; *Streptococcus pneumoniae*, Ec; *Escherichia coli*, Spy; *Streptococcus pyogenes*, St; *Salmonella typhi*, Kp; *Klebsiella pneumoniae*, Pa; *Pseudomonas aeruginosa*.

activity ranged from 8.33 to 15.5 mm zone being highest against *S. aureus* and lowest against *S. typhimurium* (Table 1, Fig. 3). The lowest MIC of *I. aucheri* was 3.12 mg ml⁻¹ in agar dilution method and 2.6 mg ml⁻¹ in agar well diffusion method (Tables 2&3 respectively). The best activity (lowest MIC) of *I. aucheri* was against *S. Aureus*, *E. coli*, *S. pyogenes* and *K. ponumoniae*.

Different species of the genus *Ferula* (Apiaceae) has shown variable antibacterial activities. The chloroform extract of *Ferula persica* roots was active against *E. coli*, *K. ponumoniae*, *S. typhi*, *S. aureus*, and *S. epidermtilis*. The active substance was known to be umbelliprenin (Shahverdi *et al.*, 2005). *Ferula rigidula* has shown a broad spectrum activity (Sener *et al.*, 1998). Root volatile oil of *Ferula hermonis* has also proved to be a strong antibacterial agent (Hilan *et al.*, 2007). In the present study, the antibacterial activity *Ferula costata* was studied, which was mildly active against all the test bacteria. In DDT, the highest zones formed by the CME of this species were 10.5mm against *S. aureus* (Table 1, Fig. 4). The lowest zone was 8.83mm against *S. pneumoniae*. The best (lowest) MIC value of *F. costata* was 6.25 mg ml⁻¹ in agar dilution method and 4.16 mg ml⁻¹ in agar well diffusion method (Tables 2&3, respectively).

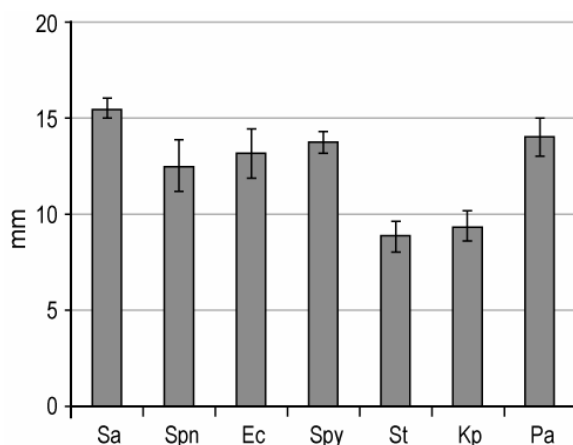


Fig. 3. Antibacterial activity of *Iphiona aucheri* by Disc Diffusion Method.

Sa; *Staphylococcus aureus*, Spn; *Streptococcus pneumoniae*, Ec; *Escherichia coli*, Spy; *Streptococcus pyogenes*, St; *Salmonella typhi*, Kp; *Klebsiella pneumoniae*, Pa; *Pseudomonas aeruginosa*.

Conclusion

The antibacterial activities of CME of four medicinal plants, *Berberis baluchistanica* (roots), *Seriphidium quettense* (aerial parts), *Iphiona aucheri* (aerial parts) and *Ferula costata* (aerial parts) were examined by disc diffusion test, agar dilution test and agar well diffusion test against *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. All the plants' extracts were variably active against all the tested bacteria. *B. baluchistanica* was the most effective species while *F. costata* was the least. Among the tested bacteria *Klebsiella pneumoniae* was comparatively resistant.

In vivo and *In vitro* activities of the tested plants are required for confirmation of its different un-exploited activities and dose determination. Bio-chemical analysis is also proposed for the isolation of active compounds for future drug development.

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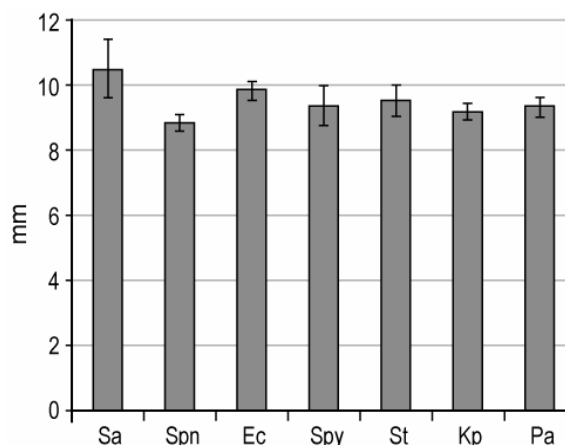


Fig. 4. Antibacterial activity of *Ferula costata* by Disc Diffusion Method.

Sa; *Staphylococcus aureus*, Spn; *Streptococcus pneumoniae*, Ec; *Escherichia coli*, Spy; *Streptococcus pyogenes*, St; *Salmonella typhi*, Kp; *Klebsiella pneumoniae*, Pa; *Pseudomonas aeruginosa*.

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