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In vitro evaluation of antibacterial and antifungal activates of *Iphiona aucheri* leaves extracts

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Abstract

The aim of the current project was to evaluate the antibacterial and antifungal activities of *Iphiona aucheri* leaves methanolic extract and its n-hexane, chloroform and aqueous fractions. Fine powder of *Iphiona aucheri* leaves were extracted in 70% methanol and then subjected to sequential fractionation through n-hexane, chloroform and water. All the fractions were assessed for their antimicrobial characteristics using agar well diffusion assay. The results revealed that the applied extracts have significant antimicrobial characteristics at 30mg/ml concentration on gram positive bacteria (*Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes*) and four gram negative bacteria (*Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae, Pseudomonas aeruginosa*) and two fungal strains, *Aspergillus flavus* and *Aspergillus niger*. The *Aspergillus fumigates* was found resistant to the applied samples. The maximum inhibition of bacterial growth caused by methanolic extract and its chloroform and aqueous fractions was found (13.7±0.57), (15.7±1.52) and (9.87±1.46) respectively against *S. aureus* while n-hexane expressed maximum inhibition (5.2±0.57) against *K. pneumonia* growth. The growth of *A. niger* was highly inhibited by chloroform fraction (47.43±1.27%), followed by methanolic extract (42.15±1.46%), aqueous fraction (34.45±0.52%) and n-hexane fraction (21.22±1.15%). The chloroform fraction with MIC values (1.6±0.35 mg/ml) and (160.75 ±0.57 µg/ml) against *S. aureus* and *A. niger* respectively was found most effective. In conclusion, the result of our study indicated that the leaves of *Iphiona aucheri* possess considerable antimicrobial activities and thus will be of great use in developing plant derived antimicrobial and chemotherapeutic agents.

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Introduction

Currently the tendency of development of antibiotic resistant bacteria and fungi is increasing owing to a number of reasons including inappropriate utilization of antibiotics in human and animal health and their extended application as growth promoters in improving the production of livestock and poultry. Rising antibiotic resistance and the scarcity of new antimicrobials has long been acknowledged and is now a global problem (Theuretzbacher and Mouton, 2011; Walsh and Toleman, 2011). To cope with this problem, it is required to explore and develop a novel, effective, accessible and affordable medicines for the treatment of microbial infections particularly in developing countries (Awouafack *et al.*, 2013; Srivastava *et al.*, 2014). A number of factors such as inappropriate and prevalent use of antibiotics in the treatment of diseases, excessive use of antibiotics as a growth enhancer in animal feed lead to the development of antimicrobial-resistant microbial species (Lowy., 2003). This problem in human and animal will persist for a long time as more species are developing resistance to the available antimicrobial medicines (Andersson and Hughes., 2011). To overcome these circumstances, it is urgently required to develop alternative drugs to treat such infectious diseases (Srivastava *et al.*, 2014).

Local information of herbal medicine is a principal source of modern knowledge. Today, thousands of plants species, conventionally used as medicines, are being searched for their antimicrobial characteristics and chemical constituents (Sofowora *et al.*, 2013). Plants possess numerous chemical compounds which may be therapeutically active or inactive. In addition to the carbohydrates, proteins and lipids, plants synthesis an array of secondary metabolites such as alkaloids, glycosides, triterpenoids, flavonoids, tannins and essential oils which exert physiological activities (Kokate *et al.*, 2002). Most of the secondary metabolite synthesized by the plants are extracted in different solvents and their therapeutic characteristics are identified (Gonzalez-Guevara *et al.*, 2004).

Plants generate a large number of secondary metabolites including flavonoids, alkaloids, tannins, glycosides, terpenoids, steroids, saponins and quinines (Das *et al.*, 2010). These active phytochemical are the source of natural plant-derived antimicrobial compounds (Srivastava *et al.*, 2014; Suresh and Nagarajan., 2009). Some natural products are very much significant in the management of bacterial infections (Fernebro., 2011). Pakistan has a number of plants varieties having many valuable bioactive compounds and its people have sufficient knowledge of herbal medicines.

Iphiona aucheri belongs to the family Asteraceae. It is scattered in Pakistan, Oman, North-East Africa, the Arabian Peninsula and Iran (Anderberg., 1985). In Pakistan, its distribution in Khuzdar, Chaghi, Mekran, Lasbela, and Loralai districts has been reported (Kakar *et al.*, 2012). Antibacterial activities of aerial parts methanolic extract of *Iphiona aucheri* against *S. Aureus, E. coli, S. pyogenes* and *K. ponumoniae* have been reported (Kakar *et al.*, 2012). The minerals i.e. Na, K, Ca, Fe and Ni were reported in *Iphiona aucheri* while Al was found absent (Lanjwani *et al.*, 2016). The aim of this project was to explore the antibacterial and antifungal activities of *Iphiona aucheri* leaves methanolic extract and its n-hexane, chloroform and aqueous fractions.

Material and methods

Plant material

The plants of *Iphiona aucheri* were collected in March 2017 from District, Bannu, Pakistan. Its taxonomic recognition was carried out by Prof. Abdur Rehman, Govt. Post Graduate College Bannu, and Khyber Pakhtunkhwa (KP) Pakistan.

Preparation of crude extract

The freshly collected plants were washed with the tap water, separated its leaves, shade dried and was pulverized into a fine powder with the help of pestle and mortar. 500g powder of leaves was put into 70% methanol (1.5 L) and kept at room temperature for 72 hours with frequent agitation and then filtered (Whatman No. 3 filter paper, Whatman Ltd., England). The filtrate was placed at room

temperature to evaporate the liquid content. The resulting gummy methanolic extract of leaves (29.63 g) was stored in falcon tube for further use.

Preparation of fractions

The stored gummy methanolic extract of leaves was subjected to fractionation. 20 g of methanolic crude extract of *Iphiona aucheri* leaves was sequentially extracted with 300 ml n-hexane, chloroform and water using separating funnel to avoid any sort of damages to the filtrate. The respective solvents were evaporated completely at room temperature. The resulting fractions of leaves (n-Hexane 2.73 g, chloroform 5.19 g and water 8.89 g) were stored for further designed assays.

Samples preparation

The crude methanolic extract of *Iphiona aucheri* leaves and its n-hexane, chloroform and aqueous fractions were dissolved in DMSO (30mg/ml) to prepare the stock solutions which were further diluted to the desired working solutions. Similarly the solutions of Levaquin (levofloxacin; positive control for bacteria) and terbinafine (positive control for fungi) were prepared. The DMSO was used as a negative control.

Test microorganism

Streptococcus pneumoniae, Streptococcus pyogenes, Staphylococcus aureus (Gram positive bacteria) *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium* (Gram negative bacteria) and *Aspergillus fumigates*, *Aspergillus flavus* and *Aspergillus niger* (fungi) were used in the current experiment.

Preparation of 0.5 McFarland standard

The requisite standard was prepared by adding together 0.5 ml. of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2 \cdot 2H_2O$) with 99.5 ml. of 0.18 M H_2SO_4 (1% w/v) whilst stirring continuously (Andrews, 2004).

Preparation of inoculums

The 24 hours old culture of the selected bacterial strains in nutrient broth (MERCK) was mixed with physiological saline (0.9% NaCl w/v.) and its turbidity was adjusted with standard turbidity of McFarland 0.5 $BaSO_4$ (10^6 CFU) by the addition of further sterile physiological saline. These inoculums were used for seeding the nutrient agar.

Antibacterial assay

The antibacterial activities of crude methanolic extract and its hexane, chloroform and aqueous fractions were determined by using agar well diffusion method (Bagamboula *et al.*, 2003). 2 g nutrient agar media and 0.8 nutrient broth media was dissolved in 100 ml water, autoclaved at 121C for 15 minutes and poured in autoclaved petri dishes up to 4cm depth. Following the solidification of media, the bacterial strains were swabbed on petri dishes by using aseptic aluminum borer in the laminar flow cabinet. Aseptic tips were used to cut five wells in the medium (growth medium) layer of each petri dish. An equal volume of Levaquin (positive control), DMSO (negative control) tested sample with different concentrations were added into separate wells in the petri dishes and incubated at 37°C for 24 hours. Following the incubation, the zone of inhibitions was measured in millimeters and recorded.

Antifungal assay

To investigate the antifungal characteristics of the crude methanolic crude extract of *Iphiona aucheri* leaves, the standard protocol of Duraipandiyan and Lgnacimuthu (Duraipandiyan and Ignacimuthu., 2009) were used. 6.5 gm Sabouraud Dextrose Agar (SDA) media was dissolved in 100ml water and autoclaved at 121 C° for 20 minutes. In laminar flow, 4ml media was poured in each autoclaved test tube marked up to 10cm and then added 55 µl working solution of terbinafine (final concentration 200 µg/ml; positive control), samples (final concentration 400 µg/ml; experimental) and DMSO (negative control) to non-solidified SDA. All the test tubes were arranged in the laminar flow in slanting position for the solidification of the media in the test tubes at the room temperature. Following the solidification, each tube was inoculated with a piece of inoculums approximately 4 mm in diameter from the 7days old

culture. Packed all the test tubes air tightly and placed them for 1 week in the incubator at 28°C. After incubation, their growth was measured, compared with the negative control and calculated the percentage inhibition with reference to the negative control (Umadevi *et al.*, 2003).

% Mycelia inhibition Gn-Gt/Gn ×100

Where Gn = Mycelial growth in normal; Gt = Mycelial growth in test

MIC determination by agar well diffusion method

The samples which revealed the antibacterial and antifungal activities were evaluated for their minimum inhibitory concentration (MIC) using agar well diffusion method (Owuama., 2017). Bacterial/ fungal suspension (1 ml) was mixed with 20 ml growth media, compared with 0.5 McFarland standards, poured into petri dishes/test tubes, allowed to solidify and made wells in agar layer of petri dishes. Two fold serial dilutions of 30mg/ml were prepared in DMSO (w/v) and introduced in the wells and approximately 10-12 fungal spores put on the slanted surface in the test tubes.

The petri dishes were incubated at 37°C for 24 hours (bacteria) and the test tubes (fungal) were placed for 7 days in the incubator at 28°C. Plates/test tubes were arranged in triplicate.

Statistical analysis

Microsoft Excel was to calculate the mean and standard deviation.

Results and discussion

Antibacterial and antifungal activities of crude methanol extracts (CME) of *Iphiona aucheri* and its n-hexane, chloroform and aqueous fractions were explored against *Streptococcus pneumoniae, Streptococcus pyogenes, Staphylococcus aureus* (Gram positive bacteria) *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium* (Gram negative bacteria) and *Aspergillus fumigates*, *Aspergillus flavus* and *Aspergillus niger* (fungi).

Table 1. Antibacterial activities of crude methanolic extract (CME) of *Iphiona aucheri* leaves and its n-hexane, chloroform and aqueous fractions.

Samples	Inhibition zones (mm)						
	Spn	Spy	Sa	Kp	Ec	Pa	St
Crude methanolic extract (CME)	11.67±1.52	12.3±1.52	13.7±0.57	8.3±0.57	13.3±1.2	12.7±0.57	7.3±1.16
n-hexane fraction	6.42±0.52	7.9±1.62	7.45±1.52	5.2±0.57	5.7±1.57	7.25±1.57	4.7±1.52
Chloroform fraction	13.67±1.57	14.8±1.52	15.7±1.52	9.6±1.52	14.7±1.2	14.0±1.73	8.6±1.15
Aqueous fraction	8.57±0.52	7.97±1.57	9.87±1.46	4.7±1.27	7.7±1.52	8.0±0.57	4.7±0.57
Levaquin®	18.75±0.52	19.83±1.57	20.8±0.50	17.7±0.57	18.25±0.75	22.4±0.57	13.3±0.52

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

The results of antibacterial activities of crude extract and fractions of *Iphiona aucheri,* Levaquin® and DMSO are articulated in Table 1.

The results indicated that all the samples were active against all the tested bacterial strains to a different extent. The antibiotic, Levaquin® (levofloxacin) was found active against tested bacterial strains while DMSO did not show any activity. The chloroform fraction expressed maximum antibacterial activities followed by the crude methanolic extract, aqueous fraction and n-hexane fraction. Among the tested samples, the chloroform fraction expressed highest antibacterial activities. Similar results were reported during the investigation of antibacterial activities of *Vernonia ambigua*, *Vernonia blumeoides* and *Vernonia oocephala* (Aliyu *et al.*, 2011) and *Aspilia latissima* (Souza *et al.*, 2015). The effectiveness of

chloroform fraction in descending order was *Staphlococcus aureus* (15.7±1.52), *Streptococcus pyogenes* (14.8±1.52), *Escherichia coli* (14.7±1.2), *Streptococcus pneumonia* (13.67±1.57), *Klebsiella pneumonia* (9.6±1.52), *Salmonella typhi* (8.6±1.15).

The n-hexane fraction exhibited the lowest antibacterial activities. Harmonious effects were determined during the antibacterial study of *Enantia chlorantha* Oliver (Ebelle Etame *et al*., 2018). The crude methanolic extract and aqueous fraction exhibited significant antibacterial activities. The methanolic extract of aerial parts of *Iphiona aucheri* has also indicated antibacterial activities against *Staphlococcus aureus, Streptococcus pyogenes, Escherichia coli, Streptococcus pneumonia, Klebsiella pneumonia* and *Salmonella typhi* (Kakar *et al*., 2012). In the previous study, the methanolic and chloroform extracts also exhibited antibacterial activities against several bacterial strains (Ahameethunisa and Hopper., 2010).

Table 2. MIC of methanolic extract (CME) of *Iphiona aucheri* leaves and its n-hexane, chloroform and aqueous fractions.

Samples	Minimum inhibitory concentration (MIC) values (mg/ml)						
	Spn	Spy	Sa	Kp	Ec	Pa	St
Crude methanolic extract (CME)	2.6 ±0.25	2.8±0.52	2.3±0.35	5.6±0.57	2.5±0.25	3.1 ±0.15	2.9±1.1
n-hexane fraction	4.2 ±0.45	4.3±0.25	4.6±0.15	6.2±0.25	5.3±0.45	4.8±0.0	3.6±0.25
Chloroform fraction	1.8±0.35	2.1±0.57	1.6±0.35	4.6±0.52	2.3±0.15	2.7±0.0	2.7±0.52
Aqueous fraction	3.1 ±1.15	4.4±1.2	2.5±1.15	5.7±0.45	3.6±0.57	4.6±0.0	3.7±0.45

In the current study, bioassays were employed to characterize the plant fractions. A quantitative method, MIC is the lowest concentration of antimicrobial agent which is capable to inhibit measurable microbial growth. According to Lambert and Pearson (Singh *et al*., 2001), in biological assays, it is a standard measure documented for the susceptibility of organisms to inhibitors. The MIC of crude extracts and its fractions are illustrated in Table 2. The lowest MIC value of chloroform fraction was found (1.6±0.35) against *Staphlococcus aureus* while the n-hexane fraction against indicate the highest MIC value (3.6±0.25) against *Salmonella typhi*. Congruent MIC values were reported for the methanolic extract of aerial parts of *Iphiona aucheri* (Kakar *et al*., 2012).

Table 3. Antifungal activities of methanolic extract (CME) of *Iphiona aucheri* leaves and its n-hexane, chloroform and aqueous fractions.

Samples	Percentage inhibition of fungal growth	
	A. flavus	*A. niger*
Crude methanolic extract (CME)	32.4±0.52	42.15±1.46
n-hexane fraction	17.35±1.27	21.22±1.15
Chloroform fraction	36.18±0.57	47.43±1.27
Aqueous fraction	33.38±1.15	34.45±0.52
Terbinafine	38.27±1.52	37.35±0.57

A; *Aspergillus*.

The results of antifungal characteristics of crude extract and fractions of *Iphiona aucheri,* terbinafine and DMSO are presented in Table 3. The crude and fraction were active to various extents against *A. niger* and *A. flavus* and did not any activity against *A. fumigates*. Terbinafine was active against all tested fungal strains. The highest inhibition of fungal growth was caused by chloroform fraction (47.43±1.27%) followed by crude methanolic extract (42.15±1.46%). The n-hexane fraction showed lowest (17.35±1.27%) antifungal activities against *A. flavus* (Omezzine *et al*., 2011).

The MIC values for antifungal activities of crude and fractions were determined and articulated in Table 4. The chloroform fraction has the lowest MIC value (160.75 ±0.57µg/ml) while the n-hexane fraction has the highest (270.25 ±0.25µg/ml) one which indicated that chloroform fraction is more effective. Synergistic results have been reported in previous studies (Habbu *et al.*, 2009).

Table 4. The MIC of methanolic extract (CME) of *Iphiona aucheri* leaves and its n-hexane, chloroform and aqueous fractions.

Samples	Minimum inhibitory concentration (MIC) values (µg/ml)	
	A. fumigates	*A. niger*
Crude methanolic extract (CME)	270.25 ±0.25	190.15±0.52
n-hexane fraction	540.25 ±0.57	49.55±0.45
Chloroform fraction	210.57 ±0.25	160.75 ±0.57
Aqueous fraction	310.45 ±0.35	230.25 ±0.75

Antimicrobial activities of *Iphiona* species have not been explored yet. However, the previous reports show that it has. Moreover, a number of substances including non-toxic pyrrolizidine alkaloid and two diterpene glycosides, atractyloside and carboxyatractyloside have been isolated from the aerial parts of *Iphiona aucheri* in which atractyloside and carboxyatractyloside were recognized as the major toxic compounds of the plant to various organisms (Stewart *et al.*, 2000). In the current project, it established to be practically efficient against all the tested bacteria.

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