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IN VITRO BACTERICIDAL AND BACTERIOSTATIC POTENTIAL OF INGRIDIENTS OF TRADITIONAL MEDICINE OBTAINED FROM KACHA AREA (RIVER INDUS) DISTRICT D.I.KHAN, KPK, AGAINST HUMAN BACTERIAL PATHOGENS

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Abstract

The aim of this study was to analyze and evaluate antimicrobial potential of medicinal plants obtained from kacha area of river indus, that are used as ingredients of traditional medicine for treatment of multiple infectious diseases. The antimicrobial activities of methanol and aqueous extracts of 5 medicinal plants of a traditional medicine were evaluated against 6 human gram positive (*Staphylococcus aureus, Micrococcos luteus*) and gram negative (*Escherichia coli, Pseudomonas aeruginosa, Enterobacter, Klebsiella pneumoniae*) pathogens. The disc diffusion and broth macro dilution assay was used to determine the zone of inhibitions and the minimum inhibitory concentration respectively. The ciprofloxacin and streptomycin were used as standard agents. Both aqueous and methanol fractions of all 5 tested plants exhibited antimicrobial activity against one or more species of microorganisms. The most active extract found was *Azadirachta indica* leaves which represented widest zone of inhibition of 16(±0.05) mm and minimum inhibitory concentration 0.19mg/ml against *Klebsiella pneumoniae. Calotropis procera* leaves was found least active representing lowest Zones of inhibition 3.13(±0.05) mm and highest minimum inhibitory concentration value (20mg/ml) against test microorganisms. Over all methanol fractions of medicinal plants represented stronger biological activity against test microorganisms than aqueous extracts. A good majority of extracts were bactericidal. These results afford the ground information for potential use of crude extracts with high MIC and MBC values. Moreover a synergistic effect is expected when used in combination. For this further attempt are in progress to investigate antimicrobial potential of combination medicine.

Introduction

Inappropriate use of antibiotics has led to an alarming increase (Hart & Karriuri, 1998) in multiple drugs resistance (MDRS) thus necessitating the need to search for new biologically active molecules (Sieradski *et al*., 1999) from different sources (microorganism, animals) including plants (Tomoko *et al*., 2002). Today the researchers believe that "green medicine" irrespective of synthetic drugs, are not only free of side effects but have and have excellent therapeutic efficacy (Iwu *et al*., 1999).

Use of plants as drugs and remedies for multiple human diseases is centuries old (Farnsworth & Loub, 1983; Gulfraz *et al.*, 2011) and even today sciences of traditional medicinal plants is practiced successfully worldwide (Qin & Xu, 1998) and are relied upon by 80% of the world's population, as they represent a vast untapped source for medicines.(Iwu *et al*., 1999). There are more than 35,000 plant species being used in various human cultures around the world for medicinal purpose (Srinivasan *et al.,* 2001), While more than 2000 species are reported with therapeutic value and are being used through generations in various traditional health care systems (Gajera *et al*., 2005; Arora, 1998). As a significant majority of world's population believe in folk medicine, the present study was designed to determine antimicrobial spectrum of traditional medicine. Five representative plants used as ingredients of folk medicine were evaluated for antimicrobial activity.

Materials and Methods

Study area: Plant species were collected from Kacha area (River Indus delta) district D.I.Khan, Khyber Pakhtoon Khawa, Pakistan. The district D.I.Khan lies in the South Zone of Khyber Pakhtoon Khawa between the latitudes 31.49°N and the longitudes70.55°E, at the elevation of 173m, 568'. The Indus River is a major river which flows through Pakistan. The total length of the river is 3,180 kilometers (1,976 miles) and is Pakistan's longest river. The river has a total drainage area exceeding 1,165,000 square kilometers (450,000 square miles). The river's estimated annual flow stands at around 207 cubic kilometers, making it the twenty-first largest river in the world in terms of annual flow. The Indus River Delta consists of clay and other fertile soils, and is very swampy. The delta receives between 10 and 20 inches of rainfall in a normal year.

Plant Material: Collection was based on information given by local inhabitants during ethno botanical surveys. Identification of plant species was performed at the department of Pharmacognosy, Faculty of Pharmacy Gomal University D.I.Khan, Khyber Pakhtoon Khawa, Pakistan. The identified species were *Azadirachta indica* (Meliaceae), *Cuseuta reflexa* (*Meliaceae)*, *Rhazya Stricta* (Apocynaceae), *Calotropis procera* (Apocynaceae) and *Melia azedarach* (Meliaceae) (Table 1).

Preparation of plant extracts: Whole plant material (excluding the root and fruit portion) collected in the month of August-September 2009 at early morning hours was subjected to sun shade drying(15-20 days) followed by powdering using Wiley Mill (mesh size 300). About 200 g of dry powdered leaves were extracted with 300ml of 95% ethanol using rotary shaker (190-200 rpm) overnight, filtering it with filter paper and concentrating it to one-fifth of the volume. Crude aqueous extract was prepared by subjecting plant material (10 g) to slow heat for 6 hours and filtered through filter paper and concentrated to one-fifth of the total volume (Vlietinck & Vanden Berghe, 1991)

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Table 1. Plants material and traditional claims.

Botanical name	Family	Common name	Traditional claim	Part used
Azadirachta indica	Meliaceae	Indian Lilac / Neem	Antimicrobial	Whole Plant
Melia azedarach	Meliaceae	Bakain / Beed tree	Antimicrobial	Leaves/Stem
Calotropis procera	Apocynaceae	Milkweed / Aak	Antimicrobial	Milk/ Leaves
Cuseuta reflexa	Convolulacae	Dodder / Amerbel	Antimicrobial	Whole Plant
Rhazya Stricta	Apocynaceae	Daryai booti / Veena	Antimicrobial	Leaves/Stem

Microorganisms: Six bacterial species viz*., Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* (clinical strain/PIMS), *Enterobacter* (clinical strain/PIMS), *Staphylococcus areus* (MRSA, clinical strain/PIMS), *Micrococcus luteus* (clinical strain/PIMS) were used in antimicrobial assay. Strains were obtained from microbiology research lab (MRL), Microbiology department, Quaid-e-Azam University, Islamabad, Pakistan, where these were identified and characterized. These strains were maintained on agar slants at 4°C in Gomal Center of Biochemistry and Biotechnology (GCBB) for antimicrobial tests. The microorganisms were incubated overnight at 37°C in Mueller-Hinton Broth (Oxoid) at pH 7.4. The reference antibiotics used were ciprofloxacin (10µg) and streptomycin (10µg) (Oxoid) (Table 1).

Inoculum preparation: A loopful of isolated colonies was inoculated into 4 ml peptone water and incubated at 37°C for 4 h. The turbidity of actively growing bacterial suspension was adjusted to match the turbidity standard of 0.5 McFarland units prepared by mixing 0.5 ml of 1.75% (w/v) Barium chloride dihydrate with 99.5 ml 1% (v/v) Sulphuric acid The concentration of suspension was standardized by adjusting optical density to 0.1 at 600nm wavelength (Shimadzu UV 1700) (TereSchuk *et al.*, 1997) This turbidity is equivalent to approximately 1–2 10^8 colony-forming units per milliliter (cfu/ml). This 24 h grown suspension was used for further testing.

Antibacterial activity

Disc diffusion method: The antimicrobial test was performed by the disc diffusion method as described by Bauer *et al.,* (1966) modified by Vlietinck & Vanden Berghe, (1991) using a cell suspension of about 1.5×106 CFU/ml obtained following a 0.5 McFarland turbidity standard. The concentration of the suspension was standardized by adjusting the optical density to 0.1 at 600nm wavelength (SHIMADZU UV-1700 spectrophotometer) (Tereschuck *et al*., 1997). Petri dishes were filled with Agar Mueller Hinton agar and inoculated with the test microorganism. Filter paper discs (6mm diameter) were prepared at the concentration of 50mg/disc for crude extracts.

Macrobroth dilution method: The minimum inhibitory concentration (MIC), considered as the lowest concentration of the sample, that inhibits the visible growth of a microbe, was determined by the macrobroth dilution method (Carbonnelle *et al*., 1987), in Muller Hinton broth supplemented with 10% glucose and 0.5% phenol red. For susceptibility testing, in a first step Mueller Hinton broth (1ml) was distributed from the first to the twelfth test tubes. Dry extracts and pure compounds were initially dissolved in DMSO (l ml) and subsequently in Mueller Hinton broth, to reach a final concentration of 20mg/ml. These solutions (1 ml) were added to the first test tube. Successive dilutions were done by transferring the mixture/solution (1 ml) from the first to the eleventh tube. An aliquot (l ml) was discarded from the eleventh tube. The twelfth tube served as growth control as no sample (extract, pure compounds, or reference antibiotics) was added. A microbial suspension (1 ml, 10^5 colony forming units), obtained from an overnight growth at 37°C was added to each test tube. The final concentration of the extracts adopted to evaluate the antimicrobial activity ranged from 20 mg/ml to 0.095mg/ml. Test tubes were incubated aerobically at 37°C for 24 h before being read. The MIC was considered as the lowest concentration of the sample that prevented visible growth or changed in color from red to yellow due to the formation of acidic metabolites corresponding to microbial growth.

Minimum inhibitory concentration (MBC): Minimum Inhibitory Concentration (MBC) of the selected plant parts was measured by the viable cell count method (Toda *et al*., 1989), and the results were expressed as number of viable cells as a percentage of the control.

Results and Discussions

A total of 20 organic and aqueous extracts from 5 different plants species were investigated. The antimicrobial activities (zones of inhibition mm) of standard drugs are listed (Table 2).The disc diffusion method was employed for determination of zone of inhibition between the edge of the filter paper and the edge of the inhibition area. The results of the *in vitro* antibacterial activity brought to light interesting facts. Organic leaf extracts of *Azadirachta indica* were found highly active with widest zones of inhibition range 6.02(±0.1) to 16(±0.05) mm, while *Calotropis procera* (white exudates) was found inactive against test strains (Table 3, 4). Almost all crude extracts (aqueous and organic) were found active against gram negative than gram positive bacteria. The MIC and MBC values are listed in Table 5 and Table 6. It was observed that plants differ significantly in their activities against tested microorganisms; most of the plants were found active against clinical isolate of *Enterobacter*, followed by *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeroginosa, Micrococcus luteus* and *Staphylococcus aureus.*

Table 2. Zone of inhibitions of reference antibiotic standards.

Antibiotic	Microorganism (mm)					
	Ec	Kp	Ent	Ps	Ml	Sta
Ciprofloxacin	14	16	16	15	5	6
Streptomycin	15	14	13	12	5	7

Ec = E.Coli, Kp = Klebsiella pneumoniae, Ent = Enterobacter, Ps = Pseudomonas aeroginosa, Ml = Micrococcus luteus, Sta = Staphylococcus areus (methicilline resistant)

Table 3. Zones of inhibitions of aqueous fractions.

Plant	Microorganism (mm)					
	EC	KP	Ent	PS	ML	Sta
Azadirachta indica (Stem)	8.06(±0.05)	na	7.09(±0.5)	na	na	na
Azadirachta indica (Leaves)	na	na	6.02(±0.1)	na	na	na
Melia azedarach (Stem)	na	9.03(±0.5)	na	na	na	na
Melia azedarach (Leaves)	8.02(±0.1)	na	na	na	na	na
Calotropis procera (Stem)	na	na	11.03(±0.05)	na	na	na
Calotropis procera (Leaves)	na	na	3.13(±0.05)	na	na	na
Calotropis procera (White exudate)	na	na	na	na	na	na
Cuseuta reflexa (Stem)	12.09(±0.1)	4.06(±0.5	na	na	na	na
Rhazya stricta (Stem)	na	na	6.03(±0.05)	na	na	na
Rhazya stricta (Leaves)	na	na	na	na	na	na

Ec = E.Coli, Kp = Klebsiella pneumoniae, Ent = Enterobacter, Ps = Pseudomonas aeroginosa, Ml = Micrococcus luteus, Sta = Staphylococcus areus (methicilline resistant), na = Not active

Table 4. Zones of inhibitions of organic fractions.

Plant	Microorganism (mm)					
	EC	KP	Ent	PS	ML	Sta
Azadirachta indica (Stem)	na	na	6.99(±0.1)	na	na	na
Azadirachta indica (Leaves)	14.03(±0.5)	16.0(±0.0)	14.09(±0.1)	9.06(±0.5)	11.0(±0.05)	9.0(±0.05)
Melia azedarach (Stem)	na	na	na	na	na	Na
Melia azedarach (Leaves)	na	na	na	na	na	9.06(±0.05)
Calotropis procera (Stem)	na	na	na	na	na	na
Calotropis procera (Leaves)	na	na	na	12.0(±0.1)	16(±0.5)	na
Calotropis procera (White exudate)	na	na	na	na	na	na
Cuseuta reflexa (Stem)	6.03(±0.05)	na	na	na	na	na
Rhazya stricta (Stem)	6.0(±0.5)	7.0(±0.5)	7.0(±0.05)	na	na	na
Rhazya stricta (Leaves)	6.1(±0.5)	5.06(±0.05)	na	na	na	na

Ec = E.Coli, Kp = Klebsiella pneumoniae, Ent = Enterobacter, Ps = Pseudomonas aeroginosa, Ml = Micrococcus luteus, Sta = Staphylococcus areus (methicilline resistant), na = Not active

Table 5. Minimum inhibitory concentration of medicinal plants.

Plant	Minimum inhibitory concentration (mg/ml)											
	Aqueous fraction						Organic fraction					
	EC	Kp	Ent	Ps	Ml	Sta	Ec	Kp	Ent	Ps	Ml	Sta
Azadirachta indica (Stem)	0.78	-	1.25	-	-	-	-	-	1.25	-	-	-
Azadirachta indica (Leaves)	-	-	1.25	-	-	-	0.19	0.19	0.19	0.78	0.19	0.156
Melia azedarach (Stem)	-	0.19	-	-	-	-	-		-	-	-	-
Melia azedarach (Leaves)	0.78	-	-	-	-	-	-	-	-	-	-	0.78
Calotropis procera (Stem)	-	-	0.19	-	-	-	-	-	-	-	-	-
Calotropis procera (Leaves)	-	-	20	-	-	-	-	-	-	0.312	0.78	-
Calotropis procera (White exudate)	-	-	-	-	-	-	-	-	-	-	-	-
Cuseuta reflexa (Stem)	2.5	>20	-	-	-	-	-	-	-	-	-	-
Rhazya stricta (Stem)	-	-	>20	-	-	-	>20	>20	-	-	-	-
Rhazya stricta (Leaves)	-	-	-	-	-	-	>20	>20	-	-	-	-

Ec = E.Coli, Kp = Klebsiella pneumoniae, Ent = Enterobacter, Ps = Pseudomonas aeroginosa, Ml = Micrococcus luteus, Sta = Staphylococcus areus (methicilline resistant), - Not determined

Table 6. Minimum bactericidal concentration (MBC) of medicinal plants.

Plant	Minimum bactericidal concentration (mg/ml)											
	Aqueous fraction						Organic fraction					
	EC	Kp	Ent	Ps	Ml	Sta	Ec	Kp	Ent	Ps	Ml	Sta
Azadirachta indica (Stem)	0.625	-	1.25	-	-	-	-	-	-	-	-	-
Azadirachta indica (Leaves)	-	-	5	-	-	-	0.156	0.156	0.39	0.78	0.19	0.156
Melia azedarach (Stem)	-	0.312	-	-	-	-	-		-	-	-	-
Melia azedarach (Leaves)	-	-	-	-	-	-	-	-	-	-	-	1.25
Calotropis procera (Stem)	-	-	0.156	-	-	-	-	-	-	-	-	-
Calotropis procera (Leaves)	-	-	20	-	-	-	-	-	-	1.25	0.78	-
Cuseuta reflexa (Stem)	2.5	-	-	-	-	-	-	-	-	-	-	-

Ec = E.Coli, Kp = Klebsiella pneumoniae, Ent = Enterobacter, Ps = Pseudomonas aeroginosa, Ml = Micrococcus luteus, Sta = Staphylococcus areus (methicilline resistant), - = Not determined

Almost all plants used in the traditional medicine exhibited moderate antimicrobial activities when tested individually as reported earlier (Wafaa *et al.,* 2007, Takazawa 1982, Parrotta, 2001) except *Rhyzea stricta* and *cuscuta reflexa***.** Little is known regarding antimicrobial activities of *Rhyzea stricta* and *Cuscuta reflexa* (Pal *et al.,* 2006). However present study confirms their antimicrobial (bacteriostatic) potential. Although their antibacterial activities were found less (Paul *et al*., 2006, Rios & Rico, 2005) compared to other plant extracts. Most of the crude extracts exhibited bactericidal activities than bacteriostatic. The bactericidal activities were recorded as three to four times higher than static activities (Gnanamani *et al.,* 2003, Kharea *et al.,* 2005) which simply highlights efftiveness of traditional medicine. It is worthy to note that MIC and MBC values of assayed plant extracts are comparatively higher as compared to reported elsewhere (Kareem *et al.,* 2008, Subapriya & Nagini, 2005). It is suggested that individual plants may be contributing to the effectiveness of traditional medicine. Moreover the dose used for treatment of infections reported was high (2 gram thrice a day) which obviates the study findings. Organic fractions were found highly active than aqueous extracts, this proves the fact that majority of active constituents are soluble in organic solvents**.** Eloff, (1998) Spectrum of activity of plants tested was limited to gram negative strains than gram positive strains. This finding reflects limited use of medicinal plants against mixed infections (Sieradski *et al.,* 1999).

Conclusion

Almost all crude extracts are already known for less or high antimicrobial activities but during present study only a few crude extracts proved good antimicrobial activities when tested individually against selected microorganisms. Rest of the extracts were although found biologically active (except one) but exhibited high bactericidal and bacteristatic activities. Therefore it is concluded that crude extracts possess good potential for antimicrobial activity. Further investigations are in progress to compare antimicrobial activities of individually tested extracts with folk medicine.

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