Pak. J. Bot., 43(1): 531-538, 2011.

IN VITRO SCREENING OF METHANOL PLANT EXTRACTS FOR THEIR ANTIBACTERIAL ACTIVITY

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Abstract

The purpose of this study was to observe the antibacterial activity of aqueous methanolic extracts of 10 plants against 2-gram negative bacteria (*Pasteurella multocida, Escherichia coli)* and 3-gram positive bacteria (*Bacillus cereus*, *Staphylococcus aureus*, *Corynebacterium bovis*) by using disc diffusion method. The minimum inhibitory concentration (MIC) was determined by agar well diffusion method and agar dilution method. All the bacteria were susceptible to different plant extracts. *Lawsonia inermis*, *Embellia ribes* and *Santalum album* showed antibacterial activity against all the tested bacteria. The extract of *Santalum album* showed maximum antibacterial activity of the 10 plant extracts used. *Bacillus cereus* and *Pasteurella multocida* were the most sensitive bacteria against most of the plant extracts. It is clear from the results of the present studies that the plant extracts have great potential as antimicrobial compounds against bacteria. However, there is a need of further research to isolate the active ingredients for further pharmacological evaluation.

Introduction

The microorganisms have developed resistance to many antibiotics because of indiscriminate use of antimicrobial drugs that create a big problem in the treatment of infectious diseases (Davis, 1994). With the increase in resistance of many microorganisms to the currently used antimicrobials and the high cost of production of synthetic compounds; in addition to many side effects; there is a need to look for the alternatives. Plants have provided a good source of antiinfective agents; emetine, quinine, berberine, tannins, terpenoids, alkaloids and flavonoids remain highly effective instruments in the fight against microbial infections (Marjorie, 1999).

Staphylococcus aureus, *Corynebacterium bovis* (Mastitis in cattle), *Pasteurella multocida* (haemorrhagic septicemia in cattle) and *Escherechia coli* (Enteric problems like diarrhoea and dysentery in cattle) are the common pathogens of our livestock (Hirsh *et al*., 2004). In developing countries like Pakistan, traditional healers claim that their medicine is cheaper and more effective than modern medicine. Therefore, the low-income people such as farmers, people of small isolated villages and native communities use ethanoveterinary medicine for the treatment of common infections (Rojas *et al*., 2006; Hammer *et al*., 1999). Furthermore, most of the live stock farmers in Pakistan have not an access to the Veterinarians. Therefore, the ethanoveterinary system is the only alternative to the Western veterinary therapy. Ethanoveterinary medicine knowledge has been transmitted orally from generation to generation (McCorkle, 1986; McCorkle & Mathias, 1989; McCorkle *et al*., 1996). This knowledge is being discouraged due to lack of scientific validation. In Pakistan, a lot of work (Akhtar & Riffat, 1987; Akthar, 1988; Akhtar & Aslam, 1997; Iqbal *et al*., 2004; Iqbal *et al*., 2005; Hayat *et al*., 1996) has been

done for the scientific validation of plants having antiparasitic activities but still no work has been done to screen out the antibacterial activities of the plants used in ethanoveterinary practices. Therefore, in the present studies ten medicinal plants have been selected on the basis of their common traditional therapeutic uses in Pakistan for the screening of their antibacterial potential (Table 1).

Material and Methods

Plant material: *Carum copticum, Mallotus philippensis, Citrullus colocynthis, Calotropis procera, Embelli ribes* and *Ricinus communis* were collected from the rural areas of district Faisalabad, Attock and Nothren areas of NWFP. *Lawsonia inermis, Amomum subulatum, Operculina turpethum* and *Santalum album* were procured from the local market. The plant species were identified by the department of Botany, University of Agriculture, Faisalabad, Pakistan. All the 10 plant species used in the present study are presented in Table 2.

Extract preparation: The plants were shade-dried at room temperature and then powdered by using electric grinder. This powdered material was soaked in 70 % hydromethanol (3:7) at 25ºC for three days. The mixture was shaken at 200 revolutions per minute (rpm) for 2 hours followed by centrifugation at 4000 rpm for 20 minutes at 4ºC. The supernatant was filtered through Whatman No.4 filter paper and then the methanol was evaporated using rotary evaporator and finally the extracts were resuspended in dimethyl sulfoxide (DMSO) to yield 200 mg/ml of the extracts.

Microorganisms: Two gram-negative bacteria *Pasteurella multocida, Escherichia coli* and three gram-positive bacteria *Bacillus cereus, Corynebacterium bovis* and *Staphylococcus aureus* were used in the present studies. The *Escherichia coli* (ATCC 29922) and *Staphylococcus aureus* (ATCC 29923) were procured from department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad,Pakistan. *Pasteurella multocida* was isolated from bone marrow of Hemorrhagic septicemic cattle. *Bacillus cereus* was isolated from soil and *Corynebacterium bovis* was isolated from mastistis milk of buffalo. All these bacteria were identified on the basis of culture, colony, morphological characteristics and also biochemical tests in the laboratory of department of microbiology, University of Agriculture, Faisalabad, Pakistan.

Antibacterial activity: The disc-diffusion assay (Nostro *et al.,* 2000) was used to determine the growth inhibition of bacteria by the plant extracts. Bacteria were maintained at 4°C on nutrient agar (NA) plates. Base plates were prepared by pouring 15 ml Mueller-Hinton (MH) agar (Biolab, UK) into sterile Petri dishes (14 cm diameter) and allowed to set. Molten MH agar held at 48°C was inoculated with a broth culture (10^5 -10^7 bacteria/ml) of the test organism and poured over the base plates forming a homogenous top layer. Fifty microliter (50 *u*l) of plant extract were applied per filter paper disc (Whatman No.3, 6 mm diameter) so that each disc contained 10 mg of material. The discs were air-dried and placed onto the seeded top layer of the agar plates. Each extract was tested in hexaruplicate (6 discs/plate), with a amoxacillin (25 *u*g Oxide UK) and teramycin (25 *u*g Oxide UK) disc as positive controls. Methanol saturated discs (air dried) and DMSO were used as negative controls. The plates were evaluated after incubation at 37°C for 24 hours. Antibacterial activity was expressed as the zone of inhibition (mm) produced by the plant extract compared with the amoxicillin and teramycin (positive controls). The experiments were repeated five times and the mean values with standard deviation were obtained.

Table 1. Traditional therapeutic uses of the selected plants in various parts of Pakistan.

S. No.	Botanical name	Common name / local name	Plant part used	Therapeutic uses
1.	*Calotropis procera* (Wild) R.Br.	Milk-weed (Aak)	Flower	Used in cold cough, asthma, malaria, cholera, intermittent fever and skin disorder (Ahmad *et al.,* 2004)
2.	*Mallotus philippensis* (Lam)	Rotllera kamela (Kamela)	Seed	As purgative, anthelmintic, colic pain and carminative. (Shradha *et al.,* 2007)
3.	*Citrullus colocynthis* (Shard)	Colocynth (Korthuma)	Fruit	As purgative, intestinal parasite, billery diseases and rheumation. (www.Botanical.com)
4.	*Ricinus communis* Linn.	Caster (Arand)	Fruit	As a Counter irritant, purgative, and also use in jaundice. (Ahmad *et al.,* 2004; Korwar *et al.,* 2006)
5.	*Lawsonia inermis* Linn	Balsam (Mehendi)	Leaves	Used as Cosmetic agent, cathartic and diuretic (Rout *et al.,* 2009)
6.	*Carum copticum* Benth. (*Trachyspermum ammi* L.)	Bishops Weed (Ajwain)	Fruit	Used as appetizers, relieve urticaria, neuralgic pain, diarrhoea and dysentry. (Dashti-Rahmatabadi *et al.,* 2007)
7.	*Amomum subulatum*	Ceylon (Moti Elaichi)	Fruit	Used as a gurgles and gastric disorders. (Daljit & Gurinder, 2007)
8.	*Santalum album* L.	Sandalwood (Sandal surkh)	Harsh Wood	Used in inflammation of bladder, gonorrhoea and cough
9.	*Operculina turpethum* L..	Turpeth (Turbad)	Root	Used as a purgative (Austin, 1982)
10.	*Embellia ribes* Burn.	Ebelia (Boabrang)	Leaves	Anthelmintic and carminative (Phulan & Khullar, 2004)

Table 2. Antibacterial activity of various plants by Disc diffusion test (Mean ± SD).

Botanical name with family	Parts used	Diameter of zone of inhibition (mm)				
		E. coli	*B. cereus*	*S. aureus*	*P. multocida*	*C. bovis*
Santalum album L. (Santalaceae)	Harsh Wood	12 ± 0.05	22 ± 0.02	16 ± 0.01	15 ± 0.28	21 ± 0.0
Lawsonia inermis Linn. (Balsaminaceae)	Leaves	12 ± 0.11	20 ± 0.31	17 ± 0.05	20 ± 0.09	18 ± 0.51
Mallotus philippensis (Lam) (Euphorbiaceae)	Seed	14 ± 0.07	16 ± 0.5	NA	14 ± 0.45	20 ± 0.21
Calotropis procera (Wild) R.Br. (Asclepiadaceae)	Flower	15 ± 0.04	17 ± 0.23	NA	12 ± 0.24	18 ± 0.18
Carum copticum Benth. (Apiaceae)	Fruit	13 ± 0.21	18 ± 0.35	NA	16 ± 0.0	19 ± 0.13
Ricinus communis L. (Euphorbiaceae)	Fruit	NA	17 ± 0.47	NA	NA	20 ± 0.08
Amomum subulatum (Zingiberaceae)	Fruit	NA	19 ± 0.09	NA	16 ± 0.03	NA
Embelia ribes Burn. (Myrsinaceae)	Leaves	13 ± 0.33	16 ± 0.03	15 ± 0.61	18 ± 0.47	14 ± 0.26
Operculina turpethum L. (Convolvulaceae)	Root	12 ± 0.02	NA	14 ± 0.05	15 ± 0.12	NA
Citrullus colocynthis (L.) Shard (Cucurbitaceae)	Fruit	13 ± 0.41	15 ± 0.07	NA	14 ± 0.07	NA
Amoxicillin		18 ± 0.03	21 ± 0.0	20 ± 0.12	21 ± 0.16	22 ± 0.01
Teramycin		20 ± 0.0	22 ± 0.0	21 ± 0.08	24 ± 0.0	23 ± 0.02
Blank (DMSO)		NA	NA	NA	NA	NA
Methanol		NA	NA	NA	NA	NA

NA - No activity, *E. coli - Escherichia coli*, *B. cereus - Bacillus cereus*, *S. aureus - Staphylococcus aureus*, *P. multocida - Pasturella multocida*, *C. bovis - Corynebacterium bovis*

Minimum inhibitory concentration: The minimum inhibitory concentration (MIC) of the extracts was measured by agar dilution method (Nostro *et al.,* 2000) and agar well diffusion method (Basu *et al.,* 2005).

a. Agar dilution method: The plant extracts, which were positive for zone of inhibition in disc diffusion test, were assayed for the determination of MIC. A stock solution of each plant extract was prepared in pure DMSO that was serially diluted two fold in pure DMSO. One ml of each dilution was incorporated in 20 ml of melted Muller Hinton agar at 45-50ºC. Plates containing only 1% (V/V) DMSO and methanol was used as control. The bacterial culture was inoculated on the surface of agar plates in the form of spotting that contained different concentrations of plant extracts. Agar plates were incubated for 24-36 hrs and observed for the presence or absence of visible bacterial growth.

b. Agar well diffusion method: The bacterial suspension was mixed @ of 1 ml/20 ml of Muller-Hinton agar at 48ºC. Wells were prepared in agar containing bacterial culture with the help of well cutter. Two fold dilutions of the stock solution of plant extracts (200 mg/ml) that were prepared in pure DMSO were introduced in wells and plates were incubated at 37ºC for 24 hours. After incubation, the plates were observed for the presence or absence of bacterial growth around wells containing various concentrations of plant extracts.

Results and Discussion

The results of the zone of inhibition determined by disc diffusion test are presented in Table 2. It is evident from the results that all the bacteria were susceptible to the plant extracts. However, only *Lawsonia inermis, Embellia ribes* and *Santalum album* showed antibacterial activity against all the tested bacteria, while the remaining plants showed varying degree of activity against different bacteria. *Mallotus philippensis, Calotropis procera, Carum copticum* and *Ricinus communis* showed no activity against *Staphylococcus aureus* but showed antibacterial activity against all the other tested bacteria. The extract of *Ricinus communis* showed antibacterial activity against *Corynebacterium bovis* and *Bacillus cereus* but no activity against *Escherichia coli, Staphylococcus aureus* and *Pasteurella multocida*. The extract of *Amomum subulatum* was effective only against *Pasteurella multocida* and *Bacillus cereus.* The extracts of *Operculina turpethum* showed no antibacterial activity against *Bacillus cereus* and *Corynebacterium bovis* but showed activity against *Pasteurella multocida, Staphylococcus aureus* and *Escherichia coli. Citrullus colocynthis* showed no effect against *Staphylococcus aureus* and *Corynebacterium bovis* but showed some effect against all other tested bacteria. The MIC of aqueous methanolic extracts of the plants was determined by Agar dilution method and Agar well diffusion method (Tables 3 and 4).

Only a few reports are available regarding the antibacterial activities of plants used in the present studies such as Toshimasa *et al*., (2005) reported the antibacterial activity of *Santalum album* against *Helicobacter pylori*. The antibacterial activity of *Lawsonia inermis* against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* was reported by Habbal *et al*., (2005). Phulan & Khullar (2004) also reported the antibacterial activity of *Embelia ribes*. Usman *et al*., (2003) reported the antibacterial activities of ethanolic extracts of fruits, leaves, stems and roots of *Citrulus colocynthi.* Taylor *et al*., (1996) reported antibacterial and antifungal efficacy of *Mallotus philippensis* (Euphorbiaceae) and *Terminalia alata* (Combretaceae) and observed a significant antibacterial activity. Some of the previous research (Mahato & Chaudary,

2005) has been conducted just to find out the zone of inhibition against microorganisms by plant extracts but the present studies have been conducted to carry out both the zone of inhibition and minimum inhibitory concentration values of effective plant extracts.

The results of the present studies regarding the antibacterial activity of *Mallotus philippensis* are in link with the recent studies conducted by Kumar *et al.*, (2006) in our neighboring country India. Like that of the results of the present studies, the antimicrobial activity of the leaves of *Lawsonia inermis* has been reported against *Staphylococcus aureus, Streptococcus* spp., *Pseudemonas aeruginosa, Candida albicans, Fusarium oxysporum* and *Aspergillus niger* by Muhammad & Muhammad. (2005) in Nigeria.

A number of reports (Taylor *et al.*, 1996; Muhammad & Muhammad, 2005; Toshimasa *et al.*, 2005; Kumar *et al*., 2006) are available regarding the antimicrobial activity of plants in different parts of the world but in Pakistan, such type of studies have been conducted very rarely. However, there is need to conduct further research on the chemical analysis of the used plant extracts to find out the active compounds.

Research into the effects of local medicinal plants is expected to boost the use of these plants in the therapy against disease caused by the test bacterial species and other microorganisms. It is possible that better therapy for many microbial diseases may be found in the bark, leaves etc. of these plants. The preliminary results of this investigation appear to indicate that a number of medicinal plants used in the present studies particularly *Lawsonia inermis, Embellia ribes* and *Santalum album* have high potential of antimicrobial activity.

Table 3. Minimum inhibitory concentrations (MIC) of aqueous methanolic extracts of plants by agar dilution method (mg/ml of agar).

Botanical name	MIC (mg/ml of agar)				
	S. aureus	***P. multocida***	***C. bovis***	***E. coli***	***B. cereus***
Santalum album L.	12.5	3.12	12.5	12.5	6.25
Lawsonia inermis Linn.	6.25	12.5	6.25	6.25	12.5
Mallotus philippensis (Lam)	NA	12.5	12.5	12.5	6.25
Calotropis procera (Wild) R.Br	NA	25	6.25	12.5	25
Carum copticum Benth.	NA	25	12.5	25	12.5
Ricinus communis Linn.	NA	NA	25	NA	25
Amomum subulatum	NA	25	NA	NA	12.5
Embilia ribes Burn.	25	12.5	25	12.5	25
Operculina turpethum L.	25	25	NA	25	NA
Citrullus colocynthis (L) Shard.	NA	12.5	NA	25	25

MIC - Minimum inhibitory concentration, NA - No activity, *S. aureus - Staphylococcus aureus, P. multocida - Pasturella multocida, C. bovis - Corynebacterium bovis, E. coli - Escherichia coli, B. cereus - Bacillus cereus*

Table 4. Minimum inhibitory concentrations (MIC) of aqueous methanolic extracts of plants by agar well diffusion method (mg/ml).

Botanical name	MIC (mg/ml)				
	S. aureus	***P. multocida***	***C. bovis***	***E. coli***	***B. cereus***
Santalum album L.	6.25	6.25	25	25	12.5
Lawsonia inermis Linn.	6.25	12.5	6.25	6.25	12.5
Mallotus philippensis (Lam)	NA	25	25	12.5	6.25
Calotropis procera (Wild) R.Br	NA	25	12.5	25	50
Carum copticum Benth.	NA	50	25	50	25
Ricinus communis Linn.	NA	NA	50	NA	50
Amomum subulatum	NA	50	NA	NA	25
Embilia ribes Burn.	50	25	50	25	25
Operculina turpethum L.	50	50	NA	50	NA
Citrullus colocynthis (L) Shard.	NA	25	NA	25	50

MIC - Minimum inhibitory concentration, NA - No activity, *S. aureus - Staphylococcus aureus, P. multocida - Pasturella multocida, C. bovis - Corynebacterium bovis, E. coli - Escherichia coli, B. cereus - Bacillus cereus*

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(Received for publication 25 July 2009)