Phytochemical, antimicrobial, insecticidal and brine shrimp lethality bioassay of the crude methanolic extract of *Ajuga parviflora* Benth

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Abstract: Methanolic extract of medicinal herb *Ajuga parviflora* Benth. was evaluated for phytochemical screening (the plant extract showed the presence of aromatic compounds, carbohydrates, glycosides, tannins, alkaloids, polyphenols, quinines and dions, aminophenols, steroids/sterols, flavonoids and terpenoids), antimicrobial activities against various strains of bacteria and fungi by using disc diffusion method and insecticidal activities against red flour beetle (*Tribolium castaneum*), wheat weevil (*Sitophilis granaries*) and their larvae. The crude extract showed anti-bacterial activity against all strains with a maximum zone of inhibition of 12mm diameter against *Citrobacter* and *Pseudomonas aurogenosa.* Standard drugs *Ampicillin*, *Gentamicin* and *Amoxicillin* were used in parallel. The crude extract did not show antifungal activity against the tested strains of fungi even at high doses. The crude methanolic extract was also used for insecticidal activity against the two types of insects and their larva. The extract showed no significant mortality in the tested strains. For brine shrimp lethality bioassay different concentrations 10, 100 and 1000ug/ml of the medicinal herb *A. parviflora* methanolic extract were used. After 24 hrs the percent mortality and LD_{50} value was calculated through probit analysis. The LD_{50} value of extract was 321.42µg/ml while that of standard drug *cyclophosphamide* was 16.09ug/ml.

Keywords: *Ajuga parviflora, Sitophilus granarius, Tribolium castaneum,* brine shrimp, phytochemical.

INTRODUCTION

Various systems of medicine i.e. Ayurveda, Unani, Tibbi and local health traditions, are practiced that utilize a large number of plants for the treatment of human diseases. Most of these medicinal plants have been identified and their uses are well documented and described by different authors, but the efficacy of many of these plants are yet to be verified (Jahan *et al.,* 2010). There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases (Mehjabeen *et al.,* 2011). Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against microbial infections.

A.parviflora Benth belongs to genus *Ajuga* and family Labiatae (also called *Lamiaceae* and *Menthaceae*, which is the largest family of order *Lamiales*) is an annual or short lived perennial herb that grows in temperate region, widely distributed in east Afghanistan, Himalaya, Kashmir and Pakistan; it flowers between March and October (Nasir *et al*., 1978; Ali and Nasir, 1990). The genus *Ajuga* is worldwide distributed and has great medicinal importance. Many plants of this genus are used as Astringent, tonic, blood purifier, anti-inflammatory, depurative, diuretic, for fever, bronchitis, pneumonia, typhoid fever, agues, diarrhea, dysentery, colic, gout, palsy, jaundice, amenorrhea (Manjunath, 1948; Chopra *et al*., 1986; His-wen and Hedge, 1994; Manandhar, 2002; Sharma *et al*., 2004a; Islam *et al*., 2006; Singh *et al*., 2006), Cancer (Johnson, 1999) arthritis (Marc *et al*., 2008), wound healing (Khalil *et al*., 2007), as a febrifuge in stomachache, rheumatic fevers, bites from insects, eye trouble, anthelmintic, hypoglycemic agent as well as diseases of the bladder (Manjunath, 1948; Perry & Metzger, 1980).

Keep in view the importance of genus *Ajuga* we decided to evaluate the methanolic extract of *Ajuga parvi flora* for phytochemical, antimicrobial, insecticidal activities and brine shrimp lethality bioassay (cytotoxicity).

MATERIAL AND METHODS

Collection and identification of plant material

The whole plant was collected in the month of June from Dir Lower (Province KPK, Pakistan) and identified by Prof. Dr. Mansoor Ahmad at Department of Pharmacognosy, University of Karachi. A voucher specimen of plant (AP-1506/2008-NR) has been deposited in the herbarium of the department.

Extraction

The plant materials were shade dried in order to avoid enzymatic degradation and fungal growth. The dried material (3.5kg) was cleaned from dust and foreign

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material, chopped and then soaked in methanol (90%) for 15 days with continuous shaking. The solution was filtered and evaporated through rotary evaporator (Büchi, Switzerland) at 40°C under reduced pressure. The process was repeated three times for extraction. After evaporation of methanol the dried extract (800g) was collected by this method. A part of it was used in experiments.

Phytochemical analysis

Phytochemical analysis for the major secondary metabolites of the plant extract was undertaken using standard qualitative methods with little modification (Trease and Evans, 1989: Harborne, 1998).

Antibacterial assay

Eight pure isolates of bacteria, *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi,* S*taphylococcus aureus, Proteus mirabilis, Micro cocus luteus, Citrobacter* were obtained from the research laboratory of PCSIR, Karachi, Pakistan. Fresh inoculums were prepared from stock cultures over nutrient agar (Merck, Germany) and incubating at 37°C for 24 hrs. Broth of each bacterium was prepared from developed (24hrs) culture of bacteria on nutrient agar. Crude extract of plant was dissolved in DMSO and various concent rations were prepared for the assay. The antibacterial potential of the crude extract was evaluated by disc diffusion method (Bauer *et al*., 1966), using above strains of bacteria. Filter paper discs (Glass Microfiber filters, Whatman; 7 mm diameter) with different concent ration of crude methanolic extract i.e. 20,40,60,80,100, 1000 and 2000 µg/disc were placed on the surface of inoculated plates. Standard drugs *Ampicillin, Gentamicin* and *Amoxicillin*, were used as positive control and DMSO soaked discs were used as negative control. Zone of inhibition in mm and the MIC were recorded in triplicates.

Antifungal assay

Nine pathogenic strains of fungi, *Aspergilus parasiticus, Aspergilus niger, Yersinia aldovae, Candida albicans, Aspergilus effuses, Fusarium solani, Macrophomina phaseolina, Saccharomyces cerevisia* and *Trichophyton rubrum* were obtained from the research laboratory of PCSIR, Karachi, Pakistan. Each inoculum was prepared by inoculating the stock culture into freshly prepared nutrient agar (Merck, Germany) and incubating at 26±1°C for 48 hrs. Broth of each fungus was prepared from 48 hrs developed culture on nutrient agar.

Different concentration of crude extract on filter paper disc, i.e. 20, 40, 60, 80, 100, 1000 and 2000 µg/disc using Kirby-Bauer method (Bauer *et al*., 1966) were prepared for the investigation of susceptibility against the above strains. Standard drugs *Itraconazole* (Mass Pharma Pak Pvt., Ltd.) and *Amphoteracin B* (Bristol Meyer Squibb Pakistan) were used as positive control and DMSO soaked filter paper discs were used as negative control. Three replicates were recorded to obtain accurate results.

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation (Jennifer *et al*., 2001).

Insecticidal activity

Two types of insects *Sitophilus granarius*, also known as Wheat weevils, grain weevils or granary weevils and *Tribolium castaneum*, also known as red flour beetle, were used for this study. These two insects and their larvae were collected from the stored grains and wheat flour from nearby market. Solutions of different concentration were prepared in methanol and poured over filter paper in Petri plates, kept for 24 hrs to get dry filter paper with extract.

Ten insects/larvae were put in each Petri dish for observing the movement, repellent activity, paralysis and death of the experimental insects / larvae at different time intervals for 24 hrs. Standard drug *Permethrin* (0.5% w/w) was used as positive control and blank paper as negative control. Three replicates were performed for accuracy.

Brine-shrimp cytotoxicity

The Brine-Shrimp cytotoxicity/Lethality test was performed against *Artemia salina* (brine-shrimp eggs). The eggs of brine shrimp were hatched in artificial sea water (prepared with sodium chloride 38 gm/l distilled water) placed under continuous illumination at 37°C for 24 hrs in a rectangular tray. Stock solution was prepared by dissolving 20 mg of the crude extract in 2 ml of 2% DMSO. With the help of Pasteur pipette 1 ml of sea water and 10 shrimp larvae were added in 3 different vials. Then 5,50 and 500ul from stock solution was transferred to each vial and the final volume was adjusted to 5ml with sea water thus making the final concentration 10,100 and 1000ug/ml respectively. The vials were incubated at 37ºC for 24 hrs. DMSO 2% was used as negative and reference cytotoxic drug Cyclophosphamide (LD_{50}16.09ug/ml) as positive control. After 24 hrs the survived brine shrimps were counted using a magnifying glass. The collected data was analyzed with Finney computer program (Probit analysis) to determine LD_{50}value with 95% confidence interval.

STATISTICAL ANALYSIS

The antimicrobial results in triplicates were calculated and presented as mean±SEM. The percentage lethality was calculated from the mean survival larvae of extract treated vials and control. The LD50 values were obtained through Fenny method of probit analysis.

RESULTS

The phytochemical screening of the plant showed the presence aromatic compounds, carbohydrates, glycosides, tannins, alkaloids, polyphenols, quinines and dions, aminophenols, steroids/sterols, flavonoids and terpenoids (table 1).

The methanolic crude extract of *A. parviflora* showed different zone of inhibition against different bacteria at various doses. The crude extract showed antibacterial activity against *E. coli* even at the minimum dose level. The 20 & 40µg doses gave no results against *S. typhi* and the higher doses inhibited the growth of the bacteria to different extent. Extract showed moderate to significant

Table 1: Qualitative phytochemical analysis of the methanolic extract of *A. parviflora*

Phytochemicals	Test performed	Observation	Results
Alkaloids	Dragendorff's test		+
	Marquis tTst	Green black	+
Aromatic compounds	Aromaticity	Yellow/Orange	+
Polyphenols	Ferric chloride Test	Green yellow	+
	Folin ciocalteu Reagent Test	Blue	+
Glycosides	Liebermann's Reagent Test	Black green	+
Tannins	Lead acetate Test	ppt formed	+
Saponins	Froth Test	Froth not .formed	-
Terpenoids	Liebermann's Reagent Test	Black green	+
Flavonides	Ferric chloride Test	Green yellow	+
Quinones and Diones	Methanolic Potassium hydroxide Test	Green orange	+
Carbohydrates	Molish Test	Black purple	+
Steroids	Salkowski Test	Black green	+
Barbiturates like compounds	Vanillin Reagent Test	Red brown/Black	-
Free salicylic acid	Mc Nally Test	Green	-
Phenothiazine derivatives	Forrest Reagent Test	Red brown	+
	FPF Reagent Test	Orange yellow	+

''Ppt'' precipitation, "+" present, "-'' absent.

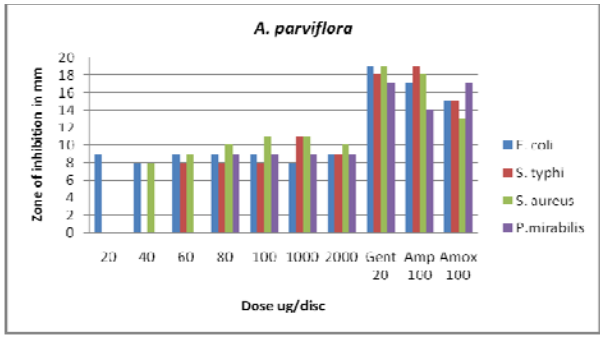

Fig. 1: Zone of inhibition (mm) showing antibacterial activity against *Escherichia coli* (*E. coli*), *Salmonella typhi* (*S. typhi*), *Staphylococcus aureus* (*S. aureus*) and *Proteus mirabilis* (*P. mirabilis*).

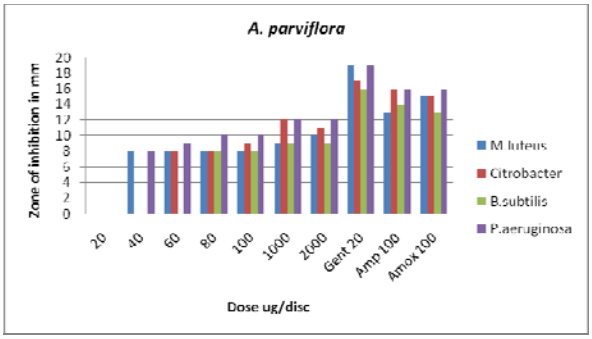

Fig. 2: Zone of inhibition (mm) showing anti-bacterial activity against *Micrococus luteus* (*M. luteus*) *Citrobacter, Bacillus subtilis* (*B. subtilis*) and *Pseudomonas aeruginosa* (*P. aeruginosa*).

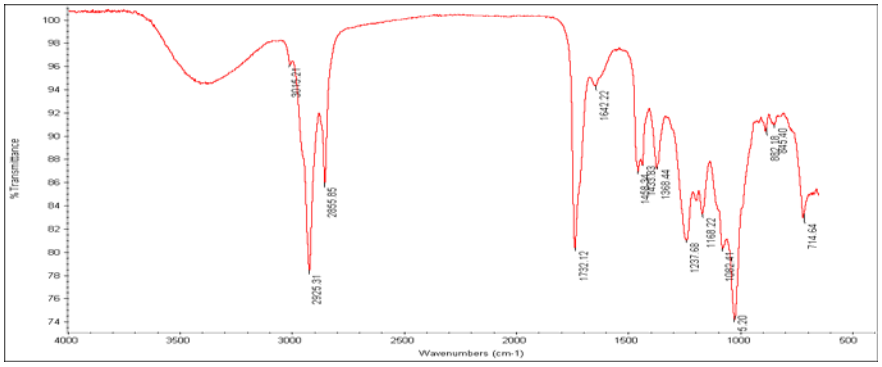

Fig. IR spectra of the crude methanolic extract of *A. parviflora*

effect against *S. aureus* (max. zone 11mm at 100 and 1000 µg/disc dose), *Citrobacter* (max. zone 12mm at 1000 µg/disc dose) *and P. aeruginosa* (max. zone 12mm at 1000 & 2000 µg/disc dose).The growth of *P. mirabilis, M. luteus* and *B. subtilis* was also inhibited at higher doses (80µg/disc) and the low concentrations of extract showed no clear zone of inhibition. All the results were compared with the standard drugs Ampicillin, Gentamicin, Amoxicillin as positive control and discs soaked in DMSO as negative control (table 2, figs. 1-2).

The extract was also used in the same doses and same method (disc diffusion) against various strains of fungi. Standard drugs Itraconazole and Amphoteracin B were used as positive control while DMSO soaked discs as negative control. The crude methanolic extract showed no antifungal activity and growth appeared on the discs as well. Fungal growth remained the same even after 72 hrs and no inhibition occurred (table 3).

The methanolic extract of the plant was used for insecticidal activity against grain weevils (*Sitophilus granaries)* and red flour beetle (*Tribolium castaneum)* and their larvae. The extract showed some repellent activity as the insects were seen to move to the non drug region of the filter paper. However, the extract resulted in no mortality or paralysis in the experimental insects (table 4).

Table 2: Anti-bacterial activity of the crude methanolic extract of *A. parviflora*

Dose µg /disc	*E. coli*	*S. typhi*	*S. aureus*	*P.mirabilis*	*M. luteus*	*Citrobacter*	*B.subtilis*	*P. aeruginosa*
20	9±0.11	0±0	0±0	0±0	0±0	0±0	0±0	0±0
40	8±0.10	0±0	8±0.11	0±0	8±0.10	0±0	0±0	8±0.10
60	9±0.11	8±0.10	9±0.12	0±0	8±0.09	8±0.11	0±0	9±0.11
80	9±0.11	8±0.10	10±0.12	9±0.13	8±0.08	8±0.12	8±0.11	10±0.22
100	9±0.10	8±0.11	11±0.11	9±0.12	8±0.12	9±0.16	8±0.10	10±0.22
1000	8±0.10	11±0.13	11±0.11	9±0.12	9±0.13	12±0.11	9±0.11	12±0.13
2000	9±0.08	9±0.14	10±0.09	9±0.12	10±0.12	11±0.12	9±0.05	12±0.13
MIC	20µg	60 µg	40 µg	80 µg	40 µg	60 µg	80 µg	40 µg
Gentamicin 20	19±0.84	18±0.14	19±0.65	17±0.2	19±0.2	17±0.55	16±0.09	19±0.11
Ampicillin 100	17±0.11	19±0.11	18±0.15	14±0.09	13±0.67	16±0.62	14±0.17	17±0.22
Amoxicillin 100	15±0.18	15±0.13	13±0.06	17±0.07	15±0.91	15±0.13	13±0.18	16±0.23

Table 3: Antifungal activity *A. parviflora* methanolic extract

Treatment	*A.parasiticus*	*A.niger*	*Y.aldovae*	*C.albican*	*A.effusus*	*F.solani*	*M.phaseolinia*	*S.cerevisia*
20 µg /disc	0	0	0	0	0	0	0	0
40 µg /disc	0	0	0	0	0	0	0	0
60 µg /disc	0	0	0	0	0	0	0	0
80 µg /disc	0	0	0	0	0	0	0	0
100 µg /disc	0	0	0	0	0	0	0	0
1000 µg/disc	0	0	0	0	0	0	0	0
2000 µg/disc	0	0	0	0	0	0	0	0
Itraconazole 2mg	19±0.67	16±0.88	21±0.63	16.5±0.31	14±0.66	12±0.34	12±0.14	14±0.25
Amphoteracin B 2mg	14±0.91	13±0.71	11±0.97	15±0.54	14±0.54	12±0.44	18±0.31	13±0.24

Table 4: Insecticide activity of *A. parviflora* methanolic extract

Dose	No. of survivor	*Tribolium castaneum*		*Sitophilus granarius*	
		Time of onset of drug action (immobility time)	% Mortality	Time of onset of drug action (immobility time)	% Mortality
1 mg	10	-	0	-	0
5 mg	10	-	0	-	0
10 mg	10	-	0	-	0
25 mg	10	-	0	-	0
50 mg	10	-	0	-	0
75 mg	10	-	0	-	0
100 mg	10	-	0	-	0

Name of insects	% Mortality	
	+ ve Control Permethrin (Coopex) at conc. 235.9 µg/cm^2	- ve Control (solvent)
Tribolium castaneum	100	0
Sitophilus granarius	100	0

During the cytotoxicity study against *Artemia salina* (nauplii) the extra-ct showed no mortality at a dose of 10ug. At a concentration of 100µg there was 20% mortality in the tested nauplii while at 1000µg tested dose 80% mortality was observed and the LD50 value calculated through probit analysis of Finney method was 321.42µg/ml (table 5).

DISCUSSION

Medicinal plants have extraordinary contribution in the ailment of human disorders. According to the WHO survey about 80% of the world population still utilizes medicinal plants for the cure of various diseases. Due to long-term side effects and overmedication of synthetic drugs people again turned to drugs of natural origin that have good efficacy and safety.

The above results showed that the plant extract having promising active constituents against micro-organisms. The crude methanolic extract of *A. parviflora* demon strated antibacterial activity against all types both gram (+ve) and gram (–ve) of the tested bacteria. The extract gave zone of inhibition against *E .coli* even at a very low dose of 20ug. Mild activity was shown against *E. coli, P. mirabilis* and *B. subtilis.* The crude extract having moderate activity against *M. luteus.* With the increase in dose the spectrum of the extract activity was also enhanced and showed high zone of inhibition against *S. typhi, S. aureus, Citrobacter* and *P. aeruginosa* as compared to other strains of bacteria. The antibacterial action of the plant may be due to the presence of tannins (Djipa *et al*., 2000; Cavanagh *et al*., 2003). Akiyama *et al*. (2001) and Funatogawa *et al*. (2004) reported tannins for antibacterial activity in plants (Doss *et al*., 2009). *M. luteus, S. aureus, P. mirabilis* and *P. aeruginosa* produce urease enzyme which hydrolyses urea to ammonia resulting in kidney stones formation (Toit *et al*., 1995). Our findings suggest that this pathway may be interrupted by this medicinal herb by inhibiting growth of aforementioned species and thus ammonia bad aroma because of bacteria can also be diminished. Most of the gram positive bacteria produce 'hyaluronidase' enzyme that cause tissue damaging effect especially skin tissues (Hynes and Walton, 2000) and we found in our study promising activity against gram positive strains, so due to dual action, various skin infections may be treated.

The crude extract gave no zone of inhibition against the tested strains of fungi even at high doses when applied. The plant demonstrating broad spectra of antibacterial activity may help to discover new chemical classes of compounds that could serve as selective agents for the maintenance of animal or human health and provide biochemical tools for the study of infectious diseases.

The plant extract was used against grain weevils (*Sitophilus granaries*) and red flour beetle (*Tribolium castaneum)* and their larvae for insecticidal activity. Three replicates were performed but give no mortality that demonstrates the plant extract has no insecticidal activity.

Table 5: Brine shrimp lethality bioassay of *A. parviflora* extract and standard drug

S. No.	Drug	Concentration (µg/ml)	No. of test Vials	No. of Shrimp test	Average Mortality(24hrs)	Percent Average mortality	LD_{50} (µg/ml)
1	Control	Without drug	1	10	00	00	00
2	*A.parviflora* methanolic extract	10	1-a	10	00	00	321.42
			1-b	10			
			1-c	10			
		100	2-a	10	02	20	
			2-b	10			
			2-c	10			
		1000	3-a	10	08	80	
			3-b	10			
			3-c	10			
3	*Cyclopho-sphamide*	10	1-a	10	03	30	16.09
			1-b	10			
			1-c	10			
		100	2-a	10	08	80	
			2-b	10			
			2-c	10			
		1000	3-a	10	10	100	
			3-b	10			
			3-c	10			

The plant crude extract showed good cytotoxicity against the nauplii (brine shrimp). Crude extract having LD_{50} greater than 1000ppm are considered to be inactive while the LD_{50} 1000ppm are considered significant for cytotoxity (Meyer *et al*., 1982). Methanolic extract of A. parviflora gave 80% mortality in 1000ppm dose and the LD_{50} value is 321.42µg/ml. The LD_{50} value of the extract demonstrates that the plant has significant cytotoxic activity and this bioassay will guide us for the isolation of cytotoxic compounds from the crude extract of the plant.

From the above study it is cleared that the plant have efficiency against bacteria both gram positive and gram negative and also have good cytotoxic activity. This study will guide researchers for the isolation of new compounds from *A. parviflora* that will provide good efficacy for the ailments of infections and diseases.

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