PHYSIO-CHEMICAL EVALUATION AND BIOLOGICAL ACTIVITY OF *AJUGA BRACTEOSA* WALL AND *VIOLA ODOROTO* LINN

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Abstract

Background: *Ajuga bracteosa* and *Viola odorata* are frequently used by the native people of Swat-Pakistan for the curing of fever, malaria, cough, urinary and stomach disorders with slightly different practice of usage like raw powdered, extracts, decoction etc.

Methods and Materials: Disc Diffusion Method was used for determination of antimicrobial activities of both plants. Nutrient Agar Media was used for the culturing and growth of all microbial strains. Vitamin C and minerals contents were determined by standard method of AOAC. Na and K were analyzed by using flame photometric technique. Micro minerals i.e. "Ni, Cr, Fe, Cu, Zn, Mn, Ca, Pb, and Mg" were determined by Atomic Absorption Spectrophotometer (AAS). Total Soluble Solid (TSS) was determined by using abbe refractometer and pH was determined by using pH meter.

Results: The present study demonstrates that both plants exhibited antibacterial activities against *P. aeruginos, E. coli, S. typhi, B. subtilis* and *S. aureus.* The examined plants showed zone of inhibition for aqueous fraction (50.90, 45.90 %) against *P. aeruginosa*; for EtOAc fraction (41.37, 57.62%) against *C. Albicans* and for hexane fraction (25.86, 40.57%) against *K. pneumoniae*, respectively. Total of 14 different minerals (Na, K, P, Ca, Mg, Fe, Zn, Mn, Co, Cr, Ni, Cu, Pb, Cd) were determined and it was also observed that both the examined plants contained significant level of these analyzed minerals. The subject plants contained highest level of magnesium (295.75, 145.85 mg 100^{-1}g) and calcium (212.49, 44.00 mg 100^{-1}g) and potassium (152.6, 437.45 mg 100^{-1}g) while moderate level P, Zn, Na and lower amount of Cd, Ni, Mn and Cu using Atomic Absorption, Flame Photometry and spectrophotometric techniques. *V. odorata* was found to contain a higher amount of vitamin C (64.05±12.37mg 100^{-1}g) as compared to *A. bracteosa* (45.45 ± 7.29 mg 100^{-1} g).

Conclusion: Findings of this study can persuade researchers for future comprehensive phytochemical study of these plants using state of art techniques and instruments, which include not only isolation of secondary metabolites from these plants but biological evaluation of isolated compounds both *in vivo and in vitro*

Key words: *Ajuga bracteosa, Viola odorata,* Antimicrobial activity, Elemental Profile

Introduction

Wild plants of Swat-Khyber Pakhtunkhwa have played its role in the dietary and therapeutic needs of the native people before the introduction of conventional food items and modern medicines. Plenty of ethno-botanical and medicinal research works were founded and it was observed that several hundred species have potential sources of therapeutic agents. *Ajuga bracteosa* Wall, an important plant of genus *Ajuga*, belongs to family Lamiaceae/Labiatae (Naheed *et al*., 2007) and its local name is *Kori Booti*, indicating its bitter taste. The plant is found in different region of China, Afghanistan, Pakistan, Bhutan and Malaysia (Subhan *et al*., 1996; Arfan *et al*., 1996). *A. bracteosa* has many folk uses including hypertension, diabetes curing, diarrhea, swollen wounds, stomach pain, dysentery, malaria, eye diseases, bites of insects, tumors, heart and bladder diseases (Ahmad and Chaoudary, 2009; Manandhar and Narayan, 2009; Perry and Metzger, 1980). The plant is also well-known for anti-plasmodic and anti-pyretic activity (Shafi *et al*., 2004). Peoples of local rural areas of Pakistan use extract of this plant for treating numerous neurological diseases (Hassan *et al*., 1994). *Ajuga bracteosa* contains diterpenoids and withanolides (Glotter 1991) which are responsible for various biological activities (Gautam *et al*., 2010; Riaz *et al*., 2007; Kuria *et al*., 2002; Ray *et al*., 1994).

Viola odorata Linn is one of the important specie of Violaceae family, commonly known as Banafshah and/or sweet violet. The genus *Voila* consist of approximately "400–500" species scattered all over the world including "South America, Northern America, Australia, Asia and some part of Europe" (Vishal *et al*., 2009). The plant has medicinal importance and is used in bronchitis, cancer, cough, fever, urinary infections, rheumatism, sneezing, kidney and liver disorders (Jetho, 2001). Minerals participated a very

considerable job against "a variety of degenerative diseases and processes, they may also prevent and reduce injury from environmental pollutants and enhance the ability to work and learn, some minerals are essential to a healthy diet (e.g. Calcium, Phosphorus, Potassium and Sodium) whereas some can be toxic (e.g. Lead, Mercury, Cadmium and Aluminium). From ancient times, Swarnabhasma (gold ash) has been used in several clinical manifestations including loss of memory, defective eyesight, infertility, overall body weakness and incidence of early aging. Qualitative analyses indicated that Swarnabhasma contained not only gold but also several microelements Fe, Al, Cu, Zn, Co, Mg, Ca, As, Pb etc" (Vermani *et al*., 2010). The knowledge elemental constituent is very important for consumers. Sometimes, lack of knowledge may lead to severe conditions due to toxic elements (Saraf and Samant, 2013). Both plant species are found in Pakistan. It's required to comprehensively study biological potential and phytochemical evaluation which becomes a lead up for our present study, these two selected plant species are investigate for such type of study for the first time.

Materials and Methods

Sampling and Sample Preparation

One kg of aerial parts of each plant (*Ajuga bracteosa* Wall. and *Viola odorata* Linn) was collected from their natural habitats at their mature stage. Both of the plants are native to Malakand district and were collected in different areas including Dargai, Sakhkot, Thana, Chakdara, Bat khala and Palay where these are separately used by local people, in indigenous system. Malakand is situated at 34.57° North latitude, 71.93° East longitude and 844 meters elevation above the sea level. The collected plant materials were first washed with distilled water for the purpose of removing any contamination dust, dirt and impurities etc. and were dried under shade for five to seven days.

The dried plant samples were then grinded with the help of mortar and pestle followed by electrical grinder (Yigan, model WF130). "The grinded samples were sieved from 80 mm mesh sieve. The powdered samples were then packed in sealed plastic bags and stored at 4°C for microbial and physiochemical analysis".

Bioassay of the Plants Extracts

Extraction and Fractionation

The powdered samples were soaked in ethanol for a period of 1 week to get extracts. The mixtures were then filtered and evaporated at room temperature by using rotary evaporator to obtain crude extract. For fractionation, each crude extract was suspended in water, first extracted with n-hexane thrice in separating funnel, followed by extraction with chloroform and ethyl acetate one by one using the same process to get their respective fractions. All the crude n-hexane, aqueous, chloroform and ethyl acetate fractions obtained from both plants were concentrated by using rotary evaporator. The initial crude extracts and its fractions were analyzed for antimicrobial activities.

Culture Media

Nutrient Agar Media was used for the culturing and growth of all microbial strains (Bauer and Kirby, 1966). "Nutrient broth media was used for standardization of these microorganisms". Nutrient agar and nutrient broth media was prepared accordingly i.e. "2.8 $g100^{-1}$mL and 1.3 $g100^{-1}$mL" respectively in distilled water. The nutrient broth media was also poured in 10 test tubes (approx. 12 mL). All the flasks along with test tubes were plugged with cotton wool and then subjected to sterilization under pressure of 1.5 pounds and 120 ^{o}C temperatures for 15 minutes by using autoclave. The sterilized nutrient agar media was poured in sterilized petri dishes in a laminar flow unit.

The antimicrobial activities of *A. bracteosa* and *V. odorata* were tested against the following microorganisms (Table-1) at "PCSIR laboratory Peshawar". Disc Diffusion Method was used for determination of antimicrobial activities of both plants (Yin *et al*., 2010).

Antimicrobial Activity Bioassay

For determination of antimicrobial activities of *A. bracteosa* and *V. odorata* extracts, the required amount of nutrient agar media and nutrient broth was prepared in flasks and was sterilized in autoclave. After sterilization, nutrient agar media was poured into the plates in a laminar flow hood and was incubated at "37 ˚C for 24 hrs" to check any contamination. Then the microbial stock cultures were freshened by streaking (known as first streak) with the help of a sterile inoculation loop on the nutrient agar plates. The first streaked cultures were again streaked on fresh media plates (known as second streak) and then incubated at "37˚C for 24 hrs".

The second streaked cultures were" inoculated into the sterilized nutrient broth in flasks which were then incubated in the shaking water bath for 18 hrs at 37 °C. Then, the microbial cultures from flasks were standardized in sterilized nutrient broth (in test tubes). The dried crude ethanol extract and different fractions of *A. bracteosa* and *V. odorata* were diluted to 1 mg $(6\ \mu L)^{-1}$ in diethyl sulfoxide (DMSO). Standardized microbial inoculums were seeded into the nutrient agar plates". Whatman filter paper discs (6 mm in diameter) was placed on agar media and extracts in different concentrations of 1 and 2 $disc^{-1}$ in 4 µl and 8 µl volumes were poured on the discs.

Vitamin C

Vitamin C contents were determined by standard method of AOAC (AOAC, 2000). About 20 mg of each plant sample was dissolved in "200 mL oxalic acid 0.4%". From the stock solution about 10 mL aliquot was titrated against dye and the end point was determined. The amount of vitamin C was calculated by using the formula

$$\text{"Vitamin C (mg } 100^{-1} \text{ g)"} = \frac{\text{"Titration reading} \times \text{dye factor} \times \text{dilution factor"}}{\text{"Volume of sample} \times \text{aliquot sample"}} \times 100$$

Mineral Analysis

The acid sample was prepared according to the standard protocol of AOAC (AOAC, 2000). About 1gm of each plant sample was digested with "HNO_3 and $HClO_4$ in 1:2 ratios". The digest was diluted with double distilled water. This acid digest was then analyzed for "Mg, Ca, Zn and Fe by using double beam atomic absorption spectrophotometer".

Micro Minerals

For determination of micro minerals i.e. "Ni, Cr, Fe, Cu, Zn, Mn, Ca, Pb, and Mg", Atomic Absorption Spectrophotometer (AAS) was used by using the standard solutions of the above minerals and their respective cathode lamp (AOAC, 2000, Khalil and Saleemullah, 2004). Data was calculated by using the following formula: "Micro mineral mg L^{-1}" = $\frac{\text{"ppm from graph x dilution factor"}}{\text{"Weight of Sample"}}$

Na and K Analysis

"Na and K were analyzed by using the standard method of AOAC" (AOAC, 2000) with slight modification, using flame photometric technique.

Physico-Chemical Analysis

Total Soluble Solid (TSS) was determined by using abbe refractometer and pH was determined by using pH meter (AOAC, 2000).

Statistical Analysis

The data was obtained in triplicate for each sample and standard error was calculated (Steel *et al*., 1997).

Results and Discussion

This study investigated antimicrobial, physico-chemical analysis and elemental profile of the two wild plants (*Ajuga bracteosa* and *Viola odorata*) which were known to be valuable for the curing of diabetes, hypertension, malaria, diarrhea, constipation, inflammation, timorous breasts kidney and liver disorders (Manandhar and Narayan, 2002).

Anti-Microbial Activity

Five different extracts of *A. bracteosa* and *V. odorata* (*n*-hexane, chloroform, ethylacetate, methanol and aqueous) were investigated for antimicrobial potential by using "*B. cereus, B. subtilus, E. coli, K. Pneumoniea, P. averugenosa S. typhi, S. aureus* and *C. Albicans"*. The examined plants extracts showed various degree of inhibition against selected microbial strains and it was observed that ethanol and ethyl acetate fractions were most potent followed by chloroform fraction (Tables 2 & 3). The highest percent zone of inhibition of hexane fraction of *A. bracteosa* was noted against "*C. albican* (25.86) followed by *S. typhi* (25.0), whereas *E. coli, S. aureus* and *B cerus* were resistance to the *A. bracteosa* plant extract".

Likewise, the maximum diameter zone of chloroform and ethyl acetate fractions was observed against "*P. auriginosa* (45.45, 38.18), *E. coli* (36.70), *B. cereus* (36.66, 31.66), *C. albican* (36.20, 41.37), *S. typhi* (26.87, 35.93) and *B. subtilis* (24.19, 46.77)", respectively. It was also observed that some fractions like aqueous fraction of *A. bracteosa* were resistant to *C. albican*, *B. cerus* and *S. typhi*. Similarly, the hexane fraction of *V. odorata* exhibited broad spectrum activity against the pathogenic microbial strains (Table-3). The highest percent inhibitory zone of hexane fraction was recorded against *B. cerus* (40.90) as compared to *K. pneumonia* (40.57) and *B. subtilis* (37.28). It was also observed that the said plant fraction has no effect on the growth of some pathogenic strains including *E. coli, S. aureus* and *P. auriginosa*. In case of chloroform fraction, all the microbial isolates were susceptible except *C. albican* which was resistant to said plant extract.

The highest inhibitory zone was examined against *E. coli* (42.66) and *P. auriginosa* (42.62) followed by *S. aureus* (32.78) and *B. subtilis* (32.20). In a similar fashion, ethyl acetate fraction retarded the growth of these harmful microbial strains to a high extent. The highest diameter inhibitory zone was noted against *C. albican* (57.62), *S. aureus* (44.26) and *S. typhi* (44.11). However, *E. coli, C. albican, B. cerus* and *S. typhi* were resistant and did not exhibit any inhibition. Various extracts of *Viola odorata* collected from India

were investigated for selected respiratory tract pathogens (Shiv *et al*., 2012), aqueous extracts of flowers part of this plant were also investigated for *E. coli, S. Aureus, S. typhi* and *B. subtilis* (Khan *et al*., 2011). Likewise, *Ajuga bracteosa* exhibited significant amount of anthelmintic, hypoglycemic, antihypertensive, anti-inflammatory, anticancer, antibacterial and antispasmodic activities (Agarwal *et al*., 2010; Akriti *et al*., 2011; Abhishek and Kaur, 2011).

Results of the current study were at par with those reported by (Jabeen *et al*., 2008). They observed that the chloroform, ethyl acetate and methanol fraction of the subject plant inhibited the growth of *S. aureus, B. subtilus* and *E. coli.* Likewise, Khatibi *et al.* (1989) observed that the subject plant possessed prominent activity against microbial strains. This research work concerning antimicrobial potential of medicinal plants leads us to the assumption that the subject plants could be the valuable source for lead bioactive compounds. The results agreed with those from the literature (Samra *et al*., 2006; Ahmad and Choudary, 2009). The crude ethyl acetate and ethanolic fraction exhibited good level of activity. Likewise, antifungal activity against the fungal strains *T. longifusus, C. albicans, C. glaberata, F. solani, A. flavus* was also evaluated in these plants and it was observed that ethanolic fractions of these crude fractions proved effectiveness against relevant fungal strains. On the basis of antimicrobial activity, it may serve the best tonic and remedy for skin infections, stomach ailments and various diseases.

Physico-Chemical Properties

Total Soluble Solids and pH of both the plants were carried out by using standard protocol while vitamin C content was determined on fresh biomass basis (mg 100 g^{-1}) by using dye reduction method (Table-4). The results revealed that vitamin C is found in *V. odorata* (64.05±12.37mg 100^{-1}g) and *A. bracteosa* (45.45 ± 7.29 mg 100^{-1} g) in a significant amount. The methanolic extract of both the plants was found slightly acidic having pH of 5.57 and 6.60 for *A. bracteosa* and *V. odorata*, respectively. It is obvious that the decaying organic matter have profound role in the pH maintenance and hence it will positively play its role in the soil fertility and in the nutrients availability for maximum growths of plants.

However, the recent agricultural practices including direct-seeded or continuously cropped land drastically altered the pH system across the world. So it is hoped that a number of wild plants will play their role in the soil and ecosystem preservation as a whole.

Mineral Profile

Fourteen minerals of both the subject plants were determined on dry biomass basis (Table-5) and the data oscillates that *A. bracteosa* and *V. odorata* were rich source of magnesium, calcium and potassium (295, 145; 212, 44; and 437, 152 mg 100^{-1}g) respectively. P, Fe and Zn were found in the least abundant level and the rest of all the eight minerals were found in low quantity. It was observed in the current study that Zn and Mn concentration of *A. bracteosa* was recorded as 2.75 ± 2.19, 1.9 ± 0.56 mg 100^{-1}g and 4.71 ± 2.27, 2.20 ± 0.17 mg 100^{-1}g for *V. odorata*, respectively. The present study confirms relatively good amount of Fe, Zn, Cr and Ni. The reasonable level of Cr appreciably increases the metabolism of proteins and carbohydrates and as a result, the efficiency of insulin is enhanced; that helped in the regulation of blood sugar levels.

Likewise, Na and K have profound role in the maintenance of body water and carry out routine nerve functions (Sealy and Laragh, 1995). Study of mineral profile is very essential due to its relation with microbes. Microbes interact with metals and minerals in natural and synthetic environments, altering their physical and chemical state, with metals and minerals also able to affect microbial growth, activity and survival. Minerals are directly and/or indirectly involved in all aspects of microbial growth, metabolism and differentiation. Metals and their compounds interact with microbes in various ways depending on the metal species, organism and environment (Gad, 2010).

Table-1: The details of microbial strains used in the present study

Bacterial stains	**Gram stain type**	**Detail of microbial strain**
Bacillus cerus	Positive	Clinical isolates obtained from Microbiology laboratory, QAU Islamabad
Bacillus subtilus	Positive	Clinical isolates obtained from Microbiology laboratory, QAU Islamabad
Staphylococcus aureus	Positive	ATCC # 6538
Escherichia coli	Negative	ACCT # 25922
Klebsiella pneumonia	Negative	Clinical isolates obtained from Microbiology laboratory, QAU Islamabad
Pseudomonas averugenosa	Negative	ATCC # 9721
Salmonella typhi	Negative	Clinical isolates obtained from Microbiology laboratory, QAU Islamabad
Candida albicans	Fungus	Clinical isolates obtained from Hayatabad Medical Complex, Peshawar

Table-2: Zone of inhibition of different extracts of *Ajuga bracteosa* Wall ex benth.

Name of Bacteria	Zone of inhibition of standard	Hexane		$CHCl_3$		EtOAc		EtOH		Aqueous	
		Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)
E. coli	39.5	00	00	14.5	36.70	9	22.78	9.5	24.05	10	25.31
S. aureus	29	00	00	00	00	10.5	36.20	11	37.93	9	31.03
B. subtilus	31	6.5	20.96	7.5	24.19	14.5	46.77	11	35.48	7	22.58
C. albican	29	7.5	25.86	10.5	36.20	12	41.37	00	00	00	00
K. pneumoniea	31	5.5	17.74	00	00	8.5	27.41	8	25.80	5	16.12
P. auriginosa	27.5	6	21.81	12.5	45.45	10.5	38.18	10.5	38.18	14	50.90
B. cerus	30	00	00	11	36.66	9.5	31.66	11	36.66	00	00
S. typhi	32	8	25.0	8.56	26.75	11.5	35.93	7	31.87	00	00

Table-3: Zone of inhibition of different extracts of *Viola odorata*.

Name of Bacteria	Zone of inhibition of standard	Hexane		CHCL3		EtOAc		EtOH		Aqueous	
		Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)	Zone of Inhibition (mm)	Inhibition (%)
E. coli	37.5	00	00	16	42.61	00	00	11	29.33	00	00
S. aureus	30.5	00	00	10	32.78	13.5	44.26	13	42.62	13	42.62
B. subtilus	29.5	11	37.28	9.5	32.20	10	33.89	00	00	7.5	25.42
C. albican	29.5	7	23.72	00	00	17	57.62	12.5	42.37	00	00
K. pneumoniea	34.5	14	40.57	8.5	24.63	13	37.68	8	23.18	12.5	36.23
P. auriginosa	30.5	00	00	13	42.62	8	26.22	11	36.06	14	45.90
B. cerus	33	13.5	40.90	8.5	25.75	00	00	12.5	37.87	00	00
S. typhi	34	9.5	27.94	9.8	28.82	15	44.11	10.5	30.88	00	00

Table-4: Vitamin C, pH and TSS of subject plants

Sample	Vitamin C	Ph	TSS
A. bracteosa	45.45 ± 7.29	5.57 ± 0.21	1.49 ± 4.41
V. odorata	64.05 ± 12.37	6.60 ± 0.34	1.50 ± 0.17

Values are for triplicate determinations (Means ± SD)

Table- 5: Mineral contents of *A. bracteosa* and *V. odorata* (mg 100^{-1}g).

S. No.	Mineral	*A. bracteosa*	*V. odorata*
1	Na	32.9 ± 7.09	246.2 ± 8.62
2	K	152.6 ± 4.24	437.45 ± 5.72
3	P	51.0 ± 4.70	45.00 ± 3.62
4	Ca	212.49 ± 8.48	44.00 ± 8.06
5	Mg	295.75 ±7.56	145.85 ± 7.84
6	Fe	3.15 ± 0.77	6.56 ± 0.94
7	Zn	2.75 ± 2.19	4.71 ± 2.27
8	Mn	1.9 ± 0.56	2.20 ± 0.17
9	Co	1.5 ± 0.84	0.69 ± 0.57
10	Cr	3.60 ± 3.81	0.64 ± 079
11	Ni	1.35 ± 0.07	1.28 ± 0.72
12	Cu	0.80 ± 0.30	0.96 ± 0.30
13	Pb	1.55 ± 0.49	0.79 ± 0.99
14	Cd	0.08 ± 0.01	0.64 ± 0.79

Values are for triplicate determinations (Means ± SD)

Conclusions

Ajuga bracteosa and *Viola odorata* were subjected to antibacterial potential determination against various microbes. Five fractions (ethanol, hexane, chloroform, ethyl acetate and aqueous fractions) of each plant were tested against *Bacillus cerus, Bacillus subtilus, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi* and *Candida albicans* and they showed potent antibacterial activities against *E. coli, S. aureus, S. typhi, B.subtilis* and *P. Aerogenosa*.

Elemental investigation was also carried out and high values of 295.75 ± 7.56 mg 100^{-1}g and 212.49 mg 100^{-1}g for magnesium and calcium, respectively and low values of 0.08 ± 0.01 mg 100^{-1}g and 0.80 ± 0.30 mg 100^{-1}g for cadmium and copper, respectively, in *A. bracteosa* were indicated. Mineral profile of *V. odorata* resulted the higher concentration of potassium (437.45 mg 100^{-1}g) followed by sodium (246.2 ± 8.62 mg 100^{-1}g), whereas concentration of chromium and cadmium was found lower (0.64 ± 0.79 mg 100^{-1}g each) followed by cobalt (0.69 ± 0.57 mg 100^{-1}g). Vitamin C contents, acidity and total soluble solids were also determined in *V. odorata* and *A. bracteosa.* The *V. odorata* was found to contain a higher amount of vitamin C (64.05±12.37mg 100^{-1}g) as compared to *A. bracteosa* (45.45 ± 7.29 mg 100^{-1} g).

Findings of this study can persuade researchers for future comprehensive phytochemical study of these plants using state of art techniques and instruments, which include not only isolation of secondary metabolites from these plants but biological evaluation of isolated compounds both *in vivo and in vitro*.

Acknowledgment

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for funding this work through Research Group no. RG 1435-014.

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