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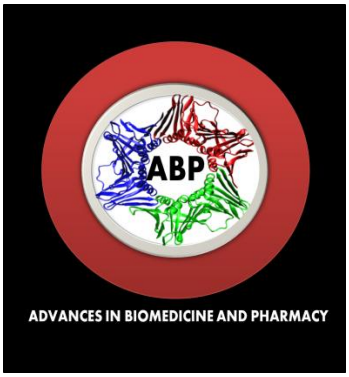

Original Article

A Study on Variation of Phytochemical Constituents with Altitude and *In vitro* antimicrobial potential of two species of *Ajuga* L.

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Abstract

The present study was aimed to assess the variation in the phytochemical constituents with altitude and the antibacterial potency of two medicinally important species, *Ajuga bracteosa* and *Ajuga parviflora*, growing in Kashmir valley. The methanolic extracts of both the species from different populations with varying altitudes were subjected to quantitative estimation of various phytochemicals by using various spectrophotometric techniques. It was observed that mean values of the analyzed phytochemical constituents varied significantly ($p \leq 0.05$) across the populations. Phytochemicals such as phenols, flavonoids, saponins, terpenoids and alkaloids were present in all the sampled populations but their quantity varied significantly across the populations. With the decrease in altitude the content of various phytochemicals gets reduced. The high altitude plants exposed to the extreme environmental conditions possess a good content of secondary metabolites altering their stress tolerance and thus enhancing their medicinal activity. The present study further revealed that the methanolic and ethyl acetate extracts of the two medicinal plant species under study; *A. bracteosa* and *A. parviflora* plant extracts possess potent antibacterial activity against two gram positive bacterial strains *(B. subtilis, S. aureus)* and two gram negative bacterial strains (*E. coli, P. vulgaris*). Therefore, these plants may prove as source of certain potent antibacterial molecules.

Keywords: *Ajuga bracteosa; Ajuga parviflora;* Phytochemical constituents; antibacterial activity.

Introduction

The plants are known to be the source of important herbal medicines that help in curing of human diseases because of the presence of phytochemical constituents [1]. The pharmacological properties of medicinal plants are attributed to these phytochemical constituents isolated from their crude extracts [2]. These phytochemicals, also known as secondary metabolite, accumulate in different parts of medicinal plants like leaves, flowers and roots. There are more than thousand known and many unknown phytochemicals and plants produce these chemicals to protect themselves, but recent researches demonstrate that many phytochemicals can also protect human against diseases [3]. The secondary metabolites particularly phenols, flavonoids, alkaloids and terpenoids, exhibit a wide array of pharmacological activities including antioxidant, antimicrobial and anticancer activities. The qualitative and quantitative estimation of the phytochemical constituents of a medicinal plant is considered to be an important step in medicinal plant research.

The biosynthesis of the secondary metabolites of the plants are determined by genetic and environmental factors as well as their interaction [4]. A variety of environmental

factors such as season, altitude and soil nutrition etc. significantly modify the secondary metabolite profile in plants [5, 6]. Altitude is one of the abiotic stresses and broad range of environmental factors such as precipitation, mean temperature, soil characteristics and atmospheric pressure etc. change with altitude [7].

Ajuga bracteosa belongs to genus *Ajuga* L. and family *Lamiaceae* (the largest family of order Lamiales). It is distributed in sub-tropical and temperate regions from Kashmir to Bhutan, Pakistan, Afghanistan, China and Malaysia [8].

It is known to cure diabetes, arthritis, rheumatism, headache, jaundice, malarial fever and hypertension [9, 10]. *Ajuga parviflora* Benth. is a small perennial herb growing wild in the temperate regions of Himalaya, Afghanistan, Kashmir and Pakistan [11, 12].

It has been used as an astringent, for the treatment of swollen wounds, diarrhea, fever, eye trouble and even cancer [13, 14]. The two species, due to their resemblances are both locally known as *Jan e adam*.

It is very common among the people who live in upper reaches of Kashmir Himalaya to use herbs for curing of various diseases [15]. In the present paper, we have comparatively analysed the phytochemical constituents (total phenolic and flavonoid content) of four samples of *Ajuga bracteosa and Ajuga parviflora;* growing at different geographical locations across the valley of Kashmir. Besides the antimicrobial activity of the ethyl acetate and methanolic extracts of the two species was also analysed with the aim to establish the similarities and differences between the two allied species especially regarding their phytochemical content and antimicrobial potential.

Materials and Methods

Study sites

An extensive field survey revealed that the two species of genus *Ajuga* grow in different regions of Kashmir valley. Four sites of varying altitudes, each of *Ajuga bracteosa* (Fig 1a) and *Ajuga parviflora* (Fig 1b) were selected for the present investigation. The salient features of the selected sites are summarized in Table 1a and 1b.

Plant Material

Fresh whole herbs each of *Ajuga bracteosa* and *Ajuga parviflora* were collected from four different natural populations of Kashmir between May-June 2016. The voucher specimes were identified and deposited in Kashmir University Herbaria (KASH), Centre of Plant Taxonomy, Department of Botany, University of Kashmir, Srinagar, for further reference.

Preparation of extract

The collected herbs were cleaned and cut into small pieces and then air dried under shade at room temperature (25°C). The dried material was ground to fine powder using a mechanical blender and powdered plant material (50 g) was packed in Soxhelt apparatus and extracted with ethyl acetate at 70- 75°C and methanol at 60-65°C successively. The extract was filtered through Whatmann filter paper No.1 and the solvent was removed under reduced pressure at 35-45°C using rotavapor. The dried extract was labelled and stored at 4°C in air tight glass bottles before experimental use.

Determination of total phenolics

The total phenolic content was determined using a modified spectrophotometric method [16]. The reaction mixture was prepared by mixing 0.5 mL of methanol solution (1 mg/mL) of each extract with 2.5 mL of 10% Folin-Ciocalteu reagent dissolved in water and 2.5 mL of 7.5% NaHCO3. The samples were incubated at 45°C for 15 min. The absorbance was measured at λmax=765 nm. The samples were prepared in triplicate and the mean value of absorbance was obtained. A blank was concomitantly prepared with methanol instead of extract solution. The same procedure was repeated for the gallic acid, and the calibration curve was constructed. The total phenolic content was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

Determination of total flavonoids

The concentration of flavonoids was determined using a spectrophotometric method with some modifications [17]. The sample contained 1 mL of methanol extract in a concentration of 1 mg/mL and 1 mL of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for 1 h at room temperature. The absorbance was measured at λ_{max}=415 nm. The samples were prepared in triplicate and the mean value of absorbance was obtained. In- stead of extract solution, methanol was used in order to prepare a blank. The same procedure was repeated for rutin, and the calibration curve was constructed. Concentration of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract).

Alkaloid estimation

2.5g of the plant powder was extracted using 100ml of 20% acetic acid in ethanol. The solution was covered for almost 4 hours. Filtrate was concentrated to 25ml.

Concentrated ammonium chloride was added stepwise for precipitation. The whole solution was kept as such so that precipitate will settle. Collected precipitate was washed with dilute ammonium hydroxide and finally filtered. Filtrate was discarded and pellet obtained was dried and weighed [18,19].

Saponin determination

10g of sample was mixed with 100ml of 20% aqueous ethanol. The mixture was kept for 4hr on water bath shaker at 55°C. The extract was concentrated to 40 ml over water bath at 90°C. Concentrate obtained was transferred into a separating funnel and 10 ml of diethyl ether was added to it. After shaking vigorously aqueous layer was recovered and ethyl layer was discarded. The process was repeated. To the aqueous layer n-Butanol was added. The whole mixture was washed in separating funnel twice with 10 ml 5% of aqueous NaCl. Upper part was retained and heated in water bath until evaporation. Later it was dried in oven to a constant weight [18] and the values were presented as mean ± SD of triplicate analysis.

Terpenoid determination

100g of plant powder were taken separately and soaked in alcohol for 24 hours. Then filtered, the filtrate was extracted with petroleum ether; the ether extract was treated as total terpenoids [20].

Antibacterial Activity

Test microorganisms

Microbial cultures of four different strains of both Gram positive and Gram negative bacteria were used for determination of antibacterial activity. Four bacterial strains; two gram negative strains viz. *Escherichia coli* (MTCC- 739)) and *Proteus vulgaris* (MTCC- 426) and two gram positive strains viz. *Bacillus subtilus* (MTCC-44) and *Staphylococcus aureus* (MTCC- 96) were standard laboratory isolates obtained from the Department of Pharmaceutical Sciences, University of Kashmir. All the bacterial strains were sub-cultured at 37°C on Mueller-Hinton agar (Himedia) slants every fifteen days and stored at 4°C.

Antimicrobial assay

Antibacterial activity of *A. bracteosa and A. parviflora* was determined by agar well diffusion method [21]. Each microorganisms were grown overnight at 37°C in Mueller-Hinton Broth. 100µl of standardized inoculum (0.5 Mac-Farland) of each test bacterium were inoculated on molten Mueller-Hinton agar in culture tubes, homogenized and poured into 90mm sterile Petri dishes. The Petri dishes were allowed to solidify inside the laminar hood. Standard cork borer of 5mm in diameter were used to make uniform wells into which was added 30µl plant extract dissolved in sterile DMSO. Standard antibiotic Kanamycin (50µg/disc) was used as positive control and DMSO alone as negative control. The plates were then incubated at 37 ± 1°C for 24h. The zone of inhibition was measured with the help of standard scale. The experiments were carried out in triplicates and results were calculated as mean ± SD.

Determination of minimum inhibitory concentrations (MICs)

The MIC was determined by macro dilution method [22]. Dilution ranges (100 – 500 µg/ml) of plant extracts and standard antibiotic were prepared from stock solution by serial dilution technique. Each sample dilutions were mixed properly with 20 ml of sterile molten Muller Hinton agar and poured into 90 mm Petri plates and allowed it to cool under laminar air flow before streaking with 10 µl of 0.5 McFarland standard inoculums of tested bacterial strains. Plates were incubated at 37 ± 1°C for 24 hours. The lowest concentration of the extract at which there was no visible growth of microorganisms was considered as minimum inhibitory concentration (MIC).

Statistical analysis

Experiments were done in three replications and the data were expressed as mean ± standard deviation (SD). The data were subjected to one way analysis of variance (ANOVA) and comparison of the means was done with the Tukey's test at $p \leq 0.05$.

Results

Phytochemical constituents

The phytochemical active compounds of *A. bracteosa* and *A. parviflora* growing at different altitudes were analysed quantitatively and the results are presented in Table 2a and 2b. It was observed during the present study that the quantity of phytochemicals varied significantly across these populations. It was found that there was an increase in the phytochemical content with an increase in the altitude. The phenols and flavonoids showed a highest concentration of 9 .56± 0.55 and 6.11± 0.64 in the crude extract of *A. bracteosa* growing at higher altitude (Betab valley) while a highest concentration of 10.16±0.08 and 6.21± 0.28 in the crude methanolic extract of *A. parviflora* growing at a higher altitude (Brinal2).The phenols and flavonoid concentration in both the species showed a

decline with a decline in their respective altitudes. The phytochemical constituents (saponins, terpenoids and alkaloids) also showed the same trend (Table 2a and 2b).

Antibacterial activity

The antimicrobial activities of crude extracts, including ethyl acetate, and methanol of *Ajuga bracteosa* and *Ajuga parviflora* were determined against two Gram negative bacterial strains (*E. coli* and *S. aureus*) and two Gram positive bacterial strains (*B. subtilis* and *P. vulgaris).* Different concentrations of the extracts ranging from 400 µg/ml to 700 µg/ml for the bacterial strains were analyzed. Kanamycin antibiotic was used as positive control for bacterial strains. Moreover, 10% aqueous DMSO (dimethyl sulfoxide) was used as negative control. The inhibitory activities of all the extracts were found to be concentration-dependent. The antimicrobial activity was recorded as Inhibition Zone Diameter (IZD), measured in "mm".

The methanolic extracts of the two species were also found to possess active antibacterial property against the tested bacterial strains (Table. 3a and 3b). The methanolic extract of *A. bracteosa* showed a significant activity against *E. coli* and *B. subtilis* with IZD of 17.93±0.54mm and 16.91±0.42mm respectively at 1000 µg/ml concentration and least activity against *P. vulgaris* with 8.94±0.74mm at 400 µg/ml concentration. While the methanolic extract of *A. parviflora* showed the maximum activity against *P. vulgaris* and *E. coli* with IZD values of 13.28±1.11mm and 13.10±0.65mm respectively at 1000 µg/ml concentration and lowest IZD of 8.17±0.67mm and 8.17±0.51mm against *S. aureus* and *P. vulgaris* respectively at a concentration of 400 µg/ml.

The ethyl acetate extract of *A. bracteosa* and *A. parviflora* showed an inhibitory effect against four bacterial strains (*E. coli, S. aureus, B. subtilis* and *P. vulgaris*) as depicted in Table 3c and 3d. The ethyl acetate extract of *A. bracteosa* exhibited widest IZD of 13.36±0.76 mm against *E. coli* at 1000 µg/ml concentration and the lowest IZD of 6.68±1.08 mm against *S. aureus* at 400 µg/ml concentration. Interestingly, the ethyl acetate extract of *A. parviflora* again showed the highest IZD of 13.57±0.61mm against *E. coli* at 1000 µg/ml concentration, however the lowest IZD of 6.98±0.68 was exhibited at 400 µg/ml concentration against *Proteus vulgaris* (Table 3c and 3d).

The MIC values of methanolic extracts of the two species were between 100 µg/ml to 300 µg/ml, while the MIC values of the ethyl acetate extracts of both the species range between 200 µg/ml to 400 µg/ml concentration (Table 3a-d).

Fig 1a: *Ajuga bracteosa* growing in its natural habitat (rocky slope).

Fig 1b: *Ajuga parviflora* growing in its natural habitat (coniferous forest).

Table 1a: Salient features of the selected sites for the collection of *Ajuga bracteosa*

S.No.	Study site	Latitude and Longitude	Altitude (m-asl)	Habitat
1.	BetabValley	34˚20'N 75˚20'E	2405	Sunny open rocky slope
2.	Drang	34˚20'N 74˚20'E	2235	Sunny open rocky slope
3.	Dachigam	34˚04'N 75˚59'E	1900	Sunny plain with partial shade
4.	Kangan	33˚37'N 75˚27'E	1775	Sunny open rocky slope

Table 1b: Salient features of the selected sites for the collection of *Ajuga parviflora*

S.No.	Study site	Latitude and Longitude	Altitude (m-asl)	Habitat
1.	Brinal 2	33˚33'N 75˚27'E	2620	Shady slopes with coniferous forest
2.	Aru	34˚30'N 75˚21'E	2510	Shady coniferous forest
3.	Gogaldara	34˚70'N 74˚28'E	2427	Shady coniferous forest
4.	Brinal1	33˚34'N 75˚48'E	2190	Shady slopes with coniferous forest

Table 2 a: Comparison of different phytochemical constituents across various populations with varying altitudes (*Ajuga bracteosa)*

Phytochemicals (g%)	Betab valley	Drang	Dachigam	Kangan	F value	LSD ≤0.05
Phenols	9.56±0.55a*	8.28±0.32b	6.08±0.12c	5.21±0.59c	61.61	0.668
Flavonoids	6.11±0.64a	4.24±0.34b	2.19±0.31c	1.16±0.48c	67.74	0.703
Saponins	3.03±0.06a	2.96±0.10a	1.89±0.04b	1.62±0.08c	247.24	0.121
Terpenoids	0.48±0.02a	0.44±0.01b	0.39±0.01c	0.39±0.02c	26.74	0.023
Alkaloids	0.46±0.01a	0.38±0.02b	0.18±0.01c	0.17±0.01c	390.61	0.019

* Means labelled with different small letters indicate thet they significantly differ from each other among different populations.

Table 2 b: Comparison of different phytochemical constituents across various populations with varying altitudes (*Ajuga parviflora)*

Phytochemicals (g%)	Brinal 2	Aru	Gogaldara	Brinal 1	F value	LSD ≤0.05
Phenols	10.16±0.08a*	8.77±0.39a	6.95±0.77b	6.01±0.60b	36.57	0.806
Flavonoids	6.21±0.28a	4.04±0.24b	3.30±0.21b	2.34±0.49c	75.01	0.499
Saponins	3.53±0.36a	3.19±0.13ab	2.92±0.07b	1.44±0.11c	61.32	0.309
Terpenoids	0.29±0.01a	0.21±0.01b	0.16±0.01c	0.14±0.02c	164.48	0.013
Alkaloids	0.18±0.01a	0.16±0.01ab	0.15±0.01bc	0.13±0.02c	17.04	0.013

* Means labelled with different small letters indicate thet they significantly differ from each other among different populations

Table 3a: Antibacterial activity of methanolic extract of *Ajuga bracteosa*

S No.	Bacterial Strain	Volume of extract used (µg/ml)	Diameter of Zone of inhibition (mm)	Diameter of Zone of inhibition(mm) *Kanamycin (50 µl)	MIC (µg/ml)
1.	*Escherichia coli*	400	10.28±0.39	24±0.21	100
		700	13.03±0.12		
		1000	17.93±0.54		
2.	*Staphylococcus aureus*	400	9.95±0.83	28±0.17	200
		700	11.61±0.95		
		1000	15.77±0.97		
3.	*Bacillus subtilis*	400	8.98±0.54	26±0.34	300
		700	11.37±1.15		
		1000	16.91±0.42		
4.	*Proteus vulgaris*	400	8.94±0.74	25±0.45	300
		700	11.43±0.76		
		1000	16.06±0.79		

* The concentration of Kanamycin was 50mg/ml

Table 3 b: Antibacterial activity of methanolic extract of Ajuga parviflora

S.No	*Bacterial Strain*	Volume of extract used (µg/ml)	Diameter of Zone of inhibition (mm)	Diameter of Zone of inhibition(mm) *Kanamycin (50 µl)	MIC (µg/ml)
1.	*Escherichia coli*	400	8.25±0.72	30±0.42	200
		700	9.66±0.58		
		1000	13.10±0.65		
2.	*Staphylococcus aureus*	400	8.17±0.67	28±0.57	300
		700	9.89±0.88		
		1000	12.89±0.85		
3.	*Bacillus subtilis*	400	8.58±0.45	24±0.23	300
		700	10.73±0.54		
		1000	12.93±0.81		
4.	*Proteus vulgaris*	400	8.17±0.51	25±0.21	300

*The concentration of Kanamycin was 50mg/ml

Table 3c: Antibacterial activity ethyl acetate extract of *Ajuga bracteosa*

S.No.	Bacterial Strain	Volume of extract used (µg/ml)	Diameter of Zone of inhibition (mm)	Diameter of Zone of inhibition(mm) *Kanamycin (50 µl)	MIC µg/ml)
1.	*Escherichia coli*	400	7.43±1.33	28±0.37	200
		700	9.71±0.42		
		1000	13.36±0.76		
2.	*Staphylococcus aureus*	400	6.68±1.08	30±0.53	400
		700	8.95±0.62		
		1000	11.6±1.21		
3.	*Bacillus subtilis*	400	8.61±0.67	28±0.64	300
		700	9.71±0.13		
		1000	13.29±1.42		
4.	*Proteus vulgaris*	400	7.04±1.03	28±0.17	400
		700	8.96±0.94		
		1000	10.38±0.56		

*The concentration of Kanamycin was 50mg/ml

Table 3d: Antibacterial activity of ethyl acetate extract of *Ajuga parviflora*

S.No	*Bacterial Strain*	Volume of extract used (µg/ml)	Diameter of Zone of inhibition (mm)	Diameter of Zone of inhibition(mm) *Kanamycin (50 µl)	MIC (µg/ml)
1.	*Escherichia coli*	400	8.81±0.83	28±0.78	200
		700	9.64±1.25		
		1000	13.57±0.61		
2.	*Staphylococcus aureus*	400	7.41±0.81	26±0.62	400
		700	9.04±0.64		
		1000	12.44±1.16		
3.	*Bacillus subtilis*	400	8.08±0.71	26±0.57	300
		700	9.73±0.64		
		1000	11.99±0.61		
4.	*Proteus vulgaris*	400	6.98±0.68	25±0.48	300
		700	8.88±0.72		
		1000	11.45±0.71		

* The concentration of Kanamycin was 50mg/ml.

Discussion

The comparative phytochemical study on the two *Ajuga* species, *Ajuga bracteosa* and *Ajuga parviflora* aimed to highlight the quantitative variations of the phenols, flavonoids, saponins, terpenoids and alkaloids fractions in dry plant material. We noticed that, in both species, the individuals from populations growing in different geographical regions, present variations of larger or more restricted limits especially quantitative for the same group of secondary metabolites. These phytochemicals serve as a major source for pharmaceutical products, so the two plant species can hold an immense potential to serve as cure for various ailments in humans. In case of *A. bracteosa* and *A. parviflora* the highest content of phytochemicals (phenols, flavonoids, saponins, terpenoids and alkaloids) was observed in the plants collected from highest altitudes viz, Betab valley at an altitude of 2405 m asl and Brinal2 at an altitude of 2620m asl. This increase in the phenolic content

with increase in altitude may be ascribed as a response of plants to radiation and decreased temperatures which elicit amplified biosynthesis of UV-absorbing and enhanced UV-B antioxidant phenolics in plants [23]. Copaja reported that saponin production is higher at high altitudes where the prevailing environmental conditions are stressful. The high production of saponins is attributed to the adaptation of the plant to survive in the adverse environmental conditions [24]. The high production of these phytochemicals may be attributed to fluctuation in temperature and non-availability of nutrients. Present investigation revealed alkaloid content increases with increase in altitude, as stress conditions induce polyamine formation which results in nitric oxide biosynthesis that moves freely through the cells acting as potential chemical elicitor of alkaloid production [25].

The present study further revealed that the two medicinal plant species under study; *Ajuga bracteosa* and *Ajuga parviflora* plant extracts possess potent antibacterial activity against four bacterial strains (*E. coli, B. subtilis*, *S. aureus* and *P. vulgaris*). Secondary metabolites of plant origin appear to be one of the alternatives for the control of antibiotic resistant human pathogens. Thus, antibacterial activity may be due to the presence of secondary metabolites [26]. Medicinal plants have their intrinsic ability to resist pathogenic microorganisms and this has led the researchers to investigate their mechanisms of action and isolation of active compounds. This has enabled exploitation of medicinal plants for the treatment of microbial infections of both plants and humans by developing new antimicrobial agents. The use of natural products for treatment of a number of diseases is due to their less harmful effects as compared to drugs synthesized in the laboratory and are safe with very little side effects if used in proper dosage [27].

Conclusions

The analysis of the phytochemical content of plants at varying altitudes can help to select elite genotype and reflect the best suited altitude for commercial cultivation of the species as these phytochemicals are considered as the basis for their medicinal activity. These findings further suggested that ethyl acetate and methanolic extracts of *Ajuga bracteosa* and *Ajuga parviflora* exhibit antimicrobial activity against both gram positive and gram negative bacteria. Therefore, these plants may prove as source of certain potent antibacterial molecules. This study would further pave way for researches to isolate new compounds from these two species with antimicrobial potential.

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