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Original Article

Antimicrobial Potential and Determination of Total Phenolic and Flavonoid Content of Aerial Part Extracts of *Ajuga bracteosa* Wall ex. Benth

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ABSTRACT

Background: Traditionally, *Ajuga bracteosa* (family Lamiaceae) has been used to cure many diseases including gout, rheumatism, amenorrhea, diabetes, malaria, inflammation and cancer in different parts of the world. The antimicrobial activity of the plant has not been validated as yet. This study aims at validation of the folklore use of the plant as a broad spectrum anti-infective agent. **Objective:** To determine the total phenolic and flavonoid contents and to evaluate the antimicrobial activity using different bacterial and fungal strains. **Materials and Methods:** Total phenolic and flavonoid content was measured spectrometrically using Folin-Ciocalteau reagent and by aluminium chloride colorimetric methods respectively. Antimicrobial activity was evaluated by standard agar well and disc diffusion methods. **Results:** The phenolic and flavonoid contents were found highest in the methanolic extract (337.26 mg GAE/g and 213.52 mg RE/g respectively). A relationship was found between flavonoid and phenolic content with biological activity. The extracts also showed good antibacterial and antifungal activity. *Salmonella typhi* (MTCC 1688) was the most sensitive organism tested. **Conclusion:** The results demonstrate that *Ajuga bracteosa* contains high content of phenolics and flavonoids making it a promising candidate for further investigation and development as a natural broad spectrum antimicrobial agent.

Key words: *Ajuga bracteosa,* Phenolic content, Flavonoid content, Antimicrobial activity, biological activity, *Salmonella typhi.*

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INTRODUCTION

There are about 300 species of *Ajuga. Ajuga bracteosa* belongs to family Lamiacea. It is distributed in temperate and subtropical regions from Kashmir to Bhutan, Afghanistan, China, Pakistan, Western Himalayas, plains of Punjab and Malaysia at altitudes below 1300 m. *Ajuga bracteosa* is a perennial erect hairy herb growing up to 5- 50 cm tall. Its flowers are blue, yellow or purple in color.[1] Traditionally the plant is used to cure many diseases. In Taiwan whole plant is used to treat various inflammatory conditions including hepatitis.[2] In the Ayurvedhic system, *Ajuga bracteosa* is cited to relieve gout, rheumatism and amenorrhea.[3] In India, a decoction of the leaves, bark and flowers is used for treatment of diabetes, malaria, inflammation and cancer.[4]

Antibiotics are employed for microbial infection caused by fungi or bacteria. Millions of people die every year due to infectious diseases.[5] Over and incorrect use of antibiotics have resulted in development of resistance.[6] The mechanisms which contribute to drug resistance are target site modification, expression of efflux pumps and metabolic inactivation.[7] In comparison to synthetic ones the antibiotics obtained from plant origin are of greater benefit due to their natural origin. The side effects like headache, abdominal pain, nausea and vomiting are not seen with the use of natural antibiotics from plants.[8]

Intense oxidative damage to lipids, proteins, DNA and enzymes is caused by reactive oxygen species, such as superoxide ion, hydroxyl ion, singlet oxygen and hydrogen peroxide, which are produced normally in cells during metabolism.[9] Various pathological conditions including Parkinson's diseases, inflammation, neuro-degeneration, arthritis, asthma, ischemia and dementia are caused by increased amount of free radicals. Herbal plants containing natural antioxidants are becoming target of a number of research studies for finding potentially useful, safe and inexpensive antioxidants.[10] Also these types of drugs are known for their therapeutic activity due to quenching of free radicals.[11]

MATERIALS AND METHODS

Plant material and preparation of extract

The whole plant of *Ajuga bracteosa* was collected from the Drang area of district Budgam, J&K, India. The plant was identified and authenticated at the Centre of Plant Taxonomy and Biodiversity, University of Kashmir under voucher specimen No. 2425-(KASH). A specimen was deposited there for future reference. The aerial parts were dried in shade at room temperature and ground to a fine powder in a mechanical blender. Hot extraction (successive) with Soxhlet apparatus was carried out. Dried powder (500 g) was packed into a Soxhlet apparatus and extracted with 500 mL petroleum ether for 18–20 h. The extract was filtered through Whatman filter paper No. 1, and the filtrate was concentrated under reduced pressure using rotary evapouration. The marc so obtained was subsequently extracted with methanol (500 ml) for 18-20 h. The extract so obtained was concentrated using rota vapour. The marc left behind was then extracted with water (400 ml) for 6-8 h and then concentrated. The extracts were dried, weighed and stored at 4°C in storage vials for experimental use.

Total phenolic content

The total phenolic content of the extract was determined spectrophotometerically using Folin Ciocalteu method.[12] The extracts were prepared at concentrations of 1 mg/ml. Briefly 200 µL of crude extracts (1 mg/mL) were made up to3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin–Ciocalteu (Analytical grade, Central drug house, New Delhi) reagent for 3 min, followed by the addition of 2 mL of sodium carbonate (20% w/v) (Analytical grade, Central drug house, New Delhi). The mixture was allowed to stand in the dark for a further 60 min and absorbance was measured at 650 nm. From the calibration curve, the total phenolic content was calculated. The results were expressed as mg of gallic acid equivalent per g dry weight.

Total flavonoid content

The aluminum chloride (Analytical grade, Central drug house, New Delhi) colorimetric method was used to determine total flavonoid content of crude extract.[13] In brief, 50µL of crude extracts (1 mg/mL) were made up to 1 mL with methanol, mixed with 4 mL distilled water and then 0.3 mL 5% NaNO2 (Analytical grade, Central drug house, New Delhi) solution was added. After 5 min of incubation, 0.3 mL of 10% $AlCl_3$ solution was added, and the mixture was allowed to stand for 6 min. 2 mL of 1 mol/L NaOH (Analytical grade, Central drug house, New Delhi) solution were added, and the final volume of the mixture was subsequently brought to 10 mL with double distilled water. After standing the mixture for 15 minutes, and absorbance was measured at 510 nm. From a calibration curve the total flavonoid content was calculated, and the results were expressed as mg rutin equivalent per g dry weight.

Antimicrobial properties

Test microorganisms

The standard bacterial strains namely *E. coli* (MTCC 443), *Salmonella typhi (*MTCC 1688), *Proteus vulgaris* (MTCC 426), *Staphylococcus aureus* (MTCC 96), were used for antibacterial activity. The strains were maintained on Muller Hinton agar by periodic subculture and preserved at 4°C prior to use. For antifungal activity S. *cerevesiae (*MTCC 443), *P. Chrysogenum* (1688), *A. fumigatus (*MTCC 426) were used. The strains were maintained on Sabouraud dextrose agar medium (SDA) (Analytical grade, TM media, New Delhi) by periodic subculture and preserved at 4°C prior to use. The microbial strains were obtained from Microbial Type Culture Collection (MTCC) Institute of Microbial technology (IM TECH) Chandigarh, India.

Antibacterial activity

Antibacterial assay for aqueous and methanolic extracts for both Gram-negative and Gram-positive bacteria by was performed by standard agar well diffusion methods.[14] Standardized 100 µl of each test bacterium was inoculated on molten Mueller Hinton Agar (Analytical grade, Hi media, Mumbai), homogenised and then poured into sterile Petri plates to yield a uniform depth of 4 mm and the Petri plates were allowed to solidify. Uniform wells of 4 mm diameter were made into each plate by sterile cork borers. 50 µl of each concentration (50 mg/ml, 100 mg/ml and 200mg/ml) of plant extracts, prepared in 10% dimethylsulfoxide (DMSO) (HybriMax™ were loaded into different peripheral wells. Streptomycin (10µg/disc) disc was placed at the centre of each Petri plate and served as positive control, while 10% dimethylsulfoxide served as negative control. The Petri plates were then incubated at 37°C for 18 to 24 hours in an incubator. The zone of inhibition was measured by vernier caliper, recorded and considered as an indication for antibacterial activity.

Antifungal activity

The assay was performed by agar disc diffusion method. Three active cultures for the experiment were prepared by transferring a loop of full of culture from the stock cultures into the boiling tubes containing SDA (sterilized and yet liquid), that were incubated at 48 hr at 25°C. Antifungal activity of the plant extract was determined by disc diffusion method on SDA medium. SDA medium was poured in to the Petri plates. After the medium solidified, discs were placed in SDA plates prior to addition of 20 µl of sample (Concentration: 50µg, 100µg and 200 µg). The plates were incubated at 25°C for 24 h. This procedure was performed in triplicate and the mean values were taken as the zone of inhibition. The antifungal activity of the plant extract was determined by measuring the diameter of the inhibitory zones using a vernier caliper and compared with those produced by the antibiotic fluconazole (Analytical grade, Hi media, Mumbai) (25µg/disc). Fluconazole was used as a positive control and the diameter of zone of inhibition less than 5 mm was considered as insignificant.[15]

Statistical Analysis

Statistical analysis was carried out with Graph Pad Instat 6 software (Graph Pad Software, Inc., USA), and results are expressed as mean ± standard error mean (SEM).

RESULTS AND DISCUSSION

Phenolic and flavonoid contents

The total phenolic content of the aerial part extracts of *Ajuga bracteosa,* calculated from calibration curve (Figure 1) are shown in Table 1. The methanolic extract showed the highest total phenolic content of 337.26 gallic acid equivalents/g. The total flavonoid content calculated from calibration curve (Figure 2) is shown in Table 2. The highest flavonoid content was shown by methanol which was equivalent to 213.52 rutin equivalents/g. Flavonoids, including flavones, flavanols and condensed tannins, are plant secondary metabolites, the antioxidant activity of which depends on the presence of free OH groups, especially 3-OH. Flavonoids are highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various other free radicals implicated in several disease.[16] They suppress reactive oxygen formation, chelate trace elements involved in free-radical production, scavenge reactive species and up-regulate and protect antioxidant defense.[17] Similarly, phenolics provide oxidative stress tolerance to plants. Crude extracts of fruits, herbs, vegetables, cereals and other plant materials rich in phenolics are nowadays very much in use in the food industry for their antioxidant properties and health benefits. The antioxidant activity of phenolic compounds is due to their redox potential.[18] Various chronic diseases including cancer, alzheimers's diseases, inflammatory bowel syndrome and cardiovascular diseases are delayed by phenolic antioxidants.[19] It has been reported that the antimicrobial effect of many herbal extracts correlates with their flavonoid contents.[20] Flavonoids have been found to be very important in the treatment of microbial infections.[21] The antimicrobial action shown by the extracts in our study may be attributed to the presence of flavonoids.

Antimicrobial activity

The antibacterial activity of methanolic and aqueous extracts of *Ajuga bracteosa in vitro* is presented in Table 3. The extracts showed antibacterial activity against both Gram positive and Gram negative bacteria. The methanolic extract showed the maximum zone of inhibition for all the four strains ranging from 12.8 - 17.0 mm. The aqueous extract showed

Table 1: Total Phenolic content of different extracts of aerial parts of *Ajuga bracteosa*.

Sample	Total phenolic content (mg gallic acid equivalent (GAE)/g DW)
Methanolic	337.26 ± 0.7
Aqueous	243.21± 1.2
Petroleum ether	159.5 ± 1.0

Values are expressed as mean ± SEM of experiment carried out in triplicate.

Table 2: Total Flavonoid Content of *Ajuga bracteosa*.

Sample	Total Flavonoid content (mg rutin equivalent/g dry weight)
Methanolic	213.52 ± 1.0
Aqueous	160.14 ± 0.8
Petroleum ether	83.03 ± 1.2

Values are expressed as mean ± SEM of experiment carried out in triplicate.

Table 3: Zones of inhibition measured against various bacterial strains for *Ajuga bracteosa* and standard streptomycin.

Bacterial Strains	Extract/standard	Conc. (µg/ml)	Zone of Inhibition(mm)
S. aureus	Aqueous	50	12.9±0.3
		100	14.3±0.3
		200	15.8±0.3
	Methanolic	50	13.3±0.33
		100	14.5±0.2
		200	16.1±0.2
	Streptomycin disc	10 µg	20.4±0.8
P. vulgaris	Aqueous	50	12.8±0.3
		100	13.9±0.1
		200	14.8±0.1
	Methanolic	50	13.8±0.1
		100	14.5±0.1
		200	15.7±0.2
	Streptomycin disc	10 µg	20.3±0.8
E. coli	Aqueous	50	13.4±0.1
		100	14.6±0.1
		200	15.7±0.3
	Methanolic	50	13.9±0.2
		100	14.8±0.2
		200	16.7±0.1
	Streptomycin disc	10 µg	21.0±0.6
S. typhi	Aqueous	50	13.9±0.5
		100	15.0±0.1
		200	16.9±0.2
	Methanolic	50	14.9±0.2
		100	15.8±0.3
		200	17.0±0.2
	Streptomycin disc	10 µg	20.5±0.8

Values are expressed as mean ± SEM of triplicate experiments

maximum activity against *S. typhi,* with a zone of inhibition 16.9 mm. The extracts were also evaluated for antifungal activity against three fungal strains as shown in Table 4.

It has been proposed that the antimicrobial potential of many plant extracts is positively related to total phenolic and flavonoid content of extracts.[22] From earlier times phenols have been reported to have a broad spectrum of biological action, including cardio-protective, vasodilator, antithrombotic and antimicrobial actions.[23] Also, it has been seen that many flavonoids are active against infection by forming complexes with different proteins present inside the cell wall of bacteria.[24] Flavonoids have also been reported to be involved in the inhibition of nucleic acid biosynthesis and other metabolic processes.[25] These have also been reported to inhibit spore germination of plant pathogens.[26] Moreover, these flavonoids are synthesized by plants in response to microbial infection. Phenolic compounds with a C_3 side chain at a lower level of oxidation and containing no oxygen have often been reported as possessing antimicrobial action.[27] The partially hydrophobic nature of these compounds has also been implicated for their antimicrobial activity. The mechanism of the toxicity of polyphenols against microbes may be related to the inhibition of hydrolytic enzymes (proteases) or other

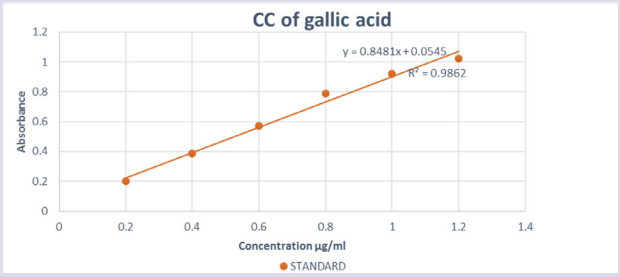

Figure 1: Calibration curve of standard gallic acid for determination of Total Phenolic Content.

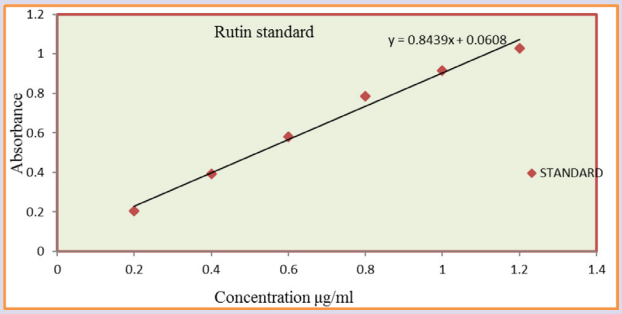

Figure 2: Calibration curve of standard Rutin for determination of Flavonoid Content.

Table 4: Zones of inhibition measured against three fungal strains for *Ajuga bracteosa* and standard Fluconazole.

Fungal strains	Extract/ standard	Concentration (mg/ml)	Zone of inhibition (mm)
P. Chrysogenum	Methanolic	50	14.2±0.1
		100	15.3±0.1
		200	16.0±0.2
	Aqueous	50	12.6±0.2
		100	14.6±0.3
		200	15.0±0.2
	Fluconazole disc	25 µg	18±0.2
A. fumigatus	Methanolic	50	14.5±0.3
		100	15.2±0.3
		200	16.7±0.2
	Aqueous	50	13.6±0.2
		100	14.7±0.2
		200	15.2±0.4
	Fluconazole disc	25 µg	18±0.2
S. cervesaie	Methanolic	50	13.8±0.1
		100	14.9±0.3
		200	16.9±0.2
	Aqueous	50	11.2±0.2
		100	14.5±0.2
		200	15.3±0.2
	Fluconazole disc	25 µg	18.1±0.5

Values are expressed as mean ± SEM of triplicate experiments.

interactions that inactivate microbial adhesions, cell envelope transport proteins and non-specific interactions with carbohydrates.[28] Various studies have reported the antifungal and antimicrobial activity of phenolic and flavonoid compounds.[29-31] Our study is in complete affirmation of these studies. Isolation of the responsible phytoconstituents is necessary for fully elucidating the antibacterial activity of these crude extracts. Also, the highest antibacterial activity in our study was achieved by the methanolic extract against *S. typhi*, with an inhibition zone of 17.2 mm. *S. typhi* causes typhoid fever against which no vaccine is available commercially, for children below 2 years of age.[32] Because of its high efficacy against *S. typhi*, the extract may be effective in the treatment of infections resulting from multi drug resistant *S. typhi.* These infections have rapidly emerged and spread worldwide, resulting in high rates of morbidity and mortality.[32-34] Our study may have implications in treating these resistant forms of microbes as the non-selective use of commercially available antimicrobial drugs has developed multiple drug resistance in humans.[35] So, there is a need of hour is to produce alternative antimicrobial drugs obtained from different sources including medicinal plants, which may counter this problem.

CONCLUSION

This study on *Ajuga bracteosa* shows that the plant is a potential source of antioxidant and antimicrobial phytoconstituents. The high content of phenols and flavonoids might be responsible for this antibacterial activity. In the future, *Ajuga bracteosa* could be used to prepare new antimicrobial drugs to treat infectious diseases caused by resistant strains. Furthermore, isolation of active constituents that show a broad spectrum of pharmacological potential should be carried out.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

DNA: Deoxyribonucleic acid; **NaNO2:** Sodium nitrite; **NaOH:** Sodium Hydroxide; **AlCl3:** Aluminium Chloride; **SDA:** Sabouraud Dextrose Agar; **DMSO:** Dimethyl sulfoxide; **OH:** Hydroxyl; **C:** Carbon; ***S. typhi:*** *Salmonella typhi;* ***S. cerevisiae:*** *Saccharomyces cerevisiae;* ***P. chrysogenum:*** *Pencillium chrysogenum;* ***A. fumigatus:*** *Aspergillus fumigatus*.

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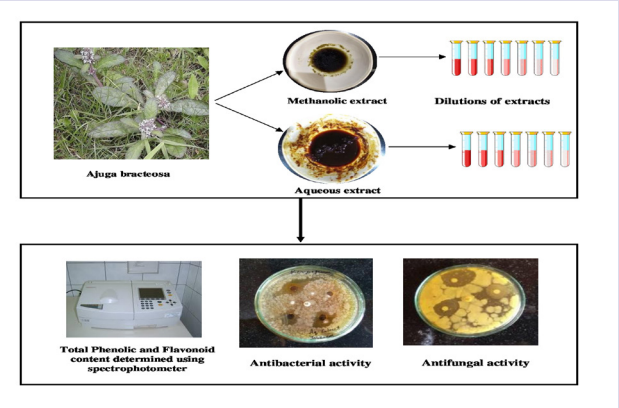
PICTORIAL ABSTRACT



SUMMARY

- Both methanolic and aqueous extracts of *Ajuga bracteosa* Wall ex. Benth showed antibacterial activity against both Gram positive and Gram negative bacteria including *E. coli* (MTCC 443), *Salmonella typhi* (MTCC 1688), *Proteus vulgaris* (MTCC 426), and *Staphylococcus aureus* (MTCC 96). The aqueous extract showed maximum activity against *S. typhi*, with a zone of inhibition 17.0 mm.
- The extracts also exhibited antifungal activity against *S. cerevesiae* (MTCC 443), *P. chrysogenum* (1688), *A. fumigatus* (MTCC 426).
- Methanol extract showed the highest antifungal activity against *S. cervesaie* with a zone of inhibition of 16.9mm.
- Methanolic extract contained the highest phenolic and flavonoid content (337.26 gallic acid equivalents/g) and (213.52 rutin equivalents/g) respectively.

ABOUT AUTHORS

G. N. Bader leads a research team in Pharmacology at Department of Pharmaceutical Sciences of University of Kashmir, Srinagar, India. Besides clinical Pharmacology, his research includes evaluation of some Himalayan medicinal herbs for their pharmacological activity/medicinal potential. Swertia Petiolata, S.tetragona, Polygonum alpinum, P.hydropiper, Arisaema propinquum, Acorus calamus, *Ajuga bracteosa*, Senecio laetus etc are some of the evaluated plants. Besides various projects, the team is having more than 20 publications in international and peer reviewed journals.