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In-Vitro Antioxidant Activity, Total Phenolic, Flavonoid and Tannin Contents in the *Ajuga Bracteosa Wall. Ex Benth*, Grown at Middle Hill Climatic Condition of Western Himalayas

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ABSTRACT

The antioxidant activity of aqueous and alcoholic extracts of different plant parts *viz,* leaves, flower, stem and root of *Ajuga bracteosa* was investigated against various *in-vitro* antioxidant assays. The total phenolic, flavonoid and tannin contents also estimated. The results revealed the significant antioxidant potential and variation in the IC_{50}, EC_{50} and phytochemical contents among all the plant parts. The aqueous extract of leaves exhibited significantly ($P<0.05$) highest antioxidant activity on 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acixd), 2,2-diphenyl-1-picryl hydrazyl, and potassium ferricyanide reducing power assay, with IC_{50} values of 0.2707±0.0008, 0.4409±0.0020, and EC_{50} value 0.3413±0.0030 mg/mL, respectively, followed by the other parts of the plant. The leaves extract also possess the highest total phenolics, flavonoid and tannin contents among all the parts. Similarly, the aqueous extract is better than the alcoholic extract of different parts as far as phytochemical contents and antioxidant activity concerned. The present study revealed that the aqueous extract of leaves had the highest antioxidant potential, which correlated with the high level of total phenolic flavonoid, and tannin contents. Therefore, higher the phytochemical contents, higher will be the antioxidant potential.

Keywords: Inhibition concentration; Phytochemical contents; Ascorbic acid

1. INTRODUCTION

An imbalance between the production of reactive oxygen species (ROS) and normal physiology of the body originates oxidative stress. The oxidation by free radicals give rise to the disintegration of the cell membrane, DNA mutation and damage of membranous protein, also a number of diseases can initiate or propagate, like diabetes, liver injury, rheumatism, cardiovascular disorders and cancer[1]. An antioxidant biomolecule can detain or interrupt the oxidation of molecules by oxidising themselves, so these are often called as reducing agents such as thiol, ascorbic acid, polyphenols etc[2]. Thus, antioxidants may improve the quality of life by preventing the origin of degenerative diseases. Due to their beneficial effects, the investigations are focused on naturally occurring antioxidant molecules, especially plant phytochemicals viz., phenolic compounds, flavonoids, carotenoids, benzoic acid derivatives, proanthocyanidins, coumarins, stilbenes and lignins to replace the synthetic antioxidants compounds, which have various side effects. The medicinal plants contain several phytochemical constituents, which attributed to the antioxidant potential and prevent chronic disease progression[3,4].

Ajuga bracteosa Wall. Ex Benth (Lamiaceae) commonly known as Neelkanthi and in Kumaon region of Uttarakhand, it is called as 'Ratapatha' or Ratpatiya. It is a perennial erect or ascending hairy herb, distributed in subtropical and temperate regions at an altitude of 1500 m[5]. Many compounds like γ-sitosterol, β-sitosterol, tri-acontanyl docosanoate, and tetra-cosanoic acid have been isolate from aerial parts[6]. This herb used since pre-historic times, and it also recommended in Ayurveda for the treatment of gout, rheumatism, amenorrhea and palsy. In the Kumaon region of Uttarakhand, the leaves of the plant used for the cure of diabetes as folk medicine, which is free radical damage to the body[5,7]. Rahman[8], *et al.* investigated the antioxidant and free radical scavenging activity in methanolic extract of different plants part viz., stem bark, root bark, leaves, and flowers of *Tabebuia pallid*. The study revealed that the highest total antioxidant capacity showed by the leaves of the plant, followed by the other parts.

The objective of the present study was to determine the total phenolic, tannin, and flavonoid contents, and also evaluate the *in vitro* antioxidant potential in terms of inhibition concentration (IC_{50} value) in the different plant parts of *Ajuga bracteosa.* Further, the correlation between the phytochemical contents and the antioxidant activity also established.

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Plant Material

The different plant parts viz. leaves, flowers, stems and roots of *Ajuga bracteosa* (100 g) were collected in the flowering season from Defence Institute of Bio-Energy Research Fd. Stn., Pithoragarh, Uttarakhand (India), located at 5500 feet altitude in 29°35'N 80°13'E in the middle

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Himalayan region of Uttarakhand, India (Fig. 1), dehydrated (in a chamber below 40 °C), powder with the help of grinder and stored for the current study. The plant material (30 gm dried powder) was extracted by cold maceration with 300 mL water by occasionally shaking, and kept for 24 h. The organic filtrate collected was then concentrated and same method was followed for the alcoholic extract using ethanol (300 mL). For the determination of antioxidant activity, Total phenolic, flavonoids and tannin contents, the solution was prepare by dissolving concentrated plant extracts with water (100 mg/10 mL).

Figure 1. ***Ajuga bracteosa*** **plant.**

2.2 Chemicals

2,6-dichlorophenolindophenol, sodium carbonate, potassium permanganate ($KMnO_4$), potassium persulfate ($K_2S_2O_8$), ferric chloride ($FeCl_3$), potassium ferricyanide (K3Fe(CN)6) aluminium chloride ($AlCl_3$), potassium acetate (CH_3COOK), Ascorbic acid, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), tannic acid, 2, 2- azino-bis (3-ethyl benzothiazoline-6-sulfonic acids) diammonium salt (ABTS), Folin-Ciocalteau reagent and oxalic acid. All the reagents were purchased from Sigma Chemicals USA and of analytical grade.

2.3 Determination of Antioxidant Activity

For the determination of antioxidant activity by different antioxidant assays, the solution of plant extracts (100 mg/10 mL) diluted according to the required concentrations. The Ascorbic acid (0.1 mg/mL) was use as the reference standard for all methods.

2.3.1 Free Radical Scavenging by ABTS Assay

The antioxidant capacity was evaluate by the reported method[9] depends on the scavenging of ABTS free radical (2,2-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid)). In this method, 7 mM ABTS solution and 2.45mM potassium persulphate solution mixed in equal volume and allow to react them for 12-16 hr in the dark to produce a blue-green chromogen, ABTS radical cation ($ABTS^{\bullet+}$). Before, performing the assay, the blue-green resultant solution diluted with the methanol for obtaining the initial absorbance of 0.702±0.001 at 734 nm. The antioxidant effects observed by mixing 1ml of ABTS solution with 00-50 µl of test samples (make up to 1 ml) and measured the absorbance after 5-7 min at 734 nm, the decrease in the absorbance observed due to hydrogen donating availability of ABTS radical cation, inducing a change in colour of $ABTS^{\bullet+}$ radical cation to colourless ABTS[10]. The percentage of free radical scavenging activity of $ABTS^{\bullet+}$ radicals were determine at different concentrations by using the formula:

FRSA (%) = [(Absorbance of control - Absorbance of test)/ Absorbance of control] ×100,

The IC_{50} value calculated as:

IC_{50} value (mg/mL) = (Concentration of test / 50% nearest FRSA) × 50

2.3.2 Free Radical Scavenging by DPPH Assay

The antioxidant capacity was estimate by the reported method[10,11] relies on the scavenging of DPPH free radical (1,1-diphenyl-2-picryl-hydrazyl). Herein, DPPH methanolic solution (2 mL, 0.135mM) mixed with different aliquots of extract, after 40 min, the absorbance was measured at 517nm against methanol as blank. The percentage of free radical scavenging activity of DPPH radicals was determine at different concentrations, also IC_{50} value calculated by using the formula mentioned above.

2.3.3 The Potassium Ferricyanide Reducing Power (PFRAP) Assay

The reducing ability was estimate by the earlier reported method[12]. The compounds having the antioxidant activity form a potassium ferrocyanide complex by reacting with potassium ferricyanide. Further, with ferric trichloride, it produces a blue coloured ferric ferrocyanide complex, at 700 nm[10]. In brief, different aliquots of extracts mixed with the phosphate buffer (6.6 pH) and 2.5 mL of $K_3Fe(CN)_6$ and then incubated for 20 min at 50 °C. After the incubation, 2.5 mL of 10% trichloroacetic acid added to the above mixture and centrifuged for 10 min. 2.5 ml of supernatant mixed with 2.5 mL of distilled water and 0.5 ml of 0.1 % $Fecl_3$ solution, and then measure the absorbance at 700 nm. EC_{50} value (mg/mL) was calculate to measure the reducing ability of the sample.

2.4 Determination of Total Phenolic Contents

The phenolic contents (TPC) of the sample determined following the Folin- Ciocalteu method[13]. In the method, the different extracts (100 µl) mixed with water up to 3 mL and 0.5 mL of Folin-Ciocalteu reagent. After 3 min, 20 % sodium carbonates (2 mL) was mixed thoroughly, and then boil the mixture for 1 min in a water bath. A blue colour was develope due to the formation of the complex by the redox reaction of phosphomol**y**bdic acid (folin ciocalteau reagent) with a maximum absorbance at 650 nm. The total phenolic contents were measure by catechol calibration curve and expressed as catechol equivalent (CE) in mg/g on a dry weight basis.

2.5 Determination of Total Flavonoid Contents

The flavonoid contents (TFC) of the sample were measure by the aluminium chloride method[14] (colourimetric method). In

the assay, test sample (100 µl) was mixed with 80% ethanol and subsequently with 95% ethanol. After that aluminium chloride solution added followed by potassium acetate solution. The mixture was thoroughly mixed and incubated for 30 min. Absorbance was then measured at 415nm versus the blank (except of Aluminium chloride). The total flavonoid contents were measure by quercetin calibration curve and expressed as quercetin equivalent (QE) in mg/g on a dry weight basis.

2.6 Determination of Total Tannin Contents

Total tannin contents (TTC) of the sample were measure by the Folin Denis method[15]. Based on the method, the test sample (100 µl) was pour to the volumetric flask (50 ml) containing 30 ml distilled water and then added Folin Denis reagent, 35% sodium carbonate and finally make up the volume. After that, test solutions heated at 10-20 °C for 30 min. Absorbance was then measure at 700 nm. The total tannin contents measured by tannic acid calibration curve and expressed as tannic acid equivalent (TAE) in mg/g on a dry weight basis.

2.7 Statistical Analysis

The data was characterise by Mean ± Standard Deviation (SD) (n=3). The obtained data were interprete by One-way analysis of variance (ANOVA) and Duncan's test at 0.05 probability level using SPSS 16.0 Software. The difference between the means ($P<0.05$) was consider to be significant.

3. RESULTS

3.1 Total Phenolic Contents (TPC)

In aqueous extract, phenolic contents varied from 5.27 to 10.46 mg CE/g d.w. Maximum phenolic contents (10.46 mg/g) found in aqueous extract of leaves followed by aqueous extract of the root (9.44 mg/g). While, least concentration of phenolics recorded from aqueous extract of stems. The phenolics contents in the alcoholic extract were ranged from 2.51 to 6.46 mg CE/g d.w. In the alcoholic extract, highest phenolics contents found in leaves (6.46 mg/g) followed by the alcoholic extract of the roots (6.11 mg/g). Maximum phenolic contents were found in aqueous extracts of different parts as compared to the alcoholic extracts. Table 1 shows the total phenolic contents present in aqueous and alcoholic extracts of different parts of the plant, and the leaf extract showed a significantly higher amount followed by the other parts.

3.2 Total Flavonoid Contents

In the present study, flavonoid contents ranged from 1.92 to 7.50 mg QE/g d.w. in aqueous extracts of *Ajuga bracteosa*. The maximum flavonoids (7.50 mg/g) were display by the aqueous extract of leaves followed by roots extract (5.80 mg/g). In the alcoholic extract of plant, flavonoids varied from 0.88 to 4.66 mg QE/g d.w. The highest flavonoid contents (4.66 mg/g) found in the leaves followed by an alcoholic extract of roots (4.19 mg/g), while, least quantity of flavonoids exhibited in the stem. Aqueous extract displayed the highest flavonoid contents followed by the alcoholic extract. Table 1 shows the total flavonoid contents present in aqueous and alcoholic extracts of different parts of the plant, and the leaf extract showed a significantly higher amount followed by the other parts.

3.3 Total Tannin Contents

In aqueous extracts of all plant parts of *Ajuga bracteos,* the tannin contents varied from 7.29 to 21.06 mg TAE/g) d.w. The maximum quantity of tannin (21.06 mg/g) recorded in the leaves followed by the root of the plant (16.66 mg/g). While the stem shows, least tannin contents. In alcoholic extracts of the plant, tannins ranged from 3.12 to 12.72 mg TAE/g) d.w. The highest tannins found in leaves (12.72 mg/g) followed by the roots (10.64 mg/g). While minimum tannin contents were present in the stem of the plant. Aqueous extract displayed the highest tannin contents followed by the alcoholic extract. Table 1 shows the total tannin contents present in aqueous and alcoholic extracts of different parts of the plant, and the leaf extract showed a significantly higher amount followed by the other parts.

3.4 Determination of Antioxidant Activity

The in-vitro antioxidant activity was assessed by three different assays viz. ABTS, DPPH, and PFRAP assay. The capacity of the extracts to inhibit the ABTS, DPPH radicals and reducing power were expressing by estimating IC_{50} value (mg/mL) for both aqueous and alcoholic extracts. Table 2 shows the antioxidant activity in aqueous and alcoholic extracts of different parts of the plant as shown in Table 2.

3.4.1 Free Radical Scavenging by ABTS Assay

The inhibition concentration (IC_{50}) was estimate in the study, minimum IC_{50} value means maximum anti-oxidant activity. In aqueous extract; minimum IC_{50} value i.e. maximum

Table 1. Total phenolics, flavonoid and tannin contents in the aqueous and alcoholic extracts of different plant parts of *Ajuga bracteosa*

Sample	Total phenolic contents (mgCE/g) d.w.		Flavonoid contents (mgQE/g) d.w.		Tannin contents (mgTAE/g) d.w.	
	Aq	Alc	Aq	Alc	Aq	Alc
Leaves	10.46±0.09a	6.466±0.0709a	7.503±0.075a	4.663±0.122a	21.06±0.3983a	12.723±0.4041a
Flowers	9.136±0.1159c	5.49±0.0519c	3.366±0.023c	1.66±0.00c	14.346±0.4041c	10.08±0.3459b
Stem	5.276±0.095d	2.516±0.1429d	1.923±0.0461d	0.886±0.023d	7.29±0.35d	3.125±0.345c
Root	9.44±0.09b	6.11±0.1558b	5.806±0.0461b	4.193±0.0461b	16.663±0.345b	10.646±0.5291b

(* AQ- Aqueous extract, Alc Alcoholic extract) The value represented as (Mean ± SD) ($n = 3$).
The values not sharing the same letter are significantly different (Duncan's test) at $P < 0.05$ probability level.

Table 2. Antioxidant activity in the aqueous and alcoholic extracts of different plant parts of *Ajuga bracteosa*

Sample	ABTS (IC_{50} = mg/mL) d.w.		DPPH (IC_{50} = mg/mL) d.w.		PFRAP (EC_{50} = mg/mL) d.w.	
	Aq	Alc	Aq	Alc	Aq	Alc
Leaves	0.2707±0.0008b	0.3521±0.0097b	0.4409±0.0020b	0.5418±0.0122b	0.3413±0.0030b	0.3743±0.0035b
Flowers	0.4931±0.0388d	0.6752±0. 056d	0.6161±0.0015d	0.6883±0.0030d	0.4573±0.0011d	0.4983±0.00577d
Stem	0.7173±0.0017e	1.1773±0.0013e	1.4323±0.0513e	1.5563±0.0466e	0.8083±0.0097e	0.9003±0.0085e
Root	0.3118±0.0005c	0.563±0.0031c	0.4933±0.0008c	0.602±0.0018c	0.372±0.0017c	0.4667±0.0045c
Ascorbic acid	0.00449±0.00001a		0.0096±0.00002a		0.0066±0.00006a	

(* AQ- Aqueous extract, Alc Alcoholic extract) the value are represented as (Mean ± SD) (n = 3).
The values not sharing the same letter are significantly different (Duncan's test) at $P < 0.05$ probability level.

antioxidant activity was exhibited by leaves (0.2707 mg/mL) followed by the root (0.3118 mg/mL). While the stem extract shows least antioxidant activity. Similarly, the alcoholic extract of leaves showed the highest antioxidant activity (0.3521mg/mL) followed by roots of the plant (0.563 mg/mL) and minimum antioxidant activity was exhibit by the stem. The ascorbic acid used as a reference standard, and the IC_{50} value was 0.00449 mg/mL.

3.4.2 Free Radical Scavenging by DPPH Assay

The DPPH assay commonly used and relatively brief method for the determination antioxidant potential[16]. The disappearance of the colour of DPPH radical at 517 nm wavelengths monitored by decreased optical density[17-19]. The aqueous extract of leaves displayed the highest antioxidant activity (0.4409 mg/mL) followed by the root extract (0.4933 mg/mL) and minimum antioxidant activity displayed by the stem of the plant. In the alcoholic extracts of the plant, IC_{50} varied from 0.5418 to 1.5563 mg/mL. The highest antioxidant activity displayed by the leaves extract (0.5418 mg/mL) followed by root extract (0.602 mg/mL). The ascorbic acid used as a reference standard, and the IC_{50} value was 0.0096 mg/mL.

3.4.3 The PFRAP Assay

The antioxidant potential by reducing power assay is associated with the reduction process, in which splitting of free radical chain occurs by contributing a hydrogen atom[18]. In the assay, the antioxidant reduces the Fe^{3+}/Ferricyanide complex to ferrous and blue colour, thus formed, which measured at 700 nm[19]. The reducing power (EC_{50}) in aqueous extracts of plants ranged from 0.3413 to 0.8083 mg/mL. The results revealed that the maximum reducing power observed in leaves extract (0.3413 mg/mL) followed by root extract (0.372 mg/mL) and minimum activity displayed by the stem. In alcoholic extract, leaves extract displayed highest reducing power (0.3743 mg/mL) followed by in roots extract (0.4667 mg/mL).

3.5 Correlation Analysis

The correlation analysis between the phytochemical contents (TPC, TFC and TTC) and antioxidant ability (IC_{50} and EC_{50} Values) of extracts represented in Table 3, the phytochemical contents exhibited highly significant correlation with the ABTS, DPPH and PFRAP assay. The results suggested that the phytochemical contents *viz,* total phenolic, flavonoid and tannin contents significantly contributed to the antioxidant activity of the different parts of the plant extracts as shown in Table 3.

4. DISCUSSION

Antioxidant compounds are known for their capabilities to prevent or delay the oxidation of other molecules. They inhibit the free radical chain reactions by donating an electron without becoming themselves free radicals, and nowadays, it becomes a vital part of our body. In the present study, antioxidant activity and phytochemical contents of the different plant parts of *Ajuga bracteosa* has been assessing. The results revealed the antioxidant potential and phytochemical contents of the aqueous and alcoholic extracts of the plant. The recent work by Kayani[19], *et al.* on *Ajuga bracteosa* to evaluate the antioxidant activity and phytochemical contents in methanolic and chloroform extract of aerial and root portion. It suggested

Table 3. Correlation Analysis between antioxidant activities (IC_{50} and EC_{50} Values) and phytochemicals content of different plant parts of *Ajuga bracteosa*

	ABTS Assay	DPPH Assay	PFRAP Assay	TPC	TFC	TTC
ABTS Assay (IC_{50})	1	0.881**	0.919**	-0.887**	-0.887**	-0.916**
DPPH Assay (IC_{50})		1	0.994**	-0.788*	-0.77*	-0.85**
PFRAP Assay (IC_{50})			1	-0.81*	-0.809*	-0.877**
TPC				1	0.853**	0.967**
TFC					1	0.923**
TTC						1

* Indicates correlation significance at P < 0.05, ** Indicates correlation significance at P < 0.01 (2-tailed).

that the aerial part shows higher antioxidant activity followed by the root. The variation observed between the antioxidant activity, and phytochemical contents depends on the part of the plant and the extraction solvent used for the study. The present study compared the antioxidant potential of aqueous and alcoholic extracts of the different parts against different free radicals. The antioxidant activity estimated by inhibition concentration (IC_{50}); the leaves displayed better antioxidant activity followed by the other parts of the plant. Similarly, the leaves showed the higher total phenolics, flavonoids and tannin contents followed by other parts. The phenolic compounds present in vegetables and fruits have attracted a lot of attention due to their potential anti-oxidant activities[22-24]. The results revealed the variation of IC_{50}, EC_{50} values, total phenolic, flavonoids and tannin contents that may attributed to different part of the plant used for the study. Further, this difference could attribute to an uneven distribution of phytochemical contents in the different parts of the plant and therefore, varying its antioxidant activity[3]. The results are in a satisfactory agreement with the previous work[19-21]. In the present study, correlation analysis represents a negative correlation among the IC_{50}, EC_{50} values and phytochemical contents, which reflects that higher the phytochemical contents, lesser the IC_{50} value, therefore higher the antioxidant activity. This result was consistent with the previous research, reported the correlation between phytochemical contents and antioxidant potential[4].

5. CONCLUSION

Hence, it can concluded from the study that among different plant parts of *Ajuga bractiosa*, leaves of the plant displayed highest antioxidant activity followed by roots and least antioxidant activity exhibited by the stem of the plant. As far as phytochemical content are concerned leaves possessed maximum phenolic, flavonoids and tannins contents followed by the root of the plant. It is further, observed that aqueous extract of the plant exhibited better antioxidant activity and phytochemical contents than alcoholic extract. Therefore, the antioxidant activity of different parts of the plants attributed to the total phenolic, tannin and flavonoid contents. Consequently, the study suggests that the leaves can employed as a direct source of antioxidants, and there is a need for further study of its isolated compounds and biological activities.

CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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CONTRIBUTIONS

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