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Variation in Antioxidant Activity and Antioxidant Constituents in Different Extracts of *Bacopa monnieri* Linn. with the Plant Maturity, Grown in Middle Hill Climatic Condition Western Himalayas'

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Abstract: A comparison of the antioxidant components and antioxidant activity of *Bacopa monnieri* Linn. in aqueous and alcoholic extracts at different growth stages (in every 15 days interval) till plant maturity (till 90 days) was carried out. In addition, a correlation analysis between antioxidant activity and antioxidant components was also performed. The research exhibited that the alcoholic extract contains greater antioxidant constituents with maximum antioxidant activity in comparison to the aqueous extracts. The results showed that the 15 days old plant exhibited the highest antioxidant activity with minimum IC_{50}/EC_{50} value by different antioxidant assays, such as DPPH (0.087 mg/ml), ABTS (0.084 mg/ml), and FRAP (0.614 mg/ml) in the alcoholic extract. In correlation analysis also found that the ABTS assay's IC_{50} value had a considerably positive correlation with the DPPH and FRAP tests' IC_{50} and EC_{50} values, respectively, and that antioxidant components had a negative correlation with IC_{50}/EC_{50}. In conclusion, the study found that 15-day-old plant extracts have the best antioxidant potential and the highest concentration antioxidant components when compared to other plant growth stages.

Keywords: ABTS, Antioxidant constituents, Correlation, Growth stages, IC_{50} value.

I. Introduction

An antioxidant is a substance that inhibits the oxidation of other compounds. An oxidizing agent gains electrons from a molecule during the chemical reaction known as oxidation. These oxidation mechanisms have the potential to produce reactive oxygen species, which can set off a series of events that can kill or injury to the cells [1]. Antioxidants have the capacity to stop chain reactions by capturing free radicals and delaying oxidation processes. To resist the harmful property of oxidants, the human body has a built-in defence mechanism [2]. Antioxidants can reduce oxidative stress and either stop or reverse damage caused by scavenging reactive oxygen species. Antioxidant enzymes that act as the defence against free radical damage during oxidative stress [3, 4]. Non-enzymatic antioxidants known as phenolic compounds provide defence by converting oxidants into non-radical end products or diverting radicals to less harmful state [2]. Vegetables and fruits rich in vitamins offer a strong defence against free radical formation [5]. Antioxidants in the diet help to reduce oxidative stress and protect against a wide range of degenerative diseases [6, 7].

Bacopa monnieri Linn (syn. *Herpestis monniera* L.) is a small creeping perennial herb of the family Scrophulariaceae, locally referred to as "Brahmi" or water hyssop. The herb found in the

tropical regions of India and neighbouring countries (Nepal, China, Sri Lanka), Florida, and Southern USA up to 1500 m altitude [8, 9]. The plant has sessile, succulent leaves which oppositely arranged with soft stems (10-30 cm long) and have white to pale blue flowers [8, 10]. It is the second most important medicinal herb and due to over exploitation enlisted as an endangered plant [8]. The plant utilised in Ayurvedic medicine for 3000 years and classified as 'medhyarasayana', which means herbs that rejuvenate memory and intelligence; therefore, the herb is recommended for memory improvement and management of various mental illness [11]. The herb was traditionally used to cure inflammation, fever, pain, epilepsy, asthma, memory loss, skin problems, and neurodegenerative diseases [8, 12]. Several compounds were isolated from the herb such as alkaloids, glycosides, saponins, stigmasterols, flavonoids, and phenolic compounds, which showed different pharmacological activities [13, 14]. The active constituents of the plant are brahmine, bacoside A, bacoside B, hersaponin, herpestine, D-mannitol, nicotine, and monnierin [8]. These active constituents especially the bacosides A & B, and steroidal saponins are responsible for the learning and memory improvement efficacy [2, 15]. Anxiolytic action, anti-arthritic, anti-diabetic, antimicrobial, antioxidant, anti-cancer, anti-depressant, anti-parkinson's, and anti-convulsive activity are some of the pharmacological properties of the herb [16, 17].

The study performed to determine how the antioxidant components and antioxidant activity of aerial portions of *Bacopa monnieri* extracts varied over the course of the plant's development at 15-day intervals till maturity up to 90 days. A correlation analysis was also performed.

II. Material and Method

A. Extraction of the Aerial Portion of the Plant

The *Bacopa monnieri* plants were grown in open fields at the DIBER (DRDO), Pithoragarh (Uttarakhand). The aerial portions of the plant (50 g) were harvested every 15 days until the plant reached maturity, and was washed properly. For the investigation, the finely chopped pieces were dried (40 °C) and grounded to a fine powder. The dried powder of aerial portions of the plant was extracted using a cold maceration process. 10 gm plant powder was extracted with ethanol and water (100 mL) for alcoholic and aqueous extract, and kept for 24 hours in dark. The final solution was concentrated to obtain the final extracts and used to assess antioxidant components and antioxidant activity (Fig. 1).

B. Chemicals

The analytical chemicals utilised in the investigation obtained from Sigma Chemicals in the United States and E Merck India Ltd. The chemicals used were ABTS, ascorbic acid, DPPH, potassium ferricyanide ($K_3Fe(CN)_6$), potassium acetate (CH_3COOK), potassium persulphate ($K_2S_2O_8$), tannic acid, ferric chloride ($FeCl_3$), trichloroacetic acid, hydrochloric acid, sodium hydroxide, aluminum chloride ($AlCl_3$), sodium carbonate, sodium dihydrogen phosphate, nitric acid, ethanol, and methanol.

Fig. 1: *Bacopa monnieri* Linn. Plants

C. Antioxidant Activity Assessment

DPPH, ABTS, and FRAP assays (n=3), were used to estimate the antioxidant properties of the aerial portions of *Bacopa monnieri*. For the assays, ascorbic acid was utilized as a standard.

(i) Antioxidant Activity by ABTS Method

According to the Re *et al.* method, the *in-vitro* antioxidant activity were assessed [18]. Various extract concentrations (20-100 µl) were utilized in this procedure to test antioxidant activity, and the activity was observed in proportion to the degree of decolorization. Due to its availability as a hydrogen donor, antioxidants convert the colored radical cation ($ABTS^{\bullet+}$) into colorless ABTS. As per the method, the extract was taken into test tubes, with distilled water to make volume upto to 1 mL, and then ABTS solution was added. The absorbance was measured at 734 nm [19, 20]. In addition, the IC_{50} value was established by calculating the % FRSA of ABTS radicals using the formula.

$$\%\ FRSA = \frac{(\mathrm{Ac} - \mathrm{At})}{Ac} \times 100$$

(Ac = control absorbance, At = test absorbance)

(ii) Antioxidant Activity DPPH Method

The *in-vitro* antioxidant activity was estimated by calculating the inhibitory concentration (IC_{50}) using the Kedare *et al.* method [21]. The DPPH methanolic solution (0.1 mmol) was applied to various aliquots (20-100 µl) of each sample. After 40 minutes, absorbance was measured at 517 nm. Furthermore, the

IC_{50} value was determined by calculating the % FRSA of DPPH radicals using the same formula as described before.

(iii) FRAP Assay

The reducing capability of antioxidants derived from plant sources was estimated according to Maruthamuthu *et al.* method [22].

Various extract concentrations (20-100 µl) were employed to determine antioxidant activity according to the method, with minor modifications, and the activity was seen in connection to the degree of decolorization [19, 20].

D. Evaluation of Antioxidant Constituent

(i) Total Phenolic Contents (TPC)

Using Folin-Ciocalteu procedure, TPC of the extracts were evaluated [23]. The test tubes were added with 100 µl of the extracts and 3 ml of water according to the protocol. The folin-ciocalteu reagent was then added and the final solution was thoroughly mixed. After 3 minutes, the aforementioned mixture was mixed with a 20% sodium carbonates solution (2 mL), and the tubes were immersed in a boiling water bath for 1 minute. As a result of the interaction between the sample and the phosphomolybdic acid, which created a blue complex, the final solution acquires a blue colour. At 650 nm, absorbance was also measured [20, 24].

(ii) Tannin Contents (TTC)

The tannin concentration of the plant extract was estimated using the folin-denis method [20, 25, 26].

(iii) Flavonoid Contents

TFC of the extracts were determined using aluminium chloride procedure [27].

E. Statistical Analysis

The data were represented as Mean ± Standard Deviation (n=3). Using the SPSS 16.0 programme, the data were evaluated using one-way analysis of variance, Duncan's test, and LSD at $P<0.05$. At the $p<0.05$ and $p<0.01$ levels, the correlation between antioxidant activity and antioxidant components such as tannin, flavonoid, and total phenolic content was estimated.

III. Results and Discussion

A. Antioxidant Activity Assessment

From the results both the extracts of aerial parts of *Bacopa monnieri* with the development at intervals of 15 days until the plant reached maturity showed variation in the antioxidant activity as well as antioxidant constituents. Dutta *et al.* [28] examined the antioxidant activity of various extracts of *Bacopa monnieri* leaves. The study revealed that the ethyl acetate extract had the utmost phenolic content, whereas, the methanolic extract had the highest flavonoid concentration. Whereas, the maximum antioxidant potential was observed in the chloroform extract. Therefore, the extraction method utilized for the study has an impact on the variation in antioxidant activity and phytochemical contents [28]. In the current study, the antioxidant activity of aerial portions in various growth stages against various free radicals was studied in aqueous and alcoholic extracts. The antioxidant activity was measured using the IC_{50}, the lowest IC_{50} value correlates to the maximum antioxidant activity. The ABTS assay demonstrated that the aqueous extract of aerial portions had an IC_{50} value ranging 0.179 to 0.259 mg/ml until the plant achieved maturity. The plants with the highest antioxidant activity (lowest IC_{50} value) was exhibited by 15 days old plants (0.179±0.002 mg/ml), followed by 30 days old plants (0.193±0.004 mg/ml). Whereas,the alcoholic extract of aerial parts showed the IC_{50} value ranged from 0.084 to 0.187 mg/ml until the plant reached maturity. The highest antioxidant activity was observed in 15 days old plants (0.084±0.001 mg/ml) followed by the 30 days old plants (0.127±0.001 mg/ml) and the lowest antioxidant activity was found in 90 days days old plants.

The DPPH assay revealed a nearly comparable trend of antioxidant activity, with the aqueous extract of aerial portions exhibiting an IC_{50} value ranging from 0.178 to 0.578 mg/ml until the plant reached maturity. The highest antioxidant potential was found in 15 days old plants (0.178±0.001 mg/ml) followed by 30 days old plants (0.186±0.001 mg/ml). Whereas, the alcoholic extract of aerial parts showed the IC_{50} value ranged from 0.087 to 0.247 mg/ml from 15 days to 90 days old plants. The maximum antioxidant activity was showed in 15 days old plants (0.087±0.001 mg/ml) followed by 30 days old plants (0.105±0.003 mg/ml) while, the least antioxidant activity was found in the 90 days old plants.

The antioxidant activity was measured using the EC_{50} (effective concentration 50) value; a lower EC_{50} value indicates greater antioxidant activity. The FRAP experiment demonstrated that the aqueous extract of aerial portions had EC_{50} value varied from 0.866 to 5.55 mg/ml until the plant reached maturity (90 days). The maximum antioxidant activity was discovered in plants 15 days old plants (0.866±0.012 mg/ml), followed by plants 30 days old plants (1.43±0.197 mg/ml). The EC_{50} value of the alcoholic extract of aerial parts ranged from 0.614 to 4.50 mg/ml from 15 to 90 days plants. The utmost antioxidant activity was reported in 15-day-old plants (0.614±0.014 mg/ml), followed by 30-day-old plants (0.87±0.010 mg/ml), and 90-day-old plants had the lowest antioxidant activity.

Among different plant growth stages, the alcoholic extract of Brahmi had much higher antioxidant activity than the aqueous extract. Furthermore, 15-day-old plants had the highest antioxidant activity, whereas, 90-day-old plants had the lowest antioxidant activity. Fig. 2 and Fig. 3 depicted the antioxidant activity of both extracts.

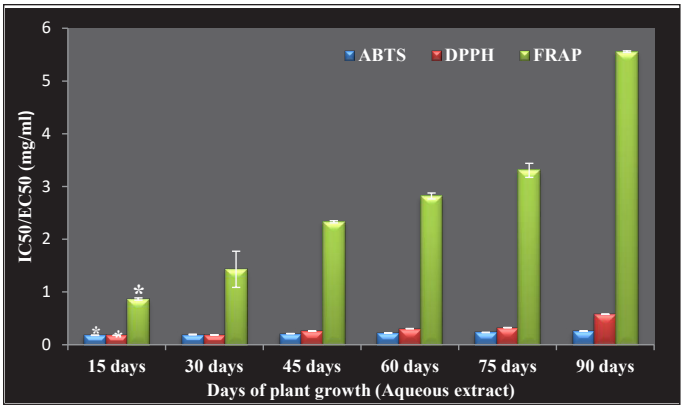

Fig. 2: Antioxidant Activity of Aqueous Extracts of Aerial Parts of *Bacopa monnieri*, the IC_{50}/EC_{50} Value with (*) are Significantly Different ($P<0.05$) According to Duncan's and LSD Test

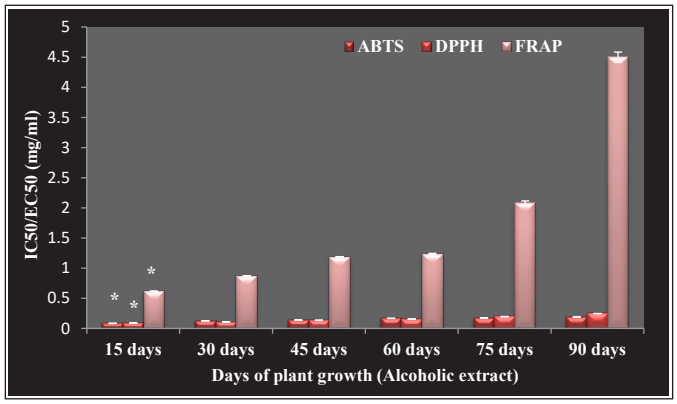

Fig. 3: Antioxidant Activity of Alcoholic Extracts of Aerial Parts of *Bacopa monnieri*, the IC_{50}/EC_{50} Value with (*) are Significantly Different ($P<0.05$) According to Duncan's and LSD Test

B. Estimation of Antioxidant Constituents

The phenolic compounds viz. flavonoid, tannin, and anthocyanin, demonstrated significant antioxidant activity in a variety of antioxidant assays [29] and have a shielding response against various reactive oxygen species [30].

The phenolic contents of both extracts ranged from 16.88 to 29.75 and 20.62 to 56.0 mg CE/gd.wt. from 15 days to 90 days, respectively. The maximum phenolic contents (29.75±**0.**235 mg/g) were showed in the aqueous extract of 15 days old plants followed by 30 days old plants (27.17±0.028 mg/g). Also, the alcoholic extract of 15 days old plant represented the highest phenolic contents (56.0±0.284 mg/g) followed by the 30 days old plants (53.28±0.065 mg/g). In different plant growth stages, the alcoholic extract of Brahmi had significantly higher phenolics concentration than the aqueous extract. Furthermore, 15-day-old plants had the highest phenolic content, whereas 90-day-old plants had the lowest phenolic level.

From 15 to 90 days, the flavonoid concentration of aqueous and alcoholic extracts of aerial portions ranged from 2.55 to 9.28 and 4.88 to 13.44 mg QE/gd.wt. plant, respectively. The aqueous extract of 15 days old plants had the highest flavonoid concentration (9.28±0.092 mg/g), followed by 30 days old plants (9.05±0.028 mg/g).

Similarly, the flavonoid concentration of the alcoholic extract of 15 days old plants exhibited highest concentration (13.44±0.235 mg/g), followed by 30 days old plants (12.52±0.051 mg/g). Among different plant growth phases, the alcoholic extract of Brahmi had significantly higher flavonoid contents than the aqueous extract. Furthermore, 15-day-old plants had the highest flavonoid concentration, whereas, 90-day-old plants had the lowest flavonoid level.

The tannin content of both extracts ranged from 11.23 to 62.03 mg TAE/gd.wt. till the plant reached maturity. The aqueous extract of 15 days old plants had the highest tannin concentration (62.03±1.59 mg/g), followed by 30 days old plants (58.07±1.79 mg/g). Similarly, the tannin content of the alcoholic extract of 15 days old plants was maximum (89.34±2.11 mg/g), followed by 30 days old plants (83.56±1.06 mg/g). Among different plant growth phases, the alcoholic extract of Brahmi had significantly higher tannin contents than the aqueous extract. Furthermore, 15-day-old plants had the highest tannin concentration, whereas, 90-day-old plants had the lowest flavonoid level. Fig. 4 and Fig. 5 depicted the total phenolic, flavonoid, and tannin concentrations of both extracts of aerial components at various growth stages.

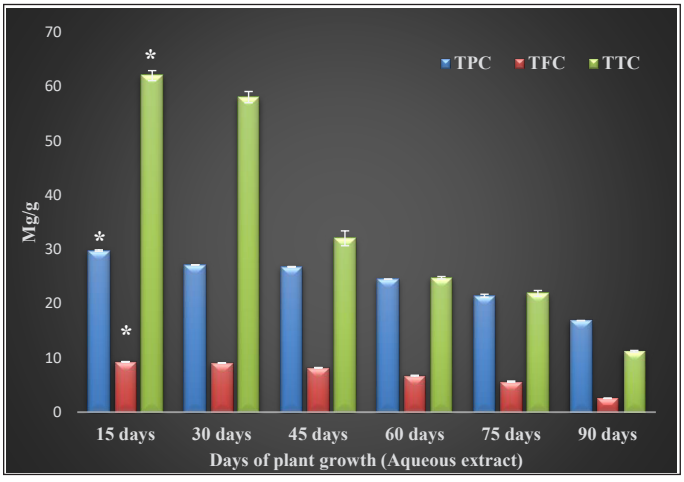

Fig. 4: Total Phenolic, Flavonoid and Tannin Contents of Aqueous Extract of Aerial Parts of *Bacopa monnieri* Grown in an Open Field Condition, the Values with (*) are Significantly Different ($P<0.05$) According to Duncan's and LSD Test

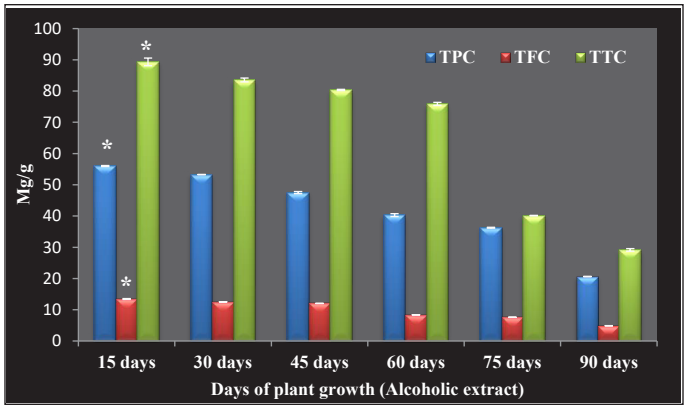

Fig. 5: Total Phenolic, Flavonoid and Tannin Contents of Alcoholic Extract of Aerial Parts of *Bacopa monnieri* Grown Inside Polyhouse Condition, the Values with (*) are Significantly Different (P<0.05) According to Duncan's and LSD Test

As a result, the current investigation demonstrated that the both extracts of aerial portions included significant amounts of antioxidant constituents. The findings showed that alcoholic extracts of plant aerial parts had more antioxidant components than water extracts. Furthermore, the both extracts of a 15-day-old plant contained much more antioxidant components than others plant stages.

Surveswaran *et al.* (2010) attribute deviation in phytochemical concentrations within plant portions to exact metabolic processes and physiological changes in the plant [29, 31]. The current study's findings agreed well with those of prior researchers [19, 32, 33].

C. Correlation Analysis

Table I shows the correlation analysis of antioxidant constituents with the antioxidant activity of both the exatracts of *Bacopa monnieri* aerial parts; the correlation coefficient found that the antioxidant constituents had a negative correlation with the IC_{50}/EC_{50} value of DPPH, ABTS, and FRAPS assays. There was also a strong positive correlation between the IC_{50} and EC_{50} values of the ABTS, DPPH, and FRAP assays (Table I).

As a result, the study concluded that the antioxidant components will be higher, the IC_{50}/EC_{50} value will be lower, and the antioxidant potential would be higher. The antioxidant components, according to the data, are the key contributors to antioxidant activity.

TABLE I: PEARSON'S CORRELATION ANALYSIS OF ANTIOXIDANT CONSTITUENTS WITH ANTIOXIDANT ACTIVITIES OF AQUEOUS AND ALCOHOLIC EXTRACTS OF *BACOPA MONNIERI*

Antioxidant Parameters	*Correlation Coefficient (r)*					
	IC_{50} ABTS Assay	*IC_{50} DPPH Assay*	*EC_{50} PFRAP Assay*	*TPC*	*FC*	*TC*
IC_{50} ABTS Assay	1	0.870**	0.766**	-0.937**	-0.900**	-0.912**
IC_{50} DPPH Assay		1	0.893**	-0.788**	-0.875**	-0.857**
EC_{50} FRAP Assay			1	-0.788**	-0.913*	-0.874*
TPC				1	0.915**	0.912**
FC					1	0.921**
TC						1

** Indicates correlation significance at P< 0.05 (2-tailed) and *Indicates correlation significance at P< 0.01 and TPC - Total Phenolic Content, FC - Flavonoid Contens, and TC - Tannin Contents.

Previous researchers had found a relationship between antioxidant components and antioxidant potential, was supported by the current findings [34, 35].

IV. Conclusion

The study concluded that alcoholic extracts of *Bacopa monnieri* had much higher antioxidant capacity and components than aqueous extracts. Furthermore, the 15-day-old plant exhibited highest antioxidant activity and antioxidant constituents, followed by the 30-day-old plant, and the 90-day-old plant had the lowest antioxidant activity and constituents. The antioxidant components of *Bacopa monnieri* aerial portions revealed a substantial negative correlation with antioxidant activity.

The antioxidant constituents are important for antioxidant activity. As a result, the conclusion provides substantial evidence that *Bacopa monnieri* can be utilized as a source of potent natural antioxidants for preparation of natural antioxidant herbal products.

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Conflict of Interest

There are no conflicts of interests.

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