

**PHYTOCHEMICAL AND ANTIOXIDANT ACTIVITY OF *BALIOSPERMUM MONTANUM* (WILLD.) MUELL. ARG.**

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**ABSTRACT**

**Objective:** The present study was carried out to evaluate the phytochemical constituents and antioxidant potential of the leaf, stem, root, and stem-derived callus extracts of *Baliospermum montanum*.

**Methods:** An *in vitro* regeneration protocol was developed for the induction of callus from stem segments cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and combination of 6-benzylaminopurine (BAP), 6-furfurylaminopurine (KIN), 1-naphthaleneacetic acid, and 2, 4-dichlorophenoxyacetic acid. The total phenol, flavonoid, and tannin content were determined according to standard methods. The antioxidant activity of methanolic extract was evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power assays.

**Results:** The maximum callus induction was observed on MS medium supplemented with a combination of KIN (0.5 mg/L) + BAP (3.0 mg/L). Methanolic extract of root and aqueous extract of leaf exhibited higher content of phenols. Whereas total flavonoids and tannin were maximum in methanolic extract of leaf compared to other extracts. The methanolic extract of *B. montanum* leaf had greater antioxidant activity than stem, root, and callus by DPPH and reducing power assays.

**Conclusion:** Our study suggests that the methanolic extracts of *B. montanum* showed potent free radical scavenging activity. Further studies are necessary for isolation and characterization of phytochemical compounds.

**Keywords:** *Baliospermum montanum*, Callus induction, 2, 2-diphenyl-1-picrylhydrazyl, Reducing power assay.

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**INTRODUCTION**

Plants have formed the basis of traditional medicine systems that have been in existence for thousands of years. Even in modern times, plant-based systems continue to play an essential role in health care. It has been estimated by the World Health Organization that approximately 80% of the world's population from developing countries rely mainly on traditional medicines (mostly derived from plants) for their primary health care [1]. The plant-based chemical compounds are classified into two classes; primary and secondary metabolites based on their chemical, biosynthetic origin, and functional groups. Primary metabolites are involved in growth and development, and secondary metabolites are in defense mechanism against harmful pests and infectious agents. Plant derived chemicals such as terpenoids, phenolics, alkaloids, flavonoids, glycosides, diterpenes, triterpenes etc., which shows better compatibility with the human body to resist against harmful diseases [2].

Most of the aromatic and medicinal plants contain chemical compounds with antioxidant properties. Several studies carried out on medicinal plants led to the development of natural antioxidant formulations for food, cosmetic, and other applications [3]. There is an increasing interest in natural antioxidants, for example, polyphenols present in medicinal and dietary plants, which might help to prevent oxidative damage. Naturally occurring antioxidants increase the antioxidant capacity of the plasma and reduce the risk of disease [4]. At present, keen interests and widespread researches on exogenous antioxidants from natural sources perhaps, due to the fact that they are less expensive, readily available and believed to have lesser side effects when compared to their synthetic counterparts [5].

*Baliospermum montanum* is commonly known as Danti, which belongs to the family Euphorbiaceae. It is found in tropical and subtropical Himalayas from Kashmir eastwards to Arunachal Pradesh. Root, leaf, and

seeds of the plants are used medicinally. The root contains phorbol ester belonging to diterpene hydrocarbon, namely, montanin, baliospermin, 12-deoxyphorbol-13-palmitate, 12-deoxy-5 $\beta$ -hydroxyphorbol-13-myristate, and 12-deoxy-16-hydroxyphorbol 13-palmitate. Leaves contain  $\beta$ -sitosterol,  $\beta$ -D-glucoside, and hexacosanol. Steroids, terpenoids, and flavonoids are also reported in this plant [6]. The root is acrid, thermogenic, purgative, antihelminthic, carminative, and anti-inflammatory. Seeds, roots, and leaves are used to treat abdominal pain, constipation, calculus, piles, helminthic manifestations, skin disorders, wound, jaundice, asthma, bronchitis, and also used in snakebite [7,8]. Thus, the aim of this study was to evaluate the phytochemical and antioxidant activity of methanol, aqueous, and chloroform extracts of *B. montanum* leaf, stem, root, and stem-derived callus.

**METHODS****Collection and sterilization of seeds**

*B. montanum* and seedlings collected from wild plants grown in Western Ghats (Karnataka), India. The plant material was authenticated at the Botanical Survey of India, Western Regional Centre, Pune, and the voucher specimen was deposited in the No. BSI/WRC/100-1/IDEN. CER./018/77. The seeds will be washed thoroughly under running tap water for 20 min and rinsed with Teepol solution 5% (v/v) and washing with sterile double distilled water 3–4 times and the sterilized seeds will be transferred to nursery trays, then pots containing a mixture of sterile soil, sand, and manure (2:1:1) and maintained in the greenhouse condition.

**Callus induction**

Nodal explants were sterilized with different sterilants (Teepol and bavistin), followed by 0.1 % HgCl<sub>2</sub> for 2 min under aseptic condition and then washed with sterile water for 3–5 times to remove the traces of sterilants. Finally, sterilized explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and

combination of 6-benzylaminopurine (BAP), 6-furfurylaminopurine (KIN), 1-naphthaleneacetic acid (NAA), and 2, 4-dichlorophenoxyacetic acid (2, 4-D). Cultures were incubated at 25±2°C in a culture room with 70–80% relative humidity and 16 h of photoperiod.

#### Preparation of extracts

The extracts were prepared according to the procedure of Samydurai and Saradha [9]. Briefly, 10 g of dried leaf, stem, root, and stem-derived callus were finely powdered using blender and extracted with 100 ml of different solvents (methanol, aqueous, and chloroform) for 24 h. Then, the extracts were concentrated by evaporation. The dried extract was stored at 4°C until further analysis.

#### Determination of total phenolics content (TPC)

The TPC of methanol, aqueous, and chloroform extracts was determined by Folin-Ciocalteu method [10] with slight modification, using gallic acid as standard. 0.5 ml of various solvent extracts was mixed with 0.5 ml of FC reagent and allowed to stand for 5 min at room temperature to allow complete reaction with FC reagent. 1 ml of 7% sodium carbonate was added and the final volume was made up to 5 ml using distilled water. The absorbance of blue color solution was measured at 725 nm using ultraviolet (UV)-visible spectrometer against the blank after the incubation of 90 min in dark. The experiments were carried out in triplicates. A calibration curve was prepared using standard solution to obtain the readings within the range of 5–50 µg gallic acid/ml. The total phenolics compounds were expressed as mg of gallic acid equivalents (mg GAE/g) of extract.

#### Determination of total flavonoids

The total flavonoids content in leaf, stem, root, and stem-derived callus extracts was estimated by modified aluminum chloride method [11]. 0.5 ml of samples were mixed with 2 ml of 95% methanol, 0.1 ml of 10% aluminum chloride, and 0.1 ml of 1 M potassium acetate and final volume was made up to 6 ml by adding distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with UV-visible spectrometer against the blank. The experiments were carried out in triplicates. The final absorbance of each sample was compared with a standard curve plotted from quercetin. The total flavonoids content was expressed in mg of quercetin equivalents per gram of extract (mg QE/g).

#### Estimation of tannins

Total tannins were determined according to the procedure of Medini *et al.* [12]. Briefly, to 0.5 ml of samples, 3 ml of 4% vanillin solution in methanol and 1.0 ml of concentrated hydrochloric acid were added. The mixture was then shaken and incubated at room temperature (RT) for 15 min; the absorbance was measured at 500 nm against blank. The experiments were carried out in triplicates. The final absorbance of each sample was compared with a standard catechin. The total tannin content was expressed in mg of catechin equivalents per gram of extract (mg CE/g).

#### Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals scavenging activity

Free radical scavenging activity was studied using 1-1-diphenyl-2-picrylhydrazyl according to Shoib and Shahid [13]. Different aliquots of

leaf, stem, root, and stem-derived callus extracts (20–100 µg/ml) were mixed with 3 ml of 0.25 mM of methanolic solution of DPPH. Incubate the reaction mixture at room temperature for 30 min in dark condition. The absorbance was measured at 517 nm using UV-visible spectrometer. Butylated hydroxytoluene (BHT) was used as standard. All estimations were performed in triplicate. The DPPH radical scavenging activity was calculated using the following equation:

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

#### Reducing power assay

The reducing power assay was done according to the protocol of Kuda *et al.* [14] with minor modifications. Different concentrations of root, stem, leaf, and callus extracts (20–100 µg/ml) were mixed with 2 ml of phosphate buffer (0.1 M pH 7) and 2 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min and kept at RT, then add 2 ml of 10% trichloroacetic acid. The mixture was centrifuged at 3000 rpm for 10 min. Finally, 1 ml of sample was mixed with same volume of d.w and 0.5 ml of ferric chloride solution (0.1%). The absorbance was measured at 700 nm. Ascorbic acid was used as a standard.

#### Statistical analysis

The results were expressed as mean ± standard deviation (SD). All the experiments were repeated thrice, and data were analyzed by one-way analysis of variance followed by Duncan's multiple range tests using SPSS software. p<0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

#### Callus induction

Stem explants were inoculated on MS medium supplemented with different concentrations of auxins and cytokinins (Table 1). Among the various concentrations and combinations, maximum creamish compact callus was achieved on MS medium supplemented with 0.5 mg/L of KIN + 3.0 mg/L of BAP (17.33±1.15) and green semi-friable callus was recorded in 0.5 mg/L of BAP + 0.5 mg/L of NAA (16.80±0.34) compared to other concentrations (Fig. 1). The previous studies also reported that maximum callus induction was observed in combination of NAA (4.0 mg/L) + BAP (0.5 mg/L) from intermodal region of *Orthosiphon stamineus* [15]. Studies by Nathar and Yattoo [16] noticed highest callus induction on combination of 2, 4-D (2.0 mg/L) + KIN (1.0 mg/L) from the *Artemisia pallens* shoot tip region compared to other concentrations and combination of growth hormones.

#### Total phenolic content

Phenolic compounds are ubiquitous secondary metabolites in plants. They have multiple biological effects such as prevention of platelet aggregation and damage of red blood cells [17,18]. They play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [19]. The results were determined using the linear regression equation ( $y = 0.058x$

**Table 1: Effect of auxins and cytokinins on callus induction from stem explants of *Baliospermum montanum***

Growth hormones	Concentration (mg/L)	Number of explants	Number of explants responded	Nature of callus
BAP+NAA	0.5+0.5	20	16.80±0.34 <sup>de</sup>	Green semi-friable callus
	0.5+1.0	20	15.60±1.21 <sup>de</sup>	Green semi-friable callus
	3.0+1.0	20	13.66±2.08 <sup>ab</sup>	Green semi-friable callus
6-furfurylaminopurine+BAP	0.5+1.0	20	12.66±2.08 <sup>b</sup>	Creamish compact callus
	0.5+2.0	20	13.66±2.51 <sup>ab</sup>	Creamish compact callus
	0.5+3.0	20	17.33±1.15 <sup>c</sup>	Creamish compact callus
2, 4-dichlorophenoxyacetic acid+NAA	0.5+0.5	20	09.33±1.52 <sup>a</sup>	Yellowish friable callus
	0.5+1.0	20	14.33±1.52 <sup>bcd</sup>	Yellowish friable callus
	0.5+2.0	20	16.20±1.70 <sup>cde</sup>	Yellowish friable callus

Values represent the Mean±SD in triplicates. One-way analysis of variance followed by Duncan's multiple range tests using SPSS software. p<0.05 was considered statistically significant. BAP: 6-benzylaminopurine, NAA: 1-naphthaleneacetic acid

+ 0.053)  $r^2=0.9978$ . The methanolic root extract (4.49±0.18 mg/g) demonstrated higher phenolic content followed by aqueous leaf extract (4.17±0.02 mg/g). Whereas aqueous and chloroform root extract showed moderate content of total phenol compared to other extracts of *B. montanum* (Table 2). Studies by Senguttuvan *et al.* [20] showed that methanolic extract of root and leaf has higher phenolic content compared to other solvent extracts in *Hypochaeris radicata*. Siddique *et al.* [21] reported that the methanolic extract of *Aegle marmelos* leaf exhibited maximum phenolic content compared to the root and stem. Anyasor *et al.* [22] observed higher total phenolic content in aqueous extract of *Costus afer* compared to the methanolic extract.

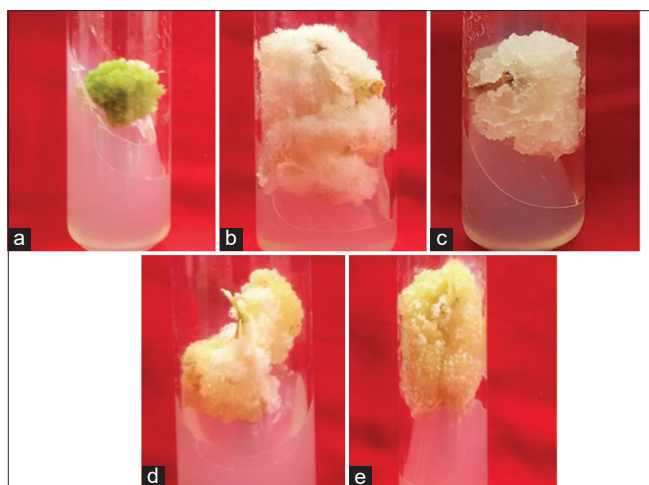
#### Total flavonoid content

Flavonoids are the most common group of polyphenolic compounds which provide health benefits through cell signaling pathways and antioxidant effects [23]. They act as chemical messengers, physiological regulators, and cell cycle inhibitors [24]. These metabolites are mostly used in plants to produce yellow and other pigments which play an important role in the colors of plants. In addition, flavonoids are readily ingested by humans and they display anti-inflammatory, antiallergic, and anticancer properties [25]. The results were determined using the linear regression equation ( $y = 0.047x + 0.0879$ )  $r^2=0.9939$ . The flavonoid content of different extracts was varying widely between 1.22 and 3.94 mg QE/g. The methanolic extract of leaf recorded maximum flavonoid content (3.94±0.07 mg/g) followed by aqueous leaf extract

(3.18±0.77 mg/g). Studies by Awah and Verla [26] revealed maximum flavonoids content in the methanolic leaf extract of *Ocimum gratissimum* compared to other phytochemical constituents. According to Basma *et al.* [27], the methanolic extract of *Euphorbia hirta* leaves exhibited a significant amount of flavonoid content followed by the flowers, roots, and stem, respectively. Ruwali *et al.* [28] also reported that methanolic extract of *Michelia champaca* leaf exhibited higher flavonoid content compared to other solvent extracts.

#### Total tannin content

Tannins are heterogeneous class of phenolic polymers in the 500–30,000 molecular weight which are water soluble and are capable of precipitating soluble proteins from solution. These compounds have been proposed as an important component of plant chemical defenses against herbivorous animals, fungi, and microbes [29]. In our present study, methanolic extract *B. montanum* leaf extract possessed high tannin content compared to other extracts (6.95±0.38 mg/g). Total tannin content was calculated using standard curve of catechin ( $y = 0.0073x + 0.0236$ )  $r^2=0.995$ . The methanolic extract of leaf showed maximum tannin content (6.95±0.38 mg/g) followed by root extract (6.66±0.06 mg/g). Whereas aqueous and chloroform extract of leaf showed moderate tannin content compared to other extracts of *B. montanum*. Studies by Rajesh *et al.* [30] reported that methanolic leaf extract of *Terminalia catappa* showed maximum tannin content compared to other extracts. According to Kassim *et al.* [31], the ethyl acetate and methanolic extracts of *Uncaria gambir* recorded higher tannin content compared to aqueous extract. Ashwini and Majumdar [32] reported that methanolic root extract showed maximum content of tannin compared to other solvent extracts in *Exacum bicolor*.



**Fig. 1: Effect of different concentrations and combination of auxins and cytokinins on callus induction from stem explants.**  
 a – MS + BAP (0.5 mg/L) + 1-naphthaleneacetic acid (NAA) (0.5 mg/L), b and c – 6-furfurylamino purine (0.5 mg/L) + BAP (2.0 and 3.0 mg/L), and d and e – 2, 4-dichlorophenoxyacetic acid (0.5 mg/L) + NAA (1.0 and 2.0 mg/L)

#### DPPH radical scavenging assay

Fig. 2 shows the dose–response curve of DPPH radical scavenging activity compared with standard BHT. In the present study, methanolic extract of leaf and root showed significant scavenging activity with an  $IC_{50}$  value around 80.0  $\mu$ g/ml compared to other extracts. According to Adebiji *et al.* [5], ethanolic extract of *Grewia carpinifolia* leaf revealed the highest scavenging activity compared to other extracts. Basma *et al.* [27] showed that methanolic extract of *E. hirta* leaves exhibited a maximum dose-dependent DPPH scavenging activity followed by the flowers, roots, and stem, respectively.

#### Reducing power assay

The reducing power assay evaluates the ability of plant fractions to reduce  $Fe^{3+}$  ions to  $Fe^{2+}$  by electron donation. This was facilitated by the presence of potassium ferricyanide ( $K_3[Fe(CN)_6]$ ) [33]. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers causes the reduction of the  $Fe^{2+}$  to the ferrous forms. Therefore, by measuring the formation at 700 nm, we can monitor the  $Fe^{2+}$  concentration [34]. The reducing power ability of methanolic extracts of leaf, stem, root, and stem-derived callus showed dose-dependent

**Table 2: Total phenolics, flavonoids, and tannin content of different extracts of *Baliospermum montanum***

Plant samples	Solvents (mg/g)	Total phenolics (mg GAE/g)	Total flavonoids (mg QE/g)	Total tannins (mg CE/g)
Leaf	Methanol	3.22±0.04 <sup>d</sup>	3.94±0.07 <sup>e</sup>	6.95±0.38 <sup>d</sup>
	Aqueous	4.17±0.02 <sup>e</sup>	3.18±0.77 <sup>d</sup>	6.25±1.89 <sup>c</sup>
	Chloroform	0.04±0.06 <sup>a</sup>	1.89±0.35 <sup>d</sup>	5.65±0.27 <sup>c</sup>
Stem	Methanol	3.13±0.10 <sup>d</sup>	2.17±0.03 <sup>c</sup>	3.77±0.51 <sup>ab</sup>
	Aqueous	2.30±0.13 <sup>b</sup>	2.00±0.04 <sup>b</sup>	3.34±0.11 <sup>b</sup>
	Chloroform	0.31±0.08 <sup>b</sup>	1.52±0.10 <sup>d</sup>	2.67±0.32 <sup>d</sup>
Root	Methanol	4.49±0.18 <sup>e</sup>	2.11±0.20 <sup>bc</sup>	6.66±0.06 <sup>d</sup>
	Aqueous	3.77±0.11 <sup>c</sup>	1.59±0.02 <sup>b</sup>	2.86±0.23 <sup>b</sup>
	Chloroform	3.66±0.23 <sup>d</sup>	1.22±0.03 <sup>b</sup>	1.99±0.12 <sup>b</sup>
Stem callus	Methanol	1.82±0.05 <sup>a</sup>	1.42±0.03 <sup>a</sup>	1.61±0.24 <sup>e</sup>
	Aqueous	2.05±0.03 <sup>a</sup>	2.21±0.01 <sup>cd</sup>	1.39±0.76 <sup>d</sup>
	Chloroform	0.48±0.01 <sup>a</sup>	1.28±0.02 <sup>b</sup>	1.16±0.27 <sup>e</sup>

Values represent the Mean±SD in triplicates. One-way analysis of variance followed by Duncan's multiple range tests using SPSS software.  $p<0.05$  was considered statistically significant. GAE: Gallic acid equivalent



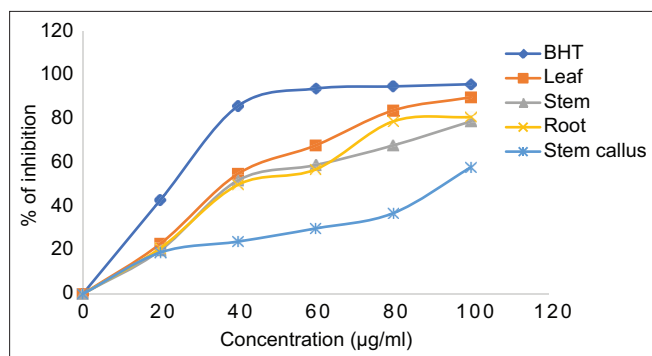


Fig. 2: 2,2-diphenyl-1-picrylhydrazyl scavenging activity of methanolic extract of *Baliospermum montanum*

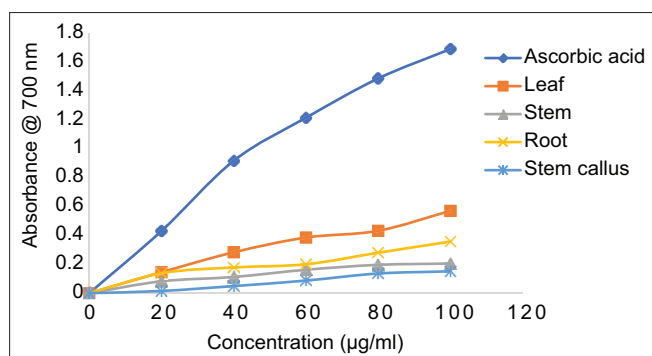


Fig. 3: Reducing power activity of methanolic extract of *Baliospermum montanum*

relationship as represented in Fig. 3. The leaf extract exhibited higher reducing power activity, whereas methanolic extract of root recorded moderated level of reducing power compared to other extracts. Simur [35] reported that the antioxidant activity was higher in methanolic extract of leaf in *Withania somnifera* compared to other extracts.

## CONCLUSION

In the present investigation, leaf extract of *B. montanum* exhibited higher phenolic, flavonoid, and tannin content compared to other extracts. The DPPH and reducing power activity were found to be higher in methanolic leaf and root extract which can correlate to higher scavenging activity. Further investigations are required to identify their active metabolites and antiproliferation activity.

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## AUTHORS' CONTRIBUTIONS

Sushma has done a phytochemical and antioxidant activity. Raveesha coordinated the work and writing of the manuscript.

## COMPETING INTEREST

The authors declared that they have no conflicts of interest.

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