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Effect of brassinosteroids on antioxidants content and radical scavenging activity of *Tinospora cordifolia* (Willd.) Miers ex Hook. F & Thoms

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Abstract
The present investigation was undertaken to determine the influence of brassinosteroids on the alkaloid and antioxidant (phenol and flavonoid) contents of the leaves, stems and roots of *Tinospora cordifolia* as well as the antioxidant potential by means of DPPH, H_2O_2 and OH radical scavenging activity and ferric reducing power. Exogenous application of brassinosteroids considerably increased alkaloid content of various plant parts. The content of phenols and flavonoids were enhanced due to brassinosteroid supplementation. Further, foliar application of the brassinosteroids improved radical scavenging activity compared to untreated control plants. Brassinosteroid feeding also elevated the reducing power capabilities of various plant parts.

Keywords: Antioxidants, radical scavenging activity, brassinosteroids, methanolic extracts

1. Introduction
Free radicals and other reactive oxygen species (ROS) are produced either from normal cell metabolism *in situ* or from external sources (pollution, cigarette smoke, radiation, medication). When an overload of free radicals cannot gradually be destroyed, their accumulation in the body generates a phenomenon called oxidative stress. This process plays a major part in the development of chronic and degenerative illness such as cancer, autoimmune disorders, aging, cataract, rheumatoid arthritis, cardiovascular and neurodegenerative diseases. The body has several mechanisms to counteract oxidative stress by producing antioxidants, either naturally generated *in situ* (endogenous antioxidants), or externally supplied through foods (exogenous antioxidants). The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention (Lien *et al*., 2008) [12]. Antioxidants can greatly reduced the damage due to oxidants by neutralizing the free radicals before they can attack the cells and prevent damage to macromolecules such as lipids, proteins, enzymes, carbohydrates and DNA and also involved in the treatment of various human disease (Sannigrahi *et al*., 2009) [20].

Natural products with medicinal value are gradually gaining importance in clinical research due to their well-known property of no side effects as compared to synthetic drugs. *Tinospora cordifolia* (Willd.) Miers ex Hook.F & Thoms commonly named as "Guduchi" is known for its immense application in the treatment of various diseases in the traditional ayurvedic literature (Saha and Ghosh., 2012) [18]. Guduchi is widely used in veterinary folk medicine/ ayurvedic system of medicine for its general tonic, antiperiodic, antispasmodic, anti-inflammatory, antiarthritic, antiallergic and anti-diabetic properties (Upadhyay *et al*, 2014) [25].

Brassinosteroids (BRs) comprises a new class of plant-specific steroid hormones characterized by their polyhydroxylated sterol structure that resembles hormones (Clouse 2011) [8]. They are known to regulate various aspects of plant growth and development, including cell elongation, photomorphogenesis, xylem differentiation, seed germination, leaf bending and epinasty, proton pump activation, regulation of gene expression, nucleic acid and protein synthesis and photosynthesis (Rao *et al* 2002, Clouse 2011 and Yang *et al*. 2011) [16, 8, 27]. Brassinosteroids enhanced growth, tuberous root yield and forskolin content in *Coleus forskohlli* (Swamy and Rao, 2011) [23]. Similar increase in growth as well as geraniol content of rose scented geranium was observed (Swamy and Rao, 2009) [22]. In an earlier study it was observed that exogenous application of brassinosteroids was found to increase the growth of *Tinospora cordifolia* (Raghu *et al*, 2015) [14].

The present study was done to find out the effect of brassinosteroids on contents of antioxidants and to asses the free radical scavenging activities of the methanolic extracts obtained from Br-treated *T. cordifolia* plants.

2. Materials and Methods

2.1 Preparation of plant extracts

Stem cuttings *T. cordifolia* were collected from Botanical Garden of Osmania University, Hyderabad. Two Bioactive brassinosteroids i.e. 24-epibrassinolide (EBL) and 28-homo brassinolide (HBL) were procured from CID tech research Inc, Mississauga, Ontario, Canada.

15 cm nodal stem cuttings of *Tinospora* with 2 leaves were excised. These were planted in nursery covers (5 kg soil capacity) filled with 3 parts of the garden soil and 1 part of the coco peat mixture (the features of the coco peat are: increase in aeration, quick wet ability and excellent potting medium and was obtained from local market). The nursery covers were watered immediately and transferred to botanical garden. These were irrigated daily during initial 20 days and subsequently once in two days.

The leafy cuttings were given brassinosteroid treatments (24-EBL and 28-HBL) as foliar spray on 30th, 45th day. Each compound was tested at 3 concentrations levels *viz* 0.5, 1.0 and 2.0µM levels. The cuttings were given stick support. The plants were allowed to grow for 90 days. The content of antioxidants and radical scavenging activity was determined.

After recording the growth indices, the plants were washed thoroughly in water to remove the soil and other foreign particles. The leaves, stems and roots of the control and brassinosteroids treated plants, were sorted out and dried under shade. All dried parts of the *T.cordifolia* plants were finely powered with mortar and pestle. This powered plant material directly used to determine the total alkaloids and phenols.

The shade dried powdered plant material was extracted successively in soxhlet extractor with different solvents (n-hexane, chloroform, ethyl acetate and methanol) according to their increasing polarity. The extraction process was carried out until the extraction was completed (the refluxing solvent became clear). All the solvent extracts were evaporated to remove the final traces of the respective solvents by using the rotary evaporators. Dried extracts were employed for estimating the radical scavenging activities (DPPH, H_2O_2 and OH radical scavenging activity) and reducing power capabilities.

2.2 Alkaloids

Total alkaloid content was determined by the method of Harborne (1973) [10]. Five grams of the sample was transfered to a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

2.3 Total phenols

Total phenol content was estimated by the method as described by Sadasivam and Manickam (2008) [17]. 1.0 g of the leaf sample was ground using a mortar and pestle with 10 times volume of 80% (v/v) ethanol. The homogenate was centrifuged at 10,000 rpm. The supernatant was evaporated to dryness, adding 5 ml of double distilled water thereafter. Later, 0.5 ml of folin ciocalteu reagent and 2 ml of 20% (v/v) Na_2CO_3 were added to each tube. The optical density of the solution was measured at 650 nm against a reagent blank. The amount of total phenol content was calculated from the standard graph prepared using different concentration of pyrocatechol.

2.4 Flavonoids

Aluminium chloride colorimetric method was used for the determination of flavonoids (Chang and Win, 2002) [5]. 0.5ml of methanolic leaf extract was mixed with 1.5ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 minutes; the absorbance of the reaction mixture was measured at 415nm with a UV Visible Spectrometer (SCHIMADZU UV-1800, Japan). Total flavonoid content was calculated as quercetin (mg QE/g dry extract), using the following equation based on the calibration curve:

$y = 0.685x$, $R^2 = 0.988$, where y was the absorbance and x is the quercetin concentration (mg mL^{-1}).

2.5 DPPH radical scavenging activity

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was estimated by measuring the decrease in the absorbance of methanolic solution of DPPH (Brand-Willams *et al.,* 1995) [4]. To 5 ml DPPH solution (3.3 mg of DPPH in 100 ml methanol), 1ml of plant extract was added, incubated for 30 min in the dark and the absorbance (A_1) was measured at 517 nm. The absorbance (A_0) of a reaction control (methanol instead of plant extract) was also recorded at the same wavelength. Ascorbic acid was used as standards. Results are expressed as percentage of inhibition of DPPH radical adopting the following formula:

DPPH radical scavenging activity (%) = $[(A_0 – A_1)/A_0] \times 100$

Where, A_0 was the absorbance of reaction control and A_1 was the absorbance of extracts and standards.

2.6 Hydrogen peroxide scavenging activity assay

Hydrogen peroxide scavenging activity of the extract was estimated by replacement titration (Zhang, 2000) [29]. Aliquot of 1.0 ml of 0.1mM H_2O_2 and 1.0 ml of extracts were mixed, followed by 2 drops of 3% ammonium molybdate, 10 ml of 2M H_2SO_4 and 7.0 ml of 1.8 M KI. The solution mixture was titrated with 5.09 mM $Na_2S_2O_3$. $5H_2O$ until yellow colour disappeared. Percentage of scavenging of hydrogen peroxide was calculated as: % inhibition = $[(V_0\text{-}V_1)/V_0] \times 100$

Where, V_0 is volume of NaS_2O_3 solution used to titrate the control sample in the presence of hydrogen peroxide (without extract), V_1 is the volume of NaS_2O_3 solution used in the presence of extract.

2.7 Hydroxyl radical scavenging activity

The scavenging activity for hydroxyl radicals was measured following the procedure described by Yu *et al*., (2004) [28]. Reaction mixture contained 60µl of 1.0 mM $FeCl_3$, 90 µl of 1mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M H_2O_2 and 1.5 ml of extract. After incubation at room temperature for 5 min, the absorbance of the mixture at 560nm was measured with a UV Visible Spectrometer (SCHIMADZU UV-1800, Japan). The hydroxyl radicals scavenging activity was calculated according to the following equation: % inhibition = $[(A_0\text{-}A_1)/A_0] \times 100$

Where A_0 is the absorbance of the control (blank, without extract) and A_1 is the absorbance in the presence of the extract.

2.8 Reducing power assay

The Fe^{3+} reducing power of the extract was determined by the method of Oyaizu (1986) [13]. The extract (0.75 ml) was mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1%, w/v), followed by incubating at 50ºC in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of 10% (w/v) trichloroacetic acid (TCA) and then centrifuged at 3000 rpm for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride solution 0.1% (w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

3. Results and Discussion

Foliar application of the brassinosteroids increased the alkaloid contents in *T. cordifolia* (Table: 1). among all the treatments, homobrassinolide at 2µM concentration proved to be highly efficient in enhancing the total alkaloid contents. Both the substances employed enhanced the total alkaloid content in all the three plant parts. The impact of brassinosteroids was much more effective on alkaloid content of stem. Auxins were found to enhance the production of steroidal alkaloids in *Solanum lyratum* (Kuo *et al*., 2012) [11]. Methyl jasmonate enhanced the accumulation of alkaloids in *Catharanthus roseus* cell suspension culture (EI-Sayed and Verpoorte, 2002) [9]. Light and phytohormones were found to influence the production of alkaloids in transformed root cultures of *Hyoscyamus albus* (Sauewein *et al*., 1992) [21]. Combinations of auxins with cytokinins stimulated for leaf callus growth and enhanced alkaloid content in *Catharanthus roseus* (Verma *et al*., 2012) [26]. The present study clearly demonstrated the positive influence brassinosteroids on alkaloid content of various plant parts of *Tinospora*.

Phenolic compounds are the most abundant secondary metabolites in plants. Phenolics have the ability to destroy radicals because they contain hydroxyl groups. These important plant components give up hydrogen atoms from their hydroxyl groups to radicals and form stable phenoxyl radicals; hence, they play an important role in antioxidant activity (Aksoy *et al*., 2013) [1]. In the present study it was observed that exogenous application of brassinosteroids led a significant increase in total phenols in *T. cordifolia* (Table: 2). There was a steep rise in phenol content in all the three parts of the plant due to brassinosteroid treatment. Highest phenol content was observed in leaves which received 1µM application of 28-homobrassinolide. The present study evidently demonstrated the elevated levels of these antioxidants in *T.cordifolia* due to brassinosteroid feeding. Flavonoid contents was also found to increase due to brassinosteroid supplementation (Table: 3). Highest levels of flavonoids in *T. cordifolia* plants were observed at 2µM concentrations of 28-homobrassinolide in leaf samples compared to other plant parts of *T. cordifolia.* Similar increase in the content of phenols, flavonoids was in tomato plants due to brassinosteroid supplementation (Ahammed *et al*., 2013) [2]. BRs also increased the production of phenolics in radish plants (Choudhary *et al*., 2011) [7].

Brassinosteroid application resulted in elevation of DPPH scavenging capabilities of *Tinospora* (Table: 4). the efficacy of brassinosteroids on DPPH scavenging was found dose dependent. There was enhancement in DPPH scavenging with increase in the concentration of brassinosteroid. Among all the treatment employed, HBL at 2µM concentration proved to be highly efficient in enhancing the DPPH scavenging in different plant parts. Brassinosteroid application also resulted in increase in the H_2O_2 radical scavenging activity of methanolic extracts of various plant parts of *Tinospora* (Table: 5). the H_2O_2 scavenging capabilities of leaf, stem, root was found to be augmented in a dose dependent manner with the application of brassinosteroids. Similarly the hydroxyl radical scavenging activity of extracts from different plant parts was enhanced due to foliar supplementation of brassinosteroids (Table: 6). It was observed that brassinosteroids furthered the OH scavenging in dose dependent manner. Among all treatments, highest hydroxyl scavenging was observed in roots from 2µM HBL treatment. Further, exogenous application of brassinosteroids resulted in steep rise in reducing power capabilities of various plant parts of *Tinospora* (Table: 7). While EBL (2µM Conc.) doubled the reducing power in leaves, HBL (2µM Conc.) account for almost 3 fold increase in reducing power of leaf extracts. In stem and root extracts also, brassinosteroids caused appreciable increase in reducing power activity.

Bhawya and Anilkumar (2010) [3] and Upadhya *et al* (2014) [25] verified the antioxidant potential of *Tinospora* by DPPH and superoxide radical scavenging activities. Sivakumar *et al* (2010) [19] evaluated antioxidant activity of methanolic extracts of *Tinospora* by reducing power assay. The present study evidently demonstrated the furtherance of radical scavenging capability of *Tinospora* by brassinosteroid supplementation. Tripathi and Sharma (2013) [24] isolated epibrassinolide from the medicinal plant *Bacopa monnieri* and showed its free radical scavenging activity. Improvement of antioxidant activity coupled with free radical scavenging due to brassinosteroid supplement was shown in case of radish seedlings growing under copper (Choudhary *et al*, 2010) [6] and zinc (Ramakrishna and Rao, 2015) [15].

Table: 1. Effect of brassinosteroids on total alkaloid content of *T.cordifolia* plants.

Treatments	Total alkaloids (mg/g dry wt)		
	Leaf	Stem	Root
Control	14.10±1.52	31.62±0.41	9.85±0.24
0.5µMEBL	17.42±1.63	34.54±1.20	11.2±1.22
1µMEBL	20.25±1.45	40.85±1.77	13.25±0.42
2µMEBL	25.41±1.45	42.73±1.63	16.35±1.85
0.5µMHBL	19.74±1.65	37.35±0.85	13.52±2.10
1µMHBL	23.52±1.55	42.31±0.75	18.52±1.52
2µMHBL	29.48±0.86	47.85±0.63	16.56±0.44

The data presented above are Mean ± S.E. (n=5). EBL=24-epibrassinolide; HBL=28-homobrassinolide.

Table: 2. Effect of brassinosteroids on total phenols content of *T.cordifolia* plants.

Treatments	Total phenols (mg/g dry wt)		
	Leaf	Stem	Root
Control	39.02±1.52	10.11±1.22	13.43±0.55
0.5µMEBL	44.32±1.08	12.02±0.45	14.03±0.45
1µMEBL	49.07±0.45	13.05±0.59	17.11±0.52
2µMEBL	55.21±0.75	17.21±1.42	20.06±1.32
0.5µMHBL	47.42±0.95	13.42±0.25	16.34±0.85
1µMHBL	61.56±0.46	19.35±1.32	20.51±2.10
2µMHBL	56.74±0.56	21.65±0.21	23.35±1.63

The data presented above are Mean ± S.E. (n=5). EBL=24-epibrassinolide; HBL=28-homobrassinolide.

Table: 3. Effect of brassinosteroids on flavonoid content of methanolic extracts of *T.cordifolia* plants.

Treatments	Flavonoids (mg/g dry wt)		
	Leaf	Stem	Root
Control	13.52±0.85	5.33±0.21	7.53±2.33
0.5µMEBL	14.43±0.11	6.42±1.22	9.75±2.01
1µMEBL	16.35±1.45	8.85±1.41	10.52±1.52
2µMEBL	19.42±1.69	11.55±1.55	12.74±0.86
0.5µMHBL	15.44±0.98	7.43±1.74	11.63±1.22
1µMHBL	18.04±1.56	10.63±2.01	14.74±1.52
2µMHBL	22.13±2.01	14.53±0.85	17.02±1.33

The data presented above are Mean ± S.E. (n=5). EBL=24-epibrassinolide; HBL=28-homobrassinolide.

Table: 4. Effect of brassinosteroids on DPPH radical scavenging activity of methanolic extracts of *T.cordifolia* plants.

Treatments	DPPH radical scavenging activity (%)		
	Leaf	Stem	Root
Control	70.01±0.1	39.02±2.01	35.20±0.21
0.5µMEBL	74.25±0.11	44.21±1.61	39.10±0.35
1µMEBL	77.52±0.20	47.32±1.26	44.03±1.52
2µMEBL	83.42±1.31	55.41±1.22	52.41±0.65
0.5µMHBL	76.02±2.01	45.42±0.21	40.3±0.86
1µMHBL	81.41±1.41	52.43±1.31	48.43±0.73
2µMHBL	89.13±0.22	63.03±1.23	73.78±1.74
Ascorbic acid	97.32±0.35	97.32±0.35	97.32±0.35

The data presented above are Mean ± S.E. (n=5). EBL=24-epibrassinolide; HBL=28-homobrassinolide.

Table: 5. Effect of brassinosteroids on hydrogen peroxide radical scavenging activity of methanolic extracts of *T.cordifolia* plants.

Treatments	H_2O_2 radical scavenging activity (%)		
	Leaf	Stem	Root
Control	68.22±2.10	61.07±1.23	70.42±1.45
0.5µMEBL	71.52±1.32	65.75±2.41	74.03±1.21
1µMEBL	77.65±0.36	68.63±0.21	83.12±0.56
2µMEBL	84.42±0.45	73.72±0.85	91.42±0.76
0.5µMHBL	73.02±0.52	67.31±0.96	77.35±0.87
1µMHBL	78.10±0.15	71.02±0.55	84.74±1.65
2µMHBL	87.31±1.74	79.33±0.39	97.56±1.08
Ascorbic acid	52.03±1.63	52.03±1.63	52.03±1.63

The data presented above are Mean ± S.E. (n=5). EBL=24-epibrassinolide; HBL=28-homobrassinolide.

Table: 6. Effect of brassinosteroids on hydroxy radical scavenging activity of methanolic extracts of *T.cordifolia* plants.

Treatments	Hydroxy radical scavenging activity (%)		
	Leaf	Stem	Root
Control	58.02±2.01	40.53±2.01	70.52±1.56
0.5µMEBL	61.12±1.04	43.08±0.22	73.01±0.58
1µMEBL	67.53±1.30	47.55±0.52	81.56±1.45
2µMEBL	74.52±1.44	55.76±0.65	89.02±1.97
0.5µMHBL	63.36±0.41	45.37±0.45	76.41±0.76
1µMHBL	73.05±0.52	48.36±1.18	87.02±0.25
2µMHBL	77.42±1.74	62.21±1.16	92.85±0.15
Ascorbic acid	93.43±0.75	93.43±0.75	93.43±0.75

The data presented above are Mean ± S.E. (n=5). EBL=24-epibrassinolide; HBL=28-homobrassinolide.

Table: 7. Effect of brassinosteroids on reducing power assay of methanolic extracts of *T.cordifolia* plants.

Treatments	Reducing Power (%)		
	Leaf	Stem	Root
Control	0.527±0.13	0.518±0.12	0.366±0.21
0.5µMEBL	0.852±0.12	0.589±0.08	0.558±0.32
1µMEBL	1.07±0.12	0.755±0.08	0.804±0.15
2µMEBL	1.26±0.31	0.929±0.66	0.824±0.08
0.5µMHBL	0.912±0.14	0.681±0.21	0.763±0.15
1µMHBL	1.105±0.12	0.879±0.12	0.916±0.18
2µMHBL	1.519±0.13	1.08±0.44	1.057±0.10
Ascorbic acid	4.02±1.15	4.02±0.11	4.02±1.15

The data presented above are Mean ± S.E. (n=5). EBL=24-epibrassinolide; HBL=28-homobrassinolide.

4. Conclusion

The outcome of the present study clearly demonstrated the positive influence of brassinosteroids on antioxidant contents and radical scavenging activity of *Tinospora cordifolia*. The foliar application of the brassinosteroids caused substantial increase in total alkaloid, phenol, flavonoid fractions and also accounted for in improvement of radical scavenging activity of *Tinospora cordifolia* plants.

5. Acknowledgement

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