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Production of Boeravinone-B, total phenolic, flavonoid content and antioxidant activity from callus cultures of Punarnava (*Boerhavia diffusa* L.)

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Abstract

Boerhavia diffusa L. (*Punarnava*) is a medicinal herb, rich in diversified plant secondary metabolites used in curing various health ailments. Boeravinone-B is one of the important phytochemicals reported in Punarnava, possessing various pharmacological activities. It belongs to the family of rotenoids, belonging to the isoflavone group. Production of Boeravinone-B from the Punarnava through conventional propagation is comparatively very low, and alternative interventions are of utmost importance to meet the growing demand. In view of this, the present study aims to develop biotechnological approaches like cell/tissue culture as a substitute strategy for the accumulation of biomass and Boeravinone-B biosynthesis. Callus was established from leaf explants of *Boerhavia diffusa* L. when cultured on MS semi solid medium fortified with varied concentrations and combinations of auxins and cytokinins. The callus induced on Murashige and Skoog medium (MS medium) supplemented with 5.0 ppm 2,4-Dichlorophenoxyacetic acid (2,4-D) favored the highest production of Boeravinone-B analyzed through High-performance Liquid chromatography (HPLC) and it was found to be 673.95 µg g^{-1} Dry weight (DW). The total phenolic and flavonoid content were determined for the callus extracts and the results showed that callus induced on 5.0 ppm 2,4-D medium showed the highest phenolic and flavonoid content, which was 63.48 mg g^{-1} Gallic acid equivalent (GAE) Dry weight (DW), and 30.22 mg g^{-1} Quercetin equivalent (QE) DW. Similarly, antioxidant activities (radical scavenging, metal chelating, and reducing power) were performed, and it was found that callus induced on 5.0 ppm 2,4-D showed the highest anti-oxidant potential. Radical scavenging activity was found to be 91.1%, and 74% of metal chelating activity was recorded, and a similar trend was observed with respect to reducing power as well. The results of the present study lay foundation for optimization and subsequent large-scale production of Boeravinone-B from callus/cell suspension cultures.

Keywords

Boerhavia diffusa, *Punarnava*, Boeravinone-B, rotenoid, callus cultures, HPLC

Introduction

There are numerous phytochemicals or secondary metabolites in medicinal plants which are used for curing various health disorders. Boeravinone-B is one such plant secondary metabolite isolated from *Boerhavia diffusa* L., commonly known as *Punarnava*, due to its ability to rejuvenate (give back) life. *Boerhavia diffusa* L., belongs to the Nyctaginaceae family which is rich in various phytochemicals like alkaloids, flavonoids, saponins, and terpenes.

Boerhavia diffusa is a medicinal herb having a typical morphology with interlocking patterns in the shoots. The name 'Boerhavia' is derived from Herman Boerhaave (1668 –1738), a notable Dutch scientist/ physiologist from the 18th century. *B. diffusa* is also commonly known as "spiderling" or "hogweed" in English. The medicinal significance of this excelsior herb is mentioned in many ayurvedic scriptures like *Sushruta Samhita*, *Charaka Samhita*, *Astanga Sangraha*, and *Adarsh Nighantu* and there are many modern scientific reports which are supportive of accepting it in alternative systems of medicine for its reduced number of side effects (1, 2).

Boeravinone-B ($C_{17}H_{12}O_6$) is a rotenoid compound (3) with the IUPAC name 6,9,11-trihydroxy-10-methyl-6H-chromeno [3,4-b] chromen-12-one, having a molecular weight of 312.27 g mol^{-1}. It belongs to the isoflavones group of flavonoids, which belong to a larger group of phenolic compounds. Boeravinone-B is reported to have various pharmacological activities like anti-inflammatory (4, 5), cardio protective (6, 7, 8), anti-cancer (9), anti-microbial (10), immunomodulatory (11), anti-oxidant (13), and other healing properties (12). Boeravinone-B is a multipotent biomolecule, and its production in plant systems through modern biotechnological approaches such as tissue-culture is very much necessary as conventional cultivation alone will not be enough to meet its demand in the coming years.

Previous studies have shown that callus was established from *B. diffusa* when leaf explants were cultured on MS medium fortified with 20 µM 2,4-D (14). Induction of compact green callus was also initiated from internode explants when cultured on MS medium fortified with 0.5 mg L^{-1} 1-Naphthaleneacetic acid (NAA) along with 1.5 mg L^{-1} 2,4-D, 1.5 mg L^{-1} 6-Benzylaminopurine (BAP), and 0.5 mg L^{-1} citric acid (15). Similarly, leaf explants of Boerhavia when cultured on MS medium fortified with 18 µM 2,4-D and 2 µM BAP resulted in the induction of friable callus (16). Studies have largely focused on the induction of callus from different explants of *B. diffusa,* but there are no reports on the production of Boeravinone-B from the callus or cell suspension cultures of *Punarnava*. In view of this, the present study aims at the production of one of the potent bioactive molecules, Boeravinone-B, from callus cultures of *B. diffusa*.

Materials and Methods

Plant material:

B. diffusa plant samples were obtained from Transdisciplinary University (TDU) nursery, authorized by Dr. N. M. Ganesh Babu and the same were grown in a polyhouse facility available in CHRIST (Deemed to be University), Bangalore, India.

Surface sterilization and media preparation:

Fresh leaf explants were collected and washed for 8 minutes with detergent under running tap water and later subjected to disinfection using 2% NaOCl (Sodium hypochlorite) for 6 minutes, followed by washing with sterile distilled water for 2 minutes, twice. The explants were then sterilized once for 45 seconds with 0.1% Mercuric chloride and twice for 2 minutes with sterile distilled water. Once the sterilization was done, the leaves were cut into 0.5 cm^2 size in the laminar airflow chamber.

Leaf explants were inoculated onto sterilized semi-solid MS medium (autoclaved at 121°C for 15 minutes) supplemented with varied concentrations (0.1, 0.5, 1.0, 2.0, 5.0 ppm) of auxins like 2,4-D, Indole-3-Acetic acid (IAA), NAA, Indole-3-Butyric acid (IBA), Picloram, and cytokinins like BAP, Kinetin, Thidiazuron (TDZ), isopentenyl adenine (2-iP). After observing a positive response on the MS medium supplemented with individual auxins and cytokinins for callus induction, the leaf explants were cultured on the MS medium supplemented with combinations of responsive auxins and cytokinins. A photoperiod of 16 hours light and 8 hours dark has been used to maintain callus from leaf explants at 25 ± 2°C.

Preliminary phytochemical screening of callus extract using GC-MS profiling:

The dried callus powder of 1 gram was subjected to hot solvent extraction using petroleum ether and later residue was concentrated to 1mL for GC-MS analysis. GC-MS analysis was carried out using SHIMADZU, GCMS-QP2010SE and the column with specifications, SH-Rxi™-5Sil MS – Low-polarity phase: Crossbond™ silarylene phase 1,4-bis(dimethylsiloxy)phenylene diemthyl polysiloxane. The instrument was equipped with Quadrupole mass analyser. Helium gas was used as carrier gas and was adjusted to column velocity flow of 1.0 ml/min with 1 µl injector.

Extraction of Boeravinone-B from induced callus:

In-vitro induced callus from leaf explants was dried at 35°C to remove the moisture content and the dry powder was collected. Boeravinone-B was extracted from the dried powder using the hot solvent extraction method using methanol as solvent (17). Once the solvent is concentrated with extraction, it is filtered and the extract is subjected to drying. The dried residue was weighed, and the desired concentrations of extracts were prepared and later subjected to quantification of Boeravinone-B.

HPLC conditions:

The RP-HPLC was used to determine the concentration of Boeravinone-B. The instrumentation is outfitted with a C-18 column (Sharpsil-U, 250 (L) X 4.6 mm ID), a SCL-40 system controller, SIL-40C XS auto-injector and a UV detector (SPD-40). Various mobile phase conditions were tried, and an optimized mobile phase system with water and acetonitrile (1:1) was employed with isocratic elution similar to that of Krishnamoorthy *et al*. 2017 (18). The standard of Boeravinone-B with concentrations of 50, 100, 200, and 1000 µg/mL were used for plotting the standard graph and the same was used for calculating the concentration of the samples. Standard Boeravinone-B was purchased from Natural Remedies Pvt. Ltd., in Bangalore, India.

Estimation of total phenol content:

Folin-Ciocalteu assay was carried out to estimate the total phenolic content of the callus extract following the method of Sembiring *et al*. (19). Where 25µL of the callus extract (10mg mL^{-1}) was added along with 25µL of 1:1 Folin -Ciocalteu reagent and 100µL of sodium bicarbonate solution (7.5 %) was added. The reaction mixture was kept at room temperature for 2 h in the dark. Later, using a microplate reader, the absorbance was measured at 765nm (BIO-RAD, iMARKTM, Japan). The total phenol content was determined using a standard curve plotted with 0-100µg mL^{-1} gallic acid and the results are expressed as mg g^{-1} GAE.

Estimation of total flavonoid content:

The aluminum chloride method developed by Sembiring *et al.* (19) was used to determine the flavonoid content of the callus extracts (19). 50 µL of callus extract (10mg mL^{-1}) was added to 150 µL of 80% methanol, 10% (w/v) aluminum chloride, and 10 µL of 1M sodium acetate. Using a microplate reader (BIO-RAD, iMARKTM, Japan), the reaction mixture's absorbance at 415 nm was measured after 45 min of incubation. The total flavonoid content was measured using a standard graph from concentration ranges of 0-100 µg mL^{-1} of quercetin, and the results are expressed as mg g^{-1} QE.

Antioxidant potential of callus extract

Radical scavenging activity:

DPPH scavenging assay was used to measure the antioxidant activity of the callus following the method of Blois (20). Where 30 µL of callus extract (10mg mL^{-1}) was taken and diluted in methanol to get final volume of 3 mL. 1 mL of DPPH (0.004%) was added to this and kept in the dark for 30 min. The absorbance at 517 nm was measured with a UV-VIS spectrophotometer (Shimadzu, UV-1900, Kyoto, Japan). Ascorbic acid was employed as standard and the blank was methanol (3 mL). The following formula was used to determine the extracts' percentage radical scavenging activity:

Radical scavenging activity (%)

$$= \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100.$$

Metal chelating activity:

Metal chelating activity was analyzed using ferrozine and ferrous sulfate method developed by Chew *et al.* (21). Where, 1 mL of callus extract (10mg mL^{-1}) was mixed with 1 mL of 0.1 mM $FeSO_4$ and 2 mL of 0.25 mM ferrozine. After the reaction mixture had been incubated for 10 min at room temperature, its absorbance was measured at 562 nm with a UV-VIS spectrophotometer (Shimadzu, UV-1900, Kyoto, Japan). The following formula was used to determine the percentage metal chelating activity of the extracts:

Metal chelating activity (%) =

$$\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

Reducing power assay:

The reducing power activity was estimated using the FRAP test in accordance with Chung *et al.* (22). 1 mL of callus extract was combined with 2.5 mL of potassium ferricyanide [$K_3Fe(CN_6)$] and 2.5 mL of phosphate buffer. The mixture was incubated at 50°C for 20 min, later 2.5 mL of 10% trichloroacetic acid was added and centrifuged for 10 min at 1000 rpm. The top layer of the reaction mixture (2.5 ml) was then mixed with 2.5 ml of distilled water and 0.5 mL of 0.1% v/v $FeCl_3$, and the absorbance at 700 nm was measured using a UV-VIS spectrophotometer (Shimadzu, UV-1900, Kyoto, Japan).

Results & Discussion

Callus induction and maintenance of callus:

Callus is an undifferentiated mass of cells that is been induced when an explant is cultured on media fortified with cytokinins, auxins alone or in combination of both auxins and cytokinins. The induction of callus depends on the type of explant cultured and the type and concentration of the plant growth regulators used. Among all the auxins and cytokinins supplemented to the MS medium for the induction of callus, 2,4-D at 1.0, 2.0, and 5.0 ppm showed positive response, and white friable callus was induced. Later, experimental setup was designed in such a way that leaves were cultured on the semi solid medium fortified with combination of 1.0ppm 2,4-D, 2.0ppm 2,4-D, and 5.0ppm 2,4-D along with different concentrations (0.1, 0.5, 1.0, and 2.0 ppm) of cytokinins like BAP, Kinetin, TDZ, and 2-iP. Table.1 shows the response of leaf explants cultured on MS media fortified with cytokinin and auxin combinations, as well as the relative fresh weights (FW) and dry weights (DW) of induced callus. Overall analysis suggests that 2,4-D (1.0, 2.0, 5.0 ppm) in combination with BAP and KN (0.5 ppm, 1.0 ppm, 2.0 ppm) showed 100% induction of callus. A mass of cells, which were induced from the margins of the explants, were sub-cultured and the white friable callus was obtained and maintained. The callus developed on MS media supplemented with varying concentration of individual auxins and cytokinins, as well as their combinations, are depicted in Fig. 1.

2,4-D is one of the few PGR's which is more suitable and has the ability to induce the callus from various explants of plant species. In *Punarnava*, 2,4-D seems to be competent for the induction of callus either alone or in combination with cytokinins like KN and BAP. Similar to our studies, Kanfade *et al.* (2011) reported that 2,4-D alone or in combination with KN induced white and friable callus (23). In *Mirabilis jalapa* L., another notable Nyctaginaceae member, the callus was induced at highest degrees when 2,4-D was supplemented in the MS medium (24). Similarly, in monocots like *Saccharum officinarum* L. and *Hymenocallis littoralis* (Jacq.) Salisb., induction of callus was seen when 2,4-D alone or in combination with BAP was supplemented into the MS medium. Growth efficiency and proliferation of cells increased with increase in 2,4-D concentration (25, 26).

Table 1. Response of leaf explants to callus induction in the presence of auxin and cytokinin combinations when cultured on MS semisolid medium.

Auxin (2,4-D)	Cytokinin	Cytokinin Conc. (PPM)	Percentage of response (%)	Nature of callus	Fresh weight (g/L)	Dry weight (g/L)
1.0 ppm	-	-	100	Friable	70.61±3.03^{def}	8.15±0.35^{efg}
2.0 ppm	-	-	100	Friable	68.54±3.19^{f}	7.75±0.36^{efgh}
5.0 ppm	-	-	100	Friable	88±1.37^{ab}	10.05±0.16^{a}
1.0 ppm	BAP	0.5	100	Friable	78.62±7.16^{bcde}	9.7±0.89^{abcd}
		1.0	100	Friable	72.17±2.97^{cdef}	8.14±0.33^{efg}
		2.0	100	Friable	80.22±1.3^{abcd}	9.76±0.16^{abcd}
	Kinetin	0.5	100	Friable	80.64±3.59^{abcd}	8.8±0.39^{bcde}
		1.0	100	Friable	80±3.69^{abcd}	8.7±0.4^{cdef}
		2.0	100	Friable	84.19±2.19^{ab}	8.65±0.22^{def}
2.0 ppm	BAP	0.5	100	Friable	70.63±4.14^{def}	9.95±0.59^{ab}
		1.0	100	Friable	58.17±4.41^{g}	8.41±0.64^{efg}
		2.0	100	Friable	82.16±2.46^{abc}	8.54±0.26^{def}
	Kinetin	0.5	100	Friable	81.82±5.14^{abc}	8.51±0.54^{def}
		1.0	100	Friable	87.81±2.2^{ab}	9.91±0.25^{abc}
		2.0	100	Friable	89.59±1.76^{a}	9.76±0.19^{abcd}
5.0 ppm	BAP	0.5	100	Friable	38.44±0.87^{h}	7.2±0.16^{gh}
		1.0	100	Friable	43.61±2.73^{h}	7.45±0.47^{fgh}
		2.0	100	Friable	55.98±1.21^{g}	7.94±0.17^{efg}
	Kinetin	0.5	100	Friable	62.85±1.82^{fg}	6.56±0.19^{h}
		1.0	100	Friable	69.24±1.57^{ef}	6.6±0.15^{h}
		2.0	100	Friable	64.11±1.38^{fg}	7.46±0.16^{fgh}

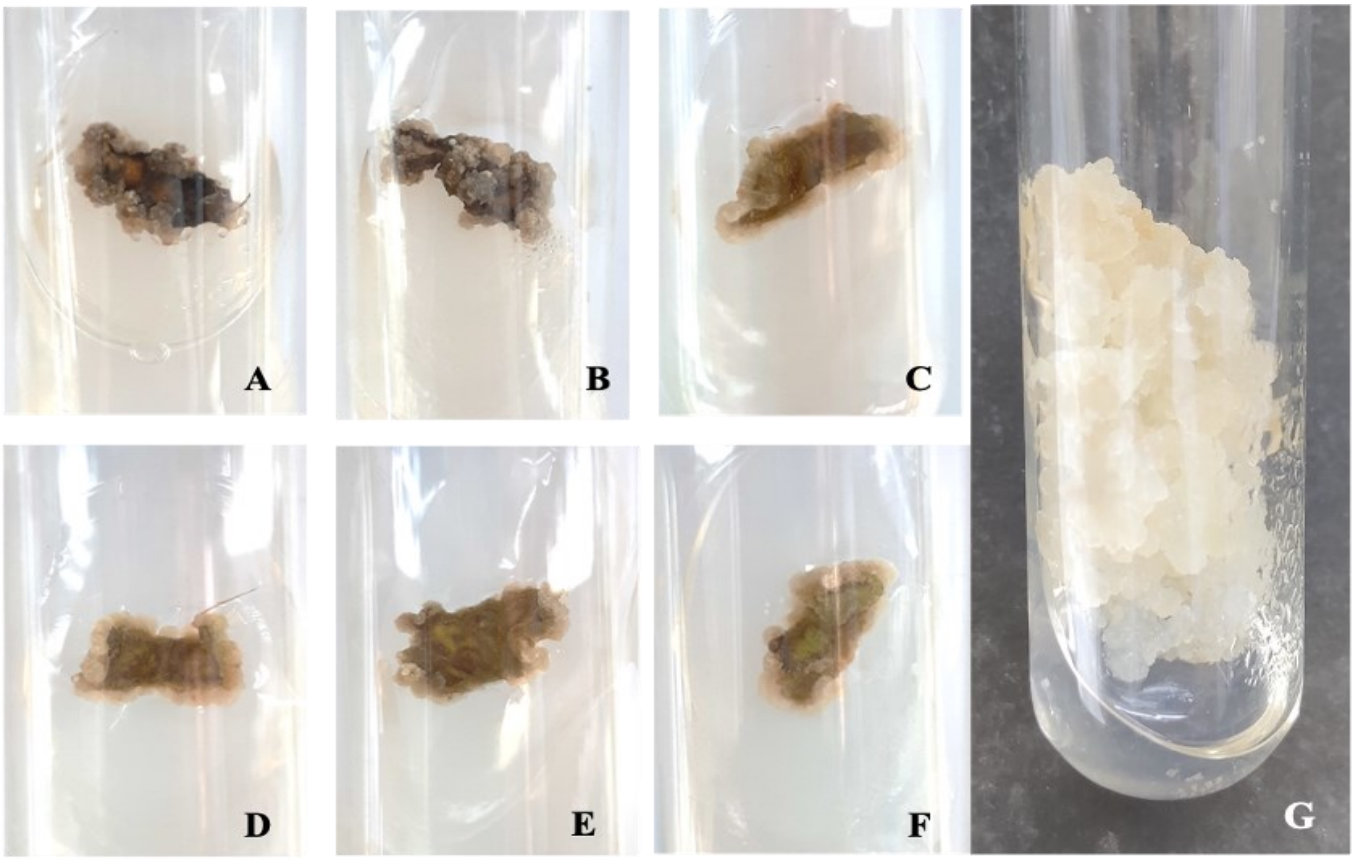

Fig. 1. Callus induced on MS medium fortified with various concentrations of cytokinins and auxins individually and in combinations: (A) 2.0 ppm 2,4-D; (B) 5.0 ppm 2,4-D; (C) 2.0 ppm 2,4-D + 2.0 ppm KN; (D) 1.0 ppm 2,4-D + 2.0 ppm KN; (E) 2.0 ppm 2,4-D + 2.0 ppm BAP; (F) 5.0 ppm 2,4-D + 2.0 ppm KN, (G) Callus cultured and maintained on MS medium with 5.0 ppm 2,4-D.

GC-MS profiling of callus extracts of Boerhavia diffusa L.

GC-MS analysis of the crude callus extracts of *Punarnava* showed various classes of phytochemicals with significant pharmacological activities. Figure-2 shows the GC-MS chromatogram, and table-2 represents the major phytochemicals, their molecular formulas, molecular weights, and area percentages in the chromatogram. Hexadecanoic acid, which was detected in the chromatogram, is important in cell proliferation and has a variety of pharmacological effects such as anti-cancer and anti-microbial properties (27). The chromatogram revealed phytochemicals such as sitosterol (28) and stigmasterol (29) that are responsible for a wide range of pharmacological actions. Squalene, a notable triterpene compound with anti-oxidant and anti-tumor properties, was also identified in the GC-MS profile (30). Along with this, several fatty acids and sterol derivatives have been reported, exemplifying *Punarnava*'s therapeutic potential.

Quantification of Boeravinone-B using RP-HPLC:

Boeravinone B was extracted using a hot solvent extraction method and the quantification was performed using RP-HPLC. It is seen that the presence of Boeravinone -B was reported in all the induced calli with significant variation. It was observed that callus induced on MS medium fortified with 5.0 ppm 2,4-D produced the highest amount of Boeravinone-B (673.95 ± 4.56 µg g^{-1} DW). It is seen that callus induced on MS medium augmented with 2,4-D and various concentrations of BAP showed increased production of Boeravinone-B followed by 5.0 ppm of 2,4-D alone. The HPLC chromatogram of Boeravinone-B standard and sample analysed are presented in figure 3. The amount of Boeravinone-B quantified using HPLC from calli induced from various plant growth regulators (PGR's) and in the leaf is represented in figure 4. The amount of Boeravinone-B from leaf is found to be 68.94 ± 1.92 µg g^{-1} DW, which is almost 10-fold less than the callus induced on 5.0 ppm 2,4-D supplemented medium.

Establishing the callus cultures are one of the most successful strategies for the production of phytochemicals. Steviol glycosides production was seen in callus induced from leaf explants of *Stevia rebaudiana* (Bertoni) Bertoni when cultured on MS medium fortified with 2.0 mg L^{-1} (31). Callus cultures of *Rhodiola imbricata* Edgew. was established on MS medium fortified with 5 mg L^{-1} NAA combined with 5 mg L^{-1} BAP. It is seen that callus produced optimal amounts of salidroside and the respective phenolics and flavonoids (32). Anti-oxidant metabolites like phenols and flavonoids were produced from the callus cultures of *Prunella vulgaris* L. Callus was induced from leaf explants of *P. vulgaris* when cultured on MS medium supplemented with 2.0 mg L^{-1} NAA (33). Bacosides were produced from the callus induced on MS medium supplemented with 1.0 µM 2,4-D combined with 5.0 µM NAA (34). Reports also suggested that production of phytochemicals from callus cultures was enhanced upon employing the optimization and elicitation strategies.

Estimation of total phenolic content:

Folin-Ciocalteu assay was employed for the estimation of total phenolic content in the callus extracts. Callus induced on MS medium fortified with 1.0, 2.0, and 5.0 ppm 2,4-D showed an increased trend in the total phenol content similar to that of Boeravinone B. The highest

Table 2. Phytochemical profiling and GC-MS analysis of callus extracts

Name of the compound	Molecular formula	Molecular weight	Peak area (%)
Hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	330.5	12.82
Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	12.53
γ-Sitosterol	$C_{29}H_{50}O$	414.7	10.20
b-Sitosterol acetate	$C_{31}H_{52}O_2$	456.7	6.96
Squalene	$C_{30}H_{50}$	410.7	4.43
Stigmasterol	$C_{29}H_{48}O$	412.7	4.08
Glycidyl Palmitate	$C_{19}H_{36}O_3$	312.5	3.68
Tetratetracontane	$C_{44}H_{90}$	619.2	3.23
Oleic acid	$C_{18}H_{34}O_2$	282.5	3.19
Octadecanoic acid	$C_{18}H_{36}O_2$	284.5	3.16
24-Norursa-3,12-diene	$C_{29}H_{46}$	394.7	2.86
Chondrillasterol	$C_{29}H_{48}O$	412.7	2.55
Dibutyl phthalate	$C_{16}H_{22}O_4$	278.3	1.70
Eicosanoic acid, 2,3-bis(acetyloxy)propyl ester	$C_{27}H_{50}O_6$	470.7	1.68
Heptadecanal	$C_{17}H_{34}O$	254.6	1.63

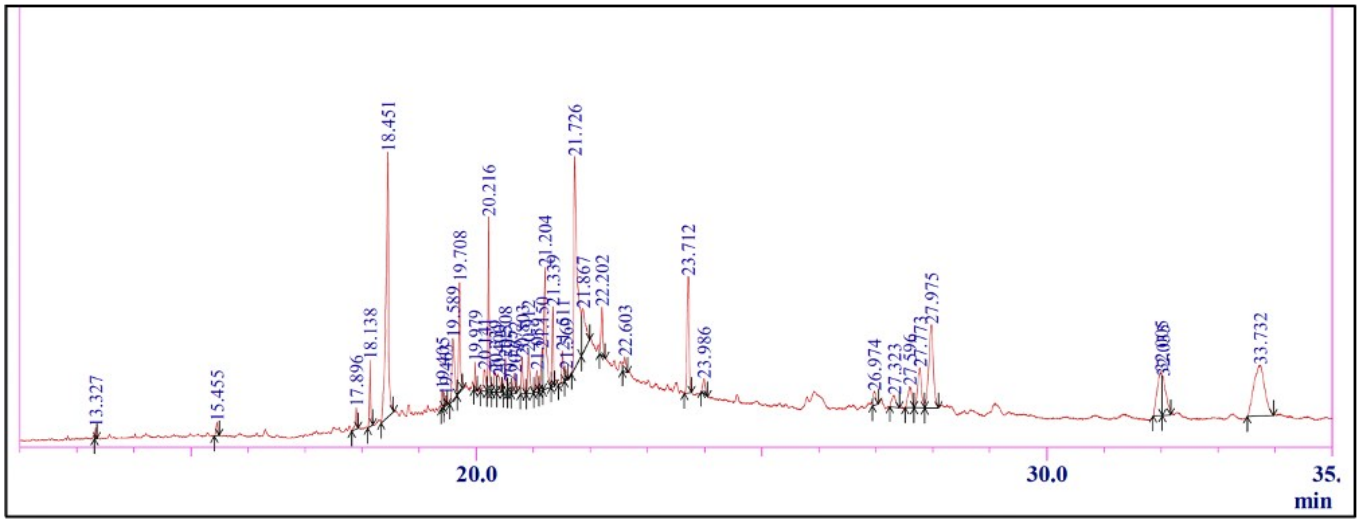

Figure 2. GCMS profile of compounds from petroleum ether extracts of callus from *Boerhavia diffusa* L.

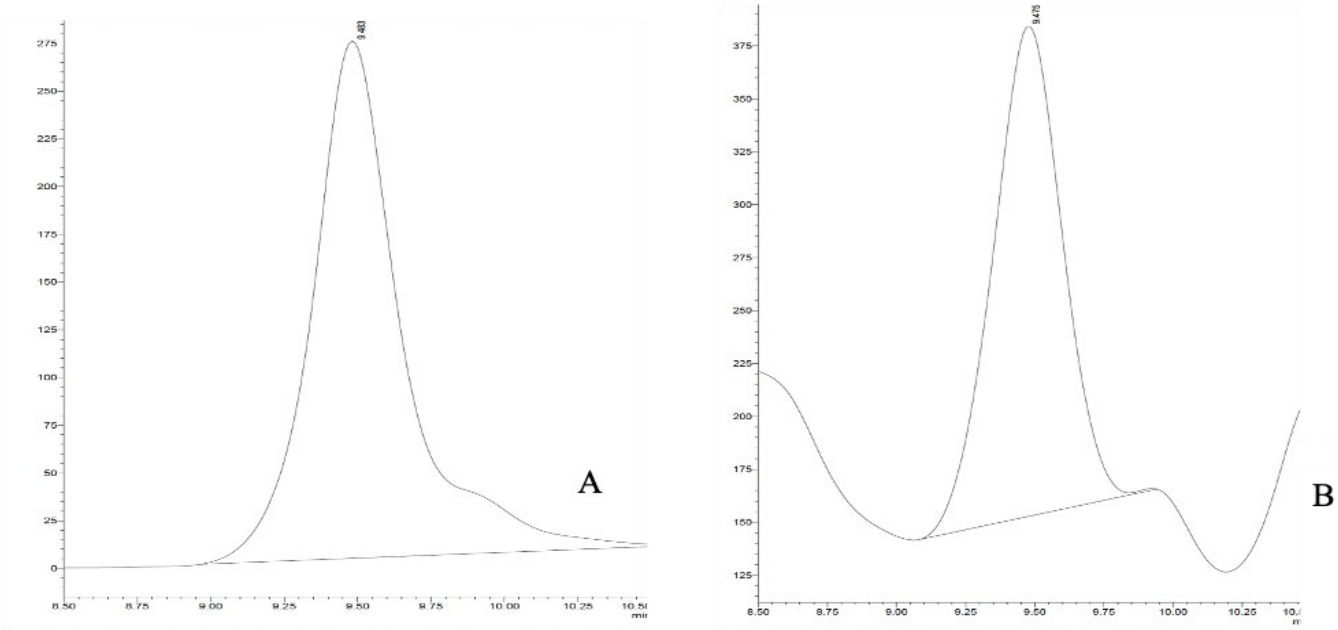

Figure 3. HPLC chromatogram of (A) Boeravinone B standard; (B) Boeravinone B quantified from extracts of callus established on MS medium supplemented with 5.0 ppm 2.4-D.

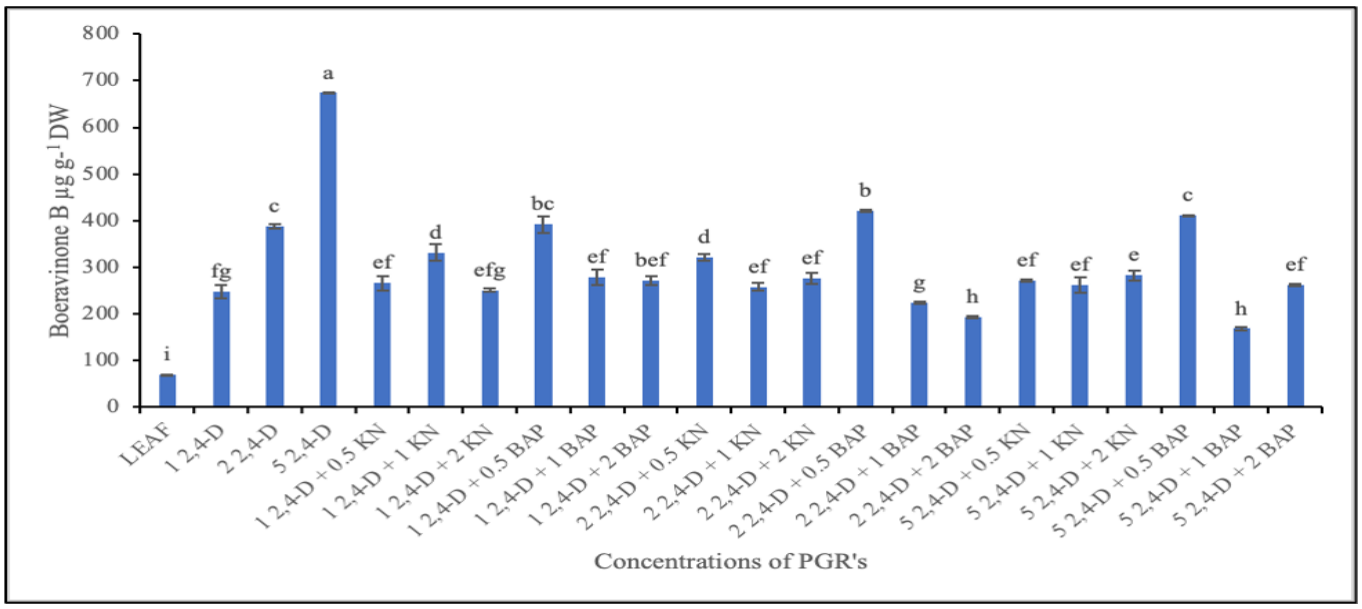

Figure 4 :Production of Boeravinone B from callus cultures of *Boerhavia diffusa* L

The values represent the mean ± SE of three replicates. The bar represents the standard error. Using IBM SPSS software, ver.21, Duncan's multiple range test shows that mean values with similar letters are not statistically different at P ≤0.05.

supplemented in combination with 2,4-D, there was comparative efficiency in the production of phenols. Figure 5 illustrates the total phenolic content of callus developed on MS medium supplemented with different PGR concentrations. The total phenolic content of the leaves of Punarnava was quantified and it was found to be 26.65 mg g^{-1} GAE DW. Studies reveal that callus extracts are reported to have rich phenolic content. It is seen that callus extracts of *Crataegus monogyna* Jacq., an important medicinal plant with neuroprotective and cardioprotective activity is reported to have rich polyphenol content, i.e., 47.70 mg g^{-1} DW (35). The callus of *Heliotropium indicum* L. was induced from internode explants when cultured on the MS medium supplemented with 2.0 mg/L NAA along with 0.5 mg L^{-1} of BA. It is seen that callus extract showed enhanced phenolic content of 9.20 mg g^{-1} GAE (36). Similarly, the callus extracts of *Zingiber zerumbet* (L.) Roscoe ex Sm., Smith reported to have a total phenolic content of 9.0 mg GAE g^{-1} (37).

Estimation of Total Flavonoid content:

The aluminum chloride method was employed for the estimation of flavonoid content. For the quantification of flavonoids from the callus extracts, quercetin was used as a standard. Since flavonoids constitute the main subgroup of phenols, their production trend when quantified is comparable to that of phenols. It is seen that flavonoids were recorded highest in callus induced on MS medium fortified with varied concentrations of 2,4-D alone and 5 ppm 2,4-D recorded highest (30.22 mg g^{-1} QE DW) amount of flavonoids. The callus established on MS medium fortified with KN and 2,4-D also revealed the increased production of flavonoid, followed by the callus induced on BAP in combination with 2,4-D supplemented media. Figure 6 represents the total flavonoid content produced from callus induced on MS medium fortified with different concentrations of PGR's. It is observed that the leaf of Punarnava showed the flavonoid content to be 19.82 mg g^{-1} QE DW. As stated earlier, flavonoids are a prominent class of plant secondary metabolites that fall within the phenol group, and their content will be proportional to that of phenols in the callus extracts as well. The callus from fenugreek (*Trigonella foenum-graecum* L.) was induced on MS medium fortified with 2 mg L^{-1} 2,4-D/NAA along with 0.5 mg L^{-1} KN was dried and the respective methanolic extracts were quantified for total flavonoid content, which is around 217.28 mg L^{-1} (38). Flavonoids were measured from *Heliotropium indicum* L. callus cultures in the same way as total phenolic content. Total flavonoids in the callus extracts were about 1.25 mg g^{-1} QE (36).

In the *in-vitro* conditions, it is known that plant growth regulators play a major role in triggering the genes involved in the biosynthesis of various classes of secondary metabolites. It is also understood from the previous studies that the production of these plant secondary metabolites by the plant growth regulators depends on various factors like types of species, their varieties, environmental and their physiological conditions (39).

Antioxidant potential of callus extracts

Radical scavenging activity:

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to assess the radical scavenging activity of callus extracts. Callus established on MS media fortified with individual 2,4-D at 2.0 and 5.0ppm concentrations showed more than 90% of radical scavenging activity. In rest of the samples, statistically there was no significant difference in their

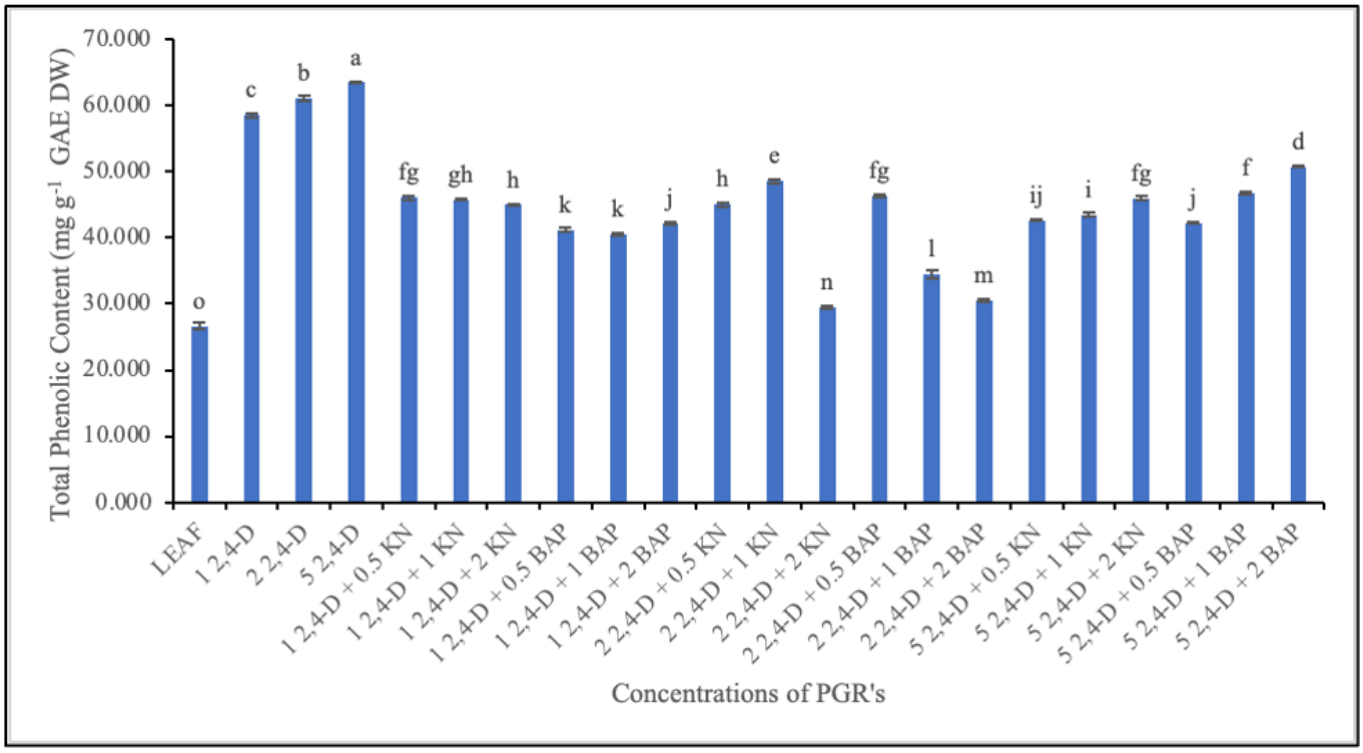

Figure 5 :Total phenolic content from callus cultures of *Boerhavia diffusa* L

The values represent the mean ± SE of three replicates. The bar represents the standard error. Using IBM SPSS software, ver.21, Duncan's multiple range test shows that mean values with similar letters are not statistically different at P ≤0.05.

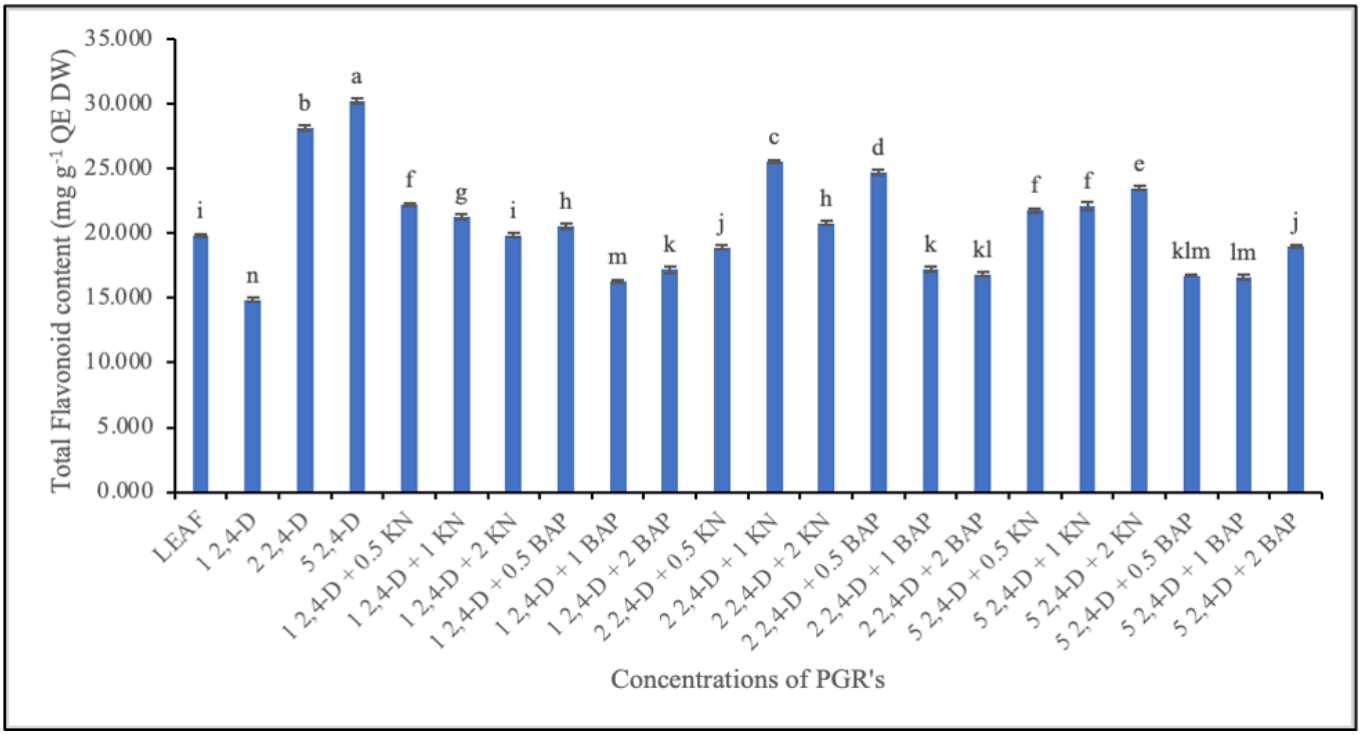

Figure 6 :Total flavonoid content from callus cultures of *Boerhavia diffusa* L.

The values represent the mean ± SE of three replicates. The bar represents the standard error. Using IBM SPSS software, ver.21, Duncan's multiple range test shows that mean values with similar letters are not statistically different at P ≤0.05.

radical scavenging property. However, callus induced on MS medium supplemented with 1.0 ppm 2,4-D along with 1.0 ppm KN showed 89.5% of radical scavenging potential. Figure 7 presents the radical scavenging activity of callus induced on MS medium fortified with various concentrations of PGR's. Radical scavenging property of *Punarnava* leaf showed 84%. The presence of phytochemicals like phenols or flavonoids in callus extracts gives them anti-oxidant properties. Callus extracts are shown to be high in such plant secondary metabolites, which contribute to their antioxidant potential. In the callus extracts of *Stevia rebaudiana* (Bertoni) Bertoni the antioxidant property was analyzed using DPPH radical scavenging activity. It is seen that methanolic extracts of callus showed the highest antioxidant property when compared to leaf extracts with 56.82% of inhibition capacity (40). Radical scavenging activity from callus (derived from cotyledons) extracts of *Argania spinosa* (L.)

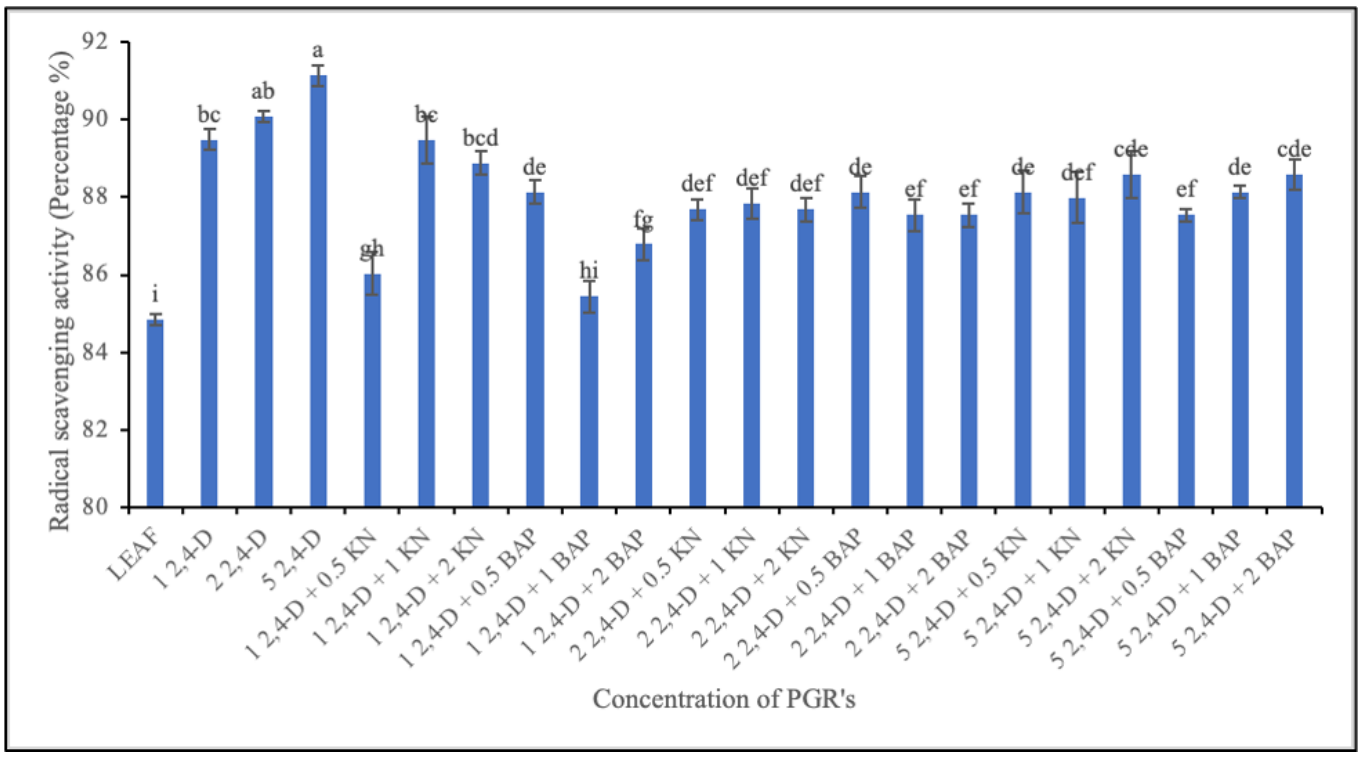

Figure 7:Radical scavenging activity from callus cultures of *Boerhavia diffusa* L.

The values represent the mean ± SE of three replicates. The bar represents the standard error. Using IBM SPSS software, ver.21, Duncan's multiple range test shows that mean values with similar letters are not statistically different at P ≤0.05.

Skeels has shown around 82% of activity (41). Similarly, *Pyrus pyrifolia* (Burm.f.) Nak. callus extracts (callus induced on MS media supplemented with 2ppm picloram) revealed 76.9% free radical scavenging activity, which is analogous to that of standard ascorbic acid (42).

Metal chelating activity:

Metal chelating activity was analyzed using ferrozine and ferrous sulfate solutions. The efficiency in chelating ferrous ions is very important to prove its antioxidant ability, as iron is an important oxidant metal ion. Callus induced on MS medium fortified with 5.0 ppm 2,4-D alone showed the highest metal chelating activity (74.06%), followed by 2.0 ppm 2,4-D and 1.0 ppm 2,4-D. It is also observed that mostly callus established on MS medium supplemented with 2,4-D combined with BAP (5.0 ppm 2,4-D + 2.0 ppm BAP, 1.0 ppm 2,4-D + 2.0 ppm BAP, and 5.0 ppm 2,4-D + 1.0 ppm BAP) showed comparative efficiency with metal chelation. Callus induced on MS medium supplemented with BAP combined with 2,4-D and KN showed 72.19% metal chelating efficiency. Figure 8 presents the metal chelating activity of callus induced on MS medium fortified with different concentrations of PGR's. Callus induced from various parts of *Ocimum sanctum* L. (*Ocimum tenuiflorum* L.) subjected to extraction and the respective callus extracts showed metal chelating activity ranging from 61 to 88 % (43). In addition to this, Song *et al.,* compared the metal chelating efficiency of callus derived from various species of *Ocimum* and they observed that callus extracts showed increased metal chelating efficiency when compared to respective plant extracts (44).

Reducing power assay:

FRAP assay was conducted to analyze the reducing power of callus extracts. The reducing power efficiency is analyzed using the absorbance at 700 nm. There is no clear significant difference recorded among the callus extracts for their reducing power. However, callus induced on MS medium fortified with 5.0 ppm 2,4-D showed the highest absorbance followed by 2.0 ppm and 1.0 ppm 2,4-D. Figure 9 presents the reducing power of callus induced on MS medium fortified with various concentrations of PGR's. In *Habenaria edgeworthii* Hook.f. ex Collett, the callus was induced and the extracts prepared were analyzed for their ferric reducing efficiency. It is seen that the callus extracts are rich in phenolic compounds and show efficient ferric reducing anti-oxidant capacity (45). Similarly, various callus extracts of *Harpagophytum procumbens* DC showed reduced ferric ion efficiency due to the presence of various phytochemicals like phenols, flavonoids, and anthocyanins (46). Thus, the callus extracts, which are rich in pharmacologically important phytochemicals, can be used as phytomedicine.

The presence of several phytochemicals belonging to distinct classes of secondary metabolites is responsible for the aforesaid anti-oxidant properties of crude extracts of callus. Total phenolic content, flavonoid content, GC-MS profile and HPLC analysis of the extract revealed the presence of phenolic and flavonoid compounds which contribute to *Punarnava*'s anti-oxidant potential.

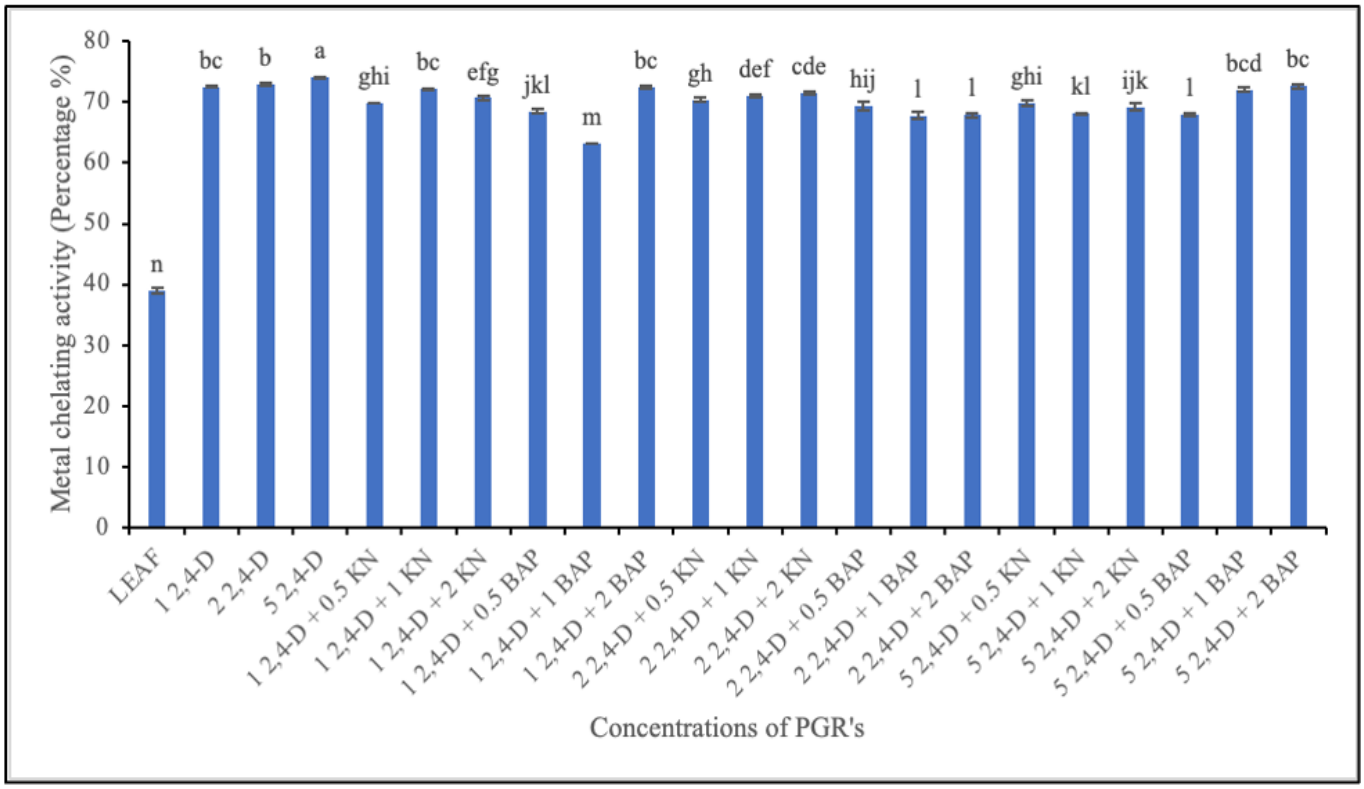

Figure 8:Metal chelating activity from callus cultures of *Boerhavia diffusa* L.

The values represent the mean ± SE of three replicates. The bar represents the standard error. Using IBM SPSS software, ver.21, Duncan's multiple range test shows that mean values with similar letters are not statistically different at P ≤0.05.

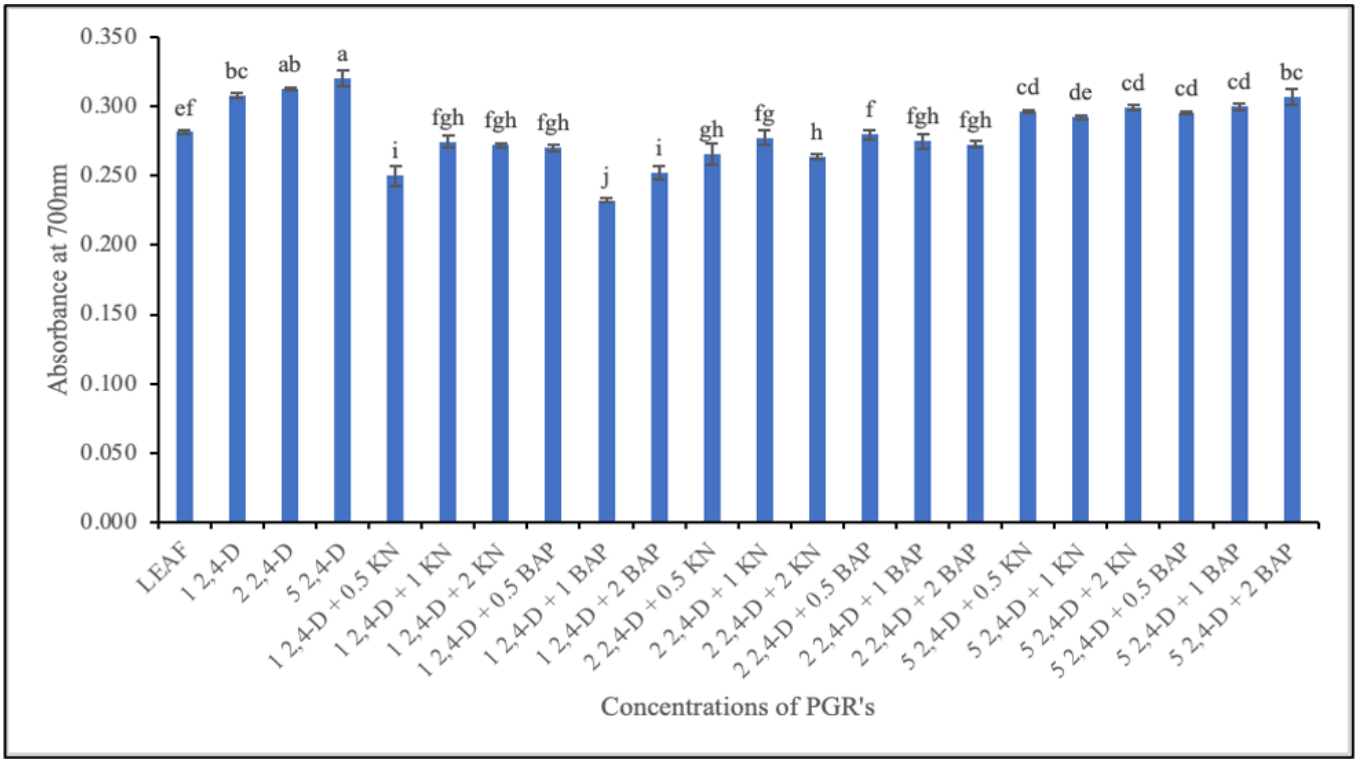

Figure 9:Reducing power from callus cultures of *Boerhavia diffusa* L.

The values represent the mean ± SE of three replicates. The bar represents the standard error. Using IBM SPSS software, ver.21, Duncan's multiple range test shows that mean values with similar letters are not statistically different at P ≤0.05.

Conclusion

It may be deduced from the current investigation that callus developed on MS media supplemented with individual 5.0 ppm 2,4-D produced highest Boeravinone-B content and also showed a similar trend when evaluated for its total phenolic content and flavonoid content. Antioxidant assays support the previous studies reported for the pharmacological efficiency of *Boerhavia diffusa* L. With this initial data for the production of Boeravinone-B from callus, the authors are further interested in establishing callus/cell suspension cultures, optimization of culture medium, and elicitation strategies for enhanced production of metabolite which would open up new arena for mass cultivation of cells and production of Boeravinone-B.

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Authors contributions

WNS conducted experiment, analysed the data, drafted the manuscript, and conducted statistical analysis. PN critically revised the manuscript, supervised and gave the final approval.

Compliance with ethical standards

Conflict of interest: The authors report no conflict of interest.

Ethical issues: None.

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