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Effect of Auxin and Cytokinin on Regeneration, Total Phenolics and *In vitro* Antioxidant Activities of *Bacopa monnieri* (L.) Pennell

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ABSTRACT

Context: Cells are protected from cellular harm by antioxidant compounds. These compounds secure cells from h Cells are protected from cellular harm by antioxidant compounds. These compounds secure cells from harmful free radicals which initiated oxidative stretch. *Bacopa monnieri* (L.) Pennell (Schropulariaceae) have medicinal properties, such as sedative, tranquilizing, antioxidant and memory-enhancing effects. **Objective:** To determine the total phenolic content and concentrations of flavonoids and also in vitro antioxidant activity in methanol extract of shoot culture from leaves explants of *Bacopa monnieri* (L.) Pennell supplemented with various combinations of auxin and cytokinin hormone. **Material and method:** Murashige and Skoog (MS) media was selected for the experiment and different concentration of auxin and cytokinin were added in media. Leaves explants were inoculated on media for callus initiation. Indirect shoot formation occurred on calli of leaf explants after subculture. Total phenolic, flavonoid content and in vitro antioxidant activities such as DPPH radical scavenging activity, hydrogen peroxide (H_2O_2) scavenging activity and ferric reducing power of the shoot cultures are quantitatively estimated by UV spectroscopy. **Result:** The highest content of total phenolics (3.14g/100g d.w.) and flavonoids (0.565 g/100g d.w.) were obtained in shoots regenerated on the MS medium supplemented with BA (2mg/lit) and IAA (1mg/lit). Shoot culture on MS medium supplemented with BA (2mg/lit) and IAA (1mg/lit) showed the highest DPPH radical scavenging activity (85.31%), highest H_2O_2 scavenging activity (75.31%) and highest ferric reducing power (25.3µg/g). **Discussion and conclusion:** Excellent in vitro antioxidant activity was given by extract of Shoot culture on MS medium supplemented with BA (2mg/lit) and IAA (1mg/lit). So they can be used as natural antioxidants after isolation and purification.

Keywords: *Bacopa monnieri*, Antioxidant activity, Auxin, Cytokinin, Regeneration

INTRODUCTION

Bacopa monnieri (L.) Pennell (Schropulariaceae) is a creeping herb of the tropics and normally located in marshy places near the rivers and lakes. Brahmi and Jalbrahmi are popular names of this plant [1]. Brahmi is utilized as the memory enhancer herb and tonicfor nerve and brain cells and, therefore, has gained more importance in ayurvedic system for the treatment of cognitive disorders of aging [2, 3]. It is utilized to reduce inflammation, pain and fever. Traditionally It is very important in treatment of epilepsy, insanity, cancer and oxidative stress [4, 5, 6].

Plant tissue culture techniques are an alternative source to regenerate whole plant for the production of very important bioactive compounds [7, 8]. Attempt has been made to enhancement of active compounds by using plant cell cultures [9, 10]. Several efforts have been made for the enhancement of bioactive compounds by *in vitro* cultures; one of them is utilizing plant growth hormones which are a important key factor for the enhancement of active compounds [11]. Auxin controls growth and morphology of roots, while the effects of cytokinin depends on nature of secondary metabolites and species concerned [12].

Generation of reactive oxygen species (ROS) and antioxidants in the body are balanced, but some time antioxidant defense mechanism is unable to compensate the high quantity of ROS which is dangerous for the body. Various toxic free radicals act through the ROS like superoxide anions (O^{2-}), hydroxyl radicals ($HO^{\cdot}$) and non free radical species such as H_2O_2, singled oxygen (O_2) and nitric oxide (NO) that are capable to generate degenerative processes such as cellular damage that cause to many body disaese viz. heart diseases, cancer and aging. Antioxidants are utilized in prevention of cellular damage.

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Catalase, superoxide dismutase and glutathione peroxidases are some of the natural antioxidants found in body. Free radicals are generated as natural by-product of normal cell metabolism and neutralize by antioxidant compounds [13]. On the basis of in experimental study on medicinal plants and vegetables it was found that natural antioxidants obtained from herbal sources are active compounds which gives a defense mechanism against oxidative stress in biological process [14].

Present research was done on effect of different ratio of auxin and cytokinin in the combination on quantitative estimation of important phytochemical compounds phenolic and flavonoid content and antioxidant activities of methanol extract of regenerated leaves of *Bacopa monnieri* (L.) Pennell.

MATERIAL AND METHOD

Collection of drugs

Fresh Leaves of *Bacopa monnieri* (L.) Pennell. were collected from herbal garden, Jamia Hamdard University, New Delhi, India in the month of March 2016. It was authenticated by Dr. Sayeed Ahmad, Department of Pharmacognosy, Faculty of Pharmacy, Jamia Hamdard, New Delhi India.

Surface sterilization of leaves explants of *Bacopa monnieri* (L.) Pennell.

Young and healthy leaf explants were used for conduct the plant tissue culture work as young cells have capacity to retained their totipotency. The explants (immature leaves) were cleaned under running tap water in glass beaker for 15 minutes, followed by immersed in the soap solution (10%) for 1 minutes, then cleaned with double distilled water for many times to remove the external dust and impurities.

The cleaned explants were immersed in Bavistine solution (1% w/v) for 30 minutes to check fungal growth and then treated with Savlon (1% v/v) for one minute as cleansing agent to decrease the surface tension between epidermis and upper fatty layer. The Savlon treated explants were washed thrice with sterile double distilled water and transferred to alcohol 70% for 30 seconds, which itself is a sterilizing agent and penetration enhancer.

Finally, it was treated with 1% concentrations of mercuric chloride for 2 minute with occasional shaking; it was then washed with the sterile double distilled water for at least six times.

Culture medium

Immature leaf explants (1-2 cm) were immunized on Murashige and Skoog media. Carbon source as 3% sucrose and solidifying agent as 0.8% agar were included in MS media with different concentrations of IAA in combination with BAP and kinetin. The pH of the medium was fixed to 5.8 before adding agar. Media was sterilized with autoclave for 20 minutes at 121ºC for 15 lbf/inch^2 pressure.

Inoculation of explants

After being sterilized, leaves explants were placed on a sterilized petridish with a sheet of millimeter graph paper underneath to allow accurate sizing of explants during dissection. The different explants were cut into 5-10 mm pieces with the help of a sterile and flamed forceps. The explants were transferred to the culture tubes containing nutrient agar media supplemented with different growth hormones for the induction of callus. All the above-mentioned processes starting from the sterilization of explants to inoculation were done in the inoculation room under a laminar air flow cabinet.

Physical conditions of cultures

The cultures were maintained in the culture room at 26 ± 2ºC. The cultures were placed under light, provided by Phillips fluorescent tubes (40 W, 220 V) with a light intensity of 1400-3300 lux at the culture level. The relative humidity (RH) was kept within the normal range (i.e. 60-70%).

After inoculation, culture tubes were kept in B.O.D. incubator at 25º C ± 2º C and the culture tubes were exposed to light for 16 hours (having light intensity of 1600 lux) and kept in the dark for 8 hours, alternatively. These inoculated cultures were observed for any growth and in those cultures; growth was found further subculture in to same hormone combinations. The remaining cultured tubes were observed for 45 days.

Subculture Technique

Subculture technique was done after three weeks of inoculation. Cultures were checked after every transfer by visual observation and the effects of different treatments were measured on the basis of percentage of cultures showing response.

Preparation of methanol extract of shoot cultures

Shoot cultures obtained from leaves explants were dried at room temperature and converted into powder form. 0.5 g of the dried shoot powder was refluxed with methanol in a water bath at 45°C for 3 h. Whatman filter paper No. 4 was used for filtration of liquid extract. Solvent was evaporated from collected filtrates under vacuum at 40°C. The extraction was rehashed twice. The dried residue was dissolved in methanol for sample solution and utilized for the quantitative estimation of phenolics and flavonoid contents and determination of antioxidant activities [15].

Determination of total phenolics content [16]

Preparation of sample: 0.5ml of extract of shoot culture (8mg/ml) was included to 5 ml of 10% Folin-Ciocalteu reagent and 4 ml of 1M Na_2CO_3 solution and blended. It was permitted to keep in dark room for 15 minutes. The absorbance of reaction mixtures were taken at 765 nm. Phytochemical compound total phenolic was communicated as mg Gallic acid equivalents / 100 g dry weight (d.w.) of the extract.

Preparation of standard: 0.5ml of standard dilution (10µg, 20 µg, 50 µg and 100 µg) was included to 5 ml of 10% Folin-Ciocalteu reagent and 4 ml of 1M Na_2CO_3 and blended. It was permitted to keep in dark room for 15 minutes. The absorbance of reaction mixtures were measured at 765 nm.

Determination of total flavonoid content [16]

Preparation of sample: 0.5 ml of extract of shoot culture (4mg/ml) and 1.5 ml methanol were blended well. After that 0.1 ml of $AlCl_3$ (0. 1mg/ml) and 0.1 ml of 1M CH_3COONa reagents were included to above solution. This reaction mixture was included to 2.8 ml of Distilled water and blended. It was maintained for 30 minute in absence of light. The absorbance of reaction mixture was taken at 415 nm. Phytochemical flavanoid was communicated as mg rutin equivalents / 100 g d.w. of the extract.

Preparation of Standard: 0.5 ml of standard dilution (10µg, 20 µg, 50 µg and 100 µg) was utilized and blended to 1.5 ml methanol. This mixture was blended with 0.1 ml of $AlCl_3$ (0. 1mg/ml) and 0.1 ml of 1 M CH_3COONa reagents. This test mixture was included to 2.8 ml of Distilled

water, blended and kept for 30 minutes in absence of light. The absorbance of test mixtures was measured at 415 nm.

Blank solutions: 0.1 ml of $AlCl_3$ and 0.1 ml of CH_3COONa reagents were blended with 2 ml methanol and this test mixture was blended with 2.8 ml distill water.

Determination of antioxidant activity

DPPH free radical scavenging activity

0.1 mM of 1, 1- diphenyl-2-picryl-hydrazyl (DPPH·) was blended in methanol and 1 mL of this mixture was blended to 3 mL of each methanol extract at one concentration (500 µg/mL). Butylated hydroxytoluene (BHT) was included as a control. Discoloration was seen at 517 nm after incubation for 30 min. absorbance were taken least in triplicate. The capacity to rummage the DPPH radical was estimated utilizing the formula: DPPH rummaging impact (%) = [ADPPH − AS / ADPPH] x100 where, ADPPH is the absorbance of the DPPH mixture and A*S* is the absorbance of the test mixture when the sample extract is utilized. The sample concentration giving 50% hindrance of radical-scavenging activity (IC_{50}) was estimated and communicated as mg/mL, d.w. [17].

Ferric reducing power determination

500µg/ml regenerated Shoot extract blend was mixed with phosphate buffer (2.5 mL, 200 mM, pH 6.6) and 1% potassium ferricyanide (2.5 mL). At that point the blend was kept in incubator for 20 min at 50 °C. Blend was mixed with 2.5 mL of 10% trichloroacetic acid and centrifuged at 10000 rpm for 10 min. The supertant fluid (5 mL) was with distilled water (5 mL) and 0.1% ferric chloride (1 mL). The absorbance of the reaction blend was adjusted at 700 nm. The final result were communicated as µg ascorbic acid equivalents / g on the basis dry weight of the extract [18].

Hydrogen peroxide scavenging activity

0.1 M, 3.4 mL phosphate buffer with pH 7.4 was mixed with and 600 µl H_2O_2 solution (43 mM) and 50 µg/mL sample blend. The absorbance of mixture was measured at 230 nm. 50µg/mL BHT was utilized as control and percentage H_2O_2 rummaging effect was measured as [$A_{Control}$ – A_{Sample} / $A_{Control}$] x 100 where $A_{Control}$ is the absorbance of the control, and A_{Sample} is the absorbance of the sample blend. The extract concentration utilizing 50% of H_2O_2 rummaging effect (IC_{50}) was measured and communicated as µg/mL on the basis of sample dry weight [19].

Statistical analysis

The tests were carried out utilizing Completely Randomized Design (CRD). Triple readings were attempted in each test. Data are maintained as means ± standard deviation (SD). Analysis of variance and significant differences among means were tried by one-way ANOVA using the COSTAT computer package (Cohort Software, 1989). The least significant difference (LSD) at $P \leq 0.05$ level was included. Correlation coefficients (R^2) from regression investigation between phenolic, flavonoid substance and antioxidant activities were moreover premeditated.

RESULTS

Induction and proliferation of callus from leaf explants

Young leaves are excised and are utilized as explants for the callus induction. Leaf explant were immunized on MS medium with auxin (IAA) and cytokinins (BAP and Kinetin) plant growth hormones in combination. Plant growth regulator with their code, nature of callus, intensities of callus were shown in table 1. F**igure 1** shows a profuse yellowish green coloured callus and indirect shoot regeneration with IAA (2.0 mg/L) and BAP (1 mg/L).

Table 1: Effect of different concentration of auxin with cytokinin on growth of callus from leaf explant of *Bacopa monnierie* (L.) Pennell.

Hormone code	Concentration (mg/L)		Intensity of callus formation	Nature of callus	Response (%)
	IAA	BA			
H_1	1.5	1.5	+++	Dark green compact	74
H_2	1.0	2.0	+++	Light green nodular	83
H_3	1.5	0.5	++	Yellowish green compact	71
H_4	1.0	4.0	++	Light green nodular	66
H_5	1.5	3.0	+	Dark green nodular	65
H_6	1.0	3.0	++	Yellowish green compact	62
H_7	0.5	4.0	++	Light brown friable	59
H_8	1.0	4.5	+	Light green copact	58
H_9	2.0	2.0	+	Yellowish green friable	65
H_{10}	0.5	3.5	++	Dark green friable	62

Indirect shoot organogenesis from leaf derived callus

Leaves are important explants and generally selected for *in vitro* regeneration from leaves explants as it maintains the genetic homozygosity of the parent genotype. Cytokinin with auxin are responsible for indirect multiple shoot regeneration from callus culture. Table 2 shows maximum shoot number (25.0 ± 0.11) and shoot length in cm (3.2 ± 0.05) were observed in MS medium with BAP (2mg/lit) with IAA (1mg/lit).

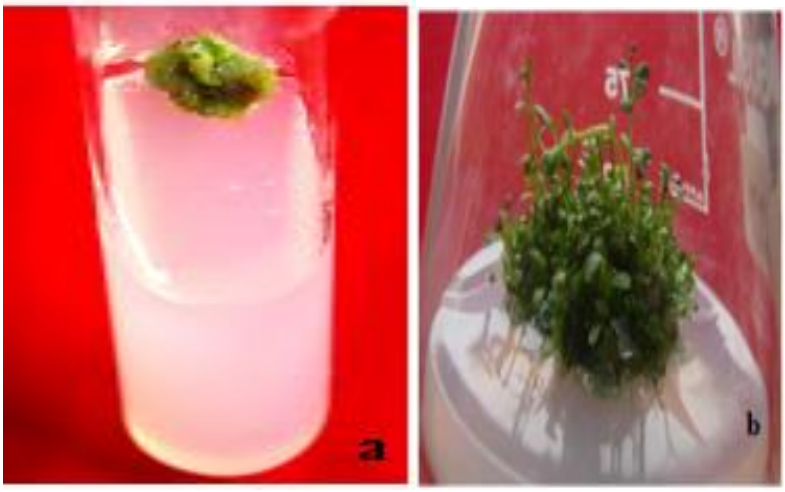

Figure 1(a): proliferation of callus on MS medium with BAP (2mg/lit) and IAA(1 mg/lit)
(b): indirect shoot regeneration from callus with BAP (2mg/lit) and IAA(1 mg/lit)

Table 2: Effect of combinations of BA and IAA on shoot regeneration from leaf explants from *Bacopa monnierie*(L.) Pennell.

Hormone code	Concentration (mg/L)		*Number of shoot/explants	*Shoot length(cm)	Response (%)
	IAA	BA			
H_1	1.5	1.5	9.20 ± 0.24^c	7.02 ± 0.41f	76
H_2	1.0	2.0	18.4 ± 0.42l	12.9 ± 0.29g	85
H_3	1.5	0.5	5.4 ± 0.42l	9. 9 ± 0.64i	70
H_4	1.0	4.0	8.12 ± 0.04j	8.6 ±0.21k	65
H_5	1.5	3.0	12.2 ± 0.15g	9.55 ± 0.42c	62
H_6	1.0	3.0	8.6 ± 1.43c	10.02 ± 0.16b	64
H_7	0.5	4.0	8.20 ± 0.24^c	6.52 ± 0.12d	56
H_8	1.0	4.5	6.14 ± 0.48d	6.23 ± 0.71f	59
H_9	2.0	2.0	13. 82 ± 0.42l	7.95 ± 0.41f	67
H_{10}	0.5	3.5	10.20 ± 0.04j	6.40 ± 0.29g	61

Data represent treatment means ± SE fallowed by different letter(s) within column indicate significant differences according. ANOVA and DMRT test ($P < 0.05$) to ANOVA and DMRT test ($P < 0.05$).

Total phenolics and flavanoid content in shoot culture of leaf explant

One-way ANOVA investigation maintainted significant differences ($p \leq 0.05$) in total phenolics and flavanoid phytochemical among the ten different combinations of growth regulators. Quantitative measurement of total phenolic content and flavanoids phytochemicals from different combination of auxin and cytokinin were tepresented in Figure 2. Highest phenolics content (3.14 g/100g d.w.) and flavanoid (565mg/100g) were observed from H_2 hormone code in methanol extract of shoot culture of *Bacopa monnieri* (L.) Pennell.

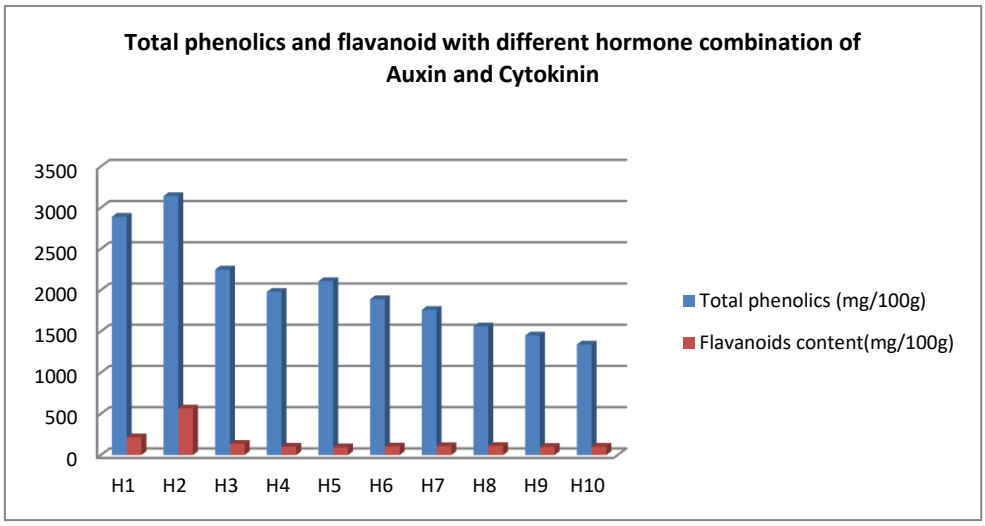

Figure 2: Quantitive estimation of total phenolics and flavanoid with presence of different combination of auxin and cytokinin.

DPPH free radical scavenging activity of regenerated shoot culture

The antioxidant activities of methanol extracts of regenerated leaves culture of of ***Bacopa monnieri*** **(L.) Penneli** and standard antioxidant BHT were determined using the DPPH method. Table 3 shows leaf-regenerated on hormone code H_2 has given relatively higher radical scavenging activity (85.31 %) with IC50 value (0.890 mg/mL) than other media code. The rummaging activity of BHT as standard represented higher activity 89.31 % with IC50 value (0.920 mg/mL) than methanol extracts of regenerated cultures.

Table 3: Evaluation of in vitro antioxidant activity of methanol extracts of regenerated shoots from with different hormone combination of *auxin and cytokinin.*

Hormone Code	*DPPH scavenging activity (%, mean ± SD)	*IC50 values (mg/mL, d.w.) of DPPH scavenging activity	*Ferric reducing power(µg/g, dw) (%, mean ± SD)	*Hydrogen peroxide scavenging activity (%, mean ± SD)	*IC50 values (µg/mL, d.w.) of H_2O_2 scavenging activity (%, mean ± SD)
H_1	79.63 ± 0.176	0.793 ± 0.034	23.4 ± 0.163	71.63 ± 0.176	81.54 ± 0.143
H_2	85.31 ± 2.685	0.890 ± 0.084	25.3 ± 0.182	75.31 ± 0.585	82.34 ± 0.353
H_3	72.7 ± 0.463	0.669 ± 0.024	18.6 ± 0.124	64.32 ± 0.213	71.70 ± 0.463
H_4	74.31 ± 0.585	0.754 ± 0.031	21.2 ± 0.113	68.63 ± 0.176	65.8 ± 0.393
H_5	71.89 ± 2.224	0.721 ± 0.074	21.7 ± 0.193	70.89 ± 0.224	73.5 ± 0.273
H_6	69.63 ± 0.176	0.513 ± 0.034	15.4 ± 0.163	67.63 ± 0.176	66.54 ± 0.143
H_7	65.31 ± 2.685	0.690 ± 0.084	19.3 ± 0.182	64.31 ± 0.585	62.34 ± 0.353
H_8	68.7 ± 0.463	0.749 ± 0.024	18.6 ± 0.124	66.32 ± 0.213	72.70 ± 0.463
H_9	61.31 ± 0.585	0.654 ± 0.031	22.2 ± 0.113	62.63 ± 0.176	63.8 ± 0.393
H_{10}	63.89 ± 2.224	0.611 ± 0.074	20.9 ± 0.193	62.89 ± 0.224	60.5 ± 0.273
Control	89.31 ± 2.685	0.920 ± 0.084	27.3 ± 0.182	78.31 ± 0.585	85.34 ± 0.353

*=average of three estimations at each level

Ferric reducing power determination of regenerated shoot culture

Changing of the Fe^{3+} form in complex to the ferrous form due to the involvement of antioxidant phytochemical compounds in sample blend. The transformation of iron (III) to iron (II)-reducing activity in the shoot culture of methanol extracts was communicated as µg ascorbic acid equivalent/g sample on the basis of dry weight. Table 3 represents Highest reducing power capacity (25.3 µg/g d.w.) was reported in shoot cultured obtained from MS media with H_2 hormone code as compared to the activity of control (27.3 µg/g d.w.).

Hydrogen peroxide scavenging activity of regenerated shoot culture

Hydrogen peroxide can enter to inside cell and may slowly oxidize a number of compounds. Thus, depleting of hydrogen peroxide and superoxide anion is very important for preservation of food systems. H_2O_2 scavenging activity of methanol extracts of shoot culture at concentration 150 µg/mL differed significantly (P≤0.05) among the different concentrations of growth regulators. Table 5 shows H_2 hormone code gave the highest H_2O_2 scavenging activity (75.31 %) with IC_{50} value 82.34 µg/mL in shoots culture.

DISCUSSION

Inoculation of explants in the culture medium containing auxin and cytokinin resulted the generation of multiple shoots and transfer of these shoots to the fresh medium containing auxin or small quantity of BAP improved the shoot multiplication and elongation [20].

Our results follow the previous studies, where BA in combination with auxins resulted in increment of shoot number and shoot length in culture media. Involvement of low concentrations of auxin along with optimum concentration of BA yielded increased shoot length and shoot number. Although all three auxins (NAA, IAA, and IBA) improved the increment of shoot number and length, BAP in combination with low concentration of IAA was reported to be efficacious for increment of shoot generation from node and shoot-tip explants of *Bacopa chamaedryoid*. With inclusion of adequate quantity of BA with less amount of IAA in culture media yielded satisfactory amount of shoot number and shoot length in *Scrophularia takesimensis*, *Momordica dioica* and *Cassia angustifolia* [21, 22, 23]. Agreeing to our inquire about, adequate auxin concentration reduced shoot number and shoot lenghth for both nodal and shoot-tip explants and adequate concentration of cytokinin along with less concentration of auxin expanded shoot number and shoot length.

The adequate amount of antioxidants in the extract of shoot culture from *Bacopa monnieri* yielded the reduction of $Fe^{3+}$and Fe^{2+} by utilizing an electron. The quantity of $Fe^{3+}$and Fe^{2+} ions can be identified by the Perl's blue colour observation and evaluated by the absorbance at 700nm [24]. Phenolics compounds as antioxidant is related to reducing capacity [25]. Reducing capacity is observed due to the presence of the high phenolic compounds of the plant [26]. Highest Ferric reducing power of shoot cultures of *Bacopa monniera* is observed by media code H_2 due to the presence of highest phenolic content and flavanoids.

The high DPPH activity could be related with high phenolics content. Reduction of absorbance of DDPH radical occurs with high phenolic content. DDPH works as a stable free radical in methanol solution that easily takes an electron or hydride radical and changed to a stable diamagnetic molecule. By interact with appropriate reducing agents DPPH radicals changed into the corresponding hydrazine. In this present study, H_2 media code showed more prominent DPPH scavenging activity than other extracts of media combination.

Involvement of low levels of H_2O_2 in biological systems may be important. Naturally found iron complexes inside the cell are generally interacted with H_2O_2 *in vivo* to produce highly reactive hydroxyl radicals which may be toxic for animal and human body [27]. Hydrogen peroxide is very responsive and may be toxic to cell because it may produce hydroxyl radical in the cells [28]. Thus, clearnce of H_2O_2 is very important for safety of our food systems. Scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water. Highest capacity for scavenging of hydrogen

peroxide is given by the methanol extracts of shoot culture supplemented with IAA (1 mg/lit) and BA (2 mg/lit).

CONCLUSION

On the basis of our research, it was observed that higher quantity of total phenolics and flavanoids were reported in regenerated shoot culture from leaf explants of *Bacopa monnieri* (L.) Pennell in MS media with IAA (1 mg/lit) and BA (2 mg/lit) than intact plant leaves. They give significant antioxidant activities to hydroxyl radical, superoxide radical, and DPPH radical. Methanol extracts of regenerated leaves with different hormonal combination possess different antioxidant activities. Content of flavonoids are directly related to antioxidant activity of sample molecules. Thus, the extract from shoots culture of *Bacopa monnieri* (L.) Pennell could be used as an antioxidant herb for adjuvant therapy. As the synthetic antioxidant Butyl Hydroxy Toluene was forbidden being used in food due to its side effects on human, development of the natural antioxidants was meaningful and prospective. In this study, antioxidant activities of regenerated leaves from *Bacopa monnieri* (L.) Pennell was found more than extract of intact leaves of plant. So they can be used as natural antioxidants after isolation and purification.

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