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Research Article

Optimization of Callus Induction with Enhancing Production of Phenolic Compounds Production and Antioxidants Activity in Callus Cultures of *Nepeta binaloudensis* Jamzad (Lamiaceae)

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Background: World Health Organization (WHO) reported that more than 80% of people in the world use herbal traditional medicines nowadays. Many endemic medicinal plants, especially *Nepeta* species, are facing to extinction as a result of high harvesting, limited distribution, and habitat destruction.Tissue culture is a successful method for plant secondary metabolites production. *Nepeta binaloudensis* is a medicinal plant belonging to family Lamiaceae.

Objective: Our study was focused on devising an optimum procedure for callus induction and phenolic compounds production in *N. binaloudensis*. First, we are focused on finding suitable explants and media for callus induction. Then, subsequent experiments were conducted to find an optimal concentration of plant growth regulators (PGRs) and reduced-glutathione for maximum biomass production, and phenolic compounds production in calli.

Material and Method: In this study, the usage of whole plant grown in Hoagland nutrient solution, were used as a source of explants. Also, different media including, ½ MS, MS, and B5 and different combination of PGRs (NAA and BAP) were used for optimization of calli induction.

Results: Based on the results of the first experiment, leaf-originated explants, and macro half strength MS (½ MS) medium were used for the next experiments. The highest FW (Fresh Weight) and DW (Dry Weight) of calli were observed in ½ MS medium, supplemented with 2 µM/L reduced-glutathione, 2 $mg.L^{-1}$ BAP, and 2 $mg.L^{-1}$ NAA. The maximum amount of total phenolic, flavonoid, tannin contents and free-radical scavenger were observed in calli which were grown in ½ MS medium supplemented with 2 µM/L reduced-glutathione, 2 $mg.L^{-1}$ BAP, and 2 $mg.L^{-1}$ NAA.

Conclusion: Our study finds the optimum condition for calli induction and phenolic compounds production in *N. binaloudensis*.

Keywords: Endangered plant, Medicinal plant, Optimization callus induction, Phenolic compounds, Radical scavenging, Tissue culture. 1/2 MS (Macro half strength MS)

1. Background

World Health Organization (WHO) reported that more than 80% of people in the world use herbal traditional medicines nowadays (1, 2). *Nepeta* species belonging to Lamiaceae contain a wide range of medicinal compounds (e.g. antispasmodic, expectorant, antiseptic, anti-asthmatic, carminative and febrifuge activities) which are used in Iranian traditional medicine (3, 4). These species contain phenolic compounds (5, 6). Among them, nepeta-lactone (3) and 1,8 cineol are the most material ones (7, 8). *Nepeta binaloudensis* Jamzad is a perennial medicinal plant which geographically grows in a restricted region in the northeast of Iran - Binaloud and Hezarmasjed Mountains (9, 10).

Many endemic medicinal plants, especially *Nepeta* species, face to extinction due to high harvesting, limited distribution, and habitat destruction (11). Plants cultivation in field is a time-consuming labor work process. Therefore, the usage of cell-tissue and organ culture is reported as a useful method for the production

of secondary metabolites (12, 13). Plant tissue and organ culture technique is an economical and suitable method for natural compound productions in laboratory conditions (14). Callus formation has been attracted a lot of attention for both research and industrial purposes. Secondary metabolites such as phenolic compounds are produced in low amounts in tissue and organ culture (15). Using chemical elicitors (e.g. Plant Growth Regulators; PGRs) and signaling molecules (e.g. methyl-jasmonate, salicylic acid, chitosan, and heavy metals) can improve secondary metabolites production (16).

Secondary metabolites of plants, such as phenolic and flavonoid compounds, play a central role in the plant defense system (17). Phenolic and flavonoid compounds are produced by the phenylpropanoid biosynthetic pathway (17, 18).

For these reasons, *in vitro* culture provides the conditions for the production of phenolic compounds. These compounds are essential targets for tissue culture in many kinds of research (19). Many studies imply phenolic compounds production under *in vitro* conditions (20-26).

2. Objective

To the best knowledge of the authors, there are a few or no reports for tissue culture and callus induction of *N. binaloudensis*. So, the objectives of this study are to:

1. Determine the best explants and media for callus induction.
2. Find the optimum combination of 6-Benzylaminopurine (BAP) + 1-Naphthylacetic Acid (NAA) for callus induction.
3. Evaluate the total phenolic, flavonoid, tannin contents, and antioxidant capacity of *N. binaloudensis* callus extracts.

3. Materials and Methods

3.1. Plant Materials Preparation

The seeds were extracted from the ripe fruits of a collected *N. binaloudensis* specimen. This specimen was stored in Ferdowsi University of Mashhad Herbarium (FUMH) under the voucher number of FUMH-1025. The seeds were surface-sterilized with 5% sodium hypochlorite for 5 minutes, ethanol 70% (v/v) for 1 minute and washed with distilled water 2-3 times. Seeds were transferred into Petri dishes filled with 5 ml distilled water. These Petri dishes were placed under the continuous dark condition for seed germination. The germinated seeds were transferred into a container supplemented with the Hoagland nutrient solution (citation needed), under a controlled condition (16 hours light (45 µ moL. m^{-2} s^{-2} irradiance level) and 25 ± 2 ºC) for three months. The Hoagland solution was renewed once a week to prevent nutrient deficiency. After three months, the whole-plants parents were used as the explant sources.

3.2. Different Potential of Explants for Callus Induction

In this study, three different types of explants (i.e., leaf, shoot apical meristem, and stem) were used for *N. binaloudensis* callus induction. These explants were cultivated in Murashige and Skoog (MS) medium, containing different concentrations of BAP and 2-4 Dichlorophenoxy acetic acid (2,4-D). The optimal explant for callus induction was selected using the Callus Induction Factor (CIF) formula.

CIF = Number of explants with callus / total number of explants used × 100

The data obtained from this step was used for the subsequent experiments.

3.3. Effect of Different Media in Callus Induction

After determining the best explant, three media, including MS, ½ MS (Macro half-strength MS), and B5 (27), were evaluated for callus induction. At this stage of the experiment, different concentrations of BAP alone were used. Ultimately, the fresh weight (FW) of each treatment was recorded. Based on FW comparison, a suitable culture medium was determined for the next experiment.

3.4. Combination of Reduced –Glutathione and PGRs to Prevent Browning

In this step, for the prevention of explant browning, we used media containing reduced-glutathione ($2\mu M.L^{-1}$) as an antioxidant compound. Different combinations of PGRs (**Table 1**) were exogenously applied to increase callus biomass production.

3.5. Preparation of the Culture Medium and Phytotron Condition for Calli and Their Harvesting Time

The aseptic explants were prepared for culture on different media. The pH of the media was adjusted to 5.8 with NaOH (1M) and HCl (1 M). These culture media (30 mL) were transferred to jars (200 mL) and then were autoclaved at 121 °C (250 ºF) 100Kpa above atmospheric pressure for 15 minutes. The calli was subcultured once a week (placed under dark condition). The fresh calli was harvested after 50 days and the callus morphological characteristics such as FW, Dry Weight (DW), and Dry Matter Content (DMC) texture and color of callus were recorded.

Table 1. The abbreviation of plant growth regulators indicated for addressing in context.

Hormonal combination	Abbreviation
Control	Free-hormone
0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	0.5 B, 0.5 N
0.5 mg L^{-1} BAP + 1 mg L^{-1} NAA	0.5 B, 1 N
0.5 mg L^{-1} BAP + 2 mg L^{-1} NAA	0.5 B, 2 N
1 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	1 B, 0.5 N
1 mg L^{-1} BAP + 1 mg L^{-1} NAA	1 B, 1 N
1 mg L^{-1} BAP + 2 mg L^{-1} NAA	1 B, 2 N
2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	2 B, 0.5 N
2 mg L^{-1} BAP + 1 mg L^{-1} NAA	2 B, 1 N
2 mg L^{-1} BAP + 2 mg L^{-1} NAA	2 B, 2 N

3.6. Preparation of Methanol Extract for Phytochemical Content Analysis

Calli were dried by the freeze-drying process. Each sample of dried calli (50 mg) was crushed to a fine powder. Then, the fine powder was added with 2 mL pure methanol. These mixtures were sonicated by (500w, 40 ºC) using Par sonic (the 2600s, IRAN) for 22 minutes and subsequent shaken for 12 hours. Supernatant of these extracts was filtered with Whatman paper (No. 1) and the solvent was evaporated under air condition. Then, dried extracts were weighted and kept at 4 ºC for biochemical analyses.

3.7. Total Phenolic Contents Assay

Total phenolic content of methanolic extracts was measured using the Singleton *et al*. method (28). Folin-Ciocalteu reagent (1 mL) (Sigma-Aldrich, Munich, Germany) and distilled water (2 ml) were added to 100 µL of the extract. After 3 minutes, 1 mL of 20% sodium carbonate (Na_2NO_3) was added, stirred rigorously in a vortex mixer, and stored in the dark condition at room temperature for 45 minutes. The absorption of the reaction mixtures was measured using spectrophotometer (Jasco UV/Vis 7800, Germany) at 725 nm. Total phenolic content was calculated as reported by a standard curve prepared with Gallic Acid Equivalent (GAE; 20-400 mg.L^{-1}) and expressed as mg of GAE per g DW.

3.8. Total Flavonoid Contents Assay

Total flavonoids content of the dried extracts was determined according to method Zhishen *et al* (28). Methanolic extracts (0.5 mL) were supplemented with 2 mL of distilled water and 150 µL of $NaNO_2$ solution (20%, w/v). After 5 minutes, 150 µL of $AlCl_3$ (10%, w/v) was added to the solution, followed by the addition of 1 mL of NaOH (1M) and 5 mL of distilled water. The mixtures were then shaken and kept in the dark condition for 30 minutes. The absorption of the reaction mixtures was read using spectrophotometer (Jasco UV/Vis 7800, Germany) at 510 nm. A standard curve was prepared using a Catechin Equivalent (CE) solution (20-100 mg.L^{-1}) and the results were expressed as mg CE per g DW.

3.9. Total Tannin Content Assay

Determination of total tannins content was performed with Sun *et al.* method (29). A 250 µL of methanolic extract was adjusted with 750 µL pure methanol. Then, 3 mL of methanol vanillin solution (1%, W/V), and 2.5 mL of methanol sulfuric Acid (9 N) were added to the mixtures. The mixtures were incubated on the water bath (38 ºC) for 15 minutes. The absorption of the mixtures was recorded by spectrophotometer (Jasco UV/Vis 7800, Germany) at 500 nm. Total tannin content was determined with the standard curve of CE solution and expressed as mg of CE solution per g DW.

3.10. Antioxidant Capacity Assay

The antioxidant capacity of calli extracts was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenger (Sigma-Aldrich, Germany). Antioxidant capacity was performed through Hatano *et al*., (1988), which was described by Cheniany *et al*., (30). Different concentrations of the extract were mixed with 1.5 mL of pure Methanol. Then, these mixtures were added to 0.5 mL of 0.1 mM methanol solution of DPPH reagent. After 30 minutes incubation, the mixtures' absorption was read by spectrophotometer (Jasco UV/vis 7800, Germany) at 517 nm against blank. The antioxidant capacity was measured based on the standard curve of ASC solution and expressed as mg of ASC solution per g DW. The inhibition percentage for scavenging DPPH free radical was calculated according to the equation:

IC50 % = {(A blank – A sample) / A blank} ×100

Here, a blank is the absorbance of the control (containing all reagents without the extract), and a sample is the absorbance of the tested sample. The extract concentration (µg.mL^{-1}) providing 50% inhibition was determined by plotting inhibition percentage against the concentration of the extract.

3.11. Statistical Analysis

All experiments were carried out in a factorial test on the basis of a completely randomized design with three independent replications. Statistical analysis was

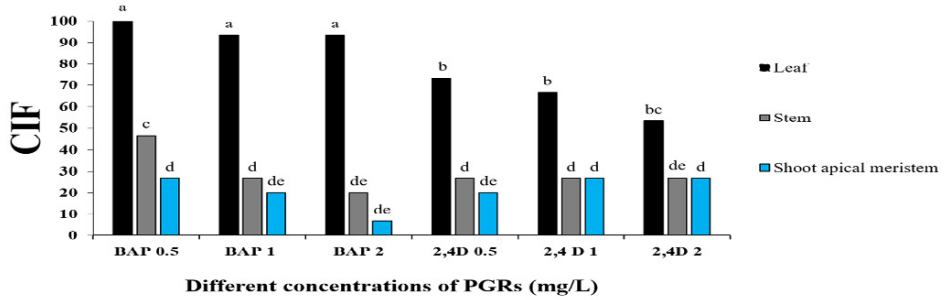

Figure 1. Comparison of potential callus induction in different explants of *N. binaloudensis*. Same letters indicate no significant difference among the means based on Duncan's multiple range test (p-value ≤ 0.05).

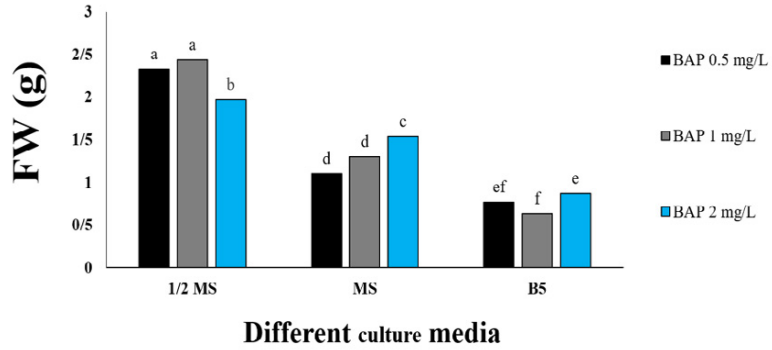

Figure 2. Comparison of different media on FW callus of *N. binaloudensis*. Same letters indicate no significant difference among the means based on Duncan's multiple range test (p-value ≤ 0.05).

performed using SPSS 25 software. Duncan's multiple range test (p-value ≤ 0.05) was used for comparison of means in all experiments.

4. Results

4.-1. Different Potential of Explants in Callus Induction

CIF results showed that the callus induction of *N. binaloudensis* was significantly influenced (p-value ≤ 0.05) by the type of explants and PGRs. In this stage, callus induction was observed after 13-16 days in all explants. Necrosis and blackening also occurred after 21 days in all explants. The study found that leaf has a higher callus induction potential than other explants (**Fig. 1**). Necrosis and blackening occurred more in explants cultured in MS medium containing 2,4 D rather than other PGRs.

4.2. Effect of Different Media on Callus Induction

Results showed that the fresh weight of calli was significantly affected (p-value ≤ 0.05) by the type of culture medium and different concentrations of BAP. Moreover, the highest fresh weight of calli was observed in ½ MS medium (**Fig. 2**). Callus formation was not observed in hormone-free media. However, toxic compounds were increased in the culture medium, which reduced the growth of calli. No callus was induced when explants were cultured on control MS and B5 media.

4-3- Effects of Reduced-Glutathione and A Combination of BAP and NAA on FW, DW, and DMC of N. binaloudensis Calli

In this study, ½ MS medium supplemented with 2 µM L^{-1} reduced-glutathione, and the combination of different concentrations of B and N was used to prevent the browning of cultured explants. Results showed that a combination of B, N had a significant effect on FW, DW, and DMC of calli. The highest FW and DW of callus (7.255 g jar^{-1} and 0.505 g jar^{-1}, respectively) were observed in the ½ MS medium, which was supplemented with the combination of 2 B, 2 N. The maximum DMC (11.273%) was related to ½ MS medium supplemented with 1 B, 2 N (**Table 2**).

The texture of the calli under different combinations of 0.5 B, 0.5 N, 0.5 B, 1 N, 0.5 B, 2 N, 1 B, 1 N, 1 B, 2 N was compact, whereas in four combinations of PGRs 1 B, 0.5 N, 2 B, 0.5 N, 2 B, 1 N, 2 B, 2 N it was fragile. The

Table 2. The comparison of callus characteristics of *N. bianloudensis* in response to different concentrations of B, N.

PGR concentration (mg L^{-1})	Callus growth parameters		
	Fresh weight (g jar^{-1})	Dry weight (g jar^{-1})	Dry matter content (%)
0.5 B, 0.5 N	3.6 ± 0.298 ^{bc}	0.275 ± 0.024 ^{c}	7.63 ± 0.271 ^{bc}
0.5 B, 1 N	1.3 ± 0.497 ^{de}	0.119 ± 0.041 ^{de}	9.156 ± 0.731 ^{b}
0.5 B, 2 N	1.05 ± 0.123 ^{e}	0.104 ± 0.010 ^{de}	9.83 ± 0.395 ^{ab}
1 B, 0.5 N	3.23 ± 0.292 ^{c}	0.25 ± 0.036 ^{c}	7.73 ± 0.678 ^{bc}
1 B, 1 N	1.26 ± 0.317 ^{de}	0.11 ± 0.011 ^{de}	8.116 ± 1.662 ^{bc}
1 B, 2 N	0.814 ± 0.186 ^{e}	0.092 ± 0.022 ^{e}	11.273 ± 0.141 ^{a}
2 B, 0.5 N	4.06 ± 0.805 ^{b}	0.33 ± 0.036 ^{b}	8.216 ± 0.802 ^{bc}
2 B, 1 N	1.98 ± 0.288 ^{d}	0.16 ± 0.045 ^{a}	7.93 ± 1.195 ^{bc}
2 B, 2 N	7.25 ± 0.456 ^{a}	0.505 ± 0.034 ^{a}	6.976 ± 0.731 ^{c}

Same letters indicate no significant difference among the means based on Duncan's multiple range test (p-value ≤ 0.05).

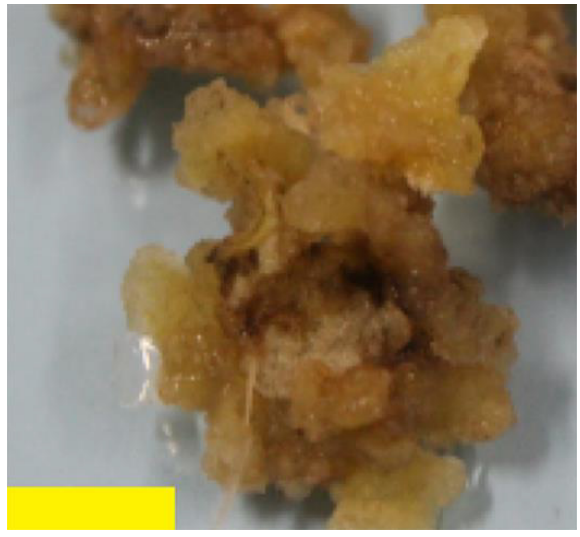

Figure 3. A close proximity of *N. binaloudensis* callus induction. Bar is 2.5 cm.

dark brown calli were observed in two combinations of PGR 0.5 B, 1 N, 0.5 B, N 2. Finally, yellow, whitish-green, and pale-yellow calli were observed under other combinations of B, N. A photograph of leaf explant callus induction is shown in **Figure 3**.

4-4- Total Phenolic, Flavonoid and Tannin Content

Results showed that PGRs had a significant effect on the content of biochemical compounds (p-value ≤ 0.05). The highest amounts of biochemical compounds, including phenolics, flavonoids, and tannins content, were observed in the callus extracts of ½ MS supplemented with 2 µM L^{-1} reduced-glutathione and 1 B, 2 N (**Fig. 4a, b, c**).

4-5- Antioxidant Capacity Assay

The total antioxidant capacity of the methanolic extracts was determined using DPPH assay. The results expressed that different combinations of PGRs had a significant effect (p-value ≤ 0.05) on total antioxidant compounds of callus extracts. In all extracts, the concentration of 80-100 µL, which was used for this test, showed the highest free radical scavenging activity than other concentrations. The results indicated that the decrease in IC50 coefficient is associated with an increase in the accumulatio of antioxidant compounds. High levels of antioxidant capacity were observed in ½ MS supplemented with 2 µM/L reduced-glutathione and 1 B, 2 N (**Fig. 5**).

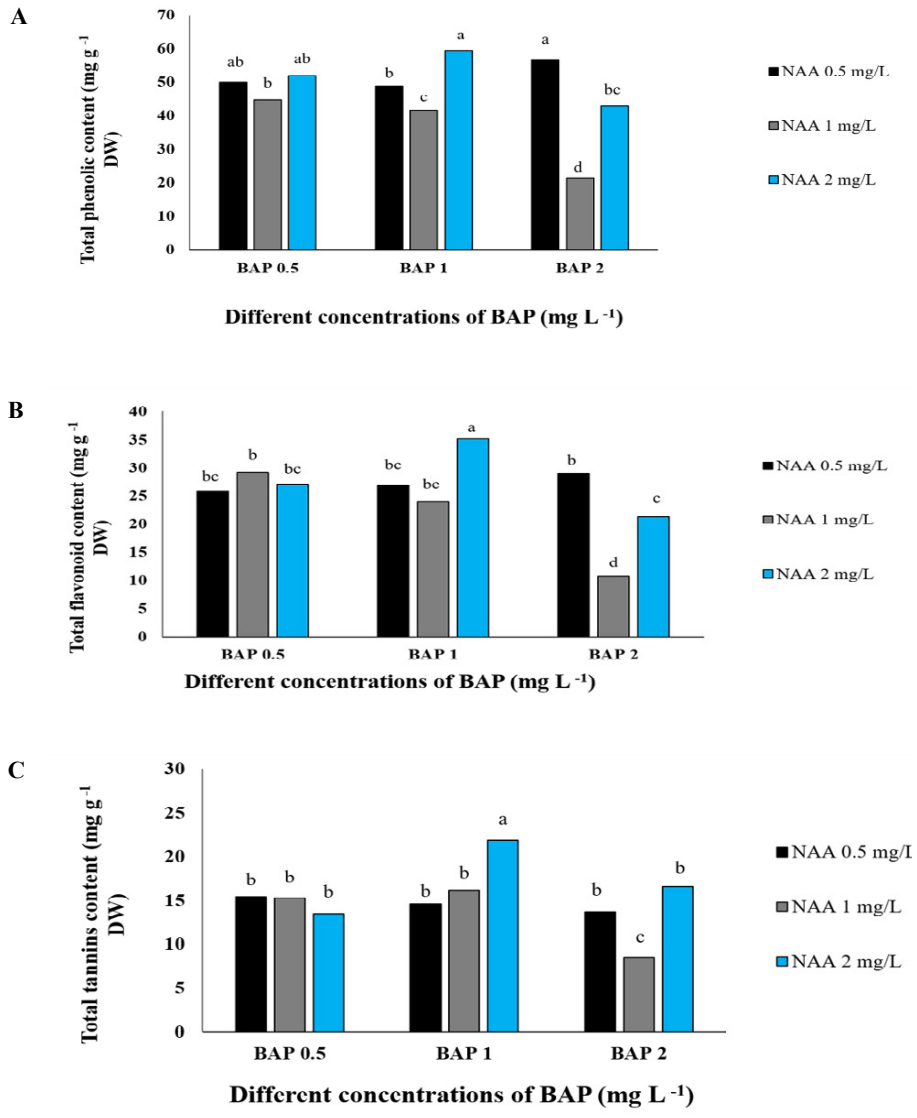

Figure 4. The effect of various combinations of PGRs (B, N) on secondary metabolites of *N. binaloudensis* callus extracts. a) Total phenolic; b) total flavonoid and c) total tannin compounds. Same letters indicate no significant difference among the means based on Duncan's multiple range test (p-value ≤ 0.05).

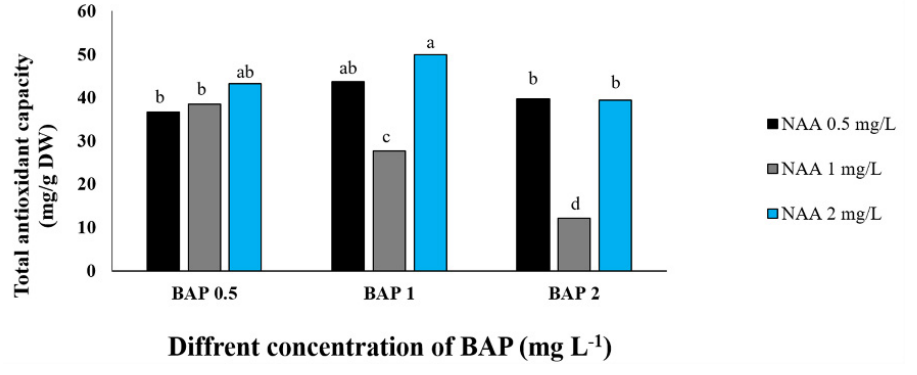

Figure 5. The effect of various combinations of PGRs (BAP and NAA) on antioxidant capacity of *N. binaloudensis* callus extracts. Same letters indicate no significant difference among the means based on Duncan's multiple range test (p-value ≤ 0.05).

5. Discuccion

In this study, the effect of different explants on callus induction was determined. The leaf explants showed the highest callus induction. Some studies have reported that the leaf explant is suitable for callus induction in plants (31). Different responses of explants to hormonal treatments are possible to lead to the type of explants, biochemical, and physiological potential (32). Our data were suggested that different culture media have a positive effect on callus induction. The growth of calli in ½ MS medium was more than that under other media. Probably, ½ MS medium may decrease phenolic and toxic compounds production in medium (33, 34).

2,4D can be led to increase browning in explants cultivated (31). In this study, 2 µM/L reduced-glutathione was used to prevent browning caused by oxidative conditions. In several studies, different antioxidants, including ascorbic acid, activated charcoal, and reduced-glutathione, have been suggested to prevent browning explants (35, 36). No calli formation was observed in free-hormones media. Similar results reported that PGRs needed for callus formation (37, 38).

In this research, B, N had an increasing effect on the callus induction and cell division *on in vitro* conditions. Exogenous application of cytokinin and auxin caused an increased callus induction in different species plants (29). There are many reports emphasized that a balance between auxin and cytokinin hormone in the culture medium is an essential for callus induction, which acts synergistically to improve cell division as a critical process for callus induction (40, 41).

Our data were suggested medium containing PGRs leads to activity phenylpropanoid pathway. Phenylpropanoid pathway activity cause enhances phenolic compounds production (32, 42).

Phenylalanine ammonia-lyase (PAL) enzyme is a key role in the biosynthesis of many phenolic compounds in plants. PAL is an important enzyme in the phenylpropanoid biosynthesis with = converting= L-phenylalanine to trans-cinnamic acid. It has a crucial role in the biosynthesis of phenolic, flavonoids, and lignin compounds (32, 43). This study found that different concentrations of PGRs had a positive effect on the synthesis of phenylpropanoid compounds. Several studies have reported that exogenous application of PGRs on the plant tissue culture can increase PAL enzyme activity, leading to the subsequent accumulation of secondary metabolites (32, 44).

Our study was determined that the antioxidant capacity has a direct relation with the number of polyphenolic compounds. several reports have proposed that there is a positive correlation between antioxidant capacity, phenolic compounds in different plants in response to biotic and abiotic elicitors (45, 46).

6. Conclusions

The tissue culture technique has the ability to produce herbal material in a short time. *N. binaloudensis* callus induction can be used for producing secondary metabolites such as phenolic, flavonoid, and tannin compounds. The maximum of FW and DW were observed in the ½ MS medium which were supplemented with the combination of 2 B, 2 N. Callus culture of this plant due to high phenolic and toxic compounds requires the presence of antioxidant compound such as reduced-glutathione. Our data emphasizes that special medium and different combinations of PGRs e.g. ½ MS supplemented with 2 $\mu M.L^{-1}$ reduced-glutathione and 1 B, 2 N had incremental effect on phenolic compounds production. we suggest such media for phenolic compounds production in *in vitro* conditions.

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Conflict of interest

The authors declare that they have no conflict of interest.

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