Research Article

Secondary Metabolite of *Bacopa monnieri* Under Plant Growth Regulators Treatment on Fibroblast and Cancer Cells

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Abstract

Background: *Bacopa monnieri* L. is a significant medicinal herb with high economic and social value.

Objectives: This study aimed to enhance the growth rate and the production of flavonoids and polyphenols using plant growth regulators (PGRs). Additionally, the study evaluated the effects of these metabolites on the viability of skin cancer cells (A375) and human fibroblast cells (HDF) in a completely randomized design with three replicates.

Methods: For flavonoid extraction, samples were dried at 50°C. A total of 0.25 g of finely powdered dried sample was extracted with 20 mL of 60% (v/v) aqueous ethanol. The total phenolic and flavonoid content of *B. monnieri* was calculated. Human fibroblast cell lines (IBRC No. C10506) and skin cancer cells (A375; IBRC No. C11337) were cultured in an incubator maintained at 37°C, with 5% CO_2 and high humidity.

Results: The study found that 1 mg/L NAA yielded the highest flavonoid (1.76 mg/g DW) and polyphenol (35.17 mg/g DW) content. Among the cytokinins, 0.8 mg/L BAP produced the highest flavonoid (1.61 mg/g DW) and polyphenol (32.51 mg/g DW) content. The maximum viability of HDF cells (135%) was observed at 0.4 mg/L 2IP. The findings suggest that increasing flavonoid content in *B. monnieri* through various hormones is not recommended for skin cancer treatment. However, the use of 0.5 μg/mL 2IP significantly enhanced the growth of fibroblast cells.

Conclusions: The results suggest that the application of 2IP in tissue culture of the *B. monnieri* plant can be beneficial for increasing fibroblast cell viability, making it a promising ingredient in cosmetics and health products. Further research is necessary to confirm these findings and optimize applications.

Keywords: Bacopa Monnieri, Flavonoids, Plant Growth Regulators, Plant Tissue Culture

1. Background

Bacopa monnieri L. Penn., commonly known as 'Brahmi,' holds significant medicinal and economic value within the Scrophulariaceae family. Initial phytochemical analysis identified the presence of alkaloids, saponins, flavonoids, tannins, carbohydrates, proteins, and anthraquinones. Quantitative estimation revealed that various parts of *B. monnieri* contain alkaloids in the root, stem, and leaf; saponins in the root, stem, and leaf; and flavonoids in the stem and leaf. This finding provides valuable pharmacobotanical information critical for the accurate identification and authentication of the crude drug and its botanical components (1).

Bacopa monnieri is one of the 32 medicinal plants prioritized for research and development by the National Medicinal Plant Board (NMPB), Government of India. Due to its medicinal properties, Brahmi has a strong market demand, with an estimated annual consumption of 1000 tons in India. More than 90% of the plant material utilized by the industry is sourced from the wild (2).

Research has demonstrated that *B. monnieri* extract possesses various therapeutic properties, including anti-depressant, anti-anxiety, anti-inflammatory, antipyretic, analgesic, sedative, free radical scavenging, and anti-

lipid peroxidative activities (3). It also exhibits anti-cancer properties effective against multiple cancer types, including prostate, breast, colon, liver, and neurological cancers. Bacosides from *B. monnieri* extract demonstrate anti-mutagenic, cytotoxic, anti-inflammatory, and anti-cancer activities. Additionally, *B. monnieri* extract has shown free radical scavenging activities in vitro on human lymphocytes without exhibiting genotoxic activity (4).

The most important active ingredients in *B. monnieri* include flavonoids, steroid compounds, alkaloids such as bromine and herpestine, and triterpene saponins known as bacosides (5). The saponins in *B. monnieri* are a combination of bacosides A and B. The plant also holds clinical significance for improving immune disorders in humans (6). As an antioxidant, *B. monnieri* enhances the activity of superoxide dismutase and glutathione peroxidase. Research further indicates that bacoside A exhibits potential anti-cancer effects by inducing cell cycle arrest and apoptosis in glioblastoma multiforme (5).

Initially, synthetic drugs and surgery were the primary methods for cancer treatment. However, due to its potent antioxidant capacity and therapeutic effects, *B. monnieri* has gained attention for its potential against a range of diseases, including cancer. Standardized extracts of the plant have demonstrated efficacy against clastogens and carcinogens. These bioactive compounds offer a promising, non-toxic, and cost-effective approach for treating cancer and improving health outcomes (4).

Natural resources of *B. monnieri* are limited, chemical synthesis is unfeasible, and seed propagation is restricted due to limited seed availability and viability. Plant tissue culture techniques provide an alternative method for the production of secondary metabolites in this plant (7). Traditional cultivation methods are time-consuming, as the plant requires several years to mature and achieve the desired level of metabolite production. Plant tissue cultures offer numerous advantages over conventional cultivation methods, including independence from geographical and seasonal variations, year-round production, relatively short growth cycles, mass propagation of uniform, high-quality plant material, and the absence of pesticides and herbicides (8).

Various approaches are employed to enhance the yield of secondary metabolites in plant cell culture, including the use of plant growth regulators (PGRs), elicitors, precursor addition, and optimization of the culture environment (9).

2. Objectives

The primary objective of this research is to investigate the effect of PGRs on the elicitation of flavonoid and polyphenol content in *B. monnieri* tissue culture and to examine their impact on the apoptosis of normal and cancerous skin cells.

3. Methods

3.1. Tissue Culture and Sample Preparation

The study was conducted by the Iranian Research Organization for Science and Technology (IROST). To enhance the production of secondary metabolites, MS medium was supplemented with five different types of PGRs: NAA, IBA, kinetin, BAP, and 2IP, each applied in concentrations ranging from 0 to 1 mg/L.

3.2. Extraction of Samples

The flavonoid extraction method followed the procedure described in (10). The samples were oven-dried at 50 °C and then ground using a mortar and pestle. A total of 0.25 g of finely dried powdered sample was extracted with 20 mL of 60% (v/v) aqueous ethanol (Merck, Germany). The extracts were agitated at 25°C for 48 hours.

3.3. Biochemical Analysis

3.3.1. Determination of Phenolic Content

The levels of phenolic compounds in the ethanolic extract of *B. monnieri* leaves were quantified using the Folin-Ciocalteu reagent assay (11). One mL of the extract in methanol was mixed with 5.0 mL of Folin-Ciocalteu reagent and 4.0 mL of sodium carbonate. The mixture was shaken thoroughly and incubated in darkness for 1 hour. The absorbance was then measured at 750 nm using a spectrophotometer. A standard curve was prepared using gallic acid monohydrate as the reference standard. The total phenolic content was calculated and expressed as mg of gallic acid equivalent per g of dry weight of the extract (mg GAE/g DW).

3.3.2. Determination of Flavonoids Content

Total flavonoids were measured following the methods described by (12) and (13). The extraction of flavonoids from *B. monnieri* required 80% ethanol, which facilitated the release and extraction of flavonoids. Quantitative determination was performed using the AlNO₃ reagent as outlined by (14). A standard curve was prepared using quercetin, and the absorbance of the samples was measured at 510 nm using a spectrophotometer. Three replicate samples were analyzed under identical conditions, and the total flavonoid content was calculated based on the standard curve.

3.4. Cell Lines

Cancer and normal cell lines used in this study were sourced from the Iranian National Center for Genetic and Biologic Resources. The cell lines included human fibroblast cells (HDF; IBRC No. C10506) and skin cancer cells (A375; IBRC No. C11337). The cells were cultured in DMEM culture medium supplemented with penicillin, streptomycin, and 10% FBS. The incubation conditions were maintained at 37°C, with 5% CO_2 and saturated humidity.

3.5. MTT Assay

The viability of both normal and cancerous skin cells was evaluated after exposure to varying concentrations (0.025, 0.5, 1, 2, 4 µg/L) of a treatment containing 0.4 mg/L 2IP, 0.8 mg/L kinetin, 25 mg/L chitosan, 1 mg/L NAA, 1 mg/L IBA, 0.8 mg/L BAP, and 25 mg/L phenylalanine for 48 hours. This assessment was performed using the MTT test, which evaluates mitochondrial function through the reduction of MTT.

The MTT viability test [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was conducted as described by (15), with modifications. The MTT assay is a quantitative colorimetric method used to measure cell viability. It involves the reduction of a yellow tetrazolium salt into dark blue formazan by mitochondrial dehydrogenases in viable cells. This reaction occurs only when mitochondrial function is intact.

After incubation, the cell medium was replaced with fresh medium (100 µL/well), followed by the addition of 50 µL of MTT solution (5 mg/mL in PBS) to each well. The plates were then incubated for 4 hours at 37°C. Formazan crystals formed during the reaction were dissolved by adding 100 µL of DMSO to each well, followed by mixing and incubation for an additional 4 hours. The concentration of formazan was determined by measuring optical density at 570 nm.

Cytotoxicity was calculated based on the survival rate of cells compared to the control group, with the survival rate of control cells set at 100%.

3.6. Statistical Analyses

The experiments were conducted in triplicate, and the results are presented as mean ± MSE. Quantitative data analysis was performed using statistical software (SPSS 16, IBM, USA). The findings revealed a significant accumulation of flavonoids and polyphenols in treated in vitro cultures compared to untreated samples after four weeks of culture. A one-way analysis of variance (ANOVA) was performed, followed by Duncan's multiple-range test to compare mean values. A significance level of $P < 0.05$ was considered statistically significant.

4. Results

4.1. Effects of Different Plant Growth Regulator on the Secondary Metabolite of Bacopa monnieri

Table 1 and Figure 1 illustrate the response of secondary metabolite production in *B. monnieri* to various PGRs, including 2,4-D, NAA, IBA, BAP, and kinetin, applied individually at concentrations ranging from 0.0 to 1 mg/L. The results indicate that these hormones positively influenced metabolite production in *B. monnieri*.

The pattern of flavonoid production was analyzed using different auxins and cytokinins on basal MS medium. The data revealed that the effects of hormones on flavonoid production were significantly greater than the control. Among the auxins tested (Figure 2), 1 mg/L NAA and IBA produced the highest flavonoid levels, at 1.76 and 1.65 mg/g DW, respectively. For cytokinins, the medium containing 0.8 mg/L BAP showed the most successful flavonoid production compared to other concentrations.

The effects of IBA and NAA at various concentrations on polyphenol accumulation revealed that the highest

Table 1. Analysis of Variance for Plant Growth Regulator Effect on Secondary Metabolite

Source of Variation	df	Mean of Square	
		Flavonoids (mg/g/DW)	Phenolic(mg/g/DW)
BAP concentration	5	0.03**	36.05**
Error	12	0.009	3.806
Kinetin concentration	5	0.013**	**17.911
Error	12	0.008	2.881
2IP concentration	5	0.014**	23.371*
Error	12	0.007	5.306
IBA concentration	5	0.049**	44.121**
Error	12	0.008	4.605
NAA concentration	5	0.041**	44.703**
Error	12	0.007	4.106

Figure 1. Response of *Bacopa monnieri* multiplication on MS medium supplemented with; right picture: 1 mg/L BAP; left: 0.4 mg/L BAP. Bar is 1.0 cm.

accumulation occurred with 1 mg/L NAA, followed by IBA. Even in the control medium, the polyphenol content was 21.26 mg/g DW, which was significantly lower than the 1 mg/L NAA medium. Additionally, the medium containing 0.8 mg/L BAP produced the highest polyphenol content among cytokinins tested.

Figure 3 highlights the variation in polyphenol productivity, which peaked at 35.17 ± 0.24 mg/g DW with 1 mg/L NAA, followed by IBA at 32.39 ± 0.31 mg/g DW. In contrast, cytokinins resulted in lower productivity. Interestingly, an increase in plant growth regulator concentration led to a decrease in polyphenol content,

though higher biomass levels resulted in increased secondary metabolite productivity.

Previous studies support these findings. For example, in *Ipomoea batatas* suspension culture, exposure to 2,4-D concentrations ranging from 0 to 4 mg/L over two weeks caused a gradual reduction in anthocyanin concentration, from 29% to 14.1%, respectively ([16]). Similarly, in *Rudbeckia hirta*, the accumulation of Pulchellin E varied with different auxins (IAA, 2,4-D, and IBA), with 2,4-D demonstrating the strongest enhancement of secondary metabolites. Conversely, NAA inhibited Pulchellin E biosynthesis ([17]). A study on *H. bonariensis* reported that 2,4-D enhanced flavonoid
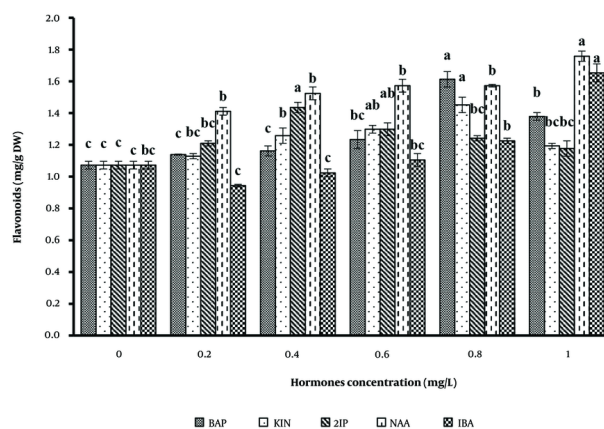
Figure 2. Effects of the plant growth regulator on flavonoids production of *Bacopa monnieri*; values are mean ± SEM of three experiments.
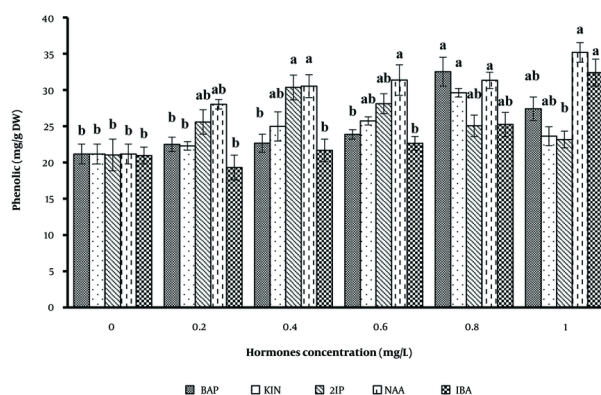

Figure 3. Effects of the plant growth regulator on phenolic production of *Bacopa monnieri*; values are mean ± SEM of three experiments.

production, while NAA produced the lowest amount. A similar comparison between NAA and IBA was observed in *Artemisia absinthium*.

4.2. Effect of Different Extracts from Bacopa monnieri on Human Fibroblast Cells and A375 Cell Lines

In this experiment, the effects of various treatments, including 2IP (0.8 mg/L), kinetin (0.8 mg/L), IBA (1 mg/L), NAA (0.8 and 1 mg/L), BAP (0.8 mg/L), chitosan (3 mg/L), and phenylalanine (20 mg/L), were investigated on A375 skin cancer cells compared to normal HDF fibroblast cells. Concentrations ranging from 0.25 to 4 µg/mL of each treatment were applied to the cells for 48 hours.

The results revealed that a concentration of 0.5 µg/mL of 2IP (0.4 mg/L) significantly increased the survival of normal cells compared to the control ($P < 0.05$). However, concentrations equal to or greater than 2 µg/mL significantly increased the survival of normal cells as well ($P < 0.05$). Additionally, concentrations exceeding 1 µg/mL significantly increased the survival of cancer cells compared to concentrations ranging from 0 to 0.5 µg/mL ($P < 0.05$). Therefore, only the 0.5 µg/mL concentration significantly enhanced the survival of
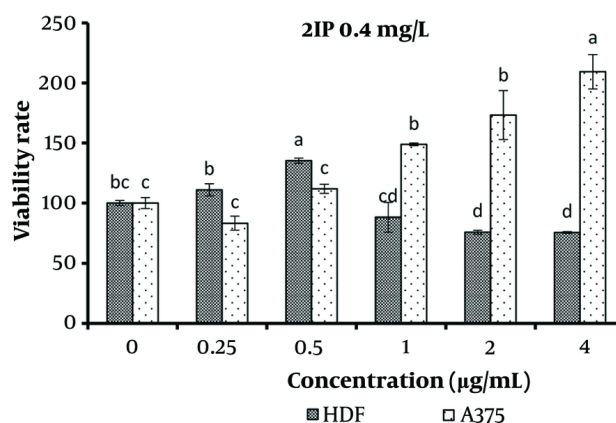
Figure 4. The effect of 0.25 to 4 µg/mL concentrations of 2IP treatment of 0.4 mg/L for 48 h on the survival of A375 and human fibroblast cells (HDF) cell lines
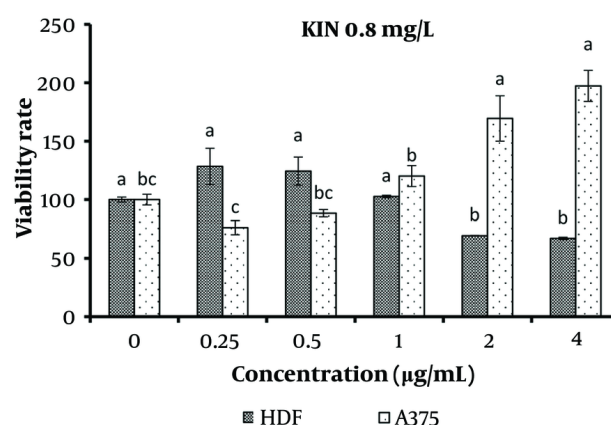

Figure 5. The effect of 0.25 to 4 µg/mL concentrations of 0.8 mg/L kinetin treatment for 48 h on the survival of A375 and human fibroblast cells (HDF) cell lines

normal cells without affecting the survival of cancer cells ($P < 0.05$) (Figure 4).

The results of kinetin (0.8 mg/L) treatment on normal and cancerous skin cells are presented in Figure 5. Concentrations ranging from 0.25 to 1 µg/mL of kinetin showed no significant difference in the viability of normal cells compared to the control. However, concentrations exceeding 2 µg/mL significantly reduced the viability of normal cells ($P < 0.05$). For cancer cells, concentrations from 0.25 to 1 µg/mL of kinetin treatment exhibited no significant difference in viability

compared to the control, while concentrations equal to or greater than 2 µg/mL significantly increased cancer cell survival ($P < 0.05$).

The concentration of 0.25 µg/mL of the treatment containing 1 mg/L IBA significantly increased the viability of normal cells ($P < 0.05$). However, concentrations equal to and greater than 1 µg/mL significantly decreased the viability of normal cells while simultaneously increasing the viability of cancer cells ($P < 0.05$) (Figure 6).
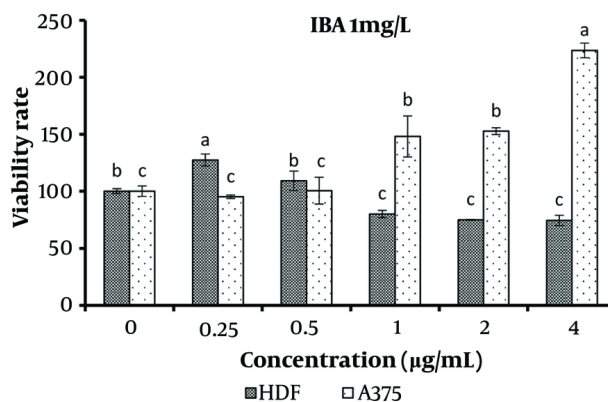
Figure 6. The effect of 0.25 to 4 µg/mL concentrations of 1 mg/L IBA treatment for 48 h on the survival of A375 and human fibroblast cells (HDF) cell lines
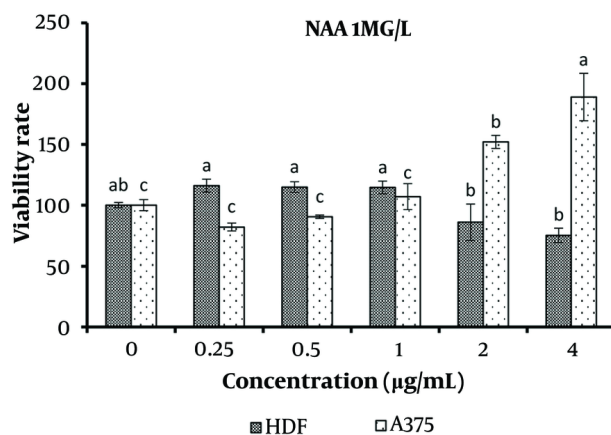

Figure 7. The effect of 0.25 to 4 µg/mL concentrations of 1 mg/L NAA treatment for 48 h on the survival of A375 and human fibroblast cells (HDF) cell lines

For the treatment with 1 mg/L NAA, concentrations ranging from 0.25 to 1 µg/mL did not show any significant difference in viability compared to the control for both normal and cancer cells. However, concentrations of 2 and 4 µg/mL caused a significant decrease in the viability of normal cells and a significant increase in the viability of cancer cells ($P < 0.05$) (Figure 7).

Increasing the treatment concentration of 0.8 mg/L BAP from 0.25 to 4 µg/mL resulted in a significant decrease in the viability of normal cells ($P < 0.05$).

Conversely, concentrations of 2 and 4 µg/mL significantly increased the survival of cancer cells compared to other treatments ($P < 0.05$) (Figure 8).

5. Discussion

The results of using effective hormone concentrations in *B. monnieri* plant cultures demonstrated that when concentrations of 2 µg/mL or higher of the specified extracts were applied to the culture medium of normal and cancerous skin cells, there was a significant increase in the number of
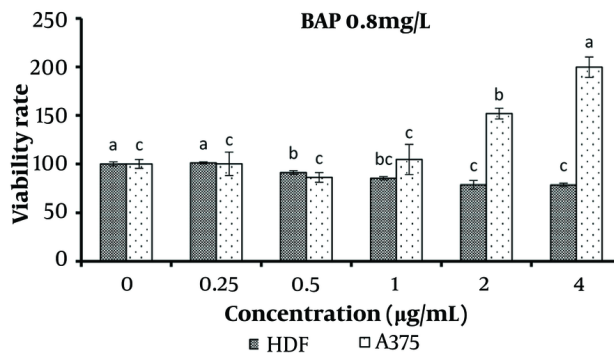
Figure 8. The effect of 0.25 to 4 µg/mL concentrations of 0.8 mg/L BAP treatment for 48 h on the survival of A375 and human fibroblast cells (HDF) cell lines

cancerous cells and a concurrent decrease in normal cells.

No tolerable upper Dietary Reference Intake (DRI) has been established for flavonoids, and consuming them in typical food amounts poses no toxicity risk. However, excessive intake through high-potency supplements may lead to flavonoid toxicity. Vulnerable populations, such as the elderly with marginal iron deficiency, may face risks since flavonoids can bind nonheme iron ([18]). While most flavonoids are generally safe, excessive consumption has been associated with adverse effects, including gastrointestinal symptoms, hemolytic anemia, hepatotoxicity, and potential interactions with medications ([19]). Long-term use of flavonoids has also been linked to conditions such as contact dermatitis in sensitive individuals and estrogen-related effects in men and breast cancer ([20]).

The results indicated that none of the studied treatments caused a significant reduction in cancer cells compared to the control. Based on this finding, the use of *B. monnieri* under the applied treatments is not recommended for controlling the growth of skin cancer cells. However, *B. monnieri* has shown promising results in studies involving other cancer cell types. The anticancer effects of *B. monnieri* remain unproven at the clinical level in humans. Currently, no clinical evidence supports the potential anticancer activity of this plant. Reports indicate that the methanolic extract of *B. monnieri* contains phenolic bioactive molecules that act by inhibiting cell motility ([4]). Studies on certain cancer cell lines have revealed that *B. monnieri* initiates DNA fragmentation after treatment. The proposed mechanism of action involves bacoside A binding to the enzyme CaMK2A. This binding triggers phosphorylation, enabling CaMK2A to interact with ryanodine receptors on the endoplasmic reticulum membrane. This interaction increases calcium ion release, leading to significant fluid absorption via macropinocytosis.

Consequently, cells swell and eventually undergo lysis ([4]). Research indicates that the ethanolic extract of *B. monnieri* regulates endogenous oxidative marker levels in the brains of pre-pubescent mice ([21]). A study by Russo et al. investigated DNA damage and cytotoxicity caused by H_2O_2 in human non-immortalized fibroblast cells treated with ethanolic *B. monnieri* extract. Their findings revealed that the extract inhibits superoxide anion formation, demonstrating its free radical scavenging ability. It provides protective effects against H_2O_2-induced cytotoxicity and DNA damage ([22]).

Histological and animal studies have shown that *B. monnieri* extract enhances the system's defense against oxidative stress by reducing free radical formation in the brain ([21]). Bacosides in *B. monnieri* inhibit lipoxygenase activity, eliminate free radicals, and protect neurons in the prefrontal cortex, hippocampus, and striatum from cytotoxicity and DNA damage associated with Alzheimer's disease. Additionally, bacosides increase glutathione peroxidase activity, facilitate iron chelation, and promote nitric oxide-

mediated cerebral vasodilation, resulting in improved memory performance (23).

It is believed that bacosides repair damaged neurons by enhancing kinase activity and neurosynthesis, restoring synaptic activity and improving nerve impulse transmission (24). Studies have demonstrated that oral administration of *B. monnieri* extract increases antioxidant levels such as SOD, GSH, and CAT, while reducing free radicals like LPO and NO. It also accelerates wound closure in mice due to its antioxidant properties (25). Furthermore, *B. monnieri* ethanol extract at 20 mg/kg body weight inhibits tumor progression by reducing LDH levels (26).

The extract of *B. monnieri* enhances autophagy, indicating a novel function that warrants further investigation (4).

The results showed that a concentration of 0.5 µg/mL 2IP significantly increased the survival of normal cells while having no notable effect on the survival of cancer cells. PGRs play a critical role in the accumulation of bioactive compounds. The type and concentration of auxins and cytokinins influence growth and product formation in callus and plant cell suspension cultures, with hormone concentration being a key factor in the accumulation of secondary metabolites such as phenolics and flavonoids (7). For example, research on *Garcinia brasiliensis* Mart. callus cultures demonstrated that treatment with 2,4-D and 2IP significantly increased total flavonoid levels (27). Similarly, studies on date palm suspension cultures found that a combination of 1 mg/L 2,4-D and 5 mg/L 2IP enhanced biomass accumulation, while treatment with 5 mg/L 2,4-D and 2.5 mg/L 2IP induced notably higher levels of phenolics and flavonoids (28). These findings highlight the importance of optimizing hormone concentrations to achieve balanced enhancement of both biomass and secondary metabolite production.

In the context of flavonoids, apigenin—a subtype of flavones—has demonstrated efficacy against various cancer types, including prostate cancer, colon cancer, breast cancer, and melanoma. Apigenin has been shown to inhibit metastasis, cell migration, invasion, and epithelial-mesenchymal transition (EMT) across these cancer models. Notably, it has been observed to reduce stem cell properties in breast cancer cells (29). This underscores the potential of flavonoid compounds in cancer treatment, further emphasizing the importance of PGR-optimized secondary metabolite production for therapeutic applications.

5.1. Conclusions

This study developed an efficient, effective, and reproducible protocol for the micropropagation of *B. monnieri*, which is at risk of extinction from its natural habitat. Additionally, the extract obtained from *B. monnieri* contained a high amount of secondary metabolites. This is the first report in which active compounds derived from tissue culture of *B. monnieri* were tested on different cell types. The use of hormones to increase flavonoid content in *B. monnieri* demonstrated that these hormones can significantly enhance flavonoid levels. However, the application of this plant under the mentioned treatments is not recommended for the treatment of skin cancer, and further research is necessary.

Regarding the effect of the studied treatments on normal cells, the results showed that a concentration of 0.5 µg/mL 2IP significantly increased the growth of fibroblast cells. Based on this finding, the use of this hormone in the tissue culture of the *B. monnieri* plant is recommended to enhance the viability of fibroblast cells for use in cosmetics and health products, although additional studies are required.

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Footnotes

Authors' Contribution: M. Z.: Taking samples, laboratory work, data collection and analysis, and drafting the manuscript, articipated in designing the evaluation and helped to draft the manuscript; M. M.: Re-evaluated the data, performed the statistical analysis, and revised the manuscript.

Conflict of Interests Statement: The authors declare that they have no conflict of interest.

Data Availability: The dataset presented in the study is available on request from the corresponding author during submission or after publication.

Ethical Approval: This research is not clinical, a medical did not do it and was done in a laboratory by researchers.

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References

1. Anju V, Naresh C, Avinash P. Anatomical markers and Phytochemical study of different plant parts of Bacopa monnieri (L.) Wettst. *Int J life sci*. 2017;**5**(3):379-86.

2. Kharde A, Kore S, Khetmalas MB. Elicitation of Bacoside Content Using Plant Growth Regulators in Cell Suspension Culture of Bacopa monnieri (L.) Wettst. *Plant Tissue Culture and Biotechnology*. 2018;**28**:191-9. https://doi.org/10.3329/ptcb.v28i2.39678.

3. Dogan MEB. Anti-cytotoxic-genotoxic influences of in vitro propagated Bacopa monnieri L. Pennell in cultured human lymphocytes. *Eurasian J Bio Chem Sci*. 2018;**1**(2):48-53.

4. Ghosh S, Khanam R, Acharya Chowdhury A. The Evolving Roles of Bacopa monnieri as Potential Anti-Cancer Agent: A Review. *Nutr Cancer*. 2021;**73**(11-12):2166-76. [PubMed ID: 33148034]. https://doi.org/10.1080/01635581.2020.1841248.

5. Moskwa J, Naliwajko SK, Markiewicz-Zukowska R, Gromkowska-Kepka KJ, Nowakowski P, Strawa JW, et al. Chemical composition of Polish propolis and its antiproliferative effect in combination with Bacopa monnieri on glioblastoma cell lines. *Sci Rep*. 2020;**10**(1):21127. [PubMed ID: 33273550]. [PubMed Central ID: PMC7712839]. https://doi.org/10.1038/s41598-020-78014-w.

6. Pothiaraj G, Manoranjani M, Pitchaikani S, Seker GK, Saravanan KM, Rajan M, et al. Investigation of therapeutic and immunomodulatory activity of Bacopa saponin from Bacopa monnieri. *South Africa J Botany*. 2022;**151**:639-50. https://doi.org/10.1016/j.sajb.2022.09.011.

7. Sanyal R, Nandi S, Pandey S, Chatterjee U, Mishra T, Datta S, et al. Biotechnology for propagation and secondary metabolite production in Bacopa monnieri. *Appl Microbiol Biotechnol*. 2022;**106**(5-6):1837-54. [PubMed ID: 35218388]. https://doi.org/10.1007/s00253-022-11820-6.

8. Clapa D, Nemeş S, Ranga F, Hârța M, Vodnar D, Călinoiu L. Micropropagation of Vaccinium corymbosum L.: An Alternative Procedure for the Production of Secondary Metabolites. *Horticulturae*. 2022;**8**(6). https://doi.org/10.3390/horticulturae8060480.

9. Zhou LG, Wu JY. Development and application of medicinal plant tissue cultures for production of drugs and herbal medicinals in China. *Nat Prod Rep*. 2006;**23**(5):789-810. [PubMed ID: 17003910]. https://doi.org/10.1039/b610767b.

10. Crozier A, Jensen E, Lean MEJ, McDonald MS. Quantitative analysis of flavonoids by reversed-phase high-performance liquid chromatography. *J Chromatograph A*. 1997;**761**(1-2):315-21. https://doi.org/10.1016/s0021-9673(96)00826-6.

11. Blainski A, Lopes GC, de Mello JC. Application and analysis of the folin ciocalteu method for the determination of the total phenolic content from Limonium brasiliense L. *Molecul J*. 2013;**18**(6):6852-65. [PubMed ID: 23752469]. [PubMed Central ID: PMC6270247]. https://doi.org/10.3390/molecules18066852.

12. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem*. 1999;**64**(4):555-9. https://doi.org/10.1016/s0308-8146(98)00102-2.

13. Xu Y, Zhang R, Fu H. Studies on the Optimal Process to Extract Flavonoids from Red-raspberry Fruits. *Natur Sci J*. 2005;**3**.

14. Liu M, Li XQ, Weber C, Lee CY, Brown J, Liu RH. Antioxidant and antiproliferative activities of raspberries. *J Agric Food Chem*. 2002;**50**(10):2926-30. [PubMed ID: 11982421]. https://doi.org/10.1021/jf0111209.

15. Shabani H, Zandi M, Ofoghi H, Sanjabi MR, Hoseini Pajooh K. The effect of combining vitamin E and C on the viability improvement of transfected ovine spermatogonial stem cells after cryopreservation and thawing. *Turk J Veterin Animal Sci*. 2017;**41**:648-55. https://doi.org/10.3906/vet-1701-38.

16. Konczak I, Terahara N, Yoshimoto M, Nakatani M, Yoshinaga M, Yamakawa O. Regulating the composition of anthocyanins and phenolic acids in a sweetpotato cell culture towards production of polyphenolic complex with enhanced physiological activity. *Trend Food Sci Tech*. 2005;**16**(9):377-88. https://doi.org/10.1016/j.tifs.2005.02.007.

17. Łuczkiewicz M, Zárate R, Dembińska-Migas W, Migas P, Verpoorte R. Production of pulchelin E in hairy roots, callus and suspension cultures of Rudbeckia hirta L. *Plant Sci J*. 2002;**163**(1):91-100. https://doi.org/10.1016/s0168-9452(02)00065-1.

18. Corcoran MP, McKay DL, Blumberg JB. Flavonoid basics: chemistry, sources, mechanisms of action, and safety. *J Nutr Gerontol Geriatr*. 2012;**31**(3):176-89. [PubMed ID: 22888837]. https://doi.org/10.1080/21551197.2012.698219.

19. Galati G, O'Brien PJ. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radic Biol Med*. 2004;**37**(3):287-303. [PubMed ID: 15223063]. https://doi.org/10.1016/j.freeradbiomed.2004.04.034.

20. Vogiatzoglou A, Mulligan AA, Lentjes MA, Luben RN, Spencer JP, Schroeter H, et al. Flavonoid intake in European adults (18 to 64 years). *PLoS One*. 2015;**10**(5). e0128132. [PubMed ID: 26010916]. [PubMed Central ID: PMC4444122]. https://doi.org/10.1371/journal.pone.0128132.

21. Shinomol GK; Muralidhara. Bacopa monnieri modulates endogenous cytoplasmic and mitochondrial oxidative markers in prepubertal mice brain. *Phytomed*. 2011;**18**(4):317-26. [PubMed ID: 20850955]. https://doi.org/10.1016/j.phymed.2010.08.005.

22. Russo A, Izzo AA, Borrelli F, Renis M, Vanella A. Free radical scavenging capacity and protective effect of Bacopa monniera L. on DNA damage. *Phytother Res*. 2003;**17**(8):870-5. [PubMed ID: 13680815]. https://doi.org/10.1002/ptr.1061.

23. Holcomb LA, Dhanasekaran M, Hitt AR, Young KA, Riggs M, Manyam BV. Bacopa monniera extract reduces amyloid levels in PSAPP mice. *J Alzheimers Dis*. 2006;**9**(3):243-51. [PubMed ID: 16914834]. https://doi.org/10.3233/jad-2006-9303.

24. Kishore K, Singh M. Effect of bacosides, alcoholic extract of Bacopa monniera Linn. (brahmi), on experimental amnesia in mice. *Indian J Exp Biol*. 2005;**43**(7):640-5. [PubMed ID: 16053272].

25. Zirmire RK, Saha D, Dey R, Tanimu H, Zaarour R, Bird D, et al. Bacopa monnieri phytochemicals regulate fibroblast cell migration via modulation of focal adhesions. *iSci J*. 2024;**27**(4):109489. [PubMed ID: 38558933]. [PubMed Central ID: PMC10981128]. https://doi.org/10.1016/j.isci.2024.109489.

26. Rohini G, Sabitha KE, Devi CS. Bacopa monniera Linn. extract modulates antioxidant and marker enzyme status in fibrosarcoma bearing rats. *Indian J Exp Biol*. 2004;**42**(8):776-80. [PubMed ID: 15573526].

27. Teixeira MG, Carvalho M, Leite MA, Barbosa S, Santos filho PRD, Santos BR. Effect of Salicylic Acid, 2,4-D and 2i-P on the Production of Secondary Metabolites in Garcinia brasiliensis Mart. Callus. *Brazil Arch Biol Tech*. 2019;**62**. https://doi.org/10.1590/1678-4324-2019170303.

28. Al-Khayri JM, Naik PM. Influence of 2iP and 2,4-D Concentrations on Accumulation of Biomass, Phenolics, Flavonoids and Radical Scavenging Activity in Date Palm (Phoenix dactylifera L.) Cell Suspension Culture. *Horticulturae*. 2022;**8**(8). https://doi.org/10.3390/horticulturae8080683.

29. Liskova A, Koklesova L, Samec M, Smejkal K, Samuel SM, Varghese E, et al. Flavonoids in Cancer Metastasis. *Cancers (Basel)*. 2020;**12**(6). [PubMed ID: 32521759]. [PubMed Central ID: PMC7352928]. https://doi.org/10.3390/cancers12061498.