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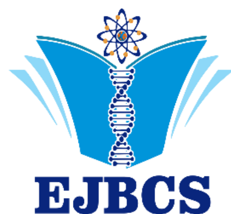

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Anti-cytotoxic-genotoxic influences of *in vitro* propagated *Bacopa monnieri* L. Pennell in cultured human lymphocytes

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Abstract: *Bacopa monnieri* L. Pennell is an important medicinal plant with antioxidant and neuroprotective role. It acts as an efficient free radical scavenger and has the potential to serve as a phytoremedy in the disease conditions involving reactive oxygen species (ROS) and oxidative stress. In addition, the protective role of *B. monnieri* on tissue antioxidant defense system and lipid peroxidative status in diabetic rats has been reported. *B. monnneri* used in the experiment was propagated by plant tissue culture techniques. The shoot tip explants of *B. monnneri* were cultured in Murashige and Skoog (MS) nutrient medium containing 1.0 mg/L 6-Benzylaminopurine (BAP). Genotoxic effects of aqueous extract obtained from *B. monnieri* in cultured human lymphocytes were determined by chromosome aberration (CA) test. In addition, cytotoxic activities of the extract on the cells were revealed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis. Different concentrations (6.25-200 mg/L) of the extract were used in the treatments. It was detected that cytotoxicty was correlated with concentration. Cell viability decreased to 69.64% in the lymphocytes exposed to maximum concentration of the extract. Based on CA/cell test, the results were statistically ($p > 0.05$) indifferent from negative control treatment. Overall, obtained data indicated that *B. monnieri* is a plant that contains natural products that are not causative of cytotoxic and genotoxic damage on human lymphocytes.

Keywords: Cytotoxicity, Genotoxicity, Shoot Regeneration

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1. Introduction

The immune system protects the body from microorganisms, cancer cells, fungi, toxins, chemicals and foreign substances. This important system recognizes molecules called antigens and neutralizes them by means of antibodies or immunoglobulins of different structure (Zumla and James, 2002; Łukasiewicz and Fol, 2018). The cells involved in the immune system are leukocytes. Leukocytes produced in various organs such as spleen, bone marrow, thymus and lymph nodes are effective on antigens. Lymphocytes, a variety of leukocytes, are the most important elements of the acquired immune system (Feng et al., 2016; Imai et al., 2017). Lymphocytes are produced to create defenses against diseases. Lymphocytes rise in some cases. This usually means that the metabolism stimulates the immune system against a disease and the lymphocyte count increases (Valdez et al., 2002; Kazmin et al., 2005). Lymphocyte deficiency is seen when the immune system can not produce enough lymphocyte cells (Mäurer et al., 2002; Wiesner et al., 2017).

The antioxidants in our body stimulate the lymphocyte proliferation and ensure that the immune system remains active. In this way, oxidative and genetic damage rates that cause many diseases will decrease (Rodríguez-Ribera et al., 2016; Jafarpour et al., 2018; Sevindik et al., 2018). Genetic damage that occurs in cells is transferred to future generations and permanent diseases can take place (Lin et al., 2018). In particular, damage to cells such as lymphocytes breaks the body's resistance (Ruehl-Fehlert et al., 2017).

In the treatment processes, it is of great importance that the side effects of the drugs taken on the body are low and do not cause serious problems such as genetic damage (Luu and Palczewski, 2018). The chemical properties of herbal products, which are frequently preferred recently, should be examined in detail. It should be determined that plant products do not adversely affect healthy cell proliferation and do not cause genetic damage (Gadano et al., 2006; Emsen et al., 2017). This is usually due to the antioxidant capacity of these natural products (Karatas et al., 2015; Sevindik et al., 2017; Emsen and Dogan, 2018).

Bacopa monnieri (L.) Pennell (Scorphulariaceae), commonly known as Brahmi (Sharma et al., 2010), grows in wetlands such as pools, streams and lakeside edges. *B. monnieri* has spread in India, Pakistan, Afghanistan, Nepal, Sri Lanka, subtropical USA, tropical Asia, Africa

and Australia (Russo and Borrelli, 2005). It has long been used in the traditional Ayurvedic medicine system to improve intelligence and memory (Uabundit et al., 2010). In an experiment conducted on seventy-six adults aged 40 and 65 years, *B.monnieri* showed a significant effect on retention of new information and memory enhancement (Roodenrys et al., 2002). It is known in the traditional medical system that *B. monnieri* is used as nerve tonic, diuretic, cardiotonic and therapeutic agents against epicopsies, insomnia, asthma and rheumatism. Many studies have shown that this plant extract has anxiolytic, anti-depression and antioxidant activity (Bhattacharya et al., 2000; Uabundit et al., 2010). *B. monnieri* has been reported to have antiinflammatory, analgesic, antipyretic, sedative, free radical scavenging and anti-lipid peroxidative activity (Mathew et al., 2010). The plant is astringent, bitter, sweet, cooling, laxative, intellect promoting, anodyne, carminative, digestive, antiinflammatory, anticonvulsant, depurative, cardiotonic, bronchodialator, diuretic, emmenagogue, sudorfic, febrifuge and tonic (Rastogi et al., 1994; Mathew et al., 2010).

To the best of our knowledge the effects of *in vitro* propagated *B. monnieri* in cultured human lymphocytes (HLs) were not investigated. Therefore, in this investigation, it was aimed to describe the cyto-genotoxic influences of aqueous extract obtained from *B. monnieri* on HLs. With this aim, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), mitotic index (MI) and chromosome aberration (CA) analyses were performed on HLs exposed to the aqueous extract of *B. monnieri*.

2. Materials and Method

2.1. In vitro plant propagation

Sterilized *B. monnieri* plants were obtained from the Biology Department of Karamanoğlu Mehmetbey University. The shoot tip explants of *B. monnieri* were cultured in MS (Murashige and Skoog, 1962) nutrient medium (Table 1) supplemented with 1.0 mg/L 6-Benzylaminopurine (BAP) at 24°C for 16 hours in light and 8 hours in dark photoperiod. MS medium and vitamins, 3% sucrose and 0.65% agar were used in all culture media. Distilled water was used for the preparation of the nurient medium. The pH of the nutrient medium was adjusted to 5.7±1 using 1N NaOH and 1N HCl, followed by sterilization at 1.2 atmospheres pressure and at 120°C for 20 minutes. In the experiments, the explants were incubated under a white LED (Light-Emitting Diode) light (1500 lux) at a temperature of 24°C and a 16 hour light photoperiod for ten weeks. Experiments were carried out in magenta containers in 3 replicates.

2.2. Extraction of bioactive ingredients

Entire samples of *B. monnieri* were used for extraction. Fine dried powdered samples were prepared using liquid nitrogen, mortar and pestle from 10 g of whole samples. The extracts were obtained in 250 mL of distilled water using Soxhlet extraction apparatus for two days. Thereafter, the solvent were evaporated using rotary evaporator under vacuum to dryness and extract with 6.25, 12.5, 25, 50, 100 and 200 mg/mL concentration were prepared. The yields were based on dry materials of plant sample. The analyses tested without extract formed the negative control (Control (-)) group.

Table 1. The content of Murashige and Skoog (1962) basic nutrient medium

Components		Concentrations (mg/L)
	NH_4NO_3	1650.000
	KNO_3	1900.000
Macroelements	$CaCI_2.2H_2O$	440.000
	$MgSO_4.7H_2O$	370.000
	KH_2PO_4	170.000
	KI	0.830
	H_3BO_3	6.200
	$MnSO_4.4H_2O$	22.300
	$ZnSO_4.7H_2O$	8.600
Microelements	$Na_2MoO_4.2H_2O$	0.250
	$FeSO_4.7H_2O$	27.850
	$CoCl_2.6H_2O$	0.025
	$CuSO_4.5H_2O$	0.025
	$Na_2EDTA.2H_2O$	37.250
	Myo-Inositol	100.000
	Nicotinic Acid	0.500
Vitamins	Pyrotinic Acid	0.500
	Thiamine-HCI	0.100
	Glycine	2.000

2.3. HLs culture

HLs in the heparinized blood samples were separated by density gradient using Ficoll-Hypaque (Sigma Aldrich, St Louis, MO, USA). Later, isolated HLs were incubated in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) with 2 mM/L L-glutamine, 10% FBS, and penicillin-streptomycin (Sigma-Aldrich, St Louis, MO, USA) in the presence of 5% CO_2 at 37°C at 1×10^6 cells/mL.

2.4. MTT assay

The cells were seeded in 96-well plates. Cells were incubated at 37°C in a humidified 5% CO_2/95% air mixture and treated with extracts at different concentrations (6.25, 12.5, 25, 50, 100 and 200 mg/mL) for 24 h. The MTT assay was carried out using a commercially available kit (Cayman Chemical Company, Ann Arbor, MI, USA). In MTT assays, mitomycin-C ($C_{15}H_{18}O_5$, Sigma, St Louis, MO, USA, at 10^{-7} M) chemotherapeutic agent was used as a positive control (Control (+)).

2.5. CA assay

Heparinized blood was cultured in 6 mL of culture medium (Chromosome Medium B; Biochrom, Berlin) with

5 mg/mL of phytohemagglutinin (Biochrom, Berlin). The cultures were incubated in complete darkness for 72 h at 37ºC. Two hours prior to harvesting, colchicine (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 2 mg/mL was added to the culture flask. Hypotonic treatment and fixation were performed. To prepare slides, 3-5 drops of the fixed cell suspension were dropped on a clean slide and air-dried. The slides were stained in 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min. For each treatment, 30 well-spread metaphases were analyzed to detect the presence of chromosomal aberrations. In CA assay, it was used mitomycin-C ($C_{15}H_{18}N_4O_5$, Sigma, St, Louis/MO, USA, at 10^{-7} M) as control (+). MI was also calculated based on number of cells in metaphase.

2.6. Statistical analyses

Mean values of different activities of the extracts were compared with analysis of variance by Duncan's multiple range test for comparing groups. A significance level of 5% was accepted for all comparisons. Median inhibitory concentration (IC_{50}) values were calculated with probit regression analysis and associated 95% confidence limits for each treatment. Pearson's correlation coefficients were used in order to determine relation levels among the variables. These calculations were carried out using the Statistical Package for Social Sciences (SPSS, version 21.0, IBM Corporation, Armonk, NY, USA).

3. Results and Discussion

Tissue culture techniques are one of the most effective techniques for propagation of plants. The production of many medicinal plants with tissue culture such as *Hypericum rumeliacum* Boiss (Danova et al., 2010), *Alternanthera sessilis* (L.) (Gnanaraj et al., 2011), *Harpagophytum procumbens* (Bairu et al., 2011), *Aloe arborescens* Mill (Amoo et al., 2012), *Coleonema pulchellum* (Baskaran et al., 2014), *Ceratophyllum demersum* L. (Dogan et al., 2015), *Rotala rotundifolia* (Buch-Ham. ex Roxb) Koehne (Dogan, 2017) *Lysimachia nummularia* L. (Dogan, 2018a), *Limnophila aromatica* (Lamk.) Merr. (Dogan, 2018b), *Actinidia melanandra* Franch. and *Actinidia rubricaulis* Dunn. (Bourrain, 2018) has been previously reported.

In this study, the shoot tip explants of *B. monnieri* for rapid and multiple production were cultured in MS medium containing 1.0 mg/L BAP for 10 weeks. Regenerated shoots on explants within two weeks have started to appear. At the end of four weeks, multiple shoot formations were clearly observed in the culture medium. High shoot regeneration frequency was obtained in all culture media. The rapid and multiple production of *B. monnieri* has been achieved successfully after 10 weeks of culture (Figure 1). Similarly, *in vitro* propagation of *B. monnieri* in previous studies have been reported (Showkat et al., 2010; Vijaykumar et al., 2010; Shrivastava et al., 2017; Naik et al., 2017; Kashyap et al., 2018).

Cytotoxic effects of different concentrations of the extract on HLs were assessed by MTT and MI tests. Results exhibited a concentration-dependent increase in growth inhibition of HLs. Cell viability percentages of HLs exposed to maximum concentrations of the extract were 69.64% (Figure 2a). In another study performed on HLs, similar results were obtained as regarding concentration dependent cytotoxicity. It was reported that olivetoric and physodic acid isolated from *Pseudevernia furfuracea* (L.) Zopf lichen decreased viability of HLs depending on the concentration (Emsen et al., 2018c). In the aforementioned study, it was detected that cytotoxicity was stable the treatments with high concentrations. At the same time, calculated MI values indicated that the treatments with high concentration were more effective on the cells. The lowest concentration (6.25 mg/L) of the extract did not significantly ($p > 0.05$) increase MI value (5.25%) in the cells compared with control (-). It was reflected in the results that cell proliferation did not change significantly in low concentration trials (Figure 2b). The cytotoxic effects of different plant species on HLs was tested by the researchers (Gadano et al., 2006). It was suggested that aqueous extracts obtained from *Chenopodium ambrosioides* L. and *C. multifidum* L. caused decreasing in MI in the cells. In a different MI-natural product study, an insignificant decrease in the MI rate in HLs was determined (Ari et al., 2015). In this study, *Parmelia sulcata* lichen extract was used and a significant difference was found between the control (+) and the extract trial results. In the present study, the values in the cells exposed to control (+) were significantly ($p < 0.05$) different from all other trial results for both MTT and MI activities.

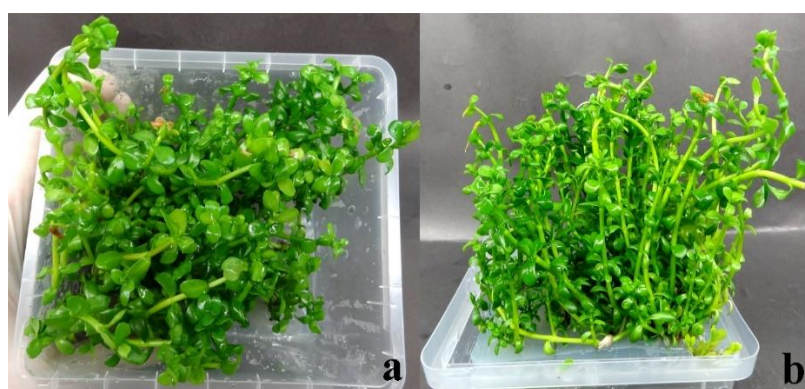

Figure 1. *In vitro* plant regeneration from shoot tip explants of *B. monnieri* (a,b) after 10 weeks of culture

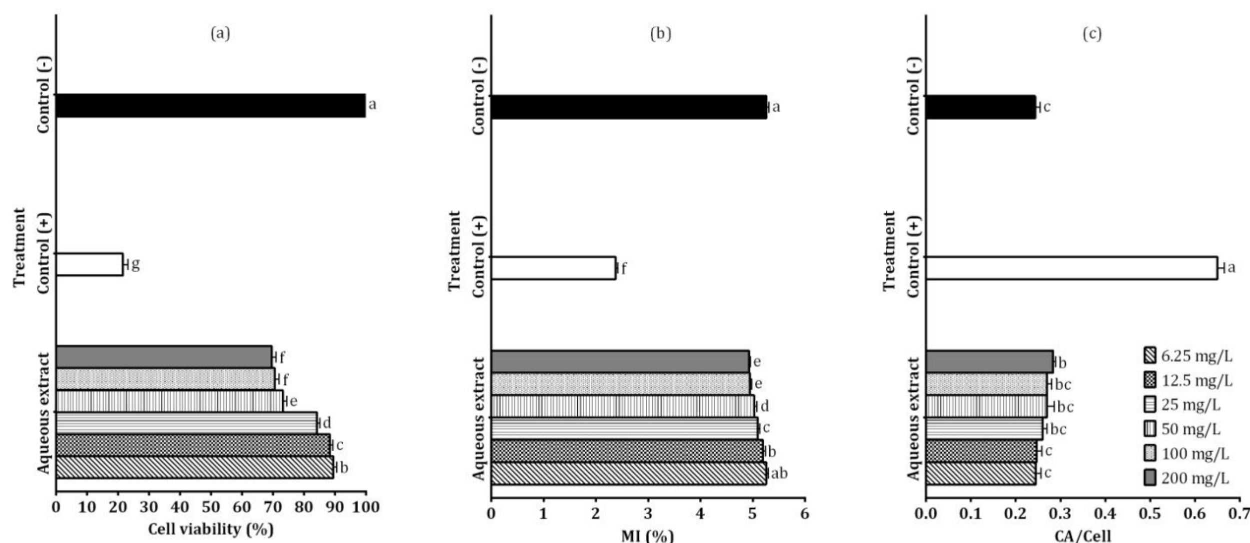

Figure 2. (a) Viability rate, (b) MI percentage and (c) CA frequency in the HPLs exposed to aqueous extract from *B. monnieri*. Different small letters indicate significant differences among treatments at $p < 0.05$.

Table 2. IC_{50} value for HLs exposed to aqueous extract from *B. monnieri* (mg/L)

IC_{50} (limits)	Slope ± standard error (limits)	X^2
1129.45 (579.92-3224.04)	0.56 ± 0.07 (0.43-0.69)	7.05

Table 3. Correlation between different variables for HLs exposed to aqueous extract from *B. monnieri*

	CA	Cell viability	Concentration	MI
CA	1[a]			
Cell viability	-0.73**	1		
Concentration	0.69**	-0.81**	1	
MI	-0.68**	0.95**	-0.82**	1

[a]Pearson correlation coefficient; **correlation is significant at the 0.01 level

Based on the experiments with high concentrations of the extracts, it was determined that there was no statistically ($p > 0.05$) significant difference between concentrations of 100 and 200 mg/L of the extracts in both MTT and MI activities. Additionally, IC_{50} value of the extract found to be 1129.45 mg/L indicated that very high concentrations of aqueous extract obtained from *B. monnieri* showed cytotoxic effect on the cells (Table 2). In another study carried out with plant-based products, it was used copaene, a tricyclic sesquiterpene. According the MTT results of previous mentioned study, copaene significantly suppressed the proliferation of HLs at high concentrations (Turkez et al., 2014). Similar relationship was found in the study performed with ascorbic acid, a strong antioxidant compound. Low proliferation index in titanium dioxide-induced HLs was increased by ascorbic acid treatment (Turkez, 2011).

Genetic damage emerged in HLs by aqueous extract obtained from *B. monnieri* was measured with CA analysis occurring in the cells. It was observed that the extracts increased CA frequency in HLs in a dose-dependent manner. However, there was no statistically ($p > 0.05$) significant difference between CA rates caused by extract treatments and control (-) except for the highest concentration. The CA data in HLs exposed to the extracts were too far and significantly ($p < 0.05$) different from the control (+) value (0.65 CA/cell) (Figure 2c). Numerous studies were conducted to inhibit the genetic damage caused by different toxic substances in the lymphocytes with natural products. Resveratrol (Turkez and Aydin, 2013), boron (Geyikoglu and Turkez, 2008), propolis (Bayram et al., 2016) and boric acid (Turkez et al., 2010) are some of these natural products. In the literature, studies on antigenotoxic activity carried out with different organism species such as *Bryoria capillaris* (Ach.) Brodo & D. Hawksw, *Xanthoria elegans* (Link) Th. Fr. (Aydin and Turkez, 2011a), *Aspicilia calcerea* (L.) Körb., *Cetraria chlorophylla* (Willd.) Poetsch (Aydin and Turkez, 2011b), *Platismatia glauca* (L.) W.L. Culb. & C.F. Culb. (Emsen et al., 2018a) and *Cladonia furcata* (Huds.) Schrad. (Emsen et al., 2018b) on HLs are also available.

When different activities of the extracts in HLs are examined, relationship between the activities was noteworthy. Bivariate correlation analysis performed on the data indicated that there were significantly ($p < 0.01$) negative correlations between several binary variables (cell viability-CA, cell viability-concentration, MI-CA and MI-concentration). In addition, concentration-CA and MI-cell viability bivariate correlations for the extract experiments were significant at the 0.01 level (Table 3).

5. Conclusions

In conclusion, we have displayed that aqueous extract obtained from *B. monnieri*, which is an important medicinal plant, does not show cytotoxic and genotoxic effects on cultured HLs especially in 6.25-50 mg/L concentration applications. So, this plant can be a new resource of therapeutics as recognized in this study against genetic damages. At the same time, thanks to the multiple production of the plant under *in vitro*, the plant will not need to be collected from its natural environment and the problem of being attached to the external environment and disrupting the balance in the ecosystem may be eliminated.

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