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Enhancing the secondary metabolite and anticancer activity of *Echinacea purpurea* callus extracts by treatment with biosynthesized ZnO nanoparticles

Narges Karimi[1], Mandana Behbahani[1], Ghasem Dini[2] and Amir Razmjou[1]

[1] Faculty of Advanced Sciences and Technologies, Department of Biotechnology, University of Isfahan, 81746-73441, Isfahan, Iran
[2] Faculty of Advanced Sciences and Technologies, Department of Nanotechnology Engineering, University of Isfahan, Isfahan 81746-73441, Iran

E-mail: ma.behbahani@ast.ui.ac.ir

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Abstract

This investigation was done to study the effect of green synthesized ZnO nanoparticles (NPs) on the anticancer activity of callus extracts of *Echinacea purpurea* in comparison with commercial ZnO microparticles (MPs). Leaf explants of *E. purpurea* were grown on the Murashinge and Skoog (MS) medium containing different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA). Callus induction at optimum concentrations were considered under both light and dark conditions. Among media with diverse concentrations of 2,4-D and NAA, fast-growing friable callus was started within three weeks after culturing on the MS medium containing 2.0 mg $2,4\text{-D}\,l^{-1}$. After adding different concentrations of synthesized ZnO NPs and ZnO MPs to the culture medium containing 2 mg $2,4\text{-D}\,l^{-1}$, the effect of ZnO NPs on the anticancer activity of plant extracts and callus biomass was found positive contrary to the control and ZnO MPs. However, these extracts did not have any cytotoxic activity on MCF-10 cells and peripheral blood monolayer cells. The frequency and intensity of CD4 expression on peripheral blood monolayer cells was not increased in the presence of all extracts. The highest flavonoid production of the extracts was also achieved in calli treated with different concentration of ZnO NPs. Therefore, it can be concluded that there is a direct relationship between the anticancer activity of *E. purpurea* and flavonoid contents.

Keywords: anticancer activity, *Echinacea purpurea*, green synthesis, ZnO nanoparticles
Classification numbers: 2.04, 4.02, 5.08

1. Introduction

Echinacea is a perennial plant that belongs to Asteraceae family and is used as a therapeutic plant [1]. Three species of *Echinacea* including *E. angustifolia*, *E. purpurea*, and *E. pallida* are presently used for their anticancer and antioxidant properties [2]. Luettig *et al* reported that *E. purpurea* has potent anticancer effect on MCF-7 cells, BT-549 and natural killer cells [3–5]. The pharmacological uses of *Echinacea* indicated that it has some effective phenolic compounds. Previous results have shown that the active compounds such as caffeic acid derivatives, alkyl amides, polysaccharides, and polyacetylenes are responsible for anti-inflammatory, immune-stimulatory, and antioxidant activities of this plant

[2, 6–8]. Plant tissue culture is a simple and economical technique for the flavonoid production with the anticancer activity compared to the direct extraction from plant biomass [9]. The callus induction was frequently used to improve the plant nutrition [10]. The positive role of nanoparticles (NPs) on callus induction has reported by the other researchers [11]. Also, there is an interest in enhancing the anticancer efficacy of *E. purpurea* callus extracts with metal oxide nanoparticles. Metal oxide NPs could change plant physiological processes, and impact plant growth. NPs can act as nutrient materials and also could enhanced bioactive components in the *in vitro* culture medium [12–14]. This potential may be differentiated by the chemical composition, structure, particle size and surface area of the NPs. The green synthesized NPs by plants are the appropriate choice because of their biocompatibility, stability and relevant nontoxicity [15]. The effects of some important metal oxide NPs such as titanium oxide (TiO_2), zinc oxide (ZnO), iron oxide (Fe_3O_4), and copper oxide (Cu_2O) have been before proved on the development and increment of secondary metabolite production in plants [16, 17]. Zafar *et al* [18] indicated that ZnO NPs may be used for the promotion of valuable plant secondary metabolites. The effects of ZnO NPs have been previously studied on the crop and horticultural plants [16]. ZnO NPs are not only essential nutrient, but also act as a co-factor for nutrient mobilizing enzymes. Raliya *et al* [19] reported that ZnO NPs could significantly increase seedling vigor, pigment, protein, and sugar contents of plants compared to ZnO microparticles (MPs). Some studies reported that ZnO NPs could improve the callus induction, genetic modification, and bioactive compounds in plant cell cultures. Although, there are different researches about the toxicity or nontoxicity of the green synthesized metal oxide NPs on the cell and tissue cultures [18], but there is no reported document showing the effects of NPs on the callus extracts. The present study aimed to determine the activity of callus extracts of *E. purpurea* which treated with synthesized ZnO NPs on the MCF-7 and MCF-10 cell lines and peripheral blood monolayer cells (PBMC).

2. Materials and methods

2.1. Ethics statement

This study was approved by the ethical committee in the University of Isfahan, Iran. All participants were provided written informed consent to participate in the study. The written consents of participation were approved by Isfahan University.

2.2. Materials

ZnO MPs were purchased from Sigma Aldrich Chemical Co. with a mean diameter of 5 μm. 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) were purchased from Sigma Aldrich (Auxin treatments). The MCF-7 and MCF-10A cell lines were obtained from the National Cell Bank of Pasteur Institute, Tehran, Iran.

2.3. Synthesis and characterization of ZnO NPs

ZnO NPs were synthesized via a green synthesis method using *Allium jesdianum* according to Karnan and Selvakumar method with some modifications [20]. Initially, 10 ml of *A. jesdianum* extract was added to 50 ml of 0.1 M zinc nitrate hexahydrate aqueous solution and stirred at 75°C for 2 h. The particles made after 2 h of stirring were collected by centrifugation at 8000 rpm for 15 min. Later, the centrifuged particles were washed with water and centrifuged again at 1000 rpm for 10 min. The centrifuged sample dried in a hot air oven at 70°C for 12 h and ground using mortar and pestle. The sample was calcined in a muffle furnace at 350°C for 2 h to achieve pure ZnO NPs. The synthesized ZnO NPs were characterized by x-ray diffraction (XRD), atomic force microscopy (AFM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and Fourier-transform infrared spectroscopy (FTIR) analyses.

2.4. Tissue culture and callus induction methods

Young leaves (4–5 cm) of *E. purpurea* were collected from 3-month old seedlings grown in a greenhouse at the University of Isfahan, Iran. These leaves were surface sterilized with a 70% (v/v) ethanol solution, then, treated with 20% (v/v) hypochlorite sodium solution with two or three drops of tween-20 for 15 min, followed by soaking in sterile distilled water. Sterile explants were cut into small pieces $0.7 \times 0.7 cm^2$ and cultured on the Murashige and Skoog (MS) medium containing different concentrations of 2,4-D and NAA ($1, 2, 5\ mg \times l^{-1}$) [21]. Each medium contained $30\ g \times l^{-1}$ sucrose and $7\ g \times l^{-1}$ agar (Sigma type A, Germany). The pH of the MS media was adjusted to 5.8 with 0.5 M NaOH solution. Then, explants were located in a growth chamber at $25 \pm 1°C$ under dark and light conditions for the callus induction. After the callus induction, the best culture medium was selected for further growth of calli.

2.5. Preparation of culture media with ZnO NPs and ZnO MPs suspensions

Different concentrations of ZnO MPs and ZnO NPs (0, 10, 25, 50, 75, 100 and $150\ mg \times l^{-1}$) were prepared and sterilized in an autoclave at 121°C for 20 min, then, sonicated for 30 min (100 W, 40 kHz), and combined with the callus induction culture medium at 45°C. In order to avoid the agglomeration of NPs, the medium was shacked well and distributed in plates to solidify. The explant samples were grouped as follows: plates fewer than 16 h light and 8 h darkness and plates under full darkness in a growth chamber at $25 \pm 1°C$ to produce callus for 30 d. Then, the weight of the *Echinacea* leaf callus was measured after drying at 55°C for 48 h. The texture (friable

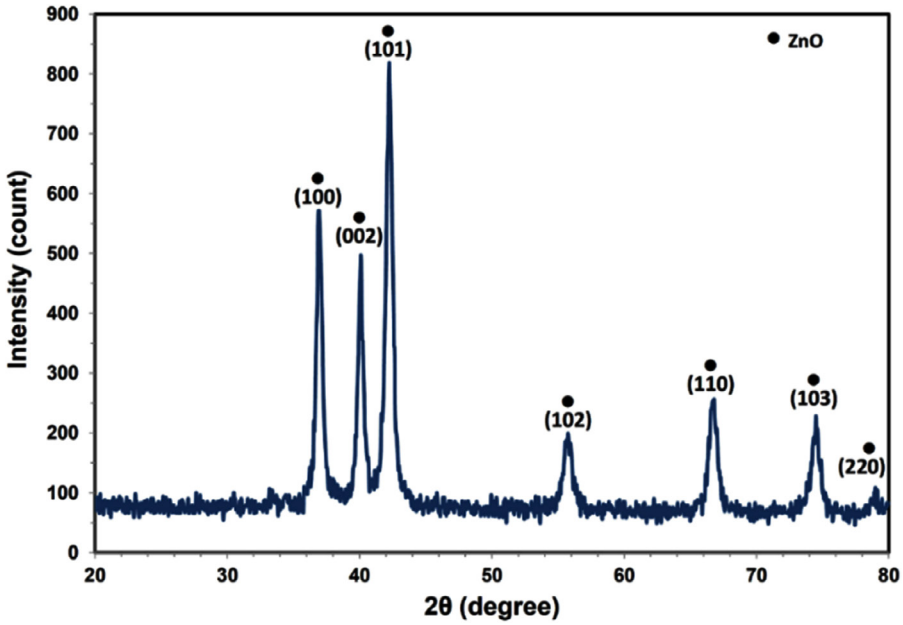

Figure 1. The XRD pattern of ZnO NPs.

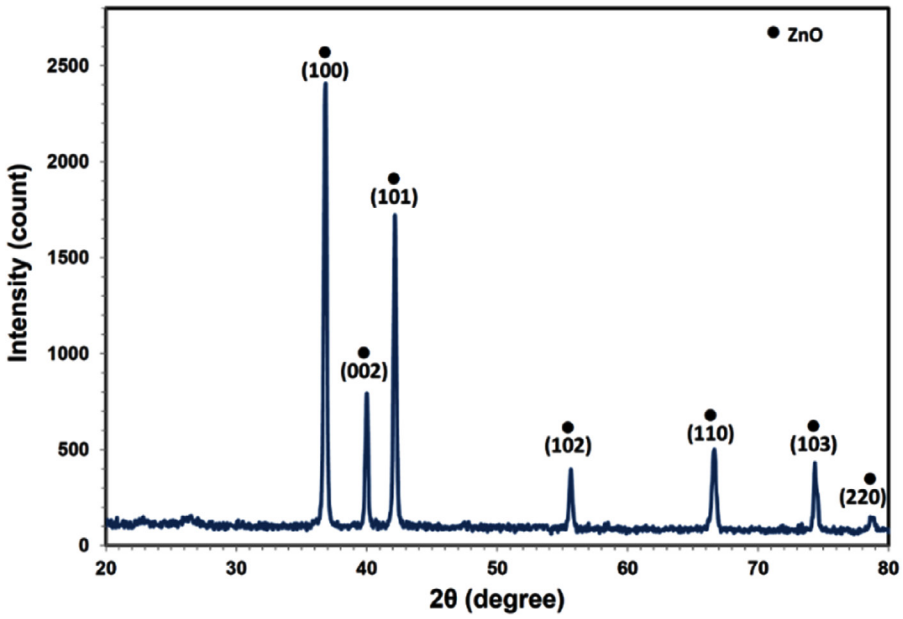

Figure 2. The XRD pattern of ZnO MPs.

and compact) and the color of the calli were studied and compared to the control.

2.6. Preparation of callus extracts

The extraction was performed three times at room temperature $(25-28°C)$ by the maceration method (72 h). The methanol extract was filtered and evaporated in a vacuum rotary evaporator (Steroglass, Italy) and freeze dried (Zirbus, Germany).

2.7. Determination of total flavonoids compounds (TFC)

The content of flavonoids in the obtained *Echinacea* callus extracts was estimated using the spectrophotometric method [22, 23]. First, callus extracts were dissolved in methanol (1 mg callus extracts ml^{-1}). Then, the solutions were added to 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for 30 min at 25°C. The absorbance was determined using a spectrophotometer at λ_{max} of 415 nm. The concentration of flavonoids was measured on the basis of the calibration curve, which was generated by a standard solution of quercetin.

2.8. Determination of anticancer activity and PBMC proliferation assay

The peripheral blood mononuclear cells (PBMCs) were attained from healthy donors by Ficol-Hypaque gradient separation. MCF-7, MCF-10 and PBMCs were respectively grown in a Dulbecco's modified eagle's medium (DMEM) and a

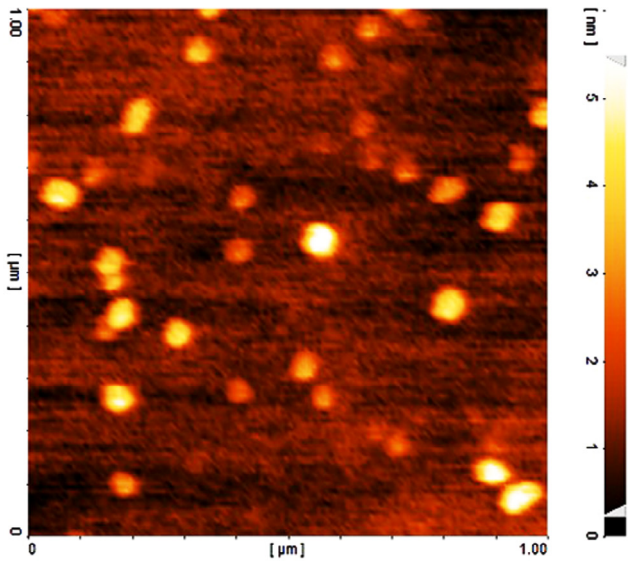

Figure 3. The AFM image of ZnO NPs.

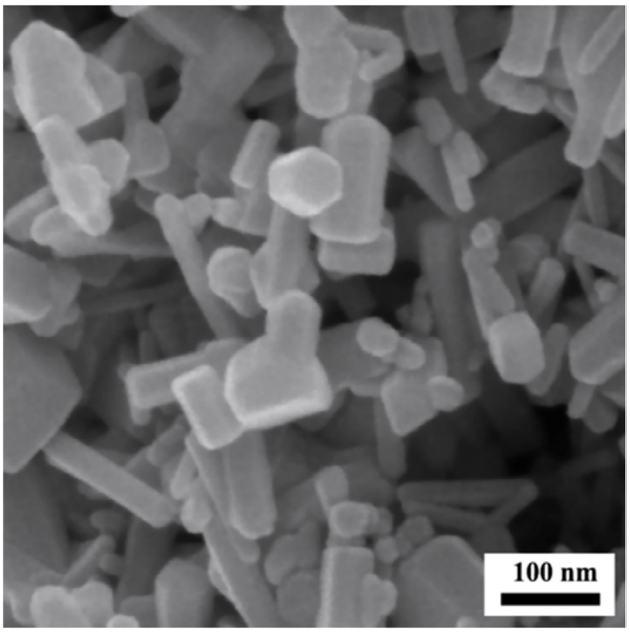

Figure 4. The SEM micrograph of ZnO NPs.

Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin, 100 mg streptomycin, and 5 mM glutamine. The cells were pre-incubated in a 96 well plate at a density of 5×10^4 cells per well for 24 h in a humidified atmosphere of 5% CO_2 and 95% of air at 37°C [24]. The cytotoxic activity of callus extracts of *E. purpurea* was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution [25, 26]. However, the cells were treated with callus extracts at different concentrations (0, 10, 25, 50, 75, 100, 250, 500, 750 and 1000 $\mu g \times ml^{-1}$) and grown for 48 h. Then, $5mg \times ml^{-1}$ MTT solution was added to each well and incubated for an additional 4 h. Finally, the medium was discarding and 100 μl of dimethyl sulfoxide (DMSO) was added to solubilize the formed formazan crystals. The absorbance was then measured at 495 nm using a microplate spectrophotometer (Awareness Technology Inc., stat fax 2100). The percentage of viability was calculated and compared with an untreated control. The 50% cytotoxic concentration to cause the death of 50% of viable cells (CC50) of the extracts was calculated.

2.9. Analysis of CD4 expression by flow-cytometry

The percentage of cluster of differentiation 4 (CD4) T-lymphocyte subsets and their expression intensities on PBMCs in the presence of callus extracts were evaluated by flow-cytometry (FAC scan from Becton Dickinson). PBMCs were cultured in 24-well plates and incubated at 37 °C for 72 h. The cells were washed with PBS and incubated with saturating concentrations of PE anti human CD4 monoclonal antibodies (Cyto Matin Gene, IRAN) for 20 min at 4 °C. Lymphocytes were gated based on their forward and side scatter properties. At least, 10000 events were acquired for each sample. Data acquisition was achieved using BD Cell Quest software.

2.10. Statistical analysis

The experiments were performed using complete randomized design (CRD) and the results were analyzed using two ways ANOVA. Statistical analysis was done by using SPSS software, version 6.12. Probability (P) less than 0.05 was considered significant. Each experiment was repeated three times.

3. Results

3.1. Characterization of ZnO NPs and ZnO MPs

The XRD patterns of ZnO NPs and MPs are shown in figures 1 and 2, respectively. In both patterns, all significant peaks correspond to ZnO crystal structure. In comparison, the XRD pattern of ZnO NPs shows the broadened diffraction peaks which might indicate the nanocrystalline nature of the synthesized ZnO particles.

The AFM image of ZnO NPs is shown in figure 3. Also, the SEM micrograph of the green synthesized ZnO NPs is given in figure 4. These figures confirm the formation of ZnO particles in the nano-scale. Additionally, the size distribution diagram of ZnO NPs (figure 5) obtained from several SEM micrographs shows the narrow size distribution with the mean particle diameter of about 40 nm. The TEM micrograph of synthesized ZnO NPs is shown in figure 6. From this figure, it can be seen that the size and the morphology of the particles are in good agreements with the results of SEM observations. The FTIR spectrum of ZnO NPs is also shown in figure 7. In the FTIR spectrum, a band observed at around 3429 cm^{-1} is due to O–H bending of absorbed water. The band of near 536 cm^{-1} corresponds to ZnO bending and confirms the presence of ZnO particles [27, 28]. The C=O peak around

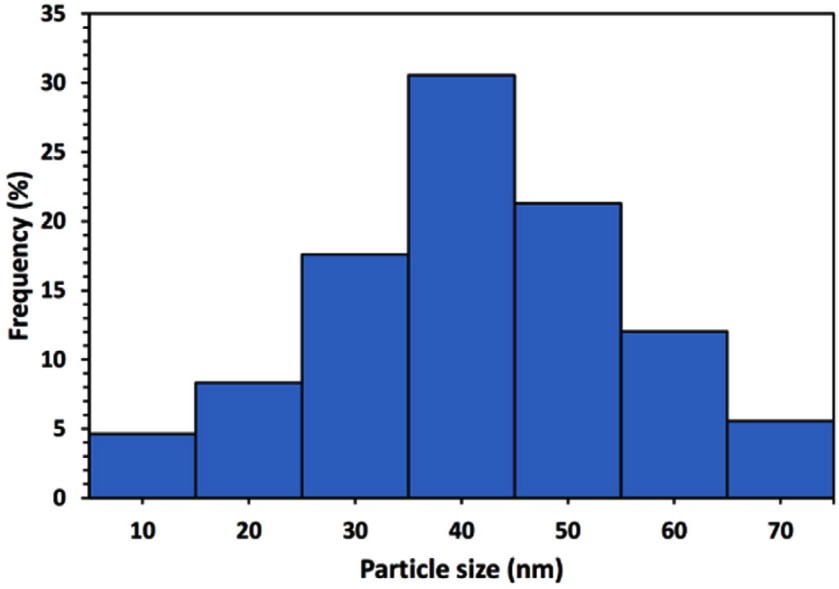

Figure 5. The size distribution diagram of ZnO NPs.

$1631\ cm^{-1}$ demonstrate the carboxylic group of modified ZnO NPs [29].

3.2. Formation of callus cultures

As mentioned before, this study was aimed to define the suitable conditions for the callus growth with a maximum flavonoid content. The growth of callus was measured via the biomass weights of the cultures (table 1). The callus induction was initiated at the cut ends and wounded sections of the leaf explants after 3 weeks of culturing. The callus grew up on the whole surface of the explants within 4 weeks of culturing. The callus merely produced on the MS medium supplemented with a growth regulator. The effect of 2,4-D on the callus induction was significantly higher than NAA hormone. The results indicated that the best medium for the callus induction from leaf explants was the MS medium supplemented with $2\,mg\ 2,4\text{-}D\,l^{-1}$ (table 1). Calli were separated and cultured on the MS medium supplemented with $2\,mg \times l^{-1}$ 2,4-D and different concentrations of ZnO NPs and ZnO MPs (table 2).The callus induction was increased with increasing the concentrations of ZnO NPs until it reached $75\,mg \times l^{-1}$ ZnO NPs, then reduced (table 2). The highest frequencies of callus induction were found in the MS medium containing 3% (w/v) sucrose supplemented with $75\,\mu g \times ml^{-1}$ ZnO NPs and $100\,\mu g \times ml^{-1}$ ZnO MPs. However, there was a significant difference between two applied NPs and MPs. Calli which obtained at the concentrations of 100 and $150\,mg \times ml^{-1}$ ZnO NPs and $150\,mg \times l^{-1}$ ZnO MPs were compact with green color and in the all other treatments, the friable white-yellow textures were produced (figure 8).

3.3. Effect of ZnO NPs and ZnO MPs on the plant biomass

The previous results demonstrated that the influence of ZnO NPs on the callus biomass of *E. purpurea* was significantly higher than ZnO MPs. Also, the maximum biomass of callus

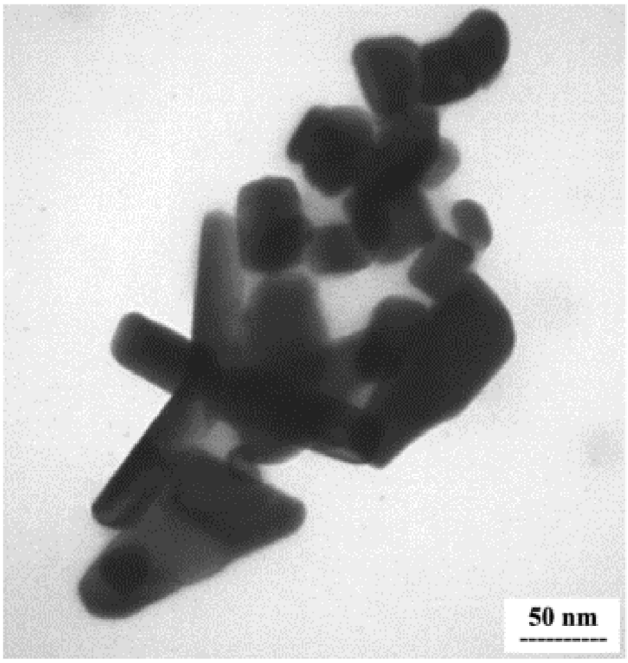

Figure 6. The TEM micrograph of ZnO NPs.

obtained in the MS medium supplemented with $75\,mg \times l^{-1}$ ZnO NPs and $100\,mg \times l^{-1}$ ZnO MPs. Therefore, at high concentrations of ZnO NPs (100 and $150\,mg \times l^{-1}$) and ZnO MPs ($150\,mg \times l^{-1}$), the callus biomass was reduced. It is evidenced that ZnO NPs and ZnO MPs at low concentrations have a positive effect on the growth parameters (table 1). The results of this investigation show that the high concentrations of zinc demonstrated a vice versa effect.

3.4. Determination of the flavonoid contents in E. purpurea callus extracts

The total flavonoid compounds in the callus extracts of *E. purpurea* are given in table 3. Using the standard plot of

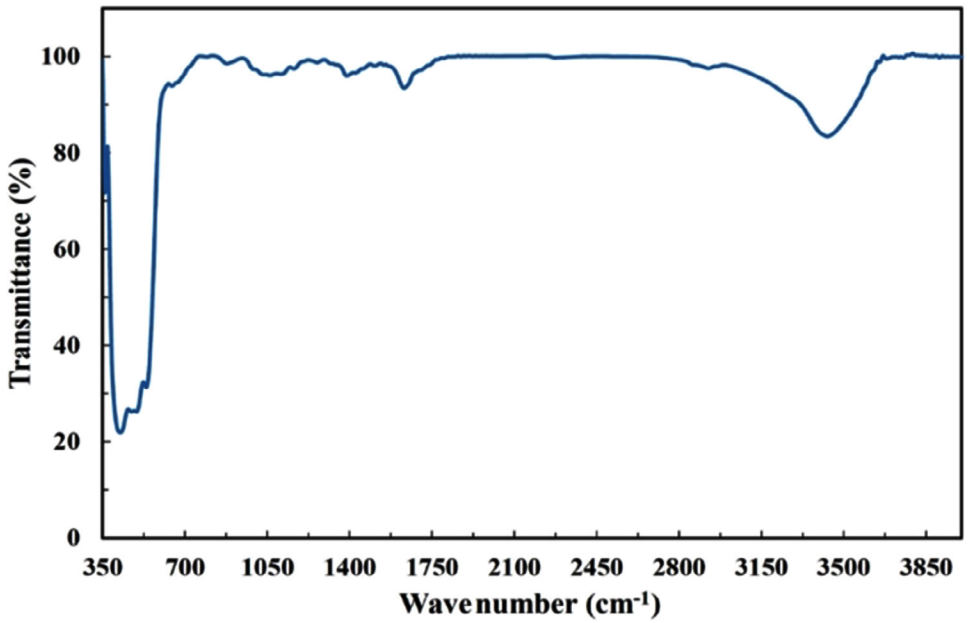

Figure 7. The FTIR spectrum of ZnO NPs.

Table 1. The effect of the different concentrations of 2,4-D and NAA on the duration of callus initiation, texture, and callus induction of *E. purpurea*.

	Days for callus initiation		Texture	
Medium	Dark	Light	Friable (yellow)	Compact (green)
Control (MS)	0	0	—	—
MS + 1 mg × l^{-1}2, 4 − D	24 ± 1[a]	35 ± 1[a]	[a]	—
MS + 2 mg × l^{-1}2, 4 − D	20 ± 1[a]	32 ± 1[a]	[a]	—
MS + 5 mg × l^{-1}2, 4 − D	17 ± 1[a]	28 ± 1[a]	—	[a]
MS + 1 mg × l^{-1}NAA	31 ± 2[a]	40 ± 2[a]	[a]	—
MS + 2 mg × l^{-1}NAA	27 ± 1[a]	33 ± 1[a]	[a]	—
MS + 5 mg × l^{-1}NAA	27 ± 1[a]	34 ± 1[a]	[a]	—

[a] The mean difference from control is significant at the 0.05 level ($P < 0.05$)

quercetin $y = 0.0148x, R^2 = 0.975$, the total flavonoid contents of untreated *E. purpurea* were calculated ranging from 0.89 to 3.7 mg quercetin equivalent per gram of dried sample. In this research, *E. purpurea* callus extracts have shown the potent cytotoxic activity and the high flavonoid compounds. The flavonoid contents in *E. purpurea* grown on ZnO NPs and ZnO MPs were significantly improved compared to the control (without particle treatment). From table 3, the highest flavonoid compounds were obtained in calli grown on culture media treated with 75mg × l^{-1} ZnO NPs, and 75 and 100mg × l^{-1} ZnO MPs. This signified that the ZnO NPs could increase the rate of flavonoid production in the calli.

3.5. Cytotoxicity and anticancer properties assay

The cytotoxic activity of *E. purpurea* callus extracts treated with ZnO NPs and ZnO MPs on MCF-7 and MCF-10 are shown in figures 9 and 10, respectively. The cytotoxic activity of callus grown on culture media treated with ZnO NPs (figure 9(a)) was remarkably higher than that of callus grown on ZnO MPs (figure 9(b)). The maximum cytotoxic effects were achieved at concentrations of 75mg × l^{-1} ZnO NPs and 100mg × l^{-1} ZnO MPs. The CC50 values of callus extracts treated with ZnO NPs and ZnO MPs were 625 and 875 μg × ml^{-1}, respectively. The results indicate that the viability of MCF-7 cells decreased with increasing the callus extract concentrations. The cytotoxic activities of these extracts on MCF-7 cells were significantly more than MCF-10A cells (figures 10(a) and (b)).

3.6. PBMCs proliferation assay

Effect of *E. purpurea* callus extracts on PBMCs proliferation is given in figure 11. The results showed that high concentrations of callus extracts increase PBMCs number in a dose-dependent manner. The maximum proliferation effect of callus extracts grown on media was respectively achieved at the concentrations of 50mg × l^{-1} ZnO NPs and 75mg × l^{-1} ZnO MPs. The ZnO NPs exhibited more effects than the ZnO MPs on the proliferation of the PBMCs.

Table 2. The effect of the different concentrations of ZnO NPs and ZnO MPs on the duration of callus initiation, texture, biomass, and callus induction of *E. purpurea*.

Medium plus 2 mg × l^{-1} 2,4-D + ZnO	Callus induction[b] after 4 weeks (%)		Biomass for dried weight after 4 weeks (g DW per culture)		Texture	
Concentration	Dark	Light	Dark	Light	Friable (pale yellow)	Gompact (light green)
Control (MS) + 2mg × l^{-1} 2,4-D	70 ± 0.2	36 ± 1	2.50 ± 0.2	0.75 ± 0.06	[a]	
10mg × l^{-1}(NPs)	79 ± 1[a]	41 ± 1[a]	2.72 ± 0.3	0.87 ± 0.08	[a]	
25mg × l^{-1} (NPs)	85 ± 1[a]	49 ± 1[a]	2.96 ± 0.2	0.93 ± 0.09	[a]	
50mg × l^{-1} (NPs)	89 ± 1[a]	51 ± 1[a]	3.28 ± 0.3	1.25 ± 0.08[a]	[a]	
75mg × l^{-1} (NPs)	96 ± 1[a]	56 ± 1[a]	3.37 ± 0.3[a]	1.37 ± 0.1[a]		[a]
100mg × l^{-1} (NPs)	77 ± 1[a]	40 ± 1[a]	3 ± 0.4	1.1 ± 0.1[a]		[a]
150mg × l^{-1} (NPs)	68 ± 1	33 ± 1	2.21 ± 0.2	0.7 ± 0.07	[a]	
10mg × l^{-1} (MPs)	72 ± 2	32 ± 2[a]	2.45 ± 0.3	0.61 ± 0.05	[a]	
25mg × l^{-1} (MPs)	75 ± 1[a]	36 ± 1	2.65 ± 0.2	0.78 ± 0.06	[a]	
50mg × l^{-1} (MPs)	80 ± 1[a]	37 ± 1	2.83 ± 0.3	1.01 ± 0.9	[a]	
75mg × l^{-1} (MPs)	86 ± 1[a]	40 ± 1[a]	3 ± 0.2	1.16 ± 0.1[a]	[a]	
100mg × l^{-1} (MPs)	91 ± 1[a]	47 ± 1[a]	3.25 ± 0.1	1.25 ± 0.1[a]	[a]	
150mg × l^{-1} (MPs)	84 ± 1[a]	27 ± 1[a]	3.04 ± 0.2	1.12 ± 0.08[a]		[a]

[a] The mean different from control less than 0.05 were considered significant ($P < 0.05$).
[b] Callus induction (%) = (Number of explants induced callus/Total number of explants inoculated) × 100.
DW: dry weight.

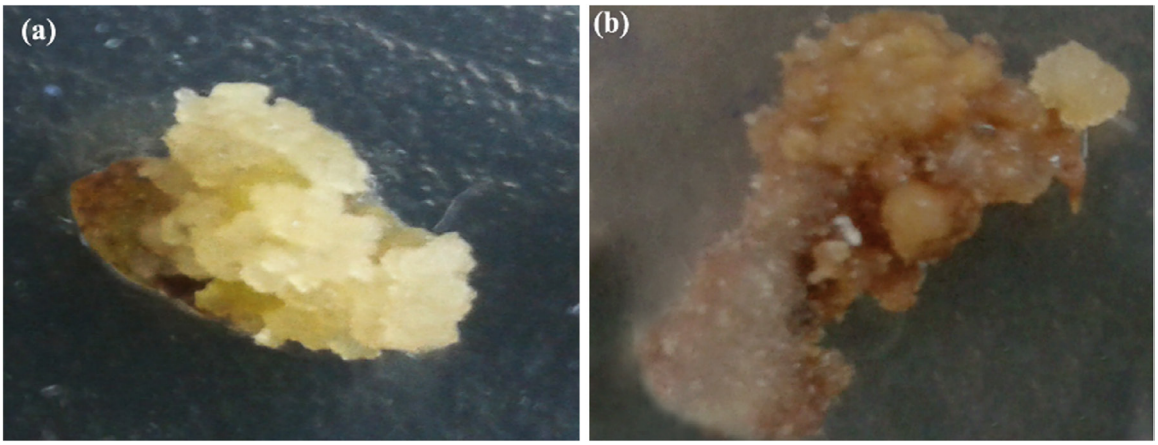

Figure 8. *E. purpurea* callus: (a) the friable callus grew on high concentration, and (b) compact callus grew on a low concentration of ZnO NPs.

3.7. Effect of callus extracts on expression of CD4

The effects of callus extracts on frequency and the average mean fluorescent intensity (MFI) of $CD4^+$ T cells in PBMCs are summarized in table 4. The results displayed that the extracts at different concentrations did not have any effects on frequency (%) and MFI value of of $CD4^+T$ cells in PBMCs. The average mean fluorescence intensity (MFI) value and frequency of CD4 lymphocytes were not significantly different for the cells treated with callus extracts treated with ZnO NPs and ZnO MPs.

4. Discussion

Plant-derived secondary metabolites are vigorous and important resources for improving nation's health. But the direct

Table 3. Total flavonoid contents of *E. purpurea* callus extracts grown on culture media treated with different concentrations of ZnO NPs and ZnO MPs.

Concentration (mg × l^{-1})	Flavonoid (mg × g^{-1} DW[b]) under ZnO NPs	Flavonoid (mg × g^{-1} DW) under ZnO MPs
control	0.89 ± 0.08	0.89 ± 0.08
10	1.50 ± 0.1[a]	0.93 ± 0.05
25	1.98 ± 0.1[a]	1.01 ± 0.08
50	3.2 ± 0.2[a]	2.63 ± 0.1[a]
75	3.7 ± 0.3[a]	3.01 ± 0.2[a]
100	3 ± 0.1[a]	3.09 ± 0.1[a]
150	2.4 ± 0.1[a]	2.55 ± 0.2[a]

[a] The mean different from control less than 0.05 were considered significant ($P < 0.05$).
[b] DW: dry weight.

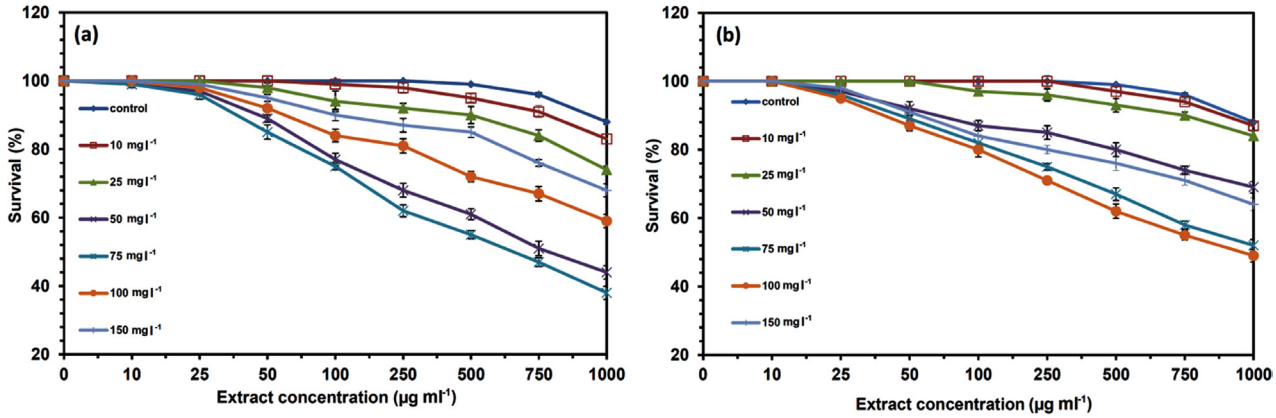

Figure 9. The cytotoxicity effect of E. purpurea callus extracts grown on culture media treated with different concentrations of (a) ZnO NPs and (b) ZnO MPs on MCF-7 cell line.

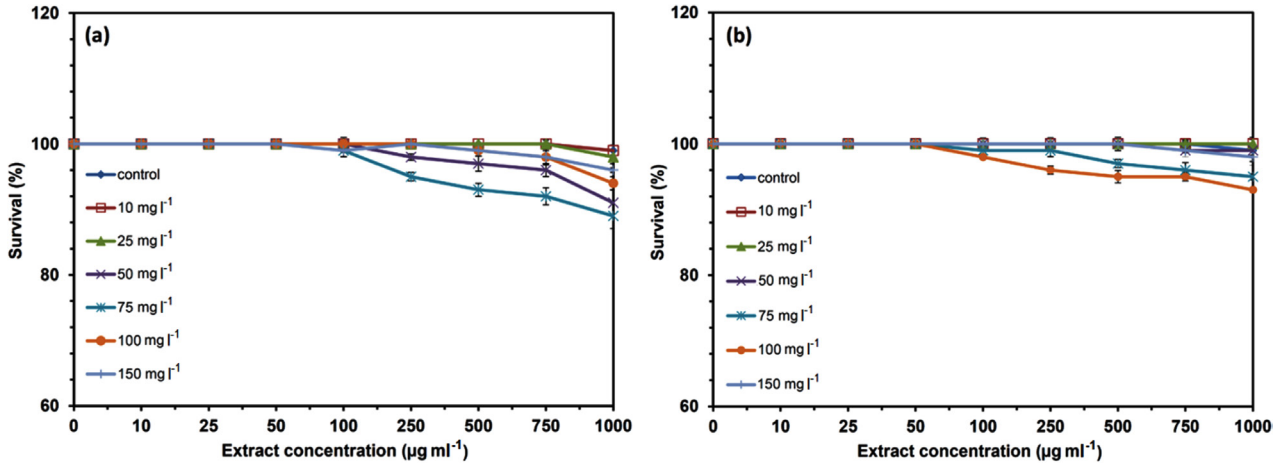

Figure 10. The cytotoxicity effect of E. purpurea callus extracts grown on culture media treated with different concentrations of (a) ZnO NPs and (b) ZnO MPs on MCF-10 cell line.

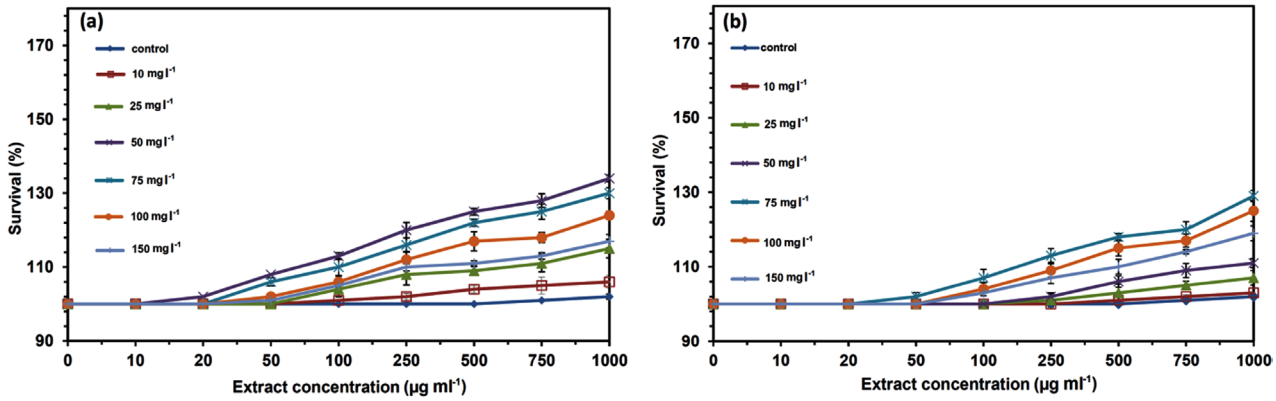

Figure 11. The effect of E. purpurea callus extracts grown on culture media treated with different concentrations of (a) ZnO NPs and (b) ZnO MPs on PBMC proliferation.

isolation of secondary metabolites from plants as well as the chemical synthesis of these compounds are expensive and effortful [30]. In order to overcome these difficulties, plant cell culture has been considered [31]. There are some anticancer compounds from superior plants which can be produced by tissue culture technique [32]. *E. purpurea* is one of the most powerful medicinal plants that have some important anticancer compounds such as polysaccharides, flavonoids, chicoric acid, alkyl amides and polyacetylenes [33]. The current study was done to study the effect of green synthesized ZnO nanoparticles (NPs) on anticancer activity and flavonoid content of callus extracts. The ZnO NPs were synthesized using *A. jesdanum* that has been reported here for the first time. *A. jesdianum* is a native plant in Iran which is found suitable for extracellular biosynthesis and production of ZnO NPs [34–38]. The formation of nanoparticles was confirmed using,

Table 4. The effect of *Echinacea* callus extract under treatment with the different concentrations of ZnO NPs and ZnO MPs on CD4.

Concentration ($mg \times l^{-1}$)	CD4 under ZnO NPs		CD4 under ZnO MPs	
	Frequency (%)	MFI	Frequency (%)	MFI
control	22.4	19.3	22.4	19.3
10	21.5	19.8	21.1	19.4
25	20	19.1	20.7	20.1
50	21.8	20.6	21.3	19.8
75	22	19.7	21.7	18.9
100	21.6	20.8	22	20.2
150	20.9	20.1	21.2	19.8

SEM, XRD and AFM techniques. In the present study the biosynthesized ZnO NPs had a size range from 14 to 45 nm, which was almost smaller than ZnO NPs which synthesized by plant extracts such as *Allium sativum*, *Rosmarinus officinalis*, *Ocimum basilicum* (14–54 nm) [27], *Aloe barbadensis* (25–40 nm) [39], *Hibiscus rosa* (30–35 nm) [40], *Nephelium lappacuml* (25–40 nm) [20], *Vitex negundo* (80 nm) [41], *Zingiber officinale* (30–50 nm) [42]. There are investigations demonstrated that NPs obtained from the green synthesis method were coated peripherally with plant polysaccharides and phenolic compounds on the surface of the NPs [43, 44]. Our results indicated that the best medium for the callus induction from leaf explants was MS medium supplemented with 2 mg 2,4-D [45, 46]. On the other hand, the effect of ZnO NPs has been evaluated more than ZnO MPs on the callus production [12–14]. However, the increasing of callus production may be dependent on the role of ZnO NPs as a co-factor for nutrient mobilizing enzymes and consequently, more production of secondary metabolite. Our results also demonstrated that ZnO NPs at low concentrations has a positive effect on the growth parameters and the high concentrations of ZnO NPs demonstrated negative effect on plant. Helaly *et al* [47] reported that the low concentrations ($100mg \times ml^{-1}$) of ZnO NP have positive effect on growth parameters of *Moringa peregrina*. However, it seems that the high concentrations of ZnO NPs exhibit the inhibitory effect on the callus and biomass production. Because the high amounts of ZnO caused the induction of oxidative stress in plants and exhibit the toxic effects [48–52]. The toxic effect also related to the possible release of Zn ions by ZnO particles in the media [53]. The present results demonstrated that ZnO NPs have positive effect on anticancer activity and flavonoid contents of *E. purpurea*. These results are in a good agreement with Chamani *et al* [11] who observed the highest content of flavonoids in a medium supplemented with $75mg \times l^{-1}$ ZnO NPs. According to our results, ZnO NPs at high concentrations decrease the flavonoid production in callus extract. These observations are likely due to the phytotoxic effects of ZnO at high concentrations. Similar results demonstrated that green synthesized AgNPs have potent anticancer activity. Prasannaraj *et al* [54] demonstrated that NPs have anticancer activity with CC50 of 75.68 and $54.68\mu g \times ml^{-1}$ against Hep-G2 and PC3 cell lines, respectively. On the other hand, the lower concentration of biosynthesized AgNPs has been reported in the other publications. Ebrahiminezhad *et al* [55] reported that the CC50 of the biosynthesized AgNPs was about $4.7\mu g \times ml^{-1}$ at Hep-G2 cell line. But there is no study about effect of AgNPs on anticancer activity of plant. Some studies demonstrated that zinc could induce biosynthesis of the plant auxin indole-3-acetic acid [53]. The effect of auxin on the accumulating of the flavonoids in callus culture of *Andrographis lineata* and *Genista tinctoria* has reported earlier [17, 56]. The good correlation was obtained between flavonoid compound and anticancer activities of *E. purpurea*. It could be indicated that ZnO NPs are primary candidate for increasing the biosynthesis secondary metabolites in plants. This study revealed that green synthesized ZnO NPs is good strategy to improve the efficacy of *E. purpurea* callus extractand reduce the toxicity of lymphocyte cells.

5. Conclusions

For the first time, the effects of different concentrations of ZnO NPs and ZnO MPs on *E. purpurea* callus induction, flavonoid production, anticancer activity and proliferation of lymphocyte have been investigated. The results showed that the medium containing ZnO NPs led to an increase of the anticancer activities and flavonoid contents of *E. purpurea*. It can be concluded that ZnO NPs improve the growth and physiological changes in tissue culture in the field of plant science.

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