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Original Article

Improvement of Hypericin and Hyperforin Production Using Zinc and Iron Nano-oxides as Elicitors in Cell Suspension Culture of St John's wort (*Hypericum perforatum* L.)

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Abstract

Zinc and iron nano-oxides (100 ppb) were promoted the hypericin and hyperforin production in *Hypericum perforatum* cell suspension culture. High performance liquid chromatography method was used for detectection and identification of hypericin and hyperforin in *H. perfuratum* cell suspension cultures elicited with different concentrations of zinc and iron nano-oxide (0, 50, 100 and 150 ppb) after 72 h. In the cultures stimulated by zinc nano-oxide, the hypericin and hyperforin production reached to the maximum (7.87 and 217.45 µg/g DW, respectively), which were 3 and 13-fold higher than the control. The amount of hypericin and hyperforin was increased from 2.07 and 16.27 µg/g DW to 11.18 and 195.62 µg/g DW in iron nano-oxide treated cultures. The cell cultures treated with zinc and iron nano-oxides showed increased hyperforin production as compared to the hypericin production. These observations suggested that nano-particles can be appropriate candidates for elicitation studies of *in vitro* secondary metabolites production.

Key words: Cell suspension. *Hypericum perfuratum*. Hypericin. Hyperforin. Nano-particles

Introduction

Hypericum perforatum L. (St. John's Wort), is an important traditional medicinal plant [1,2]. The plant is one of the most widely used treatments of several diseases, such as skin lesions cancer, burns and microbial, eczema, inflammatory, anti-oxidant and psychological disorders [3-8]. Products of *H. perforatum* are currently one of the top-selling phytopharmaceuticals in industrialized countries [9]. Plant cell culture technology has been utilized for the production of many useful secondary metabolites, including pharmaceuticals, pigments, and other fine chemicals [10]. But low metabolite production is the main problem encountered in medicinal plant cell suspension cultures. In this case, the application of elicitors to the cell suspension culture may be useful for increasing the secondary metabolites production. Elicitors are defined as molecules that stimulate defense or stress-induced responses in plants [11]. A number of elicitors, such as, Jasmonic acid [12], *Diploceras hypericinum* and *Phytophthora capsici* [13], L-tryptophan [14], have been investigated for enhancement of hypericin and hyperforin production in *H. perforatum* cell suspension cultures affecting hypericin and

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hyperforin production and increased metabolites production.

Nanoparticles are materials that are small enough to fall within the nanometric range, with at least one of their dimensions being less than a few hundred nanometers. This reduction in size brings about significant changes in their physical properties with respect to those observed in bulk materials. Most of these changes are related to the appearance of quantum effects as the size decreases, and are the origin of phenomena such as the super paramagnetism, Coulomb blockade, surface Plasmon resonance, *etc*. The increase in the surface area to volume ratio is also a consequence of the reduction in size. It leads to the appearance of surface effects related to the high number surface atoms, as well as to a high specific area, which are important from the practical point of view. A wide variety of materials can be used to make such nanoparticles, such as metal oxide ceramics and silicates, magnetic materials, lyposomes, dendrimers, emulsions, *etc* [15,16].

Iron oxide nanoparticles are iron oxide particles with diameters between about 1 and 100 nanometers. The two main forms are magnetite (Fe_3O_4) and its oxidized form maghemite (γ-Fe_2O_3). They have attracted extensive interest due to their superparamagnetic properties and their potential applications in many fields (although Cu, Co and Ni are also highly magnetic materials, they are toxic and easily oxidized) [17]. Applications of iron oxide nanoparticles include terabit magnetic storage devices, catalysis, sensors, and high-sensitivity biomolecular magnetic resonance imaging (MRI) for medical diagnosis and therapeutics. These applications require coating of the nanoparticles by agents such as long-chain fatty acids, alkyl-substituted amines and diols [18].

Zinc oxide is an inorganic compound with the formula ZnO. ZnO is a white powder and is insoluble in water. This is widely used as an additive in numerous materials and products including plastics, ceramics, glass, cement, lubricants [19], paints, ointments, adhesives, sealants, pigments, foods (source of Zn nutrient), batteries, ferrites, fire retardants, and first aid tapes [20]. Nanophase ZnO can be synthesized into a variety of morphologies including nanowires, nanorods, tetrapods, nanobelts, nanoflowers, nanoparticles *etc*. Nanostructures can be obtained with most above-mentioned techniques, at certain conditions, and also with the vapor-liquid-solid method [21,22]. The applications of zinc nano oxide powder are numerous, and the principal ones are summarized below. Most applications exploit the reactivity of the oxide as a precursor to other zinc compounds. For material science applications, zinc nano oxide has high refractive index, high thermal conductivity, binding, antibacterial and UV-protection properties. Consequently, it is added into materials and products including plastics, ceramics, glass, cement, rubber, lubricants, paints, ointments, adhesive, sealants, pigments, foods, batteries, ferrites, fire retardants, *etc* [19].

Among the latest line of technological innovations, the development of nano-materials could open up novel applications in plant biotechnology. The effects of different nano-particles have been indicated on plant growth and metabolic function [23].

The metal based nano-particles like Ag, zinc and cu have been widely used in various researches for studying their cell internalization and further translocation in plant cells [24-27]. The effects of zinc nano oxide on the germination seed and root growth of different plants have been studied [26]. The results of these researches shows that in order to reduce the risks of assessment and to clarify the eco-toxicity, more researches are needed and future studies should focus on their successful transfer to plant system.

Zinc and iron nano-oxides are novel elicitors. However, no reports are available regarding utilization of the zinc and iron nano-oxides in cell suspension culture of *H. perforatum*.

In order to examine the effects of zinc and iron nano-oxides on secondary metabolites production of *H. perforatum* cells cultures, we determined hypericin and hyperforin amounts in *H. perforatum* cell suspension culture treated with different concentrations of zinc and iron nano-oxides.

Materials and Methods

Plant material

Seeds of *Hypericum perforatum* L. were obtained from pakan seed company, Esfahan, Iran.

Callus and suspension culture initiation

Seeds were washed in running tap water and were surface sterilized using Sodium hypochlorite (5% v/v) for 20 min. Subsequently, they were washed 3 times with sterilized distilled water for 12 min. Surface sterilized seeds were placed on static MS [28]. Basal media supplemented (30 g/L) and Agar (7.5 g/L) for germination. The pH of the media was

adjusted to 5.8 before autoclaving at 121 °C for 15 min. Cultures were incubated at 25 ± 2 °C under 16 hours of photoperiod from cool white fluorescent tube giving 2400 lux at culture level.

Callus cultures were initiated by using stem segments from a 30-day- old plant grown *in vitro* and were subsequently cultured in media containing MS salts and vitamins supplemented with picloram (0.5 mg/L), N6-benzylaminopurine (BAP, 0.4 mg/L) and sucrose (30 g/L) (based on our unpublished data). Cultures were maintained under dark conditions at 23 °C (Fig. 1a).

Cell suspensions were established from callus cultures in the same medium and were maintained in 100 mL Erlenmeyer flasks with 30 mL of medium. The suspensions were shaken at 120 rpm in darkness and subcultured after 15 days in MS medium supplemented with picloram (0.5 mg/L), BAP (0.4 mg/ L) and sucrose (30 g/L) (15 mL packed cell volume to 15 mL fresh medium). These suspensions were shaken at 120 rpm in darkness and were kept for 15 days (Fig. 1b). Hypericin production was observed and recorded by microscopic investigations (Nikon Eclipse TE300, Japan) (Magnification= 40X) (Fig. 1c). Cell cultures (15-day old) were treated with four different concentrations (0, 50, 100 and 150 ppb/30 mL culture) of zinc and iron nano-oxides.

Preparation and addition of elicitor. Preparation of zinc nano-oxide suspension 1%.

A total of one mg of zinc nano-oxide powder (Plasma chem, Germany) was dissolved in 100 mL sterilized distilled water and sonicated (Misonix Co USA, Amplitude 70) for 30 min.

In this research, we have utilized stock iron nano-oxide suspension 5% (Plasma chem, Germany). The suspensions (zinc and iron nano- oxides) were sterilized by autoclaving at 121 °C for 15 min and used as an elicitor suspension. Fifteen days after initial subculture of cell suspensions, 1mL of elicitor suspensions with different concentrations (0, 50, 100 and 150 ppb/30mL culture) of zinc and iron nano-oxide were added to the cultures. The controls received an equivalent volume of culture media. The flasks were harvested 72 h after elicitation. This research was conducted as a factorial in a completely randomized design. Each experiment was accomplished with 3 replicates.

Extractions and quantification of hypericin and hyperforin

The fresh cells (0.2 g) were extracted using 9.8 mL methanol, containing 200 µl Pyridine and then sonicated in ice bath (Misonix Co USA, Amplitude 20) for 5 min. The extracts were centrifuged at 8000g for 15min [14].

The supernatants were further concentrated by freeze-drying (Model 25LL, Vir Tis, Genesis) and the powders were dissolved in 1 mL of methanol (Merck Co., Germany). Prior to HPLC (Knauer, USA) analysis, samples filtered through a 0.2 µm filter (Molinix, USA). Extracted samples (20 µl) were injected in to a TRACERE XCEL C_{18} column (5.0 µm; 4.6 mm × 150 mm) with a C_{18} guard column (10 mm × 4.6 mm). The mobile phase was ethyl acetate, 15.6 gl^{-1} solution of sodium dihydrogen phosphate, methanol (39:41:160 v/v/v) at a 1.0 mL /min flow rate. Hypericin and hyperforin were detected at 590 nm and 290 nm, respectively, utilizing an SPD-M10AV photodiode array detector. Standard curves were prepared by plotting the peak areas of standard concentrations of hypericin (0.416, 0.5, 0.625, 0.833, 1.25, 2.5 ppm) and hyperforin (1, 2, 4, 6, 8, 10 ppm); and linear regression equations ($R^2 > 0.99$) were obtained. Hypericin and hyperforin were quantified by referring peak areas (RT, retention time of 13 and 16 min, respectively) to standard curves.

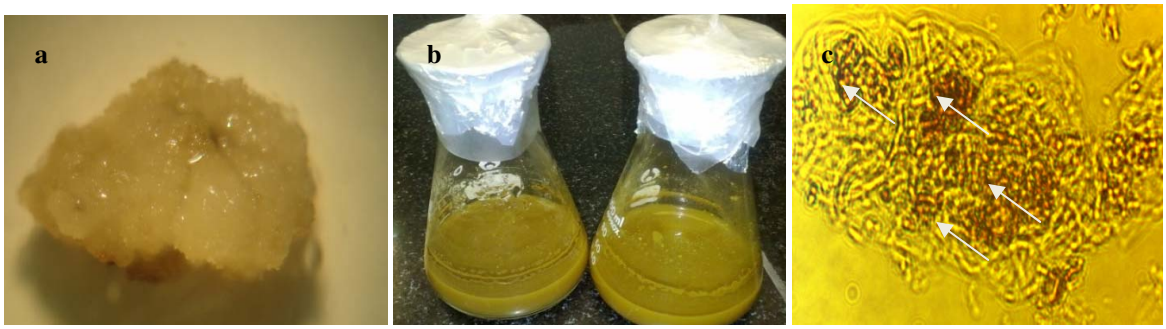

Fig. 1 Callus initiation from stem explants of *Hypericum perforatum* in media supplemented with picloram (0.5 mg/L) and N6-benzylaminopurine (BAP, 0.4 mg/L) (a). Cell suspension culture of *Hypericum perforatum* L. (b). Hypericin production in cell suspension culture of *Hypericum perforatum* L. (Magnification= 40X) (c).

Standards of hypericin and hyperforin were purchased from Sigma-Aldrich (Humburg, Germany).

Statistical analysis

The analysis of variance and duncan test (ά≤ 0.01) of mean comparison were performed using the SPSS software (version 15.0).

Results

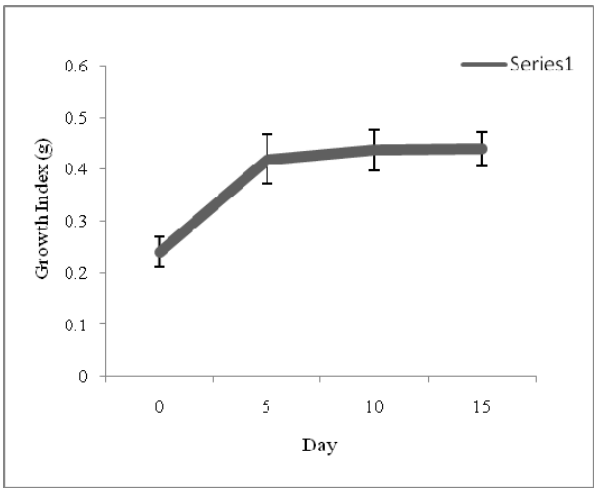

Fig. 2 Time-course for the growth (Dry Weight) of *H. perforatum* cell culture in MS medium. The cultures were harvested every five day for a 15- day culture period. Mean values marked with *different letters* are significantly different according Duncan□s multiple range test (p≤0.01).

H. perforatum cells growth

Hypericum perforatum cells showed a typical exponential growth curve during the culture period (Fig. 2).

The highest dry weight (DW) was obtained at the end of the period (10 and 15 days after culture) (0.437 and 0.438 g, respectively). Based on the obtained results, the 15 day old cell cultures were chosen for elicitation experiments.

Effects of different concentrations of zinc and iron nano-oxides

Hypericin and hyperforin accumulation were significantly increased respectively from 2.07 and 16.27 µg/g DW in the control to 7.87 and 217.45 µg/g DW, in *H. perforatum* cell suspension cultures treated with 100 ppb/30 mL culture zinc nano-oxide after 72h (Tables 1 and 2).

The superscript letters following the calculated means and standard deviations are an indication of similarity in the data. Values sharing a letter within a column are not significantly different at $p < 0.01$

In addition supplementation of 100 ppb iron nano-oxide to *H. perforatum* cell suspension cultures also caused an increase in hypericin and hyperforin production (11.18 and 195.62 µg/g DW, respectively) (Tables 3 and 4) (Fig.s 3 and 4). The effects of zinc and iron nano-oxides on dry weight of *H. perforatum* cell culture are presented in Fig. 5. Significant change in dry weight of treated and non-treated cell cultures was not detected.

After a slight dip in dry weight in media treated with 50 and 100 ppb/30 mL culture zinc and iron nano-oxides, there was a gradual increase in dry weight of cell cultures treated with 150 ppb zinc or iron nano-oxides but no marked change was observed in comparison to non-treated cell cultures.

Discussion

Hypericum perforatum L. is a well known medicinal plant [1]. Extract of *H. perforatum* is widely used to treat mild to moderate depression [2]. Numerous elicitors on hypericin and hyperforin production in cell cultures of *H. perforatum* have been tested. Iron and zinc nano oxides were used for the first time as elicitors. As previously reported, these results seem to be related to effects of elicitors on cell signaling pathway related to hypericin and hyperforin production in *H. perforatum* cell suspension.

Table 1 Analysis of variance for the effect of different concentrations of zinc nano oxide (0, 50, 100 and 150 ppb) on hypericin and hyperforin production and dry weight (g) in *Hypericum perforatum* cell suspension culture

Sourceof variation	Degreeof freedom	Mean of square		
		Hyperforin	Hypericin	Dry weight
Zincnano oxide	3	24548.345**	19.705**	0.003ns
Error	8	130.658	0.321	0.001

ns: no Significant,* Significant at p_0.05, ** Significant at p_0.01

Table 2 Hypericin and hyperforin content (µg/g DW) and dry weight (g) in zinc nano oxide-treated (50, 100 and 150 ppb) and non-treated (Control) cell suspension cultures of *Hypericum perforatum* 72h after elicitation. The Hypericin and Hyperforin were analyzed with HPLC. Data show means ± SD form triplicate experiments.

Zincnano oxide	Hyperforin	Hypericin	Dry weight
Control	16. 272^{c} ± 4.62	2.0723 ^{c} ± 0.77	0.840^{a} ± 0.02
50	49.786 ^{b} ± 0.89	3.182 ^{c} ± 0.33	0.780^{a} ± 0.03
100	217.45 ^{a} ± 4.18	7.873 ^{a} ± 0.50	0.790^{a} ± 0.02
150	136.33^{d} ± 21.97	٥٣٧.0^{b} ± 0.57	0.850^{a} ± 0.04

Table 3 Analysis of variance for the effect of different concentrations of iron nano oxide (0, 50, 100 and 150 ppb) on hypericin and hyperforin production and dry weight (g) in *Hypericum perforatum* cell suspension cultures

Sourceof variation	Degreeof freedom	Mean of square		
		Hyperforin	Hypericin	Dry weight
Ironnano oxide	3	17224.789**	56.816**	0.005*
Error	8	266.107	0٢.66	0.001

ns: no Significant,* Significant at p_0.05, ** Significant at p_0.01.

Table 4 Hypericin and Hyperforin content (µg/g DW) and dry weight (g) in iron nano oxide-treated (50, 100 and 150 ppb) and non-treated (Control) cell suspension cultures of *Hypericum perforatum* 72 h after elicitation. The Hypericin and Hyperforin were analyzed with HPLC. Data show means ± SD form triplicate experiments.

Iron nano oxide	Hyperforin	Hypericin	Dry weight
Control	16.272^{d} ± 4.62	2.072^{c} ± 0.77	0.840^{a} ± 0.02
50	64.674^{c} ± 30.55	1.856^{c} ± 0.10	0.750^{b} ± 0.05
100	195.62^{a} ± 10.00	11.181^{a} ± 0.17	0.820^{a} ± 0.03
150	89.306^{b} ± 3.06	4.571^{b} ± 0.51	0.830^{a} ± 0.02

The superscript letters following the calculated means and standard deviations are an indication of similarity in the data. Values sharing a letter within a column are not significantly different at $p < 0.01$

Walker *et al*. [12] reported significant increased in hypericin accumulation (0.35 mg/g DW) in *H. perforatum* cell suspension in response to jasmonic acid (250 µM). Gadzovska *et al*. [29] also reported enhanced production of hypericin in *H. perforatum* cell suspension (26 µg/g DW) in media supplemented with 250 µM Methyl jasmonate (MeJA). Liu *et al*. [30] observed that addition of L-tryptophan (50 mg/l) and MeJA (100 µM) in *H. perforatum* liquid cultures significantly stimulated hyperforin production (11 mg/g DW) (1.81-fold contorol). The results of our study showed that hypericin and hyperforin levels of the cells treated with zinc and iron nano-oxides are much higher than the control cells, suggesting that zinc and iron nano-oxides may trigger hypericin and hyperforin generation in the cultured cells. Therefore, zinc and iron are effective factors for hypericin and hyperforin production of *H. perforatum* cell cultures. Based on these observations, it seems that the zinc and iron nano-oxides had more effective on hyperforin production than hypericin cell suspension cultures. Jasmonate (JA) is an important plant stress hormone that induces various plant defense responses, including the biosynthesis of protective secondary metabolites [31]. Nano-particles may play an important role in signal transduction process that regulates jasmonate production genes in cells under treatment.

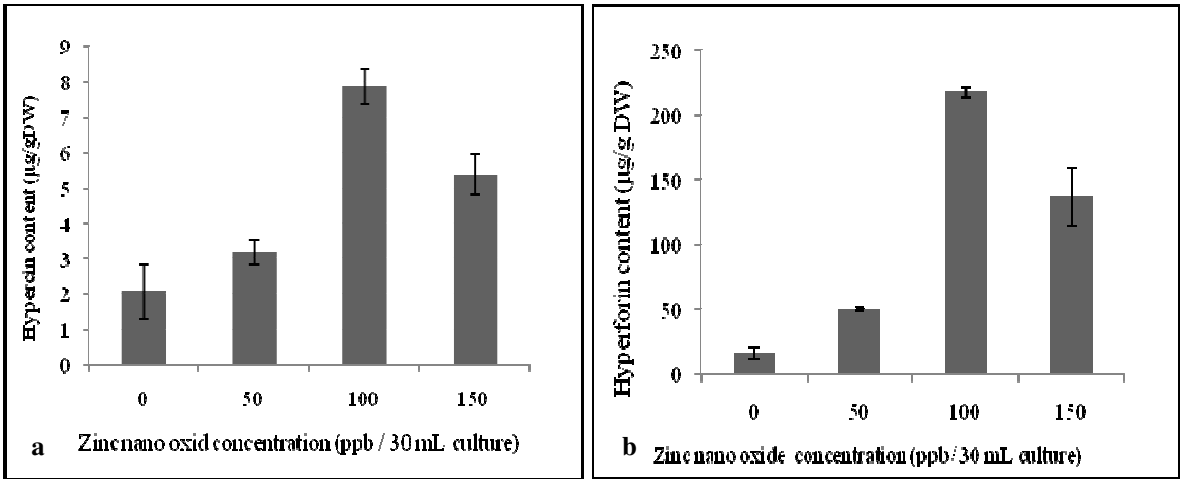

Fig. 3 Effects of zinc nano- oxide concentrations (0, 50, 100 and 150 ppb/30 mL culture) on hypericin (a) and hyperforin (b) content in cell cultures of *H. perforatum* 72 h after elicitation. The hypericin and hyperforin were analyzed with HPLC. Mean values marked with *different letters* are significantlydifferent according Duncan□s multiple range test (p≤0.01).

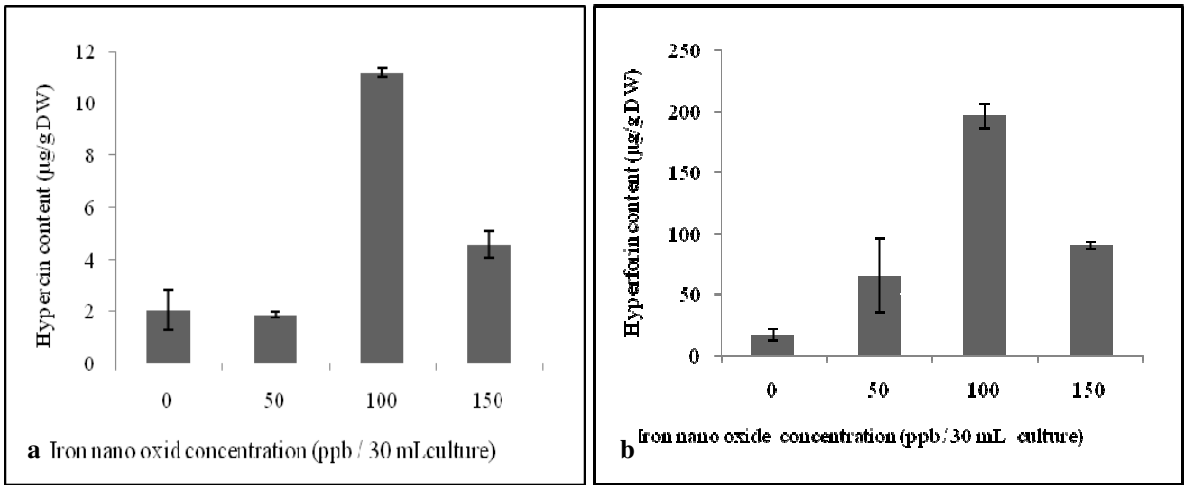

Fig. 4 Effects of iron nano- oxide concentrations (0, 50, 100 and 150 ppb/30 mL culture) on hypericin (a) and hyperforin (b) content in cell cultures of *H. perforatum* 72 h after elicitation. The hypericin and hyperforin were analyzed with HPLC. Mean values marked with *different letters* are significantly different according Duncan□s multiple range test (p≤0.01).

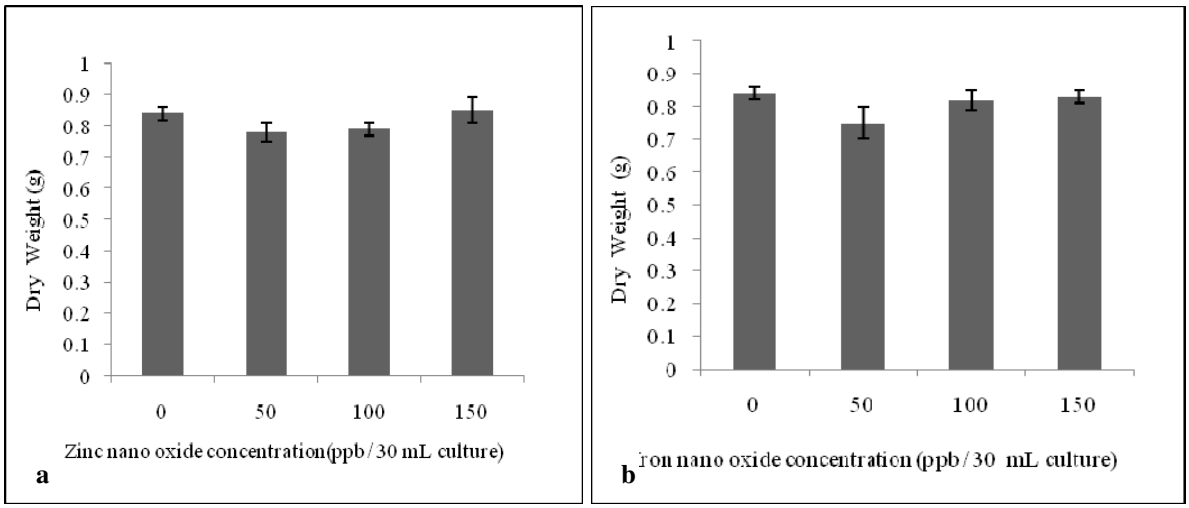

Fig. 5 Effects of zinc (a) and iron nano- oxides (b) concentrations (0, 50, 100 and 150 ppb/30 mL culture) on dry weight of *H. perforatum* cell suspension cultures 72 h after elicitation. Mean values marked with *different letters* are significantly different according Duncan□s multiple range test (p≤0.01).

Also may induce jasmonate production effective in hypericin and hyperforin production**.**

Plant cells wall act as a barrier for entry of any external materials including

nano-particles. With diameters less than the pore diameter of the cell wall, nano-particles can pass through and reach the plasma membrane. Using transport carrier proteins or ion channels, they may also cross the membrane. The nano-particles may bind with different organelles or interfere with the metabolic processes. Studies on the mechanism of uptake, transportation and binding sites of nono-particles in plant cells have also lead to more understanding of the elicitation evidences of these *in vitro* nano-particles for improving secondary metabolite production. However, higher concentrations of zinc and iron nano- oxides (150 ppb) had negative effects on hypericin and hyperforin production.

As it was pointed out, the toxic effects of higher concentrations of nano-particles have been reported by several researchers [26,32].

Pure alumina nano- particles without any modifications reduced root elongation in studied plants (*Zea mays, Cucumis sativa, Glycine max, Daucus carota, Brassica oleracea)* [32].

Zinc oxide nano-particles at high concentrations had toxic effects at high concentrations on rye grass growth [27]. Therefore, more research is needed to consider the toxic concentration of nano-particles upon their intake by cells and determination of new methods that reduce the phytotoxicity of nano-particles.

Conclusion

The present study reveals that zinc and iron nano-oxides were found to be suitable for the accumulation of hypericin and hyperforin. Therefore, the successful application of the nano particles offers as a new strategy for improving secondary metabolite production. Future research is needed to fully elucidate the effects of nano-particles on production mechanisms of secondary metabolites in medicinal plants.

Acknowledgments

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