Biocatalysis and Agricultural Biotechnology 16 (2018) 54–62

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Green synthesis of iron nanoparticles by Rosemary extract and cytotoxicity effect evaluation on cancer cell lines

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ARTICLE INFO

Keywords:
Rosemary
Green synthesize
MTT assay

ABSTRACT

Medicinal plants are of great importance in traditional medicine, in which in most part, the antioxidant activity of the plant-derived compounds is imagined responsible for treating various diseases. *Rosmarinus officinalis* L, contains several polyphenolic compounds with antioxidant activity; However, to elicit the anti-proliferative activity of *R. officinalis*, it is required to be improved *via* different strategies. Nowadays, green synthesis of metal nano- particles involving plant extract has attracted the attention of many researchers as this approach could help to derive the therapeutic benefits of the plant extracts. In this study, for the first time, the aqueous extract of rosemary was applied in green iron nanoparticle platform (Rosemary-FeNPs). Various methods, including dynamic light scattering(DLS), field emission scanning electron microscopy (FESEM), X-ray diffraction (XRD), Transmission electron microscopy (TEM), and Raman spectroscopy and Fourier Transform Infrared Spectroscopy (FTIR) were employed to characterize Rosemary-FeNPs. The mean size of the Rosemary-FeNPs were at about 100 nm with PDI of less than 0.12, which indicates a homogeneous size distribution of the nanoparticles. The cytotoxicity of Rosemary-FeNPs and total extract of rosemary was determined using MTT cytotoxic test on 4T1 and C26 cancer cell lines. The results showed that Rosemary-FeNPs could exert more cytotoxic effect than total extract on both cancer cell lines.

1. Introduction

Nanotechnology is mainly related to the synthesis of nanoparticles with variability in size, shapes, chemical compositions that can pose potential application in different purposes (Shahwan et al., 2011). Metal nanoparticles, such as iron nanoparticles (FeNPs), have been found to pose the widespread feasible application in biological and industrial fields such as diseases diagnosis and treatment, electrical components, and water decontamination (Zhang et al., 2011). Metal nanoparticles, with the desired characteristics such as size and shapes, are mainly synthesized by using various physical and chemical processes that include some important disadvantages such as high cost, labor-intensive, and being potentially hazardous to the environment and living organisms (Narayanan and Sakthivel, 2010; Gan et al., 2012). Therefore, an alternative, cost-effective, as well as safe and biocompatible method is obviously needed for nanoparticle production (Raveendran et al., 2003; Sharma et al., 2009), (Shah et al., 2014), (Zhang et al., 2011). The green synthesis of metal NPs using any secondary metabolic of the medicinal plants is quick and safe method to synthesize nanoparticles, in which plant extracts can act as both reducing and fixing agents (Oroojalian et al., 2017a). According to, chemically synthesized Fe NPs, although both share the similar degradation mechanisms, green synthesized Fe NPs showed more useful removal capability, and longevity due to the polyphenols or antioxidant in the tea extracts which protect the particles from oxidation and aggregation. Fe NPs have also been synthesized using *Terminalia chebula* aqueous extract (Kumar et al., 2013). Rosemary (*Rosmarinus officinalis* L.), belonging to Lamiaceae family, is a woody herb with fragrant needle-like leaves,which is a native plant species to the Mediterranean region (Bakırel et al., 2008). Various beneficial aspects of rosemary, including memory improvement, muscle pain and spasm relief, and hair growth stimulation, as well as antimicrobial and antioxidant properties have been frequently reported. Of the note, anticancer effects of rosemary on various cancer cells originated from skin, colon, prostate,

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https://doi.org/10.1016/j.bcab.2018.07.017
Received 10 January 2018; Received in revised form 12 July 2018; Accepted 12 July 2018

and breast are the most remarkable characteristic of the plant (Mirghaed and Yadollahi, 2013). Rosemary's anticancer properties are found to partly stem from the presence of high levels of flavonoid components such as carnosic acid, rosmarinic acid, and ursolic acid that founds in highest concentration. Carnosol is an ortho-diphenolic diterpene with an abietane carbon skeleton with hydroxyl groups (Yesil-Celiktas et al., 2010). The main anti-cancer effect of carnosol component is attributable to modulation of the cell cycle apoptotic related proteins of cancerous cells (Johnson, 2011). Rosemary extracts exhibited anti-proliferative activity in a number of different tumor cell lines (Kontogianni et al., 2013), a property which has been attributed with the presence of specific compounds, particularly carnosol, carnosic acid, rosmarinic acid (Johnson, 2011) which have been found to show antioxidant activity (Kontogianni et al., 2013). Carnosic acid inhibited proliferation of human myeloid leukemia cells without inducing apoptosis (Steiner et al., 2001). For first time, James et al., demonstrate that carnosol inhibits proliferation of the human colonic adenocarcinoma cell line Caco-2, and cause cell cycle arrest predominantly at G2/M phase (Visanji et al., 2006). Therefore, the present study was aimed to synthesize the iron nanoparticles using leaf extracts of *Rosmarinus officinalis,* and also characterize the synthesized nanoparticles by using Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscope (SEM), and X-ray Powder Diffraction (XRD). Breast cancer 4T1 and colon carcinoma C26 cell lines were also used to evaluate cytotoxic activity of the synthesized nanoparticles.

2. Material and methods

2.1. Preparation of Rosemary-FeNPs using rosemary extract

To prepare aqueous extract of rosemary (*Rosmarinus officinalis* L.), the leaves were collected locally from a botanical garden at Ferdowsi University of Mashhad, washed thoroughly in deionized water, dried, and finely crushed using a clean knife. Then, crushed leaves (60 g) were boiled in 1000 ml water with the concentration of 60 g/L at 60 °C for 1 h followed by vacuum-filtering, and storage at 4 °C for further use. To synthesis Iron nanoparticles, $FeSO_4$ was used as Fe precursor that was purchased from Sigma Chemicals. The above mentioned extracts were added to 0.1 M of $FeSO_4$ at volume ratio of (2:1) and incubated at room temperature. The immediate appearance of black color indicated the reduction of Fe^{2+} ions and indicated the formation of Fe nanoparticles.

2.2. Characterization of synthesized nanoparticles

Particle size, Zeta potential, and Polydispersity Index (PDI) were evaluated by a dynamic light scattering instrument (Nano-ZS; Malvern, UK). Transmission electron microscopy (TEM) Briefly,the samples were powder and fixed on cassette tape backing on metallic disks then covered with a thin, electric conductive gold film. Energy-dispersive spectrometer (EDS) analysis carried out to better understand the elemental composition of Rosemary-FeNPs (Weng et al., 2013). The images of samples were recorded at different magnifications at an operating voltage of 3 kV. Fourier Transform Infrared Spectroscopy (FTIR) measurements were also used to identify the possible biomolecules responsible for the reduction of the metal precursors and capping of Fe NPs (Weng et al., 2013). Raman spectroscopy (Avantes Company, Dutch) were recorded at room temperature by using 785 nm. The formation of iron nanoparticles using X-ray diffraction(XRD) method by Philips-X'Pert Pro MPD (Netherlands) with a high-power Cu-K X-ray source (= 0.154 nm) at 40 kV/40 mA and the pre and post patterns of reactions with rosemary extract were studied. All samples were scanned from 2θ∘ to 7θ∘ at a scanning rate of 2θ per min. The samples were repeatedly washed with ethanol prior to XRD analysis (Huang et al., 2014).

2.3. Culture medium and cell lines

C26 mouse colon cancer and 4T1 mouse breast cancer cell lines were supplied from the National Cell Bank of Pasture Institute, Tehran, Iran. Cells were cultured in RPMI Medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 2 mML-glutamine. Subsequently, the cell lines were grown at 37 °C in a humidified atmosphere containing 5% CO_2. All reagents and cell culture media were purchased from Gibco Company (Germany).

2.4. MTT assay

In order to study the anti-tumor activity of the nanoparticles, MTT assay using 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Sigma, USA) was done on C26 mouse colon cancer and 4T1 mouse breast cancer cell lines (Oroojalian et al., 2017b; Teymouri et al., 2015). In live cells, mitochondrial dehydrogenas enzymes can convert soluble yellow MTT to an insoluble purple formazan precipitate by cleavage of the tetrazolium ring. In order to perform the cytotoxicity assay, 100 µL of cells (5×10^4 cells.ml^{-1}) was seeded in 96 wells microplate and incubated for 24 h (37 °C, 5% CO_2 air humidified). After 24 h incubation,the 96 wells cellculture plates containing 100 µL of a cell suspension (250,000, cells/ml) were incubated with 100 µL of various iron nanoparticles concentrations from 4×10^{-15} to 4 × 10 µg/ml. The assay was performed in triplicate. After 48 h of incubation, cells were washed twice with phosphate buffered saline (PBS) and MTT (0.5 mg/ml PBS) was added to each well and incubated at 37 °C for 3 h. Finally, the formed formazan crystals were dissolved by adding dimethyl sulfoxide (DMSO) (100 µL/well), and the absorbance was read at 570 nm using a micro plate scanning spectrophotometer (OrganonTeknika, Netherlands) (Oroojalian et al., 2017c). Results were generated from three independent experiments and each experiment was performed in triplicate. IC_{50} (concentration that inhibits cell growth by 50%) values of the compounds against each tested cell line was calculated using nonlinear regression of concentration-response curves.

2.5. Statistical analysis

Data were analyzed using GraphPad Prism version 5 (GraphPadSoftware, San Diego, CA). Descriptive statistics, One-way ANOVA and Kruskal–Wallis tests were used to observe the statistical differences between groups. The average is shown as the mean ± standard deviation (SD). Values of $p \leq 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Characterizations of Rosemary-FeNPs

The color of Rosemary-FeNPs formulation was observed to be changed from yellowish brown to final dark blue color which is due to reduction of $FeSO_4$ and formation of FeNPs (Shah et al., 2014). Rosemary leaves extract has been found to mainly contain phenolic acids, carnosol derivatives, and flavonoids. The presence of these secondary metabolites can act as a capping agent with dark shades on the surface of nanoparticles, inhibiting nanoparticles from aggregation (Shah et al., 2014). As shown in the Table 1, average sizes (mean ± SD) of the Rosemary FeNPs were approximately 50 nm, revealed the successful synthesis of nanoparticles. Wang et al. concluded that the reactivity of green nanoparticles is directly related to their size, in which smaller size leads to higher reactivity (Wang et al., 2009). The PDI was found to be less than 0.14, indicating a homogeneous population of nanoparticles. Zeta potential, as shown in Fig. 1. an indicator of physical stability, was −19.03 ± 1.40. Surface charge is usually appeared after