



Antitumor efficacy of biosynthesized manganese nanoparticles

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Abstract

This study aimed to evaluate the *in vitro* and *in vivo* antitumor potential of manganese nanoparticles, in management of hepatocellular carcinoma (HCC) induced in rats. Manganese nanoparticles were biosynthesized using *Lactobacillus helveticus* cells. *In vitro* study of manganese nanoparticles on HepG-2 (human cell line of a well-differentiated HCC) revealed an IC₅₀ of 21.5 mM. The *in vivo* efficacy of manganese nanoparticles was evaluated by measuring the antioxidant activities against oxidative stress caused by diethylnitrosamine (DEN) in rat liver tissues. This treatment significantly improved the alanine aminotransferase (ALT) activity and total protein compared to DEN group. Results showed that manganese nanoparticles were effective in treatment of HCC induced by diethylnitrosamine (DEN) in rats. So manganese nanoparticles can serve as a good therapeutic agent for the treatment of hepatocellular carcinoma, and deserve further studies in the future.

Keywords: Hepatocellular carcinoma, Diethylnitrosamine, Manganese nanoparticles, Antitumor potential

1. Introduction

The liver is an essential organ with central functions in metabolic homeostasis; manufacturing triglycerides and cholesterol; glycogen synthesis; bile production; detoxification and immunity (Fox and Judith, 1976).

According to Kudo and Masatoshi, (2015), hepatocellular carcinoma (HCC) is the fifth most common cancer, and is one of the leading causes of death worldwide. This might be attributed to age;

gender; hepatitis B and C; alcohol consumption; hormone exposure; water pollutants, and parasitic infections including clonorchiasis and schistosomiasis, all of which result in liver damage or cirrhosis. In Egypt, HCC is the second most common cancer in men and the 6th in women (Elghazaly *et al.*, 2018).

Diethylnitrosamine (DEN) also known as N-nitrosodiethylamine; is widely used as a carcinogenic reagent, that is present in tobacco smoke; water, cured

and fried meals, primarily cooked bacon; beer, cheddar cheese, non-fat dry milk, fisheries agricultural chemicals, cosmetics and pharmaceutical products (Healy *et al.*, 2015). DEN is a strong hepatocarcinogenic substance, which is known to cause perturbations in the nuclear enzymes involved in DNA repair/ replication. It is normally used as a carcinogen to induce liver cancer; through generating reactive oxygen species (ROS) causing oxidative stresses, thus leading to carcinogenesis (Alyamkina *et al.*, 2010; Zeng *et al.*, 2015).

Nanoparticles (NP) are defined as particulate solid particles with a size range of 10-100 nm; have considerably unique chemical, physical, and biological properties, compared to bulk particles of the same chemical composition. This is attributed to their high surface-to-volume ratio, thus allowing them to interact easily with other particles (Pantidos *et al.*, 2014; Sahoo *et al.*, 2017).

Makarov *et al.*, (2014); Iravani, (2014); Duan *et al.*, (2015); reported that as eco-friendly alternatives of using chemical and physical synthesis of NP, biological methods using microorganisms; enzymes, and plants or plant extracts, have shown the ability to synthesize metallic NP.

Manganese (Mn) is a transition metal which is necessary in trace amounts for life processes; however it is toxic at higher doses. Manganese is also an important component of the antioxidant enzyme; manganese superoxide dismutase (MnSOD), present in the cell mitochondria. This enzyme is deficient in a number of cancer cells. MnSOD mitigates oxidative damage from reactive oxygen species, one pathway thought to participate in carcinogenesis. Manganese nanoparticles owing to their variance in morphology; structure, and oxidation states has been reported to cause these variants cancerous cells to undergo distinct oxidation or reduction reactions (Storz and Peter, 2007; Kipp *et al.*, 2017). This study aimed to evaluate the *in vitro* and *in vivo* antitumor activity of Mn NPs in management of HCC induced in rats.

2. Material and methods

2.1. Chemicals

DEN was purchased from Sigma-Aldrich Corporation (USA); Tetrahydromanganese Chloride ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$) was purchased from LOBA Chemie (India) dissolved in deionized water, and used in the preparation of Mn NPs.

2.2. Microorganism and culture conditions

Lactobacillus helveticus ATCC 7995 bacterium used in this study was obtained from the Microbiological Resources Center (Cairo, Egypt). It was cultured in de man, rogosa and sharpe (MRS) broth/ agar medium following the ATCC standard microbiological procedures in the laboratory, then culture was shaken at 150 rpm /min. for 12 h at 30°C. This culture was stored at -25°C in 20 % sterile glycerol, and liquid MRS (Oxoid Ltd, Basingstoke, UK).

2.3. Cell line

HepG2 cells (human cell line of a well-differentiated HCC) was obtained from Al-Azhar University, The Regional Center for Mycology & Biotechnology, Cairo, Egypt.

2.4. Experimental animals

About 60 adult male Swiss albino rats (150–200 g) were obtained from the animal farm of the Egyptian Holding Company for Biological Products and Vaccines (VACSERA), Cairo, Egypt. They were maintained on a standard pellet diet and tap water. These animals were housed in suitable cages under conditioned atmosphere (20°C-22°C), and kept on a standard diet.

2.5. Biosynthesis of Mn NPs

Mn NPs was green synthesized using *L. helveticus* extracellular metabolites according to the method of Das *et al.*, 2014).

L. helveticus was inoculated into sterile Erlenmeyer flask containing 100 ml sterile nutrient broth. The inoculated flasks were incubated on a rotating shaker at 200 rpm/ min. at room temperature. After incubation for 48 h, this culture was centrifuged at 6,000 rpm/ min. for 10 min. The supernatant was separated from the biomass and used for the biosynthesis of Mn NPs. The supernatant was used for studying the extracellular synthesis of Mn NPs by mixing it with filter sterilized manganese chloride solution at a final concentration of 1 mM. All the reaction mixtures were then incubated on a rotating shaker (200 rpm/ min.) at room temperature for 72 h. Visual observation was conducted periodically to check for the formation of NPs. Afterwards, characterization of these synthesized Mn NPs was carried out.

The resulting aqueous solution (the supernatant and manganese chloride solution) was filtered through a 0.22 µm Millipore filter before use.

2.6. Characterization of the synthesized Mn NPs

The recovered Mn NP's were characterized using the Transmission electron microscopy (TEM, joel, jem-2100, Japan; with an accelerating voltage of 200 kv), to assess their size and morphology. NPs were suspended in water, and one drop of this suspension was placed on a carbon –coated copper TEM grid, and then evaporated at room temperature. The Mn NPs were characterized using "advanced microscopy techniques" software, for the digital TEM camera calibrated for Mn NP size measurements. For size measurement, 100 NPs were calculated from random fields of view, and then images showing the general morphology of the Mn NPs were observed.

Ultraviolet-visible absorption of Mn NPs spectrum was analyzed using Ultraviolet-visible (UV-vis) spectrophotometer (Jenway UV, spectrophotometer model 6505) at range of 200-600 nm.

2.7. Biochemical studies

Evaluation of therapeutic *in vivo* and *in vitro* antitumor efficacy of Mn NPs was carried out as follows:

2.7.1. *In vitro* study

HepG2 cells (human cell line of a well-differentiated HCC) obtained from Al-Azhar University, The Regional Center for Mycology & Biotechnology, Cairo, Egypt, were used to determine the *in vitro* cell's cytotoxic effect of the tested Mn NPs using the crystal violet cytotoxicity assay according to Vijayan *et al.*, (2004).

2.7.2. *In vivo* studies

In screening drugs, determination of LD₅₀ using experimental animals is usually an initial step in the assessment and evaluation of the toxic characteristics of a substance. The LD₅₀ of Mn NPs was determined as described by Akhila *et al.*, (2007).

2.7.2.1. Experimental design

Animals were allowed for adaptation for 10 days. They were then randomly distributed into four equal groups, of about 15 rats each. These animal groups were categorized as follows:

1. Group 1 (Control): Normal healthy control animals.
2. Group 2 (Mn NPs): Animals were injected with Mn NPs (20 mg/ kg body weight/ day) for 6 weeks.
3. Group 3 (DEN): Each animal was injected with DEN (dissolved in 0.9% normal saline); in a dose of 20 mg/ kg, 5 times a week for 6 weeks, according to the modified method of Darwish *et al.*, (2013).
4. Group 4 (DEN + Mn NPs): Rats received DEN as in group 3; and then treated with Mn NPs for 6 weeks as in group 2, after induction.

2.7.2.2. Collection of samples

At the end of the treatment period, animals were fasted overnight prior to dissection under light ether anesthesia. Blood was drawn from the vena cava and centrifuged at 3000 g for 10 min.

2.7.2.3. Experimental parameters tested

Lipid peroxidation (malonaldehyde) was measured in liver tissue as described by Ohkawa *et al.*, (1979); whereas, superoxide dismutase activity was checked according to Nishikimi *et al.* (1972). Total protein level, and alanine aminotransferase (ALT) activity in serum were determined using KINITIK assays (Biosystem diagnostic kits) described by Gella *et al.*, (1985).

2.8. Statistical analysis

All mean values were reported as the mean ± standard deviation (SD). Data were analyzed using a one-way analysis of variance (ANOVA). The level of significance between mean values was set at $p < 0.05$ and $p < 0.01$ (significant and highly significant, respectively). All statistical analyses were performed using SPSS software (version 22.0).

3. Results

3.1. Characterization of Mn NPs

The obtained NP's were characterized by transmission electron microscopy (TEM; Fig. 1). The Mn NP's had spherical shape, and their sizes ranged between 32.67-190.1 nm.

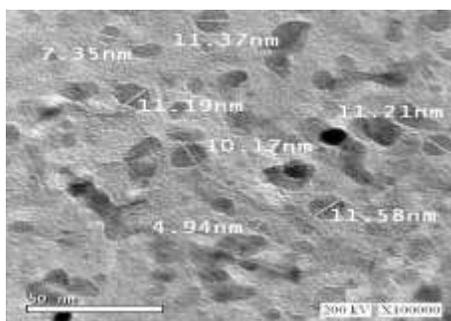


Fig. 1: Determination of size and shape of Mn NPs suspension using Transmission electron microscope (TEM)

3.2. Ultraviolet-visible (UV-vis) absorption spectra

(UV-vis) spectra of biosynthesized Mn NP's showed an absorption peak at 265 nm (Fig. 2).

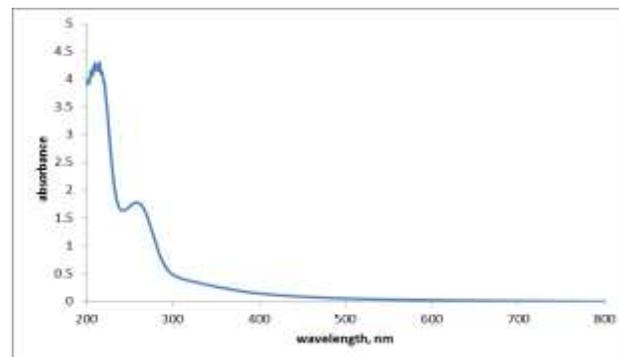


Fig. 2: Ultraviolet-visible (UV-vis) absorption spectrum of Mn NPs

3.3. In vitro studies

The antitumor potency of Mn NPs was evaluated *in vitro* using HepG2 cell lines. NPs were applied at different concentrations and results were presented in Table 1, Fig. 3. The assay was carried out twice.

Table 1: Cytotoxic activity of different concentrations of Mn NPs on HepG2 cell line

Sample conc. (mM)	Viability %
100	24.59
50	39.74
25	76.42
12.5	89.31
6.25	96.25
3.125	99.17
0	100.00

-Results were averages of 3 replicates.

3.4. In vivo studies

3.4.1. Determination of liver superoxide dismutase and malonaldehyde levels

Data presented in Table 2 indicated that liver superoxide dismutase (SOD) was significantly

decreased in DEN rats, compared with control animals. Moreover; DEN groups treated with Mn NPs resulted in significant increase in antioxidants levels, compared to DEN group. DEN animals also showed appreciable increase in malonaldehyde (MDA) level than normal rats. However; rats treated with Mn NPs presented significant decrease in MDA level compared with those treated with DEN only.

3.4.2. Determination of liver function tests in the different groups of treated rats

Data presented in Table 3 indicated that liver function tests ALT (U\l) was significantly increased in DEN rats, compared with control animals. Moreover; DEN groups treated with Mn NPs resulted in significant decreased compared to DEN group. Also total protein (g\ dl) test was significantly decreased in DEN rats, compared with control animals. Moreover; DEN groups treated with Mn NPs resulted in significant increased compared to DEN group.

Table 2. Liver antioxidant levels in the different groups of treated rats

Parameters	Control	Mn NPs	DEN	Mn NPs +DEN
SOD (U\ g tissue)	2.72 ± 0.23 ^b	2.95 ± 0.09 ^b	0.53 ± 0.01 ^a	1.25 ± 0.2 ^{ab}
MDA (mmole\ g tissue)	1.02 ± 0.04 ^b	1.20 ± 0.18 ^b	15.15 ± 1.41 ^a	4.73 ± 0.95 ^{ab}

-Where; SOD: superoxide dismutase; DEN: diethylnitrosamine; MDA: malonaldehyde. According to ANOVA analysis; each value was represented as mean ± SD (standard deviation). Data with different superscripts were significantly different at $p \leq 0.001$. ^aSignificance versus control group, ^bSignificance versus DEN group.

Table 3. Liver and kidney function tests in the different groups of treated rats

Parameters	Control	Mn NPs	DEN	Mn NPs +DEN
ALT (U\l)	21.07 ± 0.82 ^b	32.52 ± 0.46 ^{ab}	78.01 ± 1.46 ^a	33.66 ± 0.87 ^{ab}
Protein (g\ dl)	8.15 ± 0.019 ^b	7.67 ± 0.046 ^{ab}	4.47 ± 0.13 ^a	6.19 ± 0.04 ^{ab}

-Where; ALT: alanine aminotransferase; DEN: diethylnitrosamine. According to ANOVA analysis; each value was represented as mean ± SD (Standard deviation). Data with different superscripts were significantly different at $p \leq 0.001$. ^aSignificance versus control group, ^bSignificance versus DEN group.

4. Discussion

Guided treatments with NP are a new approach in cancer therapy. Silpa, (2016) pointed that NPs possess the ability to penetrate the cells efficiently; due to their small size, which facilitates *in vivo* activity and distribution. Previous study of Shah *et al.*, (2015) reported that biologically green methods of synthesizing NP's using microorganisms and plants are safe, inexpensive, and an ecofriendly alternatives.

In the current study; the biologically synthesized Mn NPs were characterized via several analytical methods such as; TEM, DLS, and UV-vis absorbance spectroscopy. Biologically synthesized Mn NPs using bacterial extracellular metabolites produced NP's of diameter size ranging from 32.67-190.1 nm. In addition, UV-vis spectroscopy showed that the absorption spectra for Mn NPs were observed at 260 nm. NPs of diameter less than 100 nm is useful for biological applications. Particle size is an important property; which may influence the biological activity of NP's, and has been suggested as a key factor in the interaction with charged surfaces.

This study was conducted to evaluate the efficiency of biologically synthesized Mn NPs for the treatment of HCC. Accordingly; the cytotoxic effects and the biological activity of Mn NPs as antitumor agent was examined *in vitro* against human liver carcinoma cell line (HepG2), using crystal violet cytotoxicity assay. Results showed potent activity of Mn NPs, where increasing their concentration resulted in an increased percentage of dead carcinoma cells. These results were in accordance with previous studies of Braydich *et al.*, (2005); Jeng and Swanson, (2006); Ngwa *et al.*, (2011), which reported that Mn NPs inhibit cell proliferation via induction of apoptotic cell death.

Mn is a transition metal; which plays an important role in a number of physiological processes, by serving as a constituent of some enzymes and an activator of others involved in the regulation of; amino acids, proteins, lipids, and carbohydrates metabolism (Zheng *et al.*, 2011). According to Baxter *et al.*, (2014); Cooke *et al.*, (2015), Mn superoxide dismutase (MnSOD) destroys the highly reactive superoxide radical by

converting it into the less reactive hydrogen peroxide (H₂O₂), that can be destroyed by catalase or glutathione peroxidase reactions.

In this study, injection of DEN into male albino rats induced significant deleterious changes in the antioxidant status. Results revealed a marked depletion in SOD activity in liver tissue; with a significant increase in MDA level, compared with the control group.

MDA; is a product of lipid peroxidation and degradation of polyunsaturated fatty acid metabolism, has been established as a mutagenic and carcinogenic entity (Sayed-Ahmed *et al.*, 2010; Ohno *et al.*, 2015). Lowering of MDA level and the increase in levels of SOD in DEN rats treated with Mn NPs; indicated that MDA acted as inhibitor of DEN-induced intracellular oxidative stress; moreover, Mn NPs removed the oxidative stress induced by DEN.

The present biochemical results revealed a decrease in total protein level; whereas, significant increase in ALT activity in DEN group was observed, compared with control rats. This might be attributed to DEN induced oxidative stress, which caused liver tissue damage and impairment of its function. Current results were in accordance with similar previous studies of Kartik *et al.*, (2010); Faten *et al.*, (2014).

In the current study, we observed that post treatment of DEN rat animals with Mn NPs resulted in improvement in liver function tests compared to DEN rats, similar to previous studies of Chen *et al.*, (2012); Abbas *et al.*, (2014).

Conclusion

From the aforementioned results; it can be concluded that treatment with Mn NPs ameliorated the measured parameters in treatment of HCC, induced in rat animals by DEN. These findings were well appreciated by histo-pathological studies; suggesting that Mn NPs can serve as a good

therapeutic agent for the treatment of HCC, and should attract further studies.

Conflict of interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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