RESEARCH ARTICLE

Comparison of the effect of Fe_3O_4 nanoparticles synthesized by green and chemical methods on liver function as well as oxidative stress and metal regulation genes expression in rats

Farzaneh Karimi[1], Kahin Shahanipour[1], Nooshin Naghsh[2], Noosha Zia-Jahromi[3]*

[1] *Department of Biochemistry, Falavarjan Branch, Islamic Azad University, Isfahan, Iran.*

[2] *Department of Biology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran.*

[3] *Department of Biology, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.*

ARTICLE INFO

Article History:

Received 2024-10-31
Accepted 2024-12-09
Published 2024-02-15

Keywords:

Fe_3O_4 nanoparticles,

MT1,

GR,

Gene expression,

Metabolism.

ABSTRACT

In biomedicine, magnetic nanoparticles (MNPs) have been used for the treatment of numerous disorders and targeted drug delivery. Among iron oxides, Fe_3O_4 can be useful in effective drug delivery to target tissues such as the liver. In this study, the side effects of Fe_3O_4 nanoparticles synthesized by the green method were compared to chemical methods in liver tissue. Gene expression analysis of metallothionein-1 and glutathione reductase-1, in addition to liver biochemical function was measured. Forty-two rats were studied in 7 groups. A control-l group with standard food (N = 6) and six treatment groups were administered 50, 100, and 150 mg/kg Fe_3O_4 nanoparticles synthesized by green and chemical methods, respectively (N = 6 for every treatment group). The destruction of liver tissue was more in the groups treated with chemicals compared to the groups treated with green synthesis nanoparticles. Also, biochemical analysis presented significant alterations in *SGPT* level in the treated groups, however, no significant finding was observed in the level of SGOT and ALK levels. Tea nanoparticles showed a significant increase in the expression level of MT1 and GR genes in the treated groups. The findings showed that the nanoparticles prepared by chemical method caused more damage to the liver tissue than the green nanoparticles, and the changes in the expression of genes involved in homeostasis in the groups treated with green synthetic nanoparticles were significantly positive.

How to cite this article
Karimi F., Shahanipour K., Naghsh N., Zia-Jahromi N., Comparison of the effect of Fe_3O_4 nanoparticles synthesized by green and chemical methods on liver function as well as oxidative stress and metal regulation genes expression in rats. J. Nanoanalysis., 2024; 11(1): 656-661.

*Corresponding Author Email: *shahanipur_k@yahoo.com*

INTRODUCTION

Green synthesis nanotechnology refers to the production of nanomaterials or nanoparticles without using dangerous chemicals that emit poisonous byproducts. Green synthesized nanoparticles have many applications in medical science, drug delivery, and chemotherapy. Many strategies have been involved in the process of their synthesis to be safe and without side effects [1,2].

The goal of creating metal oxide nanoparticles is to change the properties of metals and increase their reactivity and efficiency. Iron oxide, aluminum oxide, cerium oxide, titanium oxide, and magnetite are examples of the several utilized metal oxide nanoparticles [3]. In biomedicine, magnetic nanoparticles (MNPs) have been used to induce heat to treat hyperthermia, provide contrast effects for magnetic resonance imaging, and control targeted drug delivery remotely. Numerous iron oxides are known, which usually categorized in to three types: Fe_3O_4 (magnetite), α-Fe_3O_4 (hematite), and γ-Fe_3O_4 (maghemite). Among all iron oxides, Fe_3O_4, due to superior magnetic properties, has attracted more attention [4]. Based on their employment, Fe_3O_4 nanoparticles appear to have more excellent capability and capacity than Fe_3O_4 nanoparticles [1].

Nanoparticle size and surface charge determine its biological properties, such as absorption by cells and distribution in the body [5]. The nanoparticles deposit in essential organs like the brain, liver, or kidneys after entering the body by ingestion, inhalation, contact with the skin, or interaction with the genitourinary tract. Nanoparticles impair cellular activities by crossing membranes and interacting with biomolecules, damaging DNA and proteins or crossing the blood-brain barrier, causing neurological damage. Therefore, the need for primary research on nanoparticles' effect on biochemical parameters is emphasized [6]. This study focused on *MT1* and *GSR* genes as two appropriate markers for analyzing oxidative stress and tissue resistance to metals. Invertebrates, plants, bacteria, and vertebrates all have low-molecular-weight, cysteine-rich intracellular proteins called metallothioneins (MTs). The metallothionein molecules' α and β binding domains are made up of cysteine clusters. The peptide N-terminal region, known as the β-domain, has three binding sites for two-valent ions. Also, the C-terminal area (α-domain) can bind to four two-valent metal ions. Many organs contain *MT1* and *MT2*, including the kidneys, liver, gastrointestinal tract, and pancreas. The brain, cardiovascular system, retina, kidneys, breasts, prostate, bladder, and genital organs contain *MT3*. On the other hand, *MT4* is mainly found in squamous epithelium stratified [7,8]. These molecules can also help modulate and improve immune responses by controlling the levels of toxic metals [9]. MTs elevate the tolerance of the cells to the radical species. The liver is the main organ of the body with antioxidant weapons. Liver cells use glutathione as the most critical antioxidant in mammals that directly eliminates various oxidants, including (superoxide anion, hydroxyl radical, nitric oxide, and carbon radicals) [10,11]. An NADPH-dependent oxidoreductase called glutathione reductase (GR) expedites the transformation of oxidized glutathione (GSSG) to reduced glutathione (GSH) [12,13]. Biochemically, oxidation products are widely used to measure oxidative stress [14]. Metalloenzymatic alkaline phosphatase (ALP) [EC 3.1.3.1] consists of several isoenzymes. It is said that each isozyme evolves from a common ancestral gene and is a membrane-bound glycoprotein encoded by particular locus genes. Chromosome 2 harbors three ALP genes, while chromosome 1 harbors the fourth [15]. Therefore, in this study, by synthesizing nanoparticles using the green method, its effects on liver enzymes, liver tissue, and

antioxidant genes were investigated, and we compared the results with the effects of nanoparticles synthesized by chemical methods.

EXPERIMENTAL

Material and methods

Synthesis of green Fe_3O_4 nanoparticles

Green method

Green tea was utilized to make Fe_3O_4 nanoparticles. Green tea extract was added dropwise to a room-temperature 1:2 iron chloride solution. The creation of iron oxide nanoparticles was verified by developing a solid, black solution after the mixture had been agitated with a magnetic stirrer for 15 minutes. Centrifugation was used to separate the nanoparticles at 15,000 rpm for 10 minutes, followed by three to four rounds of washing with ethanol and water. The nanoparticles are dried in a 70 °C oven for 3 hours and kept in a covered container [16].

Chemical method

$FeCl_2$ $4H_2O$ (1.98 g), $FeCl_3$ $6H_2O$ (5.41 g), and NaOH (3.2 g) were dissolved in 100 ml of distilled water for the production of Fe_3O_4 nanoparticles. The reaction temperature was increased to 80°C and maintained there for two hours after stirring for 20 minutes. A magnetic decantation process was used to separate the resultant black precipitate, which was then washed three times with distilled water and ethanol. Magnetic nanoparticles were dried out under low pressure. The supplies were all purchased from Sigma Company (USA).

Chemical Fe_3O_4 coating with chitosan

To prepare chitosan solution, 1.74 ml of acetic acid (99%) was added to 300 ml of deionized distilled water. Then 0.125 g of chitosan powder was weighed and 300 ml of solution was added to it and stirred for 20 minutes with a magnetic stirrer until the powder was dissolved in the solution. 20 ml of this solution was removed and its pH was adjusted to 4.8 with NaCl (1 M). Then weigh 0.5 g of Fe_3O_4 that we produced by chemical method and add it to it and mix it for 30-45 minutes. With this method, we coated the Fe_3O_4 nanoparticle which was chemically synthesized with chitosan.

Particle size, PDI, and zeta potential

Dynamic light scattering (DLS) was used to gauge the size of nanoparticles. The magnetically created nanoparticles zeta potential, polydispersity index, and particle size were measured using the Zeta-sizer (Nano ZA, Malvern Instruments, UK). The temperature was 25 °C, the detecting angle was 135°, and the number of captured nanoparticles ranged from 1400 to 1930 kilo counts per second (KCPS). The Zetasizer used the same method to calculate zeta potential (Nano ZA, Malvern Instruments, and UK).

Animal treatments

In the present study, 42 male Wistar rats with an average weigh 250 ± 15.34 g and with ages of 8 to 9 weeks were involved in the study, including seven groups: 1-Control (sterile saline solution), 2- Fe_3O_4 nanoparticles made by green method at a dose of 50 mg/kg, 3- Fe_3O_4 nanoparticles made by the green way at a dose of 100 mg/kg, 4- Fe_3O_4 nanoparticles made by green method at a dose of 150 mg/kg, 5- Chemically synthesized Fe_3O_4 nanoparticles at a dose of 50 mg/kg, 6- Chemically synthesized Fe_3o_4 nanoparticles at a dose of 100 mg/kg and 7- Chemically synthesized Fe_3o_4 nanoparticles at a dose of 150 mg/kg. Animals were maintained on a 12-hour light cycle at room temperature and controlled humidity. Animals had unrestricted access to standard laboratory water and nutrition. Sterile saline-soluble iron nanoparticles were injected intraperitoneally (IP) daily for 30 days. Our study protocol was reviewed and approved by Najafabad Branch, Islamic Azad University Ethics and

Laboratory Animal Rights Monitoring Committee with the ethics code IR.IAU.NAJAFABAD.REC.1400.070.

Liver tissue biopsy, staining, and blood sampling

24 hours after discontinuation of the last treatment, rats were anesthetized with 10% ketamine and 2% xylazine. Blood samples were taken by heart perforation in sterile centrifuge bottles and rotated for 5 minutes to separate the serum. A part of the liver of each animal was removed and saved at - 80 ° C for RNA extraction and gene expression analysis. H&E staining was utilized to assess the histological shifts of rat liver tissues by a microscopic analysis based on the vain and cells morphological maintenance.

Biochemical assays

Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) are determined using the Abcam commercial kits (Cat. No. ab105135 and ab105134 respectively. Abcam Co. USA). Alkaline phosphatase (ALP) was measured using a commercial kit from Randox.

Gene expression analysis

RNA extraction and cDNA synthesis

According to the manufacturer's instructions, RNA was extracted from liver tissues using RNXplus buffer (Cinaclon Co., Iran). The first strand of cDNA was then produced with 50 ng total RNA using oligo primers (dT) and random hexamer as directed by the manufacturer using a cDNA synthesis kit (SmoBio Co., South Korea).

Primer design and real time RT-PCR

Dedicated primers for *GR* and *MT1* genes were designed utilizing Generunner software version 6.5.52, 64-bit (Table 1). Also, *ActB* gene was selected as reference gene or internal control. Realtime-RT-PCR was performed using ABI system StepOne (Applied Biosystem, USA) in final reaction mixture volume 20 µl containing 1X Master Mix, 3.5 µl of DEPC water, 0.5 µl of each of the forward and reverse primers, and 0.5 µl of template cDNA. All reactions were performed in duplicate well in 40 cycles containing 95ºC for 15 sec, 55 ºC for 30 sec, and 72 ºC for 30 sec. Also, melting curve was calculated between 65 ºC and 95 ºC with ramping rate 0.3 ºC/1min.

Data analysis

Data were provided as mean ± SEM. SPSS V.21 software was utilized to perform a one-way analysis of variance (one-way ANOVA) on the data. At $P < 0.05$, the mean values were considered statistically significant. For data normality analysis, the Kolmogorov-Smirnov test was used. Finally, Prism V.9.0.0 was utilized for graph preparation.

Table 1. Primer sequences and their properties.

Primer name	Sequence	Target length	Target gene	Accession no.
FACTBRat	5’- AGCCTTCCTTCCTGGGTATGG-3’	109bp	*ActB*	NM_031144.3
RACTBRat	5’ AGCACTGTGTTGGCATAGAGG-3’			
FMT1rat	5'-GCTCCAGCGGCTGCAAGAAC-3'	129bp	*MT1*	NM_138826.4
RMT1rat	5'-AGCACGTGCACTTGTCCGAG-3'			
FGSRrat	5'- CACTGAAGATGAAGCCGTC-3'	124bp	*GR*	NM_053906.2
RGSRrat	5'- ACAAACCATCTTCATCACGC-3'			

RESULTS AND DISCUSSIONS

Nanoparticle properties

Table 2 shows some specific properties of the produced nanoparticles. Results of the particle analysis confirmed the standard indexes for a nanoparticle. Figs. 1a and 1b shows SEM image of green and chemical nanoparticles respectively. Figs. 2a and 2b show FT-IR results on the synthesized nanoparticles.

Nanoparticles have special physical, chemical, mechanical, electrical and magnetic properties due to their small size; they easily enter the cell and interfere in its natural and vital process. The use and application of nanotechnology in various branches of science such as medicine, pharmaceuticals, imaging, environment and industry has attracted the attention of many scientists. Nanoparticles are spherical materials found in nanometer size and derivatives of iron oxide paramagnetic nanoparticles with different and variable coatings are used in drug delivery mechanism. Therefore, evaluating the bioavailability of these nanoparticles seems necessary to understand the mechanisms of action and toxicity resulting from their activity. Many researchers have used biological methods to synthesize metal nanoparticles or metal oxides from different parts of plants, especially leaves, stems, roots, and fruits. However, magnetite nanoparticles behave differently depending on how they are synthesized. In addition, the size and shape of the magnetite crystal affects its magnetic properties.

Table 2. Physical properties of the produced Fe_3O_4 nanoparticles.

Particle name	Color	Particle size Mean ± SD	Zeta potential (mV)	PDI (Polydispersity Index)
Green tea extract method	Green	21.51 ± 1.7	-25±0.87	0.4±0.45
Chemical method	Brown	13.19 ± 1.2	-19 ± 0.34	0.2 ± 0.91

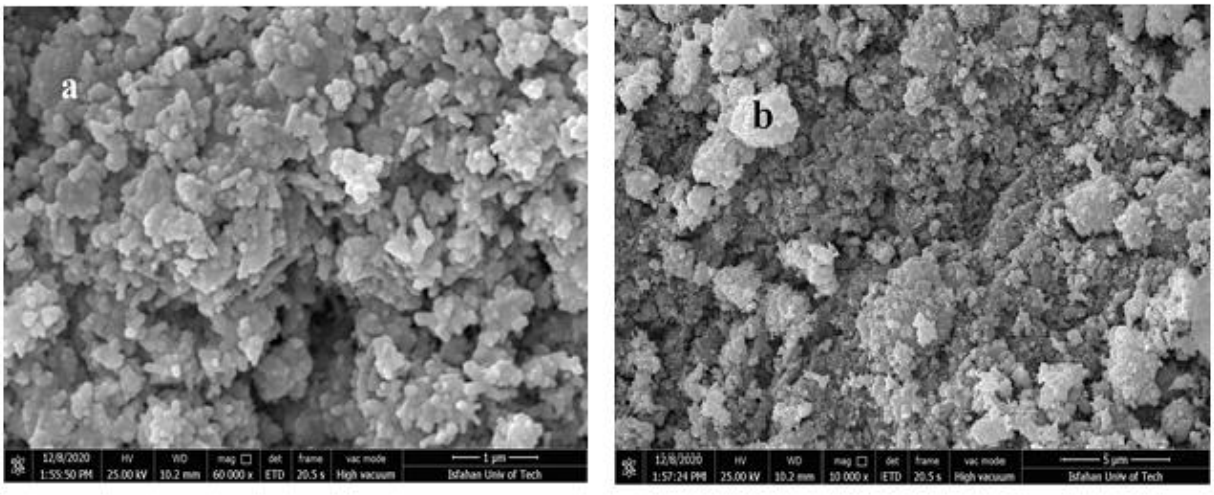

Fig. 1. a. SEM image of green tea extract method synthesized Fe_3O_4 nanoparticles. **b**. SEM image of chemical method synthesized Fe_3O_4 nanoparticles

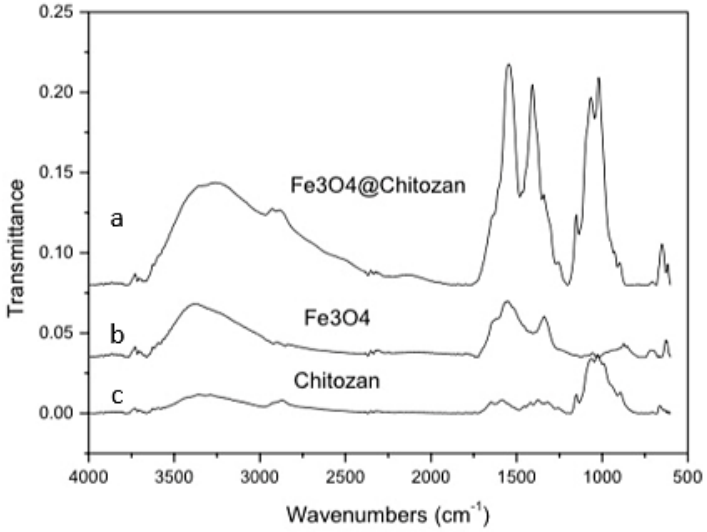

Fig. 2. FT-IR graph of the synthesized nanoparticles. a. Nanoparticle coated with chitosan and b. Fe_3O_4 nanoparticle and c. Chitosan

Pathological findings

Fig. 3 presents Hematoxylin-Eosin staining of the dissected tissue of the treated rats with the green nanoparticles compared with chemical nanoparticles.

In this study, Fe_3O_4 nanoparticles were synthesized by two chemical and green methods, and the effect of these synthetic nanoparticles as well as chitosan-coated nanoparticles in different concentrations on the activity of rat liver enzymes and gene expression was investigated. Yusefi et al. utilized Fe_3O_4 nanoparticles to destroy colon cancer cells. Fe_3O_4 nanoparticles were precipitated with the crude extract of *Garcinia mangostana* fruit in different weight percentages. HCT116 colon cancer cells were destroyed by the generated samples more than normal CCD112 colon cancer cells [17]. In the studies of Al-Karagoly et al., iron oxide nanoparticles (IONPs) were synthesized using black seed extract, and as a strong rejuvenating agent, their cytotoxic and antibacterial properties were investigated. In addition to the significant non-cytotoxic effect shown by MTT in Vero cell line, these biosynthetic nanoparticles (IONPs) showed excellent antibacterial activity against Escherichia coli and Staphylococcus aureus [18].

Biochemistry analysis

Serological analysis of the serum collected from the treated rat's blood showed some variance between the level of SGPT, SGOT and ALK enzymes between the control groups, the groups treated with the chemical synthesized nanoparticles and the groups treated with the green tea synthesized Fe_3O_4 nanoparticles (Figs. 4, 5, and 6).

No significant differences were observed between the chemical synthesized nanoparticles treated groups in comparison with the control and green tea synthesized Fe_3O_4 nanoparticle groups. C: control group, 1, 2, and 3 are 50, 100 and 150 mg dose of chemical synthesized Fe_3O_4 nanoparticle respectively. 4, 5, and 6 are 50, 100 and 150 mg dose of green tea synthesized Fe_3O_4 nanoparticle respectively.

Significant differences were observed between the chemical synthesized nanoparticle treated groups in

comparison with the control and green tea synthesized Fe_3O_4 nanoparticle groups. C: control group, 1, 2 and 3 are 50, 100 and 150 mg dose of chemical synthesized Fe_3O_4 nanoparticle respectively. 4, 5 and 6 are 50, 100 and 150 mg dose of green tea synthesized Fe_3O_4 nanoparticle respectively.

No significant differences were observed between the chemical synthesized nanoparticle treated groups in comparison with the control and green tea synthesized Fe_3O_4 nanoparticle groups. However, there is significant difference in the ALK level between the ends of course analysis compared with the middle of the course. C: control group, 1, 2 and 3 are 50, 100 and 150 mg dose of chemical synthesized Fe_3O_4 nanoparticles respectively. 4, 5 and 6 are 50, 100 and 150 mg dose of green tea synthesized Fe_3O_4 nanoparticles respectively.

The effects of silver nanoparticles on the metabolism of Wistar rats were investigated in a study. Mice were treated daily with silver nanoparticles (AgNPs). AgNPs did not affect the amount of food eaten by mice or their weight. But when mice were exposed to AgNP, serum and tissue levels of AST, ALT and ALP changed significantly. After treatment with 100 mg/kg of silver nanoparticles, the level of AST and ALT in the blood and tissues of mice decreased significantly. On the other hand, when AgNPs were given to mice, ALP levels increased in their blood and tissues [6]. In the studies of Albukhaty et al. superparamagnetic iron oxide nanoparticles were coated with poly-L-lysine (SPIONs-PLL). These nanoparticles were used to evaluate the efficiency of gene expression for SPIONs-PLL as a non-viral carrier in NSCs. Histological analysis showed that the concentration of intracellular nanoparticles is higher than that of intercellular nanoparticles. Therefore, these results indicated that SPIONs-PLL can act as a new alternative for transfection of BDNF-NSCs, which is useful for gene therapy [19].

In the blood and tissues of male Wistar rats, the toxicity of molybdenum trioxide nanoparticles was investigated by Akhundipour et al. The findings showed that high concentrations are more harmful than low values for blood and serum parameters [20]. In a study on adult male Wistar rats, TiO_2 nanoparticles were administered orally. Data showed that oral administration of TiO_2 nanoparticles (<100 nm) may lead to hepatotoxicity in mice [21]. For 28 days, Wistar rats of both sexes were gavage with small silver nanoparticles (10 nm) coated with polyvinylpyrrolidone (PVP). Oxidative stress markers and blood parameters were changed in all animals, indicating the toxicity of AgNPs even at moderate doses. Gender differences are evident in all analyzed parameters. Compared to male mice, female rats receiving moderate amounts of AgNPs eliminated nanoparticles more efficiently from their liver and kidneys.

In this research by examining the effect of Fe_3O_4 nanoparticles on the activity of liver enzymes in rats after 30 days of treatment, it was found that nanoparticles synthesized by chemical method had the most destructive effect on hepatocytes at concentrations of 100 and 150 (mg/kg). As a result, the activity of liver enzymes increased and can lead to tissue destruction. Nanoparticles synthesized by the green method did not have a destructive effect on liver cells and did not show significant results after examining the activity of the studied enzymes compared to the control group and the group treated with chemically synthesized nanoparticles.

Chitosan is considered a polymer and is obtained by deacetylation of chitin. Chitin is found naturally in the exoskeleton of crustaceans and the cell wall of fungi, which is the second most abundant natural polymer after cellulose. The length and acetyl residues of the resulting chitosan polymers are different depending on the conditions used in the deacetylation method. It may be converted into molecules with weights between 300 and over 1000 kD

In addition, the degree of chitosan acetylation, which ranges from 5 to 70%, significantly affects the physicochemical properties, including viscosity and solubility. Therefore, this non-toxic and biodegradable compound was used to coat iron oxide nanoparticles. [22].

The results showed that the coating of artificial nanoparticles with chitosan reduces the destructive effect of Fe_3O_4 nanoparticles on liver cells to such an extent that the activity of the enzymes studied in the control group decreased compared to the group treated with Fe_3O_4 nanoparticles synthesized by chemical method. Considering that the destructive effect of iron oxide nanoparticles on liver cells follows the use of magnetic fields, these magnetic fields activate processes in the body that conduct messages from the cell membrane to the nucleus and genetic content. It also affects the function of organs and changes the cell membrane potential and ion distribution in the cell. These changes affect biochemical processes and change the activity of serum enzymes and biochemical parameters [23].

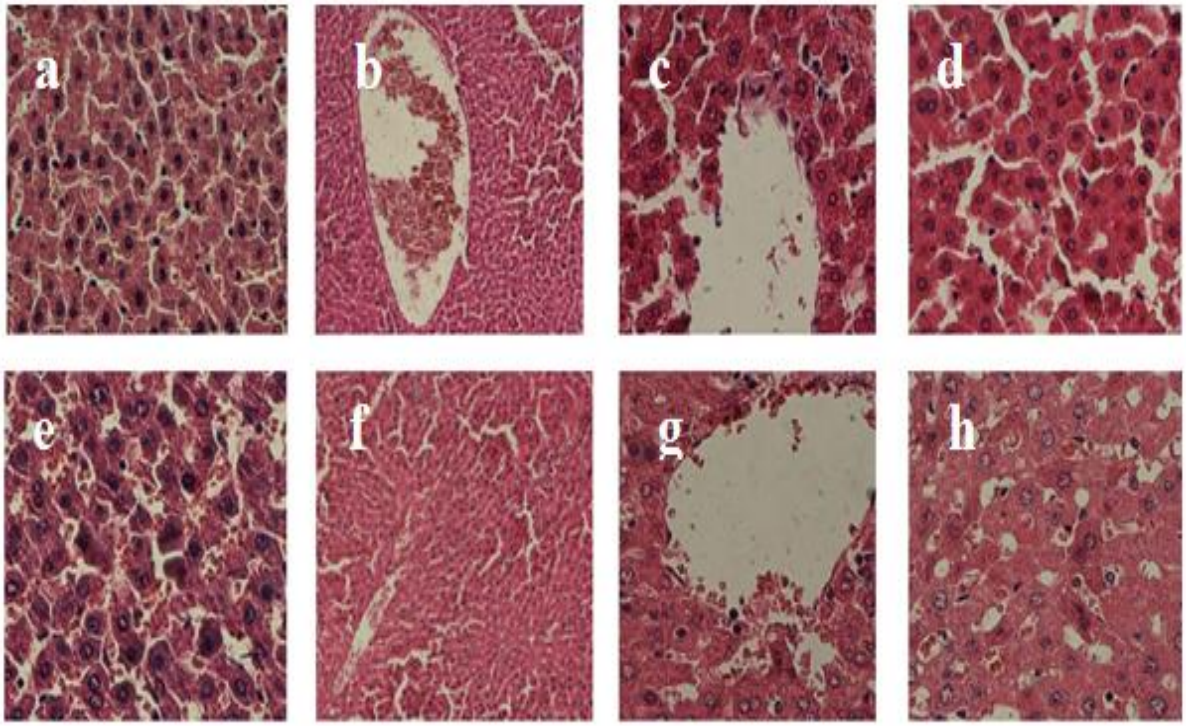

Fig. 3. Liver tissue sections treated with chemical (a, c, and d, X=400, b, X=100) compared with the rat's liver tissue sections treated green synthesized nanoparticles (e, g, and h, X=400, f, X=100) by hematoxylin-eosin staining (h, e, X=100). Lysis of hepatocytes and release of nuclei, nuclear atrophy, disorder and destruction of hepatocyte cords, disorder and dilation of sinusoids, venous congestion, and destruction of the venous wall is very intensive in treated groups treated with chemical nanoparticles relative to the green synthesized nanoparticles treatment groups.

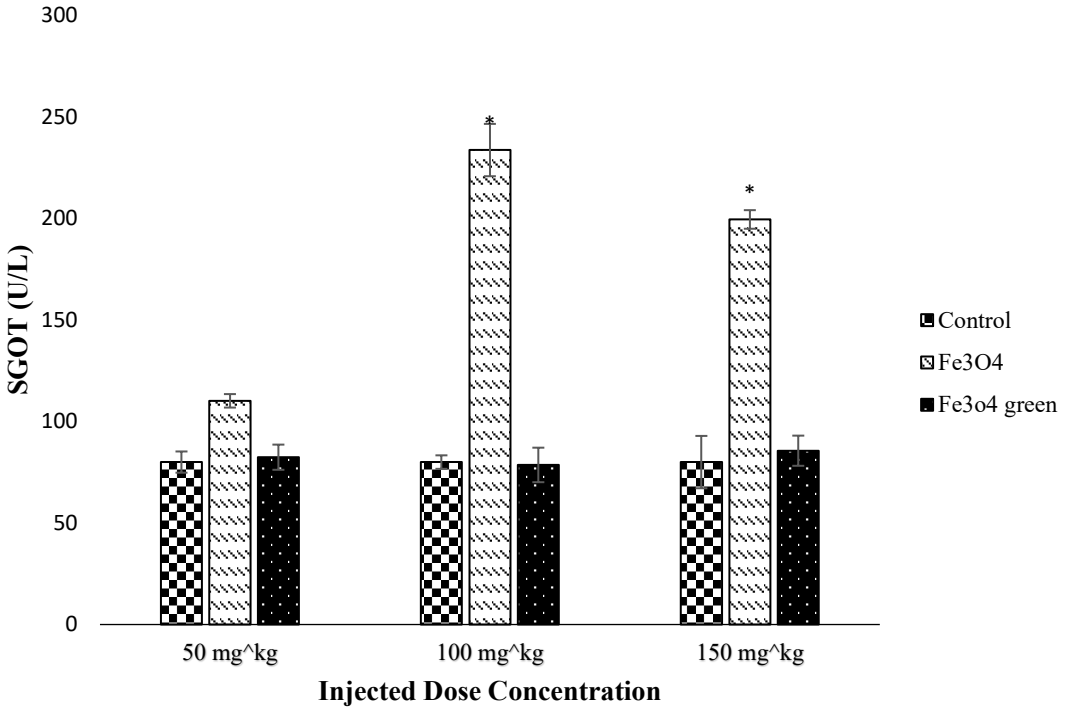

Fig. 4. SGOT levels in the treated rats.

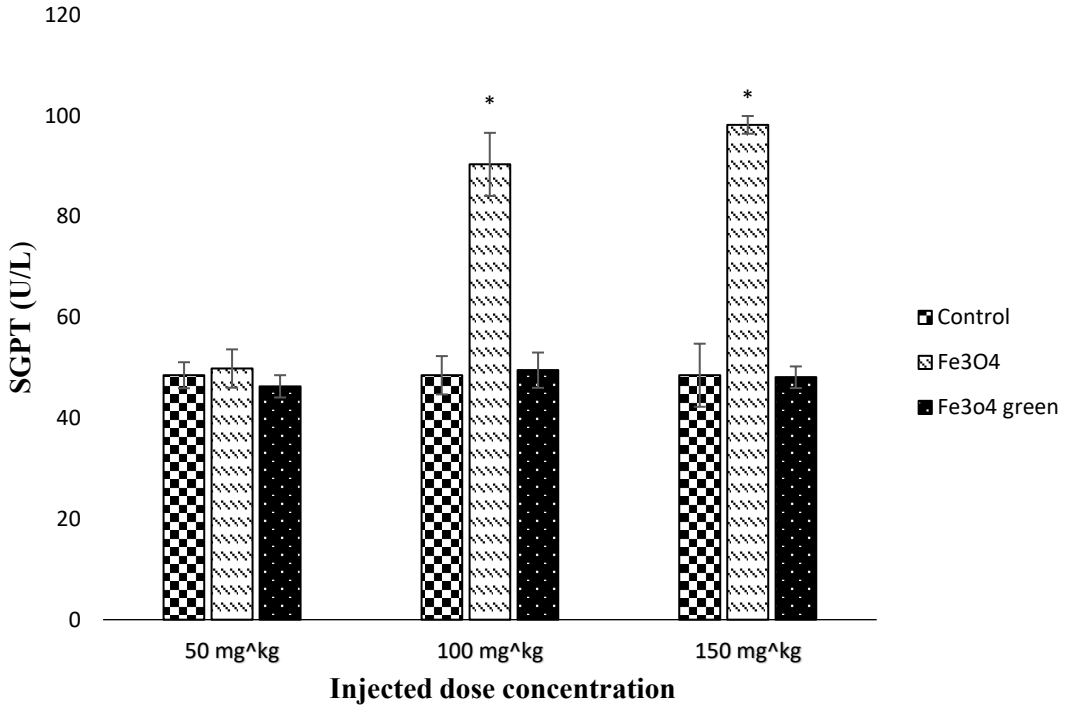

Fig. 5. SGPT levels in the treated rats.

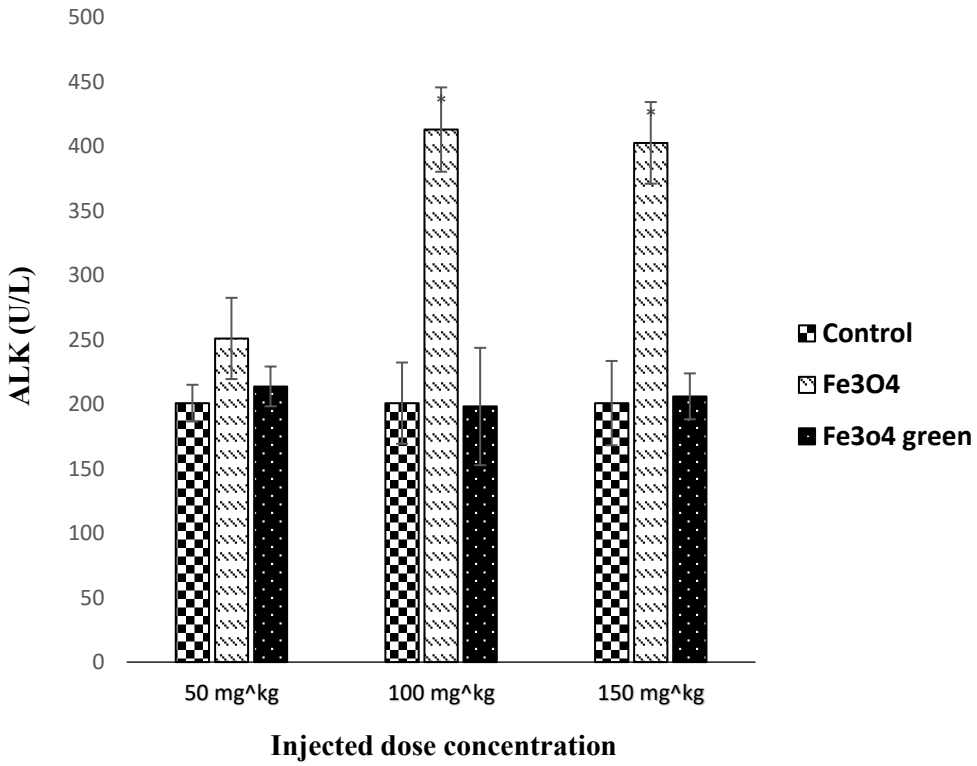

Fig. 6. ALK levels in the treated rats.

Gene expression

RNA extraction and real time RT-PCR analysis

Total RNA was extracted by using RNX plus solution and subjected to agarose gel electrophoresis (Fig. 7). 28S and 18S rRNAs show an optimal quality of the extracted RNA.

Figs. 8 and 9 is the statistical analysis of *GR* and *MT1* gene expression. As mentioned in this study, the expression changes of *Gr gene* as an antioxidant molecule and *MT1*gene as metal balance regulator in liver were measured by the $2^{-\Delta\Delta Ct}$ method. Using SPSS software ver. 18 and performing the statistical independent t-test, the research hypothesis was tested and the results are presented by GraphPad prism software. The distribution of data for the results of gene expression between control group and the rats treated with nanoparticles was performed by KS test.

Metallothioneins are essential in regulating metal homeostasis and controlling the physiological toxicity of heavy metals, DNA damage and oxidative stress [10, 11]. In living organisms, MT1 functions as an antioxidant protein and a metal carrier to maintain physiological balance and prevent damage from metal overload. Several agents, including heavy metals, antioxidants, alkylating agents, glucocorticoids, cytokines, and lipopolysaccharides, can stimulate MT1 expression to exert several immunomodulatory effects. MTs protect organisms against oxidative stress by scavenging hydroxyl radicals and superoxide radicals [24]. Glutathione synthesis occurs exclusively in liver cells. Micronutrients such as vitamin E and C and thiol-rich proteins such as metallothionein and ubiquinone are only some of the mechanisms that the liver uses to resist oxidative stress [12, 13]. Oxidative stress occurs when reactive oxygen species (ROS) are produced at a rate higher than the antioxidant capacity of living organisms. Measurement of oxidation products can be used to determine oxidative stress because most oxidants have very short half-lives [14]. Arial et al. found that the evaluation of the expression and function

of metal binding to MTs may be important for analyzing the biodegradation of Me-NPs. MTs as highly reactive thiols can also play a role in inhibiting reactive oxygen species (ROS) caused by Me-NPs. Therefore, metal ion binding to metallothioneins leads to a change from an antioxidant function to a metal ion buffer/ chaperone [25]. Also, Dai et al showed that although metal ion regulation of MT expression has been reported in several studies, the expression of MT1 creates a high capacity to bind these heavy metal ions in vivo and in vitro, and thus the detoxification process begins [9].

In our research, the results showed that MT1 gene expression in mice treated with Fe_3O_4 nanoparticles synthesized by the green method was more significant compared to mice treated with chemical nanoparticles, and a high level of MT1 gene expression was observed in green tea nanoparticles groups. Also, low concentrations of green nanoparticles in treated mice reduced GR gene expression compared to groups treated with Fe_3O_4 chemical nanoparticles. The reason for these changes is probably due to the antioxidant property of green tea, which prevented the destructive effects of the magnetic fields of iron oxide nanoparticles and exerted its antiparamagnetic effects on the expression of the studied genes.

CONCLUSION

This study was conducted in order to compare the effect of iron oxide nanoparticles synthesized by green method and concentrated nanoparticles by chemical method on functional indices of rat liver. The results of this study showed that despite the increase in the size of the nanoparticles due to the coating, this increase in size is not enough to exceed the critical size limit (30 nm for iron oxide). Iron oxide nanoparticles synthesized by the green method prevented the increase in the activity of liver enzymes in mice and had a protective effect. Chitosan coating on iron oxide nanoparticles also helped to reduce the toxic effects of nanoparticles. In the group treated with nanoparticles coated with green tea, compared to the control group and the groups treated with uncoated nanoparticles, most of the changes were decreasing and sometimes increasing, but these changes were not statistically significant. Due to the antioxidant property of nanoparticles synthesized by the green method, MT1 gene expression was increased in the groups treated with this nanoparticle, and GR gene expression was decreased in the same groups compared to the control group and the group treated with chemical nanoparticles. Probably, the short-term use of chitosan-coated iron oxide nanoparticles in biological and medical cases does not cause special toxicity in the body. Therefore, the results obtained from the present research can be the basis for future research in the direction of using nanoparticles synthesized in a green way, with less toxicity for pharmaceutical and medical purposes.

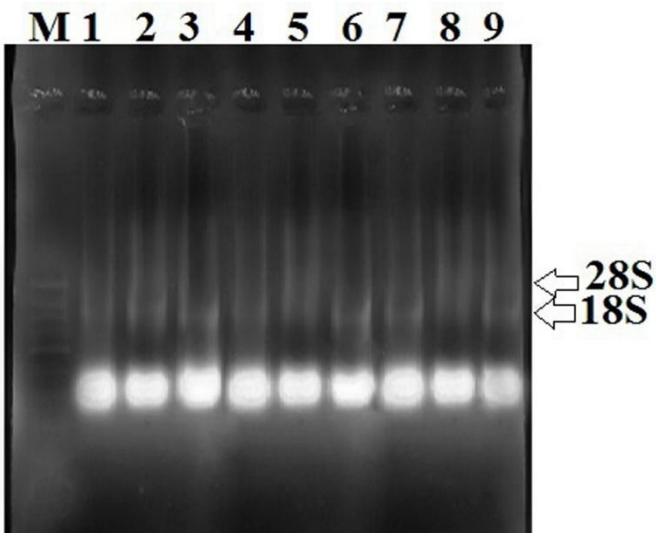

Fig. 7. Qualification of the extracted RNA. 28s and 18s rRNA shows an optimal quality of the extracted RNA. Green viewer-stained 1% agarose gel. M: 100bp DNA size marker.

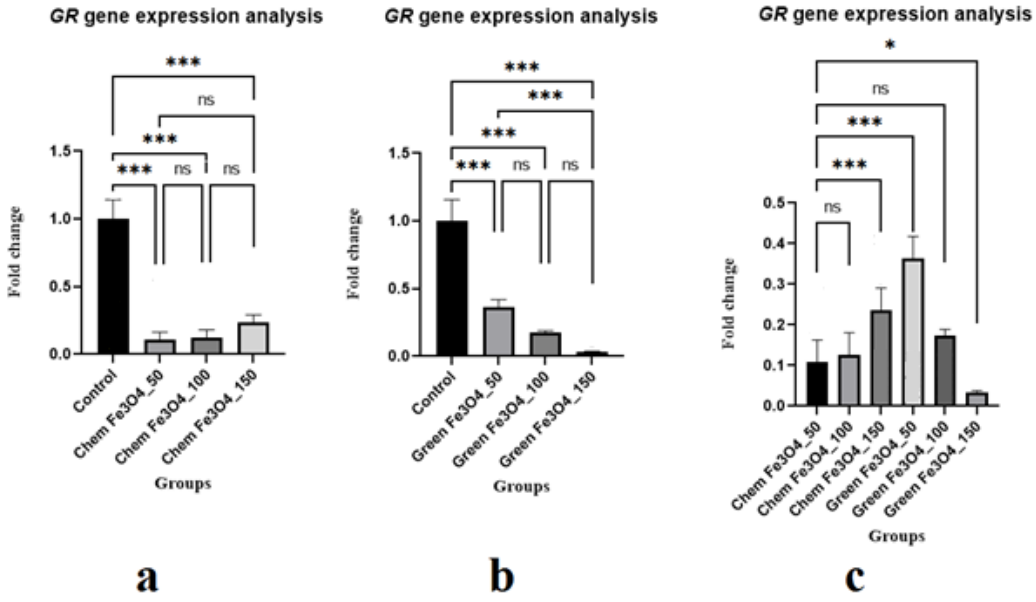

Fig. 8. One-way ANOVA analysis between the chemical Fe_3O_4 nanoparticles treated rat *GR* gene expression compared with the rat treated by green tea nanoparticles. a. *GR* gene expression changes between the treatment groups with different concentrations of chemical nanoparticles were not significant, but a significant decrease was observed between the treatment groups and the control group. b. significant decrease between the control group and green nanoparticle treated groups in the *GR* gene expression. Comparison between the chemical and green synthesized nanoparticle treated groups. Decrease in the *GR* gene expression in the green treated groups is more considerable ($P \leq 0.05$, CI: 95%).

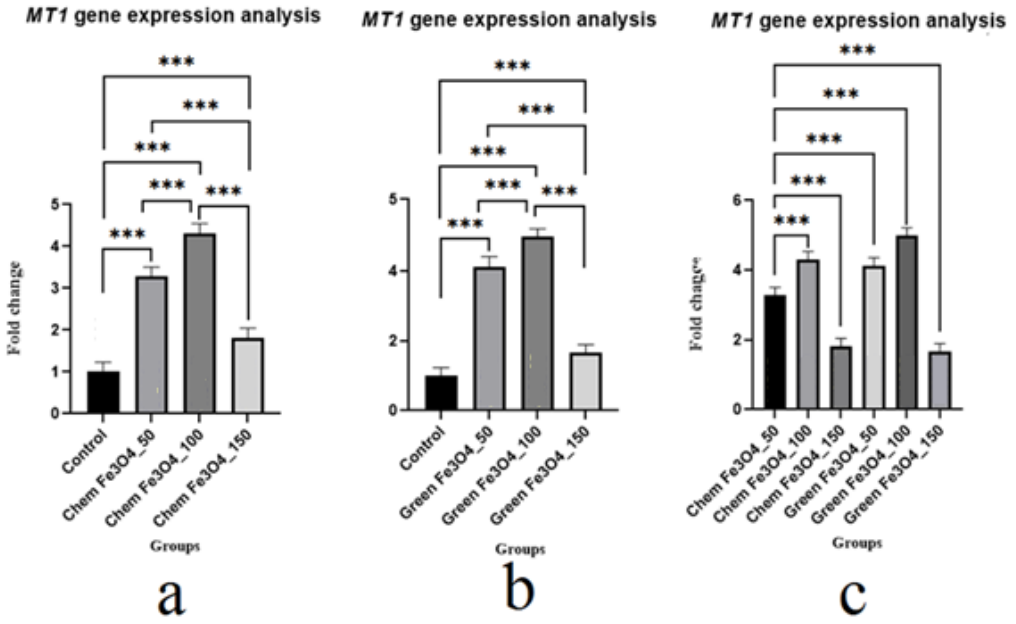

Fig. 9. One-way ANOVA analysis between the chemical Fe_3O_4 nanoparticles treated rat *MT1* gene expression compared with the rat treated by green tea nanoparticles. Significant variance was observed between the green group and the chemical ones. Elevated level of the *MT1* gene expression was observed in the green tea nanoparticle groups ($P \leq 0.05$, CI=95%).

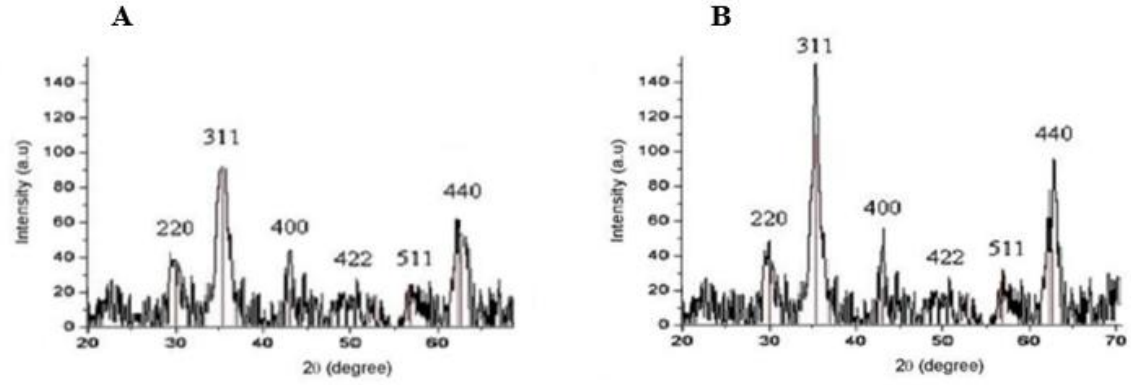

Fig. 10. XRD of Fe_3O_4 nanoparticles. A. X-ray diffraction image of chitosan coated, B. X-ray diffraction image of Fe_3O_4 nanoparticles

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

REFERENCES

[1] Yew YP, Shameli K, Miyake M, Khairudin NB, Mohamad SE, Naiki T, Lee KX. Green biosynthesis of superparamagnetic magnetite Fe_3O_4 nanoparticles and

biomedical applications in targeted anticancer drug delivery system: A review. Arabian Journal of Chemistry. 2020;13(1):2287-308.

[2] Singh J, Dutta T, Kim K-H, Rawat M, Samddar P, Kumar P. 'Green' synthesis of metals and their oxide nanoparticles: applications for environmental remediation. Journal of Nanobiotechnology. 2018;16:1-24.

[3] Ijaz I, Gilani E, Nazir A, Bukhari A. Detail review on chemical, physical and green synthesis, classification, characterizations and applications of nanoparticles. Green Chemistry Letters. 2020;13:223-45.

[4] Nguyen MD, Tran HV, Xu S, Lee TR. Fe_3O_4 nanoparticles: structures, synthesis, magnetic properties, surface functionalization, and emerging applications. Applied Sciences. 2021;11(23):11301.

[5] Saeed RM, Dmour I, Taha MO. Stable chitosan-based nanoparticles using polyphosphoric acid or hexametaphosphate for tandem ionotropic/covalent crosslinking and subsequent investigation as novel vehicles for drug delivery. Frontiers in Bioengineering and Biotechnology. 2020;8:4.

[6] Adeyemi OS, Adewumi I. Biochemical evaluation of silver nanoparticles in wistar rats. International Scholarly Research Notices. 2014;2014(1):196091.

[7] Ruttkay-Nedecky B, Nejdl L, Gumulec J, Zitka O, Masarik M, Eckschlager T, Stiborova M, Adam V, Kizek R. The role of metallothionein in oxidative stress. International Journal of Molecular Sciences. 2013;14(3):6044-66.

[8] Masiulionytė B, Valiulytė I, Tamašauskas A, Skiriutė D. Metallothionein genes are highly expressed in malignant astrocytomas and associated with patient survival. Scientific Reports. 2019;9:1-7.

[9] Dai H, Wang L, Li L, Huang Z, Ye L. Metallothionein 1: A New spotlight on inflammatory diseases. Frontiers in Immunology. 2021;12:739918.

[10] Wang W-C, Mao H, Ma D-D, Yang W-X. Characteristics, functions, and applications of metallothionein in aquatic vertebrates. Frontiers in Marine Science. 2014;1:34.

[11] Kedrowski BL, Gutow JH, Stock G, Smith M, Jordan C, Masterson DS. Glutathione reductase activity with an oxidized methylated glutathione analog. Journal of Enzyme Inhibition and Medicinal Chemistry. 2014;29(4):491-4.

[12] Vairetti M, Di Pasqua L.G, Cagna M, Richelmi P, Ferrigno A, Berardo C. Changes in glutathione content in liver diseases: An update. Antioxidants 2021;10:364.

[13] Pizzorno J. Glutathione!. Integrative Medicine: A Clinician's Journal. 2014;13(1):8.

[14] Csiszár J, Horváth E, Bela K, Gallé Á. Glutathione-related enzyme system: glutathione reductase (GR), glutathione transferases (GSTs) and glutathione peroxidases (GPXs). Redox state as a central regulator of plant-cell stress responses. 2016:137-58.

[15] Mao C, Yuan JQ, Lv YB, Gao X, Yin ZX, Kraus VB, Luo JS, Chei CL, Matchar DB, Zeng Y, Shi XM. Associations between superoxide dismutase, malondialdehyde and all-cause mortality in older adults: a community-based cohort study. BMC Geriatrics. 2019;19:1-9.

[16] Bhuiyan MS, Miah MY, Paul SC, Aka TD, Saha O, Rahaman MM, Sharif MJ, Habiba O, Ashaduzzaman M. Green synthesis of iron oxide nanoparticle using Carica papaya leaf extract: application for photocatalytic degradation of remazol yellow RR dye and antibacterial activity. Heliyon. 2020;6(8).

[17] Yusefi M, Shameli K, Su Yee O, Teow SY, Hedayatnasab Z, Jahangirian H, Webster TJ, Kuča K. Green synthesis of Fe3O4 nanoparticles stabilized by a Garcinia mangostana fruit peel extract for hyperthermia and anticancer activities. International Journal of Nanomedicine. 2021;29:2515-32.

[18] Al-Karagoly H, Rhyaf A, Naji H, Albukhaty S, AlMalki FA, Alyamani AA, Albaqami J, Aloufi S.

Green synthesis, characterization, cytotoxicity, and antimicrobial activity of iron oxide nanoparticles using Nigella sativa seed extract. Green Processing and Synthesis. 2022;11(1):254-65.

[19] Albukhaty S, Naderi-Manesh H, Tiraihi T, Sakhi Jabir M. Poly-l-lysine-coated superparamagnetic nanoparticles: a novel method for the transfection of pro-BDNF into neural stem cells. Artificial Cells, Nanomedicine, and Biotechnology. 2018;46(sup3):125-32.

[20] Akhondipour M, Faghihi Zarandi A, Amirri A, Gommnami N, Vazirinejad R. Studying the toxicity of molybdenum trioxide nanoparticles in male Wister rats. Journal of Occupational Health and Epidemiology. 2018;7(4):233-9.

[21] Vasantharaja D, Ramalingam V, Reddy GA. Oral toxic exposure of titanium dioxide nanoparticles on serum biochemical changes in adult male Wistar rats. Nanomedicine Journal. 2015;2(1).

[22] Ćurlin M, Barbir R, Dabelić S, Ljubojević M, Goessler W, Micek V, Žuntar I, Pavić M, Božičević L, Pavičić I, Vinković Vrček I. Sex affects the response of Wistar rats to polyvinyl pyrrolidone (PVP)-coated silver nanoparticles in an oral 28 days repeated dose toxicity study. Particle and Fibre Toxicology. 2021;18:1-6.

[23] Zare S, Hayatgeibi H, Alivandi S, Ebadi AG. Effects of whole-body magnetic field on changes of glucose and cortisol hormone in guinea pigs. American Journal of Biochemistry and Biotechnology. 2005;1(4):217-9.

[24] Sharifi-Rad J, Quispe C, Butnariu M, Rotariu LS, Sytar O, Sestito S, Rapposelli S, Akram M, Iqbal M, Krishna A, Kumar NV. Chitosan nanoparticles as a promising tool in nanomedicine with particular emphasis on oncological treatment. Cancer Cell International. 2021;21(1):318.

[25] Aryal BP, Neupane KP, Sandros MG, Benson DE. Metallothioneins initiate semiconducting nanoparticle cellular toxicity. Small. 2006;2(10):1159-63.