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Hepatoprotective effect of thymol against subchronic toxicity of titanium dioxide nanoparticles: Biochemical and histological evidences

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ABSTRACT

The study was aimed to investigate the protective action of thymol against nano titanium dioxide (nano-TiO_2) induced hepatotoxicity in rats. To achieve this purpose, the rats were divided into four groups (n = 6) including control, nano-TiO_2 (100 mg/kg), nano-TiO_2 + thymol (10 mg/kg) and nano-TiO_2 + thymol (30 mg/kg). Intragastric (IG) administration of nano-TiO_2 for 60 consecutive days caused widespread histological changes and significantly induced oxidative stress in the liver tissues as manifested by the rise in serum transaminase activities accompanied by marked decline of enzymatic (catalase, superoxide dismutase and glutathione peroxidase) and non-enzymatic (ferric reducing antioxidant power and glutathione) antioxidant levels, and rise of malondialdehyde levels in liver tissue. Pretreatment with thymol (IG) prior to nano-TiO_2 administration significantly ameliorated all of biochemical and histopathological alterations in a dose-dependent manner. In conclusion, thymol effectively protects against nano-TiO_2-induced hepatotoxicity in rats by its antioxidant properties.

1. Introduction

Nano-titanium dioxide (nano-TiO_2) is one of the five most widely used nanomaterials in a variety of industrial and consumer products. Nano-TiO_2 is being used in the production of toothpastes, food colorants, and nutritional supplements on large scale. Some food products such as candies, sweets and chewing gum contain higher amounts of this nanoparticle with diameters less than 100 nm (Orazizadeh et al., 2014; Shakeel et al., 2015; Weir et al., 2012). Exposure to this nanoparticle can take place through oral ingestion, injection, inhalation and skin absorption (Shakeel et al., 2015). Oral exposure mostly occurs via consumption of food products. Due to its unique physicochemical characteristics (e.g. the small size, high surface area, and high reactivity), nano-TiO_2 can easily enters the body and then cause toxic effect. It is also able to pass through biological barriers such as blood-testicular barrier, and blood-heart barrier (Meena and Paulraj, 2012). However, like most other nanoparticles, nano-TiO_2 tends to accumulate more in the liver (Attia et al., 2013; Hong and Zhang, 2016; Liu et al., 2009). In a study by Liu et al. the toxicity of nano-TiO_2 was investigated in various tissues of mice and results showed that the accumulation of nanoparticles and tissue damage in the liver was more than other tissues (Liu et al., 2009). Liver, the major site of xenobiotic metabolism, is considered as the most vulnerable target organ for nano-TiO_2 exposure (Ghaffarian-Bahraman et al., 2014; Meena and Paulraj, 2012; Vasantharaja et al., 2015). There is a vast body of evidence from mechanistic toxicology studies suggesting that nano-TiO_2 induce oxidative stress in the liver through increasing the production of reactive oxygen species (ROS) and liver lipid peroxides, which is activated by a reduction of antioxidative defense mechanisms (Hong and Zhang, 2016; Iavicoli et al., 2012; Liu et al., 2010; Meena and Paulraj, 2012; Natarajan, 2015; Rizk et al., 2017). If the endogenous antioxidant defense system fails to protect against oxidative agents, the oxidative stress can induce pro-inflammatory effects and mitochondrial dysfunction, which can trigger apoptosis and cell necrosis. The exogenous antioxidants might be able to reduce nanoparticles-induced oxidative stress via ROS elimination, which will provide a greater beneficial advantage to the use of nanoparticles (Onoda et al., 2015).

Herbal extracts derived from plant extracts are increasingly being used to treat many types of diseases. Also, special attention is paid to the protective effects of natural antioxidants on the toxicity of chemicals that humans are usually exposed to (Azim et al., 2015; Orazizadeh et al., 2014). Thymol (*p*-methyl-isopropyl-phenol) is a colorless crystalline monoterpene phenol, which is found in the oils of Thymus vulgaris and other plants such as *Thymbra spicata, Thymus ciliates,*

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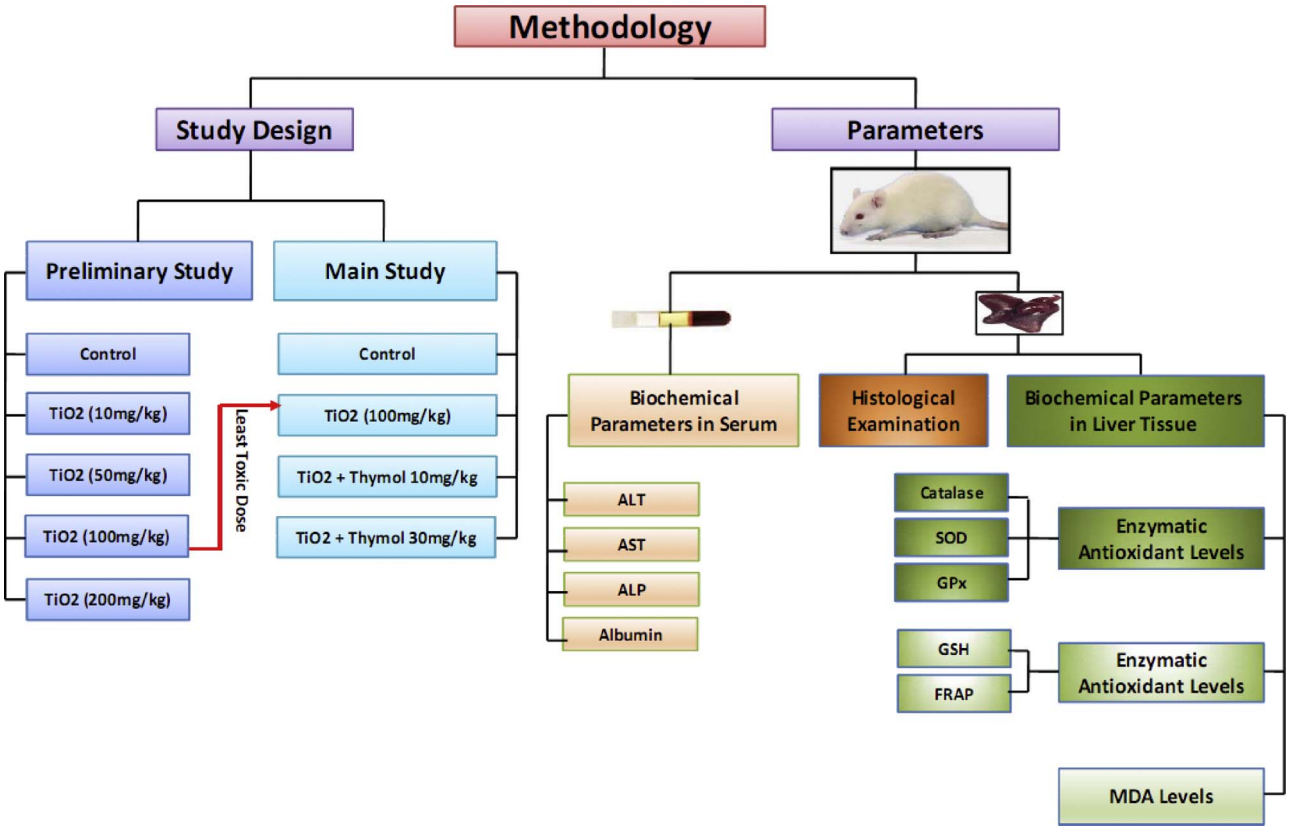

Fig. 1. The methodology of the experimental study.

Trachyspermum ammi, Monarda fistulosa, and *Nigella sativa* (Meeran and Fizur, 2012). It is an active ingredient used in food flavorings, topical ointments, toothpastes, deodorants and mouthwashes, perfumes, cosmetics, pharmaceutical products (Aboelwafa and Yousef, 2015). Thymol possesses multiple biological activities such as antibacterial, antifungal, anti-inflammatory, anti-mutagenic, analgesic, anticonvulsant, antiepileptogenic, anti-hemolytic, radioprotective, hypocholesteraemic, immunosuppressive and wound healing effects (Aboelwafa and Yousef, 2015; Meeran et al., 2017; Meeran et al., 2016; Meeran and Fizur, 2012; Saravanan and Pari, 2016). Furthermore, it exhibits free radical scavenging, anti-lipid peroxidative and antioxidant properties in both *in vivo* and *in vitro* studies (Aboelwafa and Yousef, 2015; Meeran et al., 2017; Palabiyik et al., 2016).

Considering the fact that prolonged and repetitive exposure to nano-TiO_2 can result in adverse health effects such as oxidative stress (Onoda et al., 2015; Sharifi et al., 2012), we hypothesized that antioxidants such as thymol may act as a protective agent against oxidative damage induced by this nanoparticle and facilitate its safe use. Therefore, in the current study, we aimed to investigate the probable protective effects of thymol against nano-TiO_2-induced oxidative stress and liver damage in male Wistar rats.

2. Material and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (GmbH, Munich, Germany) unless otherwise mentioned. The nano-TiO_2 powder employed in this work was PC50 (manufactured by Cristal Global, 100% anatase, 40 nm primary particle size). ALT, ALP, AST and albumin levels were evaluate by commercial reagent kit (Pars Azmoon Co. Iran). Catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were measured by kits (Zell bio, Germany).

2.2. Nano-TiO_2 characteristics

The X-ray diffraction (XRD) patterns were obtained on an X-ray micro diffractometer Rigaku D-max-RAPID, using Cu-Kα radiation. The average crystalline size of TiO_2 was determined by the Scherrer equation using full width at half maximum (FWHM) at $2\theta = 25.3^{\circ}$ corresponding to the (1 0 1) plane diffraction of anatase TiO_2. Specific surface area (SSA) of TiO_2 was measured on a Costech Sorptometer 1040 by nitrogen adsorption at $-196\,^{\circ}C$ after samples pretreatment at 150 °C for 30 min under He flow (Heterogeneous photocatalytic oxidation of methyl ethyl ketone under UV–A light in an LED-fluidized bed reactor).

2.3. Animals

All male Wistar rats (8–10 weeks old, 180–200 g) were obtained from animal house of Faculty of Medicine, Urmia University of Medical Sciences (Urmia, Iran) and housed in controlled environmental conditions of 21 ± 5 °C temperature, 55% humidity and 24 h light-dark cycle with free access to stock laboratory diet and water. They were maintained during the whole experimental period in compliance with the Ethics Committee of Urmia University of Medical Sciences.

2.4. Study design

At first, a preliminary study was designed to determine the lowest effective dose of nono-TiO_2 that can produce significant alteration in oxidative stress and biochemical parameters after intragastric administration for consecutive 60 days. Five doses of nono-TiO_2 (0, 10, 50, 100 and 200 mg/kg) were chosen and the desired dose were determined based on the liver enzymes in the blood and oxidative stress biomarkers in liver tissue. Then, in the main part of study the animals were randomly divided into five groups of six rats each, as follows;

- Group 1: control group, received corn oil by gavage for 60 consecutive days
- Group 2: nano-TiO_2 group, received 100 mg/kg nano-TiO_2 by gavage for 60 consecutive days
- Group 3: nano-TiO_2 + thymol 10 group, received 10 mg/kg thymol by gavage one hour before 100 mg/kg nano-TiO_2 administration for 60 consecutive days
- Group 4: nano-TiO_2 + thymol 30 group, received 30 mg/kg thymol by gavage one hour before 100 mg/kg nano-TiO_2 administration for 60 consecutive days

Thymol was dissolved in corn oil. Nano-TiO_2 was suspended in distilled water and treated by ultrasonic vibration for 20 min to make a homogeneous suspension. Control animals received only corn oil in appropriate volume. Doses of thymol were picked from previous studies (Cardoso et al., 2016; Ribeiro et al., 2016). The flowchart of the experimental study design is illustrated in Fig. 1.

2.5. Blood sampling and liver tissue preparation

At the end of the experiments, rats were weighed, anesthetized by intraperitoneal injection of ketamine/xylazine (60/6 mg/kg, ip) and blood samples were taken via heart puncture. The serum was centrifuged at 4000 rpm for 10 min and was kept at −80 °C for subsequent estimation of enzyme activities. Then, animals were sacrificed and liver tissues were separated, weighed and divided into two portions. One portion was kept in 10% formaldehyde for subsequent histopathological examinations. Another portion was homogenized in 4 vols of phosphate buffer (pH 7.4) for biochemical evaluation.

2.6. Coefficients of the liver

After weighing the body and liver tissue, the coefficient of liver to body weight was calculated as the ratio of tissue (wet weight, mg) to rat body weight (BW, g).

2.7. Biochemical analysis of liver tissue

To biochemical evaluation of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, a part of liver homogenate centrifuged at 12000*g* at 4 °C for 20 min. Then the supernatant was separated and stored at −80 °C and the activities of antioxidant enzymes were determined by kits, according to the manufacturer's procedure.

2.7.1. Estimation of Malondialdehyde (MDA) content

MDA is an important end product of the cellular lipid peroxidation. It can reacts with thiobarbituric acid (TBA) in acid medium to generate a pink-colored complex that can be measured spectrophotometrically, named as TBA reactive substances (TBARS).The supernatant (600 µL) was mixed with 150 µL of TBA (0.67% w/v), kept in boiling water bath (95 °C) for 30 min, extracted with *n*-butanol by vigorous shaking. Then the tube was cooled and centrifuged. Absorbance was recorded at 532 nm and the results were expressed as nmol MDA/mg protein. The method was calibrated with tetraethoxypropane, as standard solutions (Asghari et al., 2017; Jafari et al., 2015).

2.7.2. Ferric reducing antioxidant power (FRAP) assay

The total antioxidant power of supernatant was determined by measuring the ability of the homogenate to reduce Fe (III) to Fe (II). Interaction of TPTZ (2,4,6-Tri(2- pyridyl)-s-triazine) with Fe (II) leads to formation of a blue color complex, which has a maximum absorbance at 593 nm as described previously (Abdolghaffari et al., 2015; Benzie and Strain, 1996). This method was calibrated with Fe (II), as standard solutions. Data were expressed as µmol ferric ions reduced to ferrous per mg protein.

2.7.3. Determination of reduced glutathione (GSH)

Reduced GSH of supernatants was determined as described previously (Hu, 1994). In brief, 10 µL of supernatant was mixed with 200 µL of Tris–EDTA buffer (Tris base [0.25 M], EDTA [20 mM], pH 8.2) and the absorbance was measured at 412 nm (A_1). Then 4 µL of 10 mM DTNB (5, 5-dithiobis-2-nitrobenzoic acid) was added and after 15 min the absorption was measured again (A_2) together with a DTNB blank (B). Total reduced GSH was calculated as follows:

$$(A_2 - A_1 - B) \times 1.57\ \text{mM}$$

2.8. Biochemical analysis of blood serum

The serum levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and albumin were determined spectrophotometrically using commercial reagent kits, according to the manufacturer's instruction.

2.9. Histological preparations

Fragments of liver tissue were quickly removed and immersed in 10% neutral buffered formalin for at least 48 h to fix them for histopathological study. Then the samples were processed to paraffin embedded and cut into 6 µm sections. The sections were stained with haematoxylin and eosin (H&E) for examination by light microscopy.

3. Statistical analysis

All values are expressed a mean ± standard error of the mean (SEM). Data statistical analysis was performed by SPSS 16.0. Statistical significance was determined using the one-way ANOVA test, followed by the post-hoc Tukey test for multiple comparisons. The P value of less than 0.05 was considered to be statistically significant.

4. Results

4.1. Nano-TiO_2 characterization results

X-ray diffraction measurements (Fig. 2) show that the nano-TiO_2 had the anatase structure. The average crystalline size calculated from the (1 0 1) reflection, was 19 nm. SSA of TiO_2 sample was 45 m^2/g.

4.2. Preliminary study results

The effects of different doses of nano-TiO_2 (0, 10, 50, 100 and 200 mg/kg) on biochemical parameters in blood and liver tissue of rat are shown in Tables 1 and 2. The 10 mg/kg dose of nano-TiO_2 had no significant effect on liver function ($p > 0.05$). However, higher dose of nano-TiO_2 (100 and 200 mg/kg) significantly increased the MDA levels and ALT, AST and ALP activities, and decreased the total antioxidant power (FRAP values) and the activity of SOD and GPx ($p < 0.05$). Oral administration of 50 mg/kg for 60 consecutive days could not change all of the biochemical parameters compared to the control group. Thus, 100 mg/kg dose of nano-TiO_2 was chosen for main part of the study. This was the lowest effective dose of nono-TiO_2 that produced significant alterations in all oxidative stress and biochemical parameters, but that did not mean that the doses lower than 100 mg/kg did not have any toxic effects.

4.3. Coefficients of the liver

Fig. 3 illustrates the results of coefficients of the liver, which were expressed as milligrams (wet weight of liver)/grams (body weight). In terms of body weight, no significant differences were found among four treated groups (Data not shown). However, there was a significant

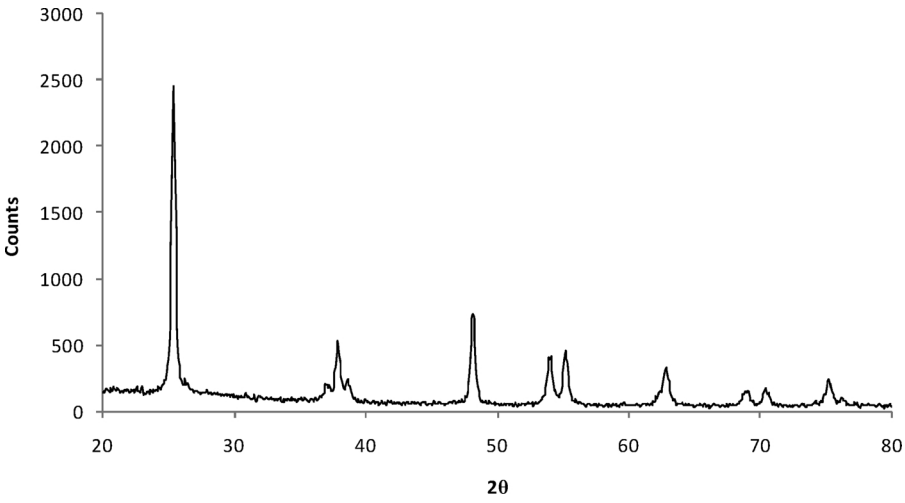

Fig. 2. The X-ray diffraction (XRD) pattern of nano-TiO_2. The average crystalline size was about 19 nm by calculation of Scherrer's equation.

increase in the coefficient of liver in the nano-TiO_2 treated group compared to the control group ($p < 0.05$). The coefficient of liver to body weight markedly decreased in rats orally treated with 30 mg/kg thymol compared with the nano-TiO_2 group ($p < 0.05$); so that there was no significant difference between the control group and 30 mg/kg thymol treated group ($p > 0.05$).

4.4. Antioxidative enzyme activities in rat liver

Oral administration of nano-TiO_2 for 60 consecutive days significantly reduced the activity of catalase, SOD and GPx in comparison with the control group ($p < 0.05$). The 10 mg/kg dose of thymol significantly improved these changes; however, there was significant change between the control and 10 mg/kg thymol treated group in terms of catalase and GPx activities. Higher dose of thymol (30 mg/kg) drastically increased catalase, SOD and GPx activities in rat-liver tissue comparing with nano-TiO_2 treated group. The activity of all three antioxidative enzymes in 30 mg/kg thymol treated group was near to that of the control group ($p < 0.05$, Table 3).

4.5. MDA, GSH levels and FRAP values in rat liver

In TiO_2 treated group, MDA levels were noticeably increased in comparison with the control group ($p < 0.05$) while the administration of thymol (at both doses) caused significant reduction in MDA levels ($P < 0.05$) and no significant difference was found between 30 mg/kg thymol treated group and the control group in terms of lipid peroxidation ($p > 0.05$).The reduced GSH and FRAP values were significantly decreased in the nano-TiO_2 treated group compared to the control ($p < 0.05$). Oral administration of thymol (especially 30 mg/kg) led to remarkable rise in the GSH levels and FRAP value as compared to nano-TiO_2 treated group ($p < 0.05$). The 10 mg/kg dose of thymol had no significant effect on these FRAP values ($p > 0.05$). These findings are depicted in Table 3.

4.6. Biochemical analysis of blood serum

As shown in Table 4, the serum levels of ALT, AST, ALP and albumin in nano-TiO_2 treated group were significantly increased compared to the control group ($p < 0.05$). In contrast, oral administration of thymol significantly ameliorated these changes in a dose-dependent manner. The 10 mg/kg dose of thymol significantly decreased the serum level of ALT and AST but had no significant effect on ALP and albumin levels ($p > 0.05$), while higher dose of thymol (30 mg/kg) significantly improved the serum levels albumin and all these enzymes to normal range.

4.7. Histopathological findings

The liver histological photomicrographs are illustrated in Fig. 4. Histological studies revealed that the liver sections from control group showed normal histoarchitecture showing hepatocytes radiating from central vein (Fig. 4A). In nano-TiO_2 treated group, widespread liver tissue damage such as, central vein and sinusoid space dilation and marked vacuolization and leukocyte infiltration were observed (Fig. 4B). In 10 mg/kg thymol administration group, hepatocellular damage was decreased (Fig. 4C). Administration of 30 mg/kg thymol with nano-TiO_2 lead to obvious improvement of hepatic tissue and only foci of hepatocellular damage with moderate leukocyte infiltration could been seen (Fig. 4D).

5. Discussion

Nanomaterials are increasingly being used in widespread applications due to their unique physicochemical properties. As a result, the potential deleterious effect of nanomaterials exposure on human health has become a matter of concern (Sha et al., 2015). Among nanomaterials, nano-TiO_2 is extensively used in a variety of consumer products such as sunscreens, cosmetics, toothpastes, food colorants, nutritional

Table 1
The changes of biochemical parameters in rat blood serum after intragastric administration of nano-TiO_2 for consecutive 60 days.

Parameters	Nano-TiO_2 (mg/kg BW)				
	0	10	50	100	200
ALT(U/L)	32.67 ± 1.31	37.13 ± 1.22	41.21 ± 2.68	61.93 ± 2.34[a]	64.56 ± 2.9[a]
AST(U/L)	143.46 ± 4.91	157.02 ± 7.58	189.31 ± 6.39[a]	203.26 ± 8.15[a]	243.56 ± 6.32[a]
ALP(U/L)	126.11 ± 6.23	124.83 ± 5.52	131.67 ± 8.36	164.32 ± 6.47[a]	197.81 ± 10.21[a]

Data are mean ± SEM of four animals in each group.
[a] Significantly different from the control (no nano-TiO_2) $p < 0.05$.

Table 2
The activities of antioxidative enzymes, MDA and FRAP value of rat liver tissue after intragastric administration of nano-TiO_2 for consecutive 60 days.

Parameters	Nano-TiO_2 (mg/kg BW)				
	0	10	50	100	200
GPx (U/mg protein)	38.74 ± 1.42	33.12 ± 2.76	26.38 ± 1.16[a]	19.31 ± 3.08[a]	14.77 ± 2.58[a]
SOD (U/mg protein)	17.37 ± 1.13	18.15 ± 1.36	15.93 ± 1.71	10.91 ± 0.93[a]	7.23 ± 2.24[a]
MDA content (nmol/mg protein)	81.32 ± 4.64	92.57 ± 6.41	125.49 ± 3.97[a]	165.28 ± 7.25[a]	191.21 ± 6.18[a]
FRAP value (μmol/mg protein)	1.43 ± 0.02	1.31 ± 0.08	1.29 ± 0.11	0.96 ± 0.04[a]	0.63 ± 0.06[a]

Data are mean ± SEM of four animals in each group.
[a] Significantly different from the control (no nano-TiO_2) $p < 0.05$.

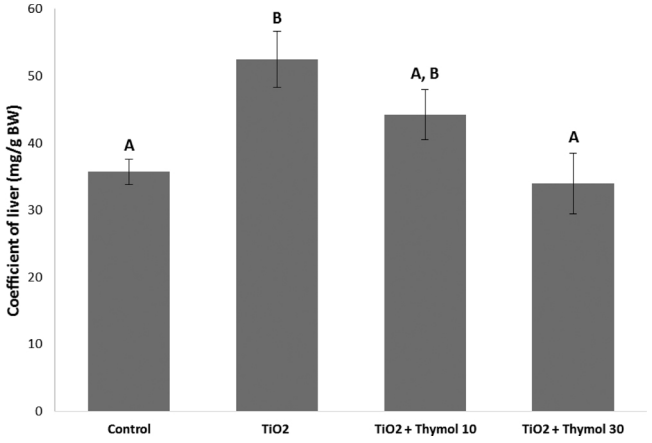

Fig. 3. Effects of various treatments on the coefficient of the liver. Data are mean ± SEM of six animals in each group. Different letters indicate significant differences among groups ($p < 0.05$).

supplements, drugs, etc. (El-Zahed et al., 2015). Exposure to high doses of this nanoparticle rarely occurs, while individuals are repetitively exposed to low doses that can be potentially harmful. There are few investigations on the long-term consequences of nano-TiO_2. Thus, we first exposed rats with low doses of TiO_2 nanoparticles daily for 60 days to determine the lowest effective dose that can produce significant alteration in all biochemical parameters. Owing to its unique properties such as very small size, large surface area and high reactivity, nano-TiO_2 can easily enter the body via different routes and accumulate in organs in a dose-dependent manner resulting in histopathological damage (Iavicoli et al., 2012). The rationale for using liver tissue lies in the fact that TiO_2 nanoparticles tend to accumulate in this organ and result in liver damage or injury (El-Zahed et al., 2015; Hong and Zhang, 2016). Exact mechanism of liver toxicity for nano-TiO2 is still not completely clear, however, several possible mechanisms have been proposed based on previous studies (Cui et al., 2010; Cui et al., 2011; Hong and Zhang, 2016; Liu et al., 2010; Meena and Paulraj, 2012; Ze et al., 2013). Oxidative stress is one of the most important toxicity mechanisms of nano-TiO_2 in the liver (Iavicoli et al., 2012; Liu et al., 2010; Meena and Paulraj, 2012; Ze et al., 2013), and that is why we examined oxidative stress biomarkers along with serum liver markers and histological parameters in this study.

Data from the present study demonstrated that oral administration of nano-TiO_2 (100 mg/kg) for 60 consecutive days caused liver damage in a dose-dependent manner as supported by the works of Rizk *et al.*, Meena et al. and Duan et al. (Duan et al., 2010; Meena and Paulraj, 2012; Rizk et al., 2017). Based on the biochemical parameters used in the present study, the 10 mg/kg dose of this nanoparticle had no significant effect on liver function, which was probably due to the fact that the rat body was able to adapt to this dose of nanoparticle after 60 days and neutralized its adverse effects. Oral administration of 50 mg/kg of nano-TiO_2 could not significantly change all of the biochemical parameters compared to the control group and only GSH and MDA levels and the activities of AST, GPx and catalase were significantly changed. However, nano-TiO_2 at doses of 100 and 200 mg/kg produced significant changes in all biochemical parameters comparing with the control group. Therefore, the 100 mg/kg dose of nano-TiO_2 was used to induce liver toxicity in the main part of this study. Although it is almost unlikely that someone are exposed to a dose of 100 mg for 60 consecutive days, this dose has been chosen in the present animal study to fully understand if thymol, as an antioxidant, was able to reverse or inhibit the biochemical and histological changes induced by long term exposure to nano-TiO_2. The significant increase in the serum level of ALT, AST and ALP following oral administration of nano-TiO_2 (100 mg/kg) indicated a liver damage, which were in agreement with previous reports on the toxicological properties of this nanoparticle (Duan et al., 2010). Duan et al. orally administered doses of 62.5, 125 and 250 mg/kg nano-TiO_2 to mice. In doses higher than 125 mg/kg, this nanoparticle caused the significant changes in the serum parameters after 30 days, while Cui et al. reported that the biochemical parameters in the blood serum of mice significantly changed at a dose of 10 mg/kg or higher following intragastric administration with nano-TiO_2 (7 nm) for consecutive 60 days (Cui et al., 2010; Cui et al., 2011). On the contrary, we found no significant changes on biochemical parameters in 10 mg/kg nano-TiO_2 treated group, which was probably due to the fact that we used TiO_2 nanoparticles of 19 nm in size (Fig. 2). In another study, the effect of different doses (50, 250 and 500 mg/kg) and exposure time durations (7, 14 and 45 days) of nano-TiO_2 were evaluated by

Table 3
Effects of various treatments on indicators of oxidative stress in rat liver tissue.

Parameters	Control	Nano-TiO_2 (100 mg/kg)	Nano-TiO_2 + Thymol 10 mg/kg	Nano-TiO_2 + Thymol 30 mg/kg
Catalase (U/mg protein)	71.31 ± 1.93	36.96 ± 2.61[a]	50.73 ± 2.76[a,b]	73.14 ± 3.38[b]
GPx (U/mg protein)	41.33 ± 1.83	18.47 ± 2.59[a]	26.71 ± 1.36[a,b]	37.83 ± 2.17[b]
SOD (U/mg protein)	19.37 ± 0.89	10.52 ± 1.13[a]	17.16 ± 0.92[b]	18.34 ± 1.94[b]
MDA content (nmol/mg protein)	78.31 ± 3.74	170.23 ± 6.35[a]	136.37 ± 5.26[a,b]	102.94 ± 9.04[b]
GSH (nmol/mg protein)	113.54 ± 4.66	49.16 ± 10.19[a]	85.31 ± 7.37[b]	96.53 ± 9.13[b]
FRAP (μmol/mg protein)	1.38 ± 0.03	0.89 ± 0.07[a]	0.97 ± 0.03[a]	1.22 ± 0.13[b]

Data are mean ± SEM of six animals in each group.
[a] Significantly different from the control $p < 0.05$.
[b] Significantly different from the nano-TiO_2 group at $p < 0.05$.

Table 4
Effects of various treatments on biochemical parameters in rat blood Serum.

Parameters	Control	Nano-TiO_2 (100 mg/kg)	Nano-TiO_2 + Thymol 10 mg/kg	Nano-TiO_2 + Thymol 30 mg/kg
ALT (U/L)	38.39 ± 1.11	59.76 ± 0.88[a]	40.33 ± 1.33[b]	39.46 ± 1.83[b]
AST (U/L)	139.54 ± 5.21	209.27 ± 7.48[a]	176.12 ± 4.86[a,b]	152.14 ± 9.31[b]
ALP (U/L)	121.14 ± 4.46	172.87 ± 6.39[a]	158.71 ± 5.42[a]	132.33 ± 9.31[b]
Albumin (g/dl)	4.21 ± 0.09	3.06 ± 0.07[a]	3.11 ± 0.21[a]	3.68 ± 0.08[b]

Data are mean ± SEM of six animals in each group.
[a] Significantly different from the control group at $p < 0.05$.
[b] Significantly different from the nano-TiO_2 group at $p < 0.05$.

histological, biochemical, oxidative stress markers and results demonstrated that the liver function were prominently influenced in a dose and time dependent manner by nano-TiO_2 (Rizk et al., 2017). It seems that besides dose, exposure time and particle size play important role in toxicological effects of nanoparticles (Rizk et al., 2017; Wang et al., 2007).

Considering the widespread application of nanoparticles and the likelihood of their adverse health effects following repeated exposure, even at low doses, it is therefore necessary to consider the ways to reduce the long-term consequences of nanoparticles on human health and facilitate their safe use. In this regard, thymol, which possesses free radical scavenging and anti-lipid peroxidation activities, was studied to reduce and prevent liver damage-induced by long-term exposure to nano-TiO_2. The results showed that thymol can effectively protect against TiO_2 nanoparticles-induced toxicity in a dose-dependent manner. Nano-TiO_2 (100 mg/kg) significantly increased the serum levels of AST, ALT and ALP, indicating hepatocellular damage (Aboelwafa and Yousef, 2015). These enzymes in the blood serum are valuable quantitative biomarkers for the extent and type of liver injury, since they are located in cytoplasm and mitochondrion and released into the blood circulation from the hepatocytes owing to the damage (Grespan et al., 2014). Also, nano-TiO_2 significantly decreased the serum levels of albumin, reflecting a decrement in protein synthesis or increment in protein catabolism (Aboelwafa and Yousef, 2015). Pretreatment with thymol for 60 days prior to nano-TiO_2 administration caused significant elevation in the serum albumin level and marked decline in the serum levels of ALT, AST, and ALP compared with the nano-TiO_2 treated group and the levels of these biomarkers in 30 mg/kg thymol treated group were near to that of the control group. The current result supports the report of Aboelwafa and Yousef (2015) that thymol decreased transaminases activities, leading to inhibition of liver toxicity induced by hydrocortisone in adult male rats (Aboelwafa and Yousef, 2015). Janbaz et al. and Raskovic et al. showed that thymol inhibited carbon tetrachloride-induced hepatic damage by preventing the alterations in the activities of liver marker enzymes (Janbaz et al., 2003; Rašković et al., 2015).

In liver tissue, the activity of catalase, SOD and GPx significantly reduced following oral administration of nano-TiO_2 for 60 consecutive days. Pretreatment of TiO_2 nanoparticles-intoxicated rats with thymol, especially at a dose of 30 mg/kg, significantly improved the activity levels of all three antioxidative enzymes. In addition, the elevated MDA levels accompanied by significant decline in levels of FRAP and reduced

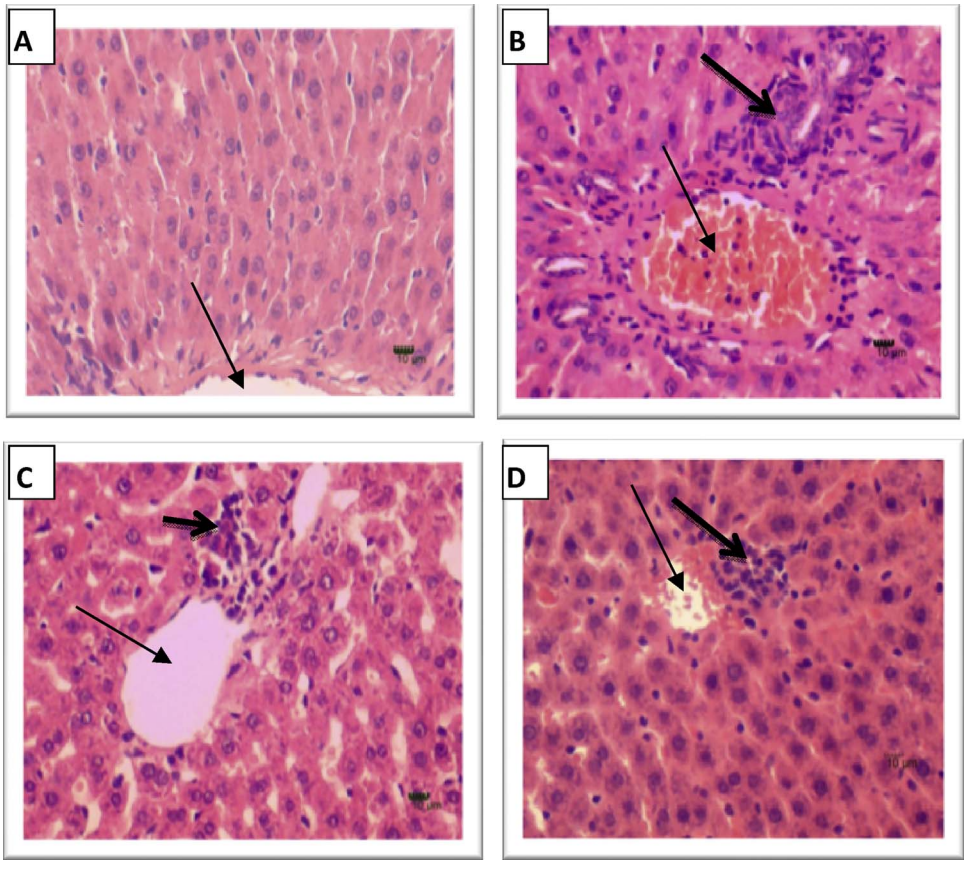

Fig. 4. Photomicrographs showing liver section of rats from different groups. (A) Control group; (B) Nano-TiO_2 (100 mg/kg) administration group; (C) Nano-TiO_2 and thymol 10 mg/kg group; (D) Nano-TiO_2 and thymol 30 mg/kg group. Thick arrows indicate leukocyte infiltration and thin arrows show central vein. (H&E stain X100).

GSH in liver tissues of rats administrated with nano-TiO_2 indicated the presence of oxidative stress and lipid peroxidation response. The administration of thymol at both doses caused significant reduction in MDA levels and in 30 mg/kg thymol treated group were near to that of the control group. Treatment by higher dose of thymol (30 mg/kg) led to remarkable rise in the GSH levels and FRAP value as compared to nano-TiO_2 treated group. The biochemical results were confirmed by the histopathological findings that revealed the widespread liver tissue damage including central vein and sinusoid space dilation, marked vacuolization and leukocyte infiltration in nano-TiO_2 treated group. Hepatocellular damage was decreased in higher dose of thymol and only foci of hepatocellular damage with moderate leukocyte infiltration could be seen. These results confirm those reported by Al-Malki et al. and Alam et al. in their studies on the antioxidant properties of thymol against carbon tetrachloride-induced oxidative stress in mice liver (Al-Malki, 2010; Alam et al., 1999). Pretreatment of carbon tetrachloride-intoxicated mice with thymol decreased lipid peroxidation and enhance the status of antioxidants thereby preventing oxidative stress mediated liver damage according to the biochemical and histological findings (Al-Malki, 2010; Alam et al., 1999; Jiménez et al., 1993). Similar results were obtained in *in vitro* studies (Kim et al., 2014; Palabiyik et al., 2016). Kim et al. showed that thymol prevented oxidative damage by decreasing ROS production, ameliorating lipid peroxidation, preventing apoptosis and restoring the antioxidant capability of liver cells which were reduced by *tert*-butyl hydroperoxide (Kim et al., 2014). Another study found that thymol enhanced both non-enzymatic and enzymatic antioxidant levels and prevented lipid peroxidation which was induced by paracetamol in HepG2 cells (Palabiyik et al., 2016).

These beneficial effects of thymol are attributed to the phenolic hydroxyl group in its structure. The phenolic compounds are potentially able to protect against the harmful effects of ROS both by absorbing or neutralizing them. Thymol is also able to enhance the activities/levels of endogenous antioxidant enzymes such as SOD, catalase, GPx together with non-enzymatic antioxidants such as reduced GSH (Meeran et al., 2017). (Kruk et al., 2000) reported that thymol can directly remove superoxide radicals like SOD *in vitro* (Kruk et al., 2000). Moreover, thymol possesses superior reducing capacity, hydroxyl radical and superoxide scavenging activity and protects cells against lipid peroxidation in a concentration dependent manner (Meeran et al., 2017; Meeran and Fizur, 2012). A well-defined mechanism for antioxidative effects of thymol is not known and need to be further elucidated. However, Sasaki et al. reported that thymol like other phenolic compounds can potentially upregulate transcription of antioxidant enzymes (e.g. glutathione S-transferase), which in turn offer protection against oxidant agents and various stress conditions (Shettigar et al., 2015).

In conclusion, the data present in this study demonstrated that thymol can effectively protect against TiO_2 nanoparticles-induced hepatotoxicity in male rats. Thymol exerts it protective effects via preventing oxidative stress, ameliorating lipid peroxidation, and augmenting non-enzymatic and enzymatic antioxidant levels of liver cells, as evidenced by all biochemical and histological parameters. Although extrapolation of these results to human is not appropriate, it seems that oral administration of thymol might be able to reduce the long-term consequences of TiO_2 nanoparticles on human health and facilitate their safe use.

Conflict of interest

The authors declare that there are no conflicts of interest.

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