Elimination of Oxidative Stress and Genotoxicity of Biosynthesized Titanium Dioxide Nanoparticles in Rats via Supplementation with Whey Protein-Coated Thyme Essential Oil

Mosaad Attia Abdel-Wahhab (mosaad_abdelwahhab@yahoo.com)
National Research Center https://orcid.org/0000-0002-7174-3341
Aziza A. El-Nekeety
National Research Centre
Hagar E Mohammed
Sinai University - El Arish Campus
Ola I. Elshafey
National Research Centre
Sekena H. Abdel-Aziem
National Research Centre
Nabila S. Hassan
National Research Centre

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Abstract

The green synthesis of metal nanoparticles is growing dramatically; however, the toxicity of these biosynthesized particles against living organisms is not fully explored. Therefore, this study was designed to synthesize and characterize TiO_2-NPs, encapsulation and characterization thyme essential oil (ETEO), determination of the bioactive constituents of ETEO using GC-MS and evaluate their protective role against TiO_2-NPs-induced the oxidative damage and genotoxicity in rats. Six groups of rats were treated orally for 30 days including the control group, TiO_2-NPs (300 mg/kg b.w)-treated group, ETEO at low (50 mg/kg b.w) or high dose (100 mg/kg b.w)-treated groups TiO_2-NPs plus ETEO at the two doses-treated groups. Blood and tissues were collected for different assays. The GC-MS results indicated the presence of 21 compounds belongs to phenols, terpene derivatives, and heterocyclic compounds. The synthesized TiO_2-NPs were 45 nm tetragonal particles with a zeta potential of -27.34 mV; however, ETEO were 119 nm round particles with a zeta potential of -28.33 mV. TiO_2-NPs administration disturbs the liver and kidney markers, lipid profile, cytokines, oxidative stress parameters, the apoptotic and antioxidant hepatic mRNA expression and induced histological alterations in the liver and kidney tissues. ETEO could improve all these parameters in a dose-dependent manner. It could be concluded that ETEO is a promising candidate for the protection against TiO_2-NPs and can be applied safely in food applications.

Introduction

Recently, nanotechnology has developed rapidly in different sectors to improve human life leading to the production of several nanomaterials which developed and extensively used in various fields including industry, food, medicine (Li et al. 2018; Sycheva et al. 2011; Wang et al. 2016; Zahin et al. 2020), personal health care (Sanders et al. 2012; Khosravi et al. 2012) toothpastes (Li et al. 2012), food packaging (Jovanović and Palić 2012; Philbrook et al. 2011) and antimicrobial agents (Martínez-Gutierrez et al. 2012). Titanium dioxide NPs (TiO_2-NPs) are the most common manufactured worldwide (Jomini et al. 2015). Inhalation of TiO_2-NPs in mice leads to accumulate these NPs in the hepatic and cardiac tissue and transfer to the circulation after 24 h (Husain et al. 2015). After oral administration, TiO_2-NPs accumulate in different organs mainly the liver, heart, brain and lung inducing damage and inflammation to these organs (Geraets et al. 2014; Kandeil et al. 2020). Additionally, the abdominal injection of mice with TiO_2-NPs accumulates these particles in different organs and induces severe damage to the liver, heart and kidneys and affects the serum lipids and sugar (Liu et al. 2009). TiO_2-NPs activate the inflammatory processes and complement cascade in the heart and innate the immune responses mediated by the complement factor 3 in the blood. However, in the liver, these particles alter the gene expression especially that related to the acute phase response (Husain et al. 2015).

Essential Oils (EOs) are the secondary metabolites extracted from different aromatic plants and are categorized as generally recognized as safe (GRAS) by the FDA (Hyldgaard et al. 2012). Thyme (*Thymus vulgaris* L) is a medicinal aromatic plant (Lamiaceae family) has been widely used in food, medicine and agriculture (Morales 2002; Tao et al. 2014). Thyme essential oil (TEO) showed a variety of beneficial biological activities which include antioxidant, antitumor, antimicrobial and anti-inflammatory properties (Nikoli et al. 2014). However, the application of EOs in the food sector is faced by some challenges such as the interaction with different food matrix including proteins, starch and fats (Hyldgaard et al. 2012), their strong volatile characteristic (Khalili et al. 2015). Besides, EOs may alter the sensory characteristics of the foods when used in high concentration, their poor solubility in the aqueous phase (Tao et al. 2014; Barbosa-Cánovas et al. 2009), their sensitivity to light, heat and oxygen during food processing, storage and utilization (Woranuch and Yoksan 2013). To overcome these problems, encapsulation process of EOs may be suitable to solve the challenges face the applications of EOs by enhancing solubility and bioavailability, protection

against thermal and chemical degradation and control the delivery release at the desired site and time (Tao et al. 2014). The current study was designed to synthesize and characterize TiO_2-NPs by green chemistry using orange peel extract, encapsulate and characterize TEO, and evaluate the potential protective activity of the encapsulated TEO (ETEO) against the oxidative damage and genotoxicity of TiO_2-NPs in rats.

Materials And Methods

Chemicals and Kits

Titanium tetra isopropoxide (TTIP) and whey protein isolate (WPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kits for transaminase (ALT and AST) were purchased from Randox Co, (Antrim, UK), triglycerides (TriG), cholesterol (Cho), low and high density lipoprotein (LDL, HDL), creatinine, urea, albumin (Alb) and total protein (TP) were purchased from FAR Diagnostics Co. (Via Fermi, Italy). Kits for nitric oxide (NO), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) were obtained from Eagle diagnostics (Dallas, TX, USA). Malondialdehyde (MDA) kit was purchased from Oxis Research TM Co. (USA). ELISA kit for alpha feta protein (AFP) was purchased from BiochemImmuno Systems Co. (Montreal, Canada). Tumor necrosis factor-alpha (TNF-α) kit was purchased from Orgenium (Helsinki, Finland). A Kit for measuring carcinoembryonic antigen (CEA) was obtained from Biodiagnostic (Giza, Egypt). PreMixc DNA Kit was obtained from iNtRON Biotechnology (Korea).TRIZOL reagent was purchased from Life Technologies Corporation (Grand Island, NY, USA).

Preparation of TiO_2-NPs

Fifty gram of the peel of orange was extracted for 2 h using 150 ml of deionised water at 90 ^oC. The extract was filtered and was used for the biosynthesis of TiO_2-NPs (Balashanmugam et al. 2013). TTIP (1.5 N) was dissolved in distilled water (100 ml) and the extract was added dropwise with constant stirring and pH was maintained to 7 by continuous washing. The mixture was stirred continuously for 6 h in the light at room temperature. TiO_2-NPs were synthesized, separated using Whatman filter paper, and washed with distilled water several times to remove any by-products. The obtained wet nanoparticles were dried at 80 ^oC overnight and were calcined at 600 ^oC for 4 h (Rao et al. 2015).

Extraction of TEO

One hundred g of the plant materials (flowering tops and stalks) were placed in 1 liter of water in a round-bottomed flask which connected with Clevenger-type apparatus and the hydro-distillation was performed for 4 h by boiling of water. The resulted essential oil was dried over anhydrous sodium sulfate and stored at 4 ^oC until used.

GC-MS analysis of TEO

GC-MS analysis was conducted using Hewlett-Packard model 5890 with a flame ionization detector (FID) and DB-5 fused silica capillary column (60 m x 0.32 mm). The temperature of the oven was maintained initially at 50°C for 5 min and then programmed from 50 to 250°C at a rate of 4°C/min. The carrier gas was helium at a flow rate of 1.1 ml/min. The temperatures of the detector and injector were 250 and 220°C, respectively, and the retention indices (Kovats index) of the separated volatile components were calculated using hydrocarbons as references (C7-C20, Aldrich Co.) as shown by Admas (2007).

Preparation of encapsulated thyme essential oil (ETEO)

Whey protein isolate (WPI) was dissolved in distilled water with stirring for 1 h. The solution was kept overnight at room temperature before emulsification. Tween 80 was added to the polymer as an emulsifier. Then, the essential oil was progressively added to the polymer solution with homogenization at 20000 rpm for 10 min to form an emulsion. The polymer concentration was 20%, and the amount of essential oil used was 10% of the mass of the polymer concentration (Jinapong et al. 2008). The emulsion solution was encapsulated by spray drying.

Characterization of TiO_2-NPs and ETEO

Scanning electron micrographs (SEM) for TiO_2NPs were recorded on JEOL JAX-840A and JEOL JEM- 1230 electron micro-analyzers, respectively. However, for ETEO, the droplets were placed onto a carbon-coated copper grid to form a thin liquid film and were negatively stained by one drop of uranyl acetate. The excess staining was removed using filter paper then the film was air-dried before the observation (Pecarski et al., 2014). The image acquisition was done using Orius 1000 CCD camera (GATAN, Warrendale, PA, USA). For measuring zeta potential, the sample of TiO_2-NPs or ETEO was sonicated for 30–60 min just before assessment. The average diameter The average diameter was calculated using zpw 388 version 2.14 nicomp software. The size distribution and the zeta potential of TiO_2-NPs and ETEO were measured using a particle size analyzer (Nano-ZS, Malvern Instruments Ltd., UK).

Animals and experimental design

Sixty sexually mature male Sprague-Dawley rats (3 months old, 150–160 g) were supplied by the Animal House Lab, National Research Center (NRC), Dokki, Cairo, Egypt. The rats were housed in stainless steel cages in an artificially illuminated (12 h dark/light cycle) and thermally controlled (25 ± 1 ^{o}C) room free from any source of chemical contamination at the Animal House Lab, NRC, Dokki, Cairo, Egypt. All animals were fed normal chow (Meladco Feed Co., Cairo, Egypt) and housed in filter top polycarbonate cages. The animals were received humane care in compliance with the guidelines of the Animal Care and Use Committee of NRC, and the National Institute of Health (NIH publication 86 – 23 revised 1985). After an acclimatization period of one week, the rats were divided into 6 groups (10 rats/group) and were treated orally using stomach tube for 30 days as follows: group1; normal control received distilled water, group 2; rats treated with TiO_2-NPs (300 mg/kg b.w) in an aqueous solution, group 3; rats treated with low dose (LD) of ETEO (50 mg/kg b.w), group 4; rats treated with high dose (HD) of ETEO (100 mg/kg b.w), groups 5 and 6; rats treated with LD or HD of ETEO plus TiO_2-NPs. Body weight was recorded each other day and at the end of the treatment period (i.e. day 31), all animals have fasted for 12 hr, weighed then blood samples were collected via the retro-orbital venous plexus under isoflurane anesthesia. Sera were centrifuged using cooling centrifuge and kept at -20 ^{O}C until used for the determination of ALT, AST, lipid profile creatinine, urea, total protein, albumin, AFP, TNF-α and CEA according to the kits instructions. Immediately after blood samples were collected, all animals were euthanized and samples of hepatic and renal tissue from each animal were dissected. A sample of liver and kidney was weighed and homogenized in phosphate buffer (pH 7.4), centrifuged at 1700 rpm and 4 ^{o}C for 10 min, and the supernatant was separated and used for the estimation of MDA, NO, CAT, GPx, and SOD (Lin et al., 1998). Other samples of these organs from each animal were used for the histological examination. The tissue samples were fixed in 10% neutral formalin and paraffin-embedded. Sections (5 µm thickness) were stained with hematoxylin and eosin (H & E) for the histological examination (Bancroft et al., 1996). However, another sample of liver of each animal was quickly frozen using liquid nitrogen and kept at -80 ^{o}C for the determination of gene expression.

Gene expression analysis

RNA was isolated from the liver samples using Trizol reagent. The concentration and the quality of RNA were determined by NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA), and only samples of high

quality, with A260/A280 ratios between 1.8 and 2.2 were used. Reverse transcription- cDNA synthesis was carried out using a PreMix cDNA Kit (iNtRON Biotechnology, Korea). The obtained cDNA was kept at -20 °C for later use or directly used as a semi-quantitative PCR (Sq-PCR) template. The expression of the selected genes was quantified using quantitative real-time PCR (RT-qPCR) performed in a One-Step SYBR Select Master Mix Kit as previously described (Kim et al. 2009). The gene-specific primer sequences for GAPDH, GPx, SOD, CAT, Bax, Bcl-2, and TNF-α are shown in Table (1). RT-qPCR was carried out on Stratagene Mx3005P RT-PCR System (Agilent Technologies) in a 20-µL reaction volume using, 1 µLcDNA, 10 µM of forward and reverse primers, 10 µL TOP real™ qPCR 2× PreMIX (SYBR Green with low ROX) (Enzynomics) and DNAse-free water. All samples were amplified in triplicates and the amplification was conducted with a 15-min denaturation at 95°C, then 40 cycles of 95°C for 12 s, 56–63°C for 15 s, and 72°C for 30 s.

The PCR cycle number (C_T) was used for the calculation of expression level where the increased fluorescence curve passes across a threshold value. However, the relative expression of the target genes was obtained using the comparative C_T ($\Delta\Delta C_T$) method. The ΔC_T was calculated by subtracting β-actin C_T from that of the target gene; whereas, $\Delta\Delta C_T$ was obtained by subtracting the ΔC_T of the calibrator from that of the test sample. The relative expression was calculated from the $2^{-\Delta\Delta CT}$ formula based on the method of Pfaffl (2001) and Abdel-Wahhab et al. (2021).

Statistical analysis

Statistical analyses were carried using SPSS 16. Data were expressed as mean ± SE. Variables were compared using one-way ANOVA; post hoc Duncan's test and the significance of differences among means were determined at $p \leq 0.05$.

Results

The GC-MS analysis of ETEO identified 21 compounds that represent 130.85 mg/g ETEO and belong to different classes including phenols, terpene, terpene derivatives, and heterocyclic compounds (Table 2). The major compounds were thymol, para-cymene, linalool, carvacrol, camphor, eucalyptol, γ-terpinene, borneol, terpinene-4-ol, β- pinene, β-Phellandrene, β-myrcene and Humuline in concentrations of 55.40, 12.1, 8.21, 7.18, 6.21, 6.11, 5.31, 5.12, 4.22, 2.31, 2.14, 2.11 and 2.10 mg/g ETEO, respectively. Besides, 8 compounds were found in concentrations less than 2 mg/g.

The present results showed that addition of the extract of orange peel to TTIP and stirring in light for 6 h at the room temperature and pH 7 resulted in the synthesizing of the tetragonal structure of TiO_2-NPs (Fig.1a) with an average size of 45 nm (Fig. 1b) and a zeta potential of -27.34 mV (Fig. 1c). Moreover, the SEM analysis of ETEO showed a round shape (Fig. 1D) with an average particle size of 110 nm (Fig. 1E) and a zeta potential of -28.33 mV (Fig. 1F).

The *in vivo* results revealed that TiO_2-NPs significantly decreased the body weight of rats; however; ETEO did not affect the body weight either at the low or high dose (Fig. 2). The co-treatment with TiO_2-NPs plus ETEO at both tested doses improved the body weight and no significant difference was observed between the two tested doses. The biochemical indices (Table 3) showed that all the tested parameters were significantly elevated in the group treated with TiO_2-NPs except TP and Alb which were decreased significantly. ALT, AST and creatinine were decreased significantly in the animals received ETEO (LD), but no significant change was observed in Alb, TP, D. BIL, urea, and uric acid. However, administration of ETEO (HD) showed a significant decrease in the liver enzymes (ALT, AST) and kidney (creatinine and uric acid) and did not affect the other parameters. Co-administration with TiO_2-NPs plus ETEO (LD) or ETEO (HD) improved all the biochemical parameters and ETEO (LD) could normalize Alb, TP, and T. BIL;

meanwhile, ETEO (HD) could normalize all these parameters except ALT and uric acid which were still higher than the control level.

The current data (Table 4) showed the effect of different treatments on lipid profile. Treatment with TiO_2-NPs increased the lipid profile parameters except HDL which was decreased significantly. Administration of ETEO (LD) increased HDL without any effect on the other lipid parameters; however, ETEO (HD) increased HDL and LDL with no effect on cholesterol or TriG. All the lipid parameters were improved significantly in the groups that received the combined treatment and the low dose could normalize TriG, HDL, and LDL; besides, the high dose could normalize HDL and LDL. Additionally, TiO_2-NPs induced a significant increase in hepatic and renal NO and MDA (Table 5). ETEO (LD) or ETEO (HD) decreased both parameters in a dose-dependent fashion. Animals that received TiO_2-NPs plus ETEO (LD) or ETEO (HD) showed a significant improvement in these markers in a dose-dependent manner. Additionally, TiO_2-NPs decreased the activity of the antioxidant enzymes (CAT, SOD, and GPx) in the hepatic and renal tissue (Table 6). Meanwhile, no significant changes were noticed in these enzymes in rats that received ETEO alone at the two doses although the renal GPx and hepatic SOD were significantly increased in these groups. A significant improvement was observed in the animals received the combined treatment of TiO_2-NPs plus ETEO, especially the hepatic GPx which was in the normal range of the control. Furthermore, TiO_2-NPs administration also increased the serum TNF-α, CEA, and AFP (Table 7). ETEO at the low or the high dose did not affect TNF-α and AFP; however, CEA was decreased significantly than the control group. TNF-α returned to normal in the rats treated with TiO_2-NPs plus ETEO (LD) or (HD); meanwhile, AFP and CEA were improved significantly especially in the group that received ETEO (HD).

A quantitative PCR was carried out to estimate the changes in hepatic mRNA gene expression of pro-apoptotic Bax (Fig. 3A), TNF-α (Fig. 3B), and the antiapoptotic Bcl-2 (Fig. 3C) in different treatment groups. TiO_2-NPs alone increased significantly the mRNA expression of Bax and TNF-α and decreased Bcl-2 mRNA expression. Moreover, GAPDH (housekeeping glyceraldehyde-3-phosphate dehydrogenase gene) indicated an insignificant difference between the untreated control group and those treated with ETEO at the two tested doses in Bax, TNF-α, and Bcl-2 expressions. However, administration of TiO_2-NPs plus ETEO at both tested doses could induce remarkable improvement in the transcript levels of these genes.

The cytogenetic study of antioxidant gene expression showed that TiO_2-NPs administration markedly reduced the mRNA expression of GPx (Fig. 4A), SOD (Fig. 4B), and CAT (Fig. 4C) compared to the control group. Treatment with ETEO (LD) or (HD) increased significantly the mRNA of GPx gene expression; however, these treatments didn't affect SOD or CAT gene expression. Administration of ETEO at the tested doses plus TiO_2-NPs improved the mRNA expression of these antioxidant genes towards the control level although these values were still differed significantly from the control group.

The examination of liver sections of the control animals showed the hepatocytes with normal cytoplasm with vesiculated nuclei and separated by blood sinusoids radiating from the central vein (Fig 5A). The liver section of the animals treated with ETEO (LD) showed no observable changes in hepatocytes architecture except few fibrous tissues around the central vein and bile duct hypertrophy (Fig. 5B); however, those treated with ETEO (HD) showed nearly normal hepatocytes architecture (Fig. 5C). The examination of liver sections of animals treated with TiO_2-NPs showed marked histological alterations in hepatocytes as disorganization, vacuolar, and fatty degeneration shrunken hepatocytes with darkly stained pyknotic nuclei, patches of necrotic cells around the dilated and congested portal tract, and numerous mononuclear cellular infiltrations localized in dilated hepatic sinusoids (Fig. 5D). The liver of rats administrated with TiO_2-NPs plus ETEO (LD) showed several histological changes in hepatocytes as vacuolar, fatty

degeneration, pyknotic nuclei and increase in mononuclear inflammatory cells (Fig. 5E). Additionally, the liver of animals in the group that received TiO_2-NPs plus ETEO (HD) showed more improvement in hepatic cells stricter but few inflammatory cells were also seen (Fig. 5F).

The examination of kidney cortex sections of the control animals showed the proximal convoluted tubules with high cuboidal acidophilic cells, narrow lumen, and distal convoluted tubules with low cuboidal cells. The renal corpuscle with the parietal layer of Bowman's capsule, glomerulus, and the urinal renal space was preserved (Fig. 6A). The kidney cortex sections of the rats treated with ETEO (LD) showed normal tubules; glomeruli with epithelial cells except some renal tubules showed a slight hyaline droplet in their lumen and widening of the Bowman's space (Fig. 6B). The kidney cortex of the animals treated with ETEO (HD) showed dilatation of tubular lumen with slight cytoplasmic hyaline but the majority of renal tubules, glomeruli, and Bowman's capsule were nearly normal (Fig. 6C). The examination of the renal cortex of animals treated with TiO_2-NPs showed vacuolar degeneration in the cytoplasm of tubular epithelial cells with deeply stained pyknotic nuclei in some renal tubules. Some of the lining epithelial cells were exfoliated into the lumen with hyaline droplets. The renal capsules were shrunken with a reduction in glomeruli mesangial cells and mononuclear cell infiltrations between degenerated tubule and renal corpuscle (Fig. 6D). The kidney cortex of animals treated with TiO_2-NPs plus ETEO (LD) showed few foci of vacuolation in the tubular epithelial cells or hyaline cytoplasmic droplet in their lumen but the majority of renal tubules and glomeruli were nearly normal (Fig. 6E). However, the renal cortex of the animals treated with TiO_2-NPs plus ETEO (HD) showed few foci of vacuolation in the tubular epithelial cells or cytoplasmic droplet in their lumen with interstitial tubular mononuclear cell infiltrations but the majority of renal tubules and glomeruli were nearly normal (Fig. 6F).

Discussion

TiO_2-NPs are widely utilized in several applications such as sauces, cheeses, ice cream, skimmed milk, food colorants, nutritional supplements, and confectionery products including chewing gum, candy, coating sweets, chocolate, and toothpaste (Bachler et al. 2015; Baranowska-Wójcik et al. 2020; Dudefoi et al. 2017; Peters et al. 2014). Weir et al. (2012) reported that chewing gums, sweets and candies contain high amounts of TiO_2-NPs (<100 nm). The absorption of TiO_2-NPs via the gastrointestinal tract leads to several organ toxicities including the liver, kidney, serum, testes, and seminal vesicle (Bu et al. 2010; Wang et al. 2007).

In the current work, the results of GC-MS analysis of ETEO revealed the presence of 21 bioactive compounds belongs to phenols, terpene, and terpene derivatives. The phenolic compounds (thymol and carvacrol) represent 62.58 mg/g; however, the terpene and its derivatives represented 65.03 mg/g. These results were in good harmony with those reported in the literature (Amiri 2012; He et al. 2020; Nieto 2020; Youdim et al. 2002). However, the number and concentration of the compounds were somewhat different which probably due to the difference in variety and the growing condition of the plant (Diniz do Nascimento et al. 2010; Mutlu-Ingok et al. 2020; El-Guendouz et al. 2019). The results also showed that the use of orange peel extract as a green approach succeeded to synthesize TiO_2-NPs in crystal shape with an average particle size was 45 nm and a zeta potential of -27.34 mV. Similar to these results, previous reports indicated that crystalline TiO_2-NPs were synthesized in different size using the orange peel extract and the size of these synthesized particles was smaller than those synthesized using chemical methods (Rao et al. 2015; Mobeen Amanulla and Sundaram 2019; Thakur et al. 2019). These authors suggested that the syntheses of TiO_2-NPs using the orange peel extract was due to its high content of insoluble polysaccharides, soluble sugars and polyphenols which act as reducing agents in addition to the amino acids, citric acid and the carboxylic groups which act as stabilizing agents (Torrado et al. 2011). In a previous work, Patra and Baek (2014) reported that the extract

concentration, reaction temperature, and pH are the main factors that affect the particles size and the irregular shape of the synthesized TiO_2-NPs may be due to the adhesion of phytochemicals in orange peel extract to particles (Swathi et al. 2019).

The synthesized ETEO reported in this current study showed a round shape with an average size of 110 nm and a zeta potential of -28.33 mV. These results suggested that WPI, which was used as a wall material in the encapsulation process, enhanced the coalescence of droplets (Abdel-Wahhab et al. 2018). Moreover, the spherical shape of the particles indicated the presence of WPI in the wall of capsules (Eratte et al. 2014; Xu et al. 2013), and the negative zeta potential is also attributed to the negatively charged WPI in neutral pH which is mostly due to the carboxylate groups as the only charged functionalities present in WPI molecule (Eratte et al. 2014). Generally, the surface properties and the particles size have a critical role in nanoparticles uptake by the mucus membranes and the size of 50-300 nm is the preferred size for uptake (Roger et al. 2010) and affect the pharmacokinetics, tissue distribution and clearance of nanoparticles (Sadat et al. 2016). Furthermore, the negative charge of zeta potential can enhance the dispersion of the droplets and increase the stability of the emulsion (McClements and Rao 2011), and the zeta potential higher than 30 mV and lower than - 30 mV stimulate the high stability and prohibit the aggregation of the particles (Mohanraj and Chen 2006).

In the *in vivo* study, animals were treated with TiO_2-NPs alone or in combination with ETEO at a low or high dose. The selected dose of TiO_2-NPs was based on Orazizadeh et al. (2014); however, the selected dose of ETEO was based on our previous work (El-Nekeety et al. 2011). The toxicity of TiO_2-NPs was manifested primarily by the effect of body weight. Animals treated with TiO_2-NPs alone showed a significant decrease in their final body weight than the control group; meanwhile, no significant change was noticed in body weight of the animals treated with ETEO at both tested doses. It was reported that ingestion of NPs induces disturbances in the digestion and the absorption of food components leading to a shortage of micro and macro elements in the body which in turn affect body weight (McClements et al. 2016). A similar decrease in body weight was reported in mice treated with different doses of TiO_2-NPs at different time intervals. The mice showed toxic symptoms including loss of appetite which leads to a reduction in body weight (Chen et al. 2009). Administration of TiO_2-NPs also reduces the number of villi in the intestine and reduces the surface responsible for nutrients absorption leading to malnutrition and the reduction of body weight (Duan et al. 2010). Furthermore, oral ingestion of TiO_2-NPs can penetrate the intestinal mucosa (Ammendollia et al. 2017) leading to the damage and chronic failure of the epithelium tissue in the intestinal wall (Brun et al. 2014).

TiO_2-NPs administration disturbed the biochemistry of the body as manifested by the significant elevation of liver and kidney function indices, serum cytokines (AFP, CEA, and TNF-α), oxidative markers (NO and MDA), cholesterol, TriG, and LDL and the significant reduction of total protein, albumin, HDL and antioxidant enzyme activity. Previous in vitro studies reported that TiO_2-NPs cause toxicity, genotoxicity (Kohen and Nyska 2002), and inflammation (Tucci et al. 2013; Wang et al. 2014; Zhao et al. 2013). The increase in liver and kidney indices reported herein indicated that TiO_2-NPs disturb the functions of these organs. The elevation of serum AST and ALT indicated the death or injury of hepatocytes (Thapa and Walia 2007). These enzymes are located in the cytoplasm of hepatic cells more than extracellular fluid and their levels elevate in the serum if the hepatocytes damaged (Dambach et al. 2005; Mohammed and Safwat 2020). Additionally, the elevation of urea, uric acid, and creatinine levels indicate the toxicity and dysfunction of the kidney (Ahamed et al. 2010). Thus, the increase of these kidney indices reported in the TiO_2-NPs-treated group indicated the injury of renal tissue (Abdelhalim and Jarrar 2011; Fartkhooni et al. 2016).

Administration of TiO_2-NPs also disturbs lipid profile markers. The increase in TriG, cholesterol, and LDL indicate that these nanoparticles affect the lipid metabolism through the effect on lipoprotein lipase enzyme (Ani et al. 2008) and/or the removing and transferring of the lipid fractions (Duan et al. 2010). Additionally, the increase of TriG and cholesterol is associated with cardiovascular disease and other metabolic syndromes (Antoni et al. 2018; Reiner 2017). In addition to the disturbance in cholesterol and TriG, the increase of LDL level is critical because it has also had a close association with arteriosclerosis; hence, TiO_2-NPs can be considered a causative factor for the incidence of cardiovascular disorders (Chen et al. 2020). Hong et al. (2017) and Chen et al. (2015) reported similar results in mice and rats.

Previous reports proposed that oxidative damage is one of the mechanisms of TiO_2-NPs-induced toxicity. These particles provoke the formation of ROS (reactive oxygen species) in different cell lines (Foroozandeh and Aziz 2015; Wang et al. 2014). In mammals, ROS induces damage to macromolecules such as proteins, lipids, carbohydrates, and nucleic acids mainly DNA (Abdel-Wahhab et al. 20220; Kelly et al. 1998; Shukla et al. 2014; Saquib et al. 2012). Lipid peroxidation (LP) probably changes the cell membrane structure resulting in disturbances in the vital functions of cells (Rikans and Hornbrook 1997). The oxidative damage induced by TiO_2-NPs is attributed mainly to the generation of hydroxyl radical (•OH) (Reeves et al. 2008). Oral exposure to TiO_2-NPs, lead to the generation of •OH a status of oxidative stress that occurs leading to the disturbances in lipids and accumulation of malondialdehyde levels and reduction of antioxidant capability in the hepatic tissue (Rajapakse et al. 2012). Also, exposure to TiO_2-NPs was reported to decrease glutathione in the liver (Federici et al. 2007). The elevation of NO and MDA and the reduction of CAT, GPx, and SOD in the hepatic and renal tissue reported herein suggested the manifestation of oxidative damage and suggesting the disturbances in redox balance in these organs (Chen et al. 2020). The increase of ROS production reflexes the damage of DNA and the up-regulation of 8-hydroxyl deoxyguanosine (8-OHdG) in the hepatic and renal tissue (Trouiller et al. 2009) and the increase of MDA and the decrease of antioxidant enzymes in these organs may contribute to the cell apoptosis. The increase of ROS was reported to increase Nrf2 which consider the master regulator of the expression of several antioxidant genes and the lack of Nrf2 increase the damage of DNA and increases the risk of cancer (Shi et al. 2015). Moreover, hydrogen peroxide (H_2O_2) in the liver and kidney is accumulated due to the decrease in SOD activity leading to the inhibition of CAT activity (Latchoumycandane and Mathur 2002). This enzyme converts H_2O_2, the harmful byproduct of the normal metabolic process, to H_2O and O_2 and thus prevents the damage of cells and tissues (Sharma et al. 2014).

The increased level of serum cytokines reported in the current study in the animals treated with TiO_2-NPs revealed the inflammatory response of these nanoparticles. These results supported the generation of ROS in TiO_2-NPs-treated rats which leads to the reduction of the cell viability and stimulates the cytotoxicity via an apoptotic process (Müller et al. 2010). Thus, the toxicity of TiO_2-NPs probably correlated to the surface chemistry of the particles which affects the inflammatory responses and the release of TNF-α and neutrophil-attracting chemokines (Iavicoli et al. 2011; Müller et al. 2010; Rossi et al. 2010) and the rate of release is the size and time-dependent (Wu and Tang 2018).

Gene expression assay is widely used as a quick and early biomarker to predict for TiO_2 NPs-induced potential liver injury and exploring the possible mechanisms of their toxicities (Li et al. 2017). The administration of TiO_2-NPs induced significant disturbances in the expression of antioxidant and apoptosis genes in hepatic tissue. The down-regulation of CAT, GPx and SOD mRNA reported herein is harmonized with the biochemical findings and supported the hypothesis that TiO_2-NPs induce oxidative stress via the exhaustion of antioxidant enzymes and suppress their gene expression. Moreover, TiO_2-NPs also decreased Bcl-2 mRNA expression and up-regulation of Bax and TNF-α mRNA in the liver. Bcl-2 proteins are family responsible for anti-apoptosis and control the mitochondrial integrity

surface, while Bax is a pro-apoptotic protein. The balance of anti-apoptotic Bcl-2 and the pro-apoptotic Bax proteins control the sensitivity of the cell to apoptotic stimuli (Ilani et al. 2018). Additionally, Bcl-2 is found on the surface of mitochondria and prevents the release of cytochrome c in the plasma; however, Bax encourages the leakage of cytochrome c through the punching of the holes of the mitochondrial membrane (Kroemer et al. 2007). The imbalance between Bax and Bcl-2 also activates the pathway of caspase-dependent apoptotic (Peng et al. 2016). Thus, the generation of ROS after TiO_2-NPs exposure disturbs the mitochondrial membrane potential as a result of apoptosis (Abdel-Wahhab et al. 2020; Zhao et al. 2009). Furthermore, the up-regulation of TNF-α mRNA in the hepatic tissue indicated the inflammatory response of TiO_2-NPs and supported the earlier findings of the previous studies (Chen et al. 2015; Gui et al. 2011; Trouiller et al. 2009).

The histological examination of the liver and kidney tissues revealed that TiO_2-NPs administration induced severe pathological changes in both tissues and confirmed the biochemical and cytogenetic results. Similar observations were reported in previous studies (Attia et al. 2013; Morgan et al. 2018) who reported disorganization of the hepatic cords with hepatocellular necrosis, macro, and microvascular steatosis. Moreover, Valentini et al. (2019) reported that the alterations on the liver tissue were mainly correlated with oxidative stress which is localized around the central vein. A close correlation between oxidative damage and anoxia of the tissue was reported in different organs (Chen et al. 2005; Pialoux et al. 2009). Moreover, the hepatic Kuffer cells are well-known to be the most impacted cells by oxidative stress which may be due to their localization around the portal area in the liver sinusoids (Olmedo et al. 2008). On the other hand, the histological changes in the kidney tissues are characterized by the accumulation of protein materials in the lumen of many distal tubules and the collecting ducts found in the medulla which is less oxygenated compared to the proximal tubules and may be more exposed to the oxidative stress generated by TiO_2-NPs (Epstein 1997).

The prophylactic activity of thyme essential oils is well documented in the literature; however, some limitations were reported for its application in the food and pharmaceutical sectors. To cope with these limitations, encapsulation technology was proposed. This technique able to enhance the oil solubility and bioavailability, protect their active components against thermal or chemical degradation and control the release of these components (Tao et al. 2014). The encapsulated thyme essential oil (ETEO) in WPI was applied for the protection against TiO_2-NPs-induced oxidative damage and genotoxicity. The antioxidant properties and the protective role of ETEO are focused on the major phenolic components mainly thymol and carvacrol (Ruberto and Baratta 2000). Beside these two major components, the other components in the oil such as linalool, myrcene, and y-terpinene enhance the antioxidant activity of the oil (Youdim et al. 2002). Animals treated with ETEO alone exhibited significant improvements in all biochemical parameters especially, the antioxidant enzyme activity and oxidative markers suggesting that ETEO enhances the antioxidant activity. Previous reports indicated that thyme oil reduces the oxidation rate through the elimination of ROS or the breakdown of the peroxides to stable substances and prevent the promotion of further oxidation (El-Newary 2017). ETEO also improved the body weight, biochemical parameters, cytokines, antioxidant enzymes, and their gene expression in rats treated with TiO_2-NPs. These improvements may be due to the elimination of ROS generation which responsible for protein damage and lipid oxidation of cell membrane as well as the disturbances in calcium homeostasis and increase the fluidity of membrane and the death of cells (Molavian et al. 2016). Furthermore, the high content of phenolic compounds in the oil was found to decrease the triglycerides and cholesterol in the hepatic and renal tissue (Ebenyi et al. 2012). These compounds also prevent the secretion of pro-inflammatory factors through the reduction of lipopolysaccharides (De Andrade et al. 2017), thus, they are potent ROS scavengers' natural products (Ebenyi et al. 2012) and increase the production of GSH, SOD, and CAT (El-Banna et al. 2013). It was also reported that thyme oil can suppress TNF-α in mouse cells and inhibited cytochrome C oxidase-2 expression (Mahran et al. 2019). Generally, the protective role of bioactive components in ETEO maybe

include the inhibition of cytochrome P450 activity, accelerate the regeneration of parenchyma cells, stabilizing the cell membrane, improvement of the antioxidant activity (Al-Fartosi et al. 2011). Previous reports indicated that encapsulation of thyme oil (TO) using different materials improve its properties. In this concern, several studies reported that the antioxidant activity, the thermal stability, and the release of the oil were improved when the oil was encapsulated using zein (Bilenler et al. 2015) chitosan (Detsi et al. 2020; Ghahfarokhi et al. 2016; Khalili et al. 2015), Arabic gum (Cai et al. 2019), gelatin-Arabic gum (Gonçalves et al. 2017) and chitosan-Arabic gum (Hassani and Hasani 2018).

In our study, WPI was used as a wall in the encapsulation process, thus, we can propose another mechanism of the protective role of ETEO. WPI is well known to possess antioxidant activity due to its high content of amino acids mainly cysteine, β-lactoglobulin, α-lactoglobulin, and bovine serum albumin (Morr and Ha 1993). The amino acid cysteine helps to replenish intracellular GSH, the endogenous antioxidant responsible for peroxide dexification (Gould and Pazdro 2019). Hence it acts as another source of antioxidants besides its role in the protection of the oil active ingredients and enhances the activity of ETEO.

Conclusion

The current results showed that 21 compounds were identified in TEO represented 130.85 mg/g oil and belong to phenols, terpene, and terpene derivatives class. Thymol, para-cymene, linalool, carvacrol, camphor, eucalyptol, γ-terpinene, borneol, terpinene-4-ol, β- pinene, β-Phellandrene, β-myrcene and Humuline in concentrations were the major compounds. TiO_2-NPs can be synthesized using orange peel extract and the resulted particles were tetragonal with an average size of 45 nm and zeta potential of -27.34 mV. The results also revealed that the encapsulation of TEEO using WPI resulted in round particles with an average size of 110 nm and a zeta potential of -28.33 mV. The biological study revealed that TiO_2-NPs induced severe toxicity to the hepatic and renal tissues as manifested by the decrease of body weight, disturbed liver and kidney function, lipid profile parameters, increased serum cytokines, NO and MDA. TiO_2-NPs also increased the mRNA of pro-apoptotic and decrease the mRNA expression of antiapoptotic and antioxidant enzymes along with the pathological alterations in the liver and kidney tissues. ETEO did not induce any significant changes in all the parameters tested or the histological picture of the liver and kidney. Co-treatment with TiO_2-NPs plus ETEO at both tested doses enhanced the antioxidant activity and alleviated the toxicity of TiO_2-NPs in a dose-dependent manner. Encapsulation of TEO in WPI enhanced its antioxidant activity and maybe a promising candidate to protect against TiO_2-NPs-induced oxidative stress and genotoxicity. ETEO can be applied to overcome the problems associated with essential oils and used in food or pharmaceutical applications for the protection against oxidative damage.

Declarations

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Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

The codes used during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

The authors declare that they have no conflict of interest.

Ethics approval The protocol of the current study was approved by the ethics Animal Care and Use Committee of the National research Center, Dokki, Cairo, Egypt (approval # 12050305/2019)

Consent for publication Not applicable

Consent to Participate Not applicable

Authors' contributions: This work was carried out in collaboration between all authors. Authors EL-Nekeety, HE Mohammed and OI Elshafey prepared and characterized the nanoparticles, carried out the experimental work, the biochemical analysis and managed the literature search. Author SH Abdel-Aziem carried out the genetic analysis. Author NS. Hassan carried out the histological part. All authors shared in writing the first draft. Author Mosaad A. Abdel-Wahhab wrote the protocol, managed the project, managed the analyses of the study, performed the statistical analysis, and wrote the final draft of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Details giving primer sequences for the genes amplified

cDNA	Accession number	Forward primer	Reverse primer	RT-PCR product size
GAPDH	NM_017008.4	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG	496
Cu-Zn SOD	FQ210282.1	GCAGAAGGCAAGCGGTGAAC	TAGCAGGACAGCAGATGAGT	477
GPx	NM_030826.4	CTCTCCGCGGTGGCACAGT	CCACCACCGGGTCGGACATAC	290
CAT	NM_012520.2	GCGAATGGAGAGGCAGTGTAC	GAGTGACGTTGTCTTCATTAGCACTG	652
Bax	NM_017059.2	AGGATGATTGCTGATGTGGATAC	CACAAAGATGGTCACTGTCTGC	300
Bcl-2	NM_016993.2	GCTACGAGTGGGATACTGGAGA	AGTCATCCACAGAGCGATGTT	446
TNF-α	NM_012675.3	CCACCACGCTCTTCTGTCTAC	ACCACCAGTTGGTTGTCTTTG	256

Table 2. Chemical constitutes of ETEO identified by GC-MS

Constituents*	mg/g	Class
Thymol	55.40	Phenols
para-Cymene	12.1	monoterpene
Linalool	8.21	Terpene
Carvacrol	7.18	Phenols
Camphor	6.21	Terpene
Eucalyptol	6.11	monoterpene
gamma-Terpinene	5.31	monoterpene
Borneol	5.12	Terpene derivative
Terpinen-4-ol	4.22	Terpene isomer
β- pinene	2.31	Terpene
β-Phellandrene	2.24	Terpene
β -myrcene	2.11	Monoterpene
Humuline	2.10	Sesquiterpene
α- Phellandrene	1.89	Terpene
Myrcene	1.67	Terpene
Linalool oxide II	1.62	Tetrahydrofurans
1, 8-cineole	1.54	Oxides
α-pinene	1.48	Terpene
β-sabinene	1.44	Terpene
α-thyjone	1.37	Oxygenated terpene
Tricyclene	1.22	Terpene
Total	130.85	
*Constituents presented in the order of concentration		
Linear retention index relative to n-alkanes (C7-C20) on DB-5 column		
Compound identified by GC/MS and / or by comparison of MS and LRI of standard compounds (ST) under the same conditions.		

Table 3. Effect of ETEO on serum biochemical parameters in rats treated with TiO_2-NPs

Groups Parameter	Control	TiO_2-NPs	ETEO (LD)	ETEO (HD)	TiO_2-NPs + ETEO (LD)	TiO_2-NPs + ETEO (HD)
ALT (U/L)	41.33 ± 1.86^a	79.00 ± 4.16^b	37.33 ± 2.60^c	37.33 ± 2.33^c	66.33 ± 1.45^d	52.00 ± 2.00^d
AST (U/L)	144.75 ± 5.81^a	209.00 ± 2.97^b	131.75 ± 5.60^c	135.75 ± 6.13^c	175.75 ± 6.17^d	150.00 ± 4.53^a
Alb (mg/dl)	2.74 ± 0.11^a	1.77 ± 0.05^b	2.59 ± 0.02^c	2.72 ± 0.06^a	2.80 ± 0.11^a	2.94 ± 0.11^a
TP (g/dl)	6.48 ± 0.21^a	5.47 ± 0.23^b	6.27 ± 0.35^a	6.85 ± 0.29^a	6.41 ± 0.17^a	6.88 ± 0.21^a
T.BIL (mg/dl)	0.09 ± 0.01^a	0.12 ± 0.01^b	0.09 ± 0.01^a	0.08 ± 0.01^a	0.08 ± 0.01^a	0.08 ± 0.01^a
D.BIL (mg/dl)	0.03 ± 0.01^a	0.16 ± 0.01^b	0.03 ± 0.01^a	0.03 ± 0.01^a	0.06 ± 0.03^c	0.03 ± 0.01^a
Cratinine (mg/dl)	0.81 ± 0.06^a	1.65 ± 0.05^b	0.72 ± 0.04^c	0.63 ± 0.03^d	0.97 ± 0.01^e	0.83 ± 0.02^a
Urea (mg/dl)	49.67 ± 4.48^a	73.67 ± 2.73^b	47.00 ± 2.65^a	45.33 ± 3.76^a	59.00 ± 5.03^c	45.33 ± 2.03^a
Uric acid (mg/dl)	1.26 ± 0.17^a	2.20 ± 0.10^b	1.22 ± 0.14^a	1.54 ± 0.13^c	1.76 ± 0.08^d	1.39 ± 0.19^e
Within each row, means superscripts with different letters are significantly different ($P < 0.05$)						

Table 4. Effect of ETEO extract on serum lipid profile parameters in rats treated with TiO_2-NPs

parameter Groups	Cholesterol (mg/dl)	TriG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Control	61.67 ± 0.88^a	82.00 ± 3.79^a	25.33 ± 0.88^a	23.67 ± 0.33^a
TiO_2-NPs	74.67 ± 1.20^b	116.67 ± 1.20^b	12.67 ± 0.33^b	34.67 ± 1.45^b
ETEO (LD)	64.67 ± 4.48^a	81.67 ± 1.20^a	33.00 ± 1.73^c	27.33 ± 0.88^c
ETEO (HD)	60.67 ± 2.19^a	72.67 ± 3.93^c	35.00 ± 2.08^c	21.00 ± 2.89^d
TiO_2-NPs + ETEO (LD)	67.33 ± 2.03^d	84.00 ± 1.53^a	23.67 ± 0.88^a	23.67 ± 1.67^a
TiO_2-NPs + ETEO (HD)	64.00 ± 4.51^c	66.33 ± 2.60^d	24.33 ± 1.20^a	23.67 ± 2.73^a
Within each column, means superscripts with different letters are significantly different ($P < 0.05$)				

Table 5. Effects of ETEO extract on NO and MDA in the liver and kidney tissues of rats treated with TiO_2-NPs

Parameter Groups	NO (µM/mg)		MDA (nmol/g)	
	Liver	Kidney	Liver	Kidney
Control	553.33 ± 3.80[a]	850.33 ± 5.67[a]	130.98 ± 4.97[a]	244.32 ± 4.46[a]
TiO_2-NPs	947.67 ± 9.94[b]	1104.00 ± 9.22[b]	233.76 ± 3.70[b]	353.42± 6.45[b]
ETEO (LD)	520.00 ± 5.28[c]	830.00 ± 5.51[c]	109.26± 5.26[c]	237.10± 3.77[c]
ETEO (HD)	510.67 ± 4.67[d]	768.00 ± 8.01[d]	101.71± 2.51[d]	227.69± 4.76[d]
TiO_2-NPs + ETEO (LD)	643.33 ± 5.96[e]	909.33 ± 5.07[e]	130.99± 3.26[a]	269.66± 5.29[c]
TiO_2-NPs + ETEO (HD)	620.00 ± 2.82[f]	806.67 ± 3.33[f]	129.54 ± 4.46[a]	253.21± 8.14[d]
Within each column for each organ, means superscripts with different letters are significantly different (P < 0.05)				

Table 6. Effect of ETEO on antioxidant enzyme activity in liver and kidney tissue of rats treated with TiO_2-NPs

Parameter Groups	CAT (mU/g tissue)		GPX (U/mg tissue)		SOD (U/mg tissue)	
	Liver	Kidney	Liver	Kidney	Liver	Kidney
Control	6.43 ± 0.27[a]	8.82 ± 0.05[a]	32.55 ± 2.07[a]	24.64 ± 0.59[a]	2.24 ± 0.14[a]	2.80 ± 0.03[a]
TiO_2-NPs	2.19 ± 0.11[b]	3.75 ± 0.03[b]	20.97 ± 3.75[b]	10.50 ± 0.32[b]	1.24 ± 0.14[b]	1.67 ± 0.06[b]
ETEO (LD)	6.76± 0.19[a]	8.20 ± 0.61[a]	35.37 ± 1.82[a]	27.91 ± 0.32[c]	2.74 ± 0.14[c]	2.86 ± 0.14[a]
ETEO (HD)	6.77± 0.45[a]	8.58 ± 0.26[a]	33.28 ± 0.54[a]	32.00± 0.43[d]	3.15 ± 0.19[d]	2.88 ± 0.01[a]
TiO_2-NPs + ETEO (LD)	4.19± 0.81[d]	6.28 ± 0.75[c]	33.38 ± 3.23[a]	16.95 ± 0.98[e]	1.49 ± 0.04[e]	1.98 ± 0.02[c]
TiO_2-NPs + ETEO (HD)	4.73± 0.56[d]	6.45 ± 0.70[c]	30.11 ± 0.14[a]	19.95 ± 0.98[f]	1.81 ± 0.08[f]	2.48 ± 0.02[d]
Within each column for each organ, means superscripts with different letters are significantly different (P < 0.05)						

Table 7. Effects of ETEO on serum cytokines in rats treated with TiO_2-NPs

parameter Groups	AFP (ng/ml)	TNF-α (ng/ml)	CEA (ng/ml)
Control	0.037± 0.02[a]	0.31 ± 0.03[a]	3.22 ± 0.14[a]
TiO_2-NPs	0.082± 0.01[b]	0.58 ± 0.02[b]	4.81± 0.31[b]
ETEO (LD)	0.035± 0.01[a]	0.30 ± 0.03[a]	3.54± 0.13[c]
ETEO (HD)	0.036± 0.01[a]	0.31 ± 0.01[a]	3.44 ± 0.32[c]
TiO_2-NPs + ETEO (LD)	0.061± 0.014[c]	0.32 ± 0.03[a]	3.85± 0.11[d]
TiO_2-NPs + ETEO (HD)	0.053± 0.021[d]	0.29 ± 0.02[a]	3.34± 0.14[c]
Within each column, means superscripts with different letters are significantly different (P < 0.05)			

Figures

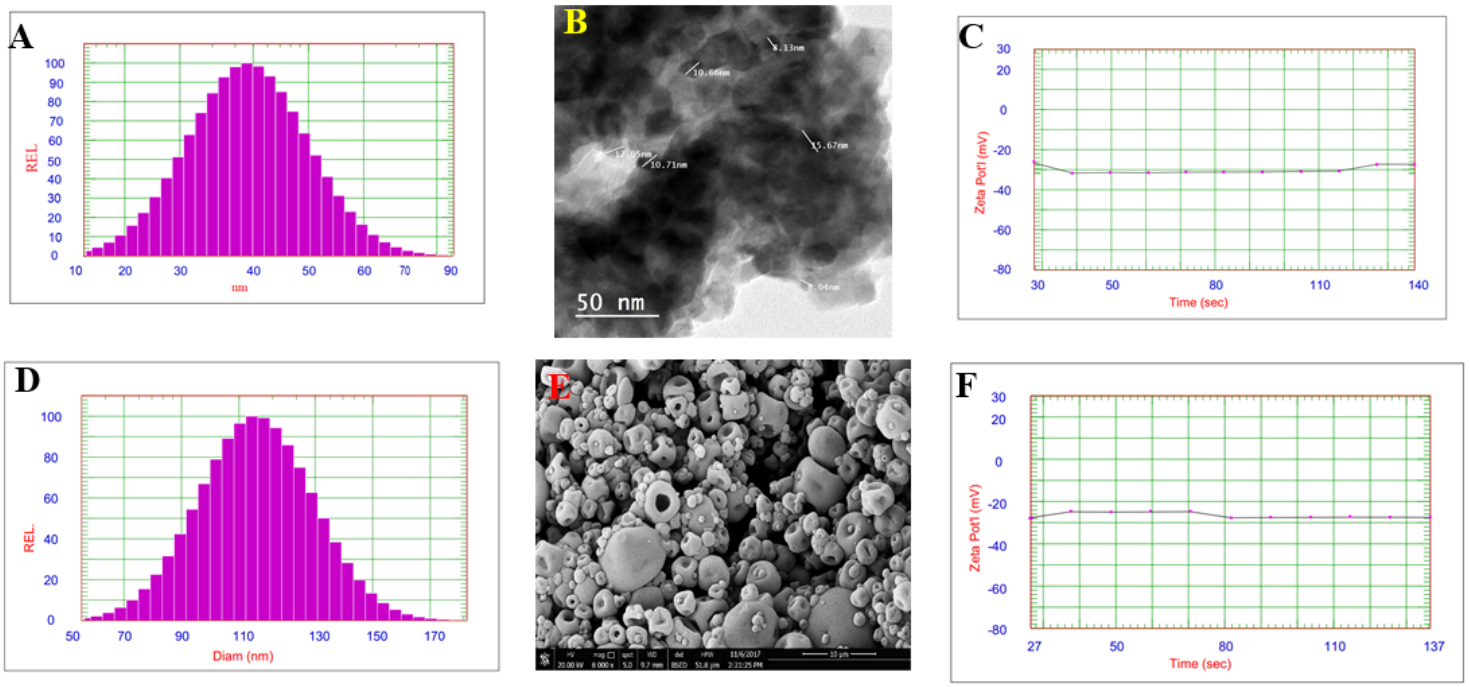

Figure 1

(A) DLS analysis showing the size distribution of TiO2-NPs, (B) TEM image of TiO2-NPs showing the particle shape and size, (C) ZetaSizer chromatogram showing the zeta potential of TiO2-NPs, (D) DLS analysis showing particles distribution of ETEO, (E) TEM image of ETEO showing the particle shape and size and (F) ZetaSizer chromatogram showing the zeta potential of ETEO.

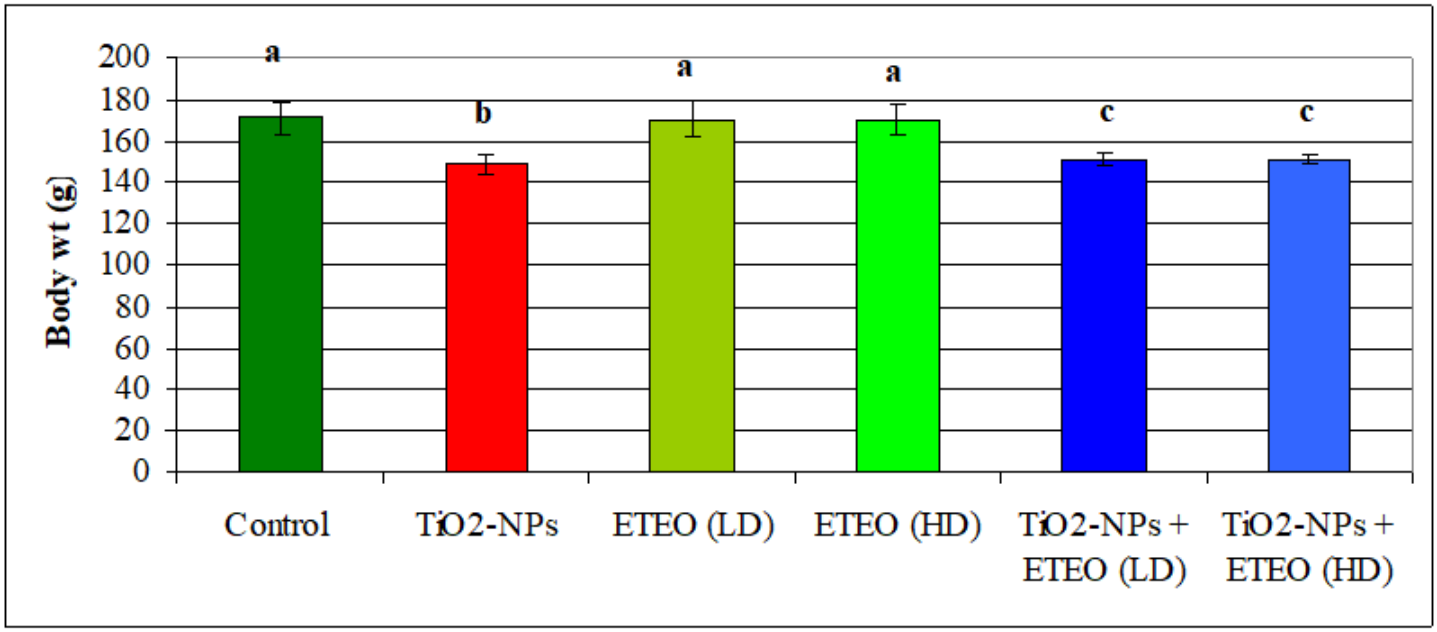

Figure 2

Effect of ETEO on body weight in rats treated with TiO2-NPs

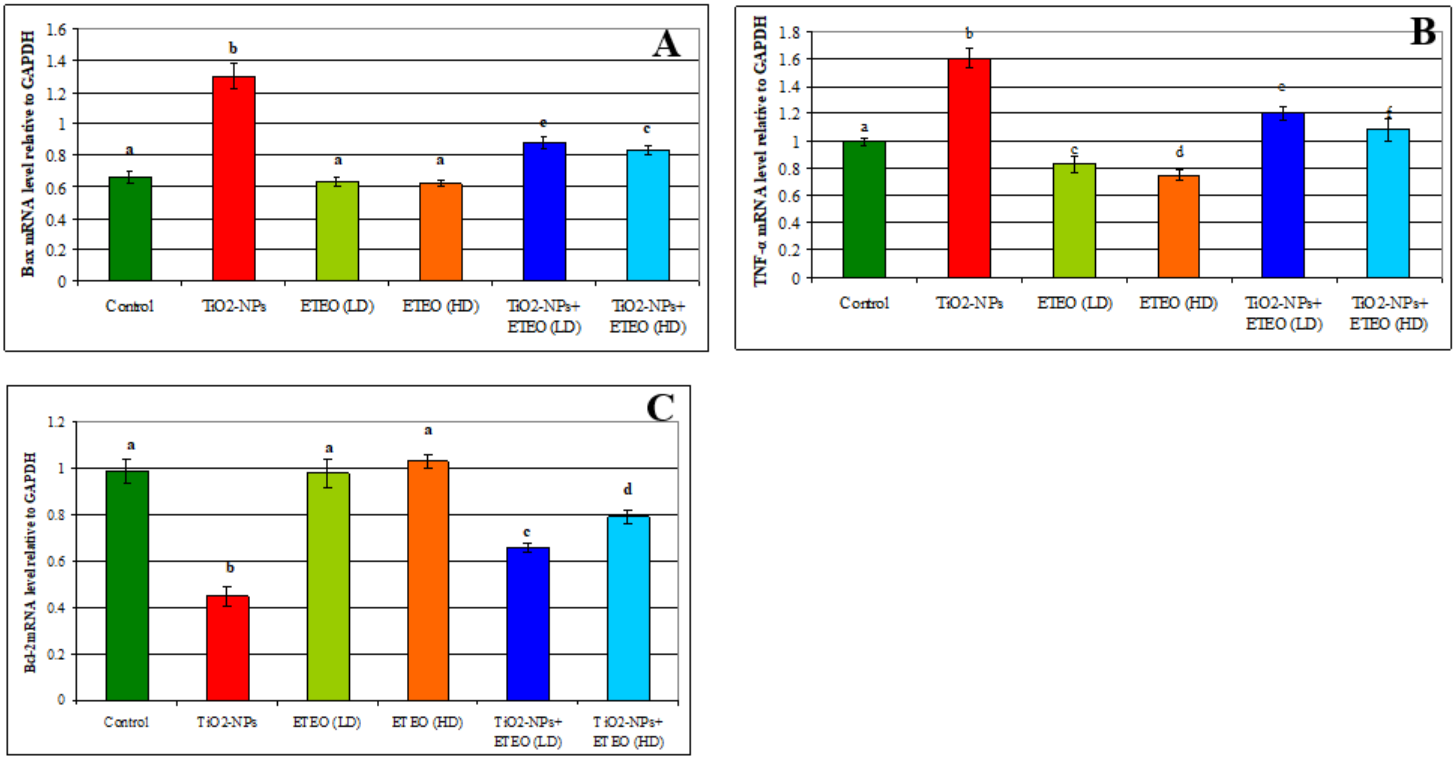

Figure 3

Effect of ETEO on relative expression of Bax (A), TNF-α (B) and Bcl-2 (C) gene in liver of rats treated with TiO2-NPs. Analyses were performed in triplicate. Data are the mean ± SE of three different liver samples in same group. Columns superscripts with different letters are significantly difference at P≤ 0.05.

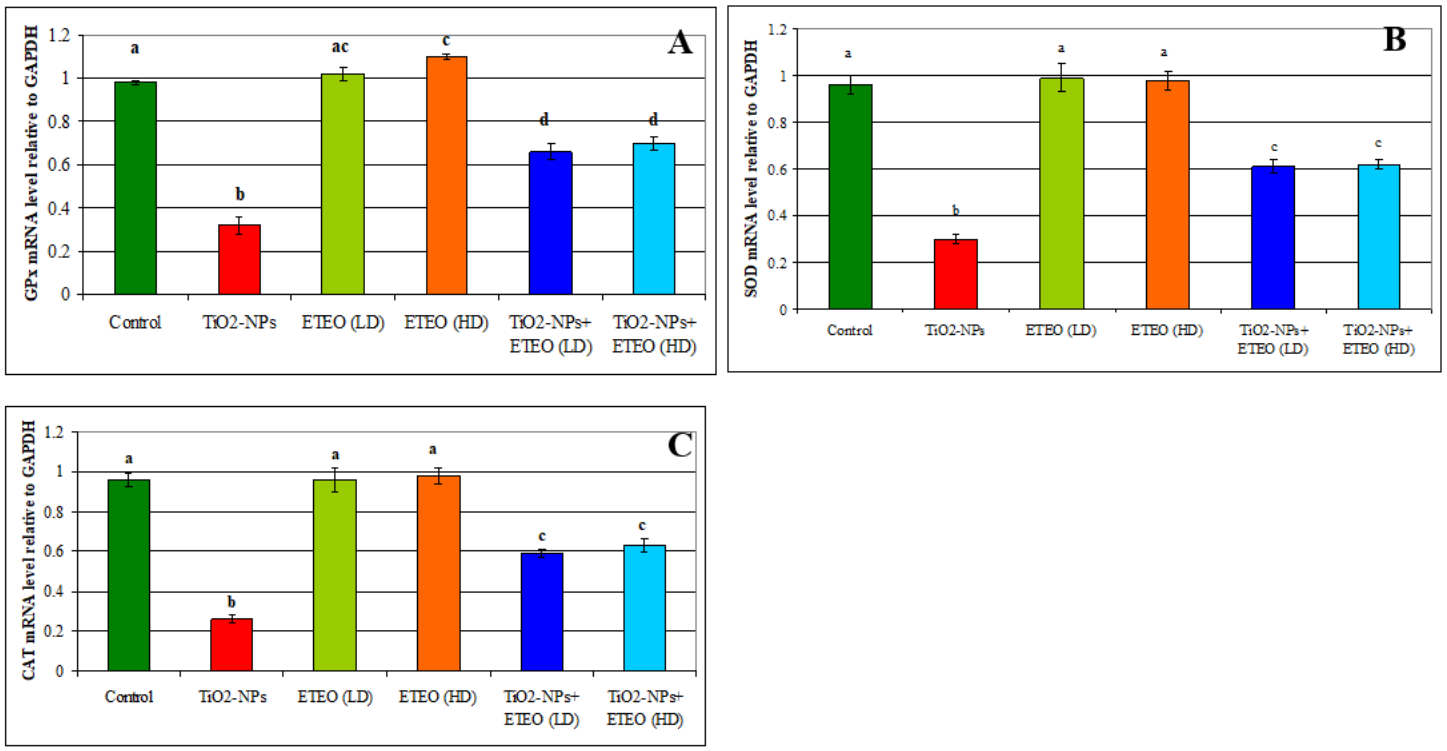

Figure 4

Effect of ETEO on relative mRNA expression of GPx (A), SOD (B) and CAT (C) gene in liver of rats treated with TiO2-NPs. Analyses were performed in triplicate. Data are the mean ± SE of three different liver samples in same group. Columns superscripts with different letters are significantly different at P≤ 0.05.

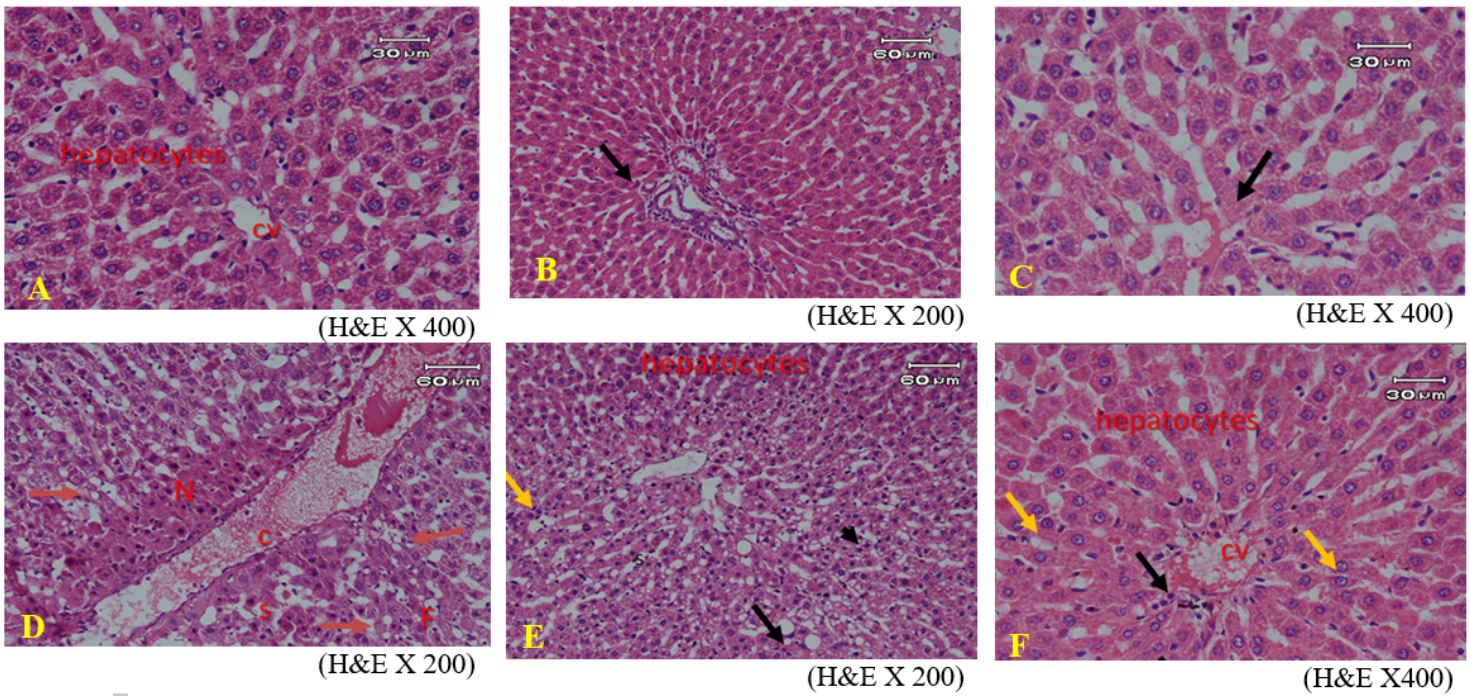

Figure 5

Photomicrographs of liver sections of (A) normal control rats showing hepatocytes with normal cytoplasm, vesiculated nuclei and separated by blood sinusoids radiating from the central vein (CV); (B) rat treated with ETEO

(LD) showing no observable changes in hepatocytes architectuer except few fibrous tissues around the central vein and bile duct hypertrophy; (C) rats treated with ETEO (HD) showing no observable changes in hepatocytes architecture except few fibrous tissues around the central vein and bile duct hypertrophy; (D) rats treated with TiO2-NPs showing marked histological alterations in the hepatocytes as disorganization, vacuolar and fatty degeneration (arrow) shrunken hepatocytes with darkly stained pyknotic nuclei (p) patches of necrotic cells around the dilated and congested portal tract also seen (N) and numerous mononuclear cellular infiltration localized in dilated hepatic sinusoids (s); (E) rats treated with TiO2-NPs plus ETEO (LD) showing several histological changes in hepatocytes as vacuolar (arrow head), fatty degeneration (black arrow), pyknotic nuclei (yellow arrow) and increase in mononuclear inflammatory cells; (F) rats treated with TiO2-NPs plus ETEO (HD) showing more improvement in hepatic cells stricter and few inflammatory cells are also seen (black arrow).

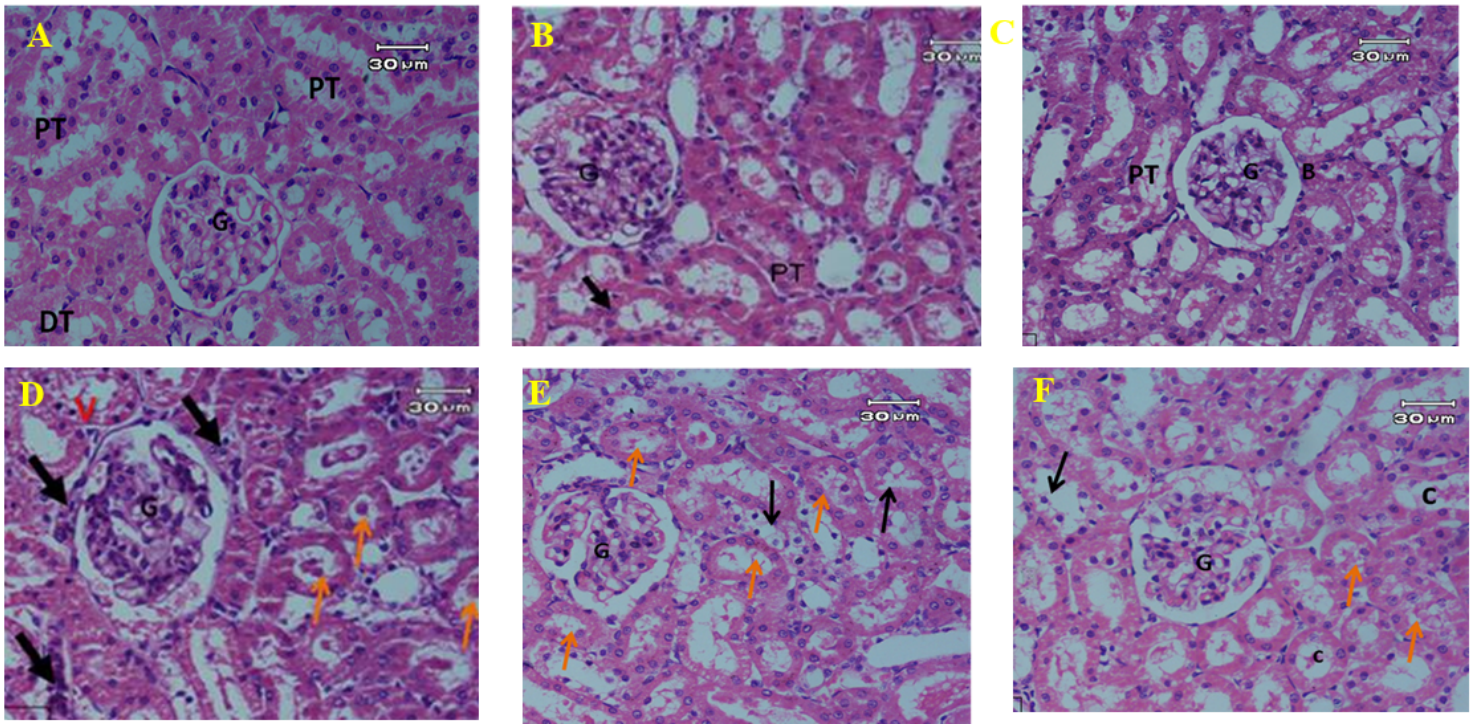

Figure 6

Photomicrographs of kidney cortex of: (A) control animals showing the proximal convoluted tubules with high cuboidal acidophilic cells and narrow lumen (arrow) and distal convoluted tubules with low cuboidal cells and wide lumen (DT). Note: Renal corpuscle with the parietal layer of Bowman's capsule (BC), glomerulus (G) and the urinally renal space are preserved; (B) Animals treated with ETEO (LD) showing the majority of the tubules (PT) and glomeruli are normal epithelial cells (arrow) except some renal tubules are still with slight hyaline droplet in their lumen and widening of the Bowman's space; (C) animals treated with ETEO (HD) showing tubular lumen dilatation with slight cytoplasmic hyaline (PT), the majority of renal tubules and glomeruli Bowman's capsule (B) are nearly normal; (D) Animal treated with TiO2-NPs showing vacuolar degeneration of the cytoplasm of tubular epithelial cells with deeply stained pyknotic nuclei in some renal tubules (V), some of the lining epithelial cells are exfoliated into the lumen with hyaline droplets (yellow arrow), the renal capsule are shrunken with reduction in glomeruli mesengial cells (G), mononuclear cell infiltrations between degenerated tubule and renal corpuscle (black-arrow); (E) Animals treated with TiO2-NPs plus ETEO (LD) showing few foci of vaculation in tubular epithelial cells (black arrow) or hyaline cytoplasmic droplet in their lumen (yellow-arrow) .the majority of renal tubules and glomeruli are nearly normal; (F) Animals treated with TiO2-NPs plus ETEO (HD) showing few foci of vaculation in tubular epithelial cells (black arrow)

or cytoplasmic droplet in their lumen (yellow-arrow), interstitial tubular mononuclear cell infiltrations but the majority of renal tubules and glomeruli are nearly normal. (H & E X 400).