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Hepatoprotective Effect of Nettle Root Extract (*Urtica dioica*) on Titanium Dioxide Nanoparticle-Stimulated Hepatotoxicity in Male Albino Rats

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ABSTRACT

Background: Nettle roots (*Urtica dioica*) are considered promising plants for treating different conditions, including liver ailments. Accordingly, we aimed to ascertain the protective implication of aqueous extract of nettle roots versus hepatotoxicity in male albino rats exposed to TiO_2 NPs.

Methods: Here, 48 adult male albino Wistar rats were randomly allocated to six groups (8 rats/group). The first group operated as controls. The second and third groups were administered *U. dioica* (100, 200 mg/kg b.w) orally. The fourth group was orally administrated TiO_2 NPs (300 mg/kg b.w). The fifth and sixth groups were treated orally with (*U. dioica* 100, 200+ TiO_2 NPs). After four weeks, biochemical, histopathological and immunohistochemical examinations of the blood samples and liver tissue were carried out.

Result: The outcomes manifested that exposure to TiO_2 NPs significantly increased ALT, AST, ALP, LDH and MDA, total protein and bilirubin, whereas albumin levels, SOD and GSH decreased significantly, causing liver tissue damage. In addition to higher expression of caspase 3. *U. dioica* treatment improved the physiological, histological and immunohistochemical results of damage caused by exposure to TiO_2 NPs. This study shows that chronic administration of *U. dioica* protects against TiO_2 NPs-induced hepatotoxicity in rats. These positive effects may appear because *U. dioica* contains antioxidants.

Key words: Antioxidant, Hepatotoxicity, Nettle roots (*Urtica Dioica*), Oxidative stress, Titanium dioxide nanoparticles.

INTRODUCTION

The use of nanotechnology, which includes nanoparticles, has experienced significant growth in various industries, including medicine, cosmetics, energy, chemistry and textiles (Malik *et al*., 2023). Nanomaterials with a size of 1 to 100 nanometers (nm) offer many applications (Farooqi *et al*., 2021). Titanium dioxide nanoparticles (TiO_2 NPs) are among the five prevailing frequently utilized nanomaterials in various commercial and consumer products. These particles are extensively used in numerous areas, encompassing plastic manufacturing, pharmaceutical pill composition as an adjuvant and as bleaching agents in the paper industry, paint manufacturing, cosmetics, sunscreen and toothpaste. They are present in higher concentrations in several culinary products (Shakeel *et al*., 2016). Although TiO_2 NPs offer numerous advantages and applications, recent research has highlighted the disadvantages associated with their frequent use. They are widely used due to their high stability, corrosion resistance and photocatalytic capabilities. Exposure to TiO_2 NPs can occur both during synthesis and during their usage, either as aerosol, suspensions, or emulsions. Therefore, inhalation, ingestion and cutaneous exposure are the main methods of exposure to TiO_2 NPs (Staroń *et al*., 2020). TiO_2, NPs can be detected in numerous internal organs after being exposed to TiO_2 NPs in different ways. According to *in vivo* experiments, TiO_2 NPs accumulate in the lungs, heart, spleen, liver, digestive system and kidneys, as well as in the myocardium, brain and testes (Baranowska-Wójcik *et al*., 2020; Kreyling *et al*., 2017; Martins *et al*., 2017). The liver is considered the most susceptible target organ to TiO_2 NPs exposure, since it is the main site of xenobiotic metabolism (Younes *et al*., 2021; Gilbert *et al*., 2021; Heringa *et al*., 2018). Hepatotoxicity was evidenced by changes in total protein concentration, total bilirubin, albumin, liver enzymes (ALT, AST, ALP, LDH) and concomitant diseases (Vargas *et al*., 2024). TiO_2 NPs act by causing damage to membrane integrity (Gholinejad *et al*., 2019), protein instability and oxidation (Jafari *et al*., 2018), nucleic acid damage (Ghareeb, 2021) and cell toxicity through the generation of reactive oxygen species (ROS) (Gholinejad *et al*., 2019).

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Therefore, we aimed to ascertain the possible physiological and histological changes as a result of the toxicological effects of TiO_2 NPs on rat's liver. As well as, the possible mitigating effect of different two doses of *U. dioica*. The physiological changes were evaluated through measuring liver function as an indicator of liver integrity and oxidative stress biomarkers as an indicator of the cellular redox status. Also, the toxic changes were further confirmed by caspase 3 immune staining as an indicator of apoptosis.

MATERIALS AND METHODS

Nettle (*U. dioica*) root extract (500 mg/capsule) was purchased from (Swanson Health Products, Fargo, North Dakota, U.S.A). Phosphate buffered saline (PBS) was purchased from (Pharmaceutical Solutions Industry, Jeddah, Saudi Arabia). Diethyl ether was purchased from Sigma-Aldrich (GMBH, Munich, Germany). Formaldehyde was purchased from (Riedel-Detain, Sleaze, Germany). The ALT, AST, ALP, LDH, albumin, total bilirubin, total protein, GSH, SOD and MDA were evaluated by Enzyme-Linked Immunosorbent Assay (ELISA) kits (My BioSource, San Diego, U.S.A).

The present experiment employs the physical grinding method to reduce the TiO_2 powder size. Specifically, 100 g of TiO_2 powder was placed in a high-speed rotator grinding machine and subjected to thorough, uniform grinding and crushing for 15 minutes, taking great care to prevent contamination. Upon completion, the finely ground powder was separated, resulting in the production of nano-sized TiO_2 powder, which was then packaged in plastic pouches and stored until needed at room temperature (Theivasanthi and Alagar, 2013).

A drop of the well-shaken solution of TiO_2 NPs was placed on carbon-coated copper grids for TEM analysis and the grids were allowed to dry until the water evaporated at ambient temperature. Electron micrographs were acquired employing a JEOL device (JEM-1010, JEOL Ltd, Tokyo, Japan) operated at an acceleration voltage of 70 kV (Baydar *et al*., 2022) (Fig 1).

The prepared sample of TiO_2 NPs underwent an XRD examination employing a Bruker-made diffractometer and Cu-Kα X-rays with a wavelength (λ) of 1.5406 Å. The data was collected with a step of 0.1972° to cover the 2θ range of 10° to 70°. The results confirmed the existence of nano-sized TiO_2 powder (Ali and Hameed, 2022) (Fig 2).

Male albino Wistar rats (*Rattus norvegicus*), with weights (134 to 225) g were selected for this study. The rats were purchased from the animal facility of the Faculty of Pharmacy at King Abdulaziz University (KAU), Jeddah, Saudi Arabia. The experimental animals underwent acclimatization for 7 days prior to the experiment. Rats were maintained under regulated laboratory conditions with a temperature (20±1°C), humidity (65%) and 12:12 h light: dark cycle and housed in standard plastic cages. Throughout the acclimation period, the rats obtained unrestricted access to water and fed with *ad libitum* on normal commercial chow on a daily basis. The interventions in the current experiment were performed as per the ethical standards established by the Animal Care and Use Committee of King Abdulaziz University.

Forty-eight rats were randomly allocated into six experimental groups (8 rats/group). The experimental groups received the following treatments:

Group 1: (Negative control) was administered saline solution (0.9% NaCl) orally on a regular basis for 4 weeks.

Group 2: Received *U. dioica* root aquatic extract (100 mg/kg b.w) orally daily for 4 weeks (Abu Almaaty *et al*., 2021; Oladimeji *et al*., 2022).

Group 3: Received *U. dioica* root aquatic extract (200 mg/kg b.w) orally daily for 4 weeks (Pashazadeh *et al*., 2013; Peyvandi *et al*., 2023).

Group 4: (Positive control) received orally TiO_2 NPs (300 mg/kg b.w) for 4 weeks daily (Hussein *et al*., 2019; Moradi *et al*., 2019; Shirdare *et al*., 2022).

Group 5: Received *U. dioica* root aquatic extract (100 mg/kg b.w) and TiO_2 NPs (300 mg/kg b.w) after 1 hour orally daily for 4 weeks.

Group 6: Received *U. dioica* root aquatic extract (200 mg/kg b.w) and TiO_2 NPs (300 mg/kg b.w) after 1 hour orally daily for 4 weeks.

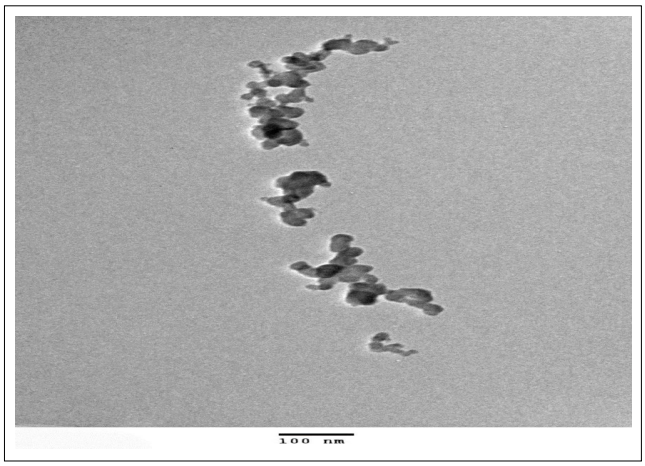


Fig 1: Transmission electron micrograph (TEM) of titanium dioxide nanoparticles (TiO_2 NPs).

Rats were anesthetized with diethyl ether and weighed following the experiment's end (fourth week). Blood samples were obtained from the retro-orbital venous plexuses. The serum was centrifuged at 2500 rpm for 15 min and stored at -80°C for biochemical analysis. The abdomen was dissected and then the liver was isolated, weighed and dissected into two parts. The first part was prepared and fixed in 10% formalin for histopathological and immunohistochemical examinations. A separate portion was homogenized for biochemical analysis in 4 volumes of phosphate buffer (pH 7.4).

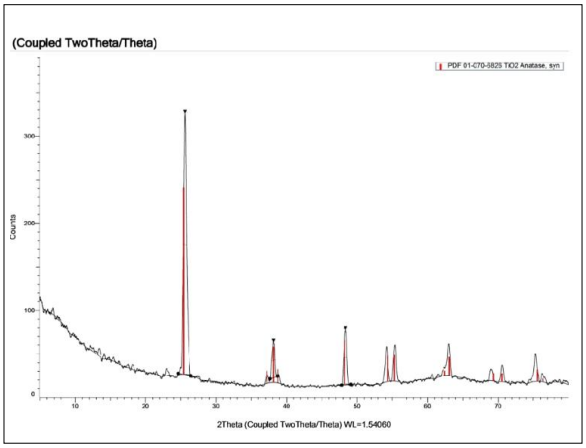


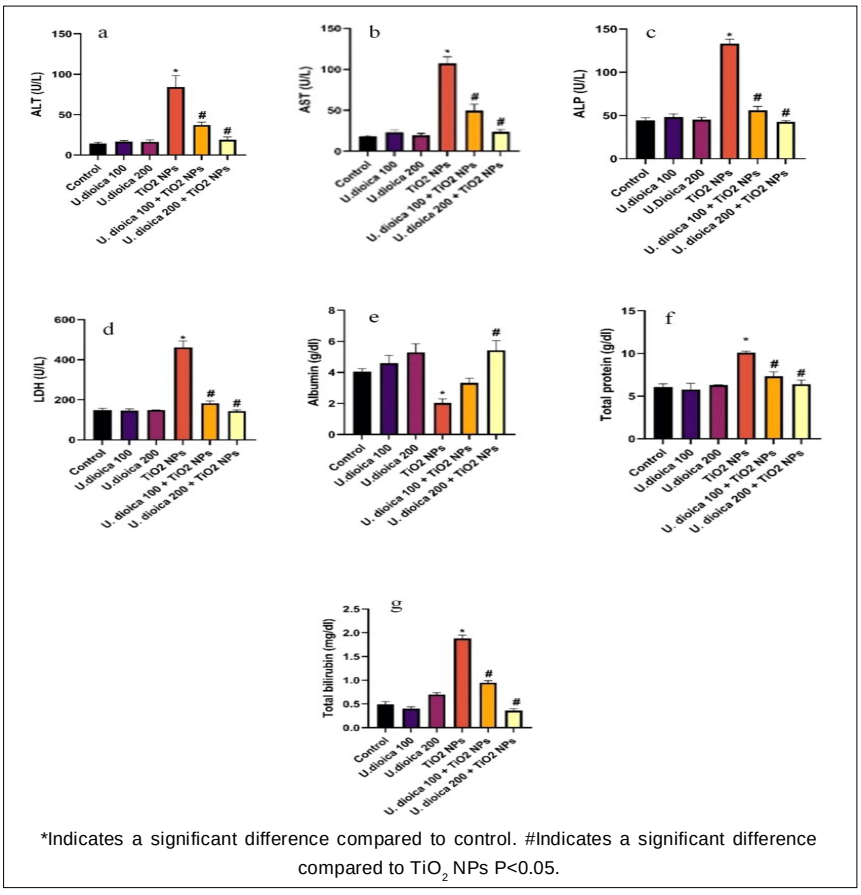
Fig 2: XRD pattern of TiO_2 NPs.

The ALT, AST, ALP, LDH, albumin, total bilirubin and total protein levels in serum were evaluated utilizing kits as per protocols.

For the biochemical assessment of GSH, SOD and MDA, a portion of liver homogenate was subjected to centrifugation at 12,000 g at 4°C for 20 min. The supernatant was then separated and preserved at -80°C, while the oxidative stress indicators activities were ascertained by employing kits, following the manufacturer's instructions.

Liver tissues fixed in formalin were embedded in paraffin blocks, sliced into 5-micrometer thickness, placed on glass slides and stained with hematoxylin and eosin (H and E). The prepared liver slices were examined using an Intellisite Ultra-Fast Scanner (Digital Pathology Slide Scanner, Philips FMT0225, Jeddah, KSA).

Furthermore, 5-µm formalin-fixed, paraffin-embedded slices were employed to conduct immunohistochemical staining via the streptavidin-biotin technique utilizing caspase 3 antibodies as an indicator for programmed cell death (apoptosis), according to a previous study (Ghonimi *et al*., 2022).



*Indicates a significant difference compared to control. #Indicates a significant difference compared to TiO_2 NPs P<0.05.

Fig 3: (a-g) Levels of serum (a) ALT, (b) AST, (c) ALP, (d) LDH, (e) Albumin, (f) Total protein and (g) Total bilirubin of different experimental groups.

The statistical analysis was performed with the Statistical Package for Social Sciences (SPSS; Windows, version 25). Data were presented as mean ± standard error. The difference across several experimental groups was assessed employing One Way ANOVA (Tukey test). A *P*-value<0.05 was deemed statistically significant. Histograms were generated with Graph Pad Prism software (v9.0.2; San Diego, CA, USA).

RESULTS AND DISCUSSION

Biochemical analyses

Increased liver enzymes levels in serum, including ALT, AST, ALP, LDH, total protein and total bilirubin, signify hepatic injury. In our study, the TiO_2 NPs intoxicated rats manifested significantly raised plasma levels of these markers compared to the control group. However, (*U. dioica* 100+ TiO_2 NPs) and (*U. dioica* 200+ TiO, NPs) treated groups exhibited a substantial decrease in the liver enzymes compared to the TiO_2 NPs-intoxicated group (Fig 3 a-f). Also, the findings indicated significantly declined albumin levels in the TiO_2 NPs intoxicated group compared to the control group. (*U. dioica* 200 + TiO_2 NPs) treated rats displayed a marked increase in the serum albumin level in comparison with the TiO_2 NPs intoxicated group (Fig 3 g).

Liver oxidative stress analysis

TiO_2 NPs intoxicated rats displayed a significant decrease in the hepatic levels of GSH and SOD in comparison to the control group (Fig 4 a-b). On the other hand, (*U. dioica* 100 + TiO_2 NPs) and (*U. dioica* 200 + TiO_2 NPs) treated rats showed a significant elevation in the hepatic levels of GSH and SOD levels in comparison to the TiO, NPs intoxicated rats. Moreover, TiO_2 NPs intoxicated rats displayed a marked increase in the hepatic MDA levels compared to control groups. On the other hand, liver MDA levels were significantly decreased in (*U. dioica* 100+ TiO_2 NPs) and (*U. dioica* 200+ TiO_2 NPs) treated rats compared with TiO_2 NPs intoxicated rats (Fig 4 c).

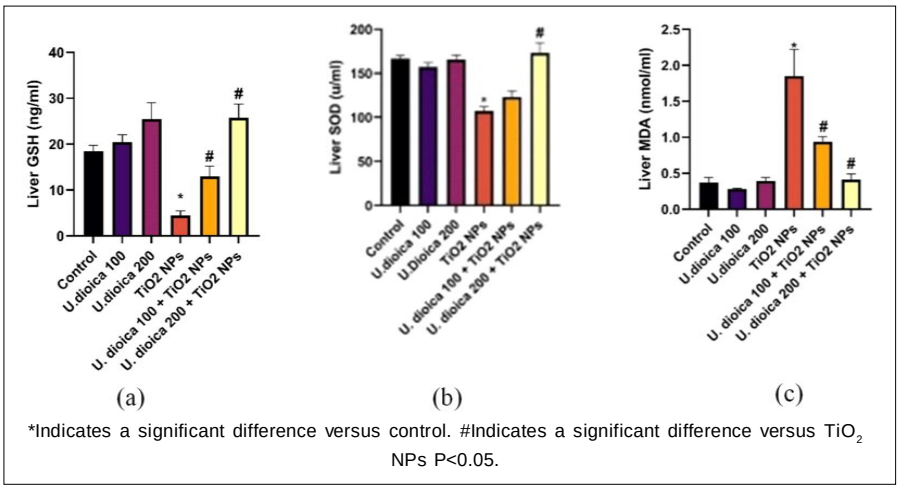
Liver histopathological examination

The histological photomicrographs below illustrate the difference between the treated groups, the differences between healthy and injured hepatocytes and the extent of damage inflicted upon the cells following exposure to TiO_2 NPs in (Fig 5 A-F). Histological studies exhibited that the control group demonstrated normal hepatic architecture (Fig 5 A). The liver structure of the *U. dioica* (100, 200 mg/kg b.w.) groups showed that the histology of the liver revealed a normal structure similar to the control group (Fig 5 B and C). TiO_2 NPs - intoxicated group showed obvious histopathological changes; these include marked centrilobular hepatic necrosis associated with hemorrhage, congestion of hepatic artery, periportal necrosis and bile duct hyperplasia (Fig 5 D). The structure of the liver treated with (*U. dioica* 100 + TiO_2, NPs) showed significantly mitigated hepatic necrosis with focal congestion of the blood sinusoids and single apoptotic cells, a notable decrease in the hepatic inflammatory changes with microfocal mononuclear cells infiltration (Fig 5 E). The structure of the liver treated with (*U. dioica* 200 + TiO_2 NPs) showed a remarkable decline in hepatic degeneration with mild congestion of the blood sinusoids and normal hepatocytes (Fig 5 F).

Immunohistochemical examination of caspase 3 in the liver

In our present study, immunostaining of caspase-3 in the liver revealed negative expression of caspase-3 in the control, (*U. dioica* 100) and (*U. dioica* 200) groups (Fig 6 a-c, Fig 7). Conversely, the TiO_2 NPs group exhibited elevated caspase-3 expression in the hepatic cells (Fig 6 d, Fig 7). Furthermore, a moderate caspase-3 expression was observed in the livers of rats administered (*U. dioica* 100 + TiO_2 NPs) (Fig 6 e, Fig 7). Also, the liver of (*U. dioica* 200 + TiO_2 NPs) groups revealed mild caspase-3 expression (Fig 6 f, Fig 7).

This research was conducted to evaluate the implication of *U. dioica* root aquatic extract on the hepatotoxicity of male

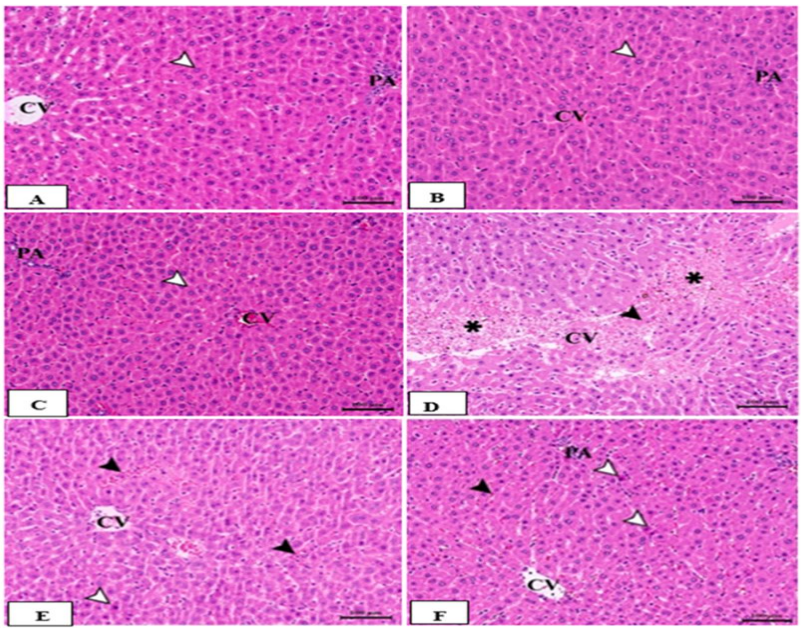


*Indicates a significant difference versus control. #Indicates a significant difference versus TiO_2 NPs P<0.05.

Fig 4 (a-c) Levels of liver homogenate (a) GSH, (b) SOD and (c) MDA of different experimental groups.

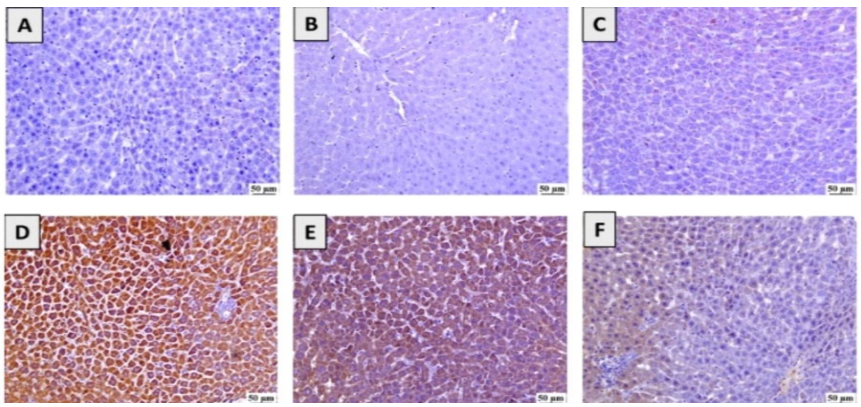
albino rats induced by TiO_2 NPs. Herein, we revealed that intake of TiO_2 NPs (300 mg/ kg b.w orally daily) for four weeks in rats led to pathological changes in the liver; these alterations are associated with liver function and oxidative stress.

Liver enzymes, including ALT, AST, ALP and LDH are found naturally in liver cells and they are released into the bloodstream when the plasma membrane is damaged or a cell dies, which raises the enzyme levels in the serum (Kumar *et al*., 2023). In our study, exposure to TiO_2 NPs



A: The control group (A) showed normal hepatocytes arranged in cords around a central vein (white arrowhead). B andC: (*U. dioica* 100, 200 mg/kg), showed normal hepatic tissues (white arrowhead). D: TiO_2 NPs-intoxicated showed marked centrilobular hepatic necrosis (black arrowhead) associated with haemorrhage (asterisk). E: (*U. dioica* 100 mg/kg, + TiO_2 NPs) showed a decrease in hepatic necrosis with focal congestion of the blood sinusoids (black arrowheads) and single apoptotic cells (white arrowheads). F: (*U. dioica* 200 mg/kg + TiO_2 NPs) showed mild congestion of the sinusoids (black arrowhead) and a marked decrease in hepatic degeneration and apoptosis (white arrowheads) (CV indicates central vein, PA indicates portal area).

Fig 5 (A-F): Liver sections (H and E; X100).



(A), (B) and (C) Control, (*U. dioica* 100), (*U. dioica* 200) negative expression of caspase-3. (D) TiO_2 NPs group showing positive expression of caspase-3. (E) (*U. dioica* 100 + TiO_2 NPs) group showing moderate expression of caspase-3 immunostaining. (F) (*U. dioica* 200 + TiO_2 NPs) group showing mild expression of caspase-3 immunostaining, scale bar 50 µm.

Fig 6 (A-F): Photomicrographs of the immunohistochemical stain of the liver against anti-caspase-3 antibody.

results in the liver enzymes release into the serum as an indirect indicator of liver injury, unlike the control group. Increased liver enzymes and oxidative stress indicate that the liver cell membranes' functional integrity has been lost and there is cellular leakage (Dash *et al*., 2019; Gomaa *et al*., 2019; Valentini *et al*., 2019).

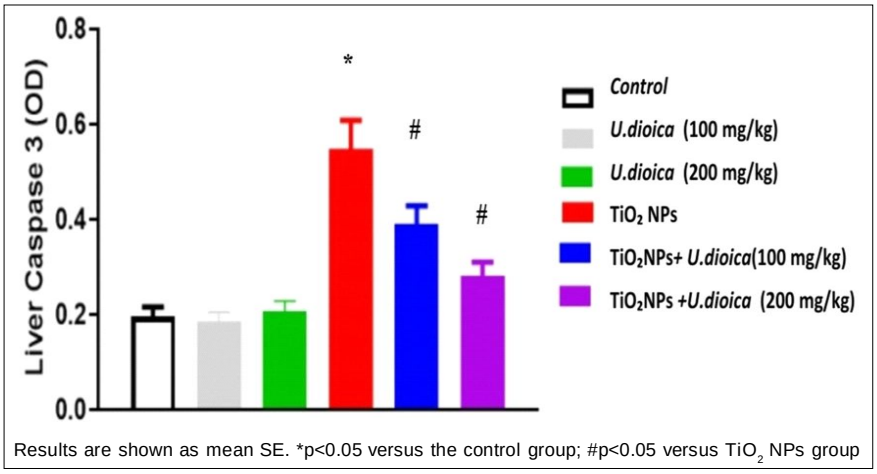
Compared to TiO_2 NPs intoxicated rats, treatment with *U. dioica* root extract (100 and 200 mg/kg) significantly improved liver enzyme levels in rats. This result is attributed to the presence of scopoletin, which is one of the most important active substances in *U. dioica* root (Chauhan *et al*., 2022). Recent research indicated that administering scopoletin (1, 5 and 10 mg/kg) to CCl_4-intoxicated rats enhances liver parameters, encompassing AST, ALT, ALP, bilirubin, total protein and albumin levels (Sharma *et al*., 2022).

Oxidative stress status is indicated by alterations in ROS, MDA levels and antioxidant enzymes (SOD, CAT and GPx) (Díaz-de-Alba *et al*., 2017; Lohiya *et al*., 2017). Herein, the TiO_2 NPs administration (300 mg/kg body weight) led to a reduction in SOD and GSH levels, while MDA levels. Abbasi-Oshaghi *et al*. (2019) demonstrated that rats treated with TiO_2 NPs (50 and 100 mg/kg) developed oxidative damage in their liver and intestine. Also, TiO_2 NPs exposure can trigger signaling cascades related to oxidative stress and ultimately result in cell oxidative damage (Dar *et al*., 2020; Moradi *et al*., 2019).

Treatment with *U. dioica* root extract (100 and 200 mg/kg) in our study led to improvement of TiO_2 NPs-stimulated oxidative stress. This result is due to the presence of antioxidants and active substances present in *U. dioica*, such as coumarin or scopoletin. Scopoletin inhibits the synthesis of MDA and rises the activity of SOD and GSH concentration (Sharma *et al*., 2022). Abu Almaaty *et al*. (2021) have stated that *U. dioica* root extracts significantly reduced oxidative stress in scopolamine-intoxicated rats. Concerning liver histological examination, rats exposed to TiO_2 NPs showed marked centrilobular hepatic necrosis associated with hemorrhage, marked congestion of the hepatic artery, periportal necrosis admixed with extensive hemorrhage and marked bile duct hyperplasia, multifocal periportal infiltration of mononuclear cells, distortion of the hepatic arrangement associated with marked hepatic degeneration and apoptosis and fibroblastic activity compared to control. This result is comparable to previous studies, including Hamed *et al*. (2021) who demonstrated inflammatory mononuclear cellular infiltrations between the hepatic tissue and around the portal area following exposure to TiO_2 NPs. Additionally, Valentini *et al*. (2019) reported that aggregated TiO_2 NPs that were visible in the liver sinusoids were phagocytosed by Kupffer cells.

On the other side, in treatment TiO_2 NPs-intoxicated rats with *U. dioica* (100 and 200 mg/kg b.w.); the liver architecture was enhanced. This is aligned with Sharma *et al*. (2022) findings in evaluating the positive effects of scopoletin; which is one of the most important active substances in *U. dioica* root; the rats treated with scopoletin exhibited alterations that seemed comparable to those of the standard control group. *U. dioica* may have a variety of biological effects, such as the capacity to initiate or inhibit important cellular metabolic processes, have antioxidant and anti-mutagenic qualities and trigger apoptotic pathways (Esposito *et al*., 2019).

In our work, treating rats with TiO_2 NPs (300 mg/kg b.w.) leads to an increase in the caspase 3 expression and consequently programmed cell death compared to the control group and this is aligned with prior outcomes (Abbasi-Oshaghi *et al*., 2019; Fadda *et al*., 2018). Nevertheless, TiO_2 NPs activated caspase-3 in animal models to cause apoptosis (Fadda *et al*., 2018). Abbasi-Oshaghi *et al*. (2019) stated that the caspase 3 expression was elevated in rat liver subjected to TiO_2 NPs (10, 50 and 100 mg/kg) for 30 days compared with the control. Nonetheless, the alteration in the 100 mg/kg group was more significant.



Results are shown as mean SE. *p<0.05 versus the control group; #p<0.05 versus TiO_2 NPs group

Fig 7: Quantitative analysis for immunohistochemical staining showing a significant increase in caspase-3 expression in the liver of rats exposed to TiO_2 NPs. (*U. dioica* 100 and 200) remarkably ameliorated caspase-3 expression in the liver tissues exposed to TiO_2 NPs.

Treatment with *U. dioica root* extract (100 and 200 mg/kg) in our study mitigated the caspase 3 expression levels and reduced apoptosis, aligning with Wu *et al*. (2022), who stated that scopoletin inhibits cell death induced by palmitate and bile acid in rat hepatocytes through alleviating endoplasmic reticulum stress, mitigating oxidative stress and downregulating caspase-3 expression.

CONCLUSION

TiO_2, NPs are one of the most important causes of liver toxicity. These NPs cumulatively cause liver damage. Medicinal herbs are one of the promising therapeutic agents because they contain effective substances with beneficial effects. In the current study, TiO_2 NPs intoxicated rats (300 mg/kg b.w.) showed physiological alterations, including liver enzyme elevation, oxidative stress and liver histological changes. These changes were associated with the elevation of caspase 3 immune expression. Treatment with *U. dioica* with two various doses (100 and 200 mg/kg) reversed all the changes caused by TiO_2 NPs.

Informed consent

The experimental design was approved by the Animal Care and Use Committee at King Abdul-Aziz University, Faculty of Pharmacy, Jeddah, Saudi Arabia (Approval number: PH-1444-17).

Conflict of interest

The authors declare no conflict of interest.

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