ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Titanium dioxide (TiO_2) nanoparticles induced apoptosis of splenocytes in adult male albino rat and the protective role of Milk thistle seeds extract

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Manuscript Info

Manuscript History:

Received: 12 July 2014
Final Accepted: 29 August 2014
Published Online: September 2014

Key words:
TiO_2 NPs toxicity, Milk thistle, splenocyte, apoptosis

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Abstract

Titanium dioxide nanoparticles (TiO_2 NPs) are used as a common additive in many kinds of foods, personal care, and other consumer products used by people everywhere. However the toxological concern of TiO_2 NPs has been linked with its ability to generate free radicals. The protection and clearance of TiO_2 NPs by natural products extracts are recent studies and need further investigations. This work was carried out to evaluate the possible protective role of Milk thistle seeds extract against TiO_2 NPs toxicological effects in the spleen of male albino rat. Rats were intraperitoneally injected with 100 mg/kg and 150 mg/kg of TiO_2 once daily for 2 weeks. Others rats were orally administrated with 300 mg/kg of Milk thistle seeds extract for 1 week before and 1 week after the treatment with TiO_2 NPs. Histologically TiO_2 NPs caused anarchic splenic architecture, vacuolar degeneration in splenic parenchyma, depletion of splenocytes and decrease in splenic follicle size, and spelencytes apoptosis. Immunohistochemically, rats interaperitonialy injected with TiO_2 NPs exhibited increase in the percentage of Caspase-3^+ apoptotic cells; the increase of the apoptotic cells was dose dependant. The treatment with Milk thistle extract ameliorates these abnormalities effects and improved the structure and appearance of the treated splenocytes. From the present study it could be concluded that TiO_2 NPs is capable of inducing splenocytes apoptosis which would be the potential mechanism underlying the toxicity of TiO_2 NPs. Milk thistle extract could help in the protection of spleen against the toxic effects of TiO_2 NPs.

Introduction

Nanoparticles (NPs) are promising new materials with potential applications in several fields such as imaging, therapy, cosmetics, and food preservation additives, ect. The European Union defined the NPs as natural or manufactured material, presence in dispersed or aggregate state and their size usually under 100 nm in diameter (Magayeet al. 2012). The NPs have several characterizations such as small size, higher surface area and surface reactive chemical groups which facilitates their endocytosis by different cells (Robertson et al. 2010). Among various types of NPs, titanium dioxide (TiO_2) NPs with special characterizations such as surface functionalization and size are used in several fields in our daily life. Currently, TiO_2 NPs are produced abundantly and used widely because of their higher stability, anticorrosive and photocatalytic properties (Riu et al. 2006). It can be used in several catalytic reactions, contaminated water purification from hazardous and industrial by-products (Ni et al. 2007), and in nanocrystalline solar cells as a photoactive material (Yuan et al. 2010; Montazer and Seifollahzadeh 2011). In the field of nanomedicine, TiO_2 NPs are under investigation as useful systems in diseases diagnosis and advanced imaging and nanotherapeutics (Yuan et al. 2010). For example, TiO_2 NPs are being used in

photodynamic therapy (Szacilowski et al. 2007) and production of skin care products and antibacterial drugs (Yuan et al. 2010; Wiesenthal et al. 2011; Montazer et al. 2011).

Many studies have shown that TiO_2 NPs accumulate in several organs such as the liver, kidneys, spleen, lungs, and heart of animals (Wang et al. 2007; Liu et al. 2009). Furthermore, several researchers have revealed the toxic impacts of TiO_2 NPs on various organs (Zhao et al. 2009; Fabian et al. 2008; Oberdorster 2001). However, when the NPs become progressively smaller, their surface areas, in turn, become progressively larger, and concerns about the harmful effects of TiO_2 NPs on human health associated with the decreased size has been raised (Maynard and Kuempel 2005; Tsuji et al. 2006). Several types of TiO_2 NPs have been reported to cause toxicity in vitro, resulting in apoptosis and formation of apoptotic bodies, mitochondrial abnormalities (Wang et al. 2007). Recently, TiO_2 NPs accumulated in the mouse spleen, leading to congestion and lymph nodule proliferation of spleen tissue, and splenocytes apoptosis. Moreover, it induces apoptosis in the mouse splenocyte via mitochondrial mediated pathway (Li et al. 2010). Also, TiO_2 NPs may have the potential to penetrate the blood–brain barriers and blood placenta barriers (Sang et al. 2012).Additionally, TiO_2 NPs could not be cleared from the liver and spleen within 2 weeks after injection, indicating that TiO2 NPs can accumulate in these organs (Chen et al. 2009).

Studies about the role of natural product in protection and clearance of TiO_2 NPs or other NPs from the body were very rare. Silymarin is one of the primary chemical constituents (80%) of Milk thistle (*Silybum marianum*) seed extract (Szilárd et al. 1998). It is composed of several polyphenolic and flavonolignans ingredients. The major ingredient (60%) of silymarin is silibinin, other ingredients include silichristin and silydianin (Kroll et al. 2007; Brantley et al. 2010). Previously, several studies revealed the protection role of silymarin against liver diseases such as cirrhosis, hepatitis and liver cancer. Extracts of Milk thistle seeds have been recognized as "liver tonics" in many centuries (Hikino et al. 1984). Some studies proved that low dosages of silymarin were ineffective (Angulo et al. 2000) and larger doses of silymarin was biologically active and had therapeutic effects in liver diseases (Lieber et al. 2003). Standardized Milk thistle seed extract was found to prevent degeneration and help repair of liver cells exposed to toxic materials (Szilárd et al. 1998). Silymarin has strong antioxidant properties and has been shown to inhibit the growth of human prostate, breast, and cervical cancer cells in vitro (Asghar andMasood2008). It has been shown that silymarin strongly prevents skin tumor promotion in mice by scavenging free radicals and reactive oxygen species and strengthening the antioxidant system (Singh and Agarwal 2002). It scavenges free radicals that can damage cells exposed to toxins as compared with vitamin E (Adzet 1986) and increases the level of the important antioxidant enzymes such as superoxide dismutase (Müzes et al. 1991). It has regenerative action as it stimulates protein synthesis in the liver, which results in an increase in the production of new liver cells to replace the damaged ones (Fiebrich and Koch 1979). Silymarin also protects cardiomyocytes against doxorubicin-induced oxidative stress via cell membrane stabilization effect, radical scavenging and iron chelating potency (Chlopcíková et al. 2004). Immunologically, silymarin has immunmodulation action as it could prevent ultraviolet-induced immunosuppression and oxidative stress by inhibiting the infiltration of leukocytes, and myeloperoxidase activity (Katiyar 2002).

There is no previous study revealed the protection effects of some natural products against the NPs toxicity. In this study we investigated the toxic impacts of different doses of TiO_2 NPs on the spleen of adult albino rats using histological and immunohistochemical studies. Thereafter, we tried to figure out the protection role of Milk thistle seed extracts against the toxic impacts caused by TiO_2 NPs. The data from our study might introduce important information about the protection and clearance of Tio_2 NPs used for medication purposes.

Materials and methods

Materials

Biotin-conjugated goat anti-rabbit IgG, titanium IV isopropoxide 97%, isopropanol 99.5% were purchased from Sigma–Aldrich Chemical Co. Rabbit anti-Caspase 3 antibody was purchased from NeoMarkers, Fremont. CA, USA. Milk thistle (*Silybum marianum*) seeds were purchased from El Masria Company for herbal medicine in Sohag, Egypt. Seeds of milk thistle were collected from Nile valley regions including El-Delta, El-Faiyum and the surrounding regions in the period from 2010 to 2012. Disposable animal feeding needles (50mm) were purchased from Fuchigami, Kyoto, Japan.

TiO_2 NPs preparation and characterization

Preparation of TiO_2 NPs was carried out following the procedure described previously (Khalil et al. 1998). Briefly, 200 ml of titanium IV isopropoxide (0.4 M) solution in isopropanol was hydrolyzed by drop wise addition of bi-distilled water under magnetic stirring for 1 hr at room temperature and then solution was aged for 3 days without stirring. The aged solution was filtered off and the resulting gel was allowed to dry overnight at 60°C for 24 hrs, and

dry further at 120°C for 24 hrs. The produced powder called titania precursor. Portion of the precursor material was calcined in muffle furnace at 400°C for 3hr in a static air atmosphere and then allowed to cool naturally till room temperature. The material thus obtained was termed as TiO_2 NPs which characterized and employed in this study.
We used X-ray diffraction techniques and transmission electron microscope (JEM-1010 Joel) to evaluate the size and calcinations of the TiO_2 NPs. TiO_2 NPs were sonicated using ultrasonic dispersion to investigate their aggregations under the light microscope before the injections into rats.

Milk thistle seeds extract

The extraction of Milk thistle seed was done based on previous literature (Gopalakrishnan and Raghu 2014; Duan et al. 2004). Milk thistle seeds were washed and dried in oven for 3 hours at 60°C.Then seeds were grinded into powder using blender to be used in this experiment. Milk thistle seeds powder (15g) was suspended in 100 ml di-ionized water, heated for 15 minutes at 100°C. Then the suspension was cooled down and filtered using Whatman filter paper. This filtered extract was kept in 4°C and used for over a week time.

Animal and treatment:

A total number of 50 adult male albino rats (*Rattus albus*) were purchased from Assuit's Experimental Animal House, Assuit University. The average weight of each rat was measured (150 ± 20g). Animals were housed in stainless steel cages in a ventilated animal room at 28° C with availability of distilled water and sterilized food. Animals were acclimated to this environment for 5 days prior to the experiment. All procedures used in this experiment were approved with the local ethics committee at Sohag University. Animals were randomly divided into five groups, 10 animals each as the following: Animals of group 1 were received standard rat chow diet and water ad libitum for 4 weeks. Animals, of group 2 were intraperitonially injected with 0.5ml of TiO_2 NPs (100mg/kg) once daily for 2 week. Animals of group 3 were intraperitonially injected with 0.5ml of TiO2 (150mg/kg) once daily for 2 week. Using animal feeding needle, animals of group 4 were orally administrated with 2ml (300mg/kg) of Milk thistle seeds extract twice daily for 1 week then intraperitonially injected with 5ml TiO_2 NPs (100mg/kg) once daily for 2 weeks. Thereafter, they orally administrated again with 2ml (300mg/kg) of Milk thistle seed extract (300mg/kg) twice daily for 1 week. Animals of group 5 were orally administrated with 2ml (300mg/kg) of Milk thistle seed extract twice daily for 1 week, then they intraperitonially injected with 5ml of TiO_2 NPs (150mg/kg) once daily for 2 weeks. Then, they orally administrated again with 2ml (300 mg/kg) of Milk thistle seed extract twice daily for 1 week. The supply of the rat chow diet was stopped before the oral administration of the extraction. Additionally, the volume of the Milk thistle extract (2ml) was orally administrated through 30 min to prevent animal stomach dilation or rupture.

Histological examinations

At the end of the experiment, the rats were anesthetized using ether inhalation, sacrificed, carefully dissected, and the spleen was taken.
For light microscopic observations, specimens of the spleen were fixed in 10% formalin for preparation of paraffin blocks. Paraffin sections with thickness 7 µm of spleen were cut using microtome, deparaffinized in xylene and hydrated in descending series of ethanol alcohol. Hydrated paraffin sections were then stained with hematoxylin and eosin, mounted in DPX mounting media, and observed under light microscope (Axio Lab.A1, Carl ZEISS, Germany) equipped with AxioCamERc5s camera.

Quantitative analysis of spleen follicle size

A total number of 15 representative images stained with hematoxylin and eosin were selected from each animal group for the quantitative analysis of the spleen follicle size. Two representative spleen follicles were selected from each representative image, the total number of spleen follicle from each group were 30 representative spleen follicles (n = 30). The average size of each representative pulp was calculated using image analysis software.

Electron microscopic examination

Specimens of the spleen were fixed in 2.5% glutaraldehyde at 4°C, washed in three to four changes of cacodylate buffer (pH 7.2) for 20 min at every change, and post-fixed in 1% osmium tetroxide for 2 h. They were then dehydrated in ascending grades of ethanol. After immersion in propylene oxide, the specimens were embedded in epoxy resin mixture. These samples were kept in an incubator at 35°C for 1 day, then at 45°C for another day, and finally at 60°C for 3 days. Semi-thin sections (1 µm thick) were prepared using an LKB (Bromma, Sweeden) ultra-microtome, stained with 1% toluidine blue, and examined by means of a light microscope. Ultrathin sections (500–800 Å) were stained with uranyl acetate and lead citrate and examined using an electron microscope Jeol JEM 1010 (Tokyo, Japan) at 80 kV at the electron microscopic unit, Sohag University.

Immunohistochemical examinations

To identify the Caspase 3^+ cells, the sections were de-paraffinezed, dehydrated, washed in 0.01 M PBS three times and incubated with blocking buffer (PBS/0.1% Tween 20 + 5% bovine serum albumin) for 3 hrs at room temperature. After washing with PBS sections were incubated for 2 hrs with rabbit anti-Caspase 3 (1:100 or 0.01mg/ml) at room temperature. After washing three times, the sections were incubated in biotin-conjugated goat anti-rabbit IgG for 2 hrs at room temperature, washed PBS three times, incubated for 1 h in ABC complex at room temperature and then visualized with a chromogen solution that contained 0.05% 3′,3′-diaminobenzadine. Immuno-stained sections then counterstained with haematoxylin, mounted with DPX mounting media and investigated under light microscope (Axio Lab.A1, Carl ZEISS, Germany, equipped with AxioCamERc5s camera) for evaluation the biodistribution and number of Caspase-3^+ cells.

Quantitative analysis of apoptotic cells in the spleen

A total number of 18 representative images immune-stained with anti-Caspase 3 antibody were selected from each animal group for the quantitative analysis of the apoptotic cell numbers in the treated and non treated spleen. The number of the apoptotic cells of each representative image was counted in 0.94 mm^2 using the image J software. Results were expressed as the average ± standard deviation, and the number of representative images used was 18 representative images (n=18)

Results:

TiO2 NPs characteristics

The aggregations of prepared TiO_2 NPs were investigated under light microscope before intraperitoneal injection in rats. The maximum size of the aggregations in the TiO_2 NPs suspensions was ranged from 0.8 -1.2 µm (Fig. 1a). Transmission electron microscope and x-ray diffraction pattern investigations demonstrated that the average particle size was 14 ± 3.5 nm in diameter (Fig. 1b).

List of figures

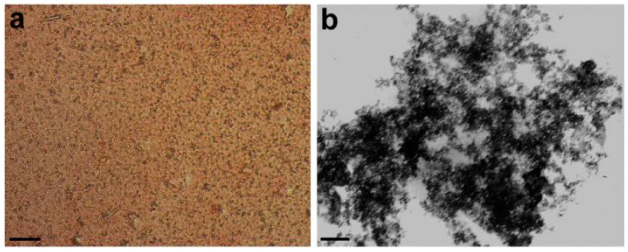

Fig. 1 light microscope image showing the aggregations of TiO_2 NPs (**a**). Transmission electron microscope image showing TiO_2 NPs (**b**). Scale bar **a** 10 µm, **b** 200 nm

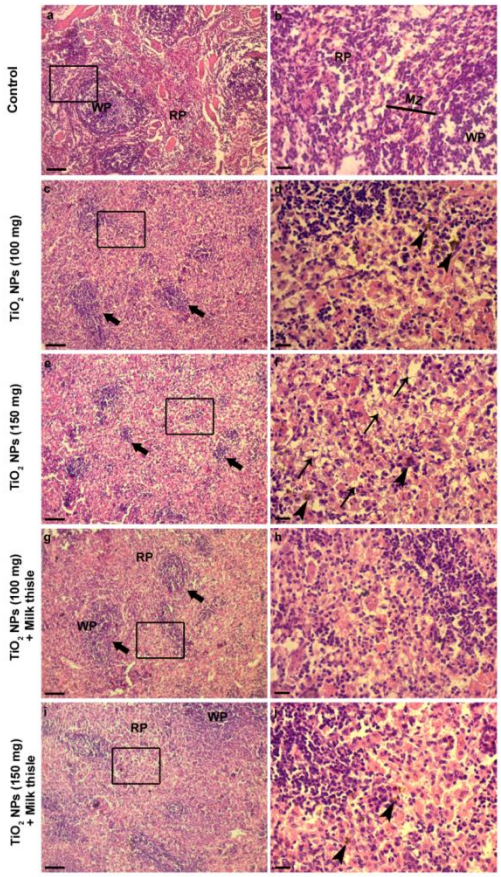

Fig. 2 Photomicrographs of rat spleen stained with H&E. Control rat spleen showing normal spleen architecture with intact follicle, white pulp (WP) surrounded with marginal zone (MZ) and clear red pulp (RP) (**a, b**).Rat spleen after interaperitoneal injection with TiO_2 NPs (100 mg/kg) showing abnormal spleen architecture, size of spleen follicle decreased (bold arrows) and cellular depletion of the follicle, the TiO_2 NPs were observed in both white and red pulps (arrow heads) (**c, d**). Rat spleen after interaperitoneal injection with TiO_2 NPs (150 mg/kg)showing vacuolar degeneration in the splenic parenchyma (arrows), marked depletion of lymphocytes with loss of demarcation between the white and red pulp (bold arrows), the TiO_2 NPs were observed in both white and red pulps (arrow heads) (**e, f**). Rat spleen after orally administration with Milk thistle seeds extract and interaperitoneally injection with TiO2 NPs (100 mg/kg) showing improvement of the spleen architecture, intact splenic architecture, increased lymphocytes and follicle size improvement (g, h). Rat spleen after orally administration with Milk thistle and interaperitoneally injection with TiO2 NPs (150 mg/kg) showing improvement of the spleen architecture with marked white pulp (WP) and red pulp (RP) with improvement of the follicle size (i, j). WP, white pulp; MZ, marginal zone; RP, red pulp. Scale bare a, c, e, h, i 50 µm, b, d, g, j 10 µm

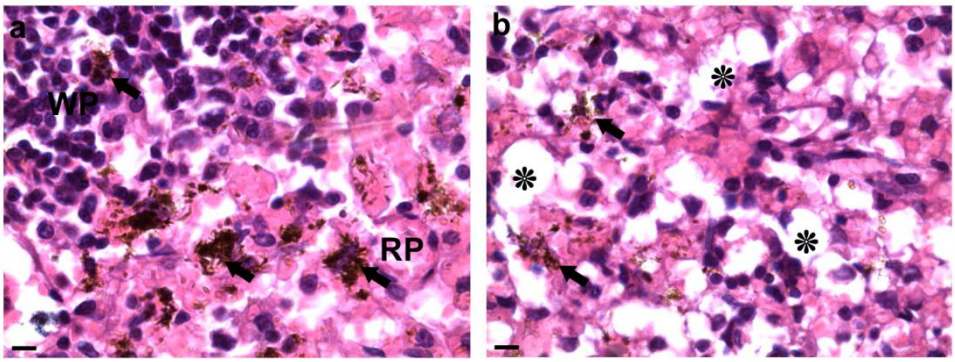

Fig. 3 Photomicrographs with higher magnifications of rat spleen treated with TiO_2 NPs and stained with H&E. Rat spleen intraperitoneally injected with 100 m/kg TiO_2 NPs showing the accumulation of TiO_2 NPs (bold arrows) in the spleen white pulp (WP) and spleen red pulp (RP) parenchyma (**a**). Rat spleen intraperitoneally injected with 150mg/kg showing the vacuolar degeneration (asters) of the splenocytes and deposition of TiO_2 NPs (bold arrows) in the spleen parenchyma (**b**). WP, white pulp; RP, red pulp. Scale bare 5 µm

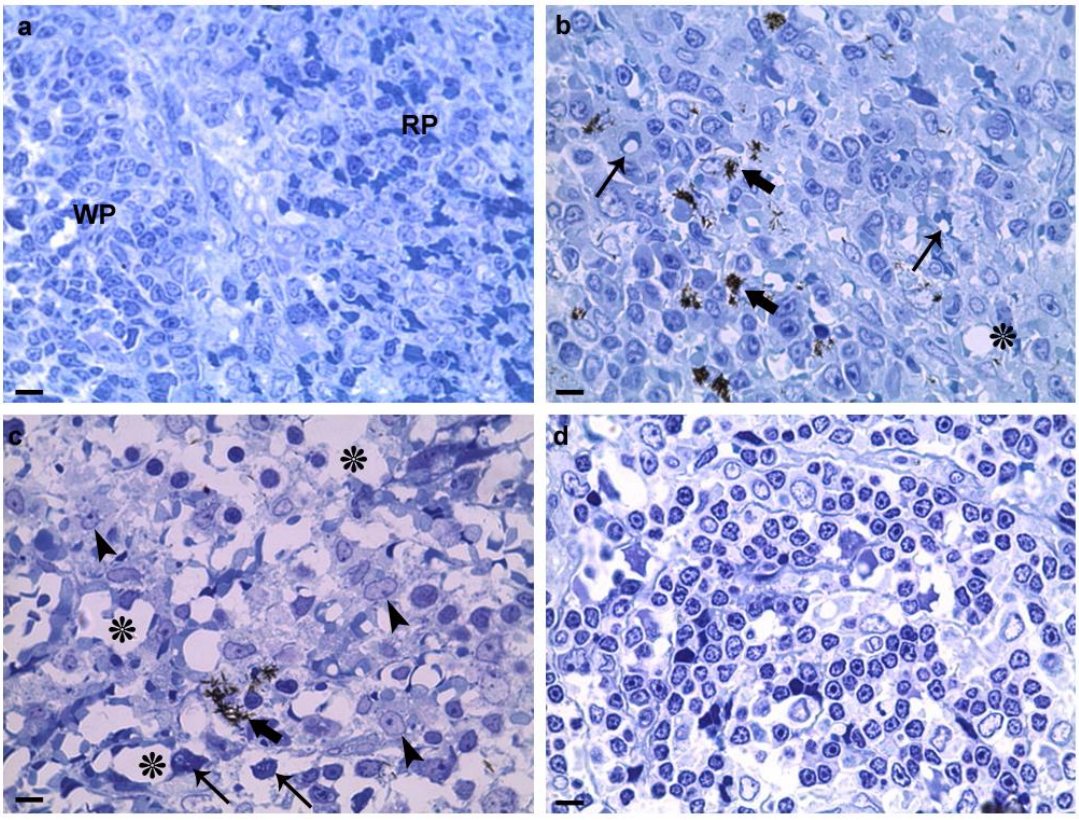

Fig. 4 Photomicrographs of semi-thin sections of control rat spleen stained with toluidine blue. Normal rat spleen showing normal architecture with intact follicle white pulp (WP) and clear red pulp (RP) (a). Rat spleen after interaperitoneal injection with TiO2 NPs (100 mg/kg) are showing TiO2 NPs deposition in the splenic parenchyma, particularly in the white pulp (bold arrows), mild vacuolar degeneration in the splenocytes cytoplasm (aster) and early stage of nuclear apoptosis (arrows) (b). Rat spleen after interaperitoneal injection with TiO2 NPs (150 mg/kg) showing severe vacuolar degeneration in the splenic parenchyma (asters), marked depletion of lymphocytes, late stage of nuclear apoptosis (arrow heads), nuclear apoptosis (arrows) and TiO2 NPs deposition in the splenic parenchyma (bold arrow) (c). Rat spleen after orally administration with Milk thistle extract and

interaperitoneallyinjection with TiO2 NPs (150 mg/kg) are showing improvement of the spleen architecture with improved lymphocytes appearance (d). WP, white pulp; RP, red pulp. Scale bar 5 µm

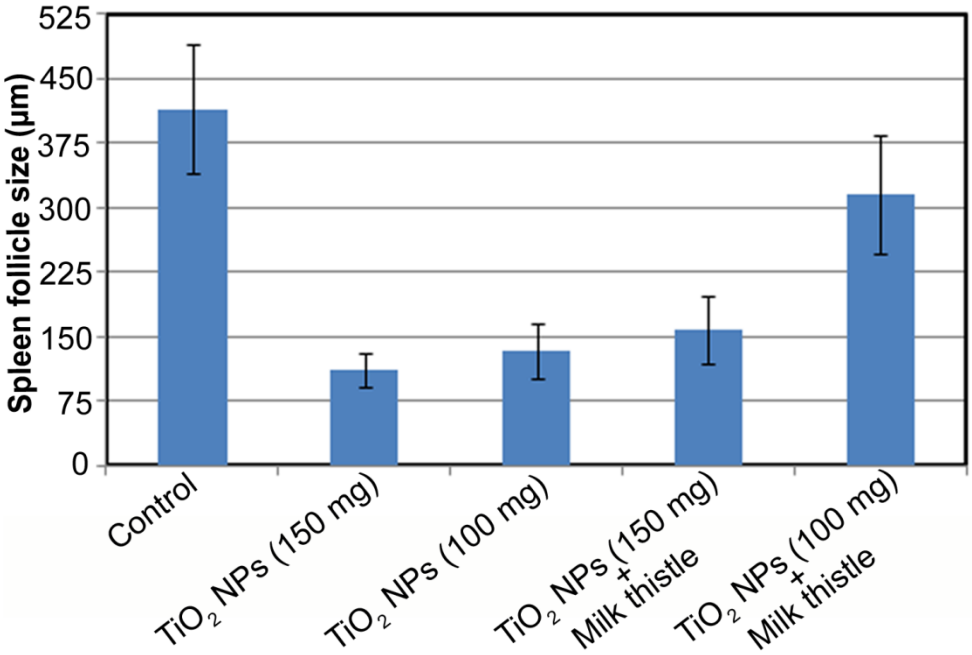

Fig. 5 A graph showing the quantitative analysis of the spleen follicle size in some representative sections of treated rats with TiO_2 NPs only or both TiO_2 NPs and Milk thistle extract. The spleen follicle size of treated rats with 150 mg and 100 mg of TiO_2 NPs was 3 times lower as compared with that of spleen follicle of control rats. There was no significant difference in the spleen follicle size between Milk thistle-TiO_2 NPs treated rats and that of control. (n = 30)

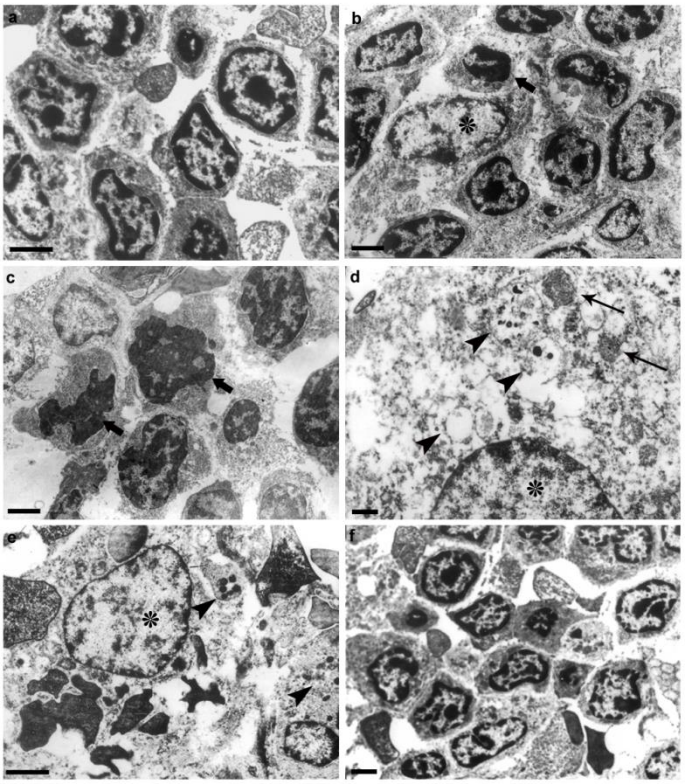

Fig. 6 Electron micrographs of rat spleen are showing normal rat splenocytes with normal nuclei, homogenous chromatin and clear nucleolus (**a**). Rat spleen after interaperitoneal injection with TiO_2 NPs (100 mg/kg) is showing apoptotic splenocytes with chromatin condensation (bold arrow) and apoptotic splenocytes (aster) with marginal chromatin (**b**). Rat spleen after interaperitoneal injection with TiO_2 NPs (150 mg/kg) is showing apoptotic splenocytes with highly condensed chromatin (bold arrow), several apoptotic bodies (arrow heads), swallow mitochondria (arrows) and marginal chromatin nucleus (asters) (**c, d, e**). Rat spleen after orally administration with Milk thistle and interaperitoneally injection with TiO_2 NPs (150 mg/kg) is showing splenocytes with normal nuclear chromatin (**f**). Scale bar **a, b, c, e, f** 2 µm, **d** 500 nm

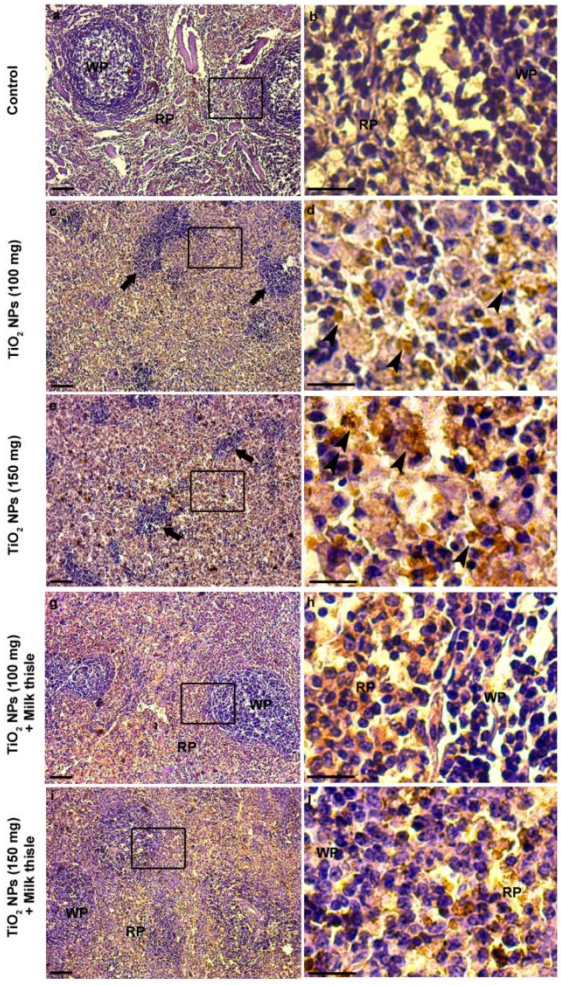

Fig. 7 Photomicrographs of rat spleen stained with anti-Caspase 3 antibody, control rat spleen showing distinct white pulp (WP), red pulp (RP) and light distribution of Caspase-3^+ cells with brown cytoplasmic deposits (**a, b**). Rat spleen after interaperitoneal injection with TiO2 NPs (100 mg/kg) is showing mild reduction in the splenic follicle size (bold arrows) and nuclear (arrow heads) and cytoplasmic positive immunoreaction for anti-Caspase 3 antibody(**c, d**). Rat spleen after interaperitoneal injection with TiO_2 NPs (150 mg/kg) showing reduction in the splenic follicle size (bold arrows) and widely distributed nuclear (arrow heads) and cytoplasmic positive immunoreaction for anti-Caspase 3 antibody (**e, f**). rat spleen after orally administration with Milk thistle seeds extract and interaperitoneally injection with TiO_2 NPs (100 mg/kg) showing mild distinct splenic white pulp (WP) and red pulp (RP) and light cytoplasmic positive immunoreaction for anti-Caspase 3 antibody (**g, h**). Rat spleen after orally administration with Milk thistle and interaperitoneally injection with TiO_2 NPs (150 mg/kg) is showing improvement of splenic follicle size and nuclear (arrows) and cytoplasmic positive immunoreaction for anti-Caspase 3 antibody (**i, j**). Scale bar **a, c, e, h, i** 50 µm, **b, d, g, j** 10 µm

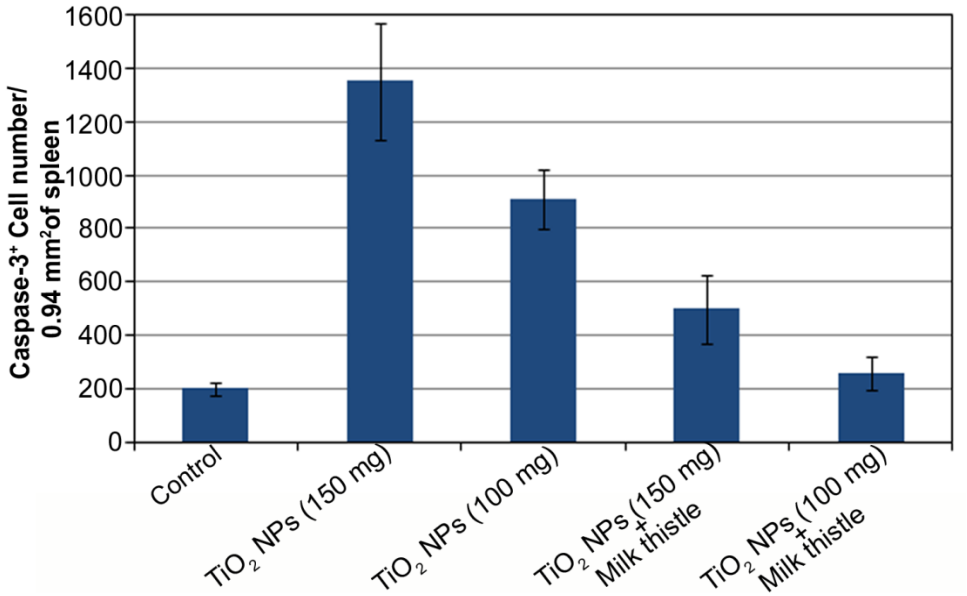

Fig. 8 A graph showing the quantitative analysis of the Caspase-3^+ cells number in spleen red pulp immunostained with anti-Caspase 3 antibody. The Caspase-3^+ cells number in the spleen treated with 150 mg and 100 mg of TiO_2 NPs was 7 times and 4 times higher respectively as compared with those in the control. After spleen treatment with both TiO_2 NPs and Milk thistle seed extract there was no significant increase of the apoptotic cells as compared with that of control. (n = 18)

Histological observations

The normal spleen of group 1 animals showed that the parenchyma of the spleen consisted of lymphoid tissues that are differentiated into white and red pulps. The white pulp was composed of lymphoid follicles (splenic Malpighian corpuscles) and a periarterial lymphoid sheath, which was composed of a sheath of many lymphocytes surrounding the central arteries (Fig. 2a, b). The lymphoid follicles consisted of a large number of lymphocytes; most of them appeared to have condensed darkly stained nuclei. Peripheral to the lymphatic follicles, marginal zones appeared separating the white pulp from the red pulp. The red pulp was composed of branching and anastomosing splenic cords (Billroth cords) and blood sinusoids in between. The cells forming the splenic cords included lymphocytes, plasma cells, macrophages, as well as other blood elements such as red blood cells, leucocytes, and platelets.

As shown in figure 2c, d spleen of group 2 rats which treated with 100mg/kg of TiO_2 NPs showed apparent decrease in the size of the lymphatic follicles compared with that of the control group. The white pulp appeared to have focal areas of cellular depletions. Spleen of group 3 which treated with 150mg/kg of TiO_2 NPs showed marked cellular depletion with loss of demarcation between the white and red pulps (Fig. 2e). The white pulp showed cells with acidophilic cytoplasm and many darkly stained pychnotic nuclei surrounded by empty space. Deposits of TiO_2 NPs were also observed clearly in the spleen red pulp and white pulp.

Spleen of group 4 which treated with 100mg/kg of TiO_2 NPs and Milk thistle extract showed apparent increase in the size of the lymphatic follicles in comparison with group 2 or 3. In some follicles, the marginal zones were well defined. However, a few lymphatic follicles still showed small areas of cellular depletion. An improvement of the cellular structure and spelenic parenchyma was observed (Fig. 2g, h). Spleen of group 4 rats which treated with 150mg/kg of TiO_2 NPs and Milk thistle extract showed apparent increase in the size of the lymphatic follicles in comparison with group 2, 3. There was an improvement of the splenic parenchyma and low density of splenic nuclei chromatin. Some follicles still showed pychnotic nuclei around the nuclei (Fig. 2i,j). Higher magnifications photographs of the rat spleen after treatment with TiO2 NPs showed depositions of TiO_2 NPs in the white pulp and red pulp (Fig. 3a). Also, the vacuolar degeneration in the spleen parenchyma was obviously observed (Fig. 3b).

For more clarification of the histological observations, toluidine blue stained sections showed the normal structure of group 1 spleen with distinct follicle and red pulp (Fig. 4a). As shown in figure 4b,c the treated spleen with 100 mg/kg and 150 mg/kg showed marked abnormal changes in the spleen follicle and red pulp. After treatment with 100 mg/kg of TiO_2, there were some early apoptotic splenic cells, and some depositions of NPs within the spleen parenchyma. Furthermore, after treatment with 150mg/kg there was a clear vacuolar degeneration of the spleen

parenchyma. Some cells also, entered in the late stages of the apoptosis. The splenic parenchyma cells appearance and structure clearly improved after administration of Milk thistle to the treated animals with TiO_2 NPs (Fig. 4d)

Quantitative analysis of spleen follicle size

The most obvious impact in the rat spleen caused by TiO_2 NPs in both group 2 (100mg/kg) and group 3 (150mg/kg) was the depletion of the spleen follicle size. As shown in figure 5 the spleen follicle size in both group 2 and group 3 significantly decreased 3 times lower as compared with that of group 1 or control. After administration of Milk thistle to the treated animals in group 2 and group 3, there was an improvement of the spleen follicle size especially that in the group 2.

Ultrastructural observations of splenocytes

As shown in Fig. 6a the untreated (control) rat spleen showed normal structure of splenocytes contained rounded nucleus with heterogenous chromatin and clear nucleolus. After spleen treatment with 100mg/kg of TiO_2 NPs, the ultrastructure of splenocyte showed early stages of splenocytes apoptosis and marginal condensed chromatin (Fig. 6b). After spleen treatment with 150 mg/kg of TiO_2 NPs spenocytes cytoplasm appeared with many vacuoles and their nuclei were characterized with highly condensed chromatin (Fig. 6c). Lysosomal vacuoles were found in the apoptotic splenocytes cytoplasm contained several apoptotic bodies (Fig. 6d, e). Some mitochondria appeared destructed but others was observed swollen and laden with heterogenous materials of variable size (Fig. 6d) the administration of Milk thistle extract to the rats treated with TiO_2 NPs showed improvement in the splenocyte structure and appearance with heterogenous cytoplasm and chromatin with clear nucleolus. The appearance and structure of splenocytes of administrated rats with Milk thistle were similar to those of control group (Fig. 6f).

Caspase-3 protein detection

Examination of Caspase-3 immmunohistochemical stained sections of control spleen showed mild distribution of Caspase-3^+ immunoreactive cells with slightly brown cytoplasmic deposits (Fig. 7a, b). Spleen sections from rats treated with 100 mg/kg TiO_2 NPs (Group 2) showed increased distribution of Caspase-3 immunoreactivity that was both nuclear and cytoplasmic (Fig. 7c,d). Furthermore after spleen treatment with 150 mg/kg of TiO_2 NPs the splenocytes showed widely distributed Caspase-3^+ nuclear and cytoplasmic immunoreactivity as compared with control group or group 2. Also, the population of Caspase-3^+ splenocytes increased by increasing the TiO_2 NPs injected dose (Fig. 7e, f). The oral administration of Milk thistle extract to the treated rats with 100 mg/Kg and 150 mg/kg of TiO_2 NPs showed lower cytoplasmic immunoreactivity between splenocytes and Caspase- 3 proteins (Fig. 7g,h,i,j). The population of Caspase-3^+ splenocytes decreased after administration of Milk thistle extract to the treated rats with TiO_2 NPs.

Quantitative analysis of apoptotic cells in the spleen

The main effect of TiO_2 NPs on splenocytes of rats was the induction of the cell apoptosis. As shown in figure 8, he Caspase-3^+ immunostained cells in spleen were counted in 18 representative images. The number of Caspase-3^+ cells in the spleen red pulp of treated rats with 150 mg/kg of TiO_2 NPs was 7 times higher as compared with that in the control spleen. Moreover, this number decreased in the treated rats with 100 mg/kg of TiO_2 NPs to be 5 times higher as compared with that of control. After administration of the Milk thistle seeds extract to the treated rats with 150mg/k and 100 mg/k of TiO_2 NPs the number of Caspase-3^+ cells in the spleen red pulp markedly increased to 2 times and 1 times higher than that of control.

Discussion

In this study we investigated the toxicological impacts of TiO2 NPs on the spleen as well as the potential role of Milk thistle seeds extract in the clearance of TiO_2 NPs from treated spleen and protection of spleen against toxic impacts caused by TiO_2 NPs. This is the first work investigates the probability of natural products extraction to protect and save tissue from the toxic impacts caused by NPs. In this work we intraperitoneally injected the animals with 100mg/kg (group 1) and 150mg/kg (group 2) of TiO_2 NPs for consecutive 15 days to study the acute toxic effects of these NPs. The data showed histopathological impacts in the spleen architecture and splenocytes structure caused by aggregations of TiO_2 NPs in spleen parenchyma. These impacts were cellular depletion with loss of demarcation between the white and red pulps, spleen follicle size reduction, and vacuolar degeneration in the splenic parenchyma. Some sub-cellular changes were also observed in splenocytes morphology and structure after treatment with TiO_2 NPs, these changes were aggregations of apoptotic bodies in the splenocytes cytoplasm resulted in acidophilic cytoplasm and many darkly stained pychnotic nuclei surrounded by empty space of splenocytes. Our results were in accordance with several recent studies which revealed that the toxicological impacts of TiO_2 NPs on the spleen are well evident. For example, intraperitoneal injection of TiO_2 NPs for consecutive 45 days caused obvious accumulation of TiO_2 NPs in the mouse spleen, leading to spleen atrophy and spleen follicle proliferation as well as splenocyte apoptosis (Li et al. 2010; Sang et al. 2013). Additionally, intravenous treated mice with TiO_2 NPs

showed signs of acute toxicity by accumulation of TiO_2 NPs in spleen parenchyma resulted in spleen damages (Chen et al. 2009). Furthermore, intravenous injection of higher dose of TiO_2 NPs (1387 mg/kg) to the mice caused mild damages in the spleen and increased white blood cells count (Jiaying et al. 2013). In recent studies, oral administration of TiO_2 NPs (10mg/kg) to the mice for 90 consecutive days caused the increases of spleen titers, immune dysfunction, and severe macrophage infiltration as well as apoptosis in the splenocytes (Sheng et al. 2014; Sang et al. 2013; Sang et al. 2012). The TiO_2 NPs caused toxic effects not only in the spleen but also other organs such as liver. Injection of 252 mg per animal of TiO2 NPs caused hepatocyte injury, cytoplasmic vacuolar degeneration and hepatocytes apoptosis (Alarifi et al. 2013). The transmission electron microscope results in our study showed that accumulation of TiO_2 NPs in the splenic parenchyma caused cellular apoptosis, swollen mitochondria, heterogeneous chromatin, cytoplasmic degeneration and apoptotic bodies aggregations. In similar studies, exposure to TiO_2 NPs caused swollen, enlarged and vacuolated mitochondria of splenocytes. The apoptotic splenocytes are characterized with condensed chromatin and vacuolated cytoplasm (Li et al. 2010; Sang et al. 2012).
The results of histopathological impacts in splenic parenchyma seen in this work suggested that the TiO_2 NPs caused spleen function alteration by interacting with minor molecules, proteins and enzymes in the spleen, interfering with the antioxidant defense mechanism and leading to generation of reactive oxygen species, which in turn might initiate inflammatory responses (Johar et al.2004). Also, these impacts might be resulted in damaged immune function and production of some cytokines and chemokines which caused splenocyte apoptosis and abnormalities. In a recent study, the exposure of TiO_2 NPs could significantly increase the levels of macrophage inflammatory protein such as interferon-γ, interleukin-13 and basic fibroblast growth factor (Sang et al. 2013). Furthermore, exposure to TiO_2 NPs aggregates in spleen caused severe macrophage infiltration as well as splenocytes apoptosis (Sheng et al. 2014;Jiaying et al. 2013). Coordinately with our study TiO_2 NPs effectively activated Caspase-3 proteins, decreased the Bcl-2 protein, and increase the levels of Bax proteins (Li et al. 2010). Intratracheal instillation of TiO2 NPs (32 mg/kg) increased proliferation of T cells and B cells and enhanced natural killer cell in spleen (Fu et al. 2014).
The novel data in this study revealed that, treatment of rats with TiO_2 NPs caused immuno-suppression due to splenocytes apoptosis, decrease spleen follicle size, decrease the lymphocytes number in the spleen and increase of Caspase-3 protein activity. But after administration of Milk thistle seeds extract to the treated rats with TiO_2 NPs there was an improvement of apoptotic splenocytes, increase of spleen follicle size, increase in the lymphocytes number and decrease of Caspase-3 protein activity. These data indicating the protection role of the Milk thistle seed extract components against TiO_2 NPs toxicity in the spleen. Several studies were done about Milk thistle seed extracts and its protective role for liver (Saller et al. 2001; Abenavoli et al. 2010). Unfortunately there is no clear study showed showed the protection role of this extract on the spleen treated with TiO_2 NPs.
Milk thistle seeds extraction usually contains a major group of components that have been identified and characterized several years ago. Silymarin is a standardized component (80%) of Milk thistle seeds extract containing several important ingredients such as silibinin (Kim et al. 2003, Szilárd et al. 1998). The most effective ingredient (60%) of silymarin is silibinin (Kroll et al. 2007). Extraction of silymarin of Milk thistle seeds using hot water was depending on the value of the temperature. The polar ingredients such as taxifolin and silychristin were preferentially extracted at 85°C, while the non-polar ingredient such as silibinin was favored at 100°C (Duan et al. 2004). Based on the previous literature data (Duan et al. 2004) the Milk thistle seeds extraction in this manuscript mainly composed from silibinin which is the most important components of silymarin extraction.
The mechanism action of silibinin is best known as antioxidant free radical for scavenging and inhibition of lipid peroxidation (Flora et al. 1998). Dose-dependent treatment with silibinin decreased the hepatic enzyme activity in patients with hepatic diseases (Kidd and Head 2005); Muzes et al. 1990). Milk thistle extract has been reported to support the immune cells through its antioxidant activity and free-radical removing action (Basaga et al. 1997). In a recent study afltoxins significantly reduced spleen weight of chicks and Milk thistle was more efficient to protect spleen against adverse effect of aflatoxin as compared to synthetic toxin binder (Chand et al. 2011). In this study, the Milk thistle extract gradients such as silibinin might be able to react with the free radicals produced from the reaction of TiO_2 NPs and the spleen proteins and enzymes. Additionally Milk thistle seeds extract could induce the immune systems in the treated rats with TiO_2 NPs. Thereafter, the splenocytes gradually turned back to the normal appearance and structure, the follicle size returned back to the normal size and lymphocytes number increased again.
The protective effects of Milk thistle extraction against TiO2 NPs not only caused by silymairn ingredients but it might be caused by other components rather than silymairn components. In the future work the effects of silymarin ingredients and other components separately should be investigated in details and approved to be used in the applied approaches. Recently Milk thistle extract was used in the field of nanobiology and nanomedicine. For example, silver and gold NPs were prepared using Milk thistle seeds extract as reducing and stabilizing agent, these NPs are eco-friendly and low toxic as compared with other NPs synthesized from chemical source (Mohammadinejad et al.

2013;Gopalakrishnan and Raghu 2014). However, Milk thistle extract characterized with strong free radical scavenging activity, inhibits lipid peroxidation, and promotes regeneration of damaged splenocytes, membrane stabilizing properties and immune system improvement (Das et al. 2011). These properties of Milk thistle extract together contribute to its spleen protective activity and TiO_2 NPs detoxification and immune system improvement.
In summary, the results of this study show that rats treated with TiO_2 NPs could result in splenic toxicity, immunological function disrupted in the spleen of rats such as reduction of follicle size, spenocytes apoptosis and increase of Caspase-3 protein activity. The administration of Milk thistle seeds extract to the treated rats with TiO_2 NPs improved the immune system in spleen rat by improving the follicle size, and reducing the Caspase-3 protein activity. Milk thistle extract has an important role to clear and react with the injected TiO_2 NPs from spleen and protect spleen from the toxic effects of the TiO_2 NPs. Using Milk thistle extract as reducing and stabilizing agent to prepare the TiO_2 NPs used as food preservation additive and in the drug delivery systems should be considered widely. Further investigations are needed to figure out the protective role of each silymarin extract ingredient and their application in the field of nanobiology and nanomedicine.

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