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Research article

Evaluation of the protective potential of hydroalcoholic extract of *Thymus daenensis* on acetaminophen-induced nephrotoxicity in rats

Soheila Ansari [a,1], Nahid Azarmehr [b,1], Zahra Barmoudeh [b], Zahra Moslemi [b], Hossein Ghahremani [c], Ali Mirzaei [a], Zeinab Salehpour [a], Mohammad Reza Rabani [a], Amir Hossein Doustimotlagh [a,d,*]

[a] Medicinal Plants Research Center, Yasuj University of Medical Sciences, Yasuj, Iran
[b] Student Research Committee, Yasuj University of Medical Sciences, Yasuj, Iran
[c] Department of Clinical Biochemistry, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[d] Department of Clinical Biochemistry, Faculty of Medicine, Yasuj University of Medical Sciences, Yasuj, Iran

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ABSTRACT

Background: Acetaminophen (APAP) is an antinociceptive and antipyretic drug that can be useful in therapeutic doses, although it can cause serious damage to the kidney if used overdose. The current study aimed to evaluate the protective effect of *Thymus daenensis* (TD) extract on APAP-induced kidney damage in rats.

Methods: Thirty female Wistar rats were randomly divided into 5 groups: control, APAP (3 g/kg), TD (500 mg/kg), APAP + TD (500 mg/kg), and APAP + N- acetylcysteine (140 mg/kg). The APAP groups received APAP on the 6th day and the rats were sacrificed on the 7th day. Plasma levels of creatinine (Cr) and urea were measured. Ferric reducing antioxidant power (FRAP), nitric oxide (NO) metabolite, total thiol (T-SH), tumor necrosis factor-α (TNF-α) and antioxidant enzymes activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) were measured in kidney tissue. The gene expression of TNF-α was also measured by real-time PCR. The histological examination of kidney tissue was also performed.

Results: Results showed that urea, Cr and FRAP markers markedly elevated in the APAP rats compared with the control group. There was a significant decrease in T-SH levels in the APAP animals in comparison with the control group. CAT activity also augmented in the APAP group compared to the control group. Urea and Cr levels were significantly decreased in the APAP + TD group in comparison with the APAP group. The administration of TD extract significantly increased the SOD enzyme activity. Histological findings were improved in the group treated with TD extract.

Conclusion: In general, the results indicate that TD extract can protect against APAP-induced nephrotoxicity by improving biochemical, histological and antioxidant effects. However, more studies are required to determine the mechanism of this extract.

1. Introduction

The kidney is a vital organ that plays an important role in filtering and controlling the concentration of various substances and chemical compounds that may cause toxicity [1]. Several environmental variables, including specific medications, can affect kidney function [2]. Acetaminophen (N-acetyl-para-aminophenol, APAP) is a commonly used antipyretic and analgesic drug. It is non-toxic in therapeutic doses, but its excessive use may cause liver and kidney failure [3]. Renal failure occurs in about 1–2% of patients with the APAP overdose [4]. Cytochrome P450 enzymes catalyze the oxidation of APAP to the NAPQI. Although the exact mechanism of renal damage induced by APAP is unclear, the oxidative stress and reactive oxygen species (ROS) produced by NAPQI have a significant role in the pathogenesis of APAP-induced nephrotoxicity [4]. NAPQI depletes cellular glutathione (GSH) stores and binds to proteins and other macromolecules causing oxidative stress and ultimately kidney damage [5]. It has been shown that APAP poisoning in experimental animals increases endoplasmic reticulum stress, lipid

* Corresponding author.
E-mail address: amirhossein.dousti@yums.ac.ir (A.H. Doustimotlagh).
[1] These authors contributed equally to this work.

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peroxidation, and inhibits antioxidant defense in kidney tissue [3, 6]. Creatinine (Cr) and urea are the most common markers used to evaluate kidney function. Cr is more specific and sensitive than urea, however several variables for instance age, sex, and muscle tissue volume effect on Cr level [7]. Several studies have shown increased serum contents of Cr and urea in APAP-induced renal injury [3, 5].

Some antioxidants have been shown to have protective effects against APAP-induced renal injury [7]. Many traditional plants are studied in animal models due to their antioxidant activity [5]. The genus *Thymus L* is a perennial aromatic plant from the Lamiaceae family and originated in the Mediterranean [8, 9, 10]. Thymus herbs mainly consist of aromatic and evergreen short stems that are commonly found in calcium-rich soils and meadows throughout Europe, Africa, and Asia. Among the 215 species of this genus that are grown in the world, there are 14 endemic species of Iran, one of which is *Thymus daenensis* Celak that grows at high altitudes on the slopes of the Zagros Mountains in western and south-western of Iran [8, 11, 12]. *Thymus daenensis* (TD) is a perennial shrub that is considered as an aromatic and medicinal plant. Aerial parts of this plant are also used as spices and flavoring compounds [8]. In traditional Iranian medicine, this herb is used in the treatment of colds and has antispasmodic, anti-inflammatory, digestive, anti-bacterial, antioxidant and anti-cough properties [8, 11]. It has also been reported that the essential oils of the leaves and flowers of this plant are potent anti-bloating, anti-rheumatic, anti-sciatic, and anti-septic [13]. All thymus species are rich in essential oils that are economically valuable due to the presence of volatile compounds such as monoterpenes, sesquiterpenes, phenolic compounds, and flavonoids. The major active ingredients in TD oil include thymol, carvacrol, p-cymene, and gamma-terpinene [8, 11]. Moreover, Alizadeh *et al* showed that the main components of the TD oil were thymol (66.62–71.49 %), p-cymene (5.52–7.12 %), and β-caryophyllene (3.91–4.09 %) [14]. Studies have shown that thymol and carvacrol have many anti-inflammatory and antioxidant properties [15, 16]. Nickavar and Esbati found that the hydroalcoholic extract of TD was rich in phenolic and flavonoid compounds, and there was a significant relationship between flavonoid content and free radical scavenging activity [17]. Flavonoids are secondary metabolites of the thymus that prevent lipid peroxidation and eliminate ROS during plant exposure to stress conditions [11]. Anti-inflammatory [18], antitumor [19] and antioxidant [20] effects of TD have been studied. The current study aimed to evaluate the protective and antioxidant effects of TD against APAP-resulted renal damage.

2. Materials and methods

2.1. Extraction

In the present study, aerial parts of TD plant were provided from the Yasuj city in Iran. The TD plant was identified by a botanist at Yasuj University of Medical Sciences. After drying in the shade at 37 °C, extraction was performed using 70% ethanol. Briefly, 100 g powder of the plant was suspended in 70% ethanol (1000 ml) at room temperature for 48 h. The whole extract was filtered through a filter paper and the collected extract was transferred to a vacuum distillation apparatus and concentrated as far as possible. Finally, the extract was dried in a 50 °C incubator and kept at -20 °C until analysis.

2.2. Experimental design

2.2.1. Animals

In the present study, 30 female Wistar rats weighing 225 ± 25 g and 8 weeks of age were obtained from Shahrekord University of Medical Sciences, Shahrekord, Iran. The rats were kept in special chambers (at 25 ± 2 °C and 55–60% relative humidity) and with a standard diet and water under a 12-hour light/dark cycle and fed. This study was performed according to the protocol of animal experiments and confirmed by the Ethics Committee of Yasuj University of Medical Sciences (Ethical code: IR.YUMS.REC.1398.096). Animals were randomly allocated into five groups of six each as follows: the control group was taken distilled water for seven days; the APAP group was taken distilled water for seven days and on the sixth day APAP (3 g/kg) [21]; the TD group was taken the TD extract (500 mg/kg) [22] for seven days; the APAP plus TD group was taken TD extract (500 mg/kg) for seven days and APAP (3 g/kg) on the sixth day; and the APAP plus N- acetylcysteine (NA) as the positive control group was taken NA (140 mg/kg) [23] for seven days and on the sixth day, APAP was injected at 3 g/kg. Administration of APAP, TD, and NA was performed orally. On the seventh day, the blood samples were drawn by cardiac puncture to assess blood urea and Cr. All animals were sacrificed and both kidneys removed, and thoroughly washed with ice-cold saline. To prepare for the assessment, one kidney kept in 10% formalin solution for histopathological analysis and the other was homogenized with a Potter-Elvehjem in PBS (10%, w/v) (10 mmol/l, pH 7.4). The kidney homogenate was centrifuged at 10000×***g*** for 5 min at 4 °C and the supernatant of renal tissues stored at − 20 °C until needed. The levels of total thiols (T-SH), ferric reducing antioxidant power (FRAP), nitric oxide (NO) metabolite, antioxidant enzymes activity [containing catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD)] and TNF-α level were determined.

2.2.2. Biochemical markers

For evaluation of biochemical parameters in renal damage, urea and Cr in plasma samples were measured by photometric methods (Pars Azmoon kit, Iran).

2.3. Oxidative stress indices

2.3.1. Measurement of ferric reducing antioxidant power (FRAP)

Benzie and Strain method was utilized to determine FRAP. In brief, 33 μL of sample was added to 1 ml of FRAP reagent [300 mmol L^{-1} acetate buffer, pH = 3.6; 10 mmol L^{-1} TPTZ (2,4,6-tripyridyl-S-triazine) in 40 mmol HCL; and 20 mmol L^{-1} $FeCl_3{\cdot}6H_2O$ in the ratio of 10:1:1] and incubated at 37 °C for 10 min. Ferric to ferrous ion reduction at acidosis pH produced a blue colored complex, which has a maximum absorption at 593 nm. The standard curve of ferrous sulfate solutions was plotted and the concentration of unknown substance was calculated using the standard curve [24].

2.3.2. Measurement of total thiol (T-SH)

Ellman's reagent (DTNB, 5,50-dithiols-2-nitrobenzoic acid) was used to measure T-SH. In this method, 150 μl of Tris-ethylenediaminetetraacetic acid, 20 μl of DTNB and 790 μl of methanol were added to microtubes containing 50 μl of the sample. The microtubes were then kept at room temperature for 15 min. After centrifugation, the supernatant was moved to a clean plate and the absorbance of the samples was read at 405 nm [25].

2.3.3. Determination of nitric oxide metabolite

NO metabolite was determined by the Griess method. Briefly, 100 μl of the sample was mixed with 100 μl Griess solution (sulfonamide and N-1-(naphthyl) ethylendiamine dihydrochloride). The samples were then kept for 30 min at 37 °C and the absorbance was read at 540 nm. NO metabolite was determined to utilize a standard curve of sodium nitrite [26].

2.3.4. Determination of antioxidant enzymes activity

Tissue homogenate of treated rats was analyzed for the activity of SOD, CAT, and GPx enzymes using ELISA kits according to the manufacturer's instructions (Zell Bio GmbH, Ulm, Germany).

2.3.5. Determination of tissue homogenate and gene expression of TNF-α

For the quantitative determination of TNF-α level in tissue homogenate, a commercial kit (Kermania pars Gene, Kerman, Iran) was used based on the manufacturer's procedure. Absorption was measured via an ELISA

reader (BioTek; Winooski, Vermont, USA) at 450 nm. To investigate gene expression, the total RNA was obtained from tissue homogenate by the RNX Plus kit (Sinaclon, Tehran, Iran) according to the manufacturer's instructions. Then, first-strand complementary DNA (cDNA) was produced using cDNA Synthesis kit (Sinaclon, Tehran, Iran), and Real-time PCR was performed using a RotorGene 3000 instrument (Bio-Rad, USA). PCR program was run as follows: denaturation stage at 95 °C for 15 s, annealing stage at 62 °C for 30 s, and elongation stage at 72 °C for 30 s in 40 cycles. The relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ formula. HPRT1 was used as the reference housekeeping gene and the findings were presented as the fold changes relative to the control group.

2.4. Histological examination

The kidney samples were washed with normal saline and fixed in 10% formalin solution. Then, the fixed tissues were inserted in paraffin and 5 μm sections were provided and stained with hematoxylin & eosin for light microscopy.

2.5. Statistical analysis

Statistical analysis was done by SPSS software. The Kolmogorov-Smirnov test was done to assessment of normality. Accordingly, quantitative variables are shown as mean ± standard error of the mean (SEM) or median (interquartile range, IQR). The One-way ANOVA and K independent sample test were performed to define differences between groups for the normally distributed and nonparametric data, respectively. Results were presented as mean ± SEM. It should be noted that $P < 0.05$ was determined as a statistically significant change.

3. Results

3.1. Biochemical parameters

To evaluate renal impairment, plasma levels of urea and Cr were measured. The results showed that plasma urea and Cr levels in the APAP rats were markedly higher than controls ($P < 0.05$). Administration of 500 mg/kg of TD extract and 140 mg/kg of NA significantly decreased urea and Cr in comparison to the APAP group ($P < 0.05$) (Figure 1A, B).

3.2. Oxidative stress indices

As presented in Figure 2, APAP-treated rats showed a significant increase in FRAP levels compared with the control group ($P < 0.05$). In the APAP + NA group FRAP levels were markedly lower than the APAP group ($P < 0.05$), although, there was no significant change in FRAP levels between the TD-treated group and the control group. Also, NO metabolite had no marked change among different groups. Furthermore, the level of T-SH in the APAP-treated group was markedly lower than the control rats ($P < 0.05$), however, there was no significant change in T-SH levels in the other groups in comparison to the APAP rats (Figure 2 A–C).

3.3. Activity of antioxidant enzymes

The results indicated that the administration of APAP slightly decreased GPx activity and increased CAT activity compared with controls ($P < 0.05$). In addition, SOD activity was markedly increased in APAP + TD and APAP + NA groups compared with APAP group ($P < 0.05$). Furthermore, TD administration had no significant impact on the activity of GPX and CAT enzymes (Figure 3 A–C).

3.4. TNF-α level

The tissue level and gene expression of TNF-α were measured. As shown in Figure 4, the relative the tissue level and gene expression of TNF-α insignificantly elevated in the APAP group in comparison to the control group. Treatment with TD extract decreased the tissue level of TNF-α against to the APAP group, however, the expression level of TNF-α was increased in the APAP + TD group compared with the APAP group.

3.5. The effect of TD extract on renal histology

Histological results showed that APAP induces inflammation and necrosis of renal glomerular cells. Granulation of white blood cells was observed in the APAP group. It was found that treatment with TD reduces these damages (Figure 5).

4. Discussion

Drug-induced nephrotoxicity is a common side effect of several medications which is closely associated with acute and chronic kidney failure [5]. APAP is the most common drug that causes hepatotoxicity and nephrotoxicity [7]. Although the hepatic injury mechanisms have been well investigated, less is known about the molecular mechanisms involved in APAP-induced nephrotoxicity [4, 27]. APAP is mostly excreted as sulfate conjugates and glucuronide, but a minor fraction is metabolized to NAPQI by the cytochrome P450 enzyme [25]. NAPQI is normally neutralized by glutathione (GSH), however, after excess intake of APAP, cellular GSH stores are depleted and then NAPQI starts to form protein adducts and causes nephrotoxicity [28, 29]. Several traditional herbs have been used in the treatment of drug-induced kidney damage [7]. In the current study, for the first time, the effect of TD on

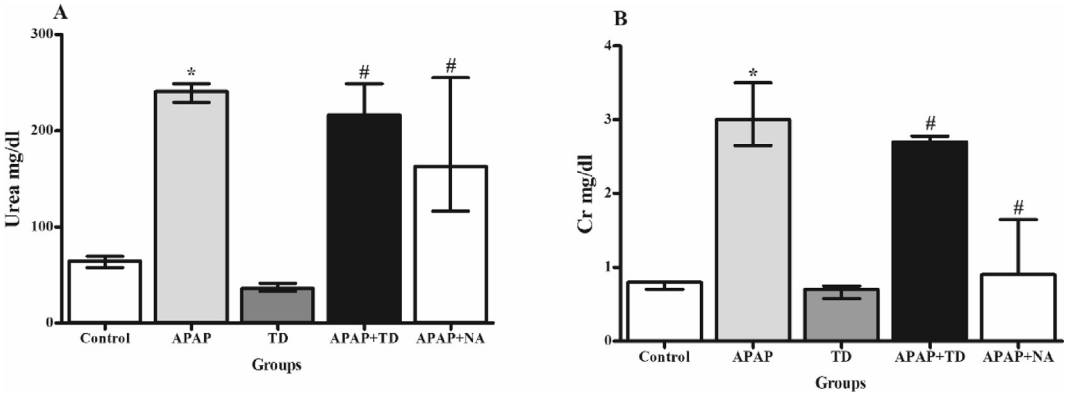

Figure 1. Effect of TD extract on plasma urea (A) and Cr (B) levels in APAP induced nephrotoxicity. APAP: acetaminophen; TD: 500 mg/kg of ethanolic extract of *Thymus daenensis*; NA: 140 mg/kg of N- acetyl cysteine. Each value represents median (Q1-Q3).; *$P \leq 0.05$ versus the control group and $^{\#}P \leq 0.05$ versus the APAP group.

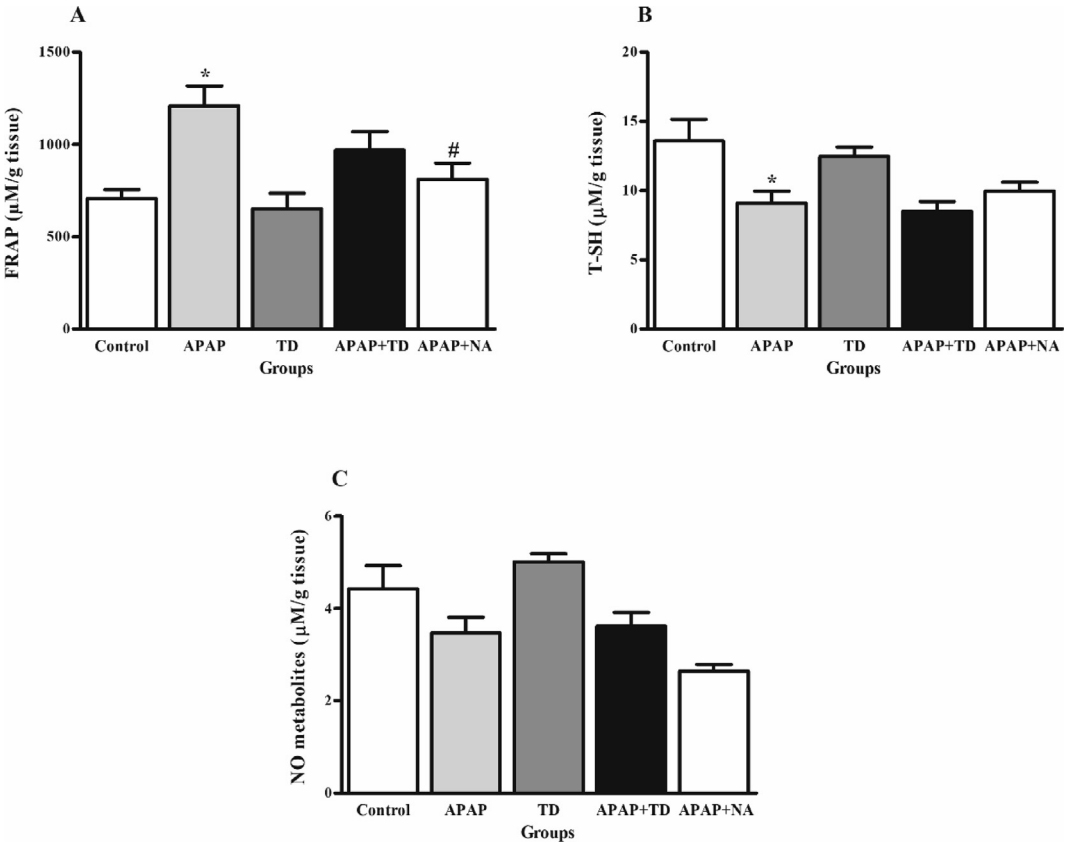

Figure 2. Effect of TD extract on kidney levels of oxidative stress markers including: FRAP (A), T-SH (B) and NO metabolite (C) in APAP-induced nephrotoxicity. APAP: acetaminophen; TD: 500 mg/kg of ethanolic extract of *Thymus daenensis*; NA: 140 mg/kg of N- acetyl cysteine. Each value represents mean ± SEM; *P ≤ 0. 05 versus the control group and #P ≤ 0. 05 versus the APAP group.

APAP-induced nephrotoxicity in rats was studied. Our findings showed that oral administration of TD extract leads to significant biochemical, histopathological, and antioxidant effects. This was demonstrated by a significant increase in SOD activity and a significant decrease in plasma urea and Cr concentrations.

Accurate assessment of kidney function before the onset of renal failure can help to reduce mortality [7]. In this regard, urea and Cr are important and reliable diagnostic markers of the renal function evaluated in drug-induced nephrotoxicity [30]. Renal proximal tubules are the site of elimination of a vast number of xenobiotics; damage to proximal tubule cells causes changes in urea and Cr levels [7]. In the present study, impaired renal function was indicated by increased plasma Cr and urea levels in the APAP group compared to the controls. In harmony with the present study, Cekmen *et al* indicated that administration of 1 g/kg APAP increased urea and Cr levels compared with the control group [27]. In addition, Fouad *et al* indicated that Cr and urea levels were markedly elevated in mice receiving the APAP at a dose of 2.5 gr/kg [31]. Studies of Hussain *et al* [5] and Ghosh *et al* [3] also confirmed the APAP caused renal injury by observing an increase in Cr and urea contents. In the present study, APAP-induced elevation of urea and Cr levels were significantly diminished in the TD receiving group. The chemical composition and drug activity of TD are similar to *Thymus vulgaris* [32]. Abu Raghif *et al* showed that aqueous extract of *Thymus vulgaris* reduced urea and Cr levels in cisplatin-induced nephrotoxicity in rabbits [33], which was in agreement with the findings of the present study.

The antioxidant system protects the body against free radicals and reactive species. When this system is disturbed by toxins, radicals, ROS and RNS, the resulting imbalance leads to oxidative stress [5]. Because the kidney is exposed to high levels of drugs and toxins, drug-induced ROSs which cause kidney damages is a serious threat among hospitalized patients, therefore, it is interesting to evaluate the role of antioxidants in drug-induced toxicity [28]. Several studies point to the potential role of oxidative stress through ROS production in APAP-induced organ toxicity [27, 34]. The first line of defense against ROS includes scavenger enzymes such as CAT, SOD, and GPx as well as antioxidant compounds such as ascorbic acid, GSH, Vitamin A and flavonoids [35]. The decrease in antioxidant status partly reflects the mechanism of APAP-resulted nephrotoxicity [30]. In the current study, injection of APAP at a dose of 3 g/kg insignificantly decreased GPx enzyme, but the catalase enzyme indicated a significant increase in the APAP group in comparison to the control rats. It has been shown that chemically produced free radicals can increase CAT activity [35]. Thus, in our study, the increase in CAT in the APAP group may represent a compensatory response to the increase in free radicals caused by the APAP overdose. Contrary to our study, Cekmen *et al* [27] and Ko *et al* [4] observed that SOD and CAT were markedly reduced in the APAP rats. Among the various antioxidant compounds, CAT and SOD act as main enzymes in the removal of ROS. The decrease in the SOD level in the APAP group might be due to the higher of superoxide anions [3]. SOD reduces the toxic effects of free radicals by trapping superoxide anion and converting it to hydrogen peroxide [36]. In the present study, treatment with TD significantly and slightly increased SOD and GPx, respectively. In agreement with the present study, Zeinab showed that thymus vulgaris significantly elevated SOD levels in APAP-induced nephrotoxicity in rats [37]. The antioxidant activity of *Thymus* species is mainly due to active compounds such as phenol and flavonoids [22]. TD is one of the Thymus species that is native to Iran and is traditionally used as a drug [20]. Thiol groups which act as sensitive indicators of oxidative stress are mainly found in proteins and GSH [25]. It has been suggested that the liver and kidney damage induced by the APAP is due to the reaction of NAPQI with

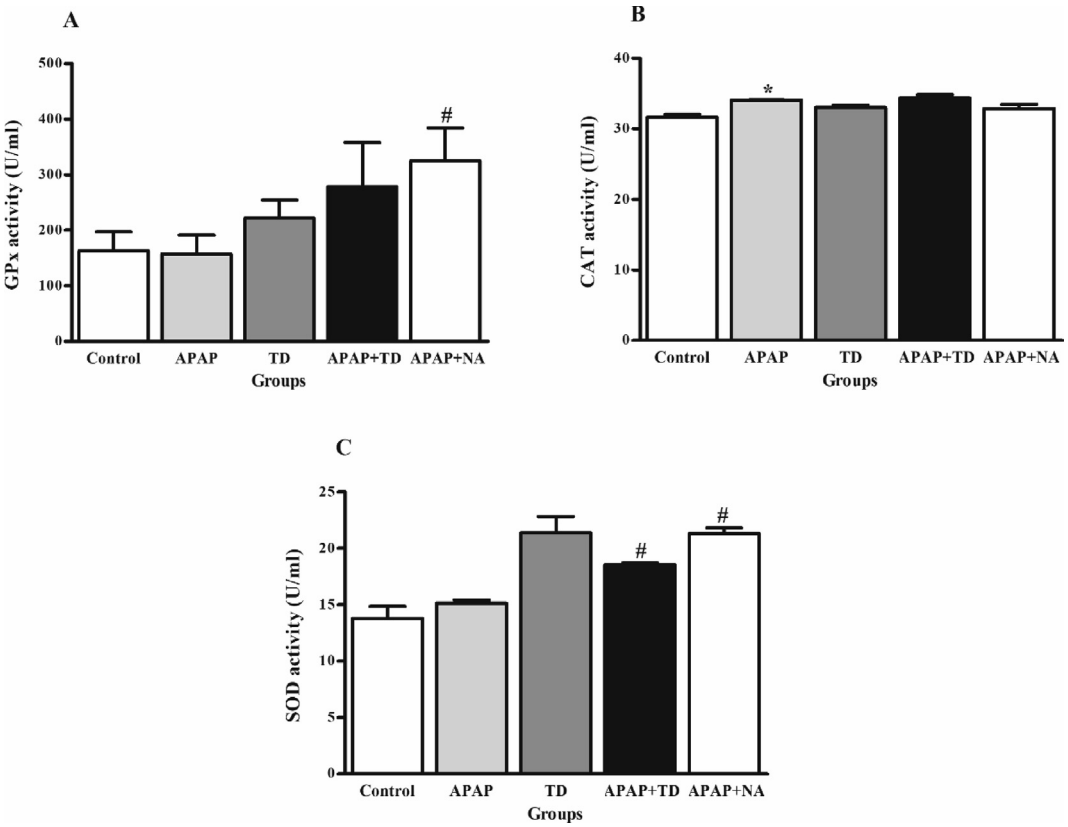

Figure 3. The effect of TD extract on the antioxidant enzymes activity of GPx (A), CAT (B) and SOD (C) in APAP-induced nephrotoxicity. APAP: acetaminophen; TD: 500 mg/kg of ethanolic extract of *Thymus daenensis*; NA: 140 mg/kg of N- acetyl cysteine. Each value represents mean ± SEM; *P < 0. 05 versus the control group and #P < 0. 05 versus the APAP group.

sulfhydryl groups and protein thiols. NAPQI depletes GSH stores and then alkylates the cellular macromolecules following APAP overdose [31]. In the present study, in the APAP group T-SH was markedly reduced compared to the control group, however, TD extract did not have a significant effect on this index.

TNF-α is one of the most important proinflammatory cytokines that induce apoptosis through binding to its receptors at the cell surface. This factor is elevated in many diseases where apoptosis is involved in their mechanisms, such as glomerular injury and acute and chronic renal impairment [38]. Oxidative stress following APAP overdose plays an imperative role in increasing proinflammatory cytokines and the incidence of inflammation and necrosis in the kidney. In fact, the increase in free radicals activates the NF-κB transcription factor and subsequently increases the expression of proinflammatory genes such as TNF-α [4, 31, 39]. The results of this study are partially similar to the studies of El-Boshy *et al* [39], Das et al [40] and Ko *et al* [4], in which APAP increased TNF-α factor compared with the control group. Treatment with TD extracts decreased and increased kidney level and gene expression of this cytokine, respectively.

Renal injury induced by APAP is associated with acute tubular necrosis [27]. However, nephrotoxicity may impact on all sections of the kidney [7]. In this study, the results of histological investigations confirmed the nephrotoxicity after APAP administration. Glomerular necrosis was the most important histological change. Also, tubular inflammation was observed. It was shown that treatment with TD extract reduced APAP-induced damage.

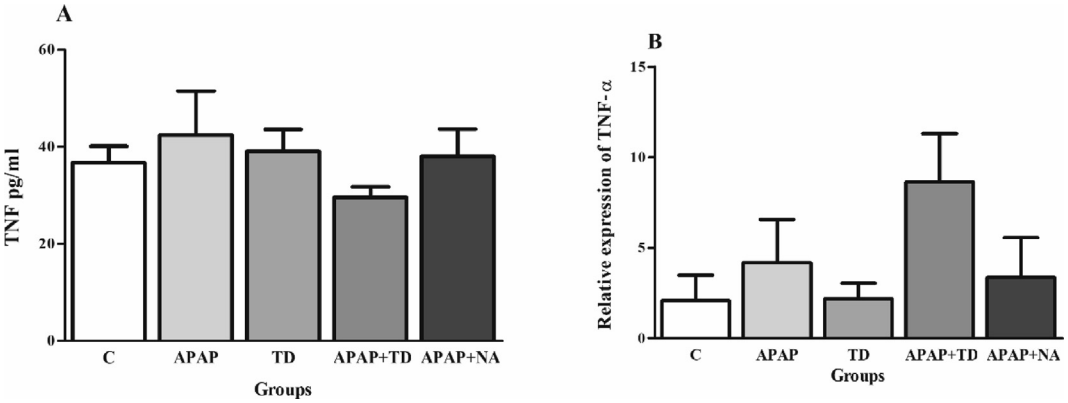

Figure 4. The effect of TD extract on the tissue level (A) and gene expression (B) of TNF-α in APAP-induced nephrotoxicity. APAP: acetaminophen; TD: 500 mg/kg of ethanolic extract of *Thymus daenensis*; NA: 140 mg/kg of N- acetyl cysteine.

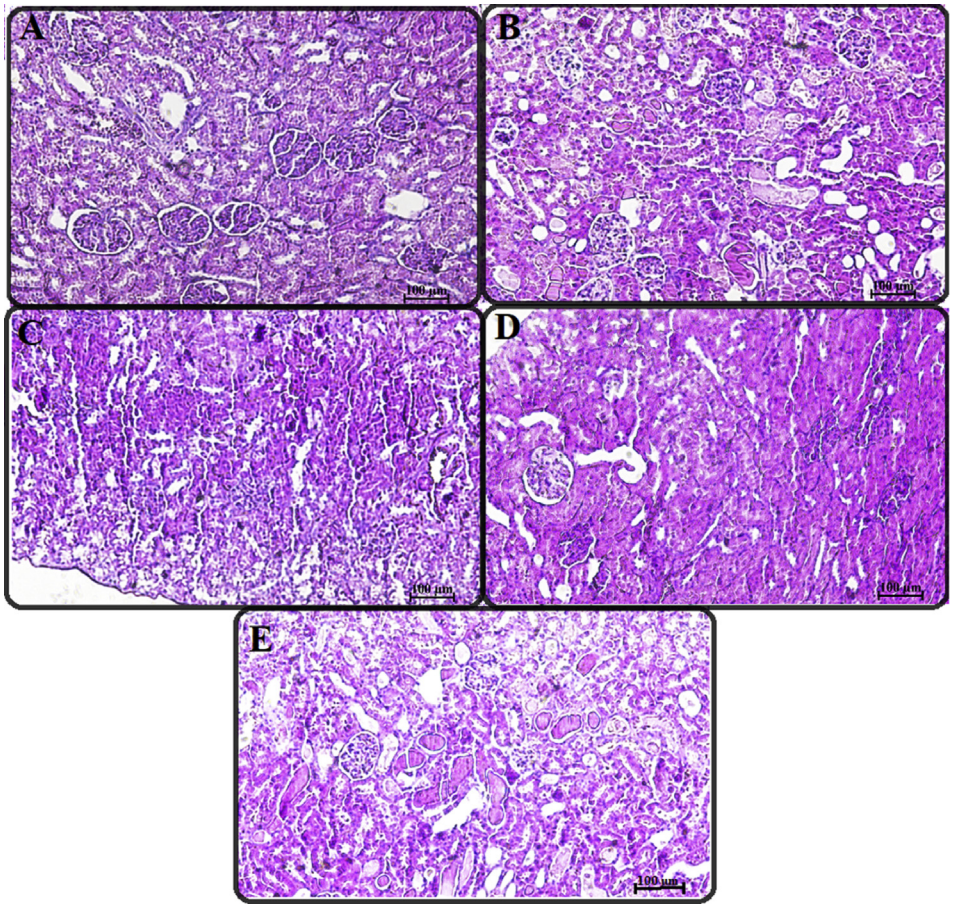

Figure 5. Histological evidence regarding the effect of *Thymus daenensis* (TD) extract on acetaminophen (APAP)-induced nephrotoxicity. A, Control. B, APAP. C, TD extract. D, TD + APAP. E, N- acetyl cysteine + APAP.

5. Conclusion

The results showed that TD extract has a significant protective potential against APAP-induced nephrotoxicity by reducing high levels of urea and Cr, histological changes in kidney tissue and increasing SOD antioxidant enzyme activity. However, more studies are needed to investigate the precise mechanism of TD function at the molecular level.

Declarations

Author contribution statement

A.H. Doustimotlagh: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

S. Ansari, N. Azarmehr, H. Ghahremani, A. Mirzaei and M.R. Rabani: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Z. Barmoudeh, Z. Moslemi and Ze. Salehpour: Performed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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