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Antioxidant effect of Abelmoschus Esculentus against acetaminophen-induced nephrotoxicity: an experimental study

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Abstract

Acetaminophen(APAP) intoxication is an important cause of nephrotoxicity and hepatotoxicity. N-acetylcysteine(NAC) is used in the treatment, but it has some serious side effects. *Abelmoschus esculentus*(AE) has various benefits as well as antioxidant effects. This study aims to investigate the effect of AE in APAP-induced acute nephrotoxicity. Forty male Wistar rats were divided into five equal groups: Control, AE, APAP, APAP+AE, and APAP+AE+NAC. Significant changes were observed in serum levels of malondialdehyde (MDA), superoxide dismutase (SOD), Neutrophil Gelatinase-Associated Lipocalin(NGAL) and Kidney Injury Molecule-1(KIM-1) after induction with APAP. NGAL and KIM-1 in the AE group remained low compared to those receiving APAP ($p=0.022$ and $p<0.001$, respectively). When the APAP group was compared with the AE and AE+NAC groups, it was found that even the administration of AE alone significantly decreased NGAL and KIM-1($p=0.036$ vs.$p=0.029$ and $p<0.001$ vs. $p<0.001$, respectively), these results were attributed to the effects of AE on reducing MDA and increasing SOD. Histopathological studies also confirmed these results. These results demonstrated that AE had protective and therapeutic effects on APAP-induced nephrotoxicity. This benefit of AE is due to its antioxidant effect. In addition, AE may also increase the regenerative capacity of the kidney, which APAP reduces.

Keywords: *Abelmoschus Esculentus; Acetaminophen; MDA; Nephrotoxicity; SOD*

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INTRODUCTION

Acetaminophen(APAP) is an antipyretic and analgesic medication that is widely used around the world. It can be purchased with or without a prescription from a pharmacy. The fact that it can be found in most households and tastes good causes childhood poisoning cases. It is a safe drug when used in recommended doses, but more than 150mg/kg at one time is considered a toxic dose.[1] According to

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studies, APAP is one of the most common medicines to cause poisoning in children. [2] APAP is 90% conjugated in the liver with glucuronic acid or sulfate to form nontoxic products, 5% is excreted unchanged in the urine, and the remaining 5% is metabolized by liver microsomal cytochrome P450 enzymes (CYP-450) to form a toxic metabolite. It is converted to N-acetyl-p-benzoquinone imine(NAPQI), converted to mercapturic acid with glutathione in NAPQI and excreted in the urine. When APAP is taken in toxic doses, the sulfate and glutathione stores are emptied so that the NAPQI produced by the CYP-450 system accumulates in the body, which leads to hepatotoxicity.[2] One of the organs affected by APAP toxicity is the kidneys. NAPQI is formed in the kidneys by CYP450 and prostaglandin synthase pathways in the medulla.[3] High doses of APAP and NAPQI are also excreted by the kidneys, leading to acute renal injury.[4] The nephrotoxicity has been reported even in the absence of hepatotoxicity.[5] Nephrotoxicity was observed in 13.5% of cases, along with hepatotoxicity.[6] Nephrotoxicity occurs in the third phase of APAP intoxication, within 72-96 hours.[4,7]

Initial symptoms of APAP intoxication are non-specific or absent. If toxic ingestion is suspected, serum APAP levels should be measured 4 hours after the indicated time of ingestion; serum APAP levels measured within the first 4 hours cannot be used to estimate toxicity potential. For individuals whose serum level is measured 4 hours after ingestion, the decision to treat should be based on where the serum APAP level is on the nomogram Rumack-Matthew.[8] N-acetylcysteine(NAC) is used in the treatment of APAP intoxication. NAC is a precursor of cysteine. It replenishes the depleted glutathione pool in the liver, thus increasing the detoxification of NAPQI and preventing hepatotoxicity and nephrotoxicity.[9] However, NAC is a chemical substance that can cause side effects such as nausea, vomiting, rash, fever, headache, hypotension, and anaphylactoid reactions.[8,10] These reactions may occur in 9.3-23.3% of patients.[11,12] Therefore, the search for alternative treatments is needed.

Abelmoschus esculentus (AE) is rich in vitamins, minerals, and fiber. Studies have shown that AE has antioxidant, anti-inflammatory, and various effects.[13,14] This study aims to investigate whether AE has renoprotective and therapeutic effects on APAP-induced acute nephrotoxicity and compares this possible therapeutic effect with standard NAC therapy.

METHOD

Experimental Animal

Rats were maintained under standard laboratory conditions (50-70% relative humidity, room temperature 19±2°C and 12-hour light, 12-hour dark cycle), fed a standard diet and water ad libitum.

Preparation of extract

The extraction of AE seeds obtained from the herbal collector was carried out by the maceration method. In the maceration method, ethanol(95%) was used as a solvent. For the extraction, 100 g of the powdered AE seeds were weighed and placed in a closed container. Six hundred ml of ethanol was added and kept in the dark at 25°C for two days. After two days, the extract mixture was filtered, and the liquid portion (ethanol) was transferred to a glass flask. The solvent was removed using a rotary evaporator set at 40°C; thus, an oily golden yellow AE extract was obtained in the flask. This extract was stored in the refrigerator at +4°C until use.

Establishment of the experimental model

Forty Male Wistar rats weighing 200-220 grams were randomly divided.

The control group received 0.3 ml of water orally for 11 days. The AE group was given a single dose of 600mg/kg BW AE orally for 11 days.[16] A single dose of APAP 1g/kg BW was administered intraperitoneally to the APAP group on the eighth day of the study. APAP administered at this dose is considered hepatotoxic.[17]

A single dose of 600mg/kg BW AE was administered to the AE+APAP group orally for 1 week. A single dose of 1g/kg BW APAP was administered intraperitoneally 1.5 hours after the administration of AE on day eight.[18] AE continued to be administered for 3 days after the treatment day.

A single dose of 600mg/kg BW of AE was administered orally to the AE+APAP+NAC group 1 week before the treatment day. On the eighth day, 1.5 hours after administration of the same dose of AE, 1 g/kg BW APAP was administered intraperitoneally as a single dose, and 1.5 hours after APAP, 300mg/kg BW NAC was administered as a single dose. It was administered intraperitoneally.[19] AE continued to be administered for 3 days after the treatment day.

All rats were placed in the supine position for the surgical procedure 72 hours after APAP administration,[20] anesthetized intramuscularly with a 90/10mg/kg BW xylazine/ketamine combination, and sacrificed. All procedures were performed in a laboratory setting and under sterile conditions. Blood was collected for biochemical analysis, and kidney tissue was removed by opening the abdomen with a midline incision. Blood samples were centrifuged at 4000 rpm for 10 minutes and stored in Eppendorf tubes at -80°C until assayed.

Kidneys collected for histomorphological examination were divided into two equal parts through the pelvis. 3 µm thick sections were taken from paraffin blocks prepared from kidney tissue and stained with hematoxylin-eosin dye. Tubular atrophy in renal tissue, tubular dilatation, cytoplasmic vacuolization in tubular epithelial cells, tubular epithelial cell necrosis, interstitial inflammation, and vascular plugging was evaluated semiquantitatively.[21,22]

Statistical Analysis

SPSS-23 program was used for statistical analysis. Data were expressed as mean+standard deviation. The Kolmogorov-Smirnov -test was used to analyze the fit of the groups to the normal distribution, and the homogeneity of variance was checked. One-way analysis of variance (ANOVA) was conducted for biochemical data between experimental groups, followed by comparisons with Tukey tests. Kruskal-Wallis analysis was used to assess the difference in histopathological scores, and a comparison of groups with significant changes was performed using the Mann-Whitney U test. $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Effects of AE on serum biochemical parameters in all groups are shown in table-1. There was no significant difference between groups in creatinine ($p=0.42$)(Table-1).

Table 1. Effects of AE on biochemical parameters in APAP-related nephrotoxicity

	Control	APAP	AE	AE+APAP	AE+APAP +NAC	P
Crea (µmol/)	132.7±23.7	174.4±29.7	136.7±34.3	162.3±84.9	159.6±38.7	0.42
MDA (mmol/l)	10.9±1.6	12.4±2.2	11.6±1	10.1±1.2	9.6±1.2	**0.014**
SOD (pg/ml)	2062.2±76.4	1881.9±106.3	1995.6±76.1	2061.4±122.7	2089.5±187.5	**0.018**
NGAL (ng/ml)	13.5±0.7	14.8±0.3	13.4±0.5	13.5±0.7	13.5±0.7	**0.024**
KIM-1 (pg/ml)	1938.2±168.9	2398.4±281.5	1816.7±341.3	1661±145.6	1643.1±149.2	**<0.001**

(APAP: Asetaminofen, AE: Abelmoschus Esculentus, NAC: N-acetyl cysteine, Crea: Creatinin, NGAL: Neutrophil gelatinase-associated lipocalin, KIM1: Kidney Injury Molecule 1, MDA: Malondialdehyde, SOD: Superoxide dismutase)

A significant difference was found between groups regarding MDA (p=0.014) (Table-1). Oxidative stress caused by APAP was lower in the APAP+AE group, but this change was not statistically significant (p=0.071). MDA was significantly reduced in the APAP+AE+NAC group (p=0.013). A significant difference was found between the groups regarding SOD (p=0.018)(Table-1). SOD was lower in the APAP group compared to the control group (p=0.038). Antioxidant activity was significantly increased in the APAP+AE and APAP+AE+NAC groups compared to the APAP group (p=0.049 and p=0.034, respectively). There was no significant difference between the use of only AE or AE+NAC in increasing serum SOD in rats exposed to APAP (p=0.66) (Figure 1a).

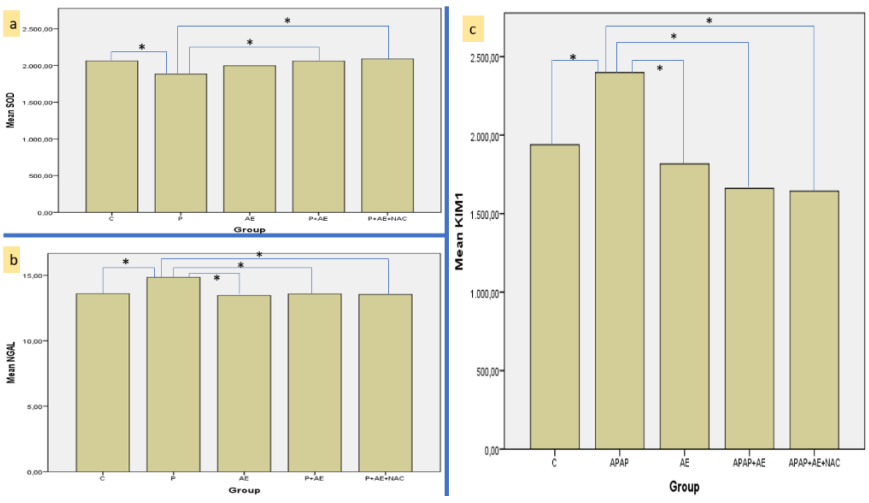

Figure 1. Effects of AE on biochemical values in APAP-related nephrotoxicity
a) Effects of AE on SOD in APAP-related nephrotoxicity
b) Effects of AE on NGAL in APAP-related nephrotoxicity
c) Effects of AE on KIM -1 in APAP-related nephrotoxicity
(APAP: Acetaminophen, AE: Abelmoschus Esculentus, NAC: N-acetylcysteine, NGAL: Neutrophil gelatinase-associated lipocalin, KIM -1:Kidney Injury Molecule-1, SOD: Superoxide dismutase, *:p<0.05)

A significant difference was found between the groups regarding NGAL (p=0.024) (Table-1). NGAL was higher in the APAP group than in the control group (p=0.039). NGAL values in the group receiving AE for prophylactic purposes were significantly lower than in the APAP group (p=0.022). Compared to the APAP group, NGAL was significantly reduced in the APAP+AE and APAP+AE+NAC groups (p=0.036 and p=0.029, respectively). There was no significant difference between using only AE or AE +NAC in reducing serum NGAL levels in rats exposed to APAP (p=0.99) (Figure 1b).

A significant difference was found between the groups in terms of KIM-1 (p<0.001) (Table-1). Higher KIM-1 was found in the APAP group compared to the control group (p=0.002). KIM-1 values in the group receiving AE for the prophylactic purpose were significantly lower than in the APAP group (p<0.001). Compared to the APAP group, the KIM -1 was significantly reduced in the APAP+AE and APAP+AE+NAC groups (p<0.001 and p<0.001, respectively). There was no significant difference between using only AE or AE+NAC in reducing serum KIM-1 in the APAP-exposed rats (p=0.99) (Figure 1c).

A significant difference was found between the groups in terms of tubular epithelial cell necrosis (p<0.001)(Table-2). Tubular epithelial cell necrosis scores were higher in the APAP group than in the control group (p<0.001) (Figure 2a, 2b). Tubular epithelial cell necrosis scores in the group given AE for prophylactic purposes were statistically and significantly lower than in the APAP group (p=0.021). Tubular epithelial cell necrosis scores did not change significantly in the APAP+AE group compared to the APAP group but could be significantly reduced in the APAP+AE+NAC groups (p=0.23 and p<0.001, respectively) (Figure 2c).

Table-2. Effects of AE on histopathological scores in APAP-related nephrotoxicity

	Control	APAP	AE	AE+APAP	AE+APAP+NAC	p
Tubular atrophy	0.6±0.5	2.2±0.4	1±0	1.1±0.3	0.8±0.3	**<0.001**
Tubular dilatation	1±0	3±0.5	1.2±0.4	1.4±0.5	1.1±0.3	**<0.001**
Cytoplasmic vacuolization	1.5±0.5	2.3±0.5	2.4±0.5	2±0.5	1.5±0.5	**0.005**
Tubular cell necrosis	1±0.5	3.1±0.8	2±0.5	2.5±0.5	1±0.5	**<0.001**
Interstitial inflammation	0.2±0.4	0±0	0.1±0.3	0±0	0±0	0.24
Vascular congestion	1.3±0.5	3±0.1	1.4±0.5	2±1	1.3±0.9	**0.016**

(APAP:Asetaminofen, AE:Abelmoschus Esculentuş, NAC:N-acetyl cysteine)

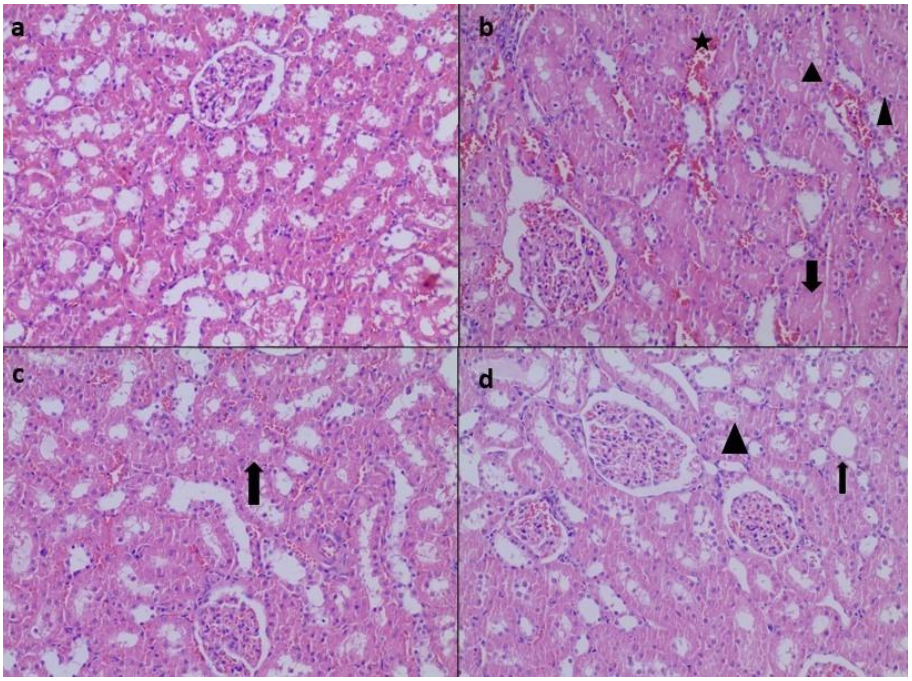

Figure 2: Histopathological changes of AE in APAP-related nephrotoxicity
a) Control group: No obvious tubular damage. Hematoxylin-eosin stain, x200
b) APAP group: Tubular necrosis (arrow), cytoplasmic vacuolization (arrowhead), vascular congestion evident (asterisk), hematoxylin-eosin stain, x200
c) APAP+AE group: Tubules without tubular necrosis and cytoplasmic vacuolization (arrow), Hematoxylin-eosin stain, x200
d) APAP+AE+NAC group: Focal tubular atrophy (arrow), tubules without necrosis (arrowhead), Hematoxylin-eosin stain, x200.
(APAP: Acetaminophen, AE: Abelmoschus Esculentus, NAC: N-acetylcysteine)

A significant difference was found between the groups in terms of tubular atrophy (p=0.001) (Table-2). The APAP group had more tubular atrophy than the control group (p<0.001). Tubular atrophy scores in the group receiving AE for prophylactic purposes were statistically and significantly lower than in the APAP group (p<0.001). Tubal atrophy scores in the APAP+AE and APAP+AE+NAC groups were significantly lower than in the APAP group (p=0.002 and p<0.001, respectively) (Figure 2d).

Furthermore, a significant difference was found between the groups in terms of tubule dilatation (p<0.001) (Table-2). Tubule dilatation scores were higher in the APAP group than in the control group (p<0.001). Tubule dilatation scores in the prophylactic AE group were substantially lower than in the APAP group (p=0.001).

Tubular dilatation scores were significantly reduced in the APAP+AE and APAP+AE+NAC groups compared to the APAP group (p=0.001 and p<0.001, respectively). A significant difference was found between the groups in terms of cytoplasmic vacuolization scores in tubular epithelial cells (p=0.005) (Table-2). Cytoplasmic vacuolization scores in tubular epithelial cells were higher in the APAP group than in the control group (p=0.021). While cytoplasmic vacuolization scores in tubular epithelial cells did not change in the APAP+AE group compared to the APAP group, they were significantly reduced in the

APAP+AE+NAC groups (p=0.33 and p=0.021, respectively).

A significant difference was found between the groups regarding vascular congestion (p=0.016)(Table-2). The APAP group had greater vascular congestion scores than the control group (p=0.007) (Figure 2b). Vascular congestion scores in the group given AE for prophylactic purposes were significantly lower than in the APAP group (p=0.009). Vascular congestion scores did not change significantly in the APAP+AE group compared to the APAP group but could be significantly reduced in the APAP+AE+NAC groups (p=0.094 and p=0.01, respectively). The anti-inflammatory activity could not be evaluated since significant inflammation was not observed in the APAP group, but the decrease in vascular congestion, one of the inflammatory indicators, also supports the anti-inflammatory effect.

The kidneys also excrete high doses of APAP.[23] Compared to other organs, its biochemical and physiological properties make the kidney more susceptible to ischemic and toxic damage. The kidney is highly susceptible to injury due to its high blood flow, ability to concentrate in the medullary interstitium and specific transporters in the tubular epithelium. Renal excretion of toxic NAPQIs that cannot be detoxified and accumulated due to glutathione stores depleted in APAP intoxication is a cause of nephrotoxicity.[4] Another cause of nephrotoxicity is oxidative stress. Oxidative stress induces the production of free oxygen radicals (SOR)s. The increase in SOR levels causes the polyunsaturated fatty acids in the cell membrane to be broken down into different products. The most important product of this process, called lipid peroxidation, is MDA. The increased oxidative stress and lipid peroxidation caused by APAP can lead to necrosis in renal tubule cells and hepatocytes. This study showed that taking AE for prophylactic purposes against APAP intoxications does not reduce lipid peroxidation. On the other hand, the result suggested that taking AE without NAC causes less oxidative stress and lipid peroxidation, thus having a therapeutic effect against APAP nephrotoxicity.

Glutathione, one of the intracellular thiols, is the most important of the non-enzymatic antioxidants. NAC is a powerful antioxidant that replenishes glutathione stores and dissolves SORs, which is why it is used in the treatment of kidney damage caused by various factors such as APAP.[9] SORs are eliminated by antioxidants such as SOD. SOD has a crucial effect in preventing the destructive effects of free radicals. Studies have shown that the activity of SOD can be increased to prevent SOR damage and maintain redox homeostasis during oxidative stress. Thus, a decrease in the activity of SOD indicates cell degeneration, while an increase in the activity of SOD indicates increasing cell regeneration.[25] This study found that one of the reasons for nephrotoxicity is increased MDA caused by oxidative stress and decreased antioxidants such as SOD. In APAP intoxications, the increase in the balance between oxidants and antioxidants in favor of antioxidants is accepted as an important indicator of the reduction of damage in the body.[3] Our results suggest that even the sole intake of AE without NAC can increase antioxidant activity and thus have a therapeutic effect against APAP nephrotoxicity.

There was no significant difference between groups in creatinine levels. It has been shown that renal injury can develop even without a change in serum creatinine levels, depending on the condition of the

kidneys and tubular creatinine secretion.[26] The fact that we found an increase in serum creatinine levels for these reasons does not mean that nephrotoxicity did not develop in our study.

Despite the late rise in serum creatinine levels, acute renal injury can be detected early to damage proteins such as KIM -1 and NGAL.[27] One of these conditions is APAP-induced nephrotoxicity.[28] NGAL, an indicator of distal tubule cell damage in acute kidney injury induced by nephrotoxic agents, is one of the proteins induced early,[29] and APAP, which stimulates the production of SOR, is the leading one among these nephrotoxins.[28] When SORs increase, cells may increase the levels of various antioxidants to protect themselves from oxidative stress. Roudkenar et al.[30] suggested that an increase in NGAL is not only an indicator of renal damage but also indicates antioxidant activity. This case shows a protective effect against cellular damage mediated by SORs.[31] It could be the reason for the increase in NGAL levels in APAP intoxications. Another property of NGAL is that it is not secreted by fully damaged cells. In this case, NGAL is a marker of active damage and salvageable nephron mass.[32] NGAL also has an anti-apoptotic effect. Through the effect of increased NGAL levels, the epithelium can be protected from damage.[33] The result of this study showed that taking AE for prophylactic purposes can decrease NGAL levels, which are an indicator of nephrotoxicity in APAP intoxications. Therefore, it may have a renoprotective effect. On the other hand, NGAL levels, which are lowered when NAC+AE are used together, have also been lowered when AE was used without NAC; thus, it may have a therapeutic effect. The therapeutic effect of AE on APAP nephrotoxicity has been attributed to its ability to prevent oxidative damage by reducing lipid peroxidation and inducing the expression of antioxidant enzymes such as SOD.

KIM-1 begins to express after renal injury. Zhou et al.,[34] reported that KIM-1 had the highest sensitivity and specificity in detecting kidney damage in rats exposed to various nephrotoxins compared to other biomarkers. KIM-1, which indicates kidney injury early, was also associated with the severity of kidney injury.[35] On the other hand, it was also found that KIM-1 mediated phagocytosis of apoptotic cells and stimulated proliferation and regeneration in proximal tubular cells.[36, 37] Thus, the inflammatory response could be controlled, and the damaged renal cells were healed.[38] All these properties make KIM-1 an important marker for reducing inflammation and damage. This study found that KIM-1 levels were significantly increased in the group exposed to APAP, which is consistent with the literature.[39] Polyphenols and flavonoids commonly found in vegetables were known to have strong antioxidant effects, and studies have shown that AE seeds were rich in polyphenols and flavonoids.[16] Furthermore, our results showed that taking AE for prophylactic purposes could reduce KIM-1 levels, an indicator of nephrotoxicity, in APAP intoxications and thus may have a renoprotective effect.

On the other hand, it has been shown that the reduced KIM-1 levels, when used together with NAC+AE, can also be reduced by the sole use of AE without NAC and thus, it may have a therapeutic effect. The therapeutic effect of AE on APAP nephrotoxicity was attributed to its ability to prevent oxidative damage by reducing lipid peroxidation and inducing the expression of antioxidant enzymes such as SOD. This result aligns with the study of Liao et al.[40] in diabetic rats. The

mentioned study showed that AE could improve renal dysfunction as antioxidant and anti-apoptotic effects.

In our study, nephrotoxicity in the APAP group was demonstrated by the increase in NGAL and KIM-1 levels and kidney damage indicators such as tubular atrophy, tubular dilatation, cytoplasmic vacuolization in tubular epithelial cells, tubular epithelial cell necrosis and vascular congestion. These results are in line with the literature.[28]

It is stated that AE has a protective feature against nephrotoxicity secondary to various toxins.[41] In this study, kidney damage such as tubular atrophy, tubular dilatation, tubular epithelial cell necrosis and vascular congestion was found to be lower in those who were given AE for prophylactic purposes, indicating the renoprotective feature of AE.

It is stated that AE has therapeutic properties against nephrotoxicity secondary to different toxins.[40] In this study, when AE+NAC was given to those exposed to APAP for treatment, histopathological kidney scores such as tubular atrophy, tubular dilatation, and cytoplasmic vacuolization in tubular epithelial cells, tubular epithelial cell necrosis and vascular congestion could be corrected. On the other hand, it has been shown that in APAP nephrotoxicities, even the administration of AE instead of NAC can improve damage indicators such as tubular atrophy and tubular dilatation, thereby accelerating regeneration.

Since it is a herbal product, AE allows starting treatment within the first 4 hours of APAP intoxication/intoxication suspicion without considering acute or repetitive APAP intake, the need for serum APAP level measurement, and the use of Rumack-Matthew nomogram.

CONCLUSION

AE had both protective and therapeutic effects for the kidneys during acute APAP nephrotoxicity with its antioxidant effects. AE, a herbal product, was as effective as NAC in treating acute APAP nephrotoxicity, suggesting that AE might be an alternative to NAC, a chemical used in treating acute APAP nephrotoxicity. Moreover, AE could also increase the regeneration ability of the kidney, which APAP reduced.

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