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Original Article

Achillea millefolium L. Extract Attenuates the Nephrotoxicity Induced by Acetaminophen in Rats

Hamid Reza Jamshidi[a], Mohamad Reza Lotfaliani[b], Shaghayegh Ghorbani[c], Jalal Hassanshahi[d*]

[a]Department of Pharmacology-Toxicology, School of Pharmacy, Shahid Sadoughi University of Medical Sciences, Yazd, Iran, [b]Department of Pharmacognosy, School of Pharmacy, Shahid Sadoughi University of Medical Sciences, Yazd, Iran, [c]School of Pharmacy, Shahid Sadoughi University of Medical Sciences, Yazd, Iran, [d]Department of Physiology and Pharmacology, School of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran.

Abstract

Acetaminophen can put the kidneys at risk of serious damage by affecting the renal antioxidant system and its cellular pathways. *Achillea millefolium L.* has been found to have antioxidant properties. This study aimed to determine the effect of *Achillea millefolium L.* (Ach) extract on acetaminophen-induced nephrotoxicity in rats. In this experimental research, 36 male Wistar rats were used. High-performance liquid chromatography (HPLC) analyzed Ach compounds. Animals were randomly assigned to six groups (n=6), which included vehicle, Ach 300 mg/kg, acetaminophen, acetaminophen + Ach (50, 150, and 300 mg/kg). Then, animals received acetaminophen (Single dose, 500 mg/kg), and after 1 hour, they received solutions containing saline/Ach as a single dose by oral gavage. After 24 hours, blood urea nitrogen (BUN) and serum creatinine were determined using the relevant kit. Also, the malondialdehyde (MDA) concentration and the activity of glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) were measured by the spectrophotometric method. Also, hematoxylin and eosin staining methods obtained renal tissue damage scores (RTDS). The results revealed that acetaminophen significantly increased the serum creatinine, BUN, RTDS, and MDA concentration and decreased the catalase, GPx, and SOD enzymes activity levels in renal tissues of the acetaminophen-administered rats versus the control ($P<0.05$). Ach treatment could significantly ameliorate these changes in acetaminophen + Ach (300 mg/kg) compared to the acetaminophen group ($P<0.05$). The present study revealed that Ach may protect against renal injury induced by acute acetaminophen poisoning, at least through its antioxidant effect.

Keywords: Acetaminophen, Oxidative stress, Acute kidney injury, *Achillea millefolium L*, Antioxidant.

Corresponding Author: Jalal Hassanshahi. Khalije Fars Blvd., Pistachio Co. Street, Rafsanjan University of Medical Sciences, Rafsanjan, Iran. E-mail: hasanshahij@gmail.com and hasanshahij@rums.ac.ir

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1. Introduction

Renal failure caused by acute acetaminophen consumption is accompanied by renal tubule necrosis, characterized by serum creatinine (Cr) elevation and a decrease in glomerular filtration [1]. On the other hand, N-acetylcysteine is used

to treat liver poisoning caused by acetaminophen. This drug leads to an increase in liver glutathione reserves, but it does not protect the kidney from acetaminophen toxicity [2-4]. Acetaminophen increases free radicals in liver and kidney tissue as a major free-radical product resulting from malondialdehyde (MDA) as a lipid peroxidation marker. It can also be a tissue damage marker following oxidative stress [4, 5]. As a result, receiving acetaminophen at high doses diminishes the antioxidant system of the kidneys and induces acute kidney injury [6]. Since kidney damage caused by acetaminophen can lead to death, finding a compound that can neutralize its effect seems necessary. Considering the damaging effects of acetaminophen on kidney tissue, the use of new drugs or protective compounds, especially natural antioxidants from plants, is of particular importance [4, 7, 8]. In general, the application of medicinal plants for disease treatment has been common in human societies for a long time, and recently, more attention has been paid to the necessity of investigating medicinal plants [9]. Because of the significant side effects associated with synthetic medications, the tendency of researchers towards medicinal plants and the use of their active compounds in treating diseases has increased [10]. Recently, many plants have been used to treat kidney disorders, and the antioxidant effects of many of these plant compounds have been identified [10, 11]. *Achillea millefolium L.* (Ach) or Ach is a stable plant, 20 to 90 cm high and even more, with long, hairy, petioleless leaves with many narrow cuts. All parts of the plant have a pungent smell and bitter taste [12, 13]. Ach grows as a car in the plains, roadsides, and mountainous areas of Europe, northern Iran, and Alborz highlands. The flowering branches of the plant and the oil extract obtained from the flower are mostly used [14]. The flowering branches of this plant contain alkaloids and flavonoids, and the flowers of this plant contain aromatic essential oil and several other medicinal substances [15, 16].

In traditional medicine, Ach has long been used to remedy various disorders, including wounds, infectious diseases, pain, and digestive complaints. In addition, it has been reported that Ach has anti-anxiety and anti-inflammatory properties [15, 16]. Also, Ach exhibits significant antioxidant and anti-apoptotic properties, which help mitigate oxidative stress and apoptosis in biological systems. Also, Ach has been found to reduce pro-inflammatory cytokines and markers of inflammation [15, 16]. In addition, the compounds of Ach oil extract have antioxidant properties and can neutralize free radicals [17]. Based on the referenced literature, the present study aims to determine the impact of Ach aqueous extract on acetaminophen-induced renal toxicity in rats. The findings from this study could lay the groundwork for future clinical investigations aimed at mitigating acetaminophen-related renal toxicity.

2. Materials and Methods

2.1. Animal

The present experimental research was conducted in a laboratory environment. This research used 36 male Wistar rats weighing 200-250 grams. Animals were kept in the cages under a controlled situation (12 h light/dark cycle at the temperature of 23 ± 1 °C) with free

access to water and food for mice in the animal room for one week before the start of the experiment. All procedures were done according to the Shahid Sadoughi University of Medical Sciences ethical committee (IR.SSU.AEC.1402.004) instructions based on the US NIH Guidelines for the Care and Use of Laboratory Animals (Publication No. 23-25).

2.2. Experimental groups

In the present study, to randomly assign the animals to various groups, healthy rats were initially weighed by an individual unaware of the study's details. They were then randomly allocated into six groups (n=6 per group), as mentioned below.

Vehicle group (animals in this group received saline solution with the same volume as other groups in a single dose and through gavage and were considered the control group). A saline solution (Vehicle) dissolved the acetaminophen and Ach extract.

Ach (300 mg/kg) group: the animals of this group received Ach extract dissolved in saline at 300 mg/kg (the highest dose used in this study) through gavage and received as a single dose.

Acetaminophen group: the animals of this group first received the drug acetaminophen (500 mg/kg, single dose) through intraperitoneal (i.p.) injection. Then, one hour later, they received saline solution with the same volume as other groups in a single dose and through gavage [4, 18].

Acetaminophen+Ach (50, 150, and 300 mg/kg) groups: the animals of this group first received acetaminophen in a single dose, then one hour later, they were treated with Ach extracts (50, 150, and 300 mg/kg) through gavage [19, 20].

2.3. Plant material

First, the Ach plant was purchased from Isfahan Herbarium after the species was confirmed by the herbarium expert (species code: 9757). Then, it was cleaned, thoroughly washed, and dried in a cool and dark place. The flowering branches and leaves of the plant were completely separated and powdered; finally, 4 grams of plant powder was placed in 100 ml of distilled water for 5-6 hours in an incubator (with a temperature of 70°C). Then, its extract was separated by filter paper and dried at a temperature of less than 40 degrees (to prevent the destruction of the effective substances of the plant); from this, an amount of plant powder equivalent to 500 mg of dry extract was obtained [21]. Therefore, other doses were calculated according to the studied groups, and the necessary amount for each group was dissolved and used.

The choice of extraction conditions was based on the following considerations; temperature: Aqueous extraction at 70°C was used to facilitate the solubilization of polar compounds and enhance the extraction efficiency.

Duration: The 5-6-hour incubation period allowed sufficient time for the plant compounds to be extracted into the aqueous solvent. Longer durations may have increased the yield but could also lead to the extraction of unwanted compounds. Solvent: Distilled water was selected as the solvent due to its safety, availability, and ability to extract a wide range of polar compounds from the plant material.

According to the high-performance liquid chromatography (HPLC) analysis, the most important flavonoid compounds of this extract were luteolin (0.29 mg / g) and apigenin (1.54 mg/g).

2.4. Experimental protocol

At first, the animals were kept for one week to adapt to the laboratory environment. After one week, the animals in the acetaminophen groups were treated with acetaminophen (Razi, Iran) at 500 mg/kg of body weight through i.p. injection. One hour after induction of poisoning, depending on which group, the rats received saline or Ach solutions (50, 150, and 300 mg/kg) as a single dose through gavage [19, 20]. It should be noted that the animals of the vehicle group received the saline solution with the same volume as the other groups in a single dose through oral gavage and were considered the control group. The animals of the 2nd group only received the solution containing Ach (300 mg/kg) received [19, 20]. Finally, 24 hours after the induction of poisoning, the rats were subjected to deep anesthesia with ketamine (100 mg/kg) and xylosin (10 mg/kg). To confirm adequate anesthesia, the paw pinch reflex was used as a measure of anesthetic depth in all animals immediately prior to decapitating the rats (**Figure 1**). Then, renal tissues were harvested, frozen in liquid nitrogen, and placed at -80 °C.

2.4.1. Serum creatinine and blood urea nitrogen measurement

After deep anesthesia, blood samples were collected via arteries in the corners of the rat's eye (about 2 ml per rat), then centrifuged (6000 g, 15 min) and subsequently measured blood urea nitrogen (BUN) and serum Cr levels using a BUN and Cr assay kit (Pars Azmoon Co., Iran).

2.4.2. Oxidative stress parameters measurement

After removing kidney tissues, one was frozen in liquid nitrogen. Some of the oxidative stress indicators such as MDA concentration (shows lipid peroxidation) and glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) activity levels were measured helping relevant commercial kits (ZellBio, Germany) [22- 24].

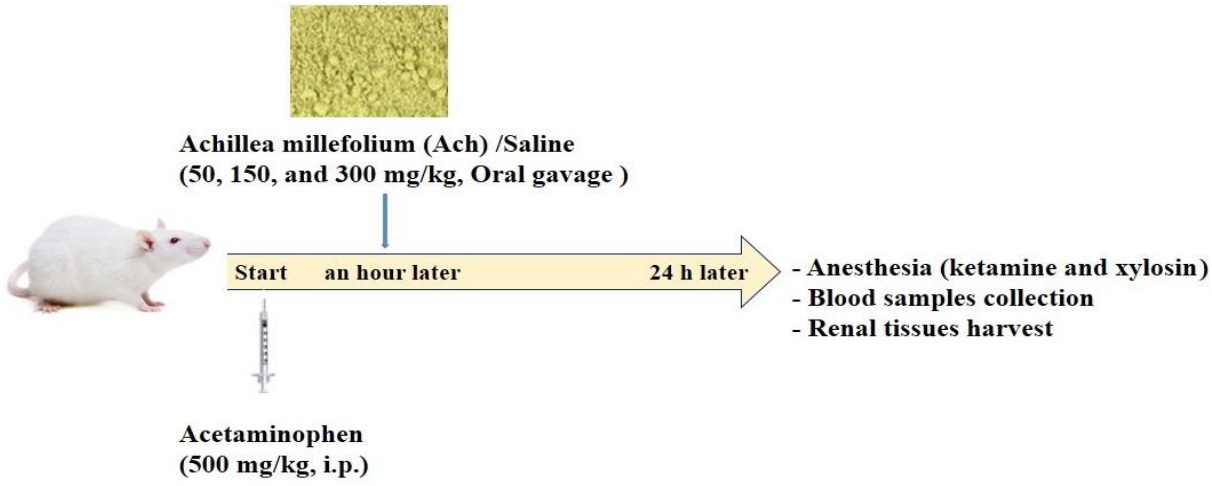

Figure 1. Schematic representation of the experimental timeline for animal studies.

2.4.3. Tissue staining

The other kidney was placed in 10% formaldehyde buffer, and the hematoxylin-eosin (H&E) staining method was applied to renal histological examination. The examination of renal tissue was conducted using light microscopy. A scoring system ranging from 0 to 3 was utilized to evaluate the severity of renal damage, with normal: (0-0.5), injury: (1: minor, 2: moderate, and 3: severe). This scoring was carried out under a blinded pathologist. Earlier studies utilized a range of criteria like tubular atrophy, tubular dilatation, tubular cell vacuolization, casts, interstitial infiltrates, and edema to evaluate the seriousness of kidney tissue damage. Each criterion was assigned a score from 0 to 3 according to the extent of the injury, and these individual scores were combined to calculate a comprehensive renal tissue damage score (RTDS) [25, 26].

2.5. Statistical analysis

The data underwent analysis, helping GraphPad Prism version 6 software. Data is presented as the mean ± standard deviation. The Shapiro-Wilk test was employed to assess the normality of the data distribution, while Levene's test was utilized to evaluate the equality of variances. Given that the data was normally distributed (P-value greater than 0.05). Group variations were assessed through one-way ANOVA following data normality confirmation, with the Tukey post hoc test applied at a significance level of 0.05 for all comparisons.

3. Results and Discussion

The current study's findings revealed significant differences in serum levels of BUN and Cr among the various groups at the conclusion of the research. Notably, the group treated with acetaminophen exhibited a marked increase in BUN and Cr levels compared to the control ($P<0.01$). Furthermore, administration of Ach at doses of 150 and 300 mg/kg significantly lowered the serum BUN levels in the acetaminophen+Ach group compared to the acetaminophen ($P<0.05$ and $P<0.001$, respectively, **Figure 2**).

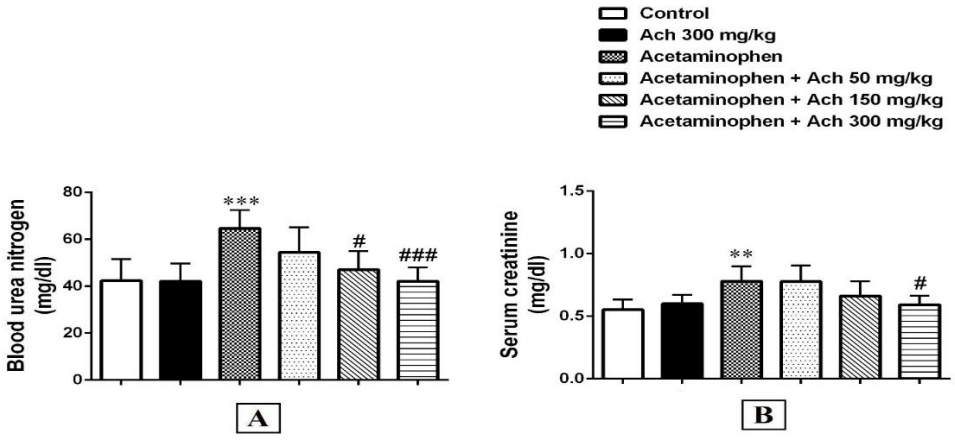

Figure 2. The effect of Ach on BUN (A) and serum Cr (B) levels in the experimental groups. Data are shown as mean ± standard deviation (SD). **P<0.01 and ***P<0.001 significant difference compared to the control. #P<0.05 and ###P<0.001 significant difference compared to the acetaminophen. Ach: Achillea millefolium, BUN: blood urea nitrogen.

Additionally, Ach at a dose of 300 mg/kg significantly reduced the serum Cr levels in the acetaminophen+Ach 300 mg/kg group relative to the acetaminophen (P<0.05). Consequently, this part of our study showed that high doses of acetaminophen in rats significantly increased serum BUN and Cr levels. Moreover, Ach (300 mg/kg) effectively decreased BUN and Cr content in animals receiving high doses of acetaminophen. Supporting this study, Najafizadeh et al. (2022) demonstrated that acute acetaminophen toxicity results in renal tubulointerstitial injury, tubular vacuolization, and dilatation characterized by necrosis of renal tubules, which correlates with elevated plasma Cr and BUN content [4].

Additionally, it has been established that acetaminophen poisoning can lead to both renal and liver failure, although kidney-specific damage has also been observed [20]. Moreover, consistent with the current findings, Karimi et al. (2021) explored the anti-diabetic properties of Ach plant extract in diabetic animals [27]. Their results indicated that Ach extract reduces the serum BUN and Cr content and elevates the antioxidant factors such as SOD and GPx in treated groups. Moreover, Ach ameliorates lipid peroxidation, kidney tissue damage, and apoptosis in this model [27]. Similarly, in line with the present study, Zangeneh (2018) investigated the renal protective effects of Ach aqueous extract in diabetic rats, reporting that treatment led to decreased BUN and Cr levels compared to the diabetic group, thereby enhancing kidney function [28].

As illustrated in **Figure 3A**, the content of MDA in the renal tissues of acetaminophen-administered rats showed a significant increase compared to the control (P<0.01).

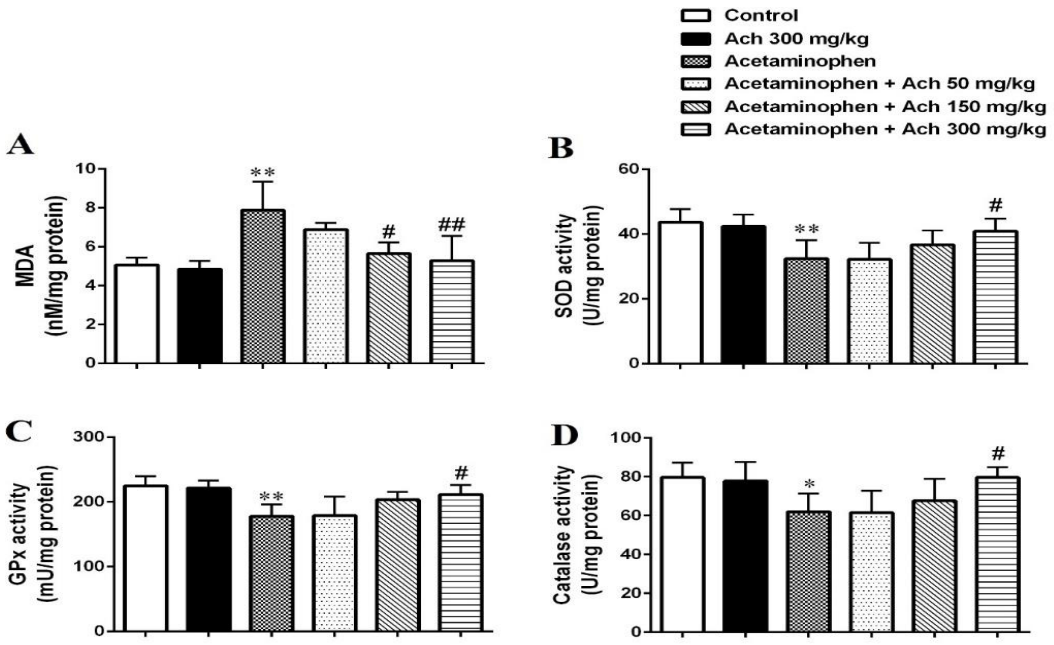

Figure 3. The effect of Ach on the MDA concentration (A), SOD (B), GPx (C), and catalase (D) activity levels in renal tissue of the experimental groups. Data are shown as mean ± SD. *P<0.05 and **P<0.01 significant difference compared to the control. #P<0.05 and ##P<0.01 significant difference compared to the acetaminophen group. Ach: Achillea millefolium, MDA: Malondialdehyde, SOD: superoxide dismutase, GPx: glutathione peroxidase.

In addition, administration of Ach at doses of 150 and 300 mg/kg effectively lowered the MDA concentration in the acetaminophen + Ach groups compared to the acetaminophen group ($P<0.05$ and $P<0.01$, respectively). The findings of this study also indicated that the activity levels of the antioxidant enzymes SOD, GPx, and catalase were significantly diminished in the acetaminophen group versus the control ($P<0.05$, **Figure 3B-D**). This suggests that acetaminophen administration in animals elevated free radical levels in the kidney, leading to oxidative stress. Notably, the 300 mg/kg dose of Ach significantly enhanced the activity of these enzymes in the acetaminophen + Ach 300 mg/kg group compared to the acetaminophen group ($P<0.05$, Figure 3B-D). In this regard, research has shown that oxidative stress induced by high doses of acetaminophen and an increase in reactive oxygen species (ROS) can cause significant DNA damage, disrupt physiological homeostasis, and ultimately result in renal tissue damage [4, 29]. The current study showed that high doses of acetaminophen led to increased lipid peroxidation in the renal tissues of the animals. The Ach extract effectively reduced MDA levels in the kidneys of those treated with high doses of acetaminophen. Supporting this, Bahbouhi and colleagues (2009) reported that the Ach extract possesses antioxidant and anti-inflammatory properties [30]. Elmann et al. (2011) found that Ach extract exhibits antioxidant and neuroprotective effects in culture cells, suggesting that Ach may protect by neutralizing free radicals and mitigating inflammation [31].

Based on hematoxylin and eosin results, the kidney tissue in the control group showed no pathological abnormalities (**Figure 4**). In contrast, the renal tissue of rats treated with acetaminophen exhibited damage compared to the control ($P<0.001$). Notably, administration of Ach (150 and 300 mg/kg) resulted in a reduction of RTDS in the groups receiving acetaminophen + Ach at both doses, compared to the acetaminophen group ($P<0.05$ and $P<0.01$, respectively, Figure 4). The results of this study suggest that high doses of acetaminophen lead to an increase in free radicals within the kidney tissue, thereby inducing oxidative stress. However, treatment with Ach (300 mg/kg) significantly ameliorated the RTDS. Supporting this, Okkay and colleagues (2021) demonstrated that Ach can mitigate oxidant levels and inflammatory factors and decrease tissue injury in a rat model of eye damage induced by high doses of cisplatin [32]. The present study showed that Ach can attenuate renal injury induced by acetaminophen. However, further investigations on the action mechanism of Ach with clinical approaches were suggested. Also, this study has some limitations: A) the assessment of renal function and oxidative stress markers was conducted 24 hours after treatment. This short duration may not capture the long-term protective effects of Ach. b) The study administered a single dose of Ach extract. This approach may not reflect the chronic exposure scenarios often encountered in clinical settings. These limitations should be considered when interpreting the study results, as they may impact the conclusions drawn about the efficacy of Ach extract in protecting against acetaminophen-induced nephrotoxicity.

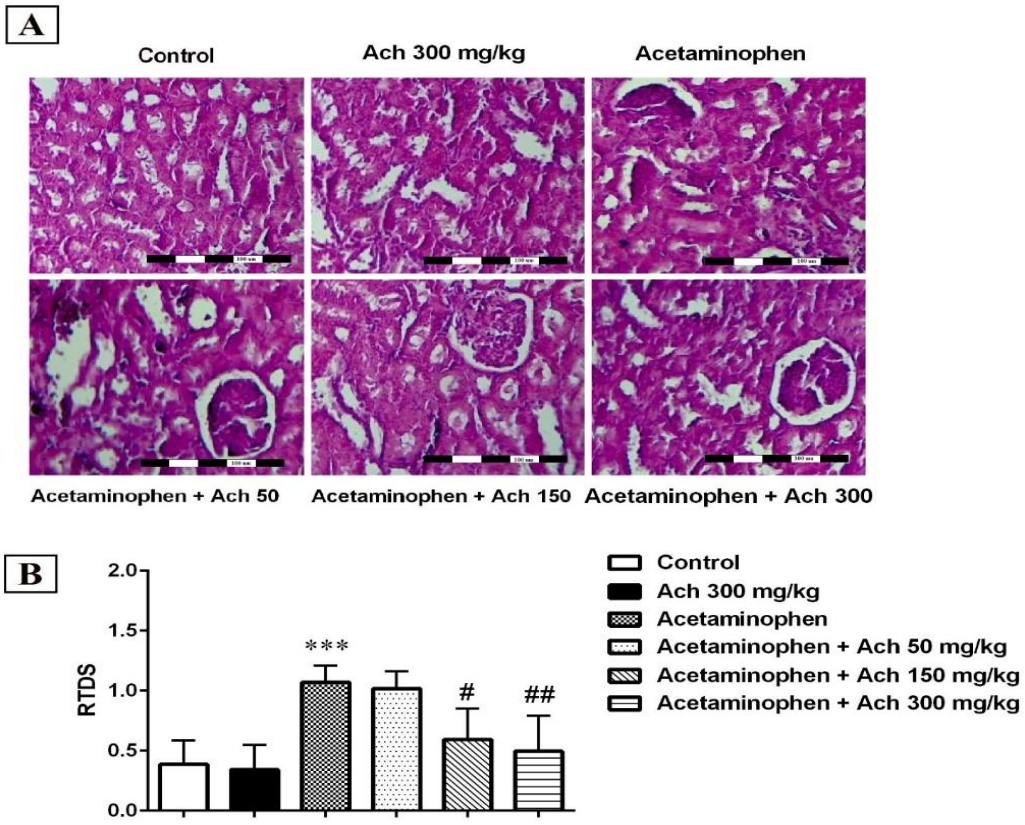

Figure 4. The renal tissue sections from the experimental groups (stained with hematoxylin and eosin at a magnification of 400×). A scoring system categorized scores 0-0.5 as normal and scores 1-3 as representing mild, moderate, and severe damage based on the presence of leukocyte infiltration, glomerular atrophy, and tubular necrosis in the kidney tissues. B: RTDS in all groups at the end of the experiment. Each value is shown as mean ± SD. ***P<0.001 compared to the control. #P<0.05, and ##P<0.01 compared to the acetaminophen. Ach: Achillea millefolium, RTDS: Renal tissue damage score.

4. Conclusion

Altogether, Ach extract showed protective properties against renal toxicity caused by high doses of acetaminophen. This protective effect is likely linked to decreased oxidative stress and RTDS associated with acetaminophen. Additionally, Ach extract mitigates the BUN and serum Cr content in this model. Therefore, it seems that Ach extract can be used after overdosing on acetaminophen besides other treatments. However, more research is required to confirm these findings. Also, the long-term effects of Ach extract and its efficacy in different models of renal injury can be explored.

Conflict of interest

The authors declare to have no conflict of interest.

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