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Albiflorin mitigates Renal Impairment in Hyperuricemic Rats by modulating HMGB1/TLR4/NF-kB signaling pathway

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Abstract

Background: It is becoming more well accepted that hyperuricemiamay be a contributing factor to hypertension, renal illness, and a number of detrimental effects of vascular disease. It has been shown that albiflorin(ALB) has potent pharmacological effects in preventing oxidation, inflammation, and apoptosis. Objective: to illustratethe underlying mechanisms and renoprotective impact of ALB in hyperuricemic rats. Material & Methods: Thirty Wister albino male rats divided into control, HU, HU+ALB groups. After 8 weeks rats were subjected to arterial blood pressure (ABP), renal blood flow velocity (RBFV) and renal artery resistance (RAR) measurement and serum levels of urea, creatinine in addition to creatinine clearance, urinary protein, renal MDA, SOD, TNF-α, IL-6, renal genes expression of HMGB1, TLR4 and NF-κB were assessed. Renal tissue was evaluated histopathologically and immunohistochemically. Results: The measured SBP,DBP , MABP, RAR, serum levels of uric acid, urea, creatinine in addition to urinary protein, renal MDA, renal TNF-α, renal IL-6, renal genes HMGB1, TLR4 and NF-kB of HU group were dramatically increased compared to control however RBFV, renal SOD and creatinine clearance values of HU group were substantially decreased compared to control. In addition there were dramatically upregulated Bax and NF-kB immunoreaction of HU group compared to control. ALB significantly enhanced the alterations brought about by HU. Conclusion: Through anti-oxidant, anti-inflammatory, and anti-apoptotic processes as well as the down-regulation of HMGB1, TLR4, and NF κB renal gene expression, ALB reduced HU-induced renal impairment.

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Introduction

A common chronic metabolic disease caused by poor purine metabolism and insufficient uric acid excretion is hyperuricemia (HUA) [1].Due to changes in eating habits and living levels, HUA is becoming more common worldwide. According to a nationally representative cross-sectional study carried out in 2018–2019, around 14% of Chinese individuals suffered from HUA [2].Gout, renal disease, type 2 diabetes mellitus, cardiovascular and cerebrovascular problems, and intestinal disorders can all be brought on by HUA, which can have a serious negative influence on a person's general health and well-being [3].

Asymptomatic hyperuricemia may have some causative impact on vascular disease, hypertension, and the advancement of chronic kidney disease (CKD), and it is a biomarker of both elevated risk for and the existence of vascular illness (such as hypertension, coronary artery disease, and kidney disease).[4].

Under some circumstances, uric acid may crystallize in the renal tubules, obstructing the tubules and increasing the production of reactive oxygen species and the renin-angiotensin-aldosterone system, which raises the risk of renal impairment [5].Therefore, there is significant scientific and practical utility in preventing and treating HUA.

One of the main chemokines generated by inflammatory cells, high-mobility group box 1 (HMGB 1), stimulates the release of cytokines by binding to receptors for AGEs (RAGE), the first HMGB1 receptor that was found, and Toll-like receptor (TLR)-4. This causes nuclear factor kappa B (NF-κB) to be activated and translocate into the nucleus [6,7].

These days, a number of processes, including endothelial dysfunction, renal angiotensin system activation, oxidative stress, and tubular epithelial cell transition, have been identified as contributing to hyperuricemic nephropathy [8].Hyperuricemia can cause chronic inflammation, which is directly linked to renal interstitial fibrosis [9]. According to recent research, toll-like receptor 4 (TLR4) is crucial for controlling inflammation [10]. TLR4's primary target gene, NF-κB, is in charge of producing inflammatory factors at the transcriptional level [11]. One of the main targets for therapeutic intervention is NF-κB signaling, which is the primary source of pathogenesis in many illness disorders.

The tissue is more susceptible to oxidative stress damage when uric acid levels are greater than typical physiological levels. While the activation of linked pro-inflammatory variables can cause inflammation, oxidative stress in vivo can trigger innate immune responses and activate associated inflammatory factors and pathways. According to reports, oxidative stress and inflammatory reactions are closely linked to the development of hyperuricemia[12].

Limiting purine consumption is now the suggested dietary strategy for preventing hyperuricemia. However, there are several practical issues with this approach since reducing consumption of purine-rich meals typically results in avoiding items with high nutritional value.[13].

Currently, the primary methods of controlling uric acid levels are medication therapy and lifestyle modifications. This condition has been extensively studied, and medications including propoxur, sulfinpyrazone, and benzbromarone have been used to treat it. Long-term use of these medications

might result in negative side effects such as skin rashes, liver damage, and possibly serious renal issues, even while they show promise in lowering uric acid levels. As a result, safer and more effective medications or natural therapeutic substances must be developed [12].

According to recent research, albiflorin (ALB) has a significant deal of potential for treating associated ailments since it has been shown to have substantial pharmacological effects in the fight against bacterial infections, cancer, oxidation, apoptosis, depression, and cognitive impairment [14].

One of the active substrates of the popular Chinese drug Paeonialactiflora is ALB, a monoterpenoid glycoside. It acts as an analgesic, antidepressant, anticonvulsant, and antiapoptotic properties [15].ALB may also have antioxidant and anti-inflammatory properties, according to earlier clinical research [16].

To the best of our knowledge, this study was created to demonstrate for the first time the renoprotective effect of ALB in hyperuricemia and the underlying mechanisms associated with referral to the HMGB1/TLR4/NF-kB signaling pathway.

Material & method

Animals

This study was conducted in compliance with the guidelines set out by Menoufia University's Faculty of Medicine's Animal Experimentation Ethics Committee with IRB NO:1/2025PATH17-2. We utilized thirty mature male albino rats that weighed between 150 and 180 g.The rats were purchased from a local providing facility and recruited for the present study and kept at 20–24 degrees Celsius with a 12-hour light–dark cycle. They were also fed regular rat chow and had unrestricted access to tap water.

Experimental design

Rats were randomly divided into 3 groups (10 rats each):

(1) Rats with normal serum uric acid levels (0.5 to 1.4 mg/dl) were chosen for the non-hyperuricemic group (Control): Each rat received a daily intraperitoneal (i.p.) injection of 1 milliliter of 0.9% sodium chloride (NacL) for eight weeks, and, each rat received an intragastric injection of 1 milliliter of NacL for the final four weeks of the trial.

(2) Hyperuricemic group (HU): potassium oxonate (PO) (Sigma Aldrich chemical business, Steinheim, Germany) as a uricase inhibitor at a dosage of 250 mg/kg/day, dissolved in 1 mL 0.9% NacL solution was administered i,p, to each rat, to induce hyperuricemia for four weeks [17]. Blood samples were obtained for the measurement of serum uric acid after four weeks. Hyperuricemic rats were defined as having a serum uric acid level more than 1.4 mg/dl [18].Rats were administered PO for four more weeks, and in the last four weeks of the trial, 1 milliliter of 0.9% NacL was administered intragastrically daily.

(3)Hyperuricemic/Albiflorin-treated group (HU+ALB): Rats were made hyperuricemic in this group, much like in the last one. Beginning in the fifth week of the experiment, they received 100 mg/kg of ALB (Chengdu HerbpurifyCo., Ltd.; purity: >98%; molecular weight: 480.46 g/mol) [19] dissolved in 0.9% normal saline intragastrially every day for four weeks.

Using metabolic cages, 24-hour urine samples were taken from all groups at the end of 8 weeks. Rats were then given anesthesia and given an ABP

measurement to evaluate renal blood flow velocity (RBFV) and renal artery resistance (RAR). Urine volume, protein content (gm/day) (NS-Biotec, Egypt), and creatinine concentration (mg/ml) were measured in order to calculate creatinine clearance. Urine creatinine concentration (mg/dL) multiplied by urine volume (mL/min) to plasma creatinine concentration (mg/dL) yielded the creatinine clearance (ml/min), with values reported in mL/min. [20].

Assessment of renal blood flow velocity and renal artery resistance

As stated earlier, RBFV and RAR were measured using a pulsed Doppler flowmeter (Hadeco, Hayashi Denki Co. Ltd., Japan) [21].To put it briefly, rats were anesthetized with xylazine and ketamine at dosages of 2 mg/kg and 60 mg/kg, respectively. A midline abdominal incision was then made to reveal the left renal artery. After filling the tip with coupling gel, the probe was positioned above the blood vessel until a steady record could be acquired.

Non-invasive blood pressure measurement

The diastolic and systolic blood pressures were measured non-invasively using the MP 36R Ultimate System R (BIOPAC, Aero Camino, USA).Before the experiment, the rat was put in the animal heating chamber's heating section while within the restrainer. The rat's temperature reached the ideal level after half an hour. The restrainer remained within the animal heating chamber, and the sensor was fastened to the rat's tail.Blood pressure measurements were taken after the rat had calmed down. Numerical data was analyzed using acknowledge software [22].

Blood sampling and biochemical analysis

Samples of fasting blood were drawn from the retro-orbital venous plexus, allowed to clot for 10 minutes at room temperature in a water bath, and then centrifuged for 20 minutes at 3000 r.p.m. For a subsequent biochemical test, the supernatant serum was collected and kept at -80. Following the sacrifice of all rats by cervical elongation and dislocation, the kidneys were removed; the right kidney was utilized for a biochemical test, while the left kidney was used for histological and immunohistochemical analyses.

Serum uric acid (El-Gomhoria company, Egypt) was measured by colorimetric method according to the manufacturer instructions. Colorimetric kits (Spectrum Diagnostics, Egypt) were used to measure the concentrations of creatinine in plasma and urine, and plasma urea

Making a Tissue Homogenate A tissue homogenizer (MPW120, MPW Medical Instruments, China) was used to homogenize each of the weighted kidney specimens separately. Before being kept at -80°C for testing, the crude tissue homogenate was spun for 15 minutes at 10,000 rpm in an ice-cold centrifuge.

ELISA Kits were used to measure renal TNF-α (TNF-α: ERT2010-1, Assaypro LLC, Saint Charles, Missouri, USA), IL-6 (IL-6: ab100772, Abcam, Cambridge, UK), Calorimetric kits were used to assess renal MDA and superoxide dismutase (SOD) in accordance with the manufacturer's instructions.

Quantitative assay of renal HMGB1, TLR4 and NF-κB genes expression using reverse transcriptase polymerase chain reaction technique (RT-PCR).

The QiagenRN easy plus Universal Kit from the USA was used to prepare renal samples for total

RNA isolation. The quality and purity of the RNA were then assured. Until it was required, RNA was stored at -80 °C. The first step therefore was to create cDNA in a single cycle using an Applied Biosystems 2720 heat cycler in Singapore using the QuantiTect Reverse Transcription Kit, which is produced by Qiagen in the USA.In RT-PCR processes, GAPDH primers were used as an RNA loading control. cDNA amplification was the second step. SensiFASTTMSYBR Lo-ROX Kit, USA, used cDNAin SYBR green-based quantitative real-time PCR for Relative Quantification (RQ) of HMGB1, TLR4, and NF-κB gene expression using the following designed primers (Midland, Texas): The forward primer for HMGB 1 was *(TGAGGGACAAAAGCCACTC)*, and the reverse primer was *(TTGGGAGGGCGGAGAATC*), The NF-kB forward primer was (TCGACCTCCACCGGATCTTTC). The reverse primer was (GAGCAGTCATGTCCTTGGGT).

The forward primer for TLR4 was (TCAGCTTTGGTCAGTTGGCT), and the reverse was (GTCCTTGACCCACTGCAAGA)

Finally, the 2.0.1 version of the Applied Biosystems 7500 software was used to finish the data analysis. The RQ of HMGB1, TLR4, and NF-κB gene expression was conducted using a comparative ΔΔCt technique, which normalizes the amount of target gene (HMGB1, TLR4, and NF-κB) mRNA to an endogenous reference gene (GAPDH) and compares it to a control.

Histological study:

The specimens were dried, cleaned, and embedded in paraffin blocks after the kidney was preserved in a 10% buffered formaldehyde solution. For routine histological analysis, serial coronal slices of 5 μm in thickness were cut and stained with hematoxylin and eosin (H & E).

Immunohistochemical study:

Anti-Bax antibody (rabbit polyclonal antibody, Dako, Carpinteria, California, USA) and anti-NF-kB antibody (monoclonal, dilution 1:200, Abcam) were incubated on kidney slices for one night at 37 °C. Finally, sections were treated for 30 minutes at room temperature with a secondary antibody conjugated with peroxidase.

Statistical analysis

The Shapiro test is used to determine whether the data is normally distributed. The results' mean ± standard deviation is displayed. The data that was gathered was evaluated using analysis of variance (ANOVA).SPSS (Version 23) (SPSS Inc., Armonk, NY, USA) was used to analyze the data. The data was expressed using the mean±SD. To determine the significance, a statistical criteria of $P < 0.05$ was applied.

Results

The measured SBP,DBP , MABP, RAR, serum levels of uric acid, urea, creatinine in addition to urinary protein, renal MDA, renal TNF-α, renal IL-6, renal genes HMGB1, TLR4 and NF-kB of HU group were dramatically increased compared to control however RBFV, renal SOD and creatinine clearance values of HU group were substantially decreased compared to control. The measured SBP, DBP ,MABP, RAR, serum levels of uric acid, urea, creatinine in addition to urinary protein, renal MDA, renal TNF-α, renal IL-6, renal genes HMGB1, TLR4 and NF-kB of HU+ALB group were substantially decreased compared to HU but still dramatically increased compared to control, however RBFV, renal SOD

and creatinine clearance values of HU+ALB were substantially increased compared to HU group but still substantially decreased compared to control (Table 1).

Table (1): The measured SBP,DBP , MABP, RBFV, RAR, serum urea, creatinine, creatinine clearance, urinary albumin, renal TNF-α, renal IL-6, renal MDA, SOD, and renal TLR4 and NF-kB genes expression in all studied groups

	Control group	HU group	HU+ALB group
SBP (mmHg)	100.5±3.1	149.2±2.1*	128±1.12*#
DBP (mmHg)	60.2±1.12	90.2±3.1	75.2±2.1*#
MABP (mmHg)	75.2±3.1	111.2±3.1*	92.2±1.94*#
RBFV (cm/second)	6.2±0.1	4.1±0.17*	5.2±0.09*#
RAR (PRU)	0.9±0.04	1.98±0.09*	1.54±0.04*#
Serum Uric acid level (mg/dl)	1.03±0.06	3.57±0.19 *	2.89±0.19 *#
Serum Urea (mg/dl)	44±2.2	88.3±1.7*	63.5±3.1*#
Serum Creatinine (mg/dl)	0.49±.07	1.51±0.18*	1.06±0.09*#
Creatinine clearance (mL/min)	2.3±0.09	1.2±0.11*	1.77±0.19*#
Urinary proteins (gm/24h urine)	0.25±0.01	2.2±0.08*	1.7±0.03*#
Renal MDA (nmol/ gm. Tissue)	4.2±0.6	16.5± 1.01*	9.8± 0.7*#
Renal SOD (U/gm. Tissue)	6.33±0.9	3.6±0.02*	4.9±0.18*#
Renal TNF-α (ng/ml)	23±0.8	38.2±2.4*	31.5±1.1*#
Renal IL-6 (pg/mL)	112.2±3.1	196.1±4.1*	148.5±4.25*#
Renal TLR4 gene expression	1	3.5±0.19*	2.31±0.12*#
Renal TLR4 gene expression	1	4.1±0.02*	2.59±0.13*#
Renal NF-κB gene expression	1	3.1±0.18*	2.31±0.09*#

* Significant compared with control, # Significant compared with HU.

Hematoxylin and Eosin staining:

The control group's kidney has normal tubular and glomerular anatomy. The HU group's kidney had periglomerular round cell infiltration, glomerulus shrinkage, and partial tubular lumen blockage. The glomerular and tubular architecture of the kidney of the HU group that received ALB treatment improved .Fig (1)

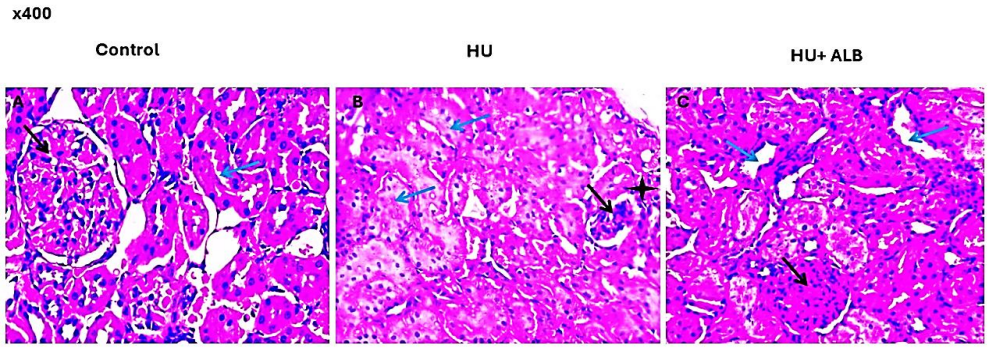

Fig 1:H& E-stained kidney sections (H&E ×400) in the groups under study:(A) The control group's kidney has normal tubular (blue arrow) and glomerular (black arrow) structure.(B) The HU group's kidney displayed periglomerular round cell infiltration (star), tubular lumen blockage (blue arrows), and glomerulus atrophy (black arrow).(C) The kidney of the HU group that received ALB treatment displayed the repair of both tubular (blue arrow) and glomerular (black arrow) architecture.

Immunohistochemical results

When compared to the control group, the HU group's percentage area of Bax increased significantly (70±0.03 vs. 10±0.55, respectively, $p<0.05$) in the Bax stain. This proportion was higher than that of the control group, but it was significantly lower in the HU+ALB group than in the HU group (23±0.05 vs. 70±0.03, respectively, $p<0.05$).(Fig. 2: A-D).

When compared to the control group, the HU group's percentage area of NF-kB increased significantly (64±0.01 vs. 11±0.03, respectively, $p<0.05$) in the NF-kB stain. Though it was still higher than the control group, the HU+ALB group's percentage was significantly lower than that of the HU group (21±0.05 vs. 64±0.11, respectively, $p<0.05$). (Fig. 2: E-H).

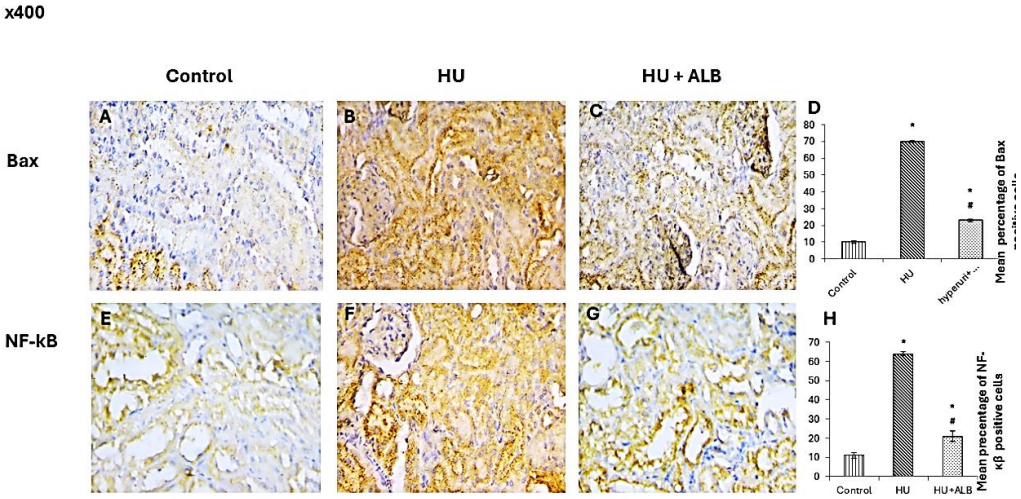

Fig (2): Representative micrographs of the different experimental groups showing significant increase of the Bax (A-D) and NF-kB (E-H) immunoreaction in the HU group and a significant decrease in the HU + ALB group.

Discussion

In our investigation, PO caused HUA and markedly raised blood uric acid levels. Simultaneously, it was shown that HUA induced deterioration in renal function; however, ALB groups considerably reduced the effects of HUA. Serum uric acid levels were first ascertained. According to control values, the HU group's serum UA levels increased considerably, suggesting that the study's primary goal—the HU model—was met, which is consistent with Rajendra et al. findings [23].Oxonic acid is an inhibitor of uricase, a hepatic enzyme found in most mammals that breaks down uric acid to allantoin, which explains why rats' blood uric acid levels were elevated after receiving PO injections [24].

Serum uric acid levels were significantly lower in the HU+ALB group than in the HU group. As far as we are aware, this is the first investigation into the role of ALB in hyperuricemia. Previous research has shown that herbaceous peonies (Paeonialactiflora Pall.) have a hypouricemic impact. One of the active components of Paeonialactiflora, a popular traditional Chinese remedy, is ALB, a monoterpenoid glycoside [25]..

Severe kidney damage can result from hyperuricemia. Oxidative stress damage and inflammation are the main processes at play [26]. .A frequent side effect of CKD, hyperuricemia is

linked to the onset and progression of CKD and has a negative correlation with renal function [27]. As demonstrated by histopathological alterations in the HU group, our data showed a drop in creatinine clearance and a substantial increase in urea, creatinine, and urine protein when compared to the control group. This is consistent with earlier research[28].

Histopathological improvement in HU+ALB compared to HU demonstrated that ALB significantly reduced renal damage brought on by hyperuricemia. In a recent work, Yu et al. [29], .showed the renoprotective effects of albiflorin. They ascribed this effect to the anti-inflammatory activity of ALB, which is achieved by inhibiting the PI3K/AKT/NF-κB pathway.

Additionally, the current study demonstrated that, as compared to control, hyperuricemia significantly raised RVR and lowered RBFV. These findings were in line with those of CristóbalGarcía et al. [30],who found that altered renal hemodynamics were linked to chronic hyperuricemia. Additionally, in hyperuricemic rats, Sanchez-Lozada et al. [31],discovered a positive association between blood uric acid and afferent and efferent arteriolar resistance.

In an animal model of hyperuricemia, research by Sanchez-Lozada et al. [32], clarified the connection between renal hemodynamics and renal impairment. Single nephron GFR (SNGFR) was reduced by 35% as a result of the simultaneous decrease in glomerular plasma flow and the ultrafiltration coefficient, which was brought on by a significant increase in afferent and efferent resistances with a decrease in renal blood flow.

SBP, DBP, and MABP were significantly higher in the hyperuricemic group compared to the control group, taking into account changes in blood pressure observed in the current investigation. This is consistent with Corry et al. [33],who linked progressive renal microvascular changes to hyperuricemic hypertension. Sanchez-Lozada et al. [31],showed that by inducing VSMC proliferation, hyperuricemia caused preglomerulararteriolopathy. Severe renal hypoperfusion will result from the afferent arteriole constriction that follows. Renal ischemia brought on by a reduction in renal blood flow or the consequence of a decline in filtered load may both lead to systemic hypertension. By activating the RAS, renal ischemia causes systemic hypertension [31]. RAS activation raises the amount of plasma renin, which results in the production of ang-II, which induces vasoconstriction and hypertension [34].

HU+ALB dramatically ameliorated renal hemodynamics and significantly lowered ABP values compared to HU group this may be attributed to the antioxidant and anti-inflammatory qualities of ALB

According to reports, UA functions as an antioxidant in plasma, but once large amounts of UA enter cells, it becomes a potent pro-oxidant [35].Our study's findings demonstrated that hyperuricemia caused oxidative stress, which helps to further explain the HU group's compromised hemodynamics and renal functions. These findings concurred with those of previous study [36].

ALB significantly reduced the oxidative damage brought on by hyperuricemia. These results are consistent with previous studies [37].ALB's ability to reduce oxidative stress is due to its ability to

scavenge free radicals, as well as its ability to up-regulate the Nrf2/HO1 system with antioxidant effects via controlling the expression of antioxidant genes [38].

Kidney tissue was analyzed in terms of inflammation, it was determined that HU increases renal TNF-a, IL-6 and upregulated NF-kB kidney immunoreaction levels compared to control this was in accordance with Xiao et al.[39].Additionally, Han et al.[40] discovered that uric acid causes the kidneys to produce systemic cytokines and activates many inflammatory transcription factors, including NF-kB.

On the other hand, in comparison to the HU group, ALB therapy dramatically reduced renal cytokines and downregulated NF-kB immunoreaction. ALB's potential to reduce proinflammatory cytokines and raise the anti-inflammatory marker IL-10 was previously shown [41].ALB also interferes with tissues' toll-like receptor-mediated signaling. Furthermore, by altering the PI3K/Akt pathway and affecting NF-κB signaling, ALB may lessen inflammation [42].

According to Awadet al. [7]), inflammatory cells generate HMGB1, which binds to TLR-4, an upstream regulatory factor of the inflammatory response, to trigger the release of cytokines. By controlling NF-κB signals, TLR4 can increase the expression of several inflammatory factors [43], which in turn mediate the inflammatory response, encourage renal tubular epithelial cells to develop into fibroblasts, and upregulate the expression of various inflammatory chemokines, all of which contribute to the development of renal inflammation [44].TLR4 can be directly activated by UA, a kind of damage-associated molecular pattern produced by ischemic tissues and dying cells [45].Research has demonstrated that TLR4 may stimulate the production of certain inflammatory components, which in turn can trigger an inflammatory response via controlling NF-κB signals. In line with previous studies, our findings demonstrated that HU increased renal gene expressions of HMGB1, TLR4, and NF-kB as well as renal NF-kB immunoreaction when compared to control [46],.

Previous research showed that ALB dramatically decreased the levels of HMGB1, p-NF-κBP65, and p-IkBa in rats and raised the levels of Nrf-2 and HO-1 in the rat hippocampal region[19], HU+ALB dramatically down-regulated HMGB1, TLR4 and NF-kB and renal NF-kB immunoreaction compared to HU indicating an additional way that ALB can help with hyperuricemia-induced renal impairment.

Apoptosis has a part in kidney damage.A poptosis's function in HU has been shown. According to reports, HU caused rats' kidneys to undergo more apoptosis. In line with previous studies,[47],our findings showed that hyperuricemia increased the proapoptotic marker Bax immunoreaction in the kidney when compared to control. Nonetheless, ALB significantly reduced Bax immunoreactivity, which is consistent with other studies [41,48].

Conclusion: Through anti-oxidant, anti-inflammatory, and anti-apoptotic processes as well as the down-regulation of HMGB1, TLR4, and NF-κB renal gene expression, ALB reduced HU-induced renal impairment.

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