**Original Article**

# The effect of daidzein on renal injury in ovariectomized rats: interaction of angiotensin receptors and long non-coding RNAs H19, GAS5, MIAT, and Rian

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## Abstract

**Background:** Renin-angiotensin system (RAS) is prominently associated with renal pathophysiology in postmenopausal women. Long non-coding RNAs (lncRNAs) H19, GAS5, MIAT, and Rian have been linked to the pathogenesis of renal injury. **Aims:** This study aimed to evaluate the beneficial effects of daidzein on unilateral ureteral obstruction (UUO) induced-renal injury in ovariectomized (OVX) rats through interaction with angiotensin AT1, Mas receptors, and lncRNAs. **Methods:** 84 female rats were ovariectomized (OVX) two weeks before performing obstruction of the left kidney ureter (UUO). The animals were then randomly divided into four main groups (n=21): Sham+DMSO, UUO+DMSO, UUO+17β-Estradiol (E2) (positive control), and UUO+daidzein. Each main group comprised three subgroups (n=7) and were treated with saline, A779 (MasR antagonist), or losartan (AT1R antagonist) for 15 days. On day 16, the animals were euthanized, and the left kidneys were harvested for histopathology and lncRNAs expression assays. **Results:** UUO significantly increased kidney tissue damage score (KTDS) in the UUO rats, increased the expression of H19 and MIAT, and decreased the expression of GAS5 and Rian. Daidzein alone and in co-treatment with losartan or A779 reversed these effects. Daidzein with 1 mg/kg dose was more effective than E2. **Conclusion:** Daidzein alone and in co-treatment with A779 and losartan improved renal injury in UUO rats and recovered dysregulated expression of UUO-related lncRNAs through modulating MasR and AT1R receptors, associating with modulation of the expression of lncRNAs. Daidzein could be considered a renoprotective phytoestrogen substitute for E2 therapy in postmenopausal women suffering from renal diseases.

**Key words:** Daidzein, Long non-coding RNAs, Rat, Renal injury, Unilateral ureteral obstruction

## Introduction

Ureteral obstruction is a common clinical case that causes renal damage in the clinical situation through inflammatory cells infiltration, activation of TGF-β1 and renin-angiotensin system (RAS) pathways, tubular injury, and extracellular matrix (ECM) overproduction and deposition (Ucero *et al*., 2014; Kaeidi *et al*., 2020). Unilateral ureteral obstruction (UUO) is approved as an experimental model to study renal injury in obstructive nephropathy (Ucero *et al*., 2014; Kaeidi *et al*., 2020).

RAS is one of the most important pathways involved in developing renal injury in the UUO model. AngII via AT1 receptors (AT1R) stimulates inflammation, TGF-β activation, ROS production, tubular atrophy, necrosis, and fibrosis in the kidney via complex mechanisms (Esteban *et al*., 2004). On the other hand, Angiotensin-converting enzyme 2 (ACE 2), the homolog of ACE,

counterbalances the ACE activity via Ang II degradation to the vasodilator peptide Ang 1-7. Ang 1-7 acts on the G-protein coupled receptor (GPCR) Mas (MasR) to show the counterbalancing effects of Ang II (Ferrario, 2011).

Female sex hormones have renoprotective effects, which decreases after menopause (Vellanki and Hou, 2018). Several studies reported that estrogen-based hormone replacement therapy (HRT) after menopause protects against chronic kidney disease (CKD), but adverse effects such as venous thromboembolism (VTE) and breast, ovarian, and endometrial malignancies (Dumanski *et al*., 2017; Vellanki and Hou, 2018). These are motivations for finding an alternative to reducing the side effects of estrogen therapy.

Phytoestrogens, a group of plant-derived chemicals, are estrogen-like compounds derived from plants, structurally similar to 17β-estradiol (Desmawati and Sulastri, 2019). Isoflavone daidzein, one of the most important phenolic phytoestrogens, is found in soybeans and other legumes (Desmawati and Sulastri, 2019). The similarity of daidzein chemical structure to E2 results in imitation of some but not all responses to E2 (Gaete *et al*., 2012; Alshehri *et al*., 2021). Daidzein showed beneficial effects against many diseases associated with estrogen regulation, such as cardiovascular diseases, osteoporosis, breast cancer, and diabetes (Vitale *et al*., 2013). Numerous basic and clinical studies have proven the antioxidant, anti-apoptosis, and anti-inflammatory effects of daidzein in nephrotoxicity and diabetic kidney diseases (Laddha and Kulkarni, 2020; Tomar *et al*., 2020). Also, it has been confirmed previously in our lab in UUO model (Askaripour *et al*., 2022a, b).

Long non-coding RNAs (lncRNAs) longer than 200 nucleotides regulate the expression of genes through transcriptional and post-transcriptional modifications (Guttman *et al*., 2013). Therefore, it is unsurprising that dysregulated expression of lncRNAs is associated with many abnormalities, including fibrotic and vascular disorders and cancer (Chen *et al*., 2012; Bijkerk *et al*., 2019). The expression of lncRNAs in kidney diseases (Ignarski *et al*., 2019), and the association between lncRNAs and processes leading to renal injury, including TGF-β/smad signaling (Zhou *et al*., 2014; Sun *et al*., 2017), oxidative stress (Xiao *et al*., 2019), and ECM production (Zhou *et al*., 2014; Sun *et al*., 2017) have been reported. LncRNAs H19, Rian (RNA imprinted and accumulated in the nucleus), MIAT (Myocardial infarction associated transcript), and GAS5 (growth arrest-specific 5) play key roles in renal inflammation and fibrosis development (Xie *et al*., 2016; Bijkerk *et al*., 2019; Yu *et al*., 2020). Inhibition of H19 in preclinical models of renal fibrosis and reduction in the expression of fibrotic-related genes suggested H19 antagonism as an anti-fibrotic therapy strategy (Xie *et al*., 2016). Rian and MIAT affect renal injury in UUO and unilateral ischemia-reperfusion injury (IRI) models under a signaling network of microRNAs-mRNAs interactions mediating kidney dysfunction and fibrosis (Bijkerk *et al*., 2019; Zhang *et al*., 2019). GAS5 in plasma and urine of CKD patients correlates with disease stages. GAS5 is highly expressed in normal kidneys of rats, and its reduction in UUO kidneys and TGF-β-induced renal damage is accompanied by inflammation, apoptosis, and fibrosis (Yu *et al*., 2020; Guo *et al*., 2021).

Since H19, GAS5, MIAT, and Rian have crucial effects on UUO-induced renal injury, and RAS is an important pathway involved in the development of the renal disorder, we investigated whether these lncRNAs might change in response to the treatment of daidzein on UUO-induced renal injury in ovariectomized (OVX) rats in the presence of losartan and A799.

## Materials and Methods

### Animals

The studies were performed on female Wistar rats (9 weeks old, weight 180-220 g) obtained from the Animal Center of Kerman University of Medical Sciences (Kerman, Iran). All experimental steps were performed by NRC Guide for the Care and Use of Laboratory Animals: 8th Edn. and approved by the Ethics Committee of Kerman University of Medical Sciences (approval No: IR.KMU.REC.1399.031.). The rats were housed on a 12-hour light/dark cycle pattern with free access to food and water.

### Bilateral ovariectomy

All experimental animals were ovariectomized two weeks before the experiment. An intraperitoneal dose of ketamine (80 mg/kg) and xylazine (10 mg/kg) was used to accomplish a bilateral ovariectomy under general anesthesia. Bilaterally, the ventral mid-lumbar region was shaved. A 2-cm incision was created through the skin and muscle of the abdominal wall. The fallopian tube and the vascular base of the ovary were ligated with a 4-0 suture in the proximal region and cut off in the distal region after the muscles were opened. We established conditions that were similar to normal menopause by removing the ovaries. After ovariectomy, two weeks of recovery are recommended to guarantee that the effects of the internal region of E2 are eliminated (Khaksari *et al*., 2015).

### Experimental design

UUO was conducted two weeks after OVX (Xie *et al*., 2016). In brief, under anesthesia with a ketamine/xylazine cocktail (80/10 mg/kg, i.p), a lateral incision was used to expose the left kidney and ureter. To prevent retrograde urinary tract infection, the left ureter was blocked at two spots with silk 4-0 and incised between ligatures. The sham procedure involved anesthesia, a flank incision, and isolation but not dissecting the ureter. Figure 1 depicts the experimental groups.

The animals were put into four groups at random (n=21): Sham+DMSO (Dimethyl sulfoxide 1%), UUO+DMSO, UUO+17β-Estradiol (E2) (5 µg/kg) (positive control group) (Dixon and Maric, 2007), and UUO+daidzein 1 mg/kg. Each main group comprised

three subgroups (n=7) of treating with saline, A779 (744 µg/kg) (Ferrario, 2011), or losartan (10 mg/kg) (Teixeira *et al*., 2017) for 15 days. The dose of daidzein was chosen as a dose-response study in our previous study (Askaripour *et al*., 2022b). To simulate the natural estrous cycle, E2 was given every four days (Guo *et al*., 2021). All treatments were performed intraperitoneally once a day from the first day of UUO (or sham operation) induction. On day 16, the left kidneys were taken from euthanized animals. The left kidney was dissected into two-part, one part was fixed in 10% formalin overnight, and the other was stored at -80°C for gene expression analysis.

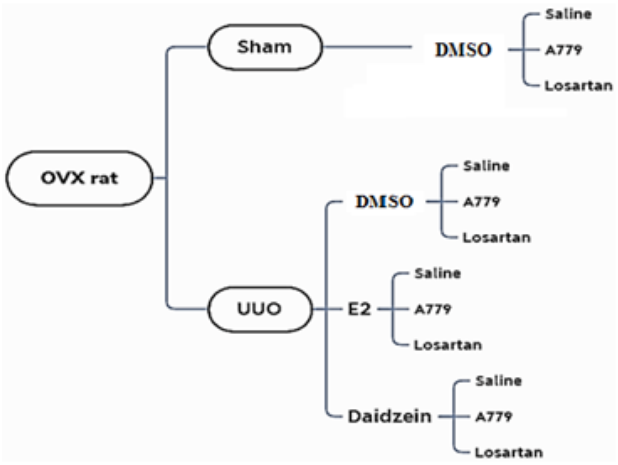



**Fig. 1:** Schematic representation of the experimental groups. OVX: Ovariectomy, UUO: Unilateral ureteral obstruction, DMSO: Dimethyl sulfoxide, E2: 17β-Estradiol, A779: Mas receptor antagonist, and losartan: AT1 receptor antagonist

## Histopathologic examination

Masson's trichrome staining of fixed tissue was performed after several preparation processes, including ethanol dehydration, paraffin embedding, and sharp cutting of embedded tissues into 5-µm thick sections. Masson's trichrome staining was performed for histopathological assay. Histologic analysis was performed by a pathological expert blindly by Olympus microscope (CX33, Tokyo, Japan). Kidney tissue damage score (KTDS) was scored from 0 to 4 (0: normal, 1= <25%, 2= 25-50%, 3= 50-75%, and 4= >75%), according to the intensity of tubulointerstitial lesions (hyaline cast, debris, vacuolization, flattening and degeneration of tubular cells, and dilatation of tubular lumen, tubular necrosis, loss of tubular brush border, interstitial mononuclear cells (MNCs) infiltration, and fibrosis (Rostami *et al*., 2014).

## Total RNA extraction and qRT-PCR

The left tissue kidney was homogenized before RNA extraction using an ultrasonic homogenizer (UP200H, 50/60Hz, Hielscher, Germany). RNA was extracted using RNA Mini-Preps kit (Bio Basic, Canada). PrimeScript 1st cDNA Synthesis kit (Takara Bio, Japan) was used for cDNA synthesis. We evaluated the expression of lncRNAs by a StepOnePlus (Applied Biosystem) instrument using RealQ Plus Master Mix Green. The Sequences of primers for H19 (Rajabi *et al*., 2022), MIAT (Zhou *et al*., 2017), GAS5 (Wu *et al*., 2019), Rian (Bijkerk *et al*., 2019), and 18S rRNA (Sawicki *et al*., 2018) are listed in Table 1. The PCR efficiency for primers was 1.9. 18S rRNA was used as the internal control to normalize the expression of target genes. The reaction mixture was 12.5 µL composed of 6 µL master mix, 0.5 µL forward primer, 0.5 µL reverse primer, 2 µL cDNA (1000 ng), and 3.5 µL distilled water. The real-time PCR was performed under the following conditions: the initial denaturation step at 95°C for 5 min followed by 40 cycles of denaturation (95°C, 5 s) annealing (60°C, 30 s), and extension (60°C, 30 s). The relative expression of genes was calculated according to $2^{-\Delta\Delta Ct}$ (Rajabi *et al*., 2020). Briefly, fold change gene expression for target genes was calculated according to the formula: Fold change = $2^{-\Delta\Delta CT}$ in which ΔΔCT is the difference between the DCT of each group and the DCT of the CTL group, with DCT equal to CT gene minus CT internal control.

$\Delta\Delta CT = [(CT\ gene - CT\ 18S\ rRNA)_{treatment} - [CT\ gene - CT\ 18S\ rRNA)]_{CTL}$

## Statistical analysis

All data were presented as mean±SEM. GraphPad Prism version 6.00 was used for statistical analysis. The

**Table 1:** Sequences of primers used for RT-PCR

| Genes | Sequence | Product size | Tm | Annealing Temp. |
|---|---|---|---|---|
| *H19* | Forward 5´-GATGGAGAGGACAGAAGGACAGT-3´ | 127 | 61.27 | 60 |
| | Reverse 5´-GAGAGCAGCAGAGATGTGTTAGC-3´ | | 61.28 | |
| *MIAT* | Forward 5´-GAGGGAAGTTCTGAGCTTGG-3´ | 138 | 57.89 | 60 |
| | Reverse 5´-CCTTTCTTCTGGGCTGAGAC-3´ | | 57.89 | |
| *GAS5* | Forward 5´-ATCCATCCAGTCACCTCTGG-3´ | 296 | 58.50 | 60 |
| | Reverse 5´-TCTCACAGGCAGTTCTGTGG-3´ | | 59.61 | |
| *Rian* | Forward 5´-CTGTTGTGCCCTCCCTGGATG-3´ | 137 | 63.01 | 60 |
| | Reverse 5´-CCAGCTAGGCTGTGTAAATCATC-3´ | | 59.19 | |
| *18S rRNA* | Forward 5´-GATCCGAGGGCCTCACTAAAC-3´ | 69 | 60.20 | 60 |
| | Reverse 5´-AGTCCCTGCCCTTTGTACACA-3´ | | 61.32 | |

Kolmogorov-Smirnov test was used to determine the normality of the variant distribution. For group comparisons, the one-way ANOVA test was performed, followed by Tukey's post-hoc test. The Kruskal-Wallis test was used to assess nonparametric data and pathology scores, followed by the Mann-Whitney U test. Statistical significance was defined as a p-value of less than 0.05.

## Results

### Histomorphological evaluation of renal tissue

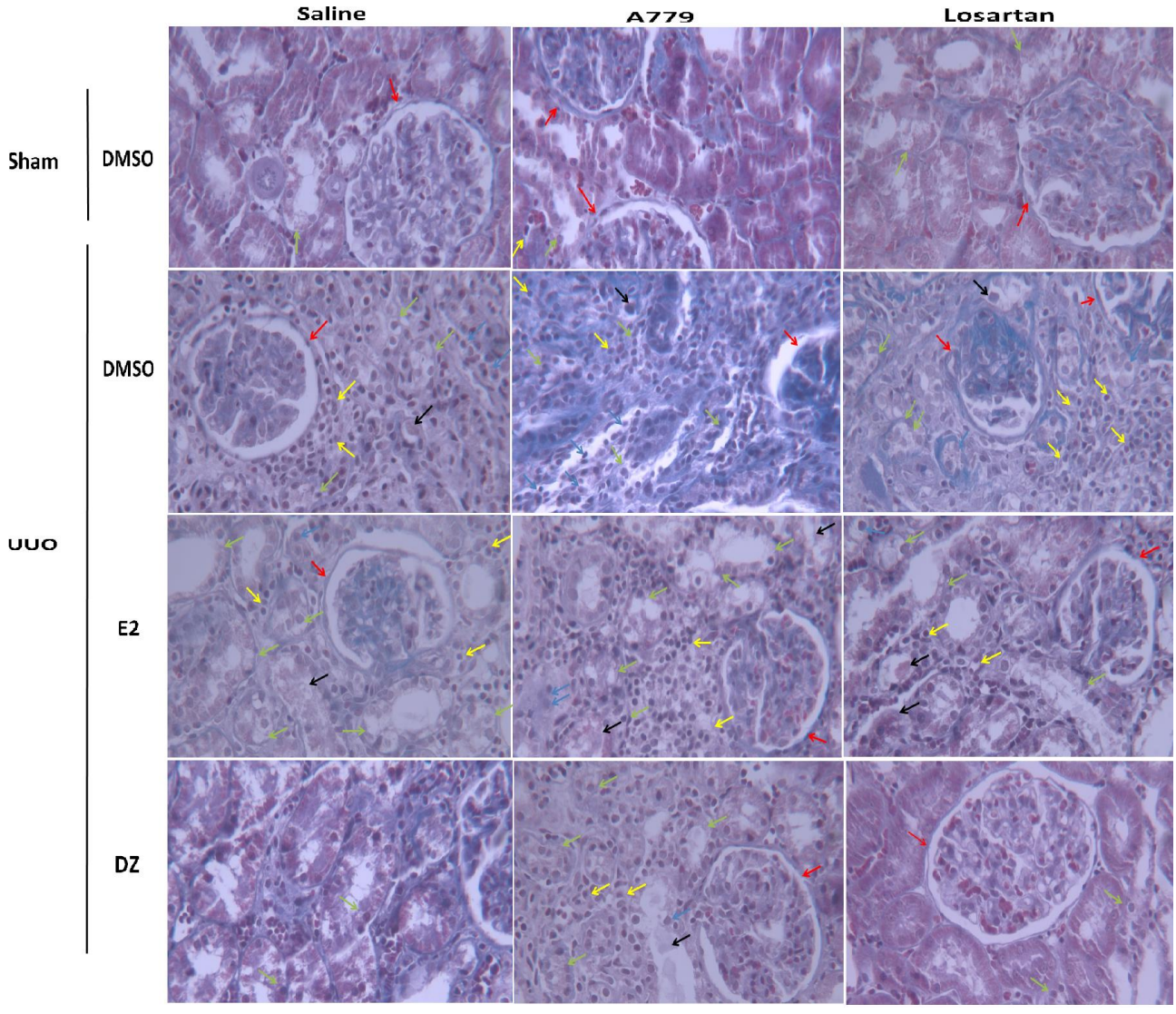
The KTDS was significantly higher in the UUO+DMSO groups compared to the sham groups (P<0.001). Treatment with daidzein (and E2) significantly reduced the KTDS compared to UUO+DMSO (P<0.05). In UUO+DMSO subgroups, A779 further increased KTDS, and losartan decreased this score compared to the sham subgroups (P<0.001).

Co-treatment daidzein (or E2) with A779 or losartan significantly reduced the KTDS compared to UUO+DMSO (P<0.05); so, co-treatment with A779 masked its harmful effects, and co-treatment with losartan further decreased KTDS. Daidzein treatment with a dose of 1 mg/kg was more effective than E2 in reducing KTDS in related groups (P<0.001) as shown in Figs. 2A, B and 3.

### The effect of daidzein on lncRNAs expression

According to real-time PCR data in the obstructed kidney groups, the expression level of H19 and MIAT was significantly increased compared to related Sham+DMSO groups (P<0.001) (Figs. 4A and B). Treatment with daidzein (and E2) significantly reduced the expression level of H19 and MIAT compared to UUO+DMSO groups (P<0.001). Daidzein 1 mg/kg significantly decreased the expression of MIAT compared to related UUO+E2 groups (P<0.01) (Fig. 4B). In the sham subgroups, A779 increased H19, and MIAT expression compared to the saline group (P<0.001). Also, losartan decreased MIAT expression compared to the saline group (P<0.001). Co-treatment daidzein (and E2) with A779 or losartan significantly reduced the expression level of H19 and MIAT compared to UUO+DMSO groups (P<0.001) (Figs. 4A and B).

**A**



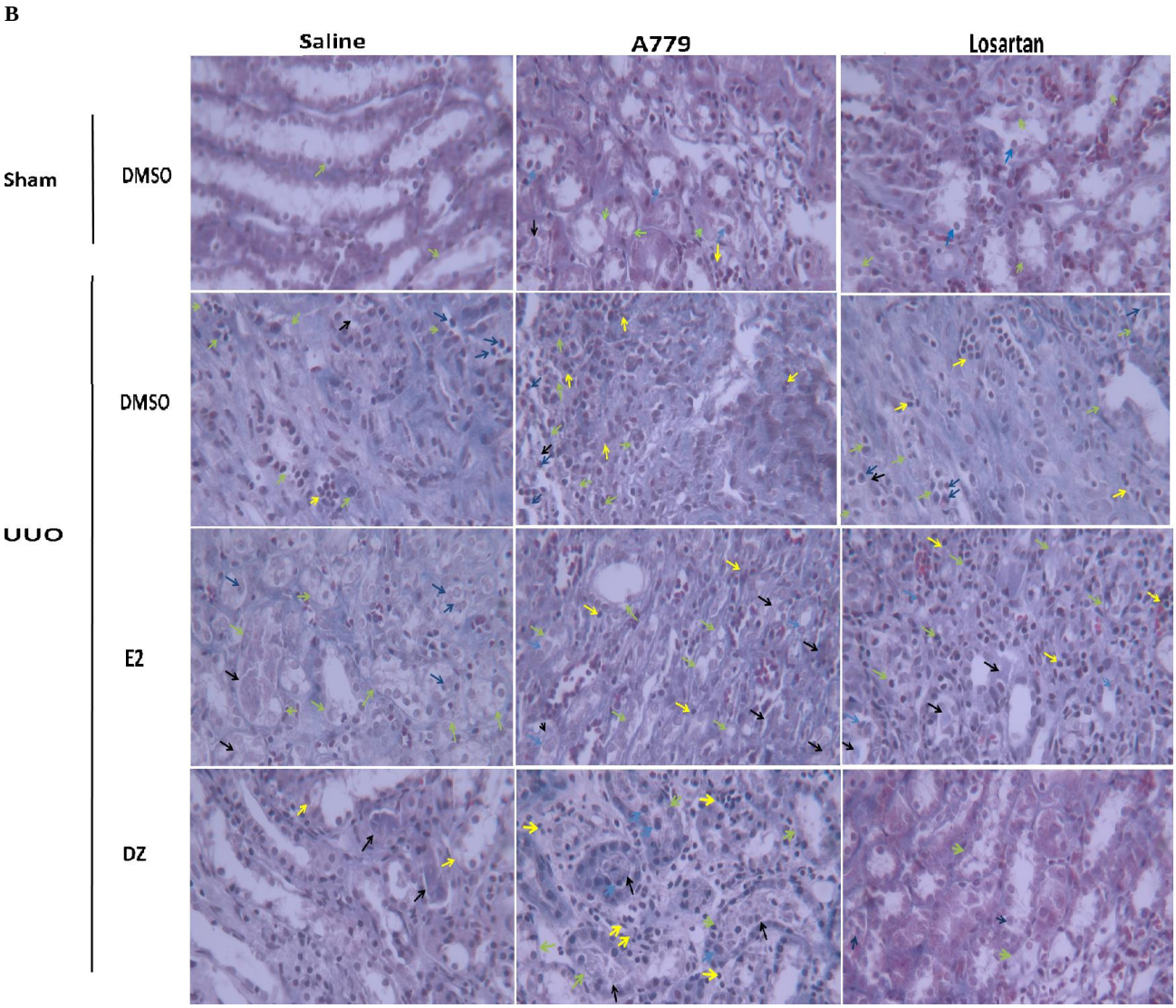



**Fig. 2:** Effect of daidzein and E2 on kidney tissue damage in experimental rats. Masson's trichrome staining showed histologic abnormalities in the cortex (**A**) and medulla (**B**) of the left kidney. Collagen content in the interstitium by changing the intensity of the blue color in the background (×400 magnification). Red arrow: glomeruli, yellow arrow: inflammation, green arrow: tubular epithelial cells vacuolization and brush border loss, bluish arrow: tubular cell necrosis and detachment, and black arrow: cast. DMSO: Dimethyl sulfoxide, E2: 17β-Estradiol, DZ: Daidzein, and UUO: Unilateral ureteral obstruction

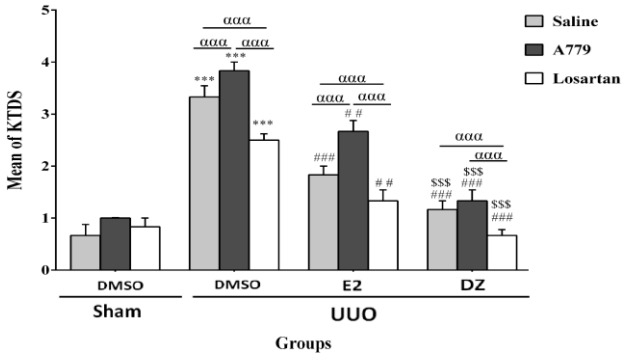



**Fig. 3:** The mean score was obtained from KTDS in sham and UUO groups. *** P<0.001 vs. Sham+DMSO groups. ## P<0.01, ### P<0.001 vs. UUO+DMSO groups. $$$ P<0.001 vs. UUO+E2 groups. ααα P<0.001 indicates a significant difference between subgroups. DMSO: Dimethyl sulfoxide, E2: 17β-Estradiol, DZ: Daidzein, UUO: Unilateral ureteral obstruction, and KTDS: Kidney tissue damage score

Figures 4C and D showed that in the groups with obstruction kidney, the level of gene expression GAS5 and Rian was significantly decreased compared to Sham+DMSO groups (P<0.05). Treatment with daidzein (and E2) significantly increased the expression of GAS5 and Rian compared to the UUO+DMSO groups (P<0.001). Daidzein 1 mg/kg is more effective than E2 in GAS5 and Rian expression (P<0.001). The results showed that in the sham groups, A779 significantly decreased the expression of GAS5 and Rian compared to saline and losartan (P<0.001). In the UUO+DMSO groups, A779 further decreased these lncRNAs than the related Sham+DMSO group (P<0.05). Co-administration of daidzein (and E2) with A779 or losartan significantly increased the expression of GAS5 and Rian compared to related groups in the UUO+DMSO (P<0.001). So, co-treatment with A779 masked its inhibitory effects and co-treatment with losartan further increased these

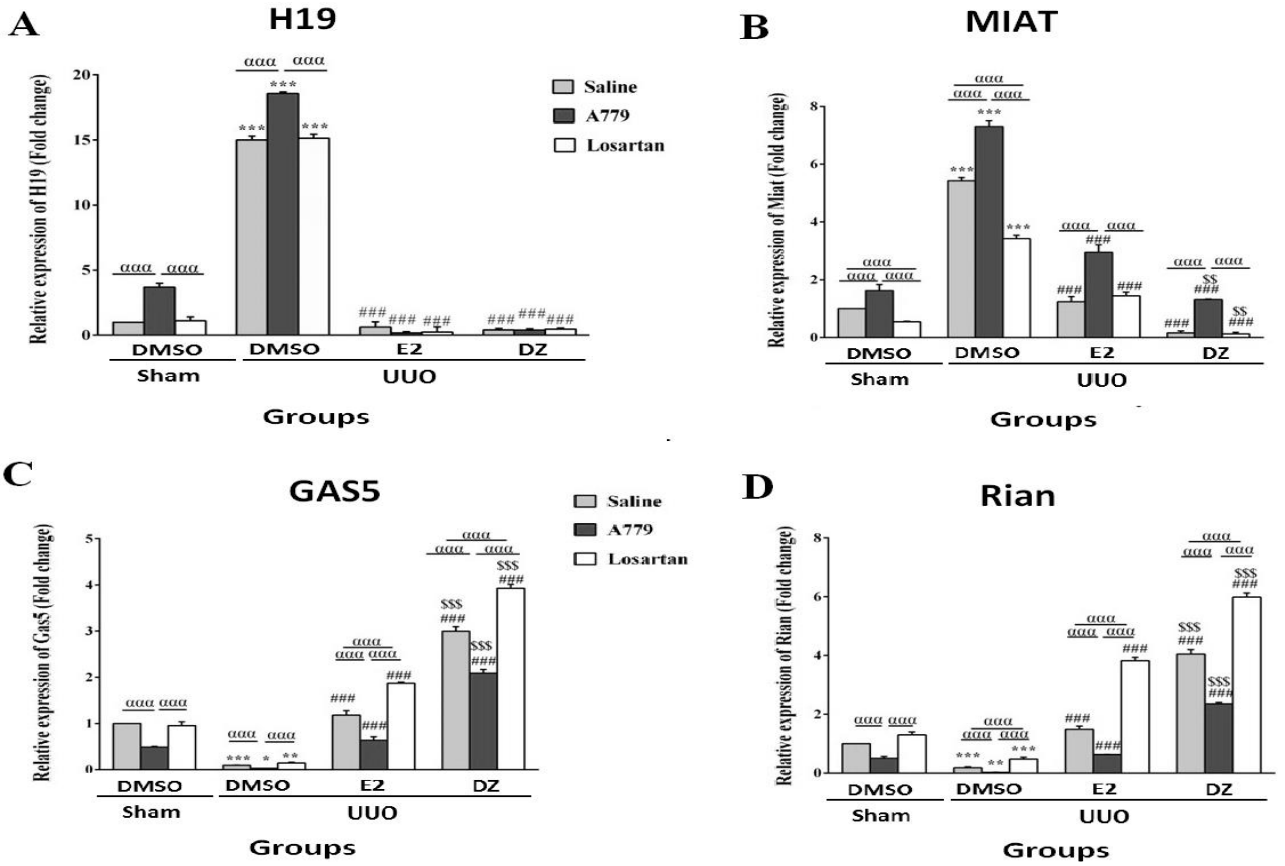



**Fig. 4:** Effect of daidzein on the relative expression of lncRNAs H19 (**A**) and MIAT (**B**), GAS5 (**C**), and Rian (**D**) in obstructed kidney tissue of sham and UUO rats. * P<0.05, ** P<0.01, *** P<0.001 vs. Sham+DMSO groups. ### P<0.001 vs. UUO+DMSO groups. $$ P<0.01, $$$ P<0.001 vs. UUO+E2 groups. ααα P<0.001 indicates a significant difference between subgroups. Mean±SEM (n=7). DMSO: Dimethyl sulfoxide, E2: 17β-Estradiol, DZ: Daidzein, and UUO: Unilateral ureteral obstruction

lncRNAs (Figs. 4C and D).

## Discussion

In the current study, we investigated whether daidzein's (or E2) positive effect on UUO-induced-renal injury in OVX rats can change the expression of UUO-related lncRNAs (H19, GAS5, MIAT, and Rian). In addition, the relationship between daidzein (or E2) treatment and the expression of lncRNAs was also investigated in the presence of two angiotensin receptor blockers losartan and A779. According to the results, daidzein treatment significantly decreased the expression of H19 and MIAT and increased the expression level of GAS5 and Rian that is beneficial against the pathogenesis of UUO. Moreover, except for H19, the effect of daidzein on the expression of lncRNAs promoted when cotreated with losartan. The daidzein had less (though significant) effect on the expression of MIAT, GAS5, and Rian when cotreated with A779. The decreased expression of H19 in response to daidzein was independent of losartan or A779 treatments.

Decreased level of estrogen after menopause is a strong risk factor for CKD progression, which may cause kidney failure at the end stages (Vellanki and Hou, 2018). To avoid the side effects of estrogen therapy, attempts to find a suitable replacement for estrogen have been considered. Herein, we showed that daidzein, as a natural compound resembling 17β-Estradiol, could improve histopathological damage and modulate the expression of UUO-related lncRNAs H19, MIAT, GAS5, and Rian similar to and even better than E2 in ovariectomized rats. The increased expression level of H19 and MIAT, and decreased levels of GAS5 and Rian have played an important role in the pathogenesis of renal injury in the obstructed kidney (Xie *et al*., 2016; Bijkerk *et al*., 2019; Yu *et al*., 2020). The use of antagonist and animal models of gene-deficient of lncRNAs reversed the expression of these lncRNAs. We observed a similar pattern of expression for these lncRNAs in UUO female rats. Our results showed that daidzein alone and co-treatment with A779 or losartan, attenuated kidney tissue damage, decreased levels of H19 and MIAT, and increased levels of GAS5 and Rian in OVX rats; therefore, it inhibited the harmful effects of A779 and co-administration with losartan potentiated its effects.

Considering the important role of RAS axes renal injury and chronic kidney disease (CKD), we examined the impact of daidzein in the presence of two RAS axes blockers, A779, and losartan, on lncRNAs. A779 blocks Ang (1-7) receptor and inhibits Ang (1-7)-MasR axis, which has anti-inflammation, anti-apoptosis, and anti-fibrotic impact on renal injury. In contrast, losartan is the

antagonist of type I angiotensin II receptor (AT1R) and inhibits Ang II-AT1R signaling, promoting renal injury (Ferrario, 2011; Wang *et al*., 2018). Didezine altered the expression of lncRNAs. Previously, Fan *et al*. (2019) showed that daidzein treatment upregulated the expression of lncRNA XLOC_098131 that subsequently regulated the Toll-like receptor signaling pathway in immune responses. Also, daidzein affected the expression of lncRNAs in lung cancer cells (Li *et al*., 2021). The regulatory effect of daidzein on the expression of lncRNAs has been considered.

In an experimental study, Su *et al*. (2018) showed that increased expression of H19 correlated with the activation of RAS signaling and promoted pulmonary artery hypertension (PAH). H19 affected RAS axes through the Let-7-AT1R axis by directly binding to Let-7 and preventing it from binding to its target AT1R. As result, AT1R increases in pulmonary arterial smooth muscle cells (PASMCs) and promotes PAH (Su *et al*., 2018). Moreover, Ang II significantly increased MIAT expression in a mouse model of cardiac hypertrophy and rat heart-derived H9c2 cells (Zhu *et al*., 2016). In contrast to H19 and MIAT, the expression of GAS5 and Rian in response to daidzein was different in the presence of RAS axes blockers. GAS5 and Rian exhibited higher expression levels when daidzein was co-treated with AT1R blocker losartan. Moreover, daidzein reverses the effect of A779. In adult rat cardiac fibroblasts, Ang II decreased lncRNA-NR024118 and Cdkn1c via AT1 receptor, while losartan almost completely reversed the down-expression of these lncRNAs (Jiang *et al*., 2015). According to the RNAInter, the binding of GAS5 to ACE2 was experimentally validated by qRT-PCR and RNA immunoprecipitation (Li *et al*., 2018). Li *et al*. (2018) confirmed a direct relation between GAS5 and ACE2 in acute respiratory distress syndrome (ARDS). They showed that decreased level of GAS5 in ARDS decreases ACE2 by increasing the expression level of miR-200c. Therefore, H19, GAS5, and Rain might relate to RAS axes, in addition to being involved in renal pathophysiology. However, more investigations are needed to clarify the detailed mechanism.

In conclusion, the beneficial effect of daidzein alone or in the presence of A779 and losartan on the renal injury was accompanied by changes in the expression of UUO-related lncRNAs (H19, GAS5, MIAT, and Rian). Phytoestrogen daidzein can be a possible treatment candidate in postmenopausal women suffering from kidney disease, however, further molecular mechanisms need to be investigated.

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## Conflict of interest

All of the authors declare that there are no conflicts of interest in this manuscript.

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