Active components from *Lagotis brachystachya* maintain uric acid homeostasis by inhibiting renal TLR4-NLRP3 signaling in hyperuricemic mice

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**Abstract**

*Lagotis brachystachya* Maxim is a herb widely used in traditional Tibetan medicine. Our previous study indicated that total extracts from *Lagotis brachystachya* could lower uric acid levels. This study aimed to further elucidate the active components (luteolin, luteoloside and apigenin) isolated from *Lagotis brachystachya* and the underlying mechanism in vitro and in vivo. The results showed that treatment with luteolin and luteoloside reversed the reduction of organic anion transporter 1 (OAT1) levels, while apigenin attenuated the elevation of urate transporter 1 (URAT1) and glucose transporter 9 (GLUT9) levels in uric acid-treated HK-2 cells, which was consistent with the finding in the kidneys of potassium oxonate (PO)-induced mice. On the other hand, hepatic xanthine oxidase activity was inhibited by the components. In addition, all of these active components improved the morphology of the kidney in hyperuricemic mice. Moreover, molecular docking showed that luteolin, luteoloside and apigenin could bind Toll-like receptor 4 (TLR4) and NLR family pyrin domain containing 3 (NLRP3). Congruently, western blot analysis showed that the components inhibited TLR4/myeloid differentiation primary response 88 (MyD88)/NLRP3 signaling. In conclusion, these results indicated that luteolin, luteoloside and apigenin could attenuate hyperuricemia by decreasing the production and increasing the excretion of uric acid, which were mediated by inhibiting inflammatory signaling pathways.

**Keywords** Luteolin · Luteoloside · Apigenin · Hyperuricemia · Uric acid transporter · Inflammation

**Introduction**

Hyperuricemia is a metabolic disease that causes increased production or decreased metabolism of uric acid (Shekelle et al. 2017). Abnormalities in either production or excretion lead to a high concentration of uric acid. If uric acid is persistently maintained at a high concentration, hyperuricemia leads to an acute course of gout. The transportation of uric acid in the kidney depends on the transport proteins in renal tubular epithelial cells. It is now clear that there are three transporters involved in the transport of urate in the proximal convoluted tubule: organic anion transporter (OAT1), urate anion transporter 1 (URAT1) and glucose transporter 9 (GLUT9) (Xu et al. 2017). OAT1 is a crucial transporter of the OAT family that is mainly responsible for the process of renal uric acid excretion (Azevedo et al. 2019). It takes up urate from blood and secretes it into tubular cells. In addition, uric acid is reabsorbed by URAT1 in the renal tubule apical membrane and then transported from the renal tubular lumen to renal tubular epithelial cells. During this process, blood uric acid levels are maintained. Clinically available drugs, such as benz bromarone and probenecid, are effective inhibitors of URAT1, which inhibit the process of reabsorption to promote uric acid excretion (Dong et al. 2019). Similar to the function of URAT1, GLUT9 is
also an important transporter in the reabsorption of urate in the proximal membrane (Zhang et al. 2016). Therefore, these three transport proteins are responsible for uric acid reabsorption and secretion in the kidney.

In addition to the function of transport proteins, inflammation is considered to be involved in the pathophysiology of hyperuricemia. High uric acid has been shown to activate the Toll-like receptor 4 (TLR4)/NLR family pyrin domain containing 3 (NLRC3) pathway and its related caspase-1 expression and the release of interleukin 1β (IL-1β) (Ma et al. 2020; Romero et al. 2017). Typically, TLR4 stimulates the activated nuclear factor kappa B (NF-κB) pathway to modulate inflammation-related genes and the secretion of the pro-inflammatory cytokines IL-1β, IL-6, and tumor necrosis factor-α (TNF-α). There was evidence that activated NF-κB phosphorylation was activated in both primary renal proximal tubule cells and mice induced by uric acid (De Nardo and Latz 2011; Wang et al. 2018). Meanwhile, NLRP3 is responsible for the process of pro-IL-1β to mature IL-1β.

*Lagotis brachystachya* Maxim is a traditional Tibetan medicine. It is primarily used to alleviate inflammation in local Tibet. Our previous study showed that the total ethanolic extract of *Lagotis brachystachya* could reduce serum uric acid levels in hyperuricemic mice, which might be induced by the active components from the flavonoid fraction (Xiong et al. 2018). Subsequently, three active components, luteolin, luteoloside and apigenin, were separated from the flavonoid fraction of *Lagotis brachystachya* (Zhu et al. 2019). The present study aimed to investigate whether these active components from *Lagotis brachystachya* could be protective against hyperuricemia in vitro and in vivo. In addition, the underlying mechanism of the hypouricemic effects of TLR4/NLRP3 was also assessed, as a previous study showed that flavonoids were expected to ameliorate the inflammatory symptoms associated with NLRP3 inflammasome activation (Lim et al. 2018).

**Materials and methods**

**Overview of the experimental design**

First, the hypouricemic effects of the active components isolated from *Lagotis brachystachya*, including luteolin, luteoloside and apigenin, were investigated in vitro and in vivo. Then, the potential targets of the active components were evaluated by molecular docking. Later, the inflammation-related mechanism underlying the hypouricemic effects was investigated in mice. The animal experiments complied with the ARRIVE guidelines and were approved by the Jiangxi University of Chinese Medicine (No. JZLLSC2019-0221 on 2019/02/28). All procedures were performed following the published guidelines of the China Council on Animal Care.

**Animals**

Six-week-old male Kunming mice (22 ± 2 g) were purchased from the Animal Center of Jiangxi University of Chinese Medicine, PR China. Animals were housed five per cage (320 × 180 × 160 cm) under a normal 12-h/12-h light/dark schedule (lights on at 07:00 a.m.) during the experiments. The animals were allowed one week to adapt before the beginning of the experiments. Ambient temperature and relative humidity were maintained at 22 ± 2 °C and 55 ± 5%. Animals have free access to food and water.

**Reagents**

Uric acid, benzbrromarone, allopurinol and potassium oxonate (PO) were purchased from Sigma (St. Louis, USA). ELISA kits for IL-1β and TNF-α were purchased from Xinbosheng (Shenzhen, China). Anti-GLUT9, anti-TLR4 and anti-NLPR3 antibodies were purchased from Bioss (Beijing, China). Anti-OAT1, anti-β-actin, anti-URAT1, anti-MyD88 and anti-IL-1β antibodies were purchased from Proteintech (Chicago, USA). Xanthine oxidase, urea nitrogen and adenosine deaminase kits were purchased from Jiancheng (Nanjing, China).

**Isolation of luteolin, luteoloside and apigenin from *Lagotis brachystachya***

*Lagotis brachystachya* Maxim, which was collected from the Sichuan Province of China in 2015, was identified by Professor Guo-Yue Zhong (Jiangxi University of Chinese Medicine). A voucher specimen (No. 01-03-23-15) was deposited at the research center. The dried *Lagotis brachystachya* (10 kg for each experiment) underwent extraction using 5 times 70% ethanol at 60 °C for 2 h. The process was repeated twice, and the total extracts were concentrated under reduced pressure. Then, the ethanol extract was passed over a porous polymer gel D101 column (20 × 150 cm). After washing with H2O, the extracts were eluted with a stepwise gradient of MeOH-H2O (3:7, 6:4, 1:0). The MeOH-H2O (3:7) eluate was subsequently chromatographed on a silica gel column (9 × 60 cm, 200–300 mesh), eluted with CH2Cl2-CH3OH (100: 2, 100: 4, 100: 6, 100:8), eluted by an ODS column chromatography gradient and repeatedly recrystallized to obtain the active compounds. 1H and 13C NMR spectroscopic analyses were performed to confirm the structures of active compounds, such as luteolin, luteoloside and apigenin (Fig. 1)—luteolin (Yellow powder, yield 0.0012%): 1H-NMR (600 MHz, CD3OD): δ: 12.49 (1H, s), 7.36 (1H, d, J = 2.0 Hz, H-2'), 7.37 (1H, J = 2.0, 8.5 Hz, J = 2.0 Hz, H-1'), 7.36 (1H, d, J = 8.5 Hz, H-3'), 6.96 (1H, d, J = 8.5 Hz, H-5), 6.88 (1H, d, J = 2.0 Hz, H-6), 6.65 (1H, dt, J = 8.5, 2.0 Hz, H-8), 6.79 (1H, dt, J = 8.5, 2.0 Hz, H-7), 3.44 (3H, s, OCH3), 3.94 (3H, s, OCH3), 3.39 (3H, s, OCH3), 3.24 (3H, s, OCH3).
Blank experiment. Third-generation HK-2 cells were randomly divided into a control group, a positive drug group (25 μmol/L benzbromarone), two luteolin groups (3.125 and 6.25 μmol/L), two luteoloside groups (3.125 and 6.25 μmol/L) and two apigenin groups (3.125 and 6.25 μmol/L).

Intervention experiment. Third-generation HK-2 cells were randomly divided into a control group, a uric acid group, a positive drug group (25 μmol/L benzbromarone), two luteolin groups (3.125, 6.25 μmol/L), two luteoloside groups (3.125, 6.25 μmol/L) and two apigenin groups (3.125, 6.25 μmol/L). All groups except the control group were treated with 400 μmol/L uric acid.

All the groups were treated and incubated for 48 h, followed by the collection of the cultures. Then, the levels of OAT1, URAT1 and GLUT9 were assessed by western blot.

**Drug treatment in vivo**

A total of 90 mice were randomly divided into 9 groups as follows: control-vehicle group, PO-vehicle group, PO-allopurinol group (10 mg/kg), PO-luteolin groups (20, 50 mg/kg), PO-luteoloside (20, 50 mg/kg), and PO-apigenin (20, 50 mg/kg). Drugs or vehicle were orally continuously administered once a day for 7 days. On the seventh day, PO (350 mg/kg) was intraperitoneally injected 1 h prior to drug administration. One hour after the last drug administration, blood was collected to obtain the serum. The liver and kidney on one side of the mouse were dissected in liquid nitrogen and stored later. The kidney from the other side of the mouse was fixed in 4% paraformaldehyde solution. Then, the kidney from one side of the mouse was cut into slices in liquid nitrogen and stored later. The kidney from the other side of the mouse was fixed in 4% paraformaldehyde solution. Then, the kidney from the other side of the mouse was cut into slices in liquid nitrogen and stored later.

The 3rd generation of HK-2 cells was incubated in a 96-well plate (5.5 x 10^3/mL for each well). After culturing for 24 h, medium containing different concentrations of reagents was added as follows: uric acid (50, 100, 200, 400, 800 μM/L), benzbromarone (25, 50, 100 μM/L), luteolin (3.125, 6.25, 12.5, 25, 50, 100 μM/L), luteoloside (3.125, 6.25, 12.5, 25, 50, 100 μM/L) and apigenin (3.125, 6.25, 12.5, 25, 50, 100 μM/L). The medium was removed 48 h later, and TCA was added. Then, the plate was transferred to a 4 °C refrigerator for 1 h, followed by the addition of SRB dye. Subsequently, the cells were rinsed with acetic acid. Finally, Tris-base lye was added, and the plate was measured with a microplate reader at 515 nm.

Measurement of HK-2 cell viability

The 3rd generation of HK-2 cells was incubated in a 96-well plate (5.5 x 10^3/mL for each well). After culturing for 24 h, medium containing different concentrations of reagents was added as follows: uric acid (50, 100, 200, 400, 800 μM/L), benzbromarone (25, 50, 100 μM/L), luteolin (3.125, 6.25, 12.5, 25, 50, 100 μM/L), luteoloside (3.125, 6.25, 12.5, 25, 50, 100 μM/L) and apigenin (3.125, 6.25, 12.5, 25, 50, 100 μM/L). The medium was removed 48 h later, and TCA was added. Then, the plate was transferred to a 4 °C refrigerator for 1 h, followed by the addition of SRB dye. Subsequently, the cells were rinsed with acetic acid. Finally, Tris-base lye was added, and the plate was measured with a microplate reader at 515 nm.
activity (Li et al. 2019; Luan et al. 2016; Nishitani et al. 2013).

**Biochemical and ELISA analysis**

Serum uric acid, urea nitrogen, adenosine deaminase and hepatic xanthine oxidase levels of the mice were measured based on the kit manufacturer’s instructions. Serum samples were used to measure the protein levels of IL-1β and TNF-α according to the instructions of the ELISA kits.

**Western blot**

The samples from cells and kidneys were first homogenized with lysis buffer. The homogenates were centrifuged at 12,000 × g at 4 °C for 15 min. Then, the supernatant was collected and used for protein determination by the bicinchoninic acid (BCA) method. Equal amounts of protein (50 μg) were loaded and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane. Later, the PVDF membrane was incubated with 5% BSA at room temperature for 2 h followed by incubation with primary antibody at 4 °C overnight (OAT1, 1: 2000; URAT1, 1:2000; GLUT9, 1:5000; TLR4, 1: 2000; NLRP3, 1: 2000; MyD88, 1: 2000; IL-1β, 1: 2000; β-actin, 1:4000). On the second day, the membrane was incubated with a secondary antibody (1:2000) at room temperature for 1 h. Finally, the membrane was exposed to enhanced chemiluminescence (ECL) solution in a gel image analyzer. ImageJ was used to analyze the gray value of the bands.

**Molecular docking**

The crystal structures of the TLR4-MD2 complex and NLRP3 were derived from the RCSB Protein Data Bank (http://www.rcsb.org/). The TLR4 complex (PDB code, 3FXI), NLRP3 (PDB code, 6NPY), luteolin, luteoloside and apigenin were processed by PyMOL and then calculated by AutoDock Vina. LigPlus was used for interaction analysis.

**Histopathological examination**

Histopathological examination was performed to detect inflammation in the kidney. The kidney was first cut into pieces after fixation in 4% paraformaldehyde. The cut tissue was rinsed with water for 2 h followed by dehydration with 50, 70, 80, 90% and absolute ethanol. Then, the tissue was dehydrated with xylene and paraffin. After embedding in paraffin, 4 μm slices were cut and collected. The slices were dewaxed with xylene, ethanol and distilled water. Subsequently, the slices were placed in hematoxylin staining solution for 8 min and eosin staining solution for 30 s. Finally, the slices were sealed and observed under a microscope. The histological examination was assessed by an observer blind to treatment.

**Statistical analyses**

The data were expressed as means ± SDs. The normal distribution of the data was first verified using the Kolmogorov–Smirnov test. Then, the data were analyzed by one-way ANOVA followed by Tukey’s post hoc test for multiple comparisons between any two treatment groups. \( P < 0.05 \) was considered a significant difference.

**Results**

**The effects of uric acid and active components on HK-2 cell viability**

As shown in Fig. 2A, there was a negative correlation between uric acid concentration and HK-2 cell viability: as the uric acid concentration increased, HK-2 cell viability decreased \((p < 0.01)\). Uric acid decreased cell viability in a concentration-dependent manner ranging from 100 to 800 μmol/L. When uric acid reached 400 μmol/L, HK-2 cell viability declined to approximately 50% \((p < 0.01)\) according to Tukey’s post hoc test. Therefore, 400 μmol/L uric acid was used to induce cell damage by hyperuricemia in HK-2 cells in the following experiment. Compared with the control group, the post hoc test indicated that benzbromarone at 25 μmol/L and active components (luteolin, luteoloside and apigenin) at 3.125 and 6.25 μmol/L did not affect HK-2 cell viability. In this respect, 25 μmol/L benzbromarone, as well as 3.125 and 6.25 μmol/L active components, was used for the following experiment.

On the other hand, the effects of uric acid (Fig. 2B, ranging from 50 to 800 μmol/L), luteolin (Fig. 2C, ranging from 6.25 to 100 μmol/L), luteoloside and apigenin were processed by PyMOL and then calculated by AutoDock Vina. In this respect, 25 μmol/L benzbromarone, as well as 3.125 and 6.25 μmol/L active components, was used for the following experiment.

**Effects of active components on the levels of OAT1, URAT1 and GLUT9 in normal HK-2 cells**

The effects of luteolin, luteoloside and apigenin on OAT1 (Fig. 3A), URAT1 (Fig. 3B) and GLUT9 (Fig. 3C) levels in normal HK-2 cells were measured first. One-way ANOVA indicated that the effect of treatment on OAT1 \((p < 0.01)\), URAT1 \((p < 0.01)\) or GLUT9 \((p < 0.01)\) levels was significant. Compared with the vehicle group, the post hoc test showed that benzbromarone decreased URAT1 levels \((p < 0.01)\) and increased OAT1 levels \((p < 0.01)\). In addition,
Fig. 2 The effects of uric acid, benzbromarone, luteolin, luteoloside and apigenin on the viability of HK-2 cells. 

**p < 0.01 versus the vehicle group

Fig. 3 The effects of luteolin, luteoloside and apigenin on OAT1 (A), URAT1 (B) and GLUT9 (C) in normal HK-2 cells. *p < 0.05 and **p < 0.01 versus the vehicle group
both luteolin (3.125 μmol/L: \( p < 0.05 \); 6.25 μmol/L: \( p < 0.05 \)) and luteoloside (3.125 μmol/L: \( p < 0.01 \); 6.25 μmol/L: \( p < 0.01 \)) but not apigenin treatment increased OAT1 levels in normal HK-2 cells.

On the other hand, apigenin did not increase OAT1 levels in normal HK-2 cells (Fig. 3A). There was a significant difference between apigenin and benz bromarone/luteoloside in OAT1 levels.

For URAT1 levels, none of the active components decreased URAT1 levels in normal HK-2 cells (Fig. 3B). There was a significant difference between luteolin/luteoloside/apigenin and benz bromarone in URAT1 levels.

Neither benz bromarone nor active components changed GLUT9 levels in normal HK-2 cells (Fig. 3C). There was a significant difference between luteolin/luteoloside/apigenin and benz bromarone in URAT1 levels.

**Effects of active components on the levels of OAT1, URAT1 and GLUT9 in HK-2 cells induced by uric acid**

Next, the effects of luteolin, luteoloside and apigenin on OAT1 (Fig. 4A), URAT1 (Fig. 4B) and GLUT9 (Fig. 4C) levels in uric acid-induced HK-2 cells were evaluated. There was a significant treatment effect on GLUT9 (\( p < 0.01 \)), URAT1 (\( p < 0.01 \)) and OAT1 (\( p < 0.01 \)) levels according to one-way ANOVA. Tukey’s test indicated that uric acid significantly increased GLUT9 (\( p < 0.01 \)) and URAT1 (\( p < 0.01 \)) levels but decreased OAT1 (\( p < 0.01 \)) levels in HK-2 cells. Compared with the uric acid-vehicle group, apigenin decreased the levels of GLUT9 (3.125 μmol/L: \( p < 0.01 \); 6.25 μmol/L: \( p < 0.05 \)) and URAT1 (3.125 μmol/L: \( p < 0.05 \); 6.25 μmol/L: \( p < 0.01 \)) while luteolin (3.125 μmol/L: \( p < 0.01 \); 6.25 μmol/L: \( p < 0.01 \)) and luteoloside (3.125 μmol/L: \( p < 0.01 \); 6.25 μmol/L: \( p < 0.01 \)) increased OAT1 levels. In addition, benz bromarone not only reduced GLUT9 and URAT1 levels but also elevated OAT1 levels.

On the other hand, apigenin did not reverse the reduction of OAT1 by uric acid (Fig. 4A). There was a significant difference between apigenin and control-vehicle/luteolin/luteoloside in OAT1 levels.

For URAT1 and GLUT9 levels, luteolin and luteoloside did not reverse the reduction of URAT1 (Fig. 4B) and GLUT9 (Fig. 4C) by uric acid. There was a significant difference between luteolin/luteoloside and control-vehicle/benz bromarone/apigenin in URAT1 and GLUT9 levels.

**Fig. 4** The effects of luteolin, luteoloside and apigenin on OAT1 (A), URAT1 (B) and GLUT9 (C) in uric acid-induced HK-2 cells. \( **p < 0.01 \) versus the control-vehicle group. \(* p < 0.05 \) and \( **p < 0.01 \) versus the uric acid-vehicle group.

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Effects of active components on serum uric acid, urea nitrogen, adenosine deaminase and hepatic xanthine oxidase levels in gouty mice

As shown in Table 1, one-way ANOVA indicated a significant treatment effect on serum uric acid ($p < 0.01$) and urea nitrogen ($p < 0.01$) levels. Post hoc tests showed that PO significantly increased serum uric acid ($p < 0.01$), urea nitrogen ($p < 0.01$) and adenosine deaminase ($p < 0.05$) levels in gouty mice compared with the vehicle control group. Compared with vehicle, luteolin, luteoloside and apigenin significantly reversed serum uric acid and urea nitrogen concentrations. In addition, luteolin and apigenin attenuated the increase in serum adenosine deaminase levels. On the other hand, there was no significant difference among the active components in serum uric acid and adenosine deaminase levels. However, compared with the vehicle control group, apigenin dramatically decreased serum uric acid nitrogen levels.

As shown in Table 2, one-way ANOVA showed a significant treatment effect on hepatic xanthine oxidase levels ($p < 0.01$). Post hoc tests showed that PO ($p < 0.05$) significantly increased xanthine oxidase levels in the liver, while luteolin, luteoloside and apigenin administration reversed the elevation. On the other hand, there was no significant difference among the active components in xanthine oxidase levels.

Effects of active components on renal transporter levels in gouty mice

According to one-way ANOVA, there was a significant effect of treatment on OAT1 ($p < 0.01$, Fig. 5A), URAT1 ($p < 0.01$, Fig. 5B) and GLUT9 ($p < 0.01$, Fig. 5C) levels in the kidney. Post hoc tests showed that PO significantly increased the levels of GLUT9 ($p < 0.01$) and URAT1 ($p < 0.01$) but decreased the levels of OAT1 ($p < 0.01$). Both luteolin (20 mg/kg; $p < 0.01$; 50 mg/kg; $p < 0.01$) and luteoloside (20 mg/kg; $p < 0.01$; 50 mg/kg; $p < 0.01$) pre-treatment prevented the reduction in OAT1 levels. Only apigenin prevented the elevation of GLUT9 (20 mg/kg; $p < 0.01$; 50 mg/kg; $p < 0.01$) and URAT1 (20 mg/kg; $p < 0.01$; 50 mg/kg; $p < 0.01$) in the kidney.

On the other hand, apigenin did not change the OAT1 levels compared with the PO-vehicle group (Fig. 5A). There was a significant difference between apigenin and control-vehicle/allopurinol/luteolin/luteoloside (20 mg/kg) in OAT1 levels.

At the URAT1 and GLUT9 levels, luteolin and luteoloside did not reverse the reduction in URAT1 and GLUT9 induced by PO (Fig. 5B, C). There was a significant difference between luteolin/luteoloside and control-vehicle/allopurinol/apigenin in URAT1 and GLUT9 levels.

### Table 1
Effects of drugs in different groups on serum uric acid, BUN and ADA levels in hyperuricemia mice (Mean ± SD, $n = 10$)

| Group            | Dose mg/kg | Uric acid (μmol/L) | Urea nitrogen (mmol/L) | Adenosine deaminase (U/L) |
|------------------|------------|--------------------|------------------------|--------------------------|
| Control-vehicle  | –          | 16.73 ± 2.76       | 4.66 ± 0.82            | 2.18 ± 0.56              |
| PO-vehicle       | –          | 79.77 ± 10.70**    | 7.12 ± 0.96**          | 3.48 ± 0.80*             |
| PO-ralpurinol    | 10         | 14.89 ± 3.39**     | 7.00 ± 0.73            | 2.87 ± 1.32              |
| PO-luteolin      | 20         | 30.09 ± 9.97**     | 5.50 ± 0.75**          | 2.21 ± 0.43*             |
| PO-luteolin      | 50         | 20.36 ± 6.31**     | 3.00 ± 0.84**          | 2.46 ± 0.95*             |
| PO-luteoloside   | 20         | 29.01 ± 11.61**    | 4.95 ± 0.76**          | 2.53 ± 0.94              |
| PO-luteoloside   | 50         | 21.25 ± 4.42**     | 5.01 ± 0.51**          | 2.85 ± 0.65              |
| PO-apigenin      | 20         | 15.52 ± 2.11**     | 2.43 ± 0.26**          | 2.49 ± 0.53*             |
| PO-apigenin      | 50         | 17.24 ± 2.85**     | 2.50 ± 0.29**          | 2.45 ± 0.73*             |

* $p < 0.05$, ** $p < 0.01$ versus control-vehicle group; * $p < 0.05$, ** $p < 0.01$ versus PO-vehicle group
Interaction between active components and TLR4/NLRP3 by molecular docking

Molecular docking simulation by AutoDock Vina demonstrated that the active components were in the pocket of the TLR4-MD2 complex (luteolin, Fig. 6A–C; luteoloside, Fig. 6D–F; apigenin, Fig. 6G–I) and NLRP3 protein (luteolin, Fig. 7A–C; luteoloside, Fig. 7D–F; apigenin, Fig. 7G–I). The simulation showed that luteolin, luteoloside and apigenin interact with TLR4/MD-2 and NLRP3 by hydrophobic and hydrogen bonding interactions. The binding energies between luteolin/luteoloside/apigenin and the proteins are shown in Table 3, indicating the putative inhibitory activity of the active components on inflammation.

Effects of active components on renal TLR4-MyD88-NLRP3-IL-1β in gouty mice

As shown in Fig. 8, one-way ANOVA showed a significant effect of treatment on TLR4 (p < 0.01), MyD88 (p < 0.01), NLRP3 (p < 0.01) and IL-1β (p < 0.01) levels. Post hoc tests showed that PO significantly increased the levels of TLR4 (p < 0.01), MyD88 (p < 0.01), NLRP3 (p < 0.01) and IL-1β (p < 0.01). Luteolin pretreatment inhibited the increase in TLR4 (20 mg/kg: p < 0.01; 50 mg/kg: p < 0.01), MyD88 (20 mg/kg: p < 0.01; 50 mg/kg: p < 0.01), NLRP3 (20 mg/kg: p < 0.01; 50 mg/kg: p > 0.05) and IL-1β (20 mg/kg: p < 0.01; 50 mg/kg: p < 0.01) in the kidney. Luteoloside inhibited the increase in NLRP3 (20 mg/kg: p < 0.01; 50 mg/kg: p < 0.01) and IL-1β (20 mg/kg: p < 0.01; 50 mg/kg: p < 0.01) in the kidney. In addition, apigenin inhibited the increase in TLR4 (20 mg/kg: p < 0.01; 50 mg/kg: p < 0.01), MyD88 (20 mg/kg: p < 0.05; 50 mg/kg: p < 0.01), NLRP3 (20 mg/kg: p < 0.01; 50 mg/kg: p < 0.01; 50 mg/kg: p < 0.01) and IL-1β (20 mg/kg: p < 0.01; 50 mg/kg: p < 0.01) in the kidney. On the other hand, luteoloside did not reverse the increase in TLR4/MyD88 induced by PO (Fig. 8A, B). There was a significant difference between luteoloside and control-vehicle/allopurinol/luteolin/apigenin in TLR4/MyD88 levels. In addition, luteoloside at 20 mg/kg did not inhibit IL-1β levels (Fig. 8D), and there was a significant difference between luteoloside (20 mg/kg) and control vehicle/allopurinol/luteolin/apigenin in IL-1β levels.

One-way ANOVA showed a significant treatment effect on IL-1β (p < 0.05) or TNF-α (p < 0.05) levels among the groups (Table 4). The post hoc test indicated that PO induced an increase in IL-1β and TNF-α in the serum. In contrast, all three active components decreased IL-1β and TNF-α levels in hyperuricemic mice. In addition, there was
Active components from *Lagotis brachystachya* maintain uric acid homeostasis by inhibiting...

no significant difference among the components in IL-1β and TNF-α levels.

**Effects of active components on kidney morphology in gouty mice**

As shown in Fig. 9, the structures of glomerular and renal tubular epithelial cells in the vehicle-treated control animals were clear. Congruently, there was no apparent inflammatory reaction in the kidneys of normal animals. In gouty mice induced by PO, necrosis of renal tubular epithelial cells was observed. Proliferation and inflammation were observed in the surrounding interstitium. Dilation and calcification occurred in renal tubules. Pretreatment with allopurinol prevented the inflammatory response, attenuated the dilation of renal tubules, and improved renal tubular epithelial cell structure. Luteolin at 20 mg/kg slightly decreased renal tubular necrosis and interstitial proliferation, while there was only slight dilation of renal tubules and inflammatory cell accumulation after administration of luteolin at 50 mg/kg. Inflammatory cell accumulation, renal tubular necrosis and interstitial proliferation were still observed by pretreatment with luteoloside at 20 mg/kg but ameliorated by pretreatment with luteoloside at 50 mg/kg. Apigenin at 20 mg/kg did not improve the accumulation of inflammatory cells, necrosis of renal tubules, or interstitial hyperplasia, while apigenin at 50 mg/kg reduced the accumulation of inflammatory cells and weakened the dilation of the renal tubule.

**Fig. 6** Molecular docking analysis of luteolin, luteoloside and apigenin interacting with TLR4/MD-2. A, D and G show that luteolin, luteoloside and apigenin insert into the TLR4/MD-2 complex. B, E and H show enlargement of the interacting structures of TLR4 and ligands. C, F, I show the detailed interaction sites between TLR4 and ligands.
Discussion

First, the present study found that the three active components from *Lagotis brachystachya* were protective against hyperuricemia (Table 1). Previously, we found that extracts from *Lagotis brachystachya* produced antihyperuricemic effects in mice and confirmed the active fractions of *Lagotis brachystachya* (Xiong et al. 2018). Then, we separated three potential components (luteolin, luteoloside and apigenin) from the active fraction (Zhu et al. 2019). Increasing evidence shows that elevated blood uric acid is an independent risk factor for kidney disease and plays an important role in the occurrence and development of kidney disease (Kanbay et al. 2017; Tsai et al. 2017). The dysfunction of uric acid homeostasis is one of the essential characteristics of hyperuricemia. In this context, the results confirmed the potential efficacy of luteolin, luteoloside and apigenin in hyperuricemia and gout treatment.

In addition, the present results showed that all three components significantly decreased hepatic xanthine oxidase activity in PO-induced hyperuricemic mice (Table 2). These results were partly consistent with a previous study showing that luteolin interacted with the primary amino acid residues located within the active site pocket of xanthine oxidase by molecular docking and biochemical analysis (Lin et al. 2018).

![Fig. 7 Molecular docking analysis of luteolin, luteoloside and apigenin interacting with NLRP3.](image)

**Fig. 7** Molecular docking analysis of luteolin, luteoloside and apigenin interacting with NLRP3. **A, D and G** show that luteolin, luteoloside and apigenin insert into NLRP3. **B, E and H** show the interacting structures of NLRP3 and ligands. **C, F, I** show the detailed interaction sites between TLR4 and ligands.

| Group          | TLR4 | NLRP3 |
|---------------|-----|-------|
| Luteolin      | −8.2| −8.4  |
| Luteoloside   | −8.8| −9.2  |
| Apigenin      | −7.9| −8.6  |
Active components from *Lagotis brachystachya* maintain uric acid homeostasis by inhibiting...

2014; Yan et al. 2013). Xanthine oxidase is the rate-limiting enzyme in the metabolic pathway of purine nucleosides. It is the final link in regulating uric acid production and plays a dominant role in the pathogenesis of hyperuricemia (Serrano et al. 2020). Therefore, the results indicated that the active components could alleviate the synthesis and release of uric acid.

On the other hand, urate transporters regulate the excretion of uric acid in the body. To elucidate the involvement of transporters in the effects of the active components, OAT1, URAT1, and GLUT9 levels were measured. In an in vitro experiment, only luteolin and luteoloside affected OAT1 levels but not URAT1 and GLUT9 levels in normal HK-2 cells (Fig. 3). When the cells were pretreated with uric acid, luteolin and luteoloside still increased OAT1 levels in

![Fig. 8](image_url)

**Fig. 8** The effects of luteolin, luteoloside and apigenin on TLR4 (A), MyD88 (B), NLRP3 (C) and IL-1β (D) in PO-induced hyperuricemic mice. 

| Group          | Dose mg/kg | IL-1β (pg/ml) | TNF-α (pg/ml) |
|----------------|------------|---------------|---------------|
| Control-vehicle| –          | 54.38 ± 2.66  | 51.21 ± 3.46  |
| PO-vehicle     | –          | 58.03 ± 3.26  | 80.77 ± 8.50  |
| PO-Allopurinol | 10         | 50.71 ± 1.84  | 48.89 ± 8.30  |
| PO-Luteolin    | 20         | 49.50 ± 2.82  | 51.22 ± 7.72  |
| PO-Luteolin    | 50         | 51.30 ± 0.50  | 51.94 ± 10.98 |
| PO-Luteoloside | 20         | 50.13 ± 3.25  | 63.06 ± 10.71 |
| PO-Luteoloside | 50         | 53.46 ± 2.03  | 61.69 ± 14.70 |
| PO-Apigenin    | 20         | 52.82 ± 4.03  | 77.01 ± 14.19 |
| PO-Apigenin    | 50         | 54.71 ± 1.65  | 68.33 ± 10.73 |

*p < 0.05; **p < 0.01 versus Control-vehicle group; *p < 0.05, **p < 0.01 versus PO-vehicle group

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Table 4: Effects of drugs in each group on IL-1β and TNF-α in PO-induced hyperuricemia mice (Mean ± SD, n = 10)
uric acid-treated HK-2 cells, while apigenin decreased the URAT1 and GLUT9 levels (Fig. 4). In an in vivo study, PO caused a reduction in OAT1 but an elevation in URAT1 and GLUT9 in the kidneys of mice. However, the three components exerted their action on the transporters differently. In detail, luteolin and luteoloside significantly increased OAT1 levels, while apigenin decreased URAT1 and GLUT9 levels in PO-induced mice (Fig. 5). The imbalance of the renal uric acid transport system is one of the leading causes of hyperuricemia (Pavelcova et al. 2020). As a membrane protein, OAT1 initially regulates renal uric acid excretion (Otani et al. 2017). URAT1, which is expressed in the proximal tubule epithelial cell brush border, is responsible for urate reabsorption. Organic anions that accumulate in renal tubular epithelial cells have a high affinity for URAT1 (Anzai and Endou 2011). The intracellular anions exchange with urate in the tubule lumen, leading to increased uric acid reabsorption. In addition to URAT1, GLUT9, which influences the renal handling of uric acid and modulates serum urate levels, responds to treatment in patients with uric acid overproduction (Torres and Puig 2018). Therefore, changes in the levels of OAT1, URAT1 and GLUT9 reflect the degree of hyperuricemia development. These results explain the excellent therapeutic efficacy of Lagotis brachystachya, as the herb has different components exerting dual antihyperuricemic actions. The active components may inhibit the production of uric acid by inhibiting hepatic xanthine oxidase and promoting renal urate excretion by regulating renal urate transporters in hyperuricemia. Thus, the presence of luteolin,
luteoloside and apigenin in *Lagotis brachystachya* could be responsible for its antihyperuricemic activity.

The potential anti-inflammatory activity of the active components was then evaluated by molecular docking as accumulating evidence demonstrating the correlation between inflammation and hyperuricemia (Amezcua-Castillo et al. 2020; Rahmi et al. 2020; Su et al. 2020). The docking results showed that all three components could enter the TLR4 and NLRP3 pocket (Figs. 6, 7) and thus obtain a high affinity for these proteins (Table 3). These observations suggested that luteolin, luteoloside and apigenin could block the activation of an inflammatory response, which could be involved in the treatment of hyperuricemia. Studies have shown that excessive urate can activate TLR4 and NLRP3 signals in hyperuricemia (So 2007a). After binding to the CD14 receptor, urate is recognized by TLR4 to form a TLR4/MD2/CD14 complex (So 2007b). Activated TLR4 transduces the signal into the cell through the intracytoplasmic domain and subsequently activates NF-κB, which initiates gene expression related to inflammation.

Subsequently, the inflammation-related mechanism was evaluated in vivo. In line with a previous study (Guo et al. 2020), PO caused the elevation of TLR4 and NLRP3 levels in the kidney. In contrast, treatment with luteolin, luteoloside or apigenin inhibited renal TLR4/MyD88/NLRP3/IL-1β levels (Fig. 8) and serum pro-inflammatory cytokine levels in PO-induced hyperuricemic mice (Table 4), indicating that the inflammation induced by hyperuricemia was effectively normalized. This finding was in line with the histopathological examination showing that these three active components reversed the accumulation of inflammatory cells (Fig. 9).

Similarly, a recent study also showed that luteolin down-regulated the TLR4/MyD88 pathway in monosodium urate-induced gouty arthritis rats (Shen et al. 2020). In addition, several studies reported the inhibitory activity of luteoloside and apigenin on the NLRP3 inflammasome in vitro (Fan et al. 2014; Yamagata et al. 2019). These observations suggested that the anti-inflammatory activity of luteolin, luteoloside and apigenin may be mediated by targeting TLR4/NLRP3.

**Conclusion**

This study demonstrated that luteolin, luteoloside and apigenin, the three active components extracted from *Lagotis brachystachya*, possessed regulatory activity of transporters against high uric acid levels in vitro. An in vivo study on PO-induced hyperuricemic mice showed that luteolin, luteoloside and apigenin exerted dual regulatory roles in xanthine oxidase activity and transporters. Moreover, the active components showed anti-inflammatory activity by targeting the TLR4/MyD88/NLRP3/IL-1β signaling pathway in the kidney. Therefore, the present study supports luteolin, luteoloside and apigenin as potential candidates for hyperuricemia treatment.

**Author’s contributions** J.Z., H.C. and L.Y. conceived of the project. J.Z., H.Y., W.H. and J.C. performed the experiments. J.Z., and Y.L. analyzed data. L.Y. did molecular docking. J.Z., H.C. and L.Y. wrote and revised the manuscript. All authors read and approved the submission.

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**Data availability** Data will be made available on reasonable request.

**Declarations**

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical approval** All animal procedure was in accordance with guidelines from China Council on Animal Care and was approved by the Animal Commission of Jiangxi University of Chinese Medicine (No. JZLSC2019-0221 on 2019/02/28).

**References**

Amezcua-Castillo LM, Juarez-Vicuna Y, Marquez-Velasco R, Amezcua-Guerra LM (2020) Activation status of NLRP3 inflammasome in peripheral blood mononuclear cells from patients with Gout flare. J Clin Rheumatol 26:5208–5212

Anzai N, Endou H (2011) Urate transporters: an evolving field. Semin Nephrol 31:400–409

Azevedo VF, Kos IA, Vargas-Santos AB, da Rocha Castelar PG, Dos Santos Paiva E (2019) Benzembramore in the treatment of gout. Adv Rheumatol 59:37

De Nardo D, Latz E (2011) NLRP3 inflammasomes link inflammation and metabolic disease. Trends Immunol 32:373–379

Dong Y, Zhao T, Ai W, Zalloum WA, Kang D, Wu T, Liu X, Zhan P (2019) Novel urate transporter 1 (URAT1) inhibitors: a review of recent patent literature (2016–2019). Expert Opin Ther Pat 29:871–879

Fan SH, Wang YY, Lu J, Zheng YL, Wu DM, Li MQ, Hu B, Zhang ZF, Cheng W, Shan Q (2014) Luteoloside suppresses proliferation and metastasis of hepatocellular carcinoma cells by inhibition of NLRP3 inflammasome. PLoS ONE 9:e89961

Guo LF, Chen X, Lei SS, Li B, Zhang NY, Ge HZ, Yang K, Lv GY, Chen SH (2020) Effects and mechanisms of Dendrobium officinalis six Nostrum for treatment of Hyperuricemia with Hyperlipidemia. Evid-Based Complement Alternate Med 2020:2914019

Kanbay M, Solak Y, Afsar B, Nistor I, Aslan G, Caglayan OH, Aylanat A, Donciu MD, Lanaspa MA, Ejar AA, Johnson RJ, Covic A (2017) Serum uric acid and risk for acute kidney injury following contrast. Angiology 68:132–144

Li Q, Tian Z, Wang M, Kou J, Wang C, Rong X, Li J, Xie X, Pang X (2019) Luteoloside attenuates neuroinflammation in focal cerebral
ischemia in rats via regulation of the PPARgamma/Nrf2/NF-kappaB signaling pathway. Int Immunopharmacol 66:309–316
Lim H, Min DS, Park H, Kim HP (2018) Flavonoids interfere with NLRP3 inflammasome activation. Toxicol Appl Pharmacol 355:93–102
Lin WQ, Xie JX, Wu XM, Yang L, Wang HD (2014) Inhibition of xanthine oxidase activity by gnaphalium affine extract. Chin Med Sci J 29:225–230
Luan RL, Meng XX, Jiang W (2016) Protective effects of Apigenin against Paraquat-induced acute lung injury in mice. Inflammation 39:752–758
Ma C, Yang X, Lv Q, Yan Z, Chen Z, Xu D, Liu X, Yang W, Xing S (2020) Soluble uric acid induces inflammation via TLR4/ NLRP3 pathway in intestinal epithelial cells. Iran J Basic Med Sci 23:744–750
Nishitani Y, Yamamoto K, Yoshida M, Azuma T, Kanazawa K, Hashimoto T, Mizuno M (2013) Intestinal anti-inflammatory activity of luteolin: role of the aglycone in NF-kappaB inactivation in macrophages co-cultured with intestinal epithelial cells. BioFactors 39:522–533
Otani N, Ouchi M, Hayashi K, Jutabha P, Anzai N (2017) Roles of organic anion transporters (OATs) in renal proximal tubules and their localization. Anat Sci Int 92:200–206
Pavelcova K, Bohata J, Pavlikova M, Bubenikova E, Pavelka K, Stiburkova B (2020) Evaluation of the influence of genetic variants of SLC2A9 (GLUT9) and SLC22A12 (URAT1) on the development of hyperuricemia and Gout. J Clin Med 9:2510
Rahmi EP, Kumolosasi E, Jalil J, Husain K, Buang F, Abd Razak AF, Jamal JA (2020) Anti-hyperuricemic and anti-inflammatory effects of Marantodes pumilum as potential treatment for Gout. Front Pharmacol 11:289
Romero CA, Remor A, Latini A, De Paul AL, Torres AI, Mukdsi JH (2017) Uric acid activates NLRP3 inflammasome in an in-vivo model of epithelial to mesenchymal transition in the kidney. J Mol Histol 48:209–218
Serrano JL, Figueiredo J, Almeida P, Silvestre S (2020) From Xanthine oxidase inhibition to in vivo hypouricemic effect: an integrated overview of in vitro and in vivo studies with focus on natural molecules and analogues. Evid-Based Complement Alternat Med 2020:9537125
Shekelle PG, Newberry SJ, FitzGerald JD, Motala A, O’Hanlon CE, Tariq A, Okunogbe A, Han D, Shanman R (2017) Management of Gout: a systematic review in support of an American College of Physicians Clinical Practice Guideline. Ann Intern Med 166:37–51
Shen R, Ma L, Zheng Y (2020) Anti-inflammatory effects of luteolin on acute gouty arthritis rats via TLR/MyD88/NF-kappaB pathway. Zhong Nan Da Xue Xue Bao Yi Xue Ban 45:115–122
So A (2007a) New knowledge on the pathophysiology and therapy of gout. Z Rheumatol 66(562):564–567
So A (2007b) Recent advances in the pathophysiology of hyperuricemia and gout. Rev Med Suisse 3(720):722–724
Su HY, Yang C, Liang D, Liu HF (2020) Research advances in the mechanisms of hyperuricemia-induced renal injury. Biomed Res Int 2020:5817348
Torres RJ, Puig JG (2018) GLUT9 influences uric acid concentration in patients with Lesch-Nyhan disease. Int J Rheum Dis 21:1270–1276
Tsai CW, Lin SY, Kuo CC, Huang CC (2017) Serum uric acid and progression of kidney disease: a longitudinal analysis and mini-review. PLoS ONE 12:e0170393
Wang K, Hu L, Chen JK (2018) RIP3-deficiency attenuates potassium oxonate-induced hyperuricemia and kidney injury. Biomed Pharmacother 101:617–626
Xiong W, Zhang H, Wen L, Wang X, Zhong G, Shi Y, DU, X, and Zhu, J. (2018) Effect of Lagotis brachystachya Maxim extract on xanthine oxidase and renal urate transporters in hyperuricemia mice. Chin J New Drugs 27:1538–1543
Xu L, Shi Y, Zhuang S, Liu N (2017) Recent advances on uric acid transporters. Oncotarget 8:100852–100862
Yamagata K, Hashiguchi K, Yamamoto H, Tagami M (2019) Dietary Apigenin reduces induction of LOX-1 and NLRP3 expression, Leukocyte Adhesion, and Acetylated low-density lipoprotein uptake in human endothelial cells exposed to Trimethylamine-N-Oxide. J Cardiovasc Pharmacol 74:558–565
Yan J, Zhang G, Hu Y, Ma Y (2013) Effect of luteolin on xanthine oxidase: inhibition kinetics and interaction mechanism merging with docking simulation. Food Chem 141:3766–3773
Zhang X, Yang X, Wang M, Li X, Xia Q, Xu S, Xu J, Cai G, Wang L, Xin L, Zou Y, Pan F (2016) Association between SLC2A9 (GLUT9) gene polymorphisms and gout susceptibility: an updated meta-analysis. Rheumatol Int 36:1157–1165
Zhu J, Shi Y, Cheng H, Wang H, Wang R, Li M (2019) Chemical constituents from Lagotis brachystachya. J Chin Med Mater 42:552–555

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