

Diuretic activity and mechanism of *Mosla chinensis* Maxim. cv. *Jiangxiangru* in normal Rats

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Research

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Abstract

Background: *Jiangxiangru*, a commonly used clinical Chinese medicine, has been investigated mainly over its effects on Summer heatstroke and the dampness inside the spleen and stomach. This study focuses on the diuretic effect of *Jiangxiangru* in normal rats as well as the underlying mechanism.

Methods: 48 rats were randomly divided into the model group (saline), the positive control group (furosemide 0.02g/kg), low and high dosage of water decoction of *Jiangxiangru* group (0.2 g/ml and 1g/ml), low and high dosage of water extract of *Jiangxiangru* group (0.1463g/ml and 0.7317g/ml), and the low and high dosage of volatile oil of *Jiangxiangru* group (0.1791g/ml and 1.4328g/ml). Each group was assigned 6 rats. Thirty minutes after the successful establishment of the model, the rats were treated orally with corresponding doses of drugs at 10 ml/kg. The control group received an equivalent amount of water. After administration, Urine volume at each time point was measured and urine K^+ , Na^+ , Cl^- content were measured. The contents of ALD, ANP, ADH, Na^+K^+ -ATPase, the gene expression and protein levels of AQP₁, AQP₂ and AQP₃ in urine of rats in each group were determined. SPSS22.0 statistical software was used to analyze the experimental data. One way ANOVA and multiple comparisons between LSD groups were performed. The difference is considered significant When $p < 0.05$ or $p < 0.01$.

Results: Our results showed that *Jiangxiangru* decoction group has the strongest diuretic effect, followed by water extract, then the volatile oil. The diuretic mechanism of *Jiangxiangru* may affect the kidney in addition to the hormone level related to the change of body fluid metabolism. The distal convoluted tubules are related to the content of aquaporin AQP in the collecting tube, thereby inhibiting water reabsorption.

Conclusion: The contents of Na^+ , K^+ , and Cl^- and PH in urine of rats don't change significantly in this experiment. The diuretic effect of *Jiangxiangru* could not cause electrolyte disturbance and other adverse reactions. This fully reflects the complex composition of traditional Chinese medicine, the role of multi-target characteristics.

Background

Diuretics have wide clinical application in treating edema caused by various reasons. They can also be used to treat hypertension(Xing hai ning, Li chen et al. 2016), kidney injury(Jeon, Kim et al. 2018), chronic heart failure(Qavi, Kamal et al. 2015), and other diseases(Alhanafy, Labeeb et al. 2018). As the medical research towards diuretics deepened, the increase in observed post-treatment clinical adverse reactions (Yang 2018) has turned the public attention to traditional Chinese medicines (TCMs)(Lin, Pan et al. 2014, Liu, Yang et al. 2019). In China, several TCMs are used as diuretic drugs (Jia and Jia 2016, Li, Cai et al. 2016, Gao, Bai et al. 2018). However, most of them are lacking pharmacological studies to reveal the molecular pathways that might be contributing to diuretic effects. *Mosla chinensis Maxim. cv. Jiangxiangru* (Chinese: *Jiangxiangru*, 羌活) is one of these TCMs. It belongs to the the Labiates family and

is a famous herbal TCM that is described in the authoritative medical book of Chinese Pharmacopoeia(CHINA 2015).

Elsholtzia is the aerial part of *Mosla chinensis Maximak* or *M. chinensis Jiangxiangru* (Liu, Wang et al. , Zhimin, Wang et al. 2016). *Jiangxiangru*, a herb mainly distributed in Yichun and Xinyu in Jiangxi Province of China, is genuinely a regional herb used in the Traditional Chinese Medicine(Liu, Wang et al. , Qi, Fang et al. 2015, Zhimin, Wang et al. 2016) (Chinese Pharmacopoeia). Elsholtzia can relieve the sweating caused by the heat in summer, and the effect is like ephedrine in winter(XianGuo 2013, lingyuan 2015). It tastes mild and warm, with effects including perspiration, diuresis, and resolving swelling, and dehumidification. Modern research shows that there are many chemical components in *Jiangxiangru*, such as flavonoids, terpenoids, glycosides, steroids, coumarins, organic acids, fats, and lignans(Hu, Xie et al. 2010). Different extracts of *Jiangxiangru* contain different active ingredients, and their pharmacological effects may also be different. Therefore, to increase the efficiency of further development of Jiangxiangru product and their usage, we tested the diuretic effects of Jiangxiangru over the decoction group, the water extract and the volatile oil. Amongst them, volatile oil is the main active ingredient and has antipyretic, anti-inflammatory and analgesic effects (Luo, Yang et al. 2006, Jiang, Lu et al. 2007, Hu, Xie et al. 2010, Li, Cui et al. 2014, Zhimin, Wang et al. 2016).

The purpose of the study was to evaluate the diuretic activities in normal rats. We assessed the urine output volume, the urinary electrolyte concentrations (Na^+ , K^+ and Cl^-) and pH value, the levels of Na^+ - K^+ -ATPase, atriopeptin (ANP), anti-diuretic hormone (ADH) and aldosterone (ALD), as well as AQPs levels for efficacy evaluation.

Methods

study chemicals and reagents

Jiangxiangru (batch number: 180823) was purchased from Jiangxi Jiangzhong Traditional Chinese Medicine Decoction Co., Ltd. The *Jiangxiangru* was placed in a round-bottom flask. After soaking in the proportion of distilled water to 1:10 for 30 minutes, the volatile oil was extracted by steam distillation for 5 hours then collected (yield: 0.67%). The volatile oil was prepared into 200 ml low and high concentration volatile oil solution with distilled water containing 2% Tween-80, respectively. The reduced crude drug amounts were 0.1791 and 1.4328 g/ ml, respectively. The water extract with low and high concentration was obtained by condensed extract part of the extract, and the equivalent crude drug dosage was 0.1463 and 0.7317g/ml, respectively. *Jiangxiangru* decoction is decocted by pharmacy group of Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine [batch number: 121223, specification: 1g (crude drug)/1ml]; Furosemide tablets (Shanghai Chaohui Pharmaceutical Co., Ltd., batch number: 1811N10, specification: 20mg/tablet); rat aldosterone (ALD) ELISA kit (batch number: 201904), rat anti-diuretic hormone (ADH) enzyme-linked immunosorbent (ELISA) kit (batch number: 201904). Rat atrial natriuretic peptide (ANP) ELISA kit (batch number: 201904), rat Na^+ - K^+ -ATPase ELISA kit (batch number: 201903), rat aquaporin 1 (AQP1) ELISA kit (batch number: 201904), rat aquaporin 2

(AQP2) ELISA kit (batch number: 201904), rat aquaporin 3 (AQP3) ELISA kit (batch number: 201904) were purchased from Shanghai Herpeng Biology Company. Technology Co., Ltd. RNA kit (Beijing Quanshijin Biotechnology Co., Ltd.); Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and Corbett Cycler (Qiagen, Valencia, CA, and USA).

Animal treatment and sample collection

Fifty SPF-grade SD male rats weight range 180 ± 20 g were provided by the Experimental Animal Science and Technology Center of Jiangxi University of Traditional Chinese Medicine with the certificate number SCXK (Gan) 2018-0003. Feeding was conducted in SPF barrier system of experimental animal science and technology center of Jiangxi University of Traditional Chinese Medicine, at a temperature of 20-23°C, humidity of 45%-55%, and the license number is SYXK (Gan) 2018-0004. All experiments were carried out in adherence with the guidelines of the Institutional Animal Care and Use Committee of China and were approved by the Animal Care and Research Committee of Jiangxi University of Traditional Chinese Medicine.

The 50 rats were isolated for one week. After the isolation rats were placed in metabolic cage for 3 days; then on the 4th day, fasting (no water) for 18 hours. The rats were given 2.5ml/100g saline water to record the urine volume for 2 hours. The rats whose urine volume was more than 40% of the water supply were successfully established as saline water load model (Chen, Feng et al. , Feng, Chen et al. , Gao, Bai et al. 2018). It can be used in the following experiments.

48 rats were randomly divided into 8 groups: the model group (saline), the positive control group (furosemide 0.02g/kg), low and high dosage of water decoction of *Jiangxiangru* group (0.2 g/ml and 1g/ml), low and high dosage of water extract of *Jiangxiangru* group (0.1463g/ml and 0.7317g/ml), and the low and high dosage of volatile oil of *Jiangxiangru* group (0.1791g/ml and 1.4328g/ml). Each group was assigned 6 rats. According to clinical TCM practice the dosage of *Jiangxiangru* for adults (60 kg/person) is 10~30 g/kg/day (Editorial Committee of Zhonghua Bencao National Traditional Chinese Herb Administration, 1999). For rats, this dosage is 0.166~1.5g/kg/day (raw herb). Thirty minutes after the successful establishment of the model, the rats were treated orally with corresponding doses of drugs at 10 ml/kg. The control group received an equivalent amount of water.

After administration, rats in each group were immediately put into metabolic cages. Urine samples were collected after 1, 2, 3, 4, 5, 6, 24, 48, 72, 96, 144 and 168 hours respectively. Urine volume at each time point was measured and urine K^+ , Na^+ , Cl^- content were measured with Beckman AU2700 biochemical analyzer. At the end of the experimental period, 4 mL of blood were collected in vacuum tubes from the abdominal aorta, allowed to clot on ice, and subsequently subjected to centrifugation at 3000 rpm at 4°C for 10 min. The supernatant was stored at -80°C for further analysis. All of the rats were sacrificed by cervical dislocation. The contents of ALD, ANP, ADH, Na^+K^+ -ATPase, the gene expression and protein levels of AQP₁, AQP₂ and AQP₃ in urine of rats in each group were determined.

Biochemical methods

Enzyme-linked immunosorbent assay (ELISA) kit was used to analyze ALD, ANP, ADH, Na⁺-K⁺-ATPase, AQP₁, AQP₂ and AQP₃ in urine. Rat urine was centrifuged at 3000 rpm for 10 min at 4 °C in an Eppendorf centrifuge. The supernatant was collected, and recombinant rat Na⁺-K⁺-ATPase, ANP, ADH, ALD, AQP₁, AQP₂ and AQP₃ were used to construct standard curves. The absorbance of standards and samples was determined spectrophotometrically at 450 nm using a Multiskan GO (Thermo scientific, USA) microplate reader. The results were plotted against the linear portion of a standard curve.

RNA-isolation and qPCR

Renal medulla samples were cleaved by Trans Zol Up homogenized and added chloroform. The solution was divided into colorless aqueous phase and pink organic phase. RNA was adsorbed in aqueous phase by silica gel membrane centrifugal column. RNA was prepared by reverse transcription using a RNA kit. Real-time PCR was performed using Power SYBR Green PCR Master Mix and Corbett Cycler. Each reaction was performed in 40 cycles with a total volume of 10 µl.

Primer sequences and PCR amplification reactions were as according to the literature (Yan, Zeng et al. 2014). The internal reference gene of the experiment was GAPDH (Zhong, Li et al. 2018). The primer sequences and reaction systems are shown in Tables 1 and 2.

Table 1 Primer sequences of genes were described for RT-qPCR

Gene	Forward primer (5' - 3')	Reverse primer (5' - 3')
AQP1	GACTACACTGGCTGTGGGATCAA	CCAGGGCACTCCCAATGAA
AQP2	GGTTGCTCCATGAATCCAG	GGGGTCCGATCCAGAAGAGGA
AQP3	ACTCCAGTGTGGAGGTGGAC	ACACTAGGAGTTGATCCCCG
GAPDH	TGGGTTTCCCGTTGATGA	AGGGCTGCCTTCTCTTGT

Table 2 Reaction system of quantitative PCR assays

Reagent	Volume (μ l)
cDNA	1.0
ddH ₂ O	3.0
S YBR GREEN II	5.0
Forward primer	0.4
Reverse primer	0.4
ROX correction fluid	0.2
Total volume	10.0

The mixed test solution was placed in a fluorescence quantitative PCR machine for reaction. The amplification reaction conditions were as following: 95 ° C for 30 s; 95 ° C for 10 s, 60 ° C for 1 min, 40 cycles; then the reaction temperature was raised from 50 ° C to 90 ° C (1 ° C every 6 s); The ct values of the reference gene and the target gene were collected; finally, the relative expression level of the target gene was calculated, and the data was processed according to the experimental method of Yang et al (Yang, Liu et al. 2015)

Statistics analysis

SPSS22.0 statistical software was used to analyze the experimental data. Data were expressed as mean \pm standard deviation ($\bar{x} \pm s$). One way ANOVA and multiple comparisons between LSD groups were performed. The difference is considered significant When $p < 0.05$ or $p < 0.01$.

Results

Effect on urinary excretion volume

In order to determine whether *Jiangxiangru* has diuretic effect, we measured the urine volume of rats treated with *Jiangxiangru* or furosemide at 1, 2, 3, 4, 5, 6, 24, 48, 72, 96, 120, 144 hours. We found that 1 hour after administration, the urine volume of the low and high dosage of decoction of *Jiangxiangru* group, the high dose of volatile oil of *Jiangxiangru* group and positive control group increased significantly compared with the model group ($P < 0.01$ or $P < 0.05$). The effect of the high dose of decoction of *Jiangxiangru* group was the most obvious, and the urine volume of other groups also increased, there was no significant difference ($P > 0.05$). At 2 hours after administration, the urine volume of rats in positive control group increased, but there was no significant difference ($P > 0.05$). At 3-5 hours after administration, the urine volume of rats in each group had no significant difference ($P > 0.05$); but at 24 hours and 96 hours after administration, the urine volume of rats in high-dose of decoction of

Jiangxiangru group increased, the difference was statistically significant ($P < 0.05$). This indicated that the water decoction of *Jiangxiangru* had a longer effect than furosemide.

From Figure 1, compared with model group, the total urine volume of rats in high dose of decoction of *Jiangxiangru* group and positive control group increased at 1h,6h,96h,120h,144h hours with statistical significance ($P < 0.05$), indicating that the water decoction of *Mosla chinensis* has obvious diuretic effect.

Effect on urinary pH and electrolyte concentrations

The changes of urinary pH and electrolytes in urine of rats at 1, 2, 3, 4, 5, 6, 24, 48, 72, 96, 120, 144 hours were observed (Fig. 2, Fig. 3). Compared with the model group, the contents of Na^+ , K^+ , and Cl^- in the urine of rats in the administration group did not change significantly, and the pH of the urine of rats in each group had no significant difference ($P > 0.05$).

Effect on urine levels of Na^+ - K^+ -ATPase, ALD, ANP, ADH

ELISA kit was used to verify whether *Jiangxiangru* could affect the contents of ALD, ANP, ADH and Na^+ - K^+ -ATPase in rat urine. Compared with model group, ALD secretion in urine of rats were increased in different treatment groups of *Jiangxiangru*, the difference was statistically significant between the high and low dosages of decoction of *Jiangxiangru* group and the high dose of volatile oil of *Jiangxiangru* group ($P < 0.01$ or $P < 0.05$). The contents of ANP in the urine of rats in the high and low dosage of decoction of *Jiangxiangru* group and the high dose of volatile oil of *Jiangxiangru* group increased. Among these groups, the high dosage of decoction has the most significantly difference ($P < 0.05$). The high dosage of water extract of *Jiangxiangru* reduced the amount of ADH ($P > 0.05$), while the high dosage of decoction of *Jiangxiangru* group increased the content of ADH insignificantly ($P > 0.05$). There was no significant change in the content of Na^+ - K^+ -ATPase in urine of different treatment groups of *Jiangxiangru* ($P > 0.05$). The contents of ALD, ANP, ADH and Na^+ - K^+ -ATPase in plasma of rats in each group were compared as shown in Fig4 and Fig5.

The levels of AQP1, AQP2 and AQP3 in Rat Urine and serum

In order to determine whether *Jiangxiangru* affects the contents of AQP₁, AQP₂ and AQP₃ in rat serum and urine, we used ELISA kit to detect the content of AQP_s proteins and RT-PCR to detect the gene expression of AQP₁, AQP₂ and AQP₃ in rat renal medulla. Compared with the model group, all groups of *Jiangxiangru* decreased the amount of AQP₁ insignificantly ($P > 0.05$). AQP₂ expression in the low dosage of decoction group (0.2g/ml) and low dosage of water extract (0.1463g/ml) of *Jiangxiangru* group have significantly decreased while the equivalent high dosage groups experienced no significant change. Water extract and volatile oil of *Jiangxiangru* decreased the amount of AQP₃ expression, and the difference was significant ($P < 0.05$ or $P < 0.01$). The changes of AQP₁, AQP₂ and AQP₃ aquaporin in rat serum are shown in Fig6A. At the same time, the protein levels of urine AQPs in each group were observed. The AQPs level results showed in Fig6B. All groups with *Jiangxiangru* administration had their

furosemide of AQP₁ level significantly lower than that of the control group (P < 0.01). Low dosage of decoction group (0.2g/ml), low dosage of water extract group(0.1463g/ml), and high dosage of volatile oil group had significant difference in AQP₂ (P < 0.01). The AQP3 protein content of low-dose group of *Jiangxiangru* decoction, volatile oil group, and furosemide group were significantly lower than that of model control group (P < 0.01).

Discussion

Urine is the most intuitive indicator to detect whether drugs have diuretic effect. Urine production includes three processes: glomerular filtration, renal tubule and collecting duct reabsorption and secretion. Diuretics are medications producing diuretic effects of varying strength by acting on different parts of the renal tubule, thus affecting the pathway of urine production. The excretion of electrolytes is also related to the site of drug action. Classic diuretics mainly exert diuretic effect by increasing the excretion of Na⁺ and Cl⁻ after drug intervention as Na⁺ and Cl⁻ excretion positively influences diuresis. For example, furosemide mainly acts on the thick segment of the ascending branch of the medullary ridge, and reduces the reabsorption of Na⁺ and Cl⁻ by inhibiting Na⁺-K⁺-2Cl⁻ co-transport, increases the excretion of NaCl, and increases the urinary H⁺ and K⁺ Excretion, which can cause hypokalemia. Therefore, it is crucial to determine the contents of Na⁺, K⁺, and Cl⁻ in urine of rats treated with *Jiangxiangru*. *Jiangxiangru* and its 3 delivery reagents had no effects on contents of Na⁺, K⁺, and Cl⁻ in urine. Therefore, we believe that the diuretic effect of Jiangxiang and each of it treatment group is not achieved by affecting Na⁺-K⁺-2Cl⁻ co-transport.

The main function of ALD synthesized and secreted by adrenocortical globular zone cells is to increase the re-absorption of Na⁺ and water, promote K⁺ excretion and maintain the balance of water-salt metabolism. The active polypeptide ANP synthesized and secreted by the atrium is also known as atrial natriuretic factor or atrial natriuretic peptide. Its main functions are natriuretic, diuretic, inhibiting rennin release and ALD secretion. ADH is secreted by the hypothalamus and its main function is to promote water reabsorption. The diuretic activity and mechanism of drugs are not only related to the levels of related diuretic hormones (ALD, ADH, ANP), but also to the levels of aquaporin in urine regulated by hormones. Therefore, this study explored the diuretic activity and its mechanism of *Jiangxiangru* related diuretic hormones (ALD, ADH, and ANP). *Jiangxiangru* and its 3 delivery reagents had no effects on the anti-metabolite enzymes. The results showed that the high dosage of decoction of *Jiangxiangru* group can increase the secretion of ALD and increase the secretion of ANP, especially the effect of ALD on dose-response relationship, suggesting that the aqueous extract of *Jiangxiangru* has diuretic effect. There was also anti-diuretic effect, which is related to the multi-component and multi-target characteristics of traditional Chinese medicine. The volatile oil of *Jiangxiangru* also has similar action characteristics.

Additionally, from the influence of gene expression and protein level of AQP, *Jiangxiangru* showed complex urinary production effects. The three preparations groups significantly down-regulated the expression of AQP₁ gene, and the protein level was significantly lower than that of the control group. The

group of decoction showed an increase in the expression of *AQP₂* and *AQP₃* genes, and the protein also increased. The other groups down-regulated the expression of *AQP₂* and *AQP₃* genes, and the protein was lower than the control group.

Conclusion

The contents of Na⁺, K⁺, and Cl⁻ and PH in urine of rats don't change significantly in this experiment. The diuretic effect of *Jiangxiangru* could not cause electrolyte disturbance and other adverse reactions. This fully reflects the complex composition of traditional Chinese medicine, the role of multi-target characteristics.

Declarations

Ethics approval and consent to participate All experiments were carried out in adherence with the guidelines of the Institutional Animal Care and Use Committee of China and were approved by the Animal Care and Research Committee of Jiangxi University of Traditional Chinese Medicine.

Consent for publication The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All participants agreed to publish.

Availability of data and materials The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests The authors have no conflicts of interest to declare.

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Authors' contributions (I) Conception and design: Zhiyong Liu; (II) Administrative support: Suyun Xiao., Dan Lei; (III) Provision of study materials: Longxue Li., Kun Shu. Weiyue Deng.; (IV) Collection and assembly of data: Yun Huang.,Weiqi Liu , Juan He., Zhongrui Wu.; (V) Data analysis and interpretation: Shouming Li ; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Figures

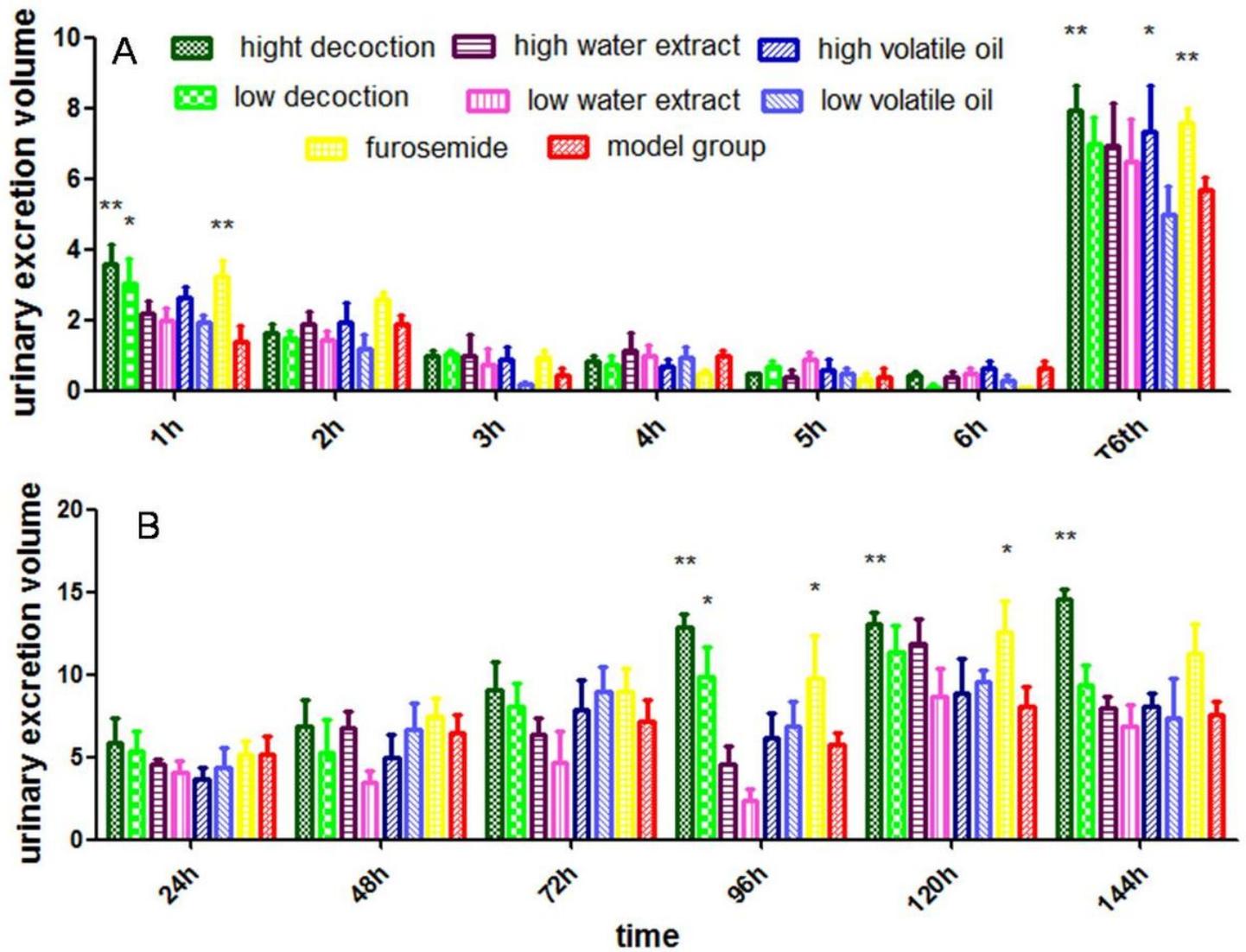


Figure 1

The volume of excreted urine was measured at 1, 2, 3, 4, 5, 6, 24, 48, 72, 96, 120, 144 hours; cumulative values were reported as mean ± SD for 6 rats in each group. *P < 0.05 and **P < 0.01 compared to the controls group.

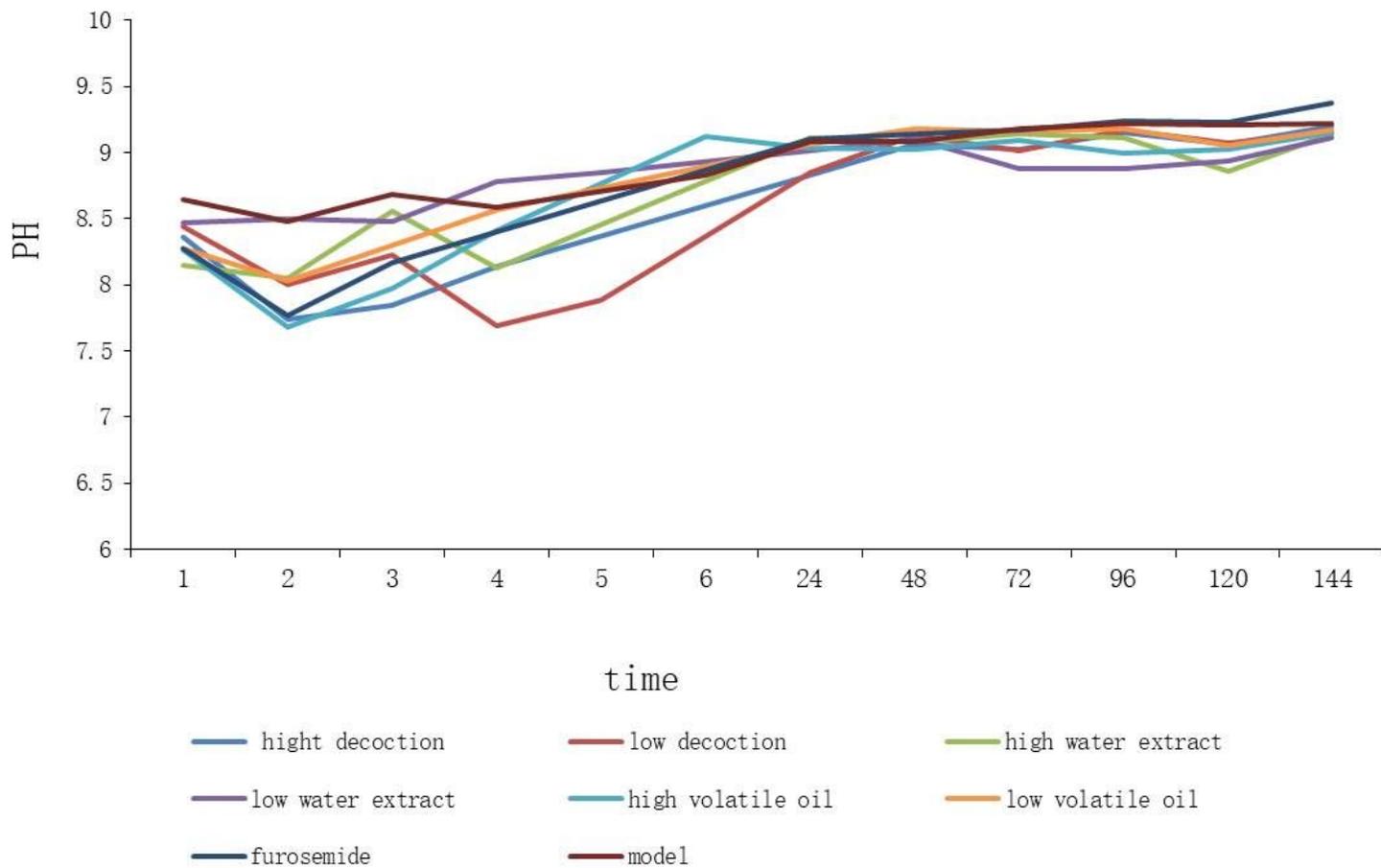


Figure 2

Effects of Jiangxiangru and its three groups together with furosemide on urinary pH at the x-axis indicates assessment time points after oral administration; the values were reported as mean \pm SD for 6 rats in each group. * $P < 0.05$ and ** $P < 0.01$ compared to the controls group.

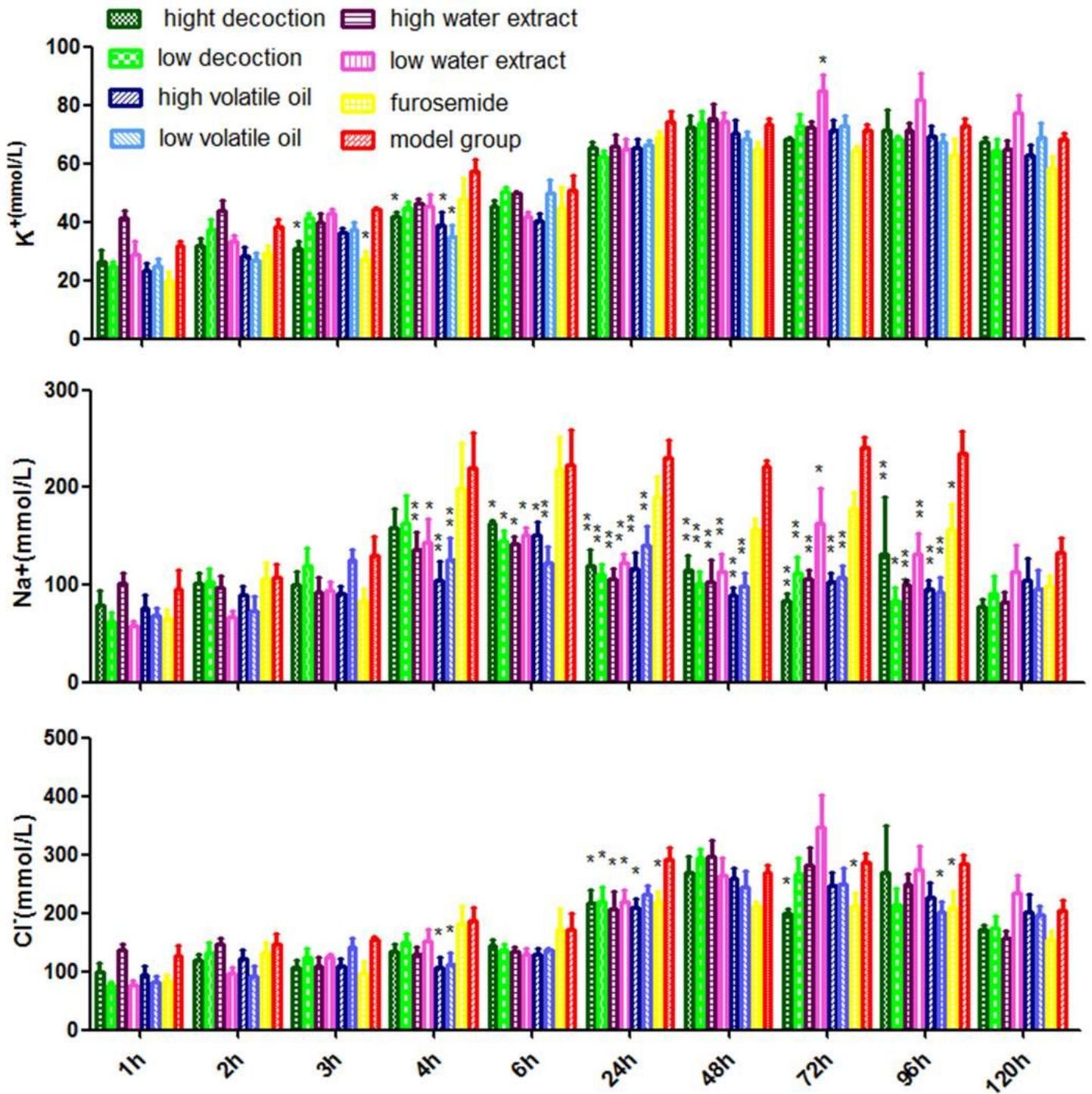


Figure 3

Effects of Jiangxiangru and its three groups together with furosemide on urinary electrolyte Na⁺·K⁺·Cl⁻ concentrations at the x-axis indicates assessment time points after oral administration; The values were reported as mean ± SD for 6 rats in each group. *P< 0.05 and **P< 0.01 compared to the controls group.

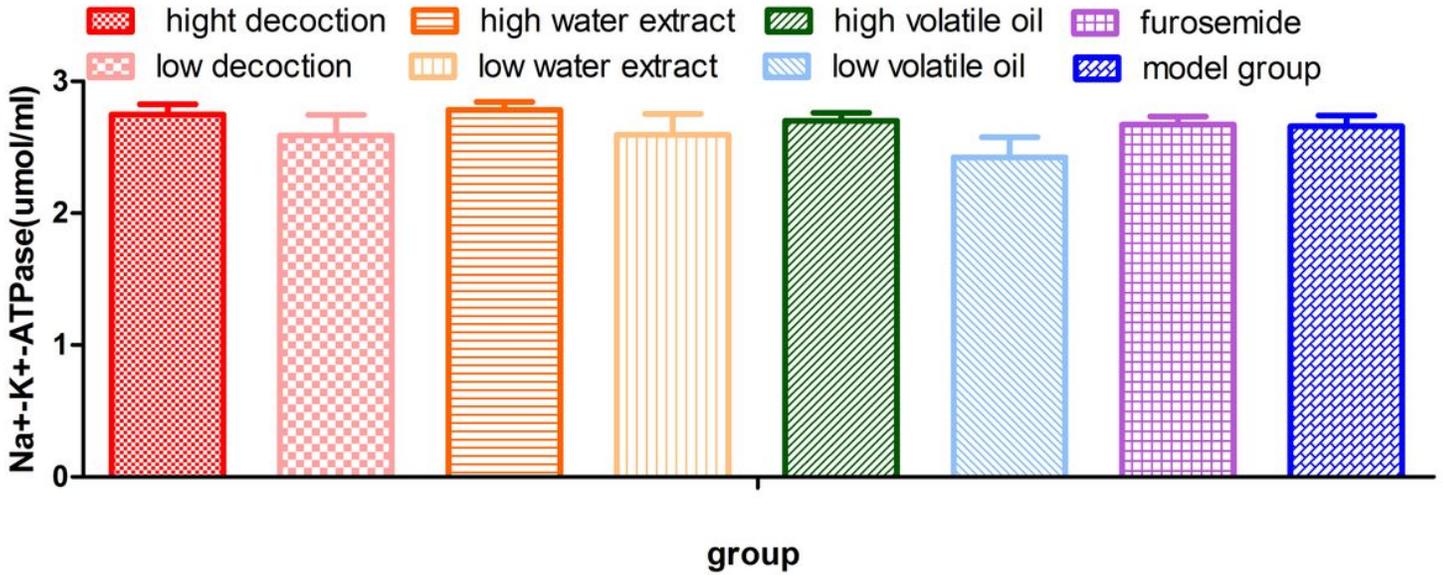


Figure 4

Effects of Jiangxiangru and its three groups together with furosemide on the levels of Na⁺-K⁺-ATPase from rat urine (mol/l) at 144 h after oral administration; the values were reported as mean ± SD for 6 rats in each group. *P< 0.05 and **P< 0.01 compared to the controls group.

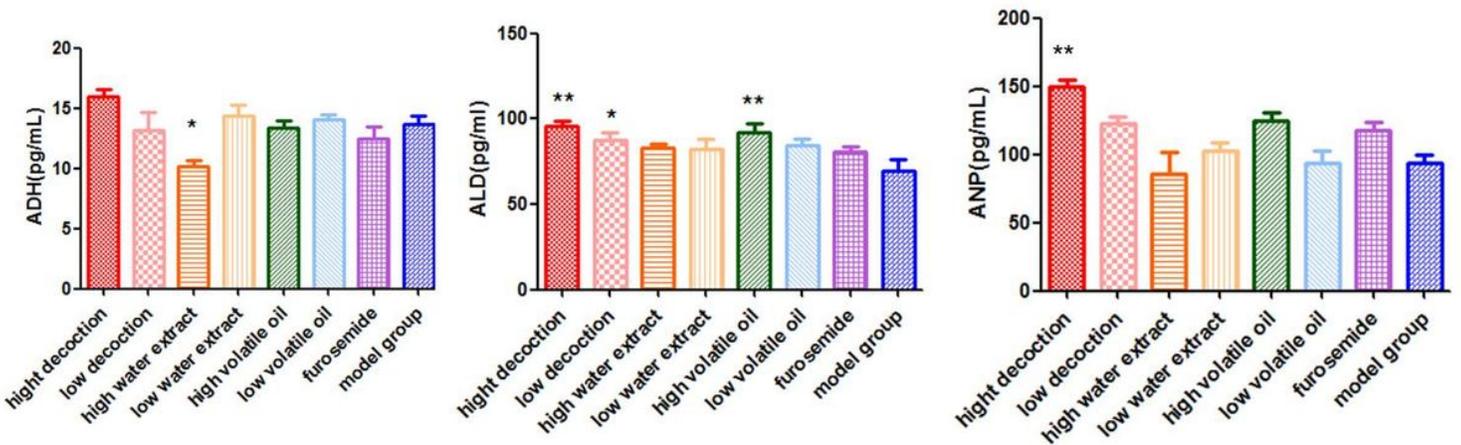


Figure 5

Effects of Jiangxiangru and its three groups together with furosemide on the levels of ANP, ADH and ALD from rat urine (pg/mL) at at 144h after oral administration; the values were reported as mean ± SD for 6

rats in each group. *P< 0.05 and **P< 0.01 compared to the controls groups.

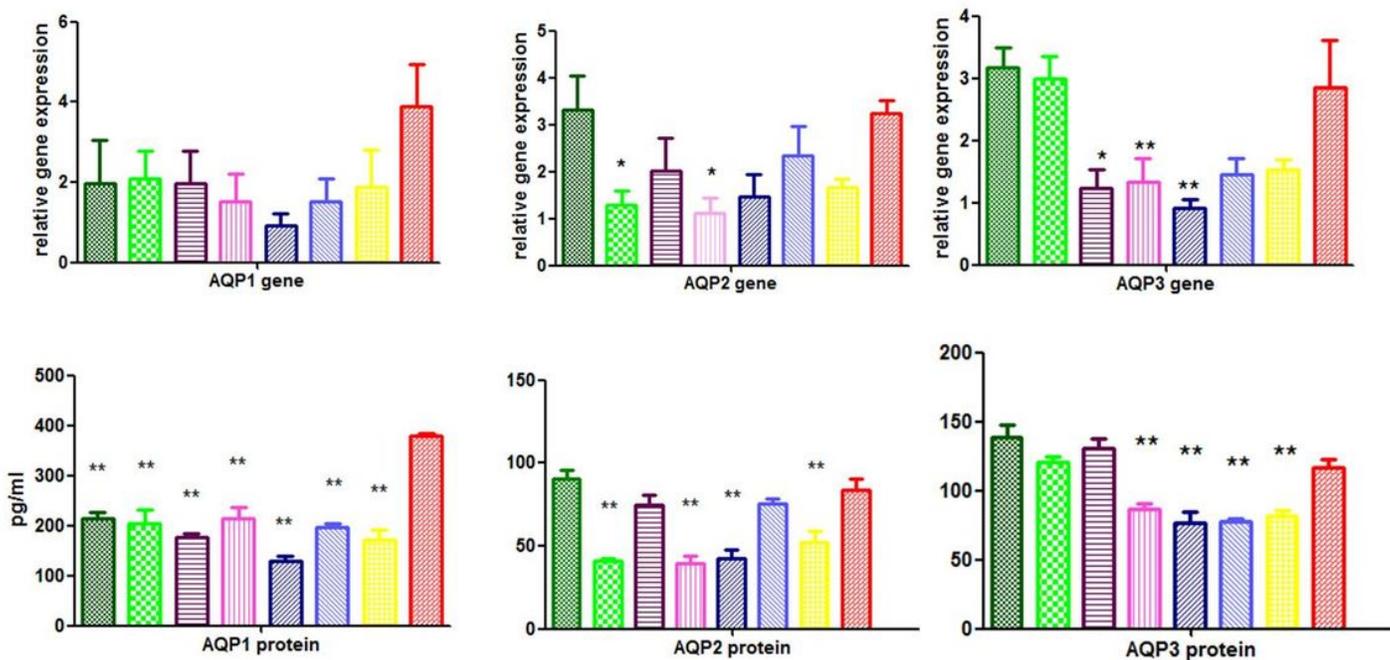


Figure 6

Effects of Jiangxiangru and its three groups together with furosemide on the levels of AQPs from rat urine and serum at 144h after oral administration; the values were reported as mean \pm SD for 6 rats in each group. *P< 0.05 and **P< 0.01 compared to the controls groups. (6A: The changes of AQP1, AQP2 and AQP3 aquaporin in rat serum, 6B: the protein levels of urine AQPs)