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Full Length Research Paper

The extraction, identification and quantification of hypoglycemic active ingredients from stinging nettle (*Urtica angustifolia*)

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Urtica angustifolia **is a traditional medicinal material which has hypoglycemic function. The objectives of this study were to isolate and identify hypoglycemic active ingredients from it. Three groups of mice were gavaged with the decoction of *Urtica* root, stem and leaf for 14 days at a dose of 20 mg/(kg·d). Area under the curve (AUC) values were 24.62±5.24, 24.33±4.66 and 25.51±4.25, respectively. The AUC in leaf decoction group was significantly lower than the other two groups ($P < 0.05$), which indicated the leaf extracts did have significant hypoglycemic action. Four kinds of extractions were obtained from leaves and fed to mice. AUC values were 33.44±3.58, 34.24±3.64, 30.44±2.57 and 9.93±1.98, respectively. AUC in group U3 ($P < 0.05$) and U4 ($P < 0.01$) were significantly lower than in the group of U1 and U2, which indicated that extraction U3 and U4 obviously decreased the blood sugar. Also, U3 was flavonoid and U4 was daucosterol, which was identified by thin-layer chromatography test, IR and ESI-MS spectral analysis.**

Key words: *Urtica angustifolia*, active component, glucose tolerance test, hypoglycemic.

INTRODUCTION

Diabetes is a common and frequently-occurring disease, seriously harm to human health. Modern medical research shows that the pathogenesis of diabetes is affected by genetic and environmental factors (Ling, 2007). With economic development, people's diet changes and population aging, diabetes is increasing rapidly. According to statistics, the incidence of diabetes in the world is 3 to 5% and the incidence of 50 is 10% (Qian, 1999). Diabetes cause many complications and the treatment cost highly. There are no effective methods and the commonly used clinical hypoglycemic agents have side-effects. Therefore, it is urgent needs to find new substances for treating diabetes, especially the active ingredients from natural plants. The direction of our study is development and characteristic of natural hypoglycemic active ingredients from natural plants.

Natural hypoglycemic foods are rich sources, simple to use and inexpensive. It is the best auxiliary therapy and will be accepted by ordinary people and most commonly used (Yuan and Guo, 2007). At present, some hypoglycemic foods have been developed, such as pumpkin polysaccharide and its capsules. It initially confirmed the hypoglycemic mechanism of pumpkin polysaccharides: the result of both the repair of damaged pancreatic islet β-cell and rejection of islet glycogen output (Zhong and Yao, 2002). Cho et al. (2007) suggested that both EPS exhibited considerable hypoglycemic effect and improved insulin sensitivity possibly through regulating PPAR-gamma-mediated lipid metabolism.

Urtica is a perennial wild herb. It is rich in natural

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resources and has very strong vitality. *Urtica* is a kind of traditional medicine edible wild plant in China. Nettle is stated to possess hypoglycemic properties (Newall et al., 1996). Moreover, it has been shown that a preparation containing various plants extraction with nettle had antidiabetic activity (Petlevski et al., 2001). *Urtica angustifolia* belongs to the family of *Urticeae*, is widely ground in China. Moreover, nettle is known in folk medicine as hypotensive and antidiabetic (Ziyyat et al., 1997). *U .angustifolia* has anti-virus, enhance immunity, the role of beauty and therapeutic health care (Bnouham et al., 2003). Nettle plant roots and rhizomes can also treat high blood pressure, hand and foot numbness, diabetes, leprosy, weak labor wounds, indigestion and constipation. It can also be used to treat polio sequelae and hernia pain and so on (Fan et al., 2010; Quan, 1997).

At present, in Europe, the United States and other developed countries have researched on the nettle, especially the *Urtica dioica*. In particular, the development of medicine in Germany had made remarkable achievements. They had specific studied on different parts of the chemical composition of *U. dioica* and obtained that the active ingredients are sterols, flavonoids, organic acids, phenols, phenylpropanoids, plant proteins and polysaccharides (Kraus and Spiteller, 1990; Kavtaradze et al, 2001; Gansser and Spiteller, 1995; Issopoulos and Manouri, 1996; Kraus and Spiteller, 1991; Neugebauer et al, 1995). Germany's "rheumatic security" (Rhenma-Hek) is the *U. dioica* leaf extraction for rheumatic pain in patients with self-treatment and it is one of five most commonly used drugs in Germany and played an important role in OTC drugs.

Lichius et al. (1999) reported the polysaccharide extracted from *U. dioica* by 20% methanol had obvious inhibitory effect on the human prostatic epithelial cell proliferation. *U. dioica* treated scientific associations in Europe album (ESCOP Monogrophs), it included as one of the botanicals. In addition, *U. dioica* has been researched in the field of agriculture and bio-engineering progress. Water extraction of *U. dioica* leaf showed hypoglycemic trend by animal experiments (Farzami et al., 2003). It was due to increased insulin secretion and thus, played a role in lowering blood sugar. In China, *U. angustifolia* has not been developed and utilized yet; hypoglycemic of *U. angustifolia* has not been studied; only the civilian applications of the clues, the mechanism is not clear, it greatly restricts its development and application (Guo and Zhang, 2006).

U. angustifolia (Urticaceae) is widely distributed in wasteland, grassland, valley, wet places and ridge. It is extremely rich in the northeast of China, such as Tonghua, Liuhe and other places in Jilin province. It has strong vitality and contiguous grow, but it was only used as feedstuff and has not been development and application yet, as well as results in a great waste in natural resources. The objectives of this study were to screen blood glucose-lowering components from different parts of *U. angustifolia* through oral glucose tolerance test in mice and further isolate and identify the hypo-glycemic active ingredients. As a raw material, *U. angustifolia* will be developed into a series of hypoglycemic drugs, potential oral hypoglycemic agents or functional foods.

Moreover, changing *U. angustifolia* into valuables is environmentally and economically significant.

MATERIALS AND METHODS

U. angustifolia was collected in Liuhe, Jilin province of China. Petroleum ether (AR) was purchased from Tianjin Tiantai Fine Chemicals Co., Ltd. Ethyl acetate; N-butanol, ethanol, methanol and chloroform (AR) were purchased from Beijing Chemical Plant. 95% ethanol (AR) was purchased from Tianjin Fuyu Fine Chemical Co., Ltd. Magnesium powder (AR) was purchased from Tianjin Guangfu Fine Chemical Research Institute. Hydrochloric acid (AR) was purchased from Laiyang Chemical Experiment Plant. Silica gel column chromatography and thin-layer chromatography silica gel was purchased from Qingdao Ocean Chemical Factory. β-Sitosterol was purchased from Shanghai Fufeng Biotech Co., Ltd. (China).

Animals

Male mice (4 weeks old, 19±2 g) were obtained from experimental center of College of Pharmacy in Jilin University. All animals were maintained in separated cages (38 cm × 60 cm × 30 cm) with tap water. Under the conditions of 21±1℃ and 50 to 60% relative humidity, they were allowed free access to food and water and kept on 12 h light/dark cycles. The care and treatment of experimental animals conformed to the guidelines for the ethical treatment of laboratory animals.

Preparation of *U. angustifolia*

The leaves, roots and stems of *U. angustifolia* were washed with water for three times and chopped, then dried natural separately. The dried leaves, roots and stems were crushed (20 to 100 mesh) separately by tilt-type high-speed universal grinder (Beijing Zhongxing Albert Instrument) and then stored at room temperature till use.

Experimental design and data analysis

Two separate experiments were conducted: The first was to determine the hypoglycemic effect of different parts (leaves, roots and stems) of *U. angustifolia* by oral glucose tolerance test. The results were compared with the saline control. The second was to separate and purify active components from the best part of *U. angustifolia* and identify their structures by thin-layer chromategraphy (TLC), infrared ray (IR) and electrospray ionization mass spectrometry (ESI-MS).

Data were analyzed using SPSS (statistical product and service solutions) 10.0 version. All data were expressed as means±SD (standard deviation). A level of $p < 0.01$ and $p < 0.05$ were considered statistically significant.

Extraction of active ingredients from *U. angustifolia*

Dried *U. angustifolia* leaves, roots and stems powder (1 kg) were extracted with water (90℃, 20 BV) for 3 times (15 min/time) and

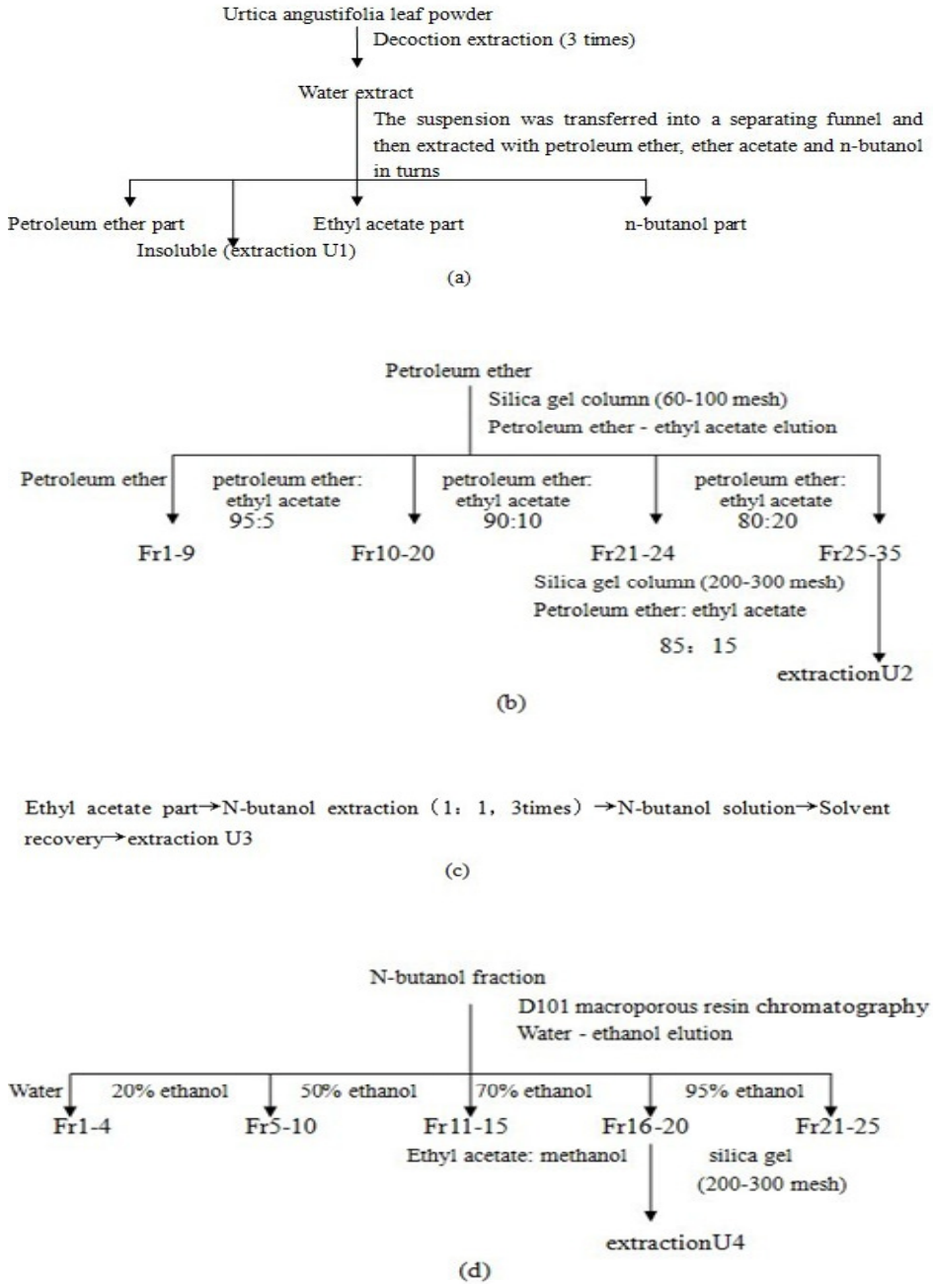

Figure 1. Extraction of U1 (a); U2 (b); U3 (c); U4 (d) from *U. angustifolia* leaves.

then, the combined filtrate were concentrated with vacuum rotary evaporator (Beijing Yarong Albert instrument) (Wang, 2004). About 100 g of decoction extract of leaves, roots and stems were collected, separately.

Separation and purification of active ingredients from *U. angustifolia* leaves

The decoction extract of *U. angustifolia* leaves (200 g) was dissolved by distilled water t o 600 ml. The suspension was transferred into a separating funnel and then extracted with petroleum ether, ether acetate and n-butanol in turns. Each extracts were evaporated at reduced pressure and the extracts obtained were 2.5, 3.9 and 16.5 g, separately, which were further separated. The insoluble substance was extraction U1 (Figure 1a).

The petroleum ether extraction was separated by silica gel column chromatography. The specific operating processes are as follows: (1) 200 g of silica gel (60 to 100 mesh) was activated (110°C, 1 h) and loaded with petroleum; (2) the petroleum ether extraction was dissolved by petroleum ether and mixed with 20 g of silica gel and then added into the column chromatography after solvent evaporate; (3) gradient elution separated with petroleum-ethyl acetates. The flow rate was 3 to 5 ml/min and each class was 20 ml. The eluent flows were combined after identified by TLC, which conditions were self-made silica gel thin-layer plate, ascen-

ding development with petroleum ether: ethyl acetate (8:2), color reaction with 20% sulfuric acid-ethanol solution. Repeated column chromatography and recrystallized in cold ethanol solution, extraction U2 was obtained (Figure 1b).

The ethyl acetate extraction was separated with n-butanol (1:1) for 3 times and n-butanol extraction was obtained. After the solvent recovered, extraction U3 was obtained (Figure 1c).

The n-butanol extraction was separated by D101 macroporous resin chromatography due to its main component, saponins identified by TLC. The specific operating processes are as follows: (1) the new resins were immersed in ethanol solution for 24 h and wet loaded into the column, which was cleaned by ethanol and distilled water; (2) the n-butanol extraction was dissolved with distilled water, and then added in drops; (3) gradient elution separated with water-ethanol. First the extract was eluted with water until Molish reaction of the outflow was negative, which indicated that sugars was removed, and then eluted with 20, 50, 70 and 95% ethanol solution. The flow rate was 3 to 5 ml/min and each class was 20 ml. The elution flows were combined and condensed after identified by TLC. In addition, the eluent of 70% ethanol solution was further gradient elution separated by silica gel chromatography with ethyl acetate-methanol and each class was 20 ml. The eluent with similar spots were combined and concentrated after detection by TLC. Extraction U4 was obtained (Figure 1d).

Oral glucose tolerance test

The blood sugar levels of normal subjects temporarily increase and return to normal levels after about two hours through a variety of regulatory mechanisms when intake a large number of glucose, which is called glucose tolerance phenomenon. Oral glucose tolerance (OGT) test is to examine the regulatory function *in vivo* glucose metabolism.

OGT test was conducted as follow: (1) the mice were randomly divided into 4 groups and each group had 10 mice and then marked, weighed; (2) each group of mice were given decoctions of root, leaf, stem and saline as control separately by gavage once daily for 14 days and then fasting blood glucose (FBG) values were measured before gavage. Fasting blood glucose (FBG): a method for learning how much glucose there is in a blood sample taken after an overnight fast of 8 to 10 h. The concentration of extracts was 15 mg/ml and dose was 20 ml/kg; (3) on the 14^{th} day, after fasting 12 h, FBG was measured. The mice were fed with 2.5 g/kg glucose, then fed by extracts and blood glucose (BG) was measured on 0, 0.5, 1 and 2 h and labeled as $BG0h$, $BG0.5h$, $BG1h$ and $BG2h$; (4) the curves were drawn according to the blood sugar levels measured at regular intervals and blood glucose area under the curve (AUC) was calculated to evaluate the changes in glucose tolerance test as follows:

$$AUC = 0.25 \times (BG0h + 4 \times BG0.5h + 3 \times BG2h) \quad (1)$$

AUC represents the bioavailability of drugs. Higher AUC indicates higher glucose concentration and lower hypoglycemic effect (Yao et al., 2006; Wolever et al, 1991).

Identification of extraction U3

Color reactions (HCl-Zn reaction and aluminum complex reaction) were conducted for flavonoids test. Thin-layer chromatography (TLC): 1% aluminum chloride ethanol solution and 5% concentrated sulfuric acid ethanol solution were used as reagent. The silica gel plate was 3.0 cm×10 cm (Qingdao Marine Chemical Plant). Extract U3 was developed by chloroform: methanol: water (7:3:0.5). The specific steps were as follows: (1) the extract U3 was pointed capillary from the end 1 to 1.5 cm, then blew drying; (2) the reagent was poured into the chromatography-cylinder and developed to required distance; (3) the silica gel plate was removed and dried and colored with 1% aluminum chloride ethanol, then blew drying; (4) fluorescence spot was observed in ultraviolet light with 365 nm. If there was no fluorescence spot, switched 5% concentrated sulfuric acid ethanol solution as color reagent.

Identification of extraction U4

HCl-Mg reaction, Molish reaction and Liebermann-Burchard reaction were conducted for flavonoids, saccharides and glycosides and steroidal saponins, sterols or triterpenoids test.

TLC: the extractions were hydrolyzed with 5% sulfuric acid ethanol solution and aglycones were extractied with chloroform, water layer treated before paper chromatography, mobile phase: butanol-pyridine-water (6:4:3), with glucose as standard contrast, the Rf values were observed, portion of aglycone was analyzed by thin-layer chromatography, with β-sitosterol as standard contrast, the Rf values were observed.

FT-IR spectroscopy was used to examine the structure of extraction U4. A Perkin–Elmer spectrum 1000 was used to obtain the spectra. Samples were ground and mixed with KBr. They were then pressed into transparent thin pellets. A FT-IR spectrum of sample was obtained in the range of 4000 and 400cm^{-1}. Spectral outputs were recorded in the transmittance mode as a function of wave number.

Electron spray ionization mass spectrometry (ESI-MS) was used to analyze the chemical structure of extraction U4. Electrospray ionization (ESI) is a technique used in mass spectrometry to produce ions. ESI-MS was recorded with LC/MSD TRAP SL mass spectrometer (Agilent, USA) set in positive ion mode (ESI+, m/z100 to 1000). The desolvation gas (nitrogen) temperature, pressure and flow rate was set to 325°C, 50Pa and 10 L/min. The sample was dissolved by methanol and membrane filtered, then on-column detected.

RESULTS AND DISCUSSION

Hypoglycemic effects of extracts from different parts of *U. angustifolia*

The hypoglycemic effect of root, stem and leaf decoctions are shown in Table 1. AUC values were calculated according to Figure 2 and listed in Table 2. According to the FBG values of mice in OGT test (Table 1), the FBG of the leaf decoction group (7.36±0.95 mmol/l) was significantly lower than the control 3 groups ($P < 0.05$) which initially proved the leaf decoction had significant hypoglycemic trend among the three parts of *U. angustifolia*.

In Figure 2, blood glucose values peaked after the glucose load for 30 min and started down from 60 to 120 min and then return to normal. The peak values of the root decoction group and stem decoction group were 16.77±5.33 and 17.48±3.85 mmol/l. The peak value of leaf decoction group was 15.11±4.96 mmol/l, which was significantly lower than other groups. Lower blood glucose in leaf decoction group after 30 min indicated the decoction of the leaves had a stronger ability to inhibit the blood glucose increasing.

Table 1. Fasting blood glucose (FBG) value of mice fed by extractions from *U. angustifolia*.

Different group	FBG (mmol/l)
Root decoction	8.88±0.71
Stem decoction	8.18±1.12
Leaf decoction	7.36±0.95*
Saline control	7.96±1.33

*Means value was significantly ($P < 0.05$) lower compared with other 3 groups.

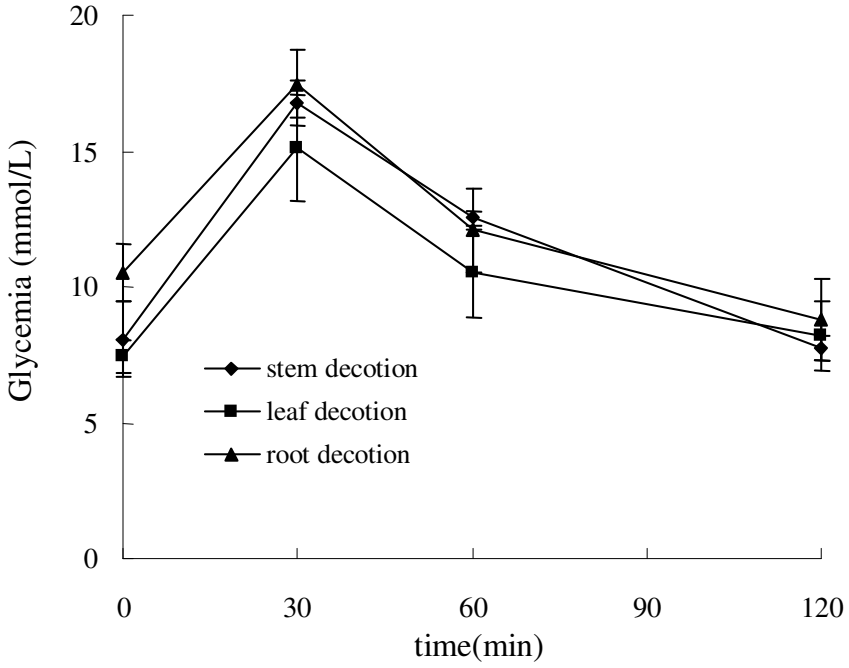

Figure 2. Effect of extractions from *U. angustifolia* different parts on hypoglycemic in mice.

Table 2. Area under the curve (AUC) values of mice fed by extractions from *U. angustifolia*.

Different group	Root decoction	Leaf decoction	Stem decoction
AUC	24.62±5.24	24.33±4.66*	25.51±4.25

*Means value was significantly ($P < 0.05$) lower compared with other 3 groups.

According to Table 2, the leaves decoction group showed the lowest AUC value (Table 2) compared with other groups ($P < 0.05$), which demonstrated that hypoglycemic ability of leaf decoction was significantly higher than root and stem decoction. Research reported that nettle root decoction could lower blood pressure and it is dose-dependent and last longer. In addition, it had significant cardiac function (Fazami et al., 2003). In diabetic mice injected *U. dioica* active substance, after 30 min, *in vivo* insulin level increased significantly, accompanied glucose concentration decreased. After 120 min, insulin level was 6 times as much as the original level (Ziyyat et al., 1997). Bnouham (2003) reported that a concentration of 250 mg/kg of *U. dioica* water extraction of diabetic was fed to mice; the results also showed that *U. dioica* had notable hypoglycemic effect; it was possibly due to inhibition of intestinal absorption of glucose in mice. Our results showed that *U. angustifolia* leaves had

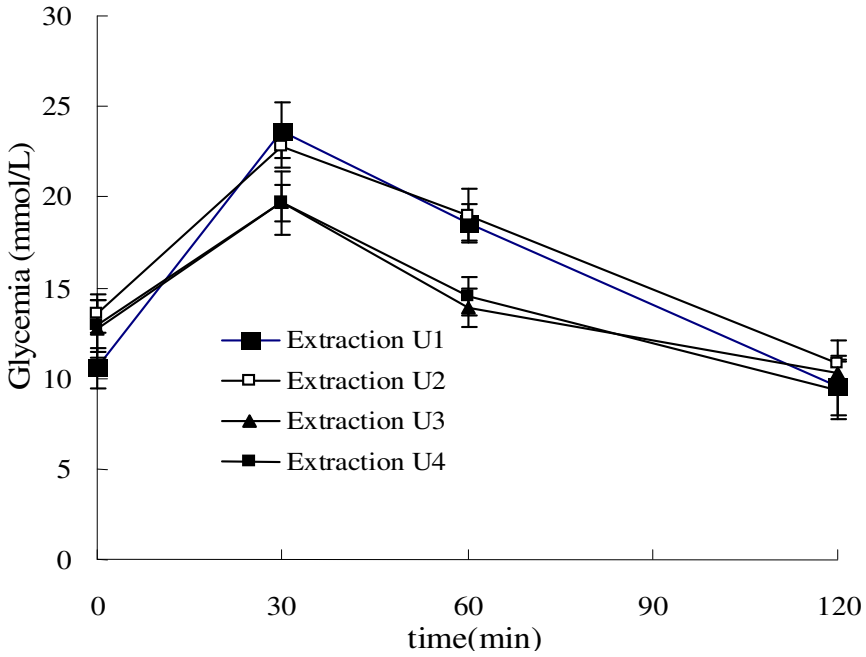

Figure 3. Effect of different extractions from *U. angustifolia* leaves on hypoglycemic in mice.

Table 3. AUC values of mice fed by four extractions from *U. angustifolia* leaves.

Different group	Extraction U1	Extraction U2	Extraction U3	Extraction U4
AUC	33.44±3.58	34.24±3.64	30.44±2.57*	29.93±1.98**

*Means value was significantly ($P < 0.05$) lower compared with other 3 groups; **means value was significantly ($P < 0.01$) lower compared with other 3 groups.

significant hypoglycemic effect, which probably due to *U. dioica* leaf extraction contained a stimulate insulin secretion substances, while it reduced glucose. So leaves were chosen for further experiments.

Hypoglycemic effects of four extracts from *U. angustifolia* leaves

On the basis of stated results, OGT test was conducted to examine the hypoglycemic effects of 4 extracts from leaves and the results are shown in Figure 3 and Table 3. In Figure 3, blood glucose values peaked after the glucose load for 30 min and started down from 60 to 120 min and then return to normal. The glucose tolerance test curves of extractions U3 and U4 were smoother compared with the curves of extractions U1 and U2. The peak values of extractions U1 and U2 test groups were 23.68±3.56 and 22.77±2.17 mmol/l, while the peak values of extractions U3 and U4 were 19.68±2.72 and 19.69±4.02 mmol/l, which was significantly ($P < 0.05$) lower. According to AUC values shown in Table 3, AUC of extraction U3 was significantly ($P < 0.05$) lower than extractions U1 and U2, AUC of extraction U4 was significantly ($P < 0.01$) lower than extractions U1 and U2, which indicated that extractions U3 and U4 had higher ability to reduce blood sugar in mice than extractions U1 and U2. All these results demonstrated that extractions U3 and U4 had strongly hypoglycemic effect.

In the following experiments, the composition and structure of extractions U3 and U4 were identified to determine its active ingredients and illustrate the mechanism of hypoglycemic effects.

Identification of extraction U3

The properties of extraction U3 were detected by color reaction and TLC method. Table 4 showed the color reaction results of extraction U3. The color of extraction U3 in HCl-zinc reaction was purple. The methanol extraction of extraction U3 was spotted on 3 filter papers and dried. The color of the filter paper spot was light yellow fluorescence under UV light (365 nm) without other treatment; 2% $A1C1_3$-ethanol solution was dropped on the second filter paper spot and it was observed fluorescent

Table 4. The color reactions of the extraction U3.

Chemical reagent	Color	Possible component		
Zn-HCl (water bath)	Fuchsia	Flavonoids	Flavonols	Dihydro-flavonols
UV (365 nm)	Yellow fluorescence	Flavonoids	Flavonols	
2% $A1Cl_3$ ethanol (sunlight)	Y- yellowish	Flavonoids	Flavonols	
2% $A1Cl_3$ ethanol (UV)	Light blue fluorescence	Flavonoids	Flavonols	Dihydro-flavonols
1% NaOH (sunlight)	Fulvous	Flavonoids	Flavonols	Isoflavones
1% NaOH (UV)	Light blue fluorescence	Flavonoids	Flavonols	Isoflavones

Table 5. The color reactions of the extraction U4.

Chemical reagent	Method	Result
Flavonoids	HCl-Mg reaction	Purple (-)
Sugar or glycoside	Molish reaction	Purple red ring (+)
Steroidal saponins, sterols triterpenoids	Liebermann-Burchard reaction	Yellow to cyan (+)

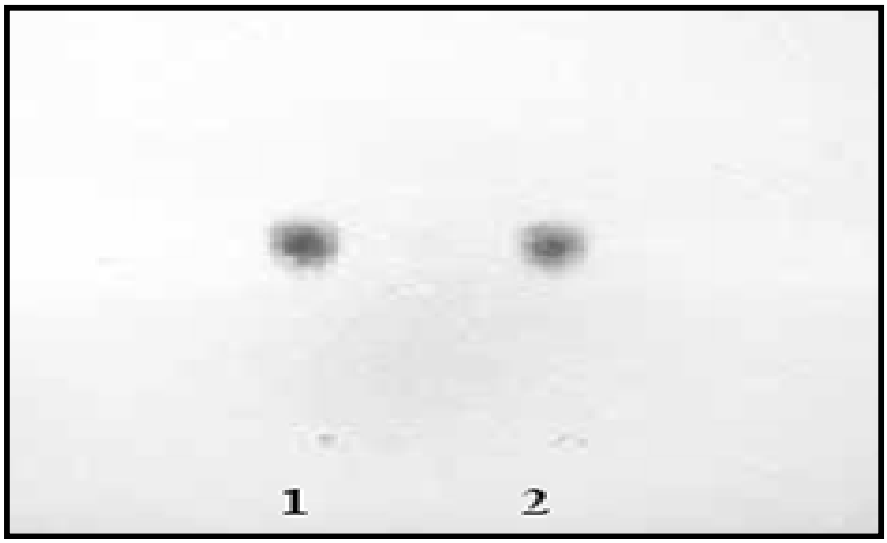

Figure 4. TLC of extraction U4. 1, Reference substance; 2, extraction U4.

yellow under daylight lamp and blue fluorescent under UV light (365 nm); 1% NaOH solution was dropped on the third filter paper spot and showed fluorescent yellow under daylight lamp and blue fluorescent under UV light (365 nm). These entire phenomenons initially proved that U3 contains falconoid.

TLC testing found that extraction U3 showed a large number of bright yellow and yellow-green spots suggesting that the main components of n-butanol extraction was flavonoid compounds, which was consistent with the results reported in other literatures (Wang et al., 2002).

Identification of extraction U4

The properties of extraction U4 were also detected by color reaction and shown in Table 5. Extraction U4 was yellow powder. There was no color change in HCl-Mg reaction, which indicated that there were no flavonoids in U4. Molish reaction was positive which demonstrated U4 contained sugar or glycoside. The color changed gradually from yellow to cyan in Liebermann-Burchard reaction indicated U4 contained steroidal saponins, sterols or triterpenoids. These results revealed that the compound of U4 was steroids, rather than triterpenes (Yao, 2001).

TLC result of U4 is shown in Figure 4. Water layer treated before paper chromatography, the result was the same with Rf values of glucose, portion of aglycone was analyzed by TLC, with β-sitosterol as standard contrast; both of the two Rf values were the same. It testified portion of aglycone was β-sitosterol and sugar was glucose.

In the infrared spectrum (Figure 5), l055 cm^{-1} was the

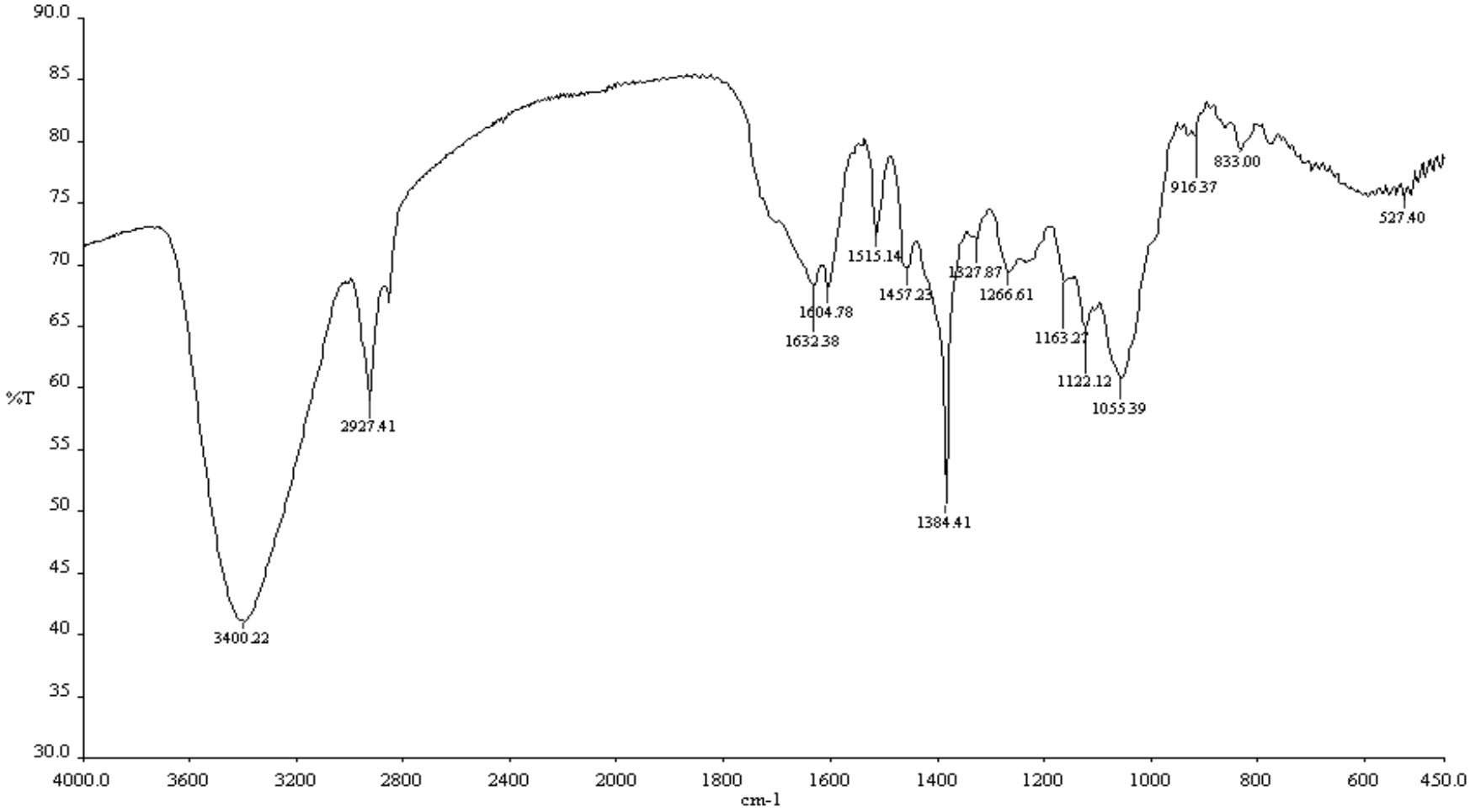

Figure 5. IR spectra of extraction U4.

absorption peak of C-O stretching vibration; 3400 cm^{-1} was absorption peak of -OH-based stretching vibration, caused by the intermolecular hydrogen bonds; 1604 cm^{-1} was caused by the strong C=O stretching vibration absorption peak; absorption peak of 1384 cm^{-1} was caused by -CH_3 stretching vibration; absorption peak 2927 cm^{-1} was generated by the -CH stretching vibration. From the mass spectrum in Figure 6, the positive ion scan m/z was 577 for the [M +1], peak for the fragment ions m/z was 415 [M+1-Glu], m/z577 lost a glucose-based (mass 162) caused. Based on the above data, the extraction U4 was daucosterol.

According to earlier mentioned identification and the structure analysis, we concluded that the active components with hypoglycemic effect in leaves were flavonoids and daucosterol. It reported that the total flavonoids from persimmon leaf

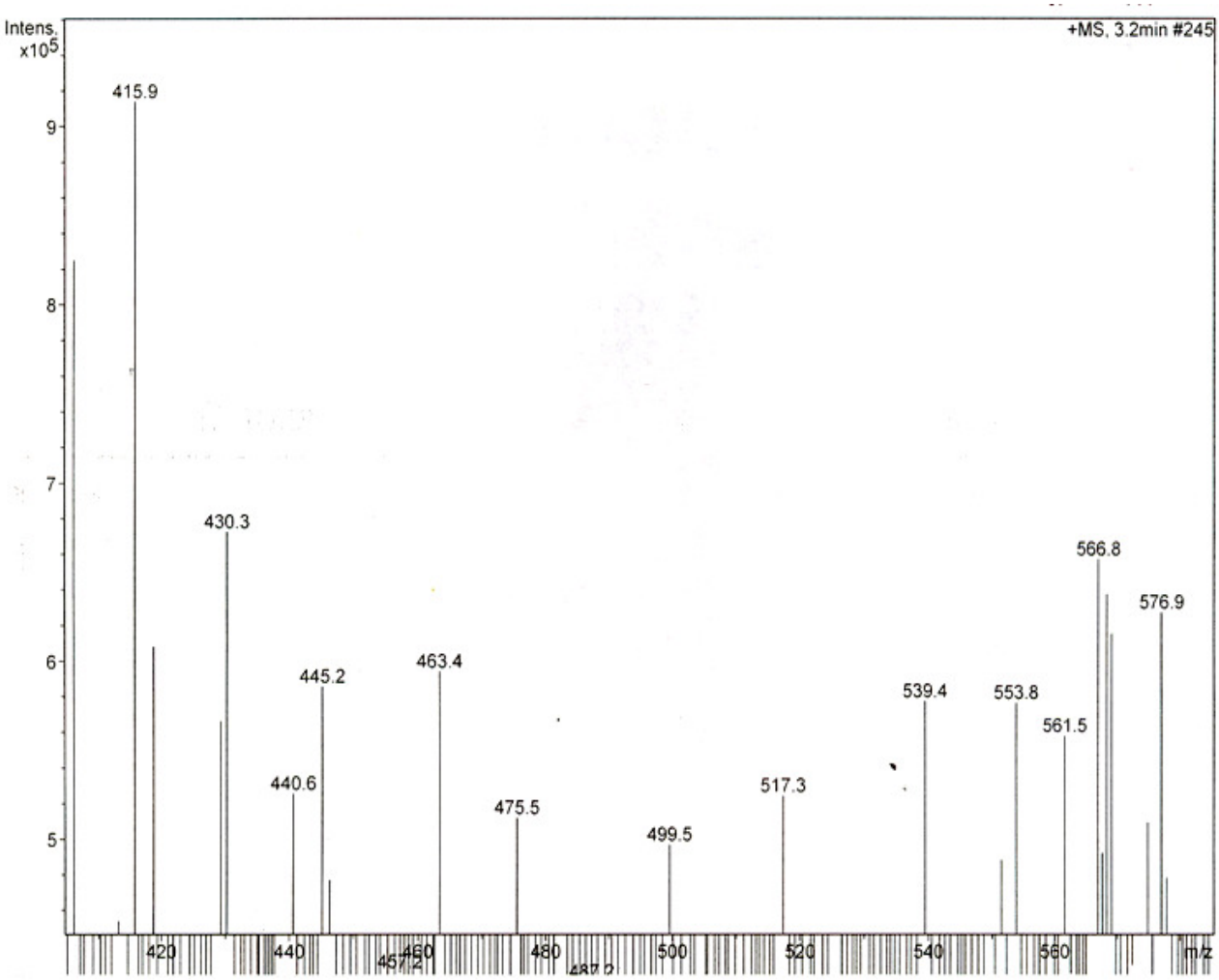

Figure 6. ESI-MS spectra of extraction U4.

had significant hypoglycemic activities on diabetic mice (Gao et al., 2009). Eagle flavonoids had hypoglycemic, which is related with its promotion of INS secretion and the regulation of lipid metabolism disorders (Lv et al., 2008). Therefore, flavonoids had a hypoglycemic effect, possibly because flavonoids could inhibit aldose reductase (rate-limiting enzyme in glucose metabolism). Daucosterol belongs to steroidal glycosides, it is similar to the role of sulfonylurea drugs, they are directly stimulating β-cells, then release insulin (Gan and Xu, 1999).

Conclusion

Through the oral glucose tolerance test in mice, leaf decoration showed significantly hypoglycemic trend among root, stem, and leaf extracts from *U. angustifolia*. For further screening of leaf hypoglycemic active ingredients, four components were extracted and extractions U3 and U4 were tested having active ingredients. Then, the chemical and structural identification were analyzed. Extraction U3 was flavonoids and U4 was daucosterol identified by TLC, IR and ESI-MS spectral analysis. The extraction from *U. angustifolia* leaves could notably lower the blood sugar levels of the normal mice. This indicated that the extractions can be used for the prevention and treatment of diabetes and be efficient as a potent functional food.

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