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Humulus Scandens Chemical Constituents Determination and Pharmacological Action Research

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Abstract

Humulus Scandens has been widely used for a long time in folk medicine, but it has not been systematically researched until now. The aims of this study were to isolate the components and simultaneously determine the contents of luteolin-7-O-*β*-D-glucoside (LGL) and apigenin-7-O-*β*-D-glucoside (AGL) in the different parts of *H. Scandens*. At the same time, our research team verified the anti-cancer activity and the alveolar fluid clearance (AFC) of *H. Scandens* total flavonoids (HSTF), LGL and AGL. The anti-cancer experiment showed that HSTH and LGL have a significant inhibition effect on the HepG2 cell. The cell viability decreased when drug concentration increased, but AGL has an indistinctive effect on HepG2 cell under the same concentration. The AFC experiment showed that AFCs of HSTF, LGL and AGL, compared to the blank control group, increased, which explained the *H. Scandens* pharmacological effect of removing edema. This result provides the theoretical basis for *H. Scandens* clinical application.

Keywords: *Humulus Scandens* total flavonoids; Luteolin-7-O-*β*-D-glucoside; Apigenin-7-O-*β*-D-glucoside; Determination; Inhibitory tumor; Alveolar fluid clearance

Introduction

The whole herb of *Humulus Scandens (Lour.)* Merr. listed in the medical literature in *Tang Ben Cao,* is effective in reducing heat, detoxicating, treating diuresis and promoting detumescence. It has been widely used for a long time in folk medicine [1]. *H. Scandens* with a strong vitality can adapt to the hardy soil texture and climate conditions of the ecological environment at an altitude of 500-1500m of barren hills, valleys, woodland and is widely distributed in northern temperate and subtropical zones, primarily in China, Korea and Japan [2]. It can be found in great abundance in nature and contains multiple flavonoids. Luteolin-7-O-*β*-D-glucoside (LGL) and apigenin-7-O-*β*-D-glucoside (AGL) are the main components of *H. Scandens* extract (HSE) [3]. Flavonoids have been confirmed by various studies to possess a wide range of pharmacological action, especially in anti-oxidation [4,5], anti-inflammation [6], prevention of coronary heart disease [7], anti-HIV pharmacological action [8] and inhibition of tumor promotion effects [9].

The aims of this study were to isolate flavonoids from the whole herb of *H. Scandens (Lour.)* Merr. and verify their structures by spectrograph. The HPLC method was adopted to determine the component contents in different parts of *H. Scandens*. Meanwhile, we chose HSTF, LGL and AGL components as the research objects and evaluated the antitumor activity *in vitro* and the removing edema effect *in vivo*. All these provided *H. Scandens* a certain theoretical basis for clinical application.

Experiments

Isolation and structure identification of flavonoids in *H. Scandens*

Plant material: The whole herbs of *H. scandens* (Lour.) Merr. were collected in Shenyang (Liaoning, China) in June 2007. The plant was identified by Prof. Xixiang Ying. Duplicate voucher specimens (Voucher number: No. 20070603) were deposited at the Central Lab of the School of Pharmaceutical Science, China Medical University (CMU).

Isolation and structure identification: A sample (5kg) of the whole herb of *H. Scandens* (Lour.) Merr. was extracted three times with 40 L of 70% aqueous ethanol. The crude extract was concentrated and then passed through a porous-polymer resin (D101, Tianjin, China). The fraction eluted with 60% ethanol was evaporated under reduced pressure to obtain the HSTF containing 51.2% LGL and 32.1% AGL.

300 g of HSTF treated with resin was chromatographed on a silica-gel column with chloroform-methanol as the gradient eluent and then purified by preparation HPLC to afford the main compounds LGL and AGL, and the low content of vitexin (VIT). The purities checked by HPLC were 99.7%, 99.2% and 99.1%, respectively. The spectrum data of LGL (Figure 1a), AGL (Figure 1b) and VIT (Figure 1c) [10].

Contents determination of LGL and AGL in different parts in *H. Scandens*

Chromatographic system and conditions: The LC system consisted of a Waters 600 solvent delivery pump, a Waters 2487 UV-vis spectrophotometric detector, an Empower system controller (Waters, USA), and an HPLC analytical column (Diamonsil™ C_{18} column, 4.6×200 mm, i.d., 5 μm particle size, Dikma) connected with a guard column filled with the same chromatographic stationary phase.

The mobile phase for HPLC analysis consisting of methanol-0.2% phosphoric acid (45:55, v/v) were filtered under reduced pressure and degassed by ultrasonic before use. HPLC analysis with UV detection at 350 nm was performed at a flow rate of 1.0 mL/min. HPLC column temperature was 35°C. The sample injection volume was 10 μL. The chromatogram was shown in Figure 2.

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Preparation of standard solution and sample solution: The suitable amounts of LGL and AGL were accurately weighed and dissolved in methanol to yield concentrations of 25 μg/mL and 18 μg/mL respectively.

One gram of each sample collected in August from different parts: root, stem, leaf, and petiole, was dried, comminuted and weighed accurately. Then each sample was put into Soxhlet Extractor. The following procedure was carried out for each sample: the Petroleumether (30~60°C) was added to the Extractor and was extracted for 1.5 h. Then the filtrate solvents were filtered out and discarded. After that, methanol was added to the Extractor and was extracted for another 4 hours. Then the methanol was reclaimed by distillation. The sediment was once again dissolved by methanol and transferred to 50 mL volumetric flask. This solution was then filtered by a (0.45μm) microfiltration membrane before use. After this procedure was completed for each sample, 10 μL of the solution of each sample was measured out and ready to use for the next step.

Performance assessment: Five-point calibration lines were constructed from peak areas of LGL and AGL versus concentrations of the calibration standards of them ranging from 0.05 to 0.40 μg (LGL) and 0.072 to 0.36 μg (AGL), respectively. Sample concentrations of LGL and AGL were calculated from the regression equations $y_1 = 3.068 \times 10^6 x_1 + 1.537 \times 10^5$, r = 0.9999 for LGL and $y_2 = 2.670 \times 10^6 x_2 + 1.273 \times 10^4$, r=0.9997 for AGL, where y_1 and y_2 were the peak areas of LGL and AGL (as longitudinal coordinate), x_1 and x_2 were the concentrations of LGL and AGL (as horizontal coordinate), and r was the correlation coefficient.

According to the method we published before [11], we determined the contents of LGL and AGL in different parts in *H. Scandens*. The contents determination results of sample (n=3) were shown in Table 1.

Anti-cancer experimental of HSTH, LGL and AGL i*n vitro*

Reagents: HSTH, LGL and AGL were obtained from The Central Lab., School of Pharmaceutical Science, China Medical University (LGL, AGL > 99% purity by HPLC, respectively). RPMI 1640 and FBS were obtained from GIBCO Life Technologies. DMSO, trypan blue and Adriamycin (ADM, Positive control) were purchased from Sigma, USA.

Cell cultures: Human hepatocellular carcinoma cell line, HepG2, was provided by the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. It was cultured with RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and maintained at 37°C in a humidified atmosphere of 5% carbon dioxide / 95% air. The cells were counted and plated at the same initial density for treatments with various concentrations of HSTF, LGL and AGL dissolved in DMSO.

Cell growth inhibition assay: The effects of HSTF, LGL and AGL treatment on the viability of HepG2 cells were measured by the trypan blue dye method. 5×10^5 cells/well were grown in 6-well plates for 24 h and the cells were further incubated with different concentrations (0, 6.25, 12.5, 25.0, 50.0, 100.0 μM, respectively) of HSTF, LGL, AGL and ADM in RPMI-1640 supplemented with 10% FBS medium. After 72 h, trypsinization, collection cells, trypan blue dye and counted under the microscope. At least three replicate experiments were performed with three wells per concentration. The relative viability effect = [total viable cells (unstained) / total cells (stained and unstained)] x 100%.

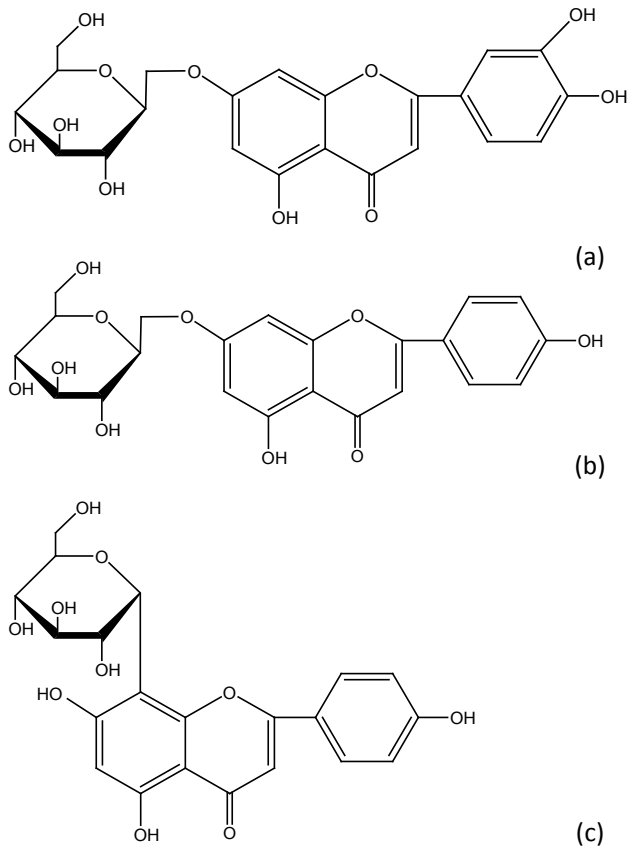

Figure 1: Chemical structures of luteolin-7-O-*β*-D-glucoside (a), apigenin-7-O-*β*-D- glucoside (b) and vitexin (c).

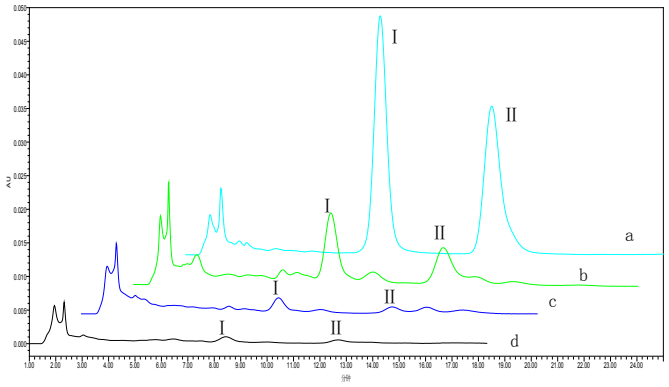

Figure 2: Typical HPLC chromatograms for the contents determination of luteolin-7-O-β-D-glucoside (I) and apigenin-7-O-β-D-glucoside (II) in different parts.
a.Chromatogram of leaves sample solution.
b.Chromatogram of stems sample solution.
c. Chromatogram of petioles sample solution.
d.Chromatogram of roots sample solution

Parts	LGL (mg/g)	AGL (mg/g)
Root	0.07	0.08
Stem	0.98	0.45
Leaf	1.31	0.91
Petiole	0.18	0.29

Table 1: Results of contents determination in different parts in *H. Scandens* (n=3).

Alveolar fluid clearance (AFC) experimental research of HSTH, LGL and AGL *in vivo*

Alveolar fluid clearance was examined *in vivo*. 8-10 weeks old, male KM mice, weighting 25-35 g, were used. All the mice were supplied by the Laboratory Animal Service Center of CMU and performed according to the requirement of the National Act on the Use of Experimental Animal (China) that was approved by the Committee of Ethics of Animal Experimentation of CMU. An isosmotic instillate containing 5% fatty-acid free bovine serum albumin (BSA) was prepared with 0.9% NaCl. Anesthetized mice were ventilated with 100% O_2 via a small animal breathing machine for a 30 minute period. 5% BSA (0.3 mL), with or without terbutaline and amiloride was instilled intratracheally as the control groups. HSTF, LGL and AGL groups, which contained 5% BSA (0.3 mL), with or without amiloride were instilled intratracheally as the experimental groups. The instilled alveolar fluid was aspirated by applying gentle suction to the tracheal catheter with a 1 mL syringe. The BSA content of the alveolar fluid was measured with a 96 well microplate reader. Alveolar fluid clearance (AFC) was calculated as follows: AFC = (Vi - Vf) / Vi * 100, where Vi and Vf denote the volume of the instilled and recovered alveolar fluid, respectively. Vf was obtained as Vf = (Vi * Pi) / Pf, where Pi and Pf represent protein concentration of instilled and collected fluid.

Result

The result of isolation and structure identification of flavonoids

Flavonoids monomer ingredients have been obtained from *H. Scandens* by the column chromatography technology and identified through UV, IR, MS, NMR technology. They are: luteolin–7–O–*β*–D-glucoside, apigenin–7–O–*β*–D-glucoside and vitexin. The structural formulas are shown in Figure 1.

The results of contents determination

Through the HPLC method, we determined the contents of LGL and AGL in different parts of *H. Scandens*. The determination result showed that the contents of LGL and AGL were 0.07 and 0.08 mg/g in root, 0.98 and 0.45 mg/g in stem, 0.18 and 0.29 mg/g in petiole, 1.31 and 0.91 mg/g in leaf, respectively. All the data were shown in the Table 1 and Figure 3.

The result of anti-cancer experimental research of HSTH, LGL and AGL i*n vitro*

The inhibitory effect of HSTF, LGL and AGL on HepG2 cell was measured by Trypan blue dye assay. Data were obtained from triplicate wells per condition and representative of at least three independent experiments.

HSTF and LGL have the best inhibition effect, with the cell viability decreasing from 100% to 4.8% and from 100% to 5.5% as the concentration increasing from 0 to 100 μM, respectively. AGL has an indistinctive effect on $HepG_2$ cell under the same concentration. The result showed in Figure 4.

AFC experimental of HSTH, LGL and AGL i*n vitro*

The AFC experiment showed that 5% BSA blank control group AFC is 29.8 %; Amiloride group AFC is 20.8 % ($P<0.05$); Terbutaline group AFC is 42.0 % ($P<0.05$); LGL group AFC is 34.3 %; LGL+Amiloride group AFC is 22.8 % ($P<0.05$); AGL group AFC is 35.4 %; AGL+Amiloride group AFC is 21.0 % ($P<0.05$); HSTF group AFC is 38.9 % ($P<0.05$); HSTF+Amiloride group AFC is 26.7 % ($P<0.05$). The results showed in the Table 2 and Figure 5.

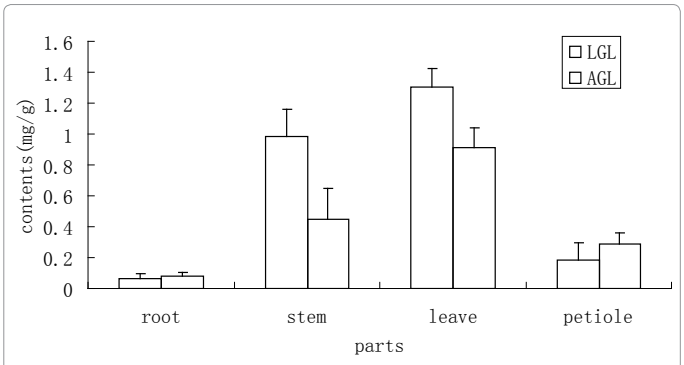

Figure 3: Contents determination results of LGL and AGL in different parts (n=3).

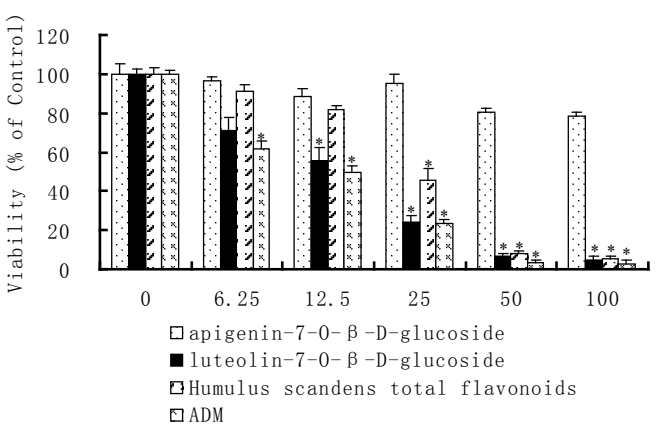

Figure 4: The viability effect of HSTF, LGL and AGL on HepG2 cell proliferation. "*" compared with the blank group.

Groups	AFC(%) Mean±SD	n
Blank group	29.8±6.9	7
Amiloride group	20.8±5.2*	8
Terbutaline group	42.0±4.0*	8
LGL group	34.3±3.3	7
LGL+Amil group	22.8±8.5#	7
AGL group	35.4±9.5	6
AGL+Amil group	21.0±2.7#	5
HSTF group	38.9±4.0*	5
HSTF+Amil group	26.7±7.7#	5

"*" compared with the blank group;
"#" compared with the same group without Amiloride

Table 2: AFC (%) experiment determination results.

Discussion

1. The experiment investigated three kinds of extraction methods - ultrasonic extraction, refluxing extraction and Soxhlet's extraction. The results showed that the sample solution treated by Soxhlet's extraction was the best which had a higher content of LGL and AGL and had a lower impurity. The ultrasonic extraction had the minimum content.

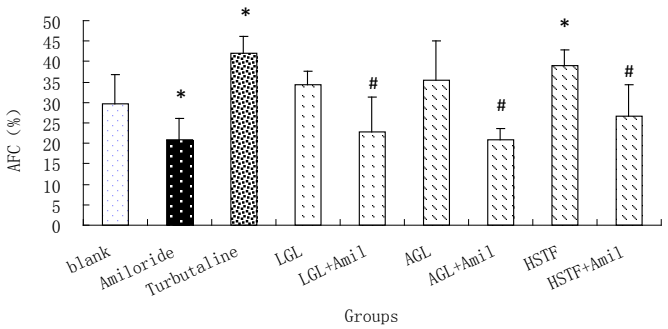

Figure 5: AFC (%) determination results.

2. The UV spectrum showed that LGL and AGL had obvious absorbent peaks at the wavelength 350 nm and 330 nm respectively after UV-scanning via an ultraviolet spectrophotometer, thus 350 nm was selected as detection wavelength. Our previous experiment results showed [11] that the best seasons for collections were from Jun. through Aug. so we collected the materials in August and divided them into different parts: root, stem, leaf and petiole. The HPLC method was adopted to determine the content of LGL and AGL. The leaf contains more LGL and AGL than other parts. The orders are: leaf > stem > petiole > root. The contents of LGL and AGL in leaf samples are dozens of times greater than that in roots. Meanwhile, a greater variety of ingredients is contained in plant parts other than a leaf, but the contents of LGL and AGL is what is most significant. We also determined the total flavonoids contents by $NaNO_2$-$Al(NO_3)_3$-NaOH colorimetry. The total flavonoids determination result showed that the root contained more flavonoids than other parts. Results of experiments on the root seem to be contradictory. We analyzed the reasons: The first is some flavonoids contained in the root might have a lower absorbance at 350 nm, so we could not find the strong peaks in the chromatograms. The second is that the colorimetry has a poor specificity [12]. Some substances like pyrocatechols may interfere with the determination. Considering the published and current test results, we draw the conclusion that the collection seasons of *H. Scandens* are best from June through August and as medicinal plant application, the parts should be separated, and the best application parts are its leaves.

3. Trypan blue dye assay is the frequently-used method in determining the anti-tumor activity *in vitro*. HepG2 cell was used to determine the activity of HSTF, LGL and AGL. The results showed that HSTF and LGL have a significant positive correlation between drug concentration and the cell viability. But AGL was obviously lower than HSTF and LGL's. The literature [13] showed that the presence of the C_2–C_3 double bond on the C ring, conjugated with the 4-oxo function, was critical for this biological activity. From a structural point of view, the double bond between C_2 and C_3 results in ring B and ring C being on the same plane, which might be crucial for access to the kinase substrate binding site, and the two adjacent polar OH groups on C_3 and C_4 of ring B are required for suppressing kinase activity. This is in accordance with our result. It demonstrates that flavonoids such as LGL exhibit potent anti-tumor bioactivity.

4. The AFC experiment showed that amiloride, as an ENaC blocker, could decrease the rate of AFC in mice about 30.2 %. Terbutaline, as an ENaC opener, could increase the rate of AFC in mice about 40.9 %. The AFC of both amiloride group and terbutaline group, compared to the blank control group, have a significant difference ($p<0.05$). These showed that the method is feasible. All of the AFCs of HSTF, LGL and AGL, compared to the blank control group, increased. which explained the *H. Scandens* pharmacological effect of removing edema. The AFCs of HSTF, LGL and AGL reduced after amiloride was added explained the water absorption is mainly through the ENaC mechanism. All these provided *H. Scandens* a certain theoretical basis for clinical application.

Acknowlegements

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