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Flash extraction of the active constituents and its antitumor activity from rehmanniae radix, achyranthes bidentatae radix, dioscoreae rhizoma and chrysanthemi flos

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Abstract: In this study, we applied the Flash extraction (FE) for the first time to the extraction of active ingredients of Sidahuaiyao (including Rehmanniae Radix, Achyranthes Bidentatae Radix, Dioscoreae Rhizoma, and Chrysanthemi Flos), and the content of active ingredients (catalpinoside, ecdysterone, chlorogenic acid and diosgenin) was determined by HPLC, and compared with Soxhlet extraction (SE) and ultrasonic extraction (UE). The results show that under the same solvent ratio, FE is used to extract the largest amount of different active ingredients. Compared with SE and UE, the extraction amount increases by 20.8% -92%. It is demonstrated for the first time that using FE to extract the active ingredients from Sidahuaiyao produced the highest extraction efficiency. In addition, we evaluated the anticancer activities of the main components. Three cancer cells and one normal cells were used to detect the anti-proliferative activity by MTT assay. The results showed that diosgenin had the strongest inhibitory effect on MCF-7 cells with IC_{50} value of 19.28±0.36μM. In short, we optimized the extraction process of Sidahuaiyao, and evaluated the anti-cancer activity of the main components, which provided a scientific theoretical basis for the application of Sidahuaiyao.

Keywords: Achyranthes bidentatae radix, chrysanthemi flos, dioscoreae rhizoma, rehmanniae radix, flash extraction.

INTRODUCTION

Extraction is a very fundamental and necessary step to utilize crude drug and provide concentrated active ingredients for manufacturing solid or semi-solid dosage medicines, such as tablet, pill, paste and capsule, and for preparing any single herbal extract for the purpose of pharmacological, phytochemical and analytical research (Dhanani *et al*., 2017; Ghasemzadeh *et al*., 2017; Xu *et al*., 2016). Traditionally, completing an extraction procedure often needs hours to days, such as boiling extraction, refluxing extraction, soxhlet extraction, soaking extraction, and so on. Even for ultrasonic extraction, half hour per time for several times is usually required (Manukovskaya *et al*., 2021; Rahman *et al*., 2021). Kaufmann reviewed microwave-assisted extraction (MAE) and pressurized solvent extraction (PSE) as recent extraction technology for natural products (Kaufmann *et al*., 2002). Through numerous examples, it was demonstrated that both MAE and PSE techniques reduced solvent consumption and shortened extraction times, while the extraction yields of the analytes are equivalent to or higher than those obtained with conventional methods. The disadvantage of MAE is, however, obvious in that the temperature must be carefully controlled to prevent the decomposition of some heat sensitive components, for example, paclitaxel that decomposed at temperatures above 115°C (Kaufmann *et al*., 2002). Flash extraction (FE) is a convenient, fast and efficient extraction process that can effectively shorten room temperature extraction and retain more effective ingredients in the extracted plants (Song *et al*., 2019). FE is based on the principle of tissue extraction, using a solvent to crush the material to the appropriate particle size in a flash extractor. At the same time, FE performshigh-speed stirring, strong vibration and negative pressure percolation to achieve extractions, which are conducive to the extraction of heat-sensitive components. According to the previous research, we hope to use FE to extract the active ingredients of Sidahuaiyao, in order to get better extraction methods and conditions.

Sidahuaiyao is specifically referred to four kinds of authentic herbs (Four kinds of traditional Chinese medicine are mainly distributed in four counties (Wenxian, Mengzhou, Qinyang and Wuzhi) in Jiaozuo, Henan). Sidahuaiyao includes Rehmanniae Radix, Achyranthes Bidentatae Radix, Dioscoreae Rhizoma and Chrysanthemi Flos. Due to their high intrinsic quality and therapeutic effects, they are well-known herbs that have been known for thousands of years. Rehmanniae Radix is a common Chinese materia medica used as a tonic agent frequently in the prescriptions of traditional Chinese medicine. Catalpol (fig. 1) is one of the major effective components of Rehmanniae roots with anti-brain ischemia, anti-senile dementia, neuroprotection, lowering

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blood glucose, anticancer, anti-viral hepatitis, spasmolysis and reducing capillary permeability etc (Meng *et al*., 2020;Yan *et al*., 2018; Zhang *et al*., 2019). The most recent research suggested that the anti-aging effect of catalpol was achieved at least partly by promoting endogenous antioxidant enzyme activities and normalizing energy disturbance (Wang *et al*., 2019). Catalpol may be a potential anti-aging agent for further preclinical study aimed for senescence or neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Bhattamisra *et al*., 2020). Therefore, quantitative determination of catalpol in Rehmanniae Radix and its extract is very important for the quality evaluation of both the crude drug and extract as an ingredient of some clinical formulae. Herein we use the content of catalpol as the quality indicator for Rehmanniae (Lin *et al*., 2005).

Achyranthes bidentatae Radix is derived from the dried roots of *Achyranthes bidentatae* Bl, a perennial herb of Amaranthaceae. Modern pharmacological studies showed that ecdysterone (fig. 1), the main active compound in Achyranthes Bidentatae Radix, can enhance immune function of host cells of the tumor-bearing rat and improve the immune system by enhancing humoral and cellular immune function (Lee *et al*., 2014). To date, there is no recorded guidence for the determination of ecdysterone in Achyranthes Bidentatae Radix in the Chinese Pharmacopoeia. To obtained ecdysterone, accelerated solvent extraction of ecdysterone from A.Bidentata had been reported (Wang *et al*., 2005). In this research, FE was successfully used for the extraction of ecdysterone. The content of ecdysterone was determined based on an HPLC method (Wang *et al*., 2005) to evaluate the extraction effectiveness of FE over the conventional Soxhlet and ultrasonic extraction methods.

Dioscoreae Rhizoma, the rhizome of *Dioscorea opposita* Thunb of Dioscoreaceae, is a well known tonic herbal medicine and health diet for its benefits to spleen, lung, and kidney. Diosgenin (fig. 1), a precursor of steroidal hormone commonly found in Dioscoreaceaeous plants, has very wide pharmacological activities including anticancer, blood lipid lowering and anti-inflamation (Mafalda *et al*., 2016). It is applicable to use diosgenin as a chemical marker for this herb to evaluate the extraction efficiencies by FE and other methods (Wang *et al*., 2017).

Chrysanthemi Flos, the dried capitulum *of Chrysanthemi morifolium* Ramat of Compositae, has been used as a traditional Chinese medicine for a long time. Modern pharmacological research shows, it has been widely used for the treatment of anemopyretic cold, headache, dizziness and acute conjunctivitis (Nie *et al*., 2019). The flavonoids and chlorogenic acid (fig. 1) are thought to be active substances of Chrysanthemi Flos with effects of antibiosis, anti-cancer and anti-inflammation. According to the Chinese Pharmacopoeia, the content of chlorogenic acid is used as quality index of Chrysanthemi Flos (Wang *et al*., 2016).

FE is performed by Herbal Blitzkrieg Extractor (HBE) (Liu *et al*., 2016). HBE combines all technological superiorities of simultaneous smashing, soaking, stirring, vibrating actions to finish the extraction within seconds to less than one minute, which is just one tenth to one hundredth of conventional methods, such as refluxing extraction. FE not only avoids the decomposition of heat-sensitive ingredients, but also provides relatively higher yield, simplicity of operation, and benefit for environment protection.

In this paper, we first used FE to extract the main components of Sidahuaiyao, compared with ultrasonic extraction (UE) and Soxhlet extraction (SE), the main components in the four kinds of traditional Chinese medicine were used as detection indexes to evaluate the extraction rate of different extraction methods and optimize the extraction process. In addition, based on the pharmacological activities of Sidahuaiyao, we evaluated the anti-cancer activities of the four main ingredients to expand the application prospects of Sidahuaiyao.

MATERIALS AND METHODS

Chemicals and reagents

Catalpol (NO.110808), diosgenin (NO. 1539-2000019) and chlorogenic acid (NO. 110753-200413) were obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); Ecdysterone was isolated in our own laboratory with purity >99%; Herbal materials Sidahuaiyao were purchased from Henan province and identified by professor Qishi Sun, Shenyang Pharmaceutical University; Methanol, acetonitrile, phosphoric acid and natrium biphosphoricum were analytical grade; Doubly distilled water was prepared in the laboratory.

Equipments

The following equipment was used: JHBE-50S Herbal Blitzkrieg Extractor (Henan Jinnai Sci-Tech Development Co., Ltd.), BS-124S precise electronic balance (Sartorius Corporation), RE-52A rotary evaporator (Shanghai Yali Rongsheng Hua Instrument Factory), Thermostatic water bath (Tianjin Taisite Instrument Co., Ltd.), KQ3200DB ultrasonic cleaning bath (Kunshan Ultrasonic Instrument Co., Ltd.), Beijing Chuangwei high performance liquid chromatogram (UV3000 ultraviolet detector and attemperator), CXTH-3000 chromatography work station, and Kromasil C_{18} (4.6×250mm) chromatographic column.

Chromatographic conditions

HPLC conditions for four herbal materials were listed in table 1. All sample solutions were filtered through 0.45μm filter before injection.

Preparation of standard solution

The following standard solutions were prepared: For Rehmanniae Radix, catalpol 1mg/mL in water; for Achyranthes Bidentatae Radix, ecdysterone 0.2mg/mL in methanol; for Dioscoreae Rhizoma, diosgenin 1mg/mL in methanol; and for Chrysanthemi Flos, chlorogenic acid 1 mg/mL in water.

Preparation of analyzing samples from extract

Soxhlet extraction (SE)

Rehmanniae Radix: 4g of Rehmanniae Radix was extracted with 250mL of methanol in a Soxhlet extractor for 1.5 hours and lost volume of methanol after cooling down at room temperature was restored as required. After filtration, the methanol solution was concentrated and dried under reduced pressure with a rotary evaporator. The residue was dissolved with mobile phase and diluted to 100mL in a volumetric flask. Achyranthes Bidentatae Radix: 5g of Achyranthes Bidentatae Radix was extracted with 100mL of 80% methanol in a Soxhlet extractor for 6 hours and the methanol solution after filtration was concentrated and dried under reduced pressure with a rotary evaporator. The residue was dissolved with methanol and diluted to 100mL in a volumetric flask. Dioscoreae Rhizoma: 2g of Dioscoreae Rhizoma was extracted with 150mL of 90% methanol in a Soxhlet extractor at 80°C for 10 hours. The methanol solution after filtration was concentrated by using the above condition to remove methanol. Then, 200ml of 5% hydrochloric acid and methanol (1:1 v/ v) was added to the residue and refluxed for 5 hours. After cooling to room temperature, it was adjusted to pH 8 with 12 mol/L sodium hydroxide. The solution was extracted three times with 200mL of chloroform. The combined chloroform extract was concentrated to dry with the above condition. The residue was dissolved in methanol and diluted to 10 mL in a volumetric flask. Chrysanthemi Flos: 1g of Chrysanthemi Flos powder was extracted with 50mL of methanol in a Soxhlet extractor at 80°C for 2 hours with same condition as Rehmanniae Radix. The methanol solution was concentrated to dryness under reduced pressure with a rotary evaporator. The residue was washed with 5mL of chloroform to remove fatty impurities. The residue was dissolved with water and diluted to 1mL in a volumetric flask. All solutions obtained above were filtered through 0.45μm filter for HPLC analysis.

Ultrasonic extraction (UE)

Rehmanniae Radix: 4g of dried Rehmanniae Radix was extracted with 250ml of methanol in a KQ3200DB Ultrasonic cleaning bath for 30min. Lost methanol was added after cooling down naturally if required. Methanol solution obtained by filtration was concentrated to dryness under reduced pressure. The residue was dissolved with the mobile phase and diluted to 100mL in a volumetric flask. Achyranthes Bidentatae Radix: 5g of dried powder of Achyranthes Bidentatae Radix was extracted with 100ml of 80% methanol in KQ3200DB Ultrasonic cleaning bath for 40 min. The methanol solution obtained was concentrated to dry under reduced pressure. The residue was dissolved with methanol and diluted to 100mL in a volumetric flask. Dioscoreae Rhizoma: The Dioscoreae Rhizoma was crashed to small peace and ground to crude powder based on extraction requirement (≤40 mesh), and then dried to consistent weight in a vacuum oven. 2g of Dioscoreae Rhizoma was extracted with 150mL of methanol in a KQ3200DB Ultrasonic cleaning bath for 40 min. After cooling down naturally and filtration, the methanol solution was concentrated with rotary vacuum evaporator and dried in water bath. The residue was dissolved with methanol and diluted to 10mL in a volumetric flask. Chrysanthemi Flos: 1g of Chrysanthemi Flos was extracted with 50mL of methanol in a KQ3200DB Ultrasonic cleaning bath for 30 min with same procedure as Rehmanniae Radix. The residue obtained was disposed as in Soxhlet extraction. All solutions obtained above were filtered through 0.45μm filter for HPLC analysis.

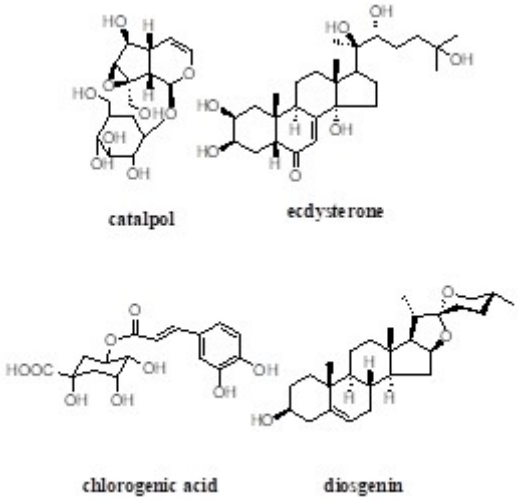

Fig. 1: The structures of detected active compounds in *Sidahuaioyao*

Flash extraction (FE)

The orthogonal experiment of three factors and three levels, i.e., FE time (A), extraction times (B) and liquid/solid ratio (C) was designed to find the optimum extraction condition of their active components. Same levels of A_1=1 min, A_2=2 min, A_3=3 min; B_1=1, B_2=2, B_3=3 and C_1=6:1, C_2=8:1, C_3=10:1 were applied for Rehmannia Radix and Achyranthes Bidentatae Radix. Level A and B were same for Dioscoreae Rhizoma and Chrysanthemi Flos, while C_1=55:1, C_2=75:1 and C_3=95:1

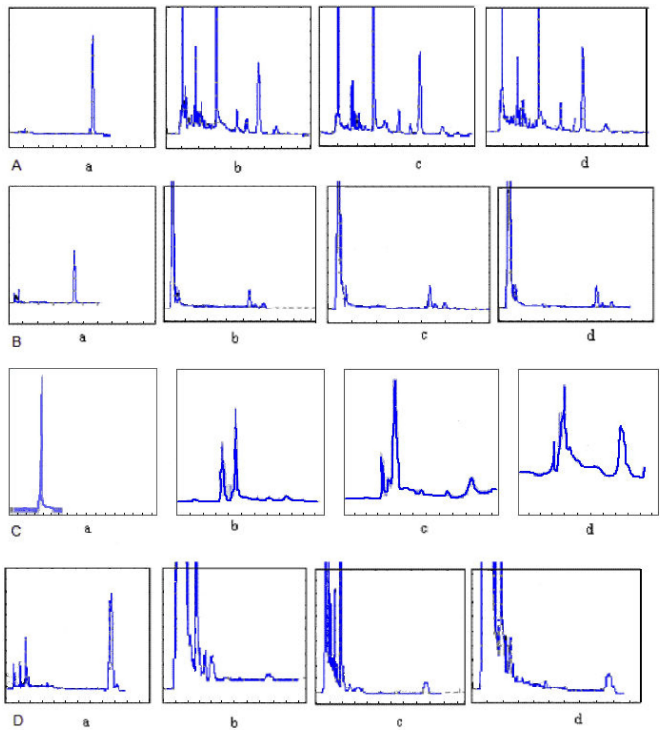

Fig. 2: HPLC Chromatograms of mark compounds from *Rehmanniae Radix* (A)*, Achyranthes Bidentatae Radix* (B), *Chrysanthemi Flos* (C) and *Dioscoreae Rhizoma* (D) by different extract methods: a standard solution; b SE; c UE; d FE.

was selected for Dioscoreae Rhizoma and C_1=30:1, C_2=50:1 and C_3=70:1 was selected for Chrysanthemi Flos based on their different physical properties.

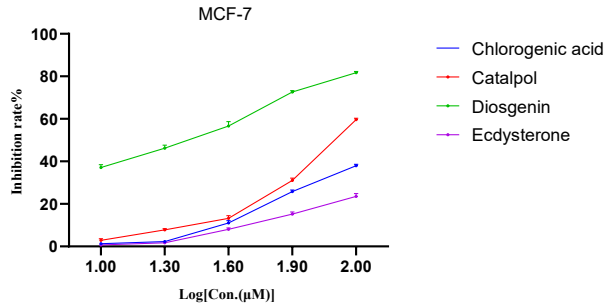

Fig. 3: The inhibition rate of the four compounds on MCF-7 cells

Cell culture

Three cancer cells (human lung cancer cell lines A549, Human colon cancer cell lines HCT-116, human breast cancer cell lines MCF-7) and a normal cell (human gastric epithelial cell lines GES-1) were bought from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute (RPMI-1640), supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity Assay

Cell viability was measured using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazo-lium bromide) assay. The cells were treated with the compound (100, 80, 40, 20, 10μM) for 48h. Then, 10μL of MTT (5 mg/mL) was added to each well and the cells were incubated at 37°C for 4h in the dark. The crystals were dissolved in DMSO (100μL/well). The solution was stirred for 10 minutes, and the absorbance was measured at 490 nm using a microplate reader to calculate a 50% inhibitory concentration (IC_{50}).

STATISTICAL ANALYSIS

Data are presented as the mean ± S.E.M. Statistical analysis was performed using Graph Pad Prism 5 software.

RESULTS

As show in table 2, it is clear that FE was successfully applied to the extraction of the active ingredients in Sidahuayao, Rehmanniae Radix, Achyranthes Bidentatae Radix, Dioscoreae Rhizoma and Chrysanthemi Flos for the first time. HPLC is used to detect the content of active ingredients (fig. 2). The content of chlorogenic acid in Chrysanthemi Flos extract obtained by FE doubled that of UE and 68.4% higher than that of SE. The content of diosgenin in Dioscoreae Rhizoma extract obtained by FE

Table 1: HPLC conditions for each of the four herbs

Herbs	Mobile phase*	Wavelength	Temperature
Rehmanniae Radix	acetonitrile/0.1%phosphoric acid,0.5:99.5	203 nm	40°C
Achyranthes Bidentatae Radix	methanol/water 40:60	242 nm	25°C
Dioscoreae Rhizoma	methanol/water, 95:5	215 nm	30°C
Chrysanthemi Flos	Natrium biphosphoricum buffer/methanol, 70:30	328 nm	25°C

Table 2: Extraction conditions for each of the four herbs by FE and contents of detected compounds (%)

Herbs	Solvent ratio	Time (min)	Extraction times	Contents of mark compounds (%)		
				FE	SE	UE
Rehmanniae Radix	8:1	3	3	0.51	0.35	0.40
Achyranthes Bidentatae Radix	10:1	3	3	0.58	0.34	0.48
Dioscoreae Rhizoma	75:1	3	3	0.50	0.26	0.32
Chrysanthemi Flos	30:1	3	3	1.65	0.98	0.82

Table 3: Anti-cancer activities of four main components

Compound	IC_{50} (μM)			
	HCT-116	A549	MCF-7	GES-1
Chlorogenic acid	>100	98.09 ± 1.08	>100	>100
Catalpol	86.35 ± 0.69	>100	94.52 ± 1.24	>100
Diosgenin	32.15 ± 0.24	22.27 ± 0.54	19.28 ± 0.36	>100
Ecdysterone	>100	>100	>100	>100

was 92% higher than that of SE and 56.3% higher than that of UE. The content of ecdysterone in Achyranthes Bidentatae Radix extract obtained by FE was 70.6% higher than that of SE and 20.8% higher than that of UE. The content of catalpol in Rehmanniae Radix extract obtained by FE was 45.7% higher than that of SE and 27.5% higher than that of UE.

DISCUSSION

FE can extract materials in a few minutes efficiently, which is impossible for the other extraction methods compared in this study. Traditional methods also apply long-time heating process, but FE operates at room temperature, while the heat generated during FE could be ignored. So, it is more suitable for extracting heat-sensitive ingredients. FE is that a set of perpendicular blade rotate at high-speed to extremely break material while strong turbulence strengthens mass transfer, so soluble components rapidly release into the solvent at low temperature (Jin *et al*., 2017).

Three cancer cell lines and one normal cell line were used to evaluate the anti-proliferative activity of the four main components by MTT assay. According to the experimental results in table 3, diosgenin showed significant anti-proliferative activity. For MCF-7 cells, the IC_{50} value was 19.28 ± 0.36 μM. The inhibition rate of the four compounds on MCF-7 cells is shown in fig. 3. In addition, Catalpol had inhibitory effect on colon cancer cells.

CONCLUSION

In this study, we use different extraction methods to detect the active ingredients in Sidahuaiyao, the results demonstrated that FE was significantly superior to both UE and SE, which have been employed in the current Chinese Pharmacopeia for the quality control. Overall, comparing with current traditional and modern extraction techniques including SE and UE, our data suggested that FE, a new method combining all the advantages of smashing, soaking, stirring, and vibrating actions, offers a faster, more effective, and practical extraction technology. It's also first report to apply FE for the extraction of Sidahuaiyao.

At the same time, we screened the anticancer activity of four main components in the Sidahuaiyao. The results showed that diosgenin had significant antitumor activity and could significantly inhibit the proliferation of MCF-7 cells with IC_{50} value of 19.28±0.36μM. The other three compounds had different degrees of inhibitory effect on cancer cells. The discovery of this study could expand the application market of the Sidahuaiyao, and provide theoretical basis for the development and research of the Sidahuaiyao. At the same time, diosgenin also had the potential to develop into a new anti-cancer drug. In future studies, further determine the therapeutic potential of diosgenin in breast cancer, including animal models and mechanisms of action.

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