Chemical constituents of Cyperus rotundus L. and their inhibitory effects on uterine fibroids.

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Abstract:

Background: Xiang Fu (Cyperus rotundus L) enters the liver, spleen and triple warmer meridians, and has qi stagnation-removing, qi circulation-promoting, menstruation-regulating and pain-relieving effects. Besides, it can improve ovarian function, and has hypolipidemic, hypoglycemic and neuroprotective actions.

Objectives: To study the biflavone constituents in Cyperus rotundus L and to investigate the effect and mechanism of amentoflavone on inhibition of uterine tumors. Modern chromatographic techniques were applied for isolation and purification of compounds, which were then structurally elucidated based on their physicochemical properties and spectral data.

Methods: Female SD rats were injected with diethylstilbestrol and progesterone to establish the pathological model of uterine fibroids. The rats were then randomly divided into amentoflavone high-, medium- and low-dose groups, mifepristone group, model group and blank control group (n=10 in each group), and these administered for six consecutive weeks. 24 h after the last administration, the rats were sacrificed, changes in uterine coefficient were observed, and morphological features of apoptotic cells in uterine smooth muscle tissues were detected. Afterwards, serum estradiol and progesterone levels were determined by radioimmunoassay, as well as NOS level in uterine fibroid tissue homogenates. Pro- and anti-apoptotic genes Bcl-2 and Bax were determined by immunohistochemical assay.

Results: Four biflavone constituents were isolated and obtained. Amentoflavone could markedly reduce the uterine coefficient in model rats, lower serum estrogen levels in rats with uterine fibroids, improve the pathological conditions of uterine tissues, markedly reduce the number of Bcl-2- and Bax-positive dots in smooth muscles, and significantly inhibit the tumor-like proliferation in model rats ($P<0.01$), which were most obvious in the amentoflavone high-dose group.

Conclusion: It concludes that amentoflavone has a significant inhibitory effect on uterine tumors in rats. Its mechanism may be by elevating Bax protein expression, down-regulating Bcl-2 expression, forming homodimers Bax/Bax, and reducing plasma estradiol and progesterone to promote apoptosis of uterine fibroid cells.

Keywords: Cyperus rotundus L., biflavone, chemical constituent, anti-tumor

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Introduction

Xiang Fu is the dried rhizome of Cyperus rotundus L. in the genus Cyperus of the family Cyperaceae, which is pungent, slightly bitter and sweet, and bland. It enters liver, spleen and triple warmer meridians, and has qi stagnation-removing, qi circulation-promoting, menstruation-regulating and pain-relieving effects. It is used in the treatment of spleen and stomach qi stagnation, hernia pain, irregular menstruation, etc.[1]. The herb mainly contains sesquiterpenoids such as patchoulenone, isopatchoulenone, sugeonyl acetate, sugetriol triacetate and sugebiol, as well as flavonoids such as kaempferol, luteolin and quercetin[2]. Pharmacological activities of Cyperus rotundus L. have been extensively studied. It can improve ovarian function; besides, it also has hypolipidemic, hypoglycemic and neuroprotective actions.

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Uterine fibroid is one of the common, frequently-occurring diseases in gynecological clinical settings, which accounts for over 90% of benign gynecological tumors[3-4].

Patients often complain of abnormal menstruation, may suffer from infertility, secondary anemia, and in a few cases, malignant transformation as well. Modern medicine believes that the main treatment of uterine fibroids is surgical removal, which causes great suffering and economic burden to the patients. Currently, effective drugs for uterine fibroids are urgently needed clinically. In this experiment, amentoflavone was applied to treat rats with uterine fibroids and to conduct related pharmacodynamic studies, then its mechanism of action was investigated, in order to provide the theoretical basis for the development and clinical application of novel drugs.

Materials and methods

Materials

60 non-pregnant female Wistar rats, weighing 180-220 g, provided by the Laboratory Animal Center of China Medical University, batch number: 22013656; mifepristone, purchased from Yunnan Zizhu Pharmaceutical Co., Ltd., batch number: 20121013; Cyperus rotundus L, purchased from Nepstar Chain Drugstore Ltd., batch number: 201302226; serum estradiol and progesterone radioimmunoassay kits, provided by Zhengzhou Biocell Bioengineering Co., Ltd., batch number: 2108965 and 2102365, respectively; NOS kit, provided by Nanjing Jiancheng Bioengineering Institute, batch number: 30301256; Bcl-2 and Bax polyclonal antibodies, purchased from Beijing Zhongbin Biotechnology Co., Ltd., batch number: 300036501 and 300032546. Diethylstilbestrol and progesterone, purchased from Dalian Melone Pharmaceutical Co., Ltd., batch number: 20120605 and 20120702.

Melting point apparatus (uncorrected temperature), Shanghai Optical Instrument Factory; Varian Inova-600 MHz NMR spectrometer (TMS as internal standard). Silica gels and silica gel plates used were products of QingdaoHaiyang Chemical Plant; Sephadex LH-20 column chromatography materials; reagents were all of analytical grade.

Methods

Cyperus rotundus. L. purchased was identified by Teacher Fang Ming of Chengdu Medical University as the dried rhizomes of Cyperus rotundus L.

Extraction and isolation

50kg of dried rhizomes of Cyperus rotundus L was extracted by heat reflux for 2 h with a 12-fold amount of 95% ethanol three times. Then solvent was recovered under reduced pressure to give extract. The extract was dispersed with hot water, and extracted twice successively with petroleum ether, dichloromethane and n-butanol with volume equivalent to water, among which the n-butanol fraction was gradient eluted by silica gel column chromatography with dichloromethane-methanol as eluent system, and chromatographed repeatedly on silica gel column and Sephadex LH-20 column to obtain four compounds.

Structure elucidation

Compound 1: yellow powder, mp 251.3-252.6, 1H-NMR (600MHz, DMSO-d6),δ: 13.15, 13.00 (2H, s, 5, 5”-OH), 10.80, 10.15 (2H, 7, 7”-OH), 8.12 (1H, d, J=7.7 Hz, 6”’-H), 8.02 (1H, d, J=1.8 Hz, 2”’-H), 7.16 (1H, d, J=9.6 Hz, 5”’-H), 6.85 (1H, s, 3”-H), 6.55 (1H, d, J=2.5 Hz, 8”-H), 6.20 (1H, d, J=1.8 Hz, 6”-H), 7.66 (2H, d, J=9.2 Hz, 2’, 6’-H), 6.80 (1H, s, 3-H), 6.76 (2H, d, J=9.0 Hz, 3’, 5’-H), 6.41 (1H, s, 6-H). 13C-NMR (150 MHz, DMSO-d6) δ: 165.1 (C-2), 102.8(C-3), 183.3 (C-4), 160.0 (C-5), 99.0 (C-6), 161.9 (C-7), 104.3 (C-8), 154.7 (C-9), 103.7 (C-10), 121.7 (C-1’), 128.5 (C-2’, 6’), 115.9 (C-3’, 5’), 162.3 (C-4’); 163.8 (C-2”), 103.2 (C-3”), 181.8 (C-4”), 161.9 (C-5”), 97.7 (C-6”),164.5 (C-7”), 94.6 (C-8”), 156.9 (C-9”), 104.1 (C-10”), 121.2(C-1”’), 128.2 (C-2”’), 122.2 (C-3”’), 159.8 (C-4”’), 116.6 (C-5”’), 132.5 (C-6”’). The above data was consistent with the spectral data of amentoflavone (4’, 4”’, 5, 5”, 7, 7”-hexahydroxy-3”’,8-biflavone) reported in the literature[5].

Compound 2: yellow powder, 1H-NMR (600MHz, DMSO-d6) δ: 13.00, 12.89 (2H, s, 5, 5”-OH), 10.79, 10.75 (1H, 7-OH), 10.30 (1H, 4’-OH), 8.22 (1H, dd, J=9.1, 2.4Hz, 6”’-H), 8.10 (1H, d, J=2.3 Hz, 2”’-H), 7.45 (1H, d, J=8.5 Hz, 5”’-H), 6.80 (1H, s, 3”-H), 6.77 (1H, d, J=2.4 Hz, 8”-H), 6.40 (1H, d, J=2.4 Hz, 6”-H), 7.50 (2H, d, J=8.5 Hz, 2’, 6’-H), 6.88 (1H, s, 3-H), 6.73 (2H, d, J=9.1 Hz, 3’, 5’-H), 6.38 (1H, s, 6-H), 3.81 (7”-OMe), 3.80 (4”’-OMe). 13C-NMR (150 MHz, DMSO-d6) δ: 163.6 (C-2), 103.5(C-3), 182.0 (C-4), 160.6 (C-5), 98.3 (C-6), 161.9 (C-7), 103.9 (C-8), 154.6 (C-9), 104.3 (C-10), 122.0 (C-1’), 128.0 (C-2’, 6’), 115.9 (C-3’, 5’), 160.9 (C-4’); 164.6 (C-2”), 103.9 (C-3”), 182.3 (C-4”), 161.2 (C-5”), 98.8 (C-6”), 165.3 (C-7”), 92.9 (C-8”), 157.6 (C-9”), 104.9 (C-10”), 122.2 (C-1”’),128.6 (C-2”’),121.9 (C-3”’),161.9 (C-4”’), 111.5 (C-5”’), 131.1 (C-6”’), 56.6 (7”-OMe), 55.2 (4”’-OMe).

The above data was consistent with the spectral data of ginkgetin (4', 4"', 5, 5", 7, 7"-hexahydroxy-4"',7"-dimethoxy-3"',8-biflavone) reported in the literatures[6-7].

Compound 3: yellow powder. mp 208.3~210.5, 1H-NMR (600MHz, DMSO-d6)δ: 13.12, 12.88 (2H, s, 5, 5"-OH), 10.78 (2H, 7, 7"-OH), 8.22 (1H, dd, J=9.0, 2.4Hz, 6"'-H), 8.09 (1H, d, J=2.4 Hz, 2"'-H), 7.33 (1H, d, J=814 Hz, 5"'-H), 6.91 (1H, s, 3"-H), 6.55 (1H, d, J=1.8 Hz, 8"-H), 6.20 (1H, d, J=1.8 Hz, 6"-H), 7.66 (2H, d, J=9.0 Hz, 2', 6'-H), 6.99 (2H, d, J=9.0 Hz, 3',5'-H), 6.92 (1H, s, 3-H), 6.42 (1H, s, 6-H), 3.66 (3H, s, 4'-OMe), 3.78 (3H, s, 4"'-OMe). 13C-NMR (150 MHz, DMSO-d6)δ: 164.2 (C-2), 103.9 (C-3), 183.2 (C-4), 161.2 (C-5), 98.9 (C-6), 161.6 (C-7), 103.8 (C-8), 154.6 (C-9), 104.1 (C-10), 122.9 (C-1'), 127.4 (C-2', 6'), 114.6 (C-3', 5'), 162.6 (C-4'), 162.9 (C-2"), 102.9 (C-3"), 181.9 (C-4"), 162.0 (C-5"), 97.9 (C-6"), 163.3 (C-7"), 94.3 (C-8"), 157.6 (C-9"), 103.9 (C-10"), 121.9 (C-1"'), 128.6 (C-2"'), 122.0 (C-3"'), 160.9 (C-4"'), 111.7 (C-5"'), 130.6 (C-6"'), 56.3 (C-OMe), 55.6 (C-OMe). The above data was consistent with the spectral data of isoginkgetin (4', 4"', 5, 5", 7, 7"-hexahydroxy-4', 4"'-dimethoxy-3"', 8-biflavone) reported in the literatures[6-7].

Compound 4: yellow powder. mp 286.9~288.4.1H-NMR (600 MHz, DMSO-d6)D: 13.09 (1H, s, OH), 13.00 (1H, s, OH), 10.91 (1H, s, OH), 88.30 (1H, dd, J=9.0, 2.5 Hz, 6"'-H), 8.11 (1H, d, J=2.5 Hz, 2"'-H), 7.61 (1H, d, J=9.0Hz, 5"'-H), 7.01 (1H, s, 3"-H), 682 (1H, d, J=2.4 Hz, 8"-H), 6.32 (1H, d, J=2.5 Hz, 6"-H), 7.66 (2H, d, J=9.0Hz, 2', 6'-H), 7.01 (2H, d, J=9.0 Hz, 3', 5'-H), 6.96 (1H, s, 3-H), 6.55 (1H, s, 6-H), 3.77 (3H, s, OMe), 3.79 (3H, s, OMe), 3.86 (3H, s, OMe). The above data was consistent with the spectral data of sciadopitysin (4', 4"', 5, 5", 7, 7"-hexahydroxy-4', 7", 4"'-trime-thyoxy-3"', 8-biflavone) reported in the literature[8].

Preparation of rat model of uterine fibroids (Yu et al., 2004)

Except for the intramuscular injection of an equal volume of distilled water in the blank control group, the other groups were intramuscularly injected with diethylstilbestrol injection at 0.02 ml/rat/d (2 mg/ml) every other day for 30 consecutive days.

Animal grouping and treatment

30 days after model establishment, rats were divided into blank control group, model group, amentoflavone high-, medium- and low-dose groups and mifepristone group, n=10 in each group. Amentoflavone high-, medium- and low-dose groups were intragastrically administered at 15 g/kg, 10 g/kg and 5 g/kg, respectively, once daily. Mifepristone group was intraperitoneally injected at a dose of 2.24 mg/kg[9], while model group and blank control group were intragastrically administered with an equal volume of normal saline. Rats in each group were administered with these for 42 days, and sacrificed 24 h after the last administration.

Comparison of uterus coefficient, cervix diameter, and corpus diameter of rats

Uterus coefficient was calculated as the uterine weight divided by the body weight of rats. Maximum diameters at the cervix and corpus were measured.

Morphological observation of apoptotic cells

The uteri were fixed in 10% neutral formalin solution, then 0.5 cm of the same position above the uterine horn was transected, embedded in paraffin, and stained with HE, followed by observation of morphological features of apoptotic cells in uterine smooth muscle tissues under a microscope.

Determination of serum estradiol and progesterone levels and tissue homogenate activity

Before sacrificing the rats, tail blood was sampled, and the serum estradiol and progesterone levels, as well as tissue homogenate activity of rats were determined using ELISA kits and NOS kit.

Determination of Bcl-2 and Bax protein expressions

A block of tissue was taken from the fibroid tumor and uterine muscle wall, respectively, fixed in neutral buffered formalin, and embedded in paraffin, followed by dewaxing and rehydration of paraffin sections. 3% hydrogen peroxide was dropped and the sections were allowed to stand for 10 min. Antigen was retrieved by high pressure boiling for 1~2 min, and the sections were placed in phosphate-buffered saline (PBS) for 5 min. 5% normal goat serum was added, and the sections were allowed to stand at room temperature for 10 min. Bax-IgG and Bcl-2-IgG were added, respectively, then the sections were washed in PBS for 3 min three times, and kept overnight at 4. Secondary antibodies were biotinylated, then the sections were washed in PBS for 3 min three times, and incubated

atroom temperature for 1 h. S-P was washed in PBS for 3 min three times, and incubated at room temperature for 1 h, then stained with freshly prepared DAB-H2O2 solution for 5~10 min, and counterstained with hematoxylin for 0.5 min, followed by mounting with neutral gum.

Evaluation of Bcl-2 and Bax protein expressions

Bcl-2 positive staining showed brownish yellow particles in the cytoplasm; non-stained cells were regarded as negative cells. Semi-quantitative evaluation was performed based on Fromowitz's 10 comprehensive scoring (proportion and staining intensity of positive cells). The system had four grades based on the presence or absence of staining: negative (-): ratio of positive cells <25%; weakly positive (+): ratio of positive cells 26%~50%, lightly stained; medium positive (++): ratio of positive cells 51%~75%, deeply stained; strongly positive (+++): positive cells >76%, deeply stained.

Statistical analysis

Data was statistically analyzed by the Wilcoxon rank-sum test and the analysis of variance. $P<0.05$ was considered statistically significant.

Results

Effect of amentoflavone on uterine coefficient

Compared with the model group, uterine coefficient, cervix diameter and corpus diameter decreased significantly in amentoflavone high-dose group; moreover, the effect was equivalent for high-dose group and mifepristone group. The experimental results showed that high-dose amentoflavone had an inhibitory effect on uterine hyperplasia in rats. The results are shown in Tab. 1

Tab.1: Effect of amentoflavone on uterus in rats with uterine fibroids

Group	Number	Uterine coefficient t (%)	Cervix diameter (cm)	Corpus diameter (cm)
Blank group	10	0.36±0.13	0.68±0. 14	0.32±0. 07
Model group	10	4.52±2. 36$^{\Delta}$	1.33±0. 89$^{\Delta}$	1.78±0. 12$^{\Delta}$
Mifepristone group	10	1.65±1. 32*	0.89±0. 32*	0.76±0. 63*
Amentoflavone high-dose group	10	1.89±1. 45*	0.88±0. 22*	0.87±0. 36*
Medium-dose group	10	2.50±2. 12	0.97±0. 23	0.85±0. 33*
Low-dose group	10	2.70±1. 36	0.95±0. 33	0.89±0. 43*

Comparison between model group and blank group, $^{\Delta}P<0.05$; comparison between treatment groups and model group, * P<0105

Morphological observation of apoptotic cells

No apoptotic cell was observed in the myometrium of rat uterine fibroids model while scattered apoptotic cells were occasionally seen in the endometrium. Marked apoptotic cells were observed in amentoflavone high-dose group and mifepristone group, which presented blue-black nuclei, pink cytoplasm, and compacted and fragmented chromatins.

Effects of amentoflavone on estradiol and progesterone levels in rats with uterine fibroids

Compared with the model group, serum estradiol concentration, serum progesterone concentration and uterine homogenate NOS activity decreased to varying degrees in each amentoflavone dose group and positive control group, and the differences were significant ($P<0.05$).

Table .2: Effects of amentoflavone on estradiol and progesterone levels and homogenate NOS activity in rats with uterine fibroids

Group	Number	Estradiol concentration (pg/mL)	Progesteroneconcentration (pg/mL)	Homogenate NOS activity
Blank group	10	234.7	3.36	18.90
Model group	10	812.3	3.89	24.60
Mifepristone group	10	255.3**	3.45*	16.65*
Amentoflavone high-dose group	10	265.2**	3.30*	8.40**
Medium-dose group	10	356.4**	3.45*	10.76**
Low-dose group	10	425.6*	3.60*	14.70*

Comparison between treatment groups and model group, * P<0.05, ** P<0101

Determination results of Bcl-2 and Bax protein expressions

Xiaozheng Pill had an obvious antitumor effect. Compared with the model group, Bax expression increased slightly, while Bcl-2 expression somewhat decreased in each dose group, and the differences were significant (P<0.05). The results are shown in Table. 3.

Table .3: Comparison of Bcl-2 and Bax expressions in the uterine muscle wall tissues of rats in each group (x±s; n=10)

Group	Number of rats (n)	Bcl-2expression				Bax expression			
		-	+	++	+++	-	+	++	+++
Blank group	10	2	8	0	0	3	7	0	0
Model group	10	0	0	0	10	0	0	0	10*
Mifepristone group	10	0	9	1	0	1	1	8	0**
amentoflavone high-dose group	10	0	9	0	1	1	0	7	2**
Medium-dose group	10	0	8	0	2	0	2	5	3**
Low-dose group	10	0	7	1	2	0	4	2	4

Note: Comparison with blank group by Ridit analysis, * P<0.01; comparison with model group by Ridit analysis, ** P<0.05

Discussion

Through the systematic study on the chemical constituents of Cyperus rotundus L, four biflavone constituents are isolated from Cyperus rotundus. L., and identified, which are amentoflavone (1), ginkgetin (2), isoginkgetin (3) and sciadopitysin (4). Biflavone constituents have anti-tumor, anti-viral and anti-inflammatory activities[11]. In this paper, the anti-uterine fibroid activity of amentoflavone was studied in rats. The results demonstrated that amentoflavone has a good anti-uterine fibroid effect, which has enormous potential for being developed as clinical therapeutic agents. In this paper, the antitumor mechanism of action of amentoflavone is studied in depth, thus providing a solid basis for clinical treatment.

Pathogenesis of uterine fibroids is complex, which involves changes in partial or total body estrogen and progesterone and their receptors, alteration of local polypeptide growth factors, cell division rate etc of which a consensus has been reached regarding ovarian hormone-dependency[12-14]. In this study, a combined protocol of exogenous estrogen and progesterone is used to establish uterine fibroids model. After gavage administration of amentoflavone, uterine coefficient, serum estrogen and progesterone levels decreased in rats, and pathomorphological changes such as uterine smooth muscle hyperplasia is improved, indicating certain anti-uterine fibroids effect of amentoflavone.

A study has shown that estrogen and progesterone can increase the vitality of uterine artery, and NOS-specifically change local circulation, thereby causing uterine vasodilation, and increasing blood flow[15]. Xiaozheng Pill can also lower uterine homogenate enzyme NOS activity in progesterone induced model rats. NOS has certain growth promoting effect on uterine fibroids, which can dilate blood vessels, increase blood supply to uterine fibroids, and promote tumor growth. Local increased levels of estrogen in uterine fibroids[16] can induce NOS activity, thus generating biological activity. Our results further suggest certain preventive and therapeutic effects of Xiaozheng Pill on uterine fibroids.

This study found positive expressions of apoptotic genes Bcl-2 and Bax in uterine fibroid tissues, yet the intensity of Bcl-2 and Bax expressions varied among groups. Positive expression of Bax in uterine leiomyoma tissues was significantly lower than that in uterine muscle tissues of blank control group ($P<0.01$), indicating that apoptotic gene Bax was in a low or a down-regulated expression state in uterine leiomyoma tissues. In contrast, positive expression of Bcl-2 in uterine leiomyoma tissues was significantly higher than that in uterine muscle tissues of blank control group ($P<0.01$), indicating that apoptotic gene Bcl-2 was in a high or an up-regulated expression state in uterine leiomyoma tissues. Bax was highly expressed in the positive control group and the high-dose group, while the expression of Bcl-2 was low.

Due to the low or down-regulated expression of Bax and high expression of Bcl-2, apoptosis program cannot be effectively initiated, causing blockage of apoptosis[17-18] and apoptotic disorders of uterine muscle cells, preventing timely updates and removal of abnormal cells, and ultimately inhibiting uterine muscle cell apoptosis. Since uterine muscle cells cannot be updated via apoptosis pathway, abnormal proliferation of uterine muscle cells occurs under the influence of certain adverse factors. At this time, the uterine muscle cells are in an unlimited or excessive proliferation state and thus lead to the incidence of uterine fibroids. In this study, amentoflavone inhibited the incidence of uterine fibroids by increasing or up-regulating Bax expression while inhibiting and down-regulating Bcl-2 expression. The therapeutic effect of XiaoZheng Pill on uterine fibroids may be achieved by promotion of leiomyoma cell apoptosis via increasing Bax expression, and inhibiting Bcl-2 expression to form homodimers Bax/Bax. Other mechanisms of action need further research.

Conflict of interest

None to declare.

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