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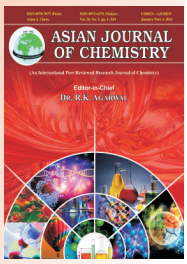

Extraction, Purification and Pharmacological Activity of Melon Pedicle Active Ingredients

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The present study was designed to characterize the effects of the extraction of melon pedicle on the contractility of isolated jejunal smooth muscle segment and its underlying mechanism. The investigation of single factor and orthogonal test was adopted to optimize the extraction and purification of melon pedicle, using the content of total saponins in entirfel preparation as the evaluation indicator; *ex vivo* assay was selected as the major method to determine the effects of the extraction separated from melon pedicle on the contractility of isolated jejunal smooth muscle segment (IJSMS). The optimum extraction conditions was to extract at 50 °C, with water as the solvent, the drug-water ratio of 1:60, extracted for 1.5 h, three times with the total saponins content of 1.8447 %; The optimal condition for purification of total saponins using 10 g HP-20 resin as follows. The resin column diameter-height ratio of 17:305, eluted with 12BV of 30 % ethanol and the elution flow rate of 1 mL/min are the optimal elution conditions, with the total saponins content of 8.3031 %. The stimulation of IJSMFS with M receptor and H1 receptor independent. No further stimulation effects of EFMP were observed in the presence of verapamil or in Ca^{2+}-free condition, indicating EFMP-induced stimulation effects are Ca^{2+}-dependent and the action of EFMP on intestinal motility was mediated by Ca^{2+} influx through L-type Ca^{2+} channel.

Keywords: Contractility, Optimize, Isolated Jejunal Smooth Muscle Segment, Total saponins, Verapamil, Ca^{2+} Influx.

INTRODUCTION

Melon pedicle is the dried stalk of cucurbitaceae plant melon (*Cucumis melo L.*), bitter clove, melon stalk from melon, the attached portion of fruit and stems, have emetic, sputum food, effectiveness of dehumidification and jaundice, can be used for stroke, epilepsy, phlegm dampness, respiratory adverse, chest abdominal pain, *etc*.

Melon pedicle contained impurity alcohol, saponins, amino acids, bitter gourd prime (cu-curbitacin) B, D, E, isocucurbitacinB, cucurbitacin B-2-O-β-D-glucopyranosi-de, also contains α-sterol (α-spinasterol).

Cucurbitacin as a class of tetracyclic triterpenoid of cucurbitaceae type, most of them are the bitter substances in plant, mainly in Turkey Fritillaria Cucurbitaceae, smelly melon, snow gall genus and bitter gourd, *etc*., such as pumpkin, water melon, bitter gourd, on other subjects also has a small amount of distribution.

Cucurbitacin triterpenoids biological activity mainly in the following: (1) Liver function; (2) Cytotoxic and anti-tumor effect; involving liver cancer[1], leukemia[2,3], squamous cell carcinoma[4,5], pancreatic cancer[6], ovarian sarcoma and breast cancer; (3) Anti-inflammatory effects; In addition, cucurbitacin also has antifertility effect. Also have an impact on immune function, cucurbitacin B can significantly enhanced the role of immune system, also has a strong effect on cellular immu-nity, humoral immunity.

EXPERIMENTAL

Plant material and preparation: Melon pedicle was bought from Xinhai Pharmaceutical Sales Co., Ltd, Bozhou City, Hebei Province, is identified as the stalk of Cucurbitaceae plant melon by the professor Yunpeng Diao of Dalian Medical University.

Animals: Research with Kunming strain mice, all of them are male, weighing 200 ± 20 g and 60 Sprague-Dawley (SD) rats, were obtained from Experimental Animal Center, Dalian Medical University.

Drug and reagents: Oleanolic acid reference (Kai Mei Reagent Company, DaLian), vanillina (Fu Chen Chemical Reagent Factory, Tianjin), perchloric acid (Kai Mei Reagent Company, DaLian), lacial acetic acid (Kai Mei Reagent Company, DaLian), methanol (Ke Mi Ou Reagent Company, TianJin), ethanol (Ke Mi Ou Reagent Company, TianJin), acetone (Ke Mi Ou Reagent Company, TianJin), macroporous

resins (Kai Mei Reagent Company, DaLian). Klinefelter buffer composition (mmol/mL): NaCl 114.0, KCl 4.7; $MgCl_2$ 1.2; $CaCl_2$ 2.5; NaH_2PO_4 1.8; Glucose 11.5; $NaHCO_3$ 4.2; PH 7.4.

Main instruments: JJ500 Precision Electronic Balance (Shuangjie Brothers Ltd. U.S.), U-3030 UV-visible (Hitachi, Japan), DHG-101-3A Heated Drum Wind Oven (Yu Gongyi City Yuhua Instrument Factory, British), HM-3s-type PH Meter Radio (Industries, Ltd., Japan East Asia), BSZ-2 Automatic Dual Water Distiller (Broadcom Medical Instrument Factory, Shanghai), HH-8 Digital Thermostat Water Bath (Cheung Chau Guohua Electric Appliance Co., Ltd.), RT-08 Eighty-two Mounted Grinder (Tianyuan Machinery Technology Co., Ltd. AA, Beijing).

Determination of total saponins extraction technology

Establishment of standard curve: Preparation of standard solutions: Mathanol was added to 4.8 mg oleanolic acid reference to a final volume of 2 mL (per 1 mL of oleanolic acid 2.4 mg), shaked, vested, accurate volume of 0, 10, 15, 20, 25, 30, 35, 40 and 45 µL oleanolic acid reference solution was added into nine 10 mL test tube, respectively, 100 µL vanillin-acetic acid (50 g/L) and 400 µL perchloric acid was added, 1.5 mL acetic acid was then added to reach a final volume of 2 mL, at water bath of 70 °C and kept for 15 min, cooled and kept for 10 min, with the corresponding reagent blank, Ultraviolet spectrophotometer, absorbance at 540 nm and draw the standard curve.

Preparation of the test solution and measurement method: After extracted the melon pedicle, filtered and to reach a certain volume, shaked, vested. Determination of total saponins: accurate volume of 50 µL test solution, placed in 10 mL tubes, according to the way established under "standard curve" item since the "Add 400 µL of vanillin-acetic acid (50 g/L)", with the corresponding reagent blank, the absorbance was measured by the UV-visible spectrophotometry and measurement of the content of total saponins was obtained from standard curve.

Determination of extract temperature: Single factor design of the assay is as follows. 5 g crushed melon pedicle were added into 500 mL flasks, with 20 times the amount of distilled water as the solvent to investigate extraction temperature 50, 65, 75 and 85 °C, respectively, extracted 1 h, measure the content of total saponins and determined the optimal extraction temperature.

Determination of extract solvent: 5 g crushed melon pedicle was added into 500 mL round bottomed flask with 20 times the amount of water, acetone, 60 % methanol, 60 % ethanol as solvent and refluenced under the condition of 70 °C, measure the content of total saponins, then determined the optimal extraction solvent.

Orthogonal design for total saponins extraction: In the design of orthogonal factor level design, selected the extraction time, drug-solution ratio, extraction times, blank four factors, each designed three levels (Table-3). According to the arrangement of L_9 (3^4) orthogonal table and 5 g crushed melon pedicle were added into 500 mL flasks, a total of nine copies, with water as the solvent extracted 1h at 50 °C, determined by the total saponins content and dry paste rate composite score which determined the optimal orthogonal condition.

Static adsorption-elution performance experiment: Macroporous resins including AB-8, HPD-400, D101, NKA-9, HP-20. This resins were pretreated with 1 M HCl and NaOH solutions sequentially and prior to adsorption experiments, pre-weighed a certain amount of resins were soaked in 95 % ethanol and washed thoroughly with deionizad water. To determine the best purification resin, the comprehensive survey of adsorption rate and resolution rate is critical. Static adsorption tests were performed as follows: 1 g treated dry resin was accurately weighed and added into 150 mL air-tight conical flask and then 30 mL melon pedicle sample solution which contained total saponins 2 mg/mL was added. The flask was shaken at 170 rpm in a constant water-bath temperature (25 °C) and shaked for 24 h. After the resin was separated from the sample solution by filtration, measure the total saponins content of the test solution after adsorption. Calculated the saturated adsorption capacity (Q) and the saturated adsorption rate; Take the saturated adsorption resins and added into 150 mL air-tight conical flask, added 30 mL of 30 % ethanol into the air-tight conical flask and at 170 rpm shaking speed of 24 h, measure the total saponins content of the analytic solution, calculated the adsorption stripping rate for each resin[7]. The results shown as belove Table-5, the purification of melon pedicle saponins, should use HP-20 macroporous resin as the carrier of enrichment and purification[8].

$$Q = (C_0\text{-}C_a) \times V_s/W$$

$$\text{Absorption rate} = (C_0\text{-}C_a)/C_0$$

$$\text{Resolution ratio} = (C_d \times V_d)/(WQ)$$

Q is the saturated adsorption capacity, C_0 is initial concentration, C_a is the concentration after adsorption, C_d is the eluent concentration, V_s is the adsorption solution volume, V_d is the eluent volume, W is the resin amount (dry mass calculation).

Determination of static adsorption capacity: Make leak curve to determine the resin adsorption capacity is also crucial. According to the above determined conditions, take the sample solution (contain saponins 1.7624 mg/mL) to flow through HP-20 resin column and make it saturated adsorption, added distilled water to rush unabsorbed solution and added 100 mL of 20 % ethanol eluted at the flow rate of 1 mL/min, each retention volume collecting an effluent, collected for 11 test tubes, measure each tube of the total saponins, draw leak curve.

Determination of eluent concentration: The investigation of eluent concentration: 10 g of pretreatment HP-20 macroporous resin was added into resin column and eluted with distilled water to non-alcoholic flavored, take the sample solution (the total saponin content of 2 mg/mL) to flow through resin column until it saturated adsorption, use 20, 30 ,40, 50 and 60 % ethanol gradiently eluted to colourless point, respectively. Measure the total saponins content of each concentration gradient elution by the established method under assaying item.

Determination of eluent flow rate: In the investigation of eluent flow rate, 10 g treated HP-20 macroporous resin was added into resin column and eluted with distilled water to non-alcoholic flavored, take the total saponins sample solution of 2 mg/mL flow through HP-20 resin column until it saturated adsorption, then use 100 mL of 30 % ethanol eluted at the flow rate of 1, 3 and 6 mL/min, respectively.

Determination of diameter-height ratio: In the investigation of diameter-height ratio of resin column, 10 g of pretreated HP-20 type resin were added into the resin column with the diameter-height ratio of 17/305, 26/305, 32/305, respectively. Then added into 50 mL of sample solution which contain the total saponins 0.6294 mg/mL, flow though the resin column until it saturatily adsorbed and then with the 30 % ethanol eluted with 1 mL/min, respectively, measure the total saponin content of each concentration gradient elution by the method described in above section. The proprietary extraction process for the highest content of total saponins was obtained in this study through the optimization of the extraction and purification, providing a chemical basis for further pharmacological studies.

Tissue preparation and contraction determination: Jejunum segments of 2 cm length were mounted in a 20 mL organ containing krebs buffer. One end of isolated jejunal segment was fixed to the wall of a tissue bath chamber (20 mL volume) and the other end was connected to a force-displacement transducer to determine the contraction. The organ bath containing krebs buffer was bubbled with a 95 % O_2 and 5 % CO_2 gas mixture and the temperature was held at 37 °C, an initial load of 1 g was applied to each of the tissue and was kept constant throughout the experiment. Each segment was allowed to equilibrate in the bath for 0.5 h and the bath solution was replaced every 10 min to obtain a regular spontaneous activity[9].

Jejunum propulsion assay: At the concentrations of 50-800 mg/L, EFMP increased contractile amplitude of IJSMS (from 100 ± 12.3 % to 207 ± 16.5 %) in a concentration-dependent manner.

Underlying mechanism involved in EFMP-induced stimulation: At the bath concentration of 200 mg/L, EFMP did not significantly affect jejunal contraction under Ca^{2+}-free condition, it did not significantly affect the contraction of jejunal smooth muscle in the presence of Ca^{2+} channel blocker verapamil (0.1 μmol/L). The EFMP can partially blocked the inhibitory effects produced by M receptor antagonist atropine and H_1 receptor antagonist diphenhydramine, this showed that EFMP induced jejunal contraction with M receptor and H_1 receptor independent. The average amplitude of contractions occurring before (5 min) and after (5 min) administration of each drug were determined. Relative changes of drug-induced contractile responses to the basal levels were calculated as percentage.

Data analysis: SPSS19.0 for Windows was used to analyze the data. Results obtained from the study were expressed as mean ± S.E.M. with *n*-denoting the number of tissue preparations tested. Paired-Sample T-Test was used to analyze and compare the results between the two groups (before and after adding drug) while a paired-way ANOVA was used to compare results between the two groups. Differences were considered statistically significant if P values were less than 0.01.

RESULTS AND DISCUSSION

Manufacturation of standard curve: From the standard curve of total saponins assaying, the linear range is 0.01-0.06 mg/mL. The regression equation of total saponins content (mg/mL) and absorbance at 540 nm is shown in Fig. 1. Y = 51.922X-0.5764, r = 0.9983.

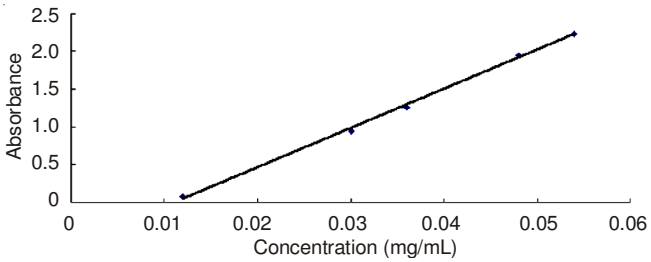

Fig.1. Regression equation of saponin content

Extraction temperature: According to Table-1, considering the content of total saponins, we determine that the optimal technology of extraction temperature is 50 °C with the content of total saponins is 1.5436 %.

TABLE-1
SINGLE FACTOR DESIGN FOR TOTAL SAPONIN EXTRACTION (TEMPERATURE LEVEL)

Extraction temperature (℃)	Total saponin content (%)
50	1.5436
65	1.1388
75	1.3225
85	1.3847

Extraction solvent: It can be seen from Table-2 that with water as the solvent, the content of total saponins is 1.5601 %, so the optimal technology of extraction solvent is water.

TABLE-2
SINGLE FACTOR DESIGN FOR TOTAL SAPONIN EXTRACTION (SOLVENT LEVEL)

Extraction solvent	Content of total saponins (%)
60 % Methanol	1.2479
Acetone	1.3792
Water	1.5601
60 % Ethanol	1.3225

Investigation on orthogonal design: The optimal process for total saponins extraction is shown in Tables 3 and 4. Intuitive analysis shows that the sequence of the factors influencing total saponins extraction is D > A > C > B, extraction times has greatest influence; therefore, we can obtain from the two analyses that the optimal extraction technology is drug-solution ratio of 1:60, extract 1.5 h, extract three times Tables 3-5.

TABLE-3
DESIGN OF ORTHOGONAL LEVEL FACTORS

A	B	C	D
Drug-water ratio	Blank	Extraction time(h)	Extraction times
1:20		1	1
1:30		1.5	2
1:40		2	3

Static adsorption and elution: Through the investigation on static adsorption and elution of total saponins in etirfel preparation of AB-8 resin, HPD-400 resin, NKA-9 resin, D101 resin and the HP-20 resin, we determine that total saponins in etirfel preparation has better adsorption and elution on HP-20 resin Table-6.

TABLE-4
RESULTS OF THE ORTHOGONAL DESIGN ON TOTAL SAPONIN TECHNOLOGY

Factor	Drug/Solution	Blank	Extract time (h)	Extracttimes	Results
Experiment 1	1	1	1	1	0.6099
Experiment 2	1	2	2	2	0.6204
Experiment 3	1	3	3	3	0.6715
Experiment 4	2	1	2	3	0.9278
Experiment 5	2	2	3	1	0.6456
Experiment 6	2	3	1	2	0.626
Experiment 7	3	1	3	2	0.6803
Experiment 8	3	2	1	3	0.7314
Experiment 9	3	3	2	1	0.7088
Average value 1	0.634	0.739	0.656	0.655	
Average value 2	0.733	0.666	0.752	0.642	
Average value 3	0.707	0.669	0.666	0.777	
Range	0.099	0.073	0.096	0.135	

TABLE-5
Analysis of variance

Factor	Squared deviations	The degrees of freedom	RatioF	Critical value F	Significant
Drug/Solution	0.016	2	0.727	5.14	-
Extraction time (h)	0.017	2	0.773	5.14	-
Extraction times	0.033	2	1.5	5.14	-
Error	0.07	6	-	-	-

TABLE-6
ADSORPTION RATE AND RESOLUTION RATE OF MACROPOROUS RESIN

Resin Type	Adsorption capacity (mg)	Adsorption rate (%)	Resolution rate (%)
AB-8	33.3611	56.12	63.98
HPD-400	27.2817	45.89	71.22
D101	31.9811	53.8	74.98
NKA-9	26.2747	44.2	48.44
HP-20	34.2303	57.58	75.57

Elution solvent: According to Table-7, considering dry extract rate and total saponins content percentage, we determine that 30 % ethanol is the optimal elution solvent with the elution rate of 14.92 %.

TABLE-7
ELUENT SELECTION

Ethanol density (%)	20	30	45	50	60
Elution rate (%)	23.44	24.05	22.73	14.85	14.92

Saturation adsorption capacity: It can be seen from Fig. 2 that when the sampling volume is 9 mL, total saponins starts to leak, so when 1.7624 mg/mL total saponins sample flow through resin column, make it dynamic adsorption at 6 mL/min, the adsorption capacity of 10 g resin is 9 mL.

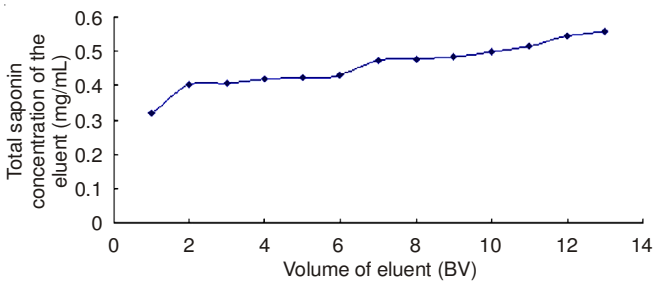

Fig. 2. Plotted leaked curve of resin column

Diameter-height ratio and eluent flow rate: Through the investigation on adsorption flow rate, dynamic adsorption, eluent concentration and the factors influencing resin elution, we finally determine that the optimal technology for the purification by 10 g HP-20 resin is that the diameter-height ratio is 17:305 with the elution rate of 93.97 % (Table-9). The elution flow rate is 1 mL/min with elution rate of 91.7225 % Table-8.

TABLE-8
DETERMINATION OF ADSORPTION FLOW RATE

Eluent flow rate (mL/min)	6	3	1
Eluting rate (%)	70.0857	81.3675	91.7225

TABLE-9
RESULTS OF THE FACTORS INFLUENCING HP-20 RESIN ELUTION

Diameter-height ratio of macroporous resin column (mm/mm)	32/305	26/305	17/305
Elution rate (%)	51.12	87.49	93.97

Effect of melon pedicle extraction on isolate jejunum smooth muscle segment: Pharmacological activity test, the results indicated that EFMP stimulate intestinal smooth muscle segment both in nomal and in low contractile states under *ex vivo* assay conditions. At bath concentration of 50-200 mg/L, EFMP increased contractile amplitude of IJSMS (from 100 ± 12.3 to 207 ± 16.5 %) in a dose dependent manner Fig. 3. In addition, sodium nitroprusside, epinephrin can attenuate the amplitude of JSMF contractions by 53.25 ± 3.50 and 77.44 ± 2 % (n = 6) over the baseline Fig. 4. It did not change the EFMP-induced smooth muscle contraction.

Results of mechanism underlying the effects: The muscarinic cholinergic receptor blocker atropine (10 µL) can partly inhibit the contraction of jejunal smooth muscle segment, the inhibition can be blocked by EFMP. And the EFMP also

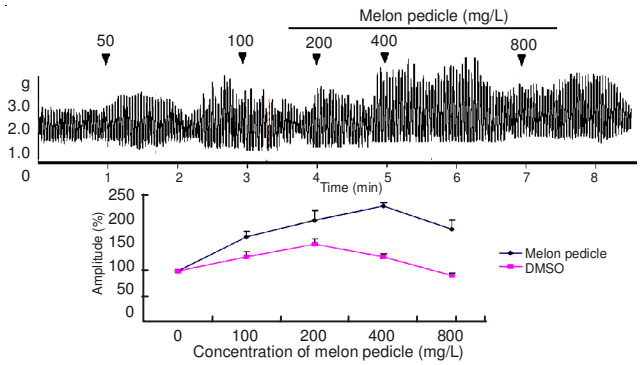

Fig. 3. Cumulative dose-response curve for the effects of melon pedicle extraction

blocked the inhibition caused by H1 receptor antagonist diphenhydramine (Fig. 4), indicating that the stimulation effects induced by EFMP with H_1 receptor and M receptor independent (Table-10).

TABLE-10
ANALYSIS OF EFMP ON THE CONTRACTION OF JLSM PRETREATED WITH SNP, NE

Agents	Before treatment*	After of treatment*
EFMP (80 µL)	0.77 ± 0.19	1.52 ± 0.19[a]
Sodium nitroprusside (10 µM)	0.91 ± 0.06	1.15 ± 0.05[a]
Atropine (10 µM)	1.31 ± 0.08	2.02 ± 0.05[a]
Diphenhydramine (10 µM)	0.83 ± 0.04	1.17 ± 0.03[a]
Verapamil	1.04 ± 0.08	0.99 ± 0.05[a]
Noradrenaline	3.60 ± 0.02	4.30 ± 0.11

Representative traces (D), (E) of melon pedicle extraction (MPE) on the contractility of isolated smooth muscle segment (IJSMS) pretreated with nitroprusside-Na, NE. Representative traces (C), (A) of MPE on the contractility of IJSMS in the conditions pretreatment with atropine, diphenhydramine, respectively. Representative traces (B) of MPE on the contractility of IJSMS in the conditions pretreatment with verapamil. **P < 0.01 compared with the nomal contractile state (control).

Effects of Ca^{2+}: Verapamil, a standard calcium-channel blocker, at the bath concentration of 0.1 µM, decreased the amplitude of jejunal smooth muscle segment contractions by 33.75 ± 2.59 % (n = 6) over baseline. The extraction of melon pedicle did not significantly changed the inhibitory state induced by verapamil, indicated that the extraction of melon pedicle on jejunum smooth muscle contraction may be related to calcium influx through L-type Ca^{2+} channel[10] (Fig. 4).

Effect of enteric nervous system: The enteric nervous system was blocked by tetrodotoxin (TTX), when pretreated with tetrodotoxin, the stimulation effects of EFMP on jejunum smooth muscle segment did not significantly affected by tetrodotoxin, indicating that the extraction of melon pedicle has a role in stimulating jejunum smooth muscle segment and with enteric nervous system independent (Fig. 5).

Effects of melon pedicle extraction on intestinal propulsion in (A) normal contractile state and in (B) the contractile state (pretreated with tetrodotoxin). Each column represents the mean ± SEM (n = 4). **P < 0.01 compared with the propulsion before the treatment of EFMP (normal control). **P < 0.01 compared with the tetrodotoxin-induced inhibition before the treatment of EFMP (tetrodotoxin control).

Melon pedicle belong to cucurbitaceae plant, containing primarily cucurbitacin class, sterols, triterpene glycosides, amino acids. The main functions are emetic, anti-treatment of liver cancer, cirrhosis, repair liver damage cells, it also can enhance cellular and humoral immune function.

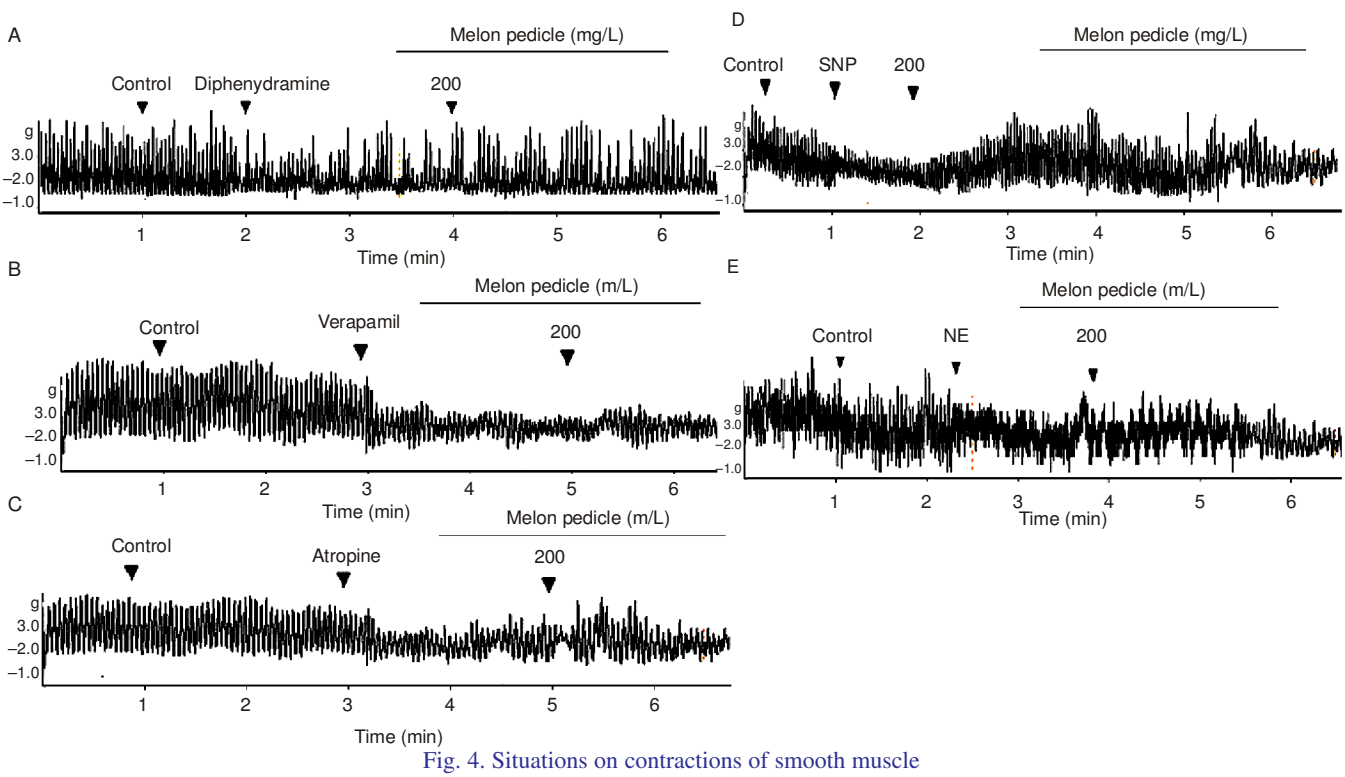

Fig. 4. Situations on contractions of smooth muscle

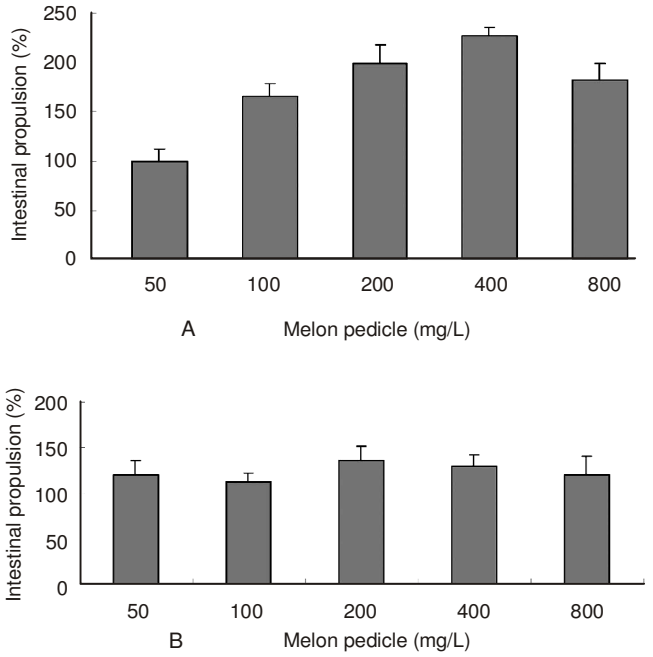

Fig. 5. Influence of melon pedicle extraction on intestinal contraction condition

The study by examining the extraction of melon pedicle on rat jejunum smooth muscle segment, the extraction of melon pedicle at low concentrations can stimulate the contraction of jejunum smooth muscle, while at high concentrations can inhibit the contraction of jejunum smooth muscle. It exerted inhibitory effects of IJSMS when pretreated with sodium nitroprusside, norepinephrine, when added in EFMP partly blocked the inhibition induced by sodium nitroprusside, norepinephrine, indicating that the extraction of melon pedicle enhanced the contraction of intestinal smooth muscle segment both in nomal and low contractile state under *ex vivo* assay conditions. When pretreated with tetrodotoxin, did not significantly affected the stimulatory action of EFMP on jejunum smooth muscle segment, indicating that the promotion effects of EFMP on the contraction of jejunum smooth muscle segment was not obviously blocked by the enteric nervous system, that means EFMP enhanced the contraction of jejunum smooth muscle segment and it was not significantly affected by enteric nervous system.

Underlying mechanism involved in EFMP-induced stimulation on the contractility of jejunal smooth muscle segment (JSMF), the extraction of melon pedicle can partially blocked the inhibitory effects induced by M receptor antagonist atropine and H_1 receptor antagonist diphenhydramine, respectively, indicating that M receptor and H_1 receptor were not found involved in the stimulatory effects induced by EFMP.

As shown in Fig. 4, EFMP at bath concentrations of 200 mg/L did not significantly affect jejunal contraction pretreated with Ca^{2+} channel blocker verapamil (0.1 μmol/L), indicated that the extraction of melon pedicle on jejunum smooth muscle contraction may be related to calcium influx through L-type Ca^{2+} channel.

The intracellular signal transduction mechanisms involved in smooth muscle contraction, Ca^{2+}-Cam-MLCK, MAPK pathway and Rho kinase, PKC kinase, MLCP *etc*[11].

Currently accepted mechanism for the contraction and relaxation of jejunum smooth muscle is the participation of Ca^{2+}, myosin light chain kinase (myosin light chain kinase, MLCK), make myosin light chain (myosin light chains) phosphorylation, contraction of smooth muscle; Themyosin light chain is dephosphorylationed[12], by the phosphatase (phosphatase), the smooth muscle is relaxed; Light chain kinase and phosphatase for the regulation of jejunal smooth muscle smooth and myosin with two-way adjustment. In addition, the calcium-binding protein (calponin) and silk protein (filamin) alone with myosin binding enhance the activity; while in the presence of actin, inhibit the activity of muscle globulin[13].

The next step, the ion and receptor mechanisms of the drugs on bidirectional regulation effects in jejunum smooth muscle will be studied.

Conclusion

The extraction of melon pedicle stimulate the contraction of isolated smooth muscle segment at low concentrations, the stimulatory effects with M receptor, H_1 receptor independent, but it is mediated by Ca^{2+} influx through L-type Ca^{2+} channel. The stimulatory effects may be with enteric nervous system independent.

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