Antidiarrheal properties of different extracts of Chinese herbal medicine formula Bao-Xie-Ning

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OBJECTIVE: Bao-Xie-Ning (BXN), a traditional Chinese herbal medicine (CHM) formula composed of Fructus Evodiae, Flos Caryophylli and Cortex Cinnamomi, and used for the treatment of infant diarrheal illness, was subject to systematic assessment for its putative multiple pharmacodynamic effects and pharmacological antidiarrheal mechanisms.

METHODS: High-performance liquid chromatography-diode array detector-electrospray ionization-mass spectrometry/mass spectrometry was developed and validated for identification and quantification of the main constituents in different extracts of BXN. Male Kunming mice weighing 20 to 25 g were used for detecting the antidiarrheal activity of the extracts. Ethanolic extract (EE), volatile oil extract (VOE), and aqueous extract (AE) of BXN were respectively subjected to pharmacodynamic and pharmacological comparison in assessing antidiarrheal effects with senna-induced diarrhea, castor oil-induced diarrhea, acetic acid-induced writhing assay, and isolated duodenum test.

RESULTS: The highest yields of three detected components of BXN, rutaecarpine, eugenol and cinnamaldehyde were observed in EE. EE showed the most remarkable antidiarrheal activity in dose-dependent and time-dependent manners in both senna- and castor oil-induced diarrhea models, and presented dose-dependent analgesic activity in acetic acid-induced algnesia model. In addition, EE extract of BXN also exhibited strong antimobility action on the intestine and strongest depression on spontaneous contraction of isolated duodenum.

CONCLUSION: Ethanol extraction is an efficient method to extract the active constituents of BXN. BXN extract demonstrated multiple pharmacological activities affecting the main mechanisms of diarrhea, which validated BXN’s usage in the comprehensive clinical treatment of diarrhea.

KEYWORDS: drugs, Chinese herbal; antidiarrhea; pain; analgesic and spasmolytic activities

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1 Introduction

Chinese herbal medicines (CHMs) are utilized worldwide and have developed a reputation for their efficacy. But usage of CHMs remains controversial in clinical practice due to the complicated and poorly-understood chemical compositions of herbs, and especially herbal combinations. Additionally, it is difficult to comprehensively assess the pharmacological activities of herbal formulas due to complications in quality control. Despite the above, there has been an increasing interest in the clinical usage and application
of herbal mixtures since the United States Food and Drug Administration (FDA) issued new guidelines in June 2004[1], which permitted the approval of herbal mixtures on the condition that their safety and efficacy could be documented.

CHM is a very important, widely-used primary health care modality in China that has historically been used in the treatment of many kinds of diseases. CHM formulae are composed of multiple constituents, and thus have the features of multiactivities, multitargets, and multimechanisms[2]. One such CHM formula, Bao-Xie-Ning (BXN), composed of Fructus Evodiae (fruits of Evodia rutaecarpa (Juss.) Benth), Flos Caryophylli (buds of Eugenia caryophyllata Thmb), and Cortex Cinnamomi (bark of Cinnamomum cassia Presl), is commonly used in the treatment of infant diarrheal diseases. Various bioactive mechanisms (including actions on the digestive system) of the crude herbal medicines of Fructus Evodiae, Flos Caryophylli, Cortex Cinnamomi and their corresponding CHM formulae have been reported, and the formula has been used extensively in clinics via oral or transdermal administration[3,4]. But no comprehensive studies exist on the effects of BXN’s antidiarrheal activities based on different extraction methods, which could be important information for the full clinical usage guidelines of BXN. This study was undertaken to fully assess the antidiarrheal effects of BXN by using novel approaches in quality control, various extraction methods, and multiple pharmacological assays that cover most aspects of the main mechanisms of the disease.

2 Materials and methods

2.1 Materials and preparation of BXN

BXN contained Fructus Evodiae 5 g, Flos Caryophylli 2 g, and Cortex Cinnamomi 1 g. All the crude herbal medicines were purchased from Pharmacy of Guangzhou University of Chinese Medicine, and identified by Prof. De-po Yang at Laboratory of Pharmacognosy and Natural Medicinal Chemistry, School of Pharmaceutical Sciences, Sun Yat-sen University, China, according to the Pharmacopoeia of the People’s Republic of China (2010 version). The crude herbal medicines were powdered to pass through a 40-mesh sieve, mixed according to the formula proportion, and stored in a closed container at room temperature before use.

2.2 Drugs and chemicals

Rutacearpine, eugenol, and cinnamaldehyde were purchased from National Institute for the Control of Pharmaceutical and Biological Products, China. The purities of all reference compounds were over 98% as determined by high-performance liquid chromatography (HPLC) analysis. HPLC-grade acetonitrile (CH₃CN) was purchased from Tedia Company, Inc. (Fairfield, OH, USA), and ultrapure water was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA, USA) and was used as mobile phase of HPLC. Other reagents were of analytical grade (Guzhou Chemical Factory, Guangzhou, China).

2.3 Preparation of BXN extracts

Three extracts of BXN were prepared as below and stored at 4 ℃ before tests were performed.

Ethanolic extract (EE): BXN powder 300 g was extracted with 95% ethanol at 10 times volume refluxing for 4 h. After filtering, the residue was extracted by refluxing for another 2 h and 10 times volume of 95% ethanol. Both filtrates were collected and evaporated to remove the solvent under reduced pressure. The yield of the EE was 19.6% from BXN powder.

Volatile oil extract (VOE): BXN powder 300 g was subjected to steam distillation with 10 times volume of water for 4 h. Volatile oil was obtained by separating the light yellow oil layer, the aqueous extract, and the residue (to be used in aqueous extraction). The yield of the VOE was 2.3% from BXN powder.

Aqueous extract (AE): The residue from the previous extraction step of VOE was subjected to decoction with 10 times volume of distilled water for another 2 h. Both of the aqueous extract solutions were collected and concentrated to an appropriate volume under reduced pressure, followed by adding 60% ethanol to precipitate the proteins and polysaccharides in the aqueous solution. After centrifuge and filtration, the filtrate was concentrated to give the AE. The yield of the AE was 14.3% from BXN powder.

In some cases, VOE and AE were mixed at a ratio of 1:6.2, or equal to the amount of crude herbal medicines in one extract sample for pharmacological testing.

2.4 Preparation of extract samples’ solutions for HPLC analysis

EE (63.60 mg), VOE (68.00 mg), and AE (148.30 mg) were weighed and transferred into three 10 mL volumetric flasks, respectively. After dissolving and mixing with 95% ethanol, all the solutions were filtered through a 0.45-μm nylon filter before HPLC analysis.

2.5 HPLC-DAD-ESI-MS/MS analysis of BXN extracts

HPLC-diode array detector-electrospray ionization-tandem mass spectrometry (HPLC-DAD-ESI-MS/MS) was employed for chemical identification and content measurement of effective ingredients of BXN and quality control.

The HPLC-DAD-ESI-MS/MS system consisted of a Surveyor MS pump, a Surveyor autosampler, a Surveyor diode array detector, and a triple-quadruple TSQ Quantum mass spectrometer (Thermo Finnigan, San José, CA, USA) with Xcalibur software for data acquisition and analysis. Separations were carried out by using a Kromasil C18 reverse phase column (250 mm×4.6 mm i.d., 5 μm, EKA Chemical, Sweden), fitted with a C18 guard cartridge.
(4 mm×3.0 mm i.d., Phenomenex, Torrance, CA, USA). Mobile phase: A, water with 0.2% acetic acid; B, acetonitrile. Gradient program: A/B 0 min, 95/5; 55 min, 40/60; 56 min, 10/90; 80 min, 10/90. Flow rate: 1.0 mL/min. A 15-min re-equilibration time was used between HPLC runs. The column was held at room temperature, and the DAD acquisition wavelength was set in the range of 200 to 400 nm. After passing through the flow cell of DAD, the column eluate was split and 0.3 mL/min was directed to a triple-quadruple tandem mass spectrometer with an electrospray interface (ESI), operating in full scan MS mode from m/z 40 to 700. Mass spectra were acquired in both positive and negative modes with ion spray voltage at 3.5 kV, capillary temperature at 350 °C, capillary voltage at 35 V, sheath gas pressure at 35 Arb, and auxiliary gas pressure at 11 Arb.

For quantitative purposes, reference standards and the extracts were analyzed by a Waters 600E HPLC system, equipped with a Waters 717 plus autosampler and a Waters 2996 photodiode array detector (Waters, Milford, MA, USA). Chromatographic conditions were the same as described above, except that the detected wave lengths were set at 343, 280, and 290 nm for rutaecarpine, eugenol, and cinnamaldehyde, respectively.

2.6 Method validation
Rutaecarpine, eugenol, and cinnamaldehyde used as reference standards were known to be the main pharmacologically active compounds of Fructus Evodiae, Flos Caryophylli, and Cortex Cinnamomi, respectively. Therefore, quantification of the three active compounds in the extracts of BXN was carried out by using external standard method. Standard solutions of rutaecarpine, eugenol, and cinnamaldehyde were prepared by dissolving them in an appropriate volume of 95% ethanol to give stock solutions with concentrations of 0.055, 0.987, and 0.200 mg/mL, respectively. Calibration curves were obtained using standard solutions at six different concentration levels. Repeatability was evaluated by intra-day (n = 6) assays. Recovery tests of the quantified constituents were determined using an extract sample, in which the corresponding constituents had been predetermined. In each case, a mixture of standards with low, medium and high concentrations of the quantified levels of constituents was spiked into the sample, and then analyzed by HPLC in duplicate.

2.7 Animals
Male Kunming mice (20 to 25 g, certificate serial number SCXK (Guangdong) 2009-0011) and New Zealand White rabbits (2.5 kg) were purchased from Experimental Animal Centre, School of Pharmaceutical Sciences, Sun Yat-sen University, China. All animals were housed at Animal Laboratory of School of Public Health, Sun Yat-sen University, China and kept in plastic cages at 23 to 25 °C with a 12 h light-dark cycle. All animals were allowed free access to pellet food and water, but food was withdrawn 10 h before experiments. All animals were observed for 3 d before experiments to exclude those suffering from diarrhea. All the animal experiments were performed in compliance with “Guide for the Care and Use of Laboratory Animals”[5], and were approved by the Ethics Committee for Animal Research of Sun Yat-Sen University, China.

2.8 Antidiarrheal activity in senna-induced diarrhea test
Diarrhea was induced in mice with a modified method developed by Xu et al[6]. Briefly, 90 mice were randomly located to nine groups. Three test dosages (low, medium, and high dosages) were selected for EE group (0.49, 0.98 and 1.47 g/kg body weight, corresponding to 2.5, 5.0 and 7.5 g crude herbal medicine of BXN, calculated from clinical human infant dosages of the BXN formula) and AE plus VOE group (0.83, 1.66 and 2.49 g/kg body weight, corresponding to 5.0, 10.0 and 15.0 g crude herbal medicine of BXN). Smecta (Smectite Powder) (3 g/pack, Batch No. 320, Beaufour Ipsen Co. Ltd., Tianjin, China) was employed as positive control with a dosage of 5.0 g/kg body weight. 0.9% NaCl was used in model control and blank control groups.

All the drugs or 0.9% NaCl were administered orally by gavage to the animals. One hour after the treatment, each animal was administered with 0.5 mL 8% aqueous extract of Folium Sennae orally except the blank control group.

Observation for defection continued up to 6 h and diarrheal index (DI) was calculated according to the following formula: DI = ratio of wet feces × grade of wet feces. Ratio of wet feces is defined to be the ratio between the frequency of wet feces and total frequency of feces in each animal, while grade of wet feces is defined to be the size contaminated by wet feces in filter paper placed beneath the individual perforated mice cages (I: diameter < 1 cm; II: diameter 1 to 1.9 cm; III: diameter 2 to 3 cm; IV: diameter > 3 cm)[6].

2.9 Antidiarrheal activity in castor oil-induced diarrhea test
Diarrhea was induced in mice with a modified method developed by Bajad et al[7]. Briefly, 70 animals were randomly divided into seven groups. Two test dosages (low and high dosages) were selected for EE group (0.49 and 1.47 g/kg body weight, corresponding to 2.5 and 7.5 g crude herbal medicine of BXN) and AE plus VOE group (0.83 and 2.49 g/kg body weight, corresponding to 5.0 and 15.0 g crude herbal medicine of BXN). Smecta (Beaufour Ipsen Co. Ltd., Tianjin, China) was employed as positive control with a dosage of 5.0 g/kg body weight. NaCl (0.9%) was used in the model control and blank control groups.

All the drugs or 0.9% NaCl were administered orally by gavage to the animals. One hour after the treatment, each animal was administered 0.3 mL of castor oil orally except the blank control group.
Observations and data analyses were carried out in the same way as mentioned above.

2.10 Analgesic activity
Acetic acid-induced writhing assay was employed to assess the potential analgesic activity of BXN. The analgesic effect was tested according to the method described by Shibata et al.[8]. Briefly, it was a model of visceral pain that was produced by an intraperitoneal injection of 0.2 mL, 0.8% aqueous solution of acetic acid to each mouse 30 min after orally administering the tested drugs or water. After administration of acetic acid injections, all the mice were immediately isolated in individual observation boxes.

Fifty mice were randomly divided into five groups: three dosages of EE (0.49, 0.98 and 1.47 g/kg body weight, administered orally by gavage to the animals), morphine control (10 mg/kg body weight by intraperitoneal injection), and 0.9% NaCl control (administered orally by gavage to the animals).

Observation for analgesic effect lasted up to 15 min. The number of mice with presence of abdominal contortions, and the period of time between administration of injecting acetic acid and presence of abdominal contortions were noted. Analgesic percentage was calculated with the formula: Analgesic percentage = (A-B)/A × 100%. A: the number of mice with presence of abdominal contortions in the 0.9% NaCl control group; B: the number of mice with presence of abdominal contortions in the treatment groups.

2.11 Spasmolytic activity
2.11.1 Preparation of isolated duodenum
Two rabbits (2.5 kg) were killed by a blow on the back of the head and 1.0 to 2.0 cm piece of the duodenum was removed, and the contents were expelled by flushing the lumen with Tyrode’s solution (NaCl 145 mmol/L, CaCl₂ 2.11.1 mmol/L, MgSO₄ 1.2 mmol/L and glucose 5 mmol/L (pH 7.4)) at 37 oC and aerated with 95% O₂-5% CO₂. The responses of isolated duodenum and the synergic effects of extracts on acetylcholine (1.66×10⁻⁵ mol/L, 0.4 mL)-induced, and BaCl₂ (20%, 0.4 mL)-induced contractions of isolated duodenum were all examined.

Inhibition percentage of extracts was calculated with the formula: Inhibition percentage (%) = (B-A)/A × 100%. A: mean of vibrant extent of isolated duodenum 3 min before administration; B: mean of vibrant extent of isolated duodenum 3 min after administration.

2.12 Statistical analysis
Data analysis was performed by one-way analysis of variance with the Dunnett post-hoc test for multiple comparisons by SPSS 12.0 software. Data were expressed as mean ± standard deviation (SD). The level of statistical significance was set at P<0.05.

3 Results
3.1 HPLC-DAD-ESI-MS/MS analysis of the extracts of BXN
Identification of the main constituents in EE of BXN was carried out by HPLC-DAD-ESI-MS/MS, comparing HPLC retention times, UV absorptions, quasi-molecular ion m/z values with those of the standards and the literature data. The HPLC chromatograms of the EE and the mixture of the reference standards are shown in Figure 1. The retention times, UV absorption wave lengths, MS data, and the identification results for the peaks numbered in the chromatograms are presented in Table 1. Peaks 1, 2 and 4 were undoubtedly identified as cinnamaldehyde, eugenol, and rutaecarpine, respectively, by comparing with the three reference standards. For peak 3, it showed the quasi-molecular ion of [M+H]+ at m/z 304, and two maximum UV absorptions at 232 and 265 nm, in agreement with those of evodiamine. Therefore, peak 3 was only tentatively identified as evodiamine because it lacked the corresponding standard.

The same analyses were employed on AE and VOE (shown in Figure 1). In the chromatogram of VOE, the peaks 1 and 2 were identified as cinnamaldehyde and eugenol respectively, while rutaecarpine and evodiamine were not detected due to their non-volatile properties. However, although all the peaks of these three active
compounds in AE could be detected, the quantification of them in AE was difficult to be accomplished due to their extremely low contents in AE.

3.2 Quantification of three active compounds in extracts of BXN

Only rutaecarpine, eugenol, and cinnamaldehyde were subjected to quantitative analysis; no analysis was done for evodiamine, which was missing the reference compound. Considering the obvious distinctions of the maximum UV absorption wavelengths of these reference compounds, the detected UV absorption wavelengths for quantitative analysis of these compounds were set at 343 nm for rutaecarpine, 280 nm for eugenol, and 290 nm for cinnamaldehyde, respectively.

The quantitative analysis of the three active compounds in EE and VOE of BXN was carried out by using the external standard method. Calibration curves were obtained using standard solutions at six different concentration levels, and were plotted with the concentrations (x: μg/mL) of standard solutions as abscissa and the corresponding peak

Table 1  Retention time, ultraviolet absorption, and mass spectrometry spectral data of constituents identified in ethanolic extracts of Bao-Xie-Ning

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Compound</th>
<th>UV&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>ESI [M+H]&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>41.30</td>
<td>Cinnamaldehyde</td>
<td>291</td>
<td>133</td>
</tr>
<tr>
<td>45.33</td>
<td>Eugenol</td>
<td>230, 282</td>
<td>163&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>53.89</td>
<td>Evodiamine</td>
<td>232, 265</td>
<td>304</td>
</tr>
<tr>
<td>56.71</td>
<td>Rutaecarpine</td>
<td>240, 329, 343, 360</td>
<td>288</td>
</tr>
</tbody>
</table>

<sup>*</sup> ESI [M-H]. ESI: electrospray ionization.

Figure 1 HPLC chromatograms of three extracts of Bao-Xie-Ning and the standard mixture 169 mm×140 mm (96 × 96 DPI)
area (y) as ordinate. The regression equations were calculated in the form of \( y = Ax + B \), where \( x \) and \( y \) were the concentration of standard solution (μg/mL) and the corresponding peak area, and \( A \) and \( B \) were the slope and the intercept, respectively. The resulting regression equations of the three active compounds were:

- For \( \text{rutaecarpine} \) with the linear range of 0.34 to 55.00 μg/mL (\( R^2 = 0.9999 \)), \( y = 141,731,405x + 12,303 \).
- For \( \text{eugenol} \) with the linear range of 6.17 to 987.50 μg/mL (\( R^2 = 0.9999 \)), \( y = 22,242,270x + 81,619 \).
- For \( \text{cinnamaldehyde} \) with the linear range of 1.25 to 200.20 μg/mL (\( R^2 = 0.9994 \)), \( y = 185,860,892x + 307,581 \).

All calibration curves were linear over the concentration ranges tested with high correlation coefficients. Repeatability was evaluated by intra-day (\( n = 6 \)) assays, and the relative standard deviation (RSD) values were found to be within the range of 0.34% to 0.48%. Recovery rates of the quantified constituents were determined by spiking a mixture of standards with the quantified levels of constituents into the sample, and then analyzed by HPLC. The results showed that the recovery rates for the three quantified compounds were within the range of 97.78% to 103.86% with RSD less than 1.02%.

This validated approach helped to determine the contents of \( \text{rutaecarpine} \), \( \text{eugenol} \), and \( \text{cinnamaldehyde} \) in the \( \text{EE} \) and \( \text{VOE} \) extracts of BXN. Results are summarized in Table 2.

3.3 Study of antidiarrheal activity

3.3.1 Senna-induced diarrhea test

All the results of antidiarrheal activity of \( \text{EE} \) and \( \text{AE plus VOE} \) in senna-induced diarrhea test are shown in Table 3, with the blank control group omitted due to the absence of diarrhea. Both \( \text{EE} \) and \( \text{AE plus VOE} \) with high dosage presented remarkable antidiarrheal activity in all the time points. For low and medium dosages, \( \text{EE} \) showed notable antidiarrheal activity at 2 h, while no antidiarrheal activity was observed at 6 h. The antidiarrheal activity of \( \text{EE} \) was more potent than \( \text{AE plus VOE} \) when they were compared with same amounts of their crude herbal materials.

3.3.2 Castor oil-induced diarrhea test

All the results of antidiarrheal activities of \( \text{EE} \) and \( \text{AE plus VOE} \) in castor oil-induced diarrhea test are shown in Table 4, with the blank control group omitted due to the

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**Table 2** Contents and yields of active compounds in different extracts

<table>
<thead>
<tr>
<th>Constituent</th>
<th>In EE Content (%)</th>
<th>In VOE</th>
<th>In EE Yield (%)</th>
<th>In VOE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutaecarpine</td>
<td>0.46±0.00</td>
<td>—</td>
<td>0.09±0.00</td>
<td>—</td>
</tr>
<tr>
<td>Eugenol</td>
<td>9.55±0.02</td>
<td>47.14±0.87</td>
<td>1.87±0.02</td>
<td>1.08±0.05</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>1.81±0.13</td>
<td>5.82±0.08</td>
<td>0.35±0.02</td>
<td>0.13±0.01</td>
</tr>
</tbody>
</table>

EE: ethanolic extract; VOE: volatile oil extract.

**Table 3** Antidiarrheal activities of different extracts at different dosages in senna-induced diarrhea test

<table>
<thead>
<tr>
<th>Groupa</th>
<th>Dosageb (g/kg body weight)</th>
<th>n</th>
<th>Diarrheal index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 h</td>
</tr>
<tr>
<td>Model control (Saline)</td>
<td>—</td>
<td>10</td>
<td>1.30±0.61</td>
</tr>
<tr>
<td>EE</td>
<td>Low dosage</td>
<td>0.49 (2.5)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Medium dosage</td>
<td>0.98 (5.0)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>High dosage</td>
<td>1.47 (7.5)</td>
<td>10</td>
</tr>
<tr>
<td>AE plus VOE</td>
<td>Low dosage</td>
<td>0.83 (5.0)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Medium dosage</td>
<td>1.66 (10.0)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>High dosage</td>
<td>2.49 (15.0)</td>
<td>10</td>
</tr>
<tr>
<td>Positive control (Smecta)</td>
<td>5.0</td>
<td>10</td>
<td>0.38±0.29</td>
</tr>
</tbody>
</table>

EE: ethanolic extract; AE: aqueous extract; VOE: volatile oil extract.

aThe values in the brackets were dosages calculated as crude herbal drugs. bP<0.05, vs model control group.
absence of diarrhea. Both EE and AE plus VOE with high dosage exhibited remarkable antidiarrheal activity at all of the time points. No antidiarrheal activities were observed at 6 h in both EE and AE plus VOE groups with low dosages. To have similar efficacy of antidiarrheal activity, the amounts of crude herbal medicines for AE plus VOE needed to be two times that of EE. The result was similar with animals in the senna-induced diarrhea test.

3.4 Study of analgesic activity

Acetic acid-induced writhing assay was performed and observation was carried out according to the method mentioned above. All the results are shown in Table 5. EE presented dose-dependent analgesic activity in all the tested parameters, which indicated antagonistic effect of EE on acetic acid-induced algesia.

3.5 Study of spasmolytic activity

EE, AE, and VOE exhibited spasmolytic effects in linear dose-dependent manners, respectively ranging from 2 to 64 mg/mL, 0.39 to 12.56 mg/mL, and 2.81 to 90 mg/mL on spontaneously contracting rabbit duodenum. Both extent and frequency of contractions decreased in a dose-dependent manner that corresponded to increasing concentrations of EE, AE, and VOE (Figure 2).

When tested on acetylcholine-induced and BaCl\textsubscript{2}-induced
compulsive contractions of isolated duodenum, EE, AE and VOE expressed remarkable antagonistic effects at the selected concentrations (EE: 32 mg/mL, VOE: 12.56 mg/mL, and AE: 90 mg/mL). The same concentrations of the three extracts were also subjected to the test of adrenaline-induced depression of contraction on isolated duodenum. Notable synergistic effects with adrenaline in adrenaline-induced depression on spontaneous contraction in isolated duodenum were found in the above selected concentration of EE, VOE and AE. All the results are shown in Figure 3. EE, VOE and AE at selected concentrations demonstrated strong antimobility of the intestine, not only independently but also in combination with adrenaline.

It should be noted in the test that equivalent concentrations of crude herbal medicines for their reference extracts were 163.2 mg/mL (for EE 32 mg/mL), 549.8 mg/mL (for VOE 12.56 mg/mL), and 629.4 mg/mL (for AE 90 mg/mL) respectively with inhibition percentage of 75%, which indicates that EE was the most effective extract of the BXN crude herbal drugs on spontaneous contraction of isolated duodenum.

Last but not least, high concentrations of all extracts could decrease all extent of swing, tensility, and frequency of spontaneous contraction of isolated duodenum, which may provide a clue for the underlying antidiarrheal mechanism.

4 Discussion

Composed of Fructus Evodiae, Flos Caryophylli, and Cortex Cinnamomi, BXN is a well-known traditional formula for the treatment of infant diarrheal diseases. This study validated the comprehensive antidiarrheal function of BXN by evaluating the formula’s active components in a laboratory setting.

Previous phytochemical studies have shown that rutaecarpine, eugenol and cinnamaldehyde are the main active pharmacodynamic constituents of Fructus Evodiae, Flos Caryophylli, and Cortex Cinnamomi, respectively. All of them have been demonstrated to possess potential antidiarrheal properties. Hence, they were considered to be the main active antidiarrheal constituents of BXN, and were selected for quality control of BXN extracts.

In order to understand CHM from a scientific perspective, it is crucial to determine the methodological approach of quality control for traditional Chinese medicine formulae. In the present study, HPLC-DAD-ESI-MS/MS was employed for the identification of the main constituents in different extracts of BXN, unambiguously identifying rutaecarpine, eugenol, and cinnamaldehyde. After being validated for precision, accuracy, and linearity with the three compounds as standards, an HPLC method was established and used to quantify the three compounds in different extracts of BXN. All the three active compounds were detected and quantified in EE of BXN, but rutaecarpine was not detected in VOE, and none of the three compounds were able to be quantified in AE due to their extremely low values.

Three diverse extraction methods were employed to ensure the potential efficacy of BXN with putative active constituents. Extracting with ethanol is believed to be more efficient for liposoluble and volatile ingredients than those with water. Most of the main active constituents of

**Figure 3** Effects of three extracts on the contractile response of isolated duodenum pretreated by acetylcholine (ACH), BaCl2 or adrenalin (ADR) respectively. A: Ethanolic extract (EE) 32 mg/mL; B: Volatile oil extract (VOE) 12.56 mg/mL; C: Aqueous extract (AE) 90 mg/mL.
BXN are liposoluble, while eugenol and cinnamaldehyde are volatile, so we hypothesized that ethanol extraction would be the most suitable extraction method for BXN. We did produce higher yields of the three active compounds with the ethanol extraction procedure as compared to the other extraction methods.

Diarrhea is defined as frequent passage of liquid faeces, along with crampy abdominal pain and loss of electrolytes (particularly sodium) and water from the intestinal tract, all of which are induced by abnormal contraction of gastrointestinal muscles and/or infections. This study investigated the anti diarrheal properties of BXN by examining a series of pharmacodynamic parameters.

Two types of diarrhea animal models (senna-induced and castor oil-induced) were utilized to assess the efficacy and mechanisms of BXN. Senna is a lenitive medicine that pharmacologically targets the large intestines, which leads to fairly wet faeces with little smell. Thus, mechanisms counteracting diarrhea in a senna-induced model should involve antisecretory pathways. It has been reported that nitric oxide (NO) is involved in the secretion mechanism of senna-induced diarrhea, and agents that successfully decrease the output of wet faeces from senna-induced models potentially include NO. On the other hand, it is well known that castor oil produces diarrhea via its most active component, recinoleic acid, which induces a hypersecretory response in the small intestines. The reduction in total number of wet faeces in the test groups of this study suggests that EE of BXN presented dose-dependent antidiarrheal activities by antisecretory action.

Due to the cramping and discomfort associated with diarrhea, pain reduction is an important aspect in the treatment of diarrhea in clinical practice. EE of BXN presented notable analgesic activity in acetic acid-induced writhing assay. All the observed parameters showed dose-dependent analgesic activities in BXN, which indicated its antagonistic effect on acetic acid-induced algesia.

Reduction of intestinal motility is one of the key mechanisms in many antidiarrheal agents, and previous studies have been done regarding the effect of herbal extracts on intestinal motility. In the rabbit-isolated duodenum, EE showed strong inhibitory effects on spontaneous contractions, as well as acetylcholine- (0.1 mmol/L) and KCl- (60 mmol/L) induced contractions in a dose-dependent manner. Synergistic effect on relaxing intestinal contraction was also observed between adrenaline and BXN extracts. Adrenaline is one of the main hormones secreted in the adrenal medulla, which accounts for around 80% to 90% of its total secretion. As a catecholamine hormone, its biological activity has close association with the sympathetic nervous system, and regulates the excitation of cholinergic fiber in abdominal organs. Exogenous adrenaline combines with the adrenoceptors located in smooth muscle cells of the intestinal tract (α-receptor and β-receptor), and leads to laxation of smooth muscle, inhibition of contracting mobility, and thereby inhibition of small intestine movement. Further research is needed to understand whether the antimobility action of BXN affects adrenoreceptors.

Smooth muscle contraction depends not only on extracellular Ca\(^2+\), but also intracellular Ca\(^2+\). In Ca\(^2+\)-free solution, the acetylcholine-induced contraction is caused by the release of intracellular Ca\(^2+\). The inhibitory action of the extract may be due to its interference with Ca\(^2+\) movement. More studies on calcium ion channels should be carried out in the future.

In conclusion, the present study suggests that multiple pharmacological activities of BXN, which address many of the main mechanisms of diarrhea, inhibit intestinal motility through interference with Ca\(^2+\) movement. Our findings validate the traditional utilization of BXN in treating diarrhea.

Perhaps an even more important finding from this study is a methodological approach for understanding complicated traditional CHMs: HPLC-DAD-ESI-ESI-MS/MS can be utilized for quality control of traditional formulae; crude herbs should undergo different extraction methods in order to optimize the content of active components before being subjected to pharmacological study; lastly, comprehensive pharmacologic assessments, using various available biochemical techniques, should be employed to validate the complex and sophisticated usage of traditional CHMs.

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6 Competing interests

The authors declare that there have no competing interests.

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