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Antidiarrhoeal mechanism study of fulvic acids based on molecular weight fractionation

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ABSTRACT

As the important component of humus, fulvic acids (FA) have a good antidiarrhoeal effect on animals and humans, and have been worldwide used in animal husbandry and even clinical practice for a long time. Due to the extremely complex chemical composition and structure of FA, the material basis and mechanism of its antidiarrhoeal activity have not been fully elucidated. In this study, we used ultrafiltration technique to fractionate this heterogeneous mixture into a series of relatively uniform fractions. The main structural features of FA and its fractions were characterized, and at the same time their antidiarrhoeal activities on drug-induced diarrhoea model mice were evaluated and the collagen content in the intestine of mice were determined. Through contrastive study of the relative variations between structure characteristics and antidiarrhoeal activities with the change of molecular weight, we found that the oxygen-containing functional groups especially phenolic hydroxyl groups, molecular weight distribution, colloidal properties and astringency were the material basis of the antidiarrhoeal activity. Fulvic acid substances had a dual antidiarrhoeal mechanism acting on the intestinal mucosa. The components with low molecular weight (< 5 K) mainly acted on the inside of intestinal mucosa and the components with high molecular weight (> 5 K) acted on the surface, and they could simultaneously exert the antidiarrhoeal effects.

1. Introduction

As the important component of humus, humic acids (HA) is a kind of complex and heterogeneous mixtures of polycarboxylic acids, which is formed by a series of biochemical and geochemical processes during the microbial decomposition and transformation of plant remains [1,2]. The relative molecular weight range of HA is generally from several hundred to several hundreds of thousands [3]. According to the relative molecular weight and the differences of solubility in different solvents, HA can be divided into three fractions: black humic acids, hymatomelanic acids and fulvic acids (FA) [2,4]. It is generally believed that FA is the part of HA with relatively low molecular weight, higher solubility, stronger biological activity, which is the most representative in chemical composition and function [4,5]. FA contains both small and large molecular substances, has high oxidation degree and many active functional groups, and is easy to be absorbed and utilized by organisms. Due to its prominent pharmacological effect, FA has a long history of application in medicine. In Europe, people spontaneously used peat or sapropel bath very early to treat skin diseases, arthritis, gynecological diseases and so on. Modern pharmacological studies have found that the physiological active ingredients in peat bath are mainly humic substances represented by FA [6]. In China, various types of coal rich in FA or HA are called “Wu-jin-shi”. As a traditional Chinese medicine, it has been used to treat pain, bleeding, diarrhoea, sores, scalds, bone injuries and so on, for > 800 years [7]. Since the 1950s, basic research and clinical practice in recent decades have basically confirmed the pharmacological properties of FA in anti-inflammatory, anti-ulcer, antidiarrhoeal, anti-virus, anti-cancer, regulating immunity and improving microcirculation [7–12]. Among them, FA has the most definite and prominent effect on digestive system diseases [13–17]. For example, FA has a strong antidiarrhoeal function and also presents a better therapeutic effect in practice. Even, FA has been active substances used in prophylaxis and as therapeutical drugs in veterinary practice in Europe (EMEA, 1999), and there are Chinese patent medicines on the market for treating diarrhoea. Unfortunately, the material basis and mechanism of its antidiarrhoeal activity have not been fully elucidated, which greatly affects the development and application of related antidiarrhoeal drugs.

FA usually has complex composition, diverse structure, wide molecular weight distribution, multi-functional groups and strong polarity.

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These characteristics not only make it exhibit many excellent physicochemical properties and biological activities, but also cause great difficulties in the study of chemical composition and molecular structure which has been lacking effective methods for separation and structure analysis [4]. In turn, it becomes an obstacle to the basic research of the biological activity of FA. As the material basis is not clear, the research results of action mechanism are often indirect speculation. Some studies suggested that the role of FA in preventing diarrhoea in animals stemmed from its anti-bacterial and anti-inflammatory properties, or its function in regulating the intestinal microbial environment [15–17]. By investigating the effect of FA on chemical drug-induced gastrointestinal propulsion in mice, other studies indicated that FA may play an antidiarrhoeal role by directly acting on multiple neuroreceptors in the intestine to inhibit small intestinal motility [13,18]. For these studies, the limitations of material basis research have led to the inadequacy of the antidiarrhoeal mechanism study or the inaccuracy of the results. Besides, the experiments carried out in vitro revealed that humic substances could bind to collagen fibres to increase their mechanical and chemical resistance, and promote their “maturity”. It seems likely that this effect of humic substances depends upon their interaction with the hydrogen bonding and covalent bonding of the collagen fibres, which is of great significance to the wound repair of skin or mucosa [19]. Considering that FA is rich in carboxyl, phenolic hydroxyl and other functional groups which easily form hydrogen bonds, we speculate that the mechanism of its antidiarrhoeal activity may also be related to the interaction between FA molecules and collagen in intestinal mucosa. Combined with more effective structural research methods, further research in this aspect may be a good direction to explore the antidiarrhoeal mechanism of FA.

Based on previous research and literature investigation, the authors found that the physicochemical properties and activities of FA or HA were generally closely related to their molecular weight [20–22]. Usually, the lower the relative molecular weight, the greater the biological activity, while sometimes it is different [23–26]. In this study, we intend to fractionate FA according to its molecular weight to contrastively study the differences of antidiarrhoeal activities from different fractions as well as the changes of intestinal physiological parameters caused by these fractions, and moreover comprehensively investigate the internal relation between the changes of structural characteristics and the changes of pharmacological activities of different fractions. This may be an effective way to study the material basis and mechanism of antidiarrhoeal effect in depth. This research model of antidiarrhoeal mechanism, including the comparative study of antidiarrhoeal activity of different FA fractions, has not been reported before.

2. Materials and methods

2.1. Raw coal and chemicals

The lignite was collected from Xiaopengzu coal mine in Eshan County, Yunnan Province, China. It was sealed and stored at 4 °C after mining, and the main physical and chemical properties were analyzed (Table 1). Total humic acids, free humic acids, and fulvic acids were determined by the volumetric methods according to the methods in reference [27–29]. 001 × 7 cation exchange resin was purchased from Shanghai Qingxi Chemical Technology Co., Ltd. Rolled ultrafiltration membrane modules with molecular weight cut offs (MWCO) of 1 K, 3 K, 5 K, 10 K and 30 K were bought from Shanghai Mosu Scientific Equipment Co., Ltd. Raw rhubarb (*Rheum officinale Baill*) was purchased from Yunnan Lvsheng Pharmaceutical Co., Ltd., and was made into 1 g/ml water decoction before use (1 ml is equivalent to 1 g of crude drug). The other reagents were analytical pure, and the laboratory water was distilled water.

Table 1
Properties of raw coal.

Sample	Moisture (%)	Ash (%)	Total humic acids (%)	Free humic acids (%)	Fulvic acids (%)
Eshan Lignite	54.71	18.00	53.41	55.65	1.58

The indicators except the moisture were calculated on a dry basis.

2.2. Animals

Male KM mice (18–22 g) were obtained from Experimental Zoology Department of Kunming Medical University, Kunming, China (License No.: SCXK 2015–002). All animals were kept under standard laboratory condition. The animals were fasting for 12 h with free access to water before the last administration for all experiments. All animals received humane care in compliance with the institutional animal care guidelines approved by the Ministry of Science and Technology of China.

2.3. Preparation, purification and fractionation

The raw lignite was ground after drying, sifted through an 80 mesh sieve and then put into the reactor. At a temperature of 30 to 40 °C, hydrogen peroxide having a mass fraction of 15% was slowly added in a ratio of 1:2.5 (weight/volume), and the mixture was stirred sufficiently until no more foam was produced. After standing at room temperature for 12 h, the mixture was centrifuged, and the residual coal was mixed with pure water of equal volume and then centrifuged once. The supernatants (degradation solution) were combined and filtered through a filter paper. The pretreated 001 × 7 type cation exchange resin was added to the filtrate and shaken at room temperature for 2.5 h, and then filtered. The filtrate was concentrated at 50 °C under vacuum, and the concentrate was dried in a freeze dryer to obtain a purified FA. Other process details can be found in reference [30].

Referring to the ultrafiltration technology described in reference [31], FA was separated into six fractions with different relative molecular weight range by using ultrafiltration membrane modules with MWCO of 1 K, 3 K, 5 K, 10 K and 30 K, respectively. The series of fractions were named FA1 (< 1 K), FA2 (1*K*−3*K*), FA3 (3 K–5 K), FA4 (5 K–10 K), FA5 (10*K*−30*K*), and FA6 (> 30 K). In pharmacological tests, when FA and its fractions were configured into solutions, it was necessary to use sodium hydroxide to adjust the pH to neutral before applying.

2.4. Structure characterization

The complex chemical composition, extremely strong polarity and wide molecular weight distribution make it difficult to separate and identify FA directly by common analytical techniques, such as HPLC and GC. In this study, elemental analysis, acidity determination, UV–Vis, IR and functional group analysis were used to characterize its overall structure. The antidiarrhoeal mechanism was explored by investigating the relative changes of structure characteristics and activities with the change of molecular weight.

The content of four elements C, H, O and N in FA was analyzed by a VARIO EL CUBE elemental analyzer (Elementar Trading Co., Ltd). The pH of 0.5% FA aqueous solution was determined by a STARTER 3100 acidity meter (Ohaus Instruments Co., Ltd.). The UV–Vis spectra of FA (0.1% aqueous solution) were obtained on a UV1102II spectrophotometer (Techcomp Scientific Instruments Co., Ltd.) by recording the absorption spectra between 200 nm and 800 nm, and the ratio of absorbance at 465 nm and 665 nm (E4/E6) was calculated. The infrared spectra were recorded by a TJ270-30A spectrophotometer (Tianjin Ruian Technology Co., Ltd.) over the 4000–400 cm^{-1} range using KBr pellets containing 1% FA or its fractions. The contents of total acidic groups, carboxyl groups and phenolic hydroxyl groups in FA and its fractions were measured by the methods described in reference [27].

The total acidic groups and carboxyl groups were determined by barium chloride method and calcium acetate method, respectively. The data of the phenolic hydroxyl groups was obtained by subtraction method.

2.5. Comparative study on antidiarrhoeal effects of FA and its fractions

2.5.1. Evacuation index of mice defecation

The method described in the references [32, 33] was used to evaluate the effect of FA and its fractions on defecation function of mice. A numerical score based on stool consistency and quantity was assigned: 3 (watery stool), 2 (semi-solid stool), and 1 (normal stool). Each test group received an evacuation index (EI) which was expressed according to the formula: EI = 3 × (number of watery stool) +2 × (number of semi-solid stool) + 1 × (number of normal stool). When EI of a test group was higher than that of control group with statistical significance ($P < .05$), the test group was considered to be diarrhoea or the drugs had a purgative effect; when lower than control group with statistical significance ($P < .05$), the group was considered to be constipation or the drugs had an antidiarrhoeal effect; in the case of other results, the drugs were considered to have no significant effect on defecation function of mice. Besides, the diarrhoea inhibition is calculated using the following formula: inhibition (%) = (EIc − EIt)/EIc × 100; the EIc is the evacuation index of control group and the EIt is the evacuation index of test group.

2.5.2. Defecation of normal and diarrhoea mice

Sixteen groups of ten mice each were respectively treated with distilled water (20 ml/kg, p.o.), diphenoxylate (5 mg/kg, p.o.), FA and FA1-FA6 (200 and 400 mg/kg, p.o.) twice a day for three days. The first two groups were used as normal control and positive control group. Each mouse was placed in an individual cage with filter paper at the bottom, which was replaced every hour. The consistency and number of the feces of each mouse were recorded for 6 h to calculate EI. Meanwhile, other nine groups of ten mice each were used for the experiment of drug-induced diarrhoea. The first group was treated with distilled water (20 ml/kg, p.o.) and served as normal control. The second group was also given distilled water (20 ml/kg, p.o.) but received castor oil (0.4 ml/mouse, p.o.) 1 h after the last administration to induce diarrhoea, as model control. The groups 3–9 were administered with FA and FA1-FA6 (400 mg/kg, p.o.) and received castor oil (0.4 ml/mouse, p.o.) 1 h after the last administration. Each mouse was given distilled water or samples twice a day for three days. The EI of each group were calculated respectively using the same method as above. In addition, two other parallel experiments were performed with the castor oil being replaced by magnesium sulfate (2 g/kg, p.o.) and raw rhubarb decoction (0.4 ml/mouse, p.o.) respectively.

2.5.3. Intestinal transit of normal and model mice

The effects of FA and its fractions on intestinal transit of normal and model mice were evaluated by using the charcoal meal test as described in previous report [34]. Eight groups of ten mice each were used for normal intestinal transit test. Treatment was carried out in different groups with distilled water (20 ml/kg, p.o.), FA and FA1-FA6 (400 mg/kg, p.o.). Each mouse was given distilled water or samples twice a day for 3 days. 0.2 ml of a suspension of charcoal meal (10% charcoal in 5% gum arabic) was given to each mouse by intragastric administration 1 h after the last administration. Then the mice were sacrificed by cervical dislocation after 25 min and the small intestines were isolated immediately. The distance traveled by the charcoal meal as well as the total length of the intestine was measured. Intestinal transit was expressed as the percentage of the distance traveled by the charcoal meal relative to the total length of the small intestine. The inhibition of intestinal transit is calculated according to the following formula: inhibition (%) = (mean distance in control group − mean distance in test group)/mean distance in control group × 100.

Nine groups of ten mice each were used for the test of small intestinal hyperfunction induced by neostigmine. The first group was treated with distilled water (20 ml/kg, p.o.) and served as normal control. The second group was also given distilled water (20 ml/kg, p.o.) but treated with neostigmine (0.15 mg/kg, s.c.) 15 min before administration of charcoal meal, as model control. The groups 3–9 were administered with FA and FA1-FA6 (400 mg/kg, p.o.), and received neostigmine (0.15 mg/kg, s.c.) 15 min before administration of charcoal meal. The other procedures were the same as normal intestinal transit test. Another nine groups of ten mice each were used for the test of small intestinal function inhibition induced by diphenoxylate. The same procedures as intestinal hyperfunction test were followed except that diphenoxylate (5 mg/kg, p.o.) was administered 30 min before administration of charcoal meal.

2.6. Collagen content in ileum

The mice were sacrificed at the completion of the castor oil-induced diarrhoea experiment. About 5 cm long intestine was cut at the fixed position in the middle of the ileum, and was weighed after being sucked dry. The hydroxyproline content of fresh tissue was analyzed according to the method described previously [35], and the collagen content in the ileum of mice was calculated by the formula in the reference [36]: collagen (mg/g) = hydroxyproline (mg/g) × 7.46.

2.7. Statistical analysis

The results were expressed as mean ± standard deviation (S.D.). The differences between two groups were analyzed by Student's *t*-test while ANOVA test was used to compare results among groups. $P < .05$ was considered statistically significant.

3. Results

3.1. Effects of FA and its fractions on mice defecation

The results of normal animal experiments were shown in Fig. 1A. The EI of the mice was reduced greatly after successive administration of FA for three days, and the group with high dose (400 mg/kg, p.o.) showed a significant difference ($P < .05$) compared with the control group. After fractionated by ultrafiltration according to relative molecular weight, all the fractions of FA could reduce EI to varying degrees, but the differences among groups were large. At the low doses (200 mg/kg, p.o.), the effects of reducing EI showed a trend of increasing first and then decreasing with the increase of molecular weight. FA3 produced significantly inhibitory effect relative to the control group ($P < .05$). Yet there was no significant pharmacological effect on mice defecation at low dose before the fractionation. At the high doses (400 mg/kg, p.o.), the trend was similar to that at low doses, but the effect of reducing EI was stronger in its totality. Compared with the control group, the EI of FA2 group and FA3 group were reduced significantly ($P < .01$ or $P < .05$), and both were lower than FA group but higher than positive control group. From the above analysis, it could be seen that FA had the effect of inhibiting defecation in mice. Through the fractionation according to molecular weight, the fractions with stronger pharmacological activity could be obtained, wherein FA2 or FA3 had the highest activity but lower than diphenoxylate. And the pharmacological effects of FA and its fractions could be better reflected at high doses, which had the obvious dose-dependence.

In the castor oil-induced diarrhoea model experiment (Fig. 1B), FA showed a significant antidiarrhoeal activity ($P < .05$) as compared to the model control group. After fractionation, the antidiarrhoeal activity was more pronounced in low molecular weight fractions, and especially the difference between FA2 group and model control group reached a very significant level ($P < .01$). With the increase of molecular weight, the antidiarrhoeal activity gradually decreased. As presented in Fig. 1C,

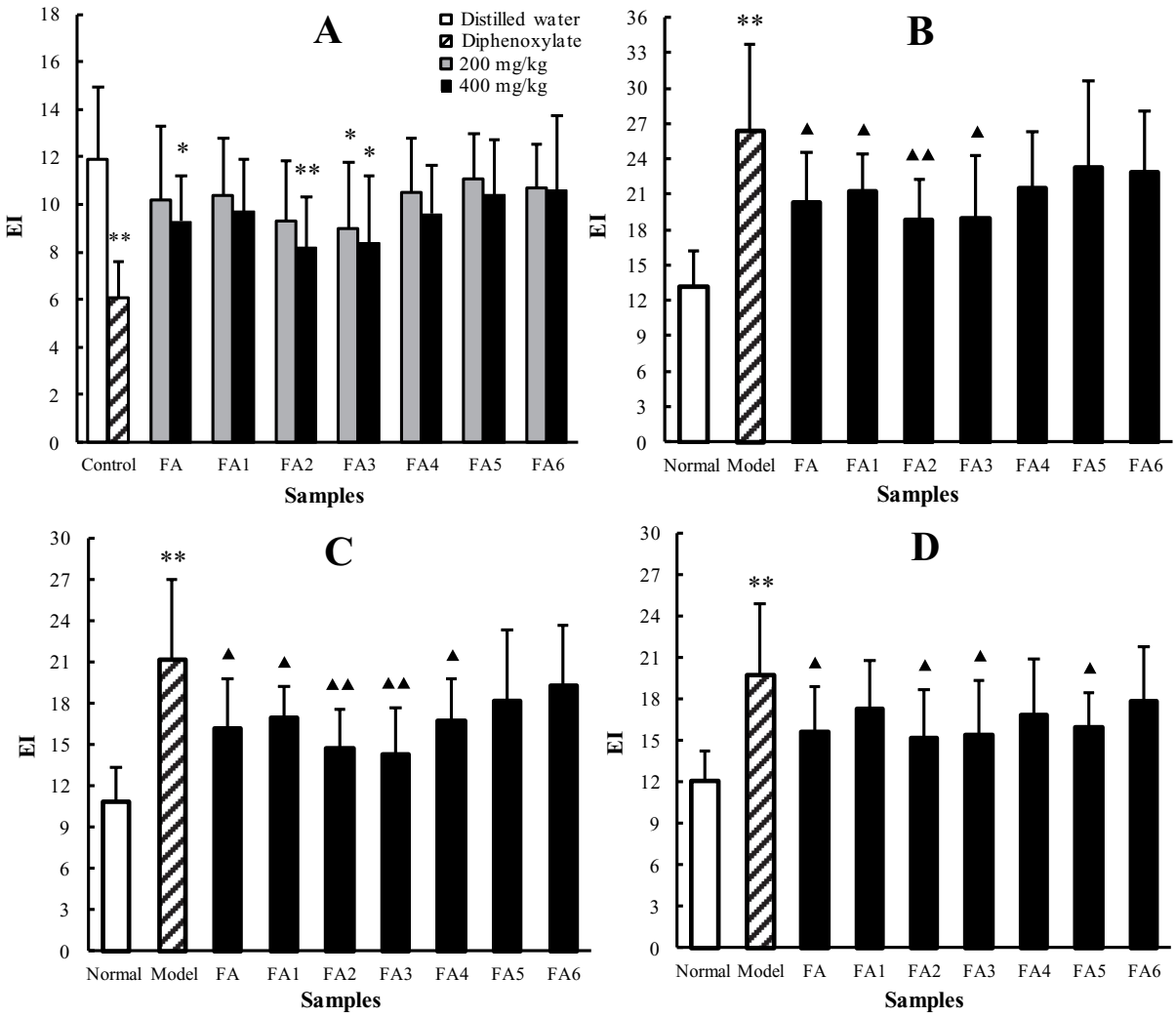

Fig. 1. Different effects of FA and its fractions on the defecation of normal mice (Fig. 1A), diarrhoea mice treated with castor oil (Fig. 1B), diarrhoea mice treated with magnesium sulfate (Fig. 1C), and diarrhoea mice treated with raw rhubarb (Fig. 1D). In diarrhoea model experiments, the doses of FA and its fractions were all 400 mg/kg (p.o.). Evacuation index (EI) was expressed as mean ± S.D. ($n = 10$). $^{*}P < .05$ vs. Normal control; $^{**}P < .01$ vs. Normal control; ▲$P < .05$ vs. Model control; ▲▲$P < .01$ vs. Model control; Student's *t*-test.

FA and its fractions had better inhibitory effects on diarrhoea induced by magnesium sulfate. Not only FA2 and FA3 produced extremely significant antidiarrhoeal effects ($P < .01$), but also FA4 as the high molecular weight fraction showed significant inhibitory effect on mice defecation ($P < .05$). It indicated that FA and its fractions were more sensitive to the diarrhoea induced by magnesium sulfate. Fig. 1D illustrated that the inhibitory effects of FA and its fractions on diarrhoea induced by raw rhubarb were weaker on the whole than their performances in the other two diarrhoea model experiments, and the regularity of antidiarrhoeal activity with the change of molecular weight was not as obvious as that in the other three experiments. Nevertheless, there were still three fractions which showed significant antidiarrhoeal effects ($P < .05$), and the activity of FA2 was the best and stronger than FA. It revealed that the sensitivity of fulvic acid substances to rhubarb-induced diarrhoea was not as strong as that to the other two types of diarrhoea, but the sensitivity and adaptability could be improved through the fractionation according to molecular weight. The above results exposed that in the three types of drug-induced diarrhoea model experiments, the regularity of pharmacological effects of FA and its fractions on mice defecation was similar to that in normal animal experiment. But the inhibitory effect on EI was more easily reflected in the model animals. Although FA and some fractions had significant antidiarrhoeal activity, it may be necessary to increase the dose or administration times in order to restore EI to normal levels in diarrhoea mice.

3.2. Effects of FA and its fractions on intestinal transit

As shown in Table 2, FA could significantly inhibit the intestinal transit of normal mice compared with the control group ($P < .05$). After fractionation, the effects of FA and its fractions on intestinal transit were very similar to that on mice defecation, and the inhibitory effects of FA2 and FA3 were stronger. FA could inhibit neostigmine-induced intestinal hyperfunction to some extent, but there was no significant difference relative to the model control group. FA2 and FA3 produced significant inhibition on the intestinal hyperfunction ($P < .05$), and the latter had the strongest activity. FA and its fractions could also reduce the intestinal transit rate of the mice with intestinal function inhibition induced by diphenoxylate. However, none of them showed significant effect compared with the model control group, and their inhibition rates were substantially smaller than the corresponding data for each sample in the normal animal experiment. Overall, the inhibitory effects of FA and its fractions on intestinal transit in two kinds of model animal experiments were less intensive than their performances in normal animal experiment. Therefore, there might be no synergism or antagonism between fulvic acid substances and neostigmine or diphenoxylate.

Table 2
Different effects of FA and its fractions on intestinal transit of normal and model mice.

Group	Normal		Neostigmine		Diphenoxylate	
	Intestinal transit (%)	Inhibition (%)	Intestinal transit (%)	Inhibition (%)	Intestinal transit (%)	Inhibition (%)
Normal control	70.79 ± 15.27	0.00	64.57 ± 10.57	–	68.78 ± 13.46	–
Model control	–	–	83.80 ± 16.62**	0.00	49.04 ± 7.77**	0.00
FA	58.66 ± 9.84*	17.14	72.30 ± 11.93	13.72	45.57 ± 8.91	7.08
FA1 (< 1 K)	60.07 ± 13.12	15.13	74.90 ± 14.18	10.62	45.80 ± 7.72	6.61
FA2 (1 K–3 K)	55.36 ± 10.19*	21.79	70.42 ± 11.00▲	15.96	44.08 ± 6.44	10.12
FA3 (3 K–5 K)	57.27 ± 11.77*	19.10	68.21 ± 14.02▲	18.60	42.75 ± 8.00	12.83
FA4 (5 K–10 K)	60.78 ± 13.16	14.14	73.96 ± 12.71	11.74	46.52 ± 6.46	5.14
FA5 (10 K–30 K)	63.09 ± 11.87	10.87	77.12 ± 15.42	7.97	45.90 ± 6.46	6.40
FA6 (> 30 K)	64.08 ± 14.03	9.47	75.90 ± 13.03	9.43	47.14 ± 7.51	3.89

In all experiments, the doses of FA and its fractions were 400 mg/kg (p.o.). Intestinal transit (%) was expressed as mean ± S.D. (n = 10). * $P < .05$ vs. Normal control; ** $P < .01$ vs. Normal control; ▲ $P < .05$ vs. Model control; Student's *t*-test.

3.3. Element composition, pH and UV–vis spectral characteristics

The element composition, pH and UV–Vis spectral characteristics of FA and its fractions were shown in Table 3. Compared with the contents of C and H, their atom ratio H/C changed more regularly with the change of molecular weight, and decreased from 1.83 to 1.36 with the increasing of molecular weight. This result indicated that the aromatic condensation degree of the fractions increased as the molecular weight increased. The content of O and the atomic ratio O/C increased first and then decreased, revealing the corresponding change of oxygen-containing functional groups in samples. This trend had a good positive correlation with the change trend of antidiarrhoeal activity of the fractions; and had a certain negative correlation with the change of pH, suggesting that the oxygen-containing functional groups were mainly acidic groups. The UV–Vis absorption properties of FA are usually characterized by the ratio E4/E6, which is a characteristic function of the molecular weight of humic acid substances and independent of the concentration of the solution. The higher the E4/E6, the more chromophores in the sample, and the lower the aromatic condensation degree of FA (the lower the degree of humification). At the same time, it is negatively correlated with the apparent molecular weight of FA. In this study, the change trend of the E4/E6 of the fractions fitted well with that of their molecular weight, and the fractions with high molecular weight may have undergone a relatively long humification process. In a word, the above analysis showed that the FA fractions with low molecular weight contained more oxygen-containing functional groups and had higher antidiarrhoeal activity, while the fractions with high molecular weight contained more aromatic ring structure and had lower antidiarrhoeal activity. In order to determine the types of oxygen-containing functional groups, the samples were analyzed by IR.

3.4. IR

The infrared spectra of FA and its fractions were shown in Fig. 2, and their interpretations were based on the known structural characteristics of fulvic acid substances and the literature data [37,38]. Different samples presented roughly similar infrared spectral characteristics and the main signal (absorption peak) could be assigned to four bands. The spectra of some fractions exhibited obvious differences in individual bands or in certain peak locations.

Band 1 (3600–3000 cm^{-1}): all the spectra contained a strong and broad absorption peak in this band attributed to stretching vibration of hydrogen-bonded OH. Combining with the characteristic peaks in the range of 2700–2500 cm^{-1} and 955–915 cm^{-1}, it was inferred that a part of the OH groups must belong to the carboxyl groups. Different from other samples, the peak shape of FA1 in this band became narrow and the absorption intensity increased, which might be related to the changes of hydroxyl species and content. With the increase of molecular weight, the intensity of absorption peak of the fractions decreased. Band 2 (1750–1600 cm^{-1}): a sharp and intensive peak centred at about 1710 cm^{-1} could be assigned to C═O stretching of various carbonyl groups, mainly including COOH. The absorption peak at 1630 cm^{-1} could be ascribed to the stretching vibration of aromatic ring skeleton, even though the stretching vibration of hydrogen bonded C═O cannot be completely excluded. Considering the distinctive signal at 1210 cm^{-1}, the carbonyl group forming the ester bond might also contribute to the absorption produced by FA1 and FA2 in band 2. We speculated that the formation of the ester group caused a relative decrease of the hydroxyl belonging to carboxyl group, resulting in the big peak of FA1 in band 1 becoming sharp. The intensity of absorption peak of the fractions decreased with the increase of molecular weight, which suggested that the carboxyl content of each fraction may also gradually decrease possibly. Band 3 (1420–1250 cm^{-1}): combining with the signal of OH stretching in band 1, the absorption peak at 1410 cm^{-1} was probably the combination band of C—O stretching and O—H bending of the phenolic compound. As the molecular weight increased, the peak height first rose and then reduced, indicating a variation trend of the phenolic hydroxyl content in the fractions. The C—O stretching and O—H deformation vibration of carboxyl group occurred at 1290 cm^{-1}. Band 4 (830–800 cm^{-1}): The absorption peak at 817 cm^{-1}

Table 3
Element composition, pH and UV–Vis spectral characteristics of FA and its fractions.

Samples	Yield (%)	Elemental composition (%)						Acidity	UV–Vis
		C	H	O	N	H/C	O/C	pH*	E_4/E_6▲
FA	–	35.06	3.69	58.92	1.88	1.26	1.26	2.31	12.97
FA1 (< 1 K)	20.80	32.26	4.93	58.43	2.74	1.83	1.36	2.18	20.23
FA2 (1 K–3 K)	20.37	29.90	3.94	64.76	0.94	1.58	1.62	2.65	18.18
FA3 (3 K–5 K)	12.23	34.90	4.31	59.54	1.05	1.48	1.28	3.01	13.45
FA4 (5 K–10 K)	3.90	35.19	4.18	59.34	1.10	1.42	1.26	3.24	12.37
FA5 (10 K–30 K)	1.30	40.18	4.68	53.78	1.20	1.40	1.00	3.72	10.99
FA6 (> 30 K)	8.67	41.99	4.77	51.67	1.40	1.36	0.92	4.11	8.19

*Refers to the pH of FA aqueous solution at the concentration of 0.5%. ▲ refers to the ratio of the absorbance at 465 nm and 665 nm.

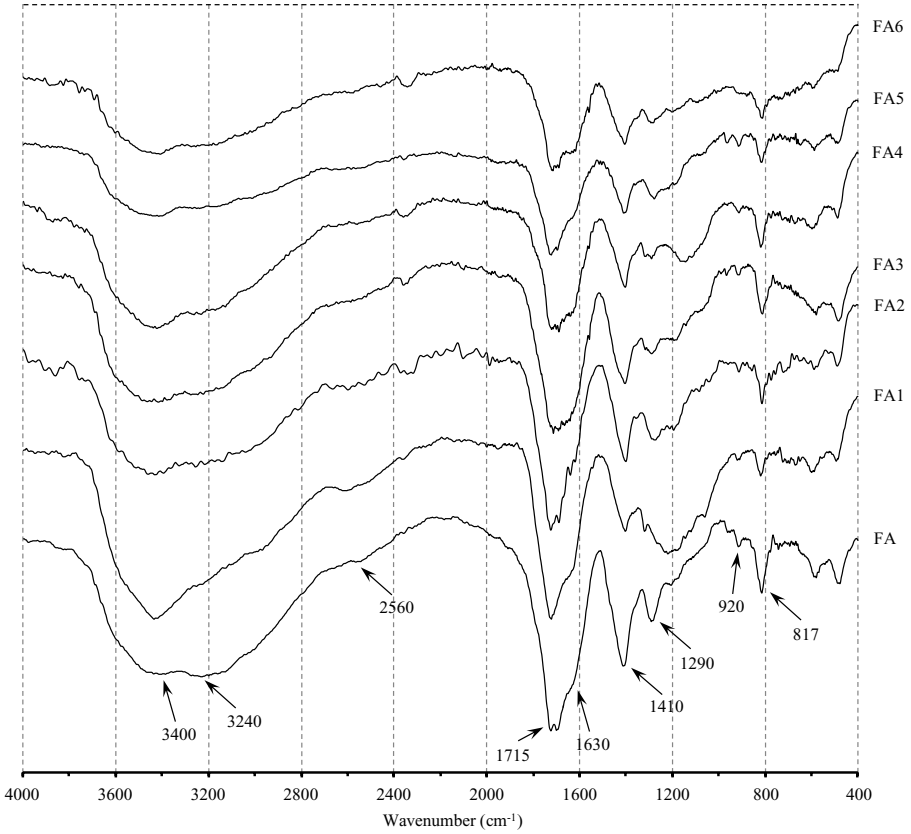

Fig. 2. The infrared spectra of FA and its fractions.

was generally attributed to the = C-H deformation vibration of the substituted aromatic ring. The peak height in Band 4 showed a trend of increasing first and then decreasing with the increase of molecular weight. The absorption produced by FA1 was the lowest, which was probably related to its smaller molecular weight and weaker aromaticity.

From the above analysis, it could be seen that the FA and its fractions from Esan lignite had aromatic structure and contained a large amount of phenolic hydroxyl groups and carboxyl groups. Some fractions also had other oxygen-containing functional groups such as ester groups. The above structural informations and their tendency to change with molecular weight were in good agreement with the informations obtained from elemental analysis, acidity determination and UV–Vis. As the main oxygen-containing functional groups of FA and its fractions, the content of phenolic hydroxyl groups and carboxyl groups was further analyzed by chemical quantitative method to explore the material basis and mechanism of antidiarrhoeal activity.

3.5. Comparative study on functional groups and activities

For the convenience of comparative study, the antidiarrhoeal activity of FA and its fractions was expressed by the inhibition rate of EI, and the data of the castor oil experiment was taken as an example in Fig. 3 (the original data was in Fig. 1B). As shown in Fig. 3A, the carboxyl and total acidic group content of the fractions decreased continuously with the increase of molecular weight. Overall, the proportion of carboxyl groups in total acidic groups was generally larger than that of phenolic hydroxyl groups, especially in FA1. This was roughly consistent with the functional group information reflected by the infrared spectra and pH. The content of phenolic hydroxyl showed a trend of increasing first and then decreasing with the increase of molecular weight, which was very close to that presented by diarrhoea inhibition. The univariate linear regression analysis showed that there was a high correlation between carboxyl content and diarrhoea inhibition (Fig. 3C). The comparative analysis among activity, molecular weight and functional groups indicated that the antidiarrhoeal activity of fulvic acid substances was the highest when their molecular weight was in the range of 2 K–4 K (the median value of molecular weight range of FA2 and FA3). Correspondingly, their phenolic hydroxyl content was the highest, and the content of total acidic groups was also at the higher position. In the main, from FA2 to FA6 the change trend in activity was consistent with that in the content of these three kinds of functional groups. Therefore, we believed that without considering the effects of molecular weight the antidiarrhoeal activity was positively correlated with the content of acidic functional groups. Among them, phenolic hydroxyl groups had the greatest influence, and the lower activity of FA1 might be due to its lower content of phenolic hydroxyl groups. In addition, the antidiarrhoeal activity was also affected by the molecular weight. From FA1 to FA3, the antidiarrhoeal activity was positively influenced by the increase of molecular weight, and negatively impacted by the decrease of functional group content. These two effects reached a balance in the red shadow region. In other words, fulvic acid substances obtained the maximum activity in the interval of FA2 to FA3 under the combined influence of acidic functional groups and molecular weight. Based on the above analysis, we believed that acidic functional groups as well as molecular weight distribution were the material basis of the antidiarrhoeal activity, with phenolic hydroxyl groups having greater influence. The activity intensity was the result of the comprehensive influence of the type and content of acidic functional groups, and molecular weight distribution.

3.6. Collagen content in the intestine

After continuous administration for three days and induction of diarrhoea with castor oil, the collagen content in the connective tissue of mice ileum was shown in Fig. 4. Compared with normal animals, the collagen content model control group was decreased (no significant

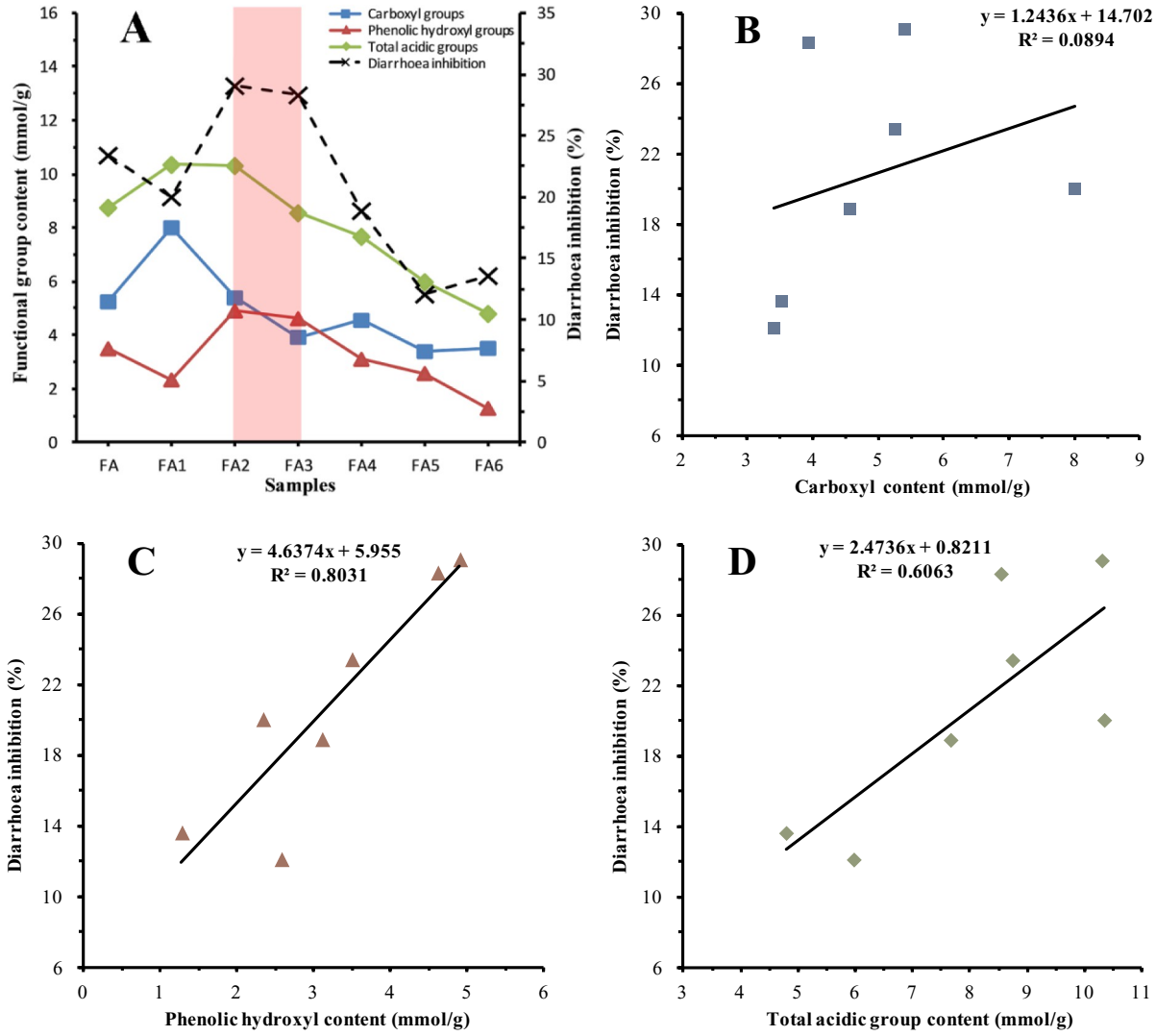

Fig. 3. The acidic functional group content and antidiarrhoeal activity of FA and its fractions (Fig. 3A); the univariate linear regression analysis between diarrhoea inhibition and carboxyl content (Fig. 3B), between diarrhoea inhibition and carboxyl content (Fig. 3C), and between diarrhoea inhibition and total acidic group content (Fig. 3D).

difference), probably due to intestinal mucosal damage caused by the diarrhoea induced by castor oil (an irritant laxative) [39]. For the mice treated with FA, the reduction in collagen content caused by castor oil was greatly attenuated and only slightly below normal levels, but there was no significant difference compared with the model control group. With the change of molecular weight, the ability of the fractions to alleviate the decrease of collagen content was very similar to their antidiarrhoeal activity, and the activity of the fraction with low molecular weight was higher. In particular, FA1 group and FA2 group not only had significant differences with the model group ($P < .05$), but also their collagen content was higher than normal animal group. This indicated that FA1 and FA2 could not only alleviate the decrease of collagen content caused by diarrhoea, but also increase the collagen content in intestinal tract of mice. Other samples might have the same effects as above. These results suggested that the antidiarrhoeal activity of FA and its fractions was closely related to their ability to alleviate the decrease of intestinal collagen content.

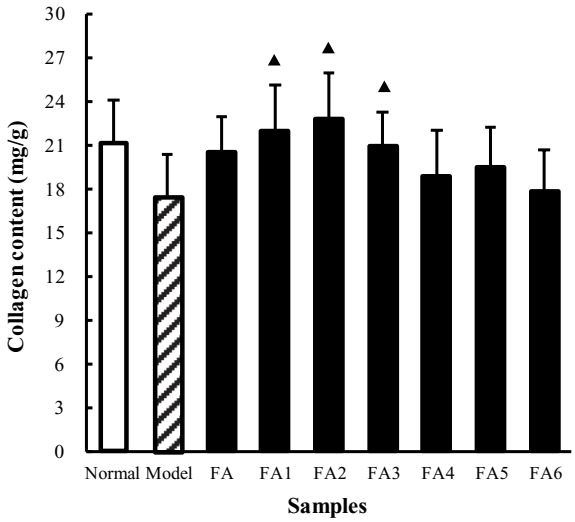

Fig. 4. The effects of FA and its fractions on the collagen content in mice ileum. Collagen content was expressed as mean ± S.D. ▲ $P < .05$ vs. Model control, Student's *t*-test.

4. Discussion

In this paper, the effects of FA and its fractions on intestinal function in mice were studied. The results showed that fulvic acid substances

had the pharmacological activity of inhibiting defecation and intestinal transit in normal mice and significant antidiarrhoeal activity against many different types of drug-induced diarrhoea or intestinal hyperfunction in a dose-dependent manner. Through the fractionation according to molecular weight, the fractions with stronger pharmacological activity than FA could be obtained. The antidiarrhoeal activity of different fractions varied regularly with the change of molecular weight. Generally, the activity of the fractions with low molecular weight was stronger and some fractions were particularly prominent, especially FA2 and FA3. It indicated that the antidiarrhoeal activity of fulvic acid substances was affected by molecular weight distribution and the active components in the mixture were mainly in the range of molecular weight 2 K–4 K. The correlation analysis between structure characteristics and antidiarrhoeal activity showed that the oxygen-containing functional groups especially phenolic hydroxyl groups and the molecular weight distribution were the material basis of antidiarrhoeal activity of fulvic acid substances. The activity intensity was the result of the comprehensive influence of the type and content of acidic functional groups, and molecular weight distribution.

FA and its fractions could inhibit the diarrhoea induced by castor oil, magnesium sulfate and raw rhubarb, and the intestinal hyperfunction caused by neostigmine, showing non-specific anti-diarrhoea activity. However, these drugs have quite different mechanisms of action. Castor oil, a stimulant laxative, is hydrolyzed in the upper small intestine to ricinoleic acid, a local irritant, which irritates the gastrointestinal mucosa resulting in enhancement of intestinal motility [40]. Anthraquinones are usually considered as the chemical basis of purgative activity of raw rhubarb, and cause diarrhoea by stimulating the gastrointestinal tract and increasing the paracellular permeability across the colonic mucosa [41]. The purgative action of castor oil mainly occurs in the small intestine while anthraquinones act in the large intestine, so the antidiarrhoeal action of fulvic acid substances occurs in the whole intestinal tract. Neostigmine, as a cholinesterase inhibitor, can inhibit the cholinesterase and cause much acetylcholine accumulating in the synaptic cleft to excite cholinergic receptors, promoting intestinal transit [42]. Diphenoxylate is an opioid receptor agonist, which directly acts on intestinal smooth muscle and plays an antidiarrhoeal role by inhibiting intestinal peristalsis [43]. In both intestinal hyperfunction mice induced by neostigmine and in intestinal suppression mice induced by diphenoxylate, the inhibition of intestinal transit produced by FA and its fractions was weaker than that in normal animals. In other words, fulvic acid substances did not show significant antagonism or synergism with neostigmine or diphenoxylate. The phenomena mentioned above implied that the antidiarrhoeal activity of fulvic acid substances was not mediated by the cholinergic nervous system or opioid receptors. Besides, Magnesium sulphate belonging to osmotic laxative induces diarrhoea by preventing the re-absorption of water to increase the volume of intestinal contents [44]. The macromolecules of fulvic acid substances rich in carboxyl and phenolic hydroxyl groups are liable to complex with metal ions to reduce intestinal osmotic pressure and the chemical stimulation of Mg and SO_4 ions on intestinal tract, thereby playing an auxiliary role in inhibiting diarrhoea. This may be the reason why FA and its fractions were more sensitive to diarrhoea induced by magnesium sulfate. It can also serve one of the evidences for the above-mentioned conclusion about the material basis of antidiarrhoeal activity of fulvic acid substances.

Previous studies by Riede et al. [19] had shown that as carboxylated phenolic compounds, humic substances can bind to collagen fibres to increase their mechanical and chemical resistance, and promote their “maturity” (a process bound up with the increase in cross-links). The importance of this in traumatology is obvious, because newly formed collagen fibres are not, during tissue repair, at first completely resistant to mechanical stress. The results of this study also indicated that the antidiarrhoeal activity of FA and its fractions was closely related to their ability to alleviate the decrease of intestinal collagen content. It could be inferred that the function of promoting the “maturity” of collagen in intestinal connective tissue resisted intestinal mucosal damage caused by diarrhoea, thus exhibiting antidiarrhoeal activity. The stabilizing effects of fulvic acid substances on collagen can be explained on the basis of a tanning process [45]. Meanwhile, the above studies also suggested that this effect of humic substances seemed to depend upon their hydrogen bonding interactions with collagen, which had been indirectly confirmed by the experiments in vivo in this study. The results of the experiments showed that the samples with high content of phenolic hydroxyl groups and carboxyl groups had stronger antidiarrhoeal activity. These functional groups are easy to form hydrogen bonds. This was another evidence that the oxygen-containing functional groups were the material basis of antidiarrhoeal activity of fulvic acid substances. Moreover, the results of this study exposed that compared with normal animals, FA also increased the collagen content in the intestinal tract of diarrhoea mice. Yasar et al. [46] believed that the increase of collagen content in intestinal connective tissue may protect the epithelial cells from sloughing and keep the integrity of ileal epithelium in a whole. Accordingly, one of antidiarrhoeal mechanisms was considered that fulvic acid substances could protect intestinal mucosal damage, prevent epithelial cells from sloughing and maintain the integrity of intestinal epithelium by promoting the maturity and increasing the content of collagen in the intestinal tract. For low molecular weight fractions, this mechanism was more pronounced due to the higher content of functional groups. On the other hand, although the antidiarrhoeal activity of the high molecular weight fractions was generally weak, a certain fraction also exhibited significant activity for rhubarb-induced diarrhoea. When the average molecular weight of the mixture is large, an obvious characteristic of FA solution is its colloidal properties [4]. In the treatment of ulcerative colitis with FA, through colonoscopy Su Bingwen et al. [47] observed that FA formed a gelatinous protective film on the wound surface after contact with the ulcer surface, which reduced the exudation of secretions. They considered that the formation of this film was consistent with the colloidal properties and astringency of fulvic acid substances. Islam et al. [17] believed that HA are able to form a protective film on the mucous epithelia of the gastro-intestinal tract against infections and toxins, and the macro-colloidal structure of HA ensures a good shielding on the mucous membrane of the stomach and gut, the peripheral capillaries and damaged mucous cells. Therefore, we speculated that the antidiarrhoeal mechanism of high molecular weight fraction may be as follows: fulvic acid substances formed a gelatinous protective layer on the intestinal mucosa, passivating the role of local irritants, hindering water leakage, thus playing an antidiarrhoeal role. The formation of the protective layer was mainly due to the flocculation and precipitation of fulvic acid substances with colloidal properties. The formation of this protective layer might also benefit from a partial contribution of the astringency, which binds to proteins on the surface of the intestinal mucosa to precipitate on epithelial surface. The mechanism of the astringent antidiarrhoeal effect was similar to that of tannins, which was also related to the polar functional groups such as phenolic hydroxyl and carboxyl groups [45].

In conclusion, this study demonstrated that fulvic acid substances had non-specific antidiarrhoeal activity against the whole intestinal tract. The oxygen-containing functional groups especially phenolic hydroxyl groups, molecular weight distribution, colloidal properties and astringency were the material basis of the antidiarrhoeal activity. The activity intensity was the result of the comprehensive influence of these factors. For low molecular weight components (< 5 K), the antidiarrhoeal effect could be exerted by promoting the maturity and increasing the content of collagen in the connective tissue of intestinal mucosa to protect the mucosal damage, prevent the epithelial cells from sloughing and maintain the integrity of intestinal epithelium. This mechanism was dominated by oxygen-containing functional groups such as phenolic hydroxyl and carboxyl groups. For high molecular weight components (> 5 K), the mechanism was considered that fulvic acid substances formed a gelatinous protective layer on the surface of intestinal mucosa to passivate the role of local irritants and hinder water leakage. The colloidal properties and astringency of the mixture

played a dominant role. That is to say, fulvic acid substances had dual antidiarrhoeal mechanism acting on the intestinal mucosa. One mainly acted on the inside of intestinal mucosa and the other acted on the surface, which could simultaneously exert the antidiarrhoeal effects. The non-specific antidiarrhoeal effect caused by the dual mechanism would perfectly explain the fact that fulvic acid substances could inhibit several different types of diarrhoea. The antidiarrhoeal material basis and mechanism of FA were confirmed and elucidated directly for the first time, which could provide important theoretical support for the development and clinical application of antidiarrhoeal drugs. This study also verified that molecular weight grading was a very effective sample pretreatment method for the activity and mechanism study of fulvic acid substances. Researchers may improve the activity efficiency of samples or regulate their antidiarrhoeal mechanism through fractionation technique, which has important guiding significance for the activity study and product development of similar natural macromolecular mixtures.

Declaration of Competing Interest

The authors declare no conflict of interest.

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