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Main components of ethyl acetate extract of *Chimonanthus salicifolius* and its effects on intestinal mucositis in mice induced by 5-fluorouracil

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Abstract

This study aimed to analyze the main components of ethyl acetate extract of *Chimonanthus salicifolius* (EAECS) and investigate its effect on intestinal mucositis in mice induced by 5-fluorouracil. EAECS was prepared, and its main components were analyzed. Fifty mice were randomly divided into control, model and 3, 6 and 9 g/kg EAECS groups. The latter three groups were intragastrically administrated with 3, 6 and 9 g/kg EAECS for 7 days, respectively. The intestinal mucositis model was established in latter four groups from the 1st to 7th day of administration. Results showed that, the main components in EAECS were rutin, scopolamine, isofraxidin, scoparone, quercetin and kaempferol. After administration, compared with model group, in 9 g/kg EAECS group the body weight was increased; the serum tumor necrosis factor α (TNF-α) and interleukin 6 levels were decreased, and the interleukin 10 level was increased; the intestinal tissue superoxide dismutase level was increased, and the malondialdehyde and myeloperoxidase levels wer decreased; the liver tissue TNF-α level was significantly decreased (all $P < 0.05$). In conclusion, EAECS has rich flavonoids and coumarins. It can alleviate the intestinal mucositis in mice induced by 5-FU. The mechanism may be related to the anti-inflammatory and antioxidant effects.

Keywords: *Chimonanthus salicifolius*; extract; ethyl acetate; intestinal mucositis.

Practical Application: This study has provided a basis for preparing ethyl acetate extract of *Chimonanthus salicifolius* and applying it to treatment of intestinal mucositis.

1 Introduction

Chemotherapy is one of the most common and effective treatment methods for patients with malignant tumors. However, the long-term use of chemotherapeutic drugs often causes the serious damage to normal tissues of body (Vriesendorp et al., 1987). 5-fluorouracil (5-FU) is a basic drug for the treatment of rectal cancer and breast cancer (Longley et al., 2003). The clinical studies have shown that 5-FU has strong toxic and side effects, such as gastrointestinal mucositis, immune hypofunction, hematopoietic dysfunction, etc. (Takatsuki et al., 1990; Randall & Weissman, 1997; Saegusa et al., 2008; Hu et al., 2020). Studies have shown that, some substances extracted from natural plants have good pharmacological activities (Lin et al., 2019; Milagres et al., 2020). It is found that some Chinese medicinal herbs with anti-inflammatory and antioxidant activities can be used to treat the 5-FU induced intestinal mucositis (Wright et al., 2009; Ávila et al., 2015; Santos et al., 2015). It is found that, the water extract of *Chimonanthus salicifolius*, a kind of Chinese medicinal herb, can alleviate the mucositis caused by 5-FU, but the specific active site of extract is not clear (Liu et al., 2013). In addition, the previous studies have shown that, the ethyl acetate extract of *Chimonanthus salicifolius* (EAECS) mainly contains flavonoids and coumarins, and has better bacteriostatic effect in vitro than its chloroform extract and petroleum ether extract (Wang et al., 2012, 2016; Wen et al., 2013). In the present study, the EAECS was prepared, and its main components were preliminarily determined. In addition, the protective effects of EAECS on intestinal mucositis induced by 5-FU in mice were investigated. The objective was to provide an experimental basis for the development of EAECS related medicines for the adjuvant anti-cancer therapy.

2 Materials and methods

2.1 Materials and animals

Chimonanthus salicifolia leaves were obtained from Songyang, Lishui, Zhejiang Province, China, and was identified as by Professor Chengxin Fu in Zhejiang University. Fifty ICR mice (half male and half female; 18-20 g; license number SCXK (Zhejiang) 2016-0001) were purchased from Jinhua Inspection and Research Institute for Food and Drug (Jinhua, China). Acetonitrile (chromatographic grade), scoparone and scopoletin were purchased from Beijing Zhongke Quality Inspection Biotechnology Co., Ltd. (Beijing, China). Rutin, quercetin, kaempferol and isofraxidin were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). 5-FU was provided by Sigma-Aldrich Corp. (MO, USA). The kits of superoxide dismutase (SOD), malondialdehyde (MDA) and myeloperoxidase (MPO) were purchased from Nanjing Institute of Bioengineering (Nanjing, China). The enzyme-linked immunosorbent assay (ELISA) kits of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and interleukin 10 (IL-10) were purchased from Wuhan Huamei Bioengineering Co., Ltd. (Wuhan, China).

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2.2 Preparation of EAECS

A 2 kg of dry crushed and sieved *Chimonanthus salicifolia* was added to the extraction kettle, followed by adding 15 times volume of 80% ethanol. The extraction was performed at 90 °C for 2 h in water bath. After concentrating to no alcoholic taste by decompression, the residues were successively extracted with equal-volume petroleum ether, chloroform and ethyl acetate. After concentrating the ethyl acetate fraction at 60 °C, 56 g EAECS was obtained.

2.3 Determination of components of EAECS

A 0.2 g EAECS was placed in a 10 mL volumetric flask. The methanol was added to dissolve EAECS and dilute the solution to the scale. After shaking, the sample solution was filtered by 0.45 microporous membrane. The components of EAECS were determined by high performance liquid chromatography (HPLC) (Agilent 1260). The HPLC conditions were as follows: Agilent ZORBAX Extend-C18 liquid chromatography column (250 mm x 4.6 mm, 5 μm); mobile phase: acetonitrile (A)-0.2% glacial acetic acid water (B); detection wavelength 365 nm; flow rate: 1 mL/min; column temperature: 35 °C; injection volume 10 μL. The linear gradient elution started at 15.0%-16.5% (A) within 0-15 min, changed to 16.5-40.0% (A) within 15-30 min, and changed to 40.0-15.0% (A) within 30-35 min.

The standard sample rutin, scopolamine, isofraxidin, scoparone, quercetin and kaempferol were precisely weighed, and then were put into 10 mL volumetric flask, respectively. The methanol was added to dissolve the standard sample and dilute the solution to the scale. The concentration of above standards was 0.32, 0.39, 0.25, 0.14, 0.49 and 0.29 mg/mL, respectively. A 1 mL of each standard sample solution was taken, and was added to the 10 mL volumetric flask, followed by adding methanol to the scale. Thus the mixed standard sample solution was prepared. It was diluted with different multiples, and the standard curve of standard sample was drawn.

The mixed standard sample solution was continuously injected with 6 needles into HPLC instrument. The relative standard deviation was calculated to inspect the precision of the HPLC instrument. The EAECS test solution was injected into HPLC instrument at 2, 4, 6, 8, 10 and 12 h, respectively, and the relative standard deviation was calculated to inspect the stability of test sample. Six parts of one EAECS test solution were taken and were injected into HPLC instrument. The relative standard deviation was calculated to inspect the repeatability.

2.4 Pharmacological experiments

EAECS was dissolved in 2% Tween 80 solution to different concentrations for pharmacological experiments. Fifty ICR mice were randomly divided into control group, model, 3 g/kg EAECS, 6 g/kg EAECS and 9 g/kg EAECS groups, 10 mice in each group. After a week of adaptation, the mice in 3 g/kg EAECS, 6 g/kg EAECS and 9 g/kg EAECS groups were intragastrically administrated with 3, 6 and 9 g/kg EAECS, respectively. The control group and model group were simultaneously intragastrically administrated with equal volume of normal saline. The administration was performed once a day, for 7 successive days. The intestinal mucositis model was established in model, 3 g/kg EAECS, 6 g/kg EAECS and 9 g/kg EAECS groups. From the first day of administration, the mice were intraperitoneally injected with 25 mg/kg 5-FU. The injection was performed once a day, for 7 successive days. During the experimental period, the body weight of mice was continuously observed. On the eighth day, the orbital blood of mice was obtained, followed by centrifugation at 2000 rpm for 15 min. The serum was collected. The serum levels of TNF-α, IL-6 and IL-10 were determined by ELISA. The mice were executed. The small intestine and liver tissues were taken, and the tissue homogenate was prepared, respectively. The intestinal tissue SOD, MDA and MPO levels were detected according to the instruction of kits. The liver tissue TNF-α level was determined by ELISA. In addition, the hematoxylin-eosin staining of liver tissue was performed, and the pathological changes were observed.

2.5 Statistical analyses

All statistical analysis was carried out using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The data were presented as mean ± SD. The differences among different groups were analyzed using one-way ANOVA with LSD post hoc test. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Feasibility of determination method

The results of HPLC instrument precision inspection showed that, the standard deviations of rutin, scopolamine, isofraxidin, scoparone, quercetin and kaempferol were 0.29%, 0.47%, 0.82%, 1.50%, 1.60% and 0.46%, respectively, which were less than 3%, indicating that the precision of the HPLC instrument was good. The inspection results of test sample solution stability showed that, the standard deviations of rutin, scopolamine, isopyrimidine, scoparone, quercetin and kaempferol were 0.45%, 0.42%, 0.79%, 0.33%, 1.90% and 0.53% respectively, which indicated that the test sample solution was stable at room temperature for 12 h. The results of repeatability inspection showed that, the standard deviations of rutin, scopoletin, isofraxidin, scoparone, quercetin and kaempferol were 2.3%, 0.97%, 1.18%, 1.90%, 1.59%, 0.46%, respectively, which indicated that the determination method had good repeatability. Above results suggested that the determination method was stable and could be used for the content analysis of EAECS.

3.2 Main components in EAECS

The HPLC showed that, the main components in EAECS were rutin, scopolamine, isofraxidin, scoparone, quercetin and kaempferol (Figure 1). The content of rutin, scopolamine, isofraxidin, scoparone, quercetin and kaempferol in EAECS were 72.03, 29.34, 32.95, 5.36, 7.68 and 12.59 mg/g, respectively (Table 1).

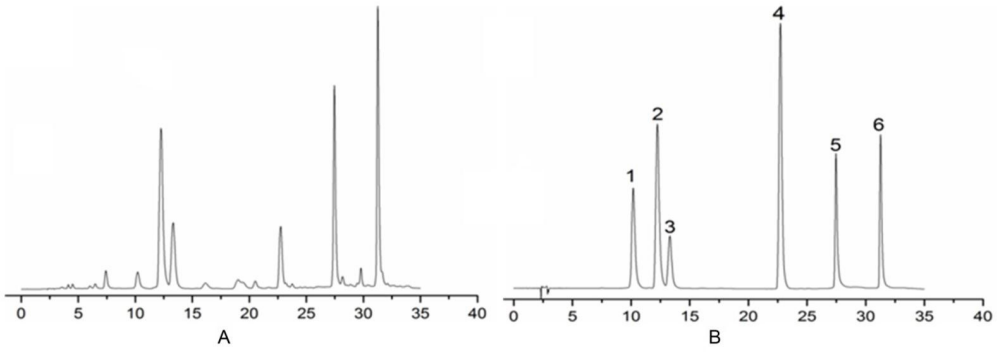

Figure 1. High performance liquid chromatography of EAECS (A) and mixed standard samples (B). (1) rutin; (2) scopolamine; (3) isofraxidin; (4) scoparone; (5) quercetin; (6) kaempferol.

Table 1. Main components of EAECS.

Component	Linear relationship	Relevance	Linear range (mg/mL)	Content (mg/g)
Rutin	y = 2451.5x + 0.0458	0.9994	0.0032-0.032	72.03
Scopolamine	y = 15042x + 0.4978	0.9998	0.0039-0.039	29.34
Isofraxidin	y = 12655x + 10.983	0.9994	0.0014-0.014	32.95
Scoparone	y = 14751x + 8.2425	0.9993	0.0024-0.024	5.36
Quercetin	y = 18096x + 17.756	0.9995	0.0049-0.049	7.68
Kaempferol	y = 13862x + 8.3043	0.9993	0.0029-0.029	12.59

EAECS: ethyl acetate extract of *Chimonanthus salicifolius*.

3.3 Effect of EAECS on body weight of mice with intestinal mucositis

As shown in Figure 2, from the modeling beginning, the body weight of mice in control group gradually increased. The body weight in each of other groups gradually increased, followed by gradual decreasing. On the 5th, 6th and 7th day, the body weight in model, 3 g/kg EAECS, 6 g/kg EAECS and 9 g/kg EAECS groups was significantly lower than that in control group, respectively ($P < 0.05$). Compared with model group, the body weight in 3 g/kg EAECS, 6 g/kg EAECS and 9 g/kg EAECS groups were significantly increased, respectively ($P < 0.05$).

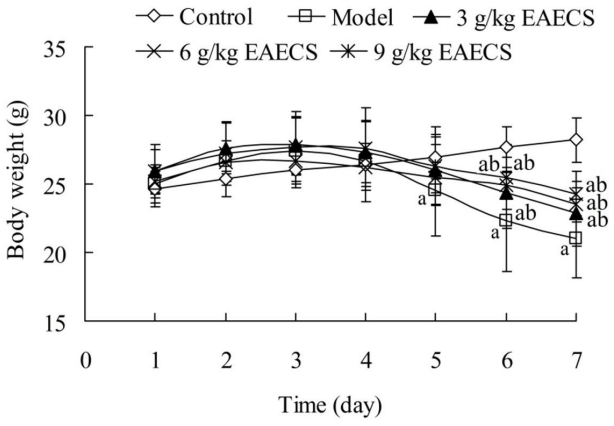

Figure 2. Changes in body weight of mice during experiment in five groups. [a]$P < 0.05$ compared with control group; [b]$P < 0.05$ compared with model group. EAECS: ethyl acetate extract of *Chimonanthus salicifolius*.

3.4 Effect of e EAECS on serum TNF-α, IL-6 and IL-10 level in mice with intestinal mucositis

As shown in Table 2, at the end of experiment, compared with control group, in model group the serum TNF-α and IL-6 levels were significantly increased, respectively ($P < 0.05$), and the serum IL-10 level was significantly decreased ($P < 0.05$). Compared with model group, in 3 g/kg EAECS, 6 g/kg EAECS and 9 g/kg EAECS groups the serum TNF-α and IL-6 levels were significantly decreased, respectively ($P < 0.05$), and the serum IL-10 level was significantly increased ($P < 0.05$).

3.5 Effect of e EAECS on intestinal tissue SOD and MDA levels in mice with intestinal mucositis

Table 3 showed that, at the end of experiment, compared with control group, in model group the intestinal tissue SOD level was significantly decreased ($P < 0.05$), and the intestinal tissue MDA level was significantly increased ($P < 0.05$). Compared with model group, the intestinal tissue SOD level in 6 g/kg EAECS and 9 g/kg EAECS groups was significantly increased, respectively ($P < 0.05$), and the intestinal tissue MDA level in 9 g/kg EAECS group was significantly decreased ($P < 0.05$).

3.6 Effect of EAECS on intestinal tissue MPO level in mice with intestinal mucositis

As shown in Figure 3, at the end of experiment, the intestinal tissue MPO level in control, model, 3 g/kg EAECS, 6 g/kg EAECS and 9 g/kg EAECS groups was 0.98 ± 0.19, 1.28 ± 0.25, 1.20 ± 0.23, 1.06 ± 0.15 and 0.85 ± 0.14 U/mg prot, respectively. Compared with control group, the intestinal tissue MPO level in model group

Table 2. Serum TNF-α, IL-6 and IL-10 level in mice in five groups.

Group	n	TNF-α (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)
Control	10	169.89 ± 30.35	60.86 ± 23.46	96.46 ± 18.65
Model	10	413.51 ± 169.79[a]	150.07 ± 11.03[a]	56.33 ± 11.88[a]
3 g/kg EAECS	10	282.71 ± 69.16[ab]	124.81 ± 31.13[ab]	90.76 ± 26.57[b]
6 g/kg EAECS	10	260.00 ± 43.59[ab]	91.92 ± 21.48[abc]	108.40 ± 32.36[b]
9 g/kg EAECS	10	199.66 ± 49.59[bc]	77.83 ± 19.48[bc]	111.41 ± 24.72[b]
F		11.476	26.215	8.488
P		< 0.001	< 0.001	< 0.001

[a]P < 0.05 compared with control group; [b]P < 0.05 compared with model group; [c]P < 0.05 compared with 3 g/kg EAECS group. EAECS: ethyl acetate extract of *Chimonanthus salicifolius*; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; IL-10: interleukin-10.

Table 3. Intestinal tissue SOD and MDA levels in mice in five groups.

Group	n	SOD (U/mg prot)	MDA(nmol/mg prot)
Control	10	101.73 ± 23.81	8.95 ± 2.29
Model	10	76.20 ± 16.26[a]	12.61 ± 2.79[a]
3 g/kg EAECS	10	87.87 ± 17.34	10.44 ± 2.47
6 g/kg EAECS	10	96.83 ± 12.79[b]	10.92 ± 3.37
9 g/kg EAECS	10	94.73 ± 19.47[b]	9.51 ± 1.64[b]
F		2.917	3.019432957
P		0.031	0.027

[a]P < 0.05 compared with control group; [b]P < 0.05 compared with model group. EAECS: ethyl acetate extract of *Chimonanthus salicifolius*; SOD: superoxide dismutase; MDA: malondialdehyde.

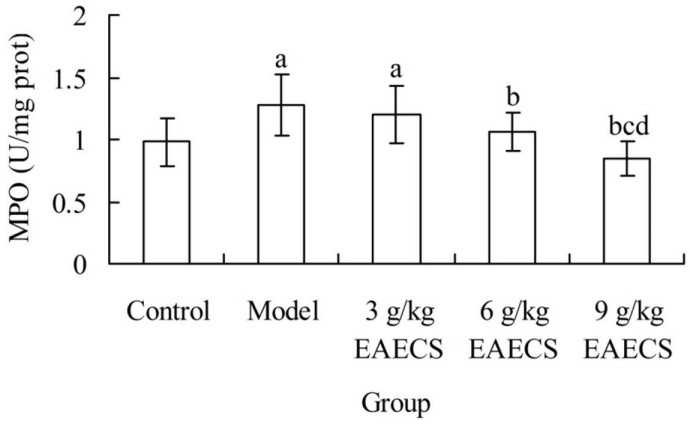

Figure 3. Intestinal tissue MPO level in mice in five groups. [a]P < 0.05 compared with control group; [b]P < 0.05 compared with model group; [c]P < 0.05 compared with 3 g/kg EAECS group; [d]P < 0.05 compared with 6 g/kg EAECS group. EAECS: ethyl acetate extract of *Chimonanthus salicifolius*; MPO, myeloperoxidase.

was significantly increased (P < 0.05). Compared with model group, the intestinal tissue MPO level in 6 g/kg EAECS and 9 g/kg EAECS groups was significantly decreased, respectively (P < 0.05).

3.7 Effects of EAECS on liver tissue histopathology in mice with intestinal mucositis

At the end of experiment, compared with control group, there was obvious intrahepatic bile duct inflammation in model group. Compared with model group, the inflammatory reaction in 3 g/kg EAECS, 6 g/kg EAECS and 9 g/kg EAECS groups was significantly mitigated, respectively (Figure 4).

3.8 Effect of e EAECS on liver tissue TNF-α level in mice with intestinal mucositis

At the end of experiment, the intestinal tissue liver tissue TNF-α level in control, model, 3 g/kg EAECS, 6 g/kg EAECS and 9 g/kg EAECS groups was 41.88 ± 11.53, 215.38 ± 66.73, 199.57 ± 71.30, 178.46 ± 59.94 and 143.30 ± 44.19 pg/mg prot, respectively. Compared with control group, the liver tissue TNF-α level in model group was significantly increased (P < 0.05). Compared with model group, the liver tissue TNF-α level in 9 g/kg EAECS group was significantly decreased (P < 0.05) (Figure 5).

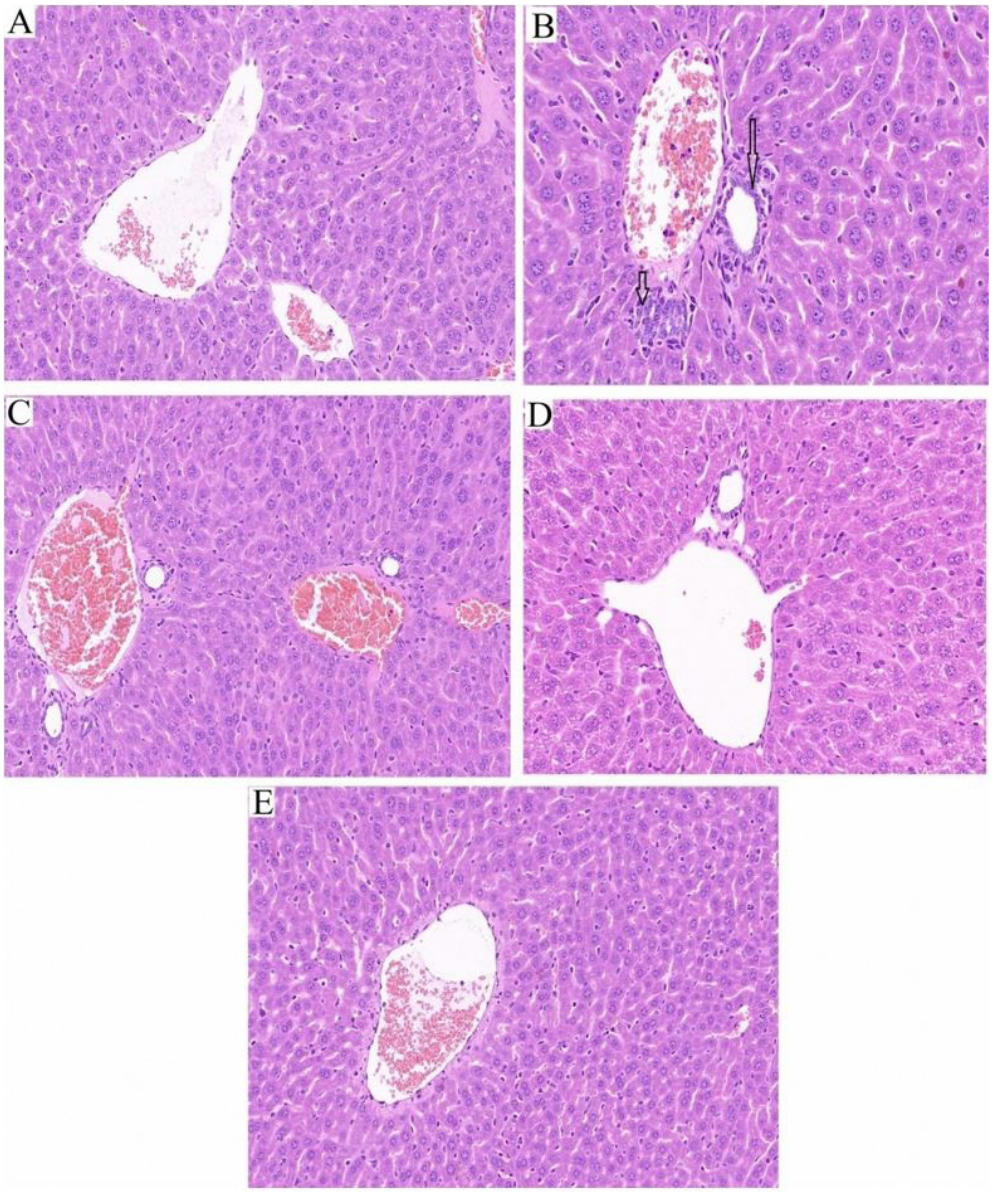

Figure 4. Liver tissue histopathology in mice in five groups (× 10). (A) control; (B) model; (C) 3 g/kg EAECS; (D) 6 g/kg EAECS; (E) 9 g/kg EAECS. EAECS: ethyl acetate extract of *Chimonanthus salicifolius*.

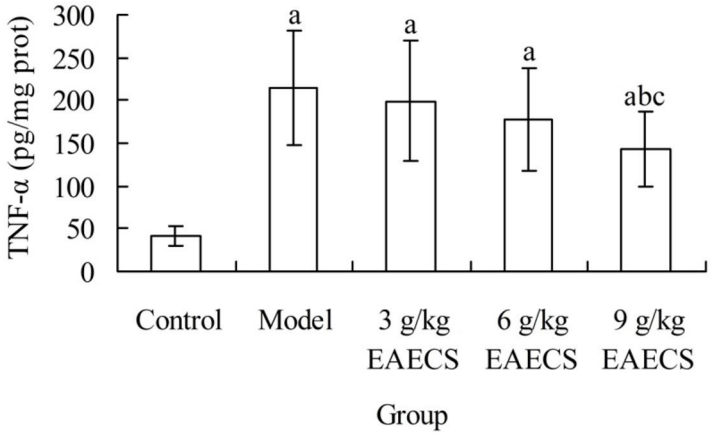

Figure 5. Liver tissue TNF-α level in mice in five groups. [a]P < 0.05 compared with control group; [b]P < 0.05 compared with model group; [c]P < 0.05 compared with 3 g/kg EAECS group. EAECS: ethyl acetate extract of *Chimonanthus salicifolius*; TNF-α, tumor necrosis factor α.

4 Discussion

Chimonanthus salicifolius belongs to Calycanthaceae family and is considered as a unique plant species in China. This plant is generally distributed in the mountain areas of southeast China and it is a semievergreen shrub with solitary and small yellowish flowers (Hu & Xiao, 2005). This study prepared the EAECS and determined its main components. Results showed that, the main components in EAECS were rutin, scopolamine, isofraxidin, scoparone, quercetin and kaempferol. In Liu et al.'s (2013) study, the main components of *Chimonanthus salicifolius* aqueous extract are rutin, quercetin and kaempferol. In can be found that, the ethyl acetate extract can obtain more active ingredients from *Chimonanthus salicifolius*, compared with aqueous extract.

Excessive activation of inflammatory cytokines has a significant causal relationship with the development of systemic inflammatory response. TNF-α is an important regulator of physiological and pathological changes in the body during infection. Excessive TNF-α can promote the production of other cytokines, leading to the linkage and amplification effects (Zelová & Hošek, 2013). IL-6 is one of the key cytokines in inflammatory response, which is also called proinflammatory factor. After the inflammation occurs, the production of IL-6 is increased, which induce the formation of C-reactive protein and procalcitonin (Vincent et al., 2015). IL-10 is a multifunctional and multicellular inflammatory inhibitor, and is one of the most important and effective anti-inflammatory cytokines. It plays a key role in down-regulating inflammatory response. IL-10 can inhibit the adhesion and infiltration of inflammatory cells, inhibit the synthesis of monocytes/macrophages, and release the proinflammatory cytokine (Ljungberg et al., 2010). Results of this study showed that, at the end of experiment, compared with control group, in model group the serum TNF-α and IL-6 levels were significantly increased, respectively, and the serum IL-10 level was significantly decreased. Compared with model group, in three EAECS groups the serum TNF-α and IL-6 levels were significantly decreased, and the serum IL-10 level was significantly increased. This indicates that, EAECS can reduce the inflammatory response in mice with intestinal mucositis.

SOD is an important antioxidant enzyme in body, which specifically scavenges the superoxide anions. SOD can resist and block the damage caused by oxygen free radicals to cells and repair the damaged cells in time (Kalra et al., 1988). MDA is an important product of lipid peroxidation. The increased content of MDA can damage the integrity of cell membrane and increase its permeability, thus causing the tissue damage (Janero, 1990). MPO is a heme protease containing heme prosthetic group secreted by neutrophils, monocytes and macrophages of some tissues. The MPO activity can indirectly reflect the content of neutrophils (Tsumbu et al., 2012). In the present study, at the end of experiment, compared with control group, in model group the intestinal tissue SOD level was significantly decreased, and the intestinal tissue MDA and MPO levels were significantly increased. Compared with model group, the intestinal tissue SOD level in 6 g/kg EAECS and 9 g/kg EAECS groups was significantly increased, respectively, and the intestinal tissue MDA and MPO levels in 9 g/kg EAECS group were significantly decreased, respectively. This indicates that, EAECS can increase the intestinal tissue SOD activity in mice with intestinal mucositis, and decrease the intestinal tissue MDA and MPO activities. The mechanism may be related to the high content of rutin, kaempferol, scopolamine and isofraxidin in EAECS, which have obvious anti-inflammatory effects (Nieman et al., 2007, 2009; Grabs et al., 2014).

It is found that 5-FU can easily lead to abnormal liver function in mice (Loibl et al., 2004). In our study, the effects of EAECS on liver tissue histopathology in mice were investigated. Results showed that, compared with control group, there was obvious intrahepatic bile duct inflammation in model group. Compared with model group, the inflammatory reaction in three EAECS groups was significantly mitigated. In addition, compared with control group, the liver tissue TNF-α level in model group was significantly increased. Compared with model group, the liver tissue TNF-α level in 9 g/kg EAECS group was significantly decreased. Previous study (Gelen et al., 2017) has shown that, quercetin and rutin can reduce the hepatotoxicity induced by 5-FU, which further explains that EAECS can alleviate 5-FU-induced liver dysfunction in mice.

5 Conclusion

In conclusion, the main components in EAECS were rutin, scopolamine, isofraxidin, scoparone, quercetin and kaempferol. EAECS can alleviate the intestinal mucositis in mice induced by 5-FU, which is related to its rich flavonoids and coumarins which have the anti-inflammatory and antioxidant effects. This study has provided an experimental basis for the development of EAECS related medicines for the adjuvant anti-cancer therapy.

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