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# Immuno-enhancement effects of ethanol extract from *Cyrtomium macrophyllum* (Makino) Tagawa on cyclophosphamide-induced immunosuppression in BALB/c mice

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ABSTRACT

*Ethnopharmacological relevance:* *Cyrtomium macrophyllum* (Makino) Tagawa has been traditionally used as a herbal medicine for the treatment of various infectious diseases such as tapeworm infestation, colds, and viral diseases. However, no systematic study of the immunity of *Cyrtomium macrophyllum* ethanol extracts (CM) has yet been reported. The present work evaluates these traits.

*Materials and methods:* 120 male BALB/c mice were divided into 6 groups of 20 mice each: (1) normal group (sterile physiological saline), which served as a blank control; (2) model group (Cyclophosphamide, CY) group (sterile physiological saline), which served as a negative control; (3) low-dose CM (50 mg/kg BW); (4) intermediate-dose CM (100 mg/kg BW); (5) high-dose CM (200 mg/kg BW); (6) CM group (200 mg/kg BW). CY (0.2 ml) was administered via intraperitoneal injection. The other regimens were administered via gavage in 0.2 ml solution. Phytochemical of CM was characterized by HPLC–LTQ-Orbitrap. The acute toxicity effect of the ethanol extract of *Cyrtomium macrophyllum* was also investigated.

*Results:* The spleen and thymus indices of mice receiving low, intermediate, and high doses of CM recovered more quickly than those of CY mice, and they did so in a dose-dependent manner. These mice also showed higher T cell and B cell proliferation responses and macrophage function than those of CY mice, and their serum levels of interleukin-6 and interferon-γ had become normal. In acute toxicity test, CM exhibited no mortality and behavioral changes in mice. Quantitative phytochemical analysis showed flavonoids, polyphenols, and tannins to be the major compounds present in the extract, at 27.64%, 30.87%, and 11.22%, respectively. We found that 16 compounds were characterized by the interpretation of their mass spectra obtained by the MS/MS.

*Conclusion:* The current study demonstrates that *Cyrtomium macrophyllum* ethanol extract improved immune function in CY-treated mice.



## 1. Introduction

A large number of plants used in traditional medicines have been shown to possess immunomodulating activities (Choi et al., 2004; Ben Sghaier et al., 2011). Recently, complementary or alternative medicines have become popular for treating different immune disorders. Increasingly among these are extracts from medicinal plants. Evaluation of the immunomodulatory activity of plant extracts is an interesting and growing area of research. However, little is known about its immunomodulatory activities, in particular, for less frequently studied or previously unknown (medically) plants.

*Cyrtomium macrophyllum* (Makino) Tagawa f. muticum (Christ) Ching et Shing (Family: Dryopteridaceae) is a common medicinal plant found in the southwest of China. It is recorded as Daye Guanzhong in Traditional Chinese Medicines (Wu and Song, 1996).

*Abbreviations:* CM, *Cyrtomium macrophyllum* rhizome ethanol extract; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; IFN-γ, interferon-γ; IL-6, interleukin-6

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Decoctions of *Cyrtomium macrophyllum* are used in folk medicine for the treatment of a variety of diseases including, epidemic flu, epidemic encephalitis B, homeostasis and dysfunctional uterine bleeding (Wu and Song, 1996; Lou and Qin, 2003; Liu et al., 2004) and their efficacy is widely acclaimed among the Guizhou Province of China. Previous studies suggest that the rhizome of *Cyrtomium* may contain various pharmacological actions such as anti-oxidative, anti-bacterial, anti-parasite, anti-viral and anti-cancer (Yang and Li, 1993; Kapadia et al., 1996; Ito et al., 2000; Zhou et al., 2009; Da Silva et al., 2010; Cao et al., 2013).

These activities are mediated by active components such as flavonoids, phloroglucinol derivatives, triterpenes, tannins, essential oils and polysaccharides (Hisada et al., 1972). Major flavonoids were flavonol O-glycosides based on kaempferol, quercetin, and sometimes myricetin, and C-glycosylflavones, such as isovitexin, vitexin, isoorientin, orientin and their O-glycosides (Xiang et al., 2002; Iwashina et al., 2006).

However, there is too little information on the components and bioactivities of most fern species including *Cyrtomium macrophyllum*. In this study, we aimed to demonstrate in vivo ethnomedicinal benefits of ethanolic extract from *Cyrtomium macrophyllum* against immunosuppressive conditions. The major compounds in the tested extracts were tentatively identified by high performance liquid chromatography–linear ion trap-Orbitrap hybrid mass spectrometry (HPLC–LTQ-Orbitrap) analysis for the first time for the rhizome of this species. In particular, this is the first report of immunomodulatory activities of this plant collected from Guizhou province.

## 2. Materials and methods

### 2.1. Material

RPMI 1640 was purchased from Gibco (Invitrogen Corporation, U.S.). The T-cell mitogen concanavalin A (ConA) was purchased from Sigma. Lipopolysaccharide (LPS, from *Escherichia coli* 055:B5, L2880, lyophilized powder) and dimethyl sulfoxide (DMSO) were acquired from the Yixin Institute of Chemical Engineering (Jiangsu, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Gibco (Grand Island, NY, U.S.). Cyclophosphamide (CY) was purchased from Jiangsu Hengrui Medicine Co., Ltd. (Lianyungang, Jiangsu, China). Interleukin-6 (IL-6) and interferon-γ (IFN-γ) Enzyme-Linked Immunosorbent Assay (ELISA) kit was purchased from R&D (Minneapolis, MN, U.S.). All chemicals used in the experiments were of analytical grade.

### 2.2. Plant extract and quantitative analysis

Rhizome of *Cyrtomium macrophyllum* was collected in May 2013 from Yanjing, a village in Guizhou Province, China (latitude, 27°70′ N; longitude, 108°01′ E; altitude, 834 m). The identities of the plants were verified by Dr. Wenliang Liu and deposited (Voucher no. 20130510) at the herbarium of Nanjing University College of Traditional Chinese Medicine (NJUCM), Nanjing, Jiangsu, P.R. China.

Dried rhizomes of *Cyrtomium macrophyllum* (100 g) were extracted twice with 70% ethanol (with a 2-h reflux), and the extract was then concentrated under reduced pressure. The decoction was filtered, lyophilized, and stored at 4 °C. The yield of dried extract from crude starting materials was 22.5% (w/w).

The polyphenol content of *Cyrtomium macrophyllum* ethanol extract was quantified by the Folin–Ciocalteau method (Yuan et al., 2005; Chattopadhyay and Kumar, 2006). Aliquots of test sample (100 μl) were mixed with 2.0 ml of 2% $Na_2CO_3$ and incubated at room temperature for 2 min. After the addition of 100 ml of 50% Folin–Ciocalteau phenol reagent, the reaction tube was incubated for 30 min at room temperature, and finally absorbance was read at 720 nm. Gallic acid (0.2 mg/ml) was used as standard. Polyphenol content was expressed according to the following formula (OD stands for optical density):

$$\text{Polyphenols } (\%) = \frac{\text{OD extract} \times 0.2}{\text{OD Gallic acid} \times \text{Extract concentration}} \times 100,$$

A known volume of each extract was placed in a 10 ml volumetric flask to estimate flavonoid content according to the modified method of Zhishen et al. (1999). After addition of 75 ml of $NaNO_2$ (5%), 150 ml of freshly-prepared $AlCl_3$ (10%), and 500 ml of NaOH (1 N), the volume was adjusted with distilled water until 2.5 ml. After 5 min incubation, the total absorbance was measured at 510 nm. Quercetin (0.05 mg/ml) was used as a standard.

Flavonoid content was expressed according to the following formula:

$$\text{Flavonoids } (\%) = \frac{\text{OD extract} \times 0.05}{\text{OD Quercetin} \times \text{Extract concentration}} \times 100$$

The method described by Pearson (1976) was used for the determination of tannin content of samples. Extraction of tannins was achieved by dissolving 5 g of sample in 50 ml of distilled water in a conical flask, allowing the mixture to stand for 30 min with shaking the flask at 10 min intervals, and then centrifuging at 5000*g* to obtain a supernatant (tannin extract). The extract was diluted to 100 ml in a standard flask using distilled water. 5 ml of the diluted extract and 5 ml of standard tannic acid (0.1 g/l) were measured into different 50 ml volumetric flasks. 1 ml of Folin–Denis reagent was added to each flask followed by 2.5 ml of saturated sodium carbonate solution. The solutions were made up to the 50 ml mark with distilled water and incubated at room temperature (20–30 °C) for 90 min. The absorption of these solutions was measured against the reagent blank (containing 5 ml distilled water in the place of the extract or the standard tannic acid solution) in a Genesys (Wisconsin, USA) spectrophotometer at 760 nm wavelength. Tannin content was calculated in triplicate (Nwabueze, 2007) according to the following formula:

$$\text{Tannins } (\%) = \frac{\text{OD extract}}{\varepsilon \times L \times \text{Extract concentration}} \times 100$$

where $\varepsilon$ = molar extinction coefficient ($L\,g^{-1}\,cm^{-1}$) of tannic acid ($=3.27\ L\,g^{-1}\,cm^{-1}$) and $L=1$ cm.

### 2.3. Analysis of the main compounds in ethanolic extract

HPLC analyses were performed using a Thermo Scientific UltiMate 3000, equipped with a binary pump system (Thermo). The separation was performed on a Hypersil Gold column (100 mm × 2.1 mm, i.d., 3 μm) and the column temperature was set at 35 °C. The mobile phases were 0.02% formic acid in water (A) and acetonitrile (B). Gradient elution was linearly programmed as follows: 0–5 min 10% (v/v) B, 5–25 min 90% B, 25–30 min 90% B, at a constant flow rate of 0.2 ml/min. The injection volume was 5 μl.

Mass spectra were analyzed on a LTQ-Orbitrap instrument with a heated electrospray ionization (HESI) source (Thermo, Bremen, Germany). The MS was operated in negative mode to analyze the ethanolic extract. Both analyses were done in a single run. The data were acquired in scan mode using a *m/z* range of 150–800. The ionization source working conditions were as follows: sheath gas (nitrogen) flow rate of 35 arb, aux gas (nitrogen) flow rate of 10 arb, spray voltage of 3.0 kV, capillary temperature of 300 °C, capillary voltage of −5 V. Nitrogen (>99% purity) and argon (99% purity) were used as nebulizing and collision (product ion scan,

MS/MS) gases, respectively. Data acquisition was carried out with Xcalibur 2.1 software.

### 2.4. Animal and experimental design

Male BALB/c mice (8 weeks old, 18–20 g) were purchased from the Yangzhou University Laboratory Animal Center (Yangzhou, China). The mice were housed at 22 ± 1 °C, with 12 h-light/12-h dark cycle and 50–60% relative humidity, free access to food and water during the experiments. All procedures involving animal care were approved by the Ethics Committee of the Chinese Academy of Agricultural Sciences. After being adapt to environment for 1 week, these mice were randomly divided into six groups (20 for each). Two groups of healthy mice were used as normal control and positive control groups and treated once daily with physiological saline solution and 200 mg/kg body weight CM, respectively, for 18 days. From days 1 to 3, the other four groups of mice were given 80 mg/kg/d cyclophosphamide (CY) via intraperitoneal injection. From days 4 to 18, the mice were given the following treatments: model groups (CY) were treated with physiological saline solution; three CM groups were treated with 50, 100, and 200 mg/kg CM. CY (0.2 ml) was administered via intraperitoneal injection. The other regimens were administered via gavage in 0.2 ml solution. 24 h after the last dose, the animals were weighed and then killed via decapitation. The heart, liver, kidney, spleen, and thymus were excised; the spleen and thymus were immediately weighed. The thymus and spleen indices were calculated according to the following formula: index (mg/g)= (weight of thymus or spleen)/body weight.

### 2.5. Assay of splenic lymphocyte proliferation induced by T cell and B cell mitogens concanavalin A and lipopolysaccharide

The mouse spleens were aseptically removed from the mice using scissors and forceps in 0.1 M cold PBS, gently homogenized, and passed through a 40 μm nylon cell strainer to produce single-cell suspensions in accordance with the method used by Yuan et al. (2006). The erythrocytes in the cell mixture were washed via hypo-osmotic hemolysis, and the cells were resuspended to a final density of $5 \times 10^6$ cells/ml in RPMI 1640 medium supplemented with 10% newborn bovine serum (Invitrogen Corp., Carlsbad, CA, U.S.), 100 U/ml streptomycin, and 100 U/ml penicillin. Spleen cells (100 μl/well) were seeded into a 96-well plate containing ConA (1 μg/well) or LPS (2 μg/well). The spleen cells were then cultured for 3 days in 5% $CO_2$ atmosphere at 37 °C, and then further incubated for 4.5 h with 10 μl MTT (5 mg/ml) per well. The plate was centrifuged at 200 × *g* for 15 min, and the supernatant was discarded. DMSO (100 μl) was added to each well, which was then shaken until all crystals dissolved. The absorbance at 570 nm was measured on a microplate reader (Thermo Multiskan MK3, U.S.).

### 2.6. Phagocytic index

The function of the macrophage cells was assessed via a carbon clearance test performed on three mice from each group according to the procedure of Wang et al. (2011). Each mouse was intravenously injected with diluted India ink at 100 μl/10 g body weight. Blood specimens were collected at 2 min ($t_1$) and 10 min ($t_2$) from the retinal venous plexuses, and 20 μl blood was then mixed with 2 ml 0.1% $Na_2CO_3$. The absorbance at 600 nm was measured on a UV–visible spectrophotometer with 0.1% $Na_2CO_3$ as the blank. The liver and the spleen were weighed, and the phagocytic index was calculated as follows:

$$K = (\lg OD_1 - \lg OD_2)/(t_2 - t_1)$$

Here $OD_1$ represents $t_1$ and $OD_2$ represents $t_2$.

Phagocytic index $a = \sqrt[3]{K} \times A/(B+C)$, where $A$ is the body weight, $B$ is the liver weight, and $C$ is the spleen weight.

### 2.7. Serum IFN-γ and IL-6 assay

Whole blood was obtained from BALB/c mice killed by cervical dislocation, under sterile conditions. Whole blood was centrifuged at 1000 × *g* and 4 °C for 20 min, while the upper layer contained the serum. The amount of IFN-γ and IL-6 in the serum was analyzed by the mouse IFN-γ and IL-6 ELISA kit according to the manufacturer's instructions (R&D, Minneapolis, MN, U.S.).

### 2.8. Acute toxicity study

This protocol was performed as reported previously (Atsamo et al., 2011). After overnight fasting, groups of ten BALB/c male mice were administered *Cyrtomium macrophyllum* ethanol extract in graded doses up to 2000 mg/kg. The normal control group received only water. The groups were observed for 7 days and at the end of the experiment mortality was recorded for each group.

### 2.9. Statistical analysis

All data are presented as the mean ± SD, analyzed using SPSS for Windows version 15.0 (SPSS Inc., Chicago, IL, U.S.). The statistical analysis was evaluated via one-way ANOVA followed by Scheffe's test to detect the intergroup differences. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Quantitative analysis of CM extract

*Cyrtomium macrophyllum* ethanol extract was studied chemically to determine its composition. Results showed the presence of flavonoids, tannins, and polyphenols. Quantitative phytochemical analysis showed flavonoids, polyphenols, and tannins to be the major compounds present in the extract, at 27.64%, 30.87%, and 11.22%, respectively. Other compounds of the ethanol extract remain unclear and need to be further investigated and clarified.

### 3.2. Characterization of ethanolic extracts from Cyrtomium macrophyllum by HPLC–LTQ-Orbitrap

Fig. 1 shows the base peak chromatogram ethanolic extract from *Cyrtomium macrophyllum* by HPLC–LTQ-Orbitrap. The 16 compounds characterized are presented in Table 1, identified considering the elution order. All the compounds were characterized by the interpretation of their mass spectra obtained by the MS/MS and also taking into account the data provided by the literature.

### 3.3. The mouse spleen and thymus indices

Spleen and thymus indices can reflect the immune function and prognosis of an organism. As shown in Table 2, the spleen and thymus indices of the CY-treated group showed lower values than in the normal group. CM200 group showed significantly higher spleen and thymus indices than other groups ($P < 0.05$). The spleen and thymus indices of the CM+CY-treated groups (CM100+CY and CM200+CY) were significantly higher than those

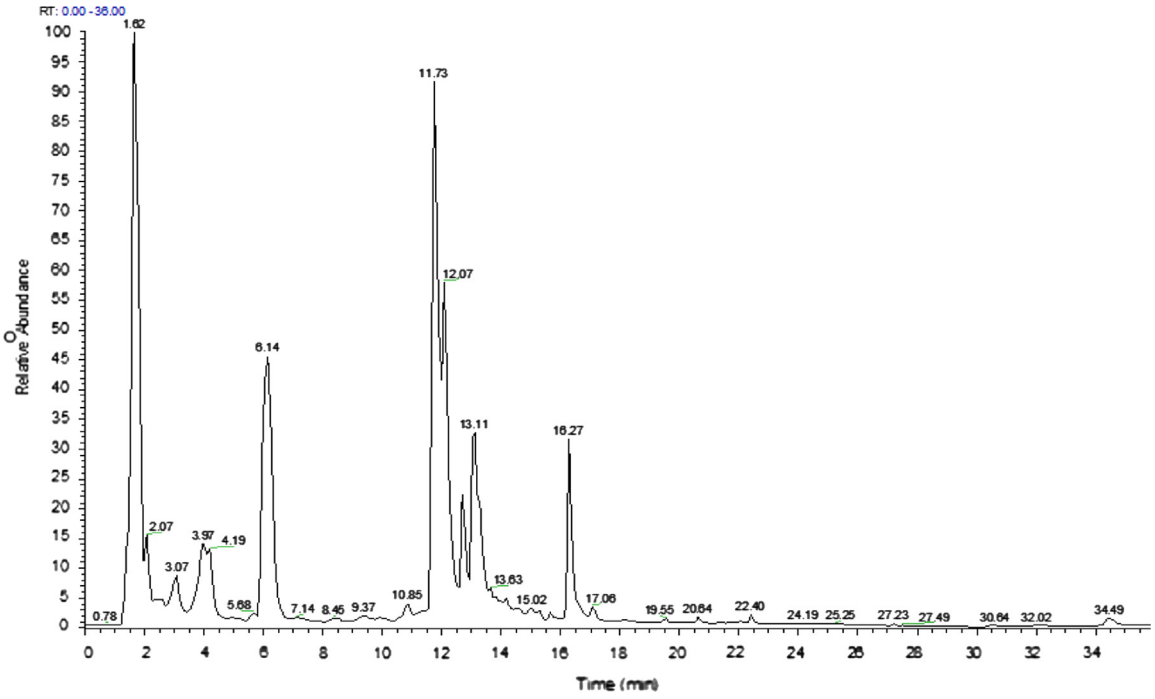


**Fig. 1.** TIC of ethanol extract from *Cyrtomium macrophyllum*.

**Table 1**
Relevant analytical data for compounds isolated in rhizome of *Cyrtomium macrophyllum* ethanol extract.

| Peak | RT (min) | Parent ion | MS/MS | Error (ppm) | Mol. formula | Proposed compound |
|---|---|---|---|---|---|---|
| 1 | 1.62 | 195.0503 | 176.9515, 158.9404, 128.8641, 98.8543 | 1.953 | $C_6H_{12}O_7$ | Hamamelonic acid |
| 2 | 2.07 | 191.0192 | 173.0831, 126.9273, 110.9442, 84.8312 | 3.984 | $C_6H_8O_7$ | Citric acid |
| 3 | 2.25 | 205.0347 | 172.9829, 160.9288, 158.9091, 110.9170 | 1.907 | $C_7H_{10}O_7$ | Citric acid Me ester |
| 4 | 2.34 | 243.0502 | 225.0817, 199.1000, 155.0023, 110.9371, 86.8467 | 1.814 | $C_{10}H_{12}O_7$ | 1-O-Galloylglycerol |
| 5 | 2.43 | 315.071 | 296.9396, 271.1269, 195.0247, 152.8542, 108.9266 | −0.154 | $C_{13}H_{16}O_9$ | Ginnalin B |
| 6 | 2.6 | 447.1129 | 429.3446, 403.2545, 315.1623, 297.2086, 271.1322, 207.0412, 178.9783, 163.0275, 151.9585 | −3.632 | $C_{18}H_{23}O_{13}$ | 1-O-(3,4-Dihydroxybenzoyl)-6-O-[(2S,3S,4S)-3,4-dihydroxy-4-(hydroxymethyl)tetrahydro-2-furanyl]-β-L-glucopyranose |
| 7 | 3.07 | 153.0194 | 109.0297 | −0.993 | $C_7H_6O_4$ | Resorcylic acid |
| 8 | 3.92 | 577.1329 | 559.1234, 467.0958, 451.1021, 425.0864, 407.0761, 372.9723, 299.0552, 289.0709, 273.0392 | −2.996 | $C_{20}H_{26}O_{12}$ | Procyanidin B4 |
| 9 | 4.19 | 353.0502 | 176.9447, 174.9537, 156.9658, 112.8752 | −3.965 | $C_{15}H_{14}O_{10}$ | Daphnetin 7-glucuronoside |
| 10 | 6.14 | 209.0451 | 190.971, 147.0450 | −2.567 | $C_{10}H_{10}O_5$ | 2-Methyl-3-(2,4,6-trihydroxyphenyl)acrylic acid |
| 11 | 6.41 | 289.0708 | 245.0815, 205.0502, 179.0346, 165.0191 | −1.763 | $C_{15}H_{14}O_6$ | Epicatechin |
| 12 | 11.73 | 543.043 | 254.9810, 175.0244 | −3.653 | $C_{21}H_{20}O_{15}S$ | Quercetin 3-(3-sulfoglucoside) |
| 13 | 12.07 | 463.0862 | 287.0553, 175.0245, 151.0036 | −4.36 | $C_{21}H_{20}O_{12}$ | 3′,4′,5,7-Tetrahydroxyflavanone,7-O-Glucuronopyranoside |
| 14 | 12.69 | 593.1486 | 285.0397, 257.0448, 229.0501 | −4.439 | $C_{27}H_{30}O_{15}$ | Kaempferol 3-rutinoside |
| 15 | 13.11 | 447.0918 | 301.0343, 300.0273 | −3.321 | $C_{21}H_{20}O_{11}$ | Quercetin 7-O-L-Rhamnopyranoside |
| 16 | 16.27 | 285.0395 | 229.0506, 151.0037 | −3.513 | $C_{15}H_{10}O_6$ | Kaempferol |

of the CY-treated group ($P<0.05$), indicating that CM can counteract the immunosuppressive effect of CY.

### 3.4. The concanavalin-A- and lipopolysaccharide-induced splenic lymphocyte proliferation in cyclophosphamide-treated mice

The normal splenic lymphocyte proliferation ratio induced by ConA or LPS in mice treated with RPMI-1640 medium was considered 100%. The proliferative responses of splenic lymphocytes to both T cell and B cell mitogens (ConA and LPS, respectively) were significantly lower than the normal splenic lymphocyte proliferation ratio in CY-treated mice, as shown in Fig. 2 ($P<0.05$). Treatment with CM50+CY, CM100+CY and CM200+CY groups significantly enhanced dose-dependently both T cell and B cell proliferation compared with CY treated group ($P<0.05$). The treatment of CM200 promoted both T cell and B cell proliferative responses. These findings indicate that CM counteracts the immunosuppressive effect of CY.

### 3.5. Effect of CM on macrophage phagocytosis in cyclophosphamide-treated mice

Carbon clearance tests were performed to determine the effect of CM on macrophage activation. As shown in Fig. 3, the phagocytic index $a$ of the CY-treated group was significantly lower than that of the normal group ($P<0.05$). This inhibitory effect of CY was dramatically reversed by pretreatment with CM. In CM-treated group (200 mg/kg), phagocytic activity was higher than that of the normal group ($P<0.05$); in the CM-treated groups (all three dosages), the values were significantly higher than those in the CY-treated group ($P<0.05$), demonstrating that CM can enhance the macrophage function in CY-treated mice.

**Table 2**
The changes of immune organ index in every group.

| Group | Spleen index | Thymus index |
|---|---|---|
| Normal | $5.32 \pm 0.31^{a}$ | $1.69 \pm 0.16^{a}$ |
| CY | $3.51 \pm 0.27^{b}$ | $0.94 \pm 0.25^{c}$ |
| CM50+CY | $4.43 \pm 0.41^{c}$ | $1.17 \pm 0.30^{c}$ |
| CM100+CY | $5.15 \pm 0.28^{a}$ | $1.65 \pm 0.23^{a}$ |
| CM200+CY | $5.29 \pm 0.43^{a}$ | $1.72 \pm 0.38^{a}$ |
| CM200 | $5.72 \pm 0.37^{d}$ | $2.21 \pm 0.49^{d}$ |

[a–d] Data within a column without the same superscripts differ significantly ($P < 0.05$).

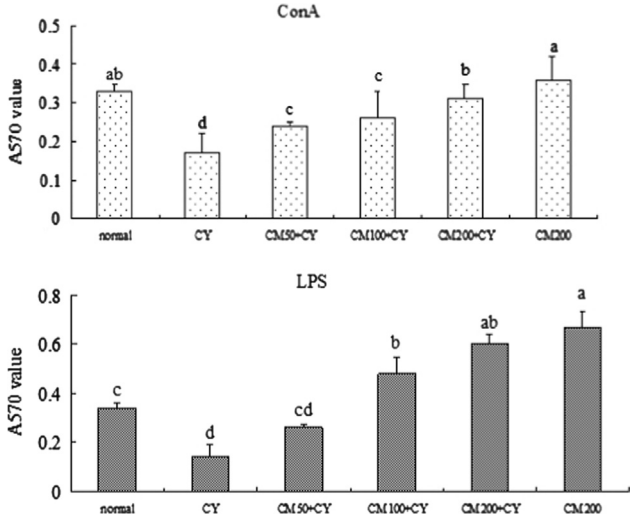


**Fig. 2.** Spleen lymphocyte proliferation in CY-treated mice.
[a–d]Data within a column without the same superscripts differ significantly ($P < 0.05$).

### 3.6. Serum IFN-γ and IL-6 assay

To assess the immunomodulating effect of CM, cytokines (IFN-γ and IL-6) levels in the supernatants of serum cultures from CY-treated mice were determined by ELISA. As shown in Fig. 4, the serum levels of cytokines (IFN-γ and IL-6) were significantly lower in CY-treated mice than those in normal group ($P < 0.05$). Treatment with CM100+CY and CM200+CY groups recovered the amount of IFN-γ and IL-6 compared with CY-treated group ($P < 0.05$), rather than CM 100+CY group ($P > 0.05$). The serum from the CM+CY-treated group (200 mg/kg) showed more secreted cytokines IFN-γ and IL-6. Concentrations reached 123.08 and 69.42 pg/ml, respectively. After CM administration, a dose-dependent increase of IFN-γ and IL-6 production was observed in the supernatants of cultured serum.

### 3.7. Acute oral toxicity study

The result of the acute toxicity study showed that a single oral administration CM (2000 mg/kg) did not produce any mortality, behavioral changes (gait, posture, fur, depression, and panting) in the mice as compared to the control group. Similarly, no significant changes were recorded in serum biochemical (total bilirubin, creatinine, triglycerides, SGOT, and glucose) as well as hematology parameters (RBCs, WBCs) of the treated group when compared to the control group.

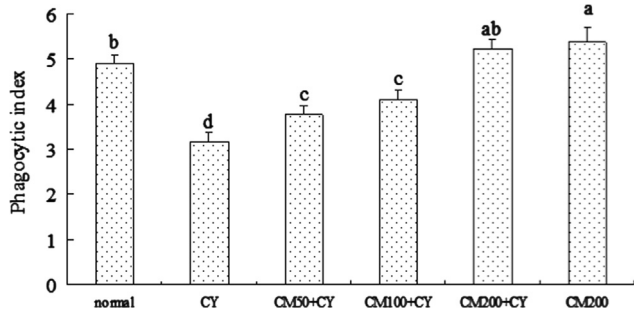


**Fig. 3.** The phagocytic index of CY-treated mice.
[a–d]Data within a column without the same superscripts differ significantly ($P < 0.05$).

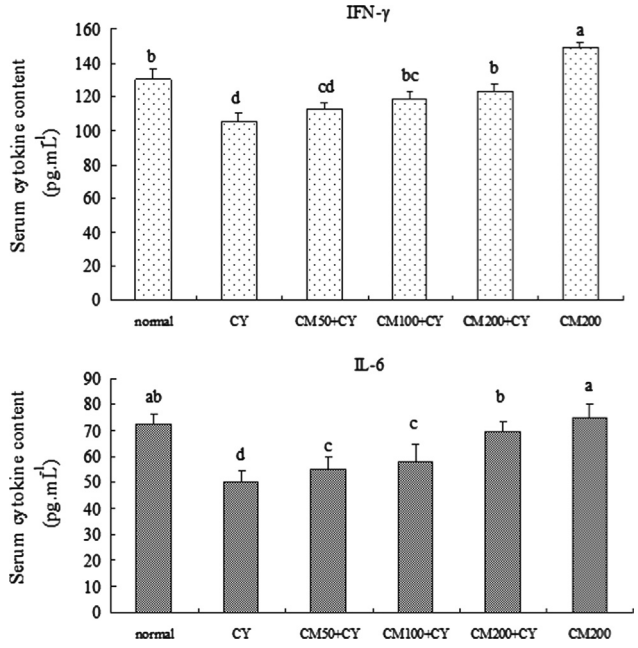


**Fig. 4.** IFN-γ and IL-6 activity in immunosuppressed mice.
[a–d]Data within a column without the same superscripts differ significantly ($P < 0.05$).

## 4. Discussion

The immune system is the human's ultimate defense against infectious diseases, tumor and cancer growth. A healthy immune system contains elements that are in balance with one another and if this balance is broken, our immune system will be unable to protect the body against harmful agents or processes. For above reasons, we investigated several aspects of immunomodulatory activity.

Cyclophosphamide is an immunosuppressant and its mechanism is similar to that of virus immunosuppression. CY can damage the structure of DNA, kill immune cells, interfere with the proliferation and differentiation of macrophages, T, and B mother cells, and restrain the humoral and cellular immune response (El-Abasy et al., 2004; Cruz-Chamorro et al., 2007; Atsamo et al., 2011; Wang et al., 2011). Animals treated with CY are often used as immune deficiency or immunosuppression models. CY-immunosuppressed mice are used for the study of drug immune regulation. Such a model was used in the present study. Through palpation and the subcutaneous injection of 80 mg/kg of cyclophosphamide, a model of low immune function was established in mice. These mice showed significantly lower concentrations of ring disk amides and lower values in the thymus and spleen indices than those in the normal group ($P < 0.05$).

The spleen and thymus are important immune organs in mammals. They are the sites of growth and proliferation of immunological cells. The development status of immune organs directly affects immune function and the ability to resist disease. The immune organ index reflects immune organ development and the immune function. The results of the present experiment showed the spleen and thymus indices in the high, medium and low dose groups to be significantly higher than those of in the CY-treat group. This indicated that CM could resist the effects of immunosuppression on the development of immune organs. It has been reported that some Chinese herbal medicines and their ingredients can also significantly improve the immune organ indices of mice and inhibit atrophy of immune organs (Chen et al., 2012; Wang et al., 2012).

The proliferation of T and B lymphocytes is a response to the stimulation induced by antigens or mitogens. This is a typical, non-specific immune reaction with a well-understood mechanism. This assay has been used extensively as an immune parameter in investigations of lymphocyte responsiveness because of its considerable sensitivity. Cellular proliferation induced by ConA is commonly used to detect T lymphocyte immunity, and LPS-induced activation of B cells indicates B lymphocyte immunity (Wang et al., 2011). Previous reports have shown that cyclophosphamide can suppress both humoral and cellular immune responses (Bear, 1986; Hoover et al., 1990). Results showed that treatment with CM can promote the recovery of splenocyte proliferative responses to both T and B lymphocytes in CY-treated mice organisms and that this response is dose-dependent (Fig. 2).

Preliminary results of phytochemical (data not shown) screening revealed a presence of numerous polyphenol, tannins and flavonoids, a class of agents already known to be active immunomodulatory substances (Feldman et al., 1999). The stimulation of lymphocytes could also be attributed to flavonoids present in the extract. Flavonoid compounds have been previously shown to modulate the immune system mechanistically, due to hydroxyl groups in their structures (Lopez-Posadas et al., 2008).

Phagocytes (neutrophils, monocytes, and macrophages) are key participants in the innate immune response, and phagocytic function is commonly used in evaluating the non-specific immune status of animals (Chen et al., 2010). Macrophages are the most important phagocytes. They play an essential role in host defense against all types of invasive cells, including tumor cells (Katsiari et al., 2010). Macrophages can directly release factors harmful to microorganisms and cancer cells, such as oxygen radicals and tumor necrosis factor. They also play an indirect role in antimicrobial and anti-tumor activities by secreting cytokines and by processing and presenting antigens, thereby regulating the immune system. Activated macrophages play an important role in both the innate and adaptive immune responses (Klimp et al., 2002). Within a certain range, the rate of removal of carbon particles from the body and the concentration of carbon in the blood are related through an exponential function (Zhang et al., 2008). The present study showed that CM significantly enhanced the phagocytosis of peritoneal macrophages in a dose-dependent manner, suggesting that CM could enhance the non-specific immune function in CY-treated mice.

T lymphocytes can recognize and present antigens. $T_H$ cells and $T_S$ cells can regulate the proliferation and differentiation of B cells (Minato et al., 2004). IFN-γ is secreted by Th1. They mainly promote cellular immune response and play a key role in the regulation of the immune system besides the broad-spectrum antiviral function (Raymond and Wilkie, 2004). It can connect the natural and adaptive immune responses, facilitate the recognition of pathogens by natural immune cells and induce the generation of the specific immune response. IL-6 is secreted by Th2 cells and plays an important role in humoral immunity. It can regulate diverse cell functions including proliferation and differentiation of B-cells and T-cells (Salgame et al., 1991). In the present experiment, the concentrations of the two cytokines in high and medium doses of CM groups were found to be significantly higher than those in the CY-treat group. This indicated that CM could promote the secretion of some cytokines and so reduce the severity of immunosuppression. The dynamic balance and mutual adjustment between Th1 and Th2 has been reported to play an important role in the maintenance of normal immunologic function (Lee et al., 2004).

## 5. Conclusions

The current study demonstrates that the ethanol extract of *Cyrtomium macrophyllum* rhizome could improve immune function. Antineoplastic immunotherapy and chemical treatment require good agents with immunomodulatory activity. These results suggest that CM contains potent components such as polyphenol, tannins and flavonoids, which could be used to modulate immune cell functions in the manner required by these therapies. However, the pharmacologically-active ingredients in *Cyrtomium macrophyllum* and the signaling pathways involved in the modulation of both macrophage and lymphocyte responses also remain to be further elucidated.

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## References

Atsamo, A.D., Nguelefack, T.B., Datté, J.Y., Albert Kamanyi, A., 2011. Acute and subchronic oral toxicity assessment of the aqueous extract from the stem bark of *Erythrina senegalensis* DC (*Fabaceae*) in rodents. Journal of Ethnopharmacology 134, 697–702.

Bear, H.D., 1986. Tumor-specific suppressor T-cells which inhibit the in vitro generation of cytolytic T-cells from immune and early tumor-bearing host spleens. Cancer Research 46, 1805–1812.

Ben Sghaier, M., Krifa, M., Mensi, R., Bhouri, W., Ghedira, K., Chekir-Ghedira, L., 2011. in vitro and in vivo immunomodulatory and anti-ulcerogenic activities of *Teucrium ramosissimum* extracts. Journal of Immunotoxicology 8, 288–297.

Cao, J.G., Xia, X., Dai, X.L., Xiao, J.B., Wang, Q.X., Andrae-Marobela, K., Okatch, H., 2013. Flavonoids profiles, antioxidant, acetylcholinesterase inhibition activities of extract from *Dryoathyrium boryanum* (Willd.) Ching. Food and Chemical Toxicology 55, 121–128.

Chattopadhyay, S.K., Kumar, S., 2006. Identification and quantification of two biologically active polyisoprenylated benzophenones xanthochymol and isoxanthochymol in *Garcinia* species using liquid chromatography-tandem mass spectrometry. Journal of Chromatography B 844, 67–83.

Chen, J.R., Yang, Z.Q., Hu, T.J., Yan, Z.T., Niu, T.X., Wang, L., Cui, D.A., Wang, M., 2010. Immunomodulatory activity in vitro and in vivo of polysaccharide from *Potentilla anserina*. Fitoterapia 81, 1117–1124.

Chen, X.M., Nie, W.J., Fan, S.R., Zhang, J.F., Wang, Y.F., Lu, J.X., Jin, L.Q., 2012. A polysaccharide from *Sargassum fusiforme* protects against immunosuppression in cyclophosphamide-treated mice. Carbohydrate Polymers 90, 1114–1119.

Choi, E.M., Koo, J.K., Hwang, J.K., 2004. Immune cell stimulating activity of mucopolyssaccharide isolated from yam (*Dioscorea batatasi*). Journal of Ethnopharmacology 91, 1–6.

Cruz-Chamorro, L., Puertollano, M.A., Puertollano, E., Alvarez, D.C.G., de Pablo, M.A., 2007. Examination of host immune resistance against *Listeria monocytogenes* infection in cyclophosphamide-treated mice after dietary lipid administration. Clinical Nutrition 26, 631–639.

Da Silva, A.A., Magalhaes, L.G., Kapadia, G.J., Tonuci, L.R.D., Caixeta, S.C., Parreira, N. A., Rodrigues, V., 2010. in vitro schistosomicidal effects of some phloroglucinol derivatives from *Dryopteris* species against *Schistosoma mansoni* adult worms. Parasitology Research 106, 395–401.

El-Abasy, M., Motobu, M., Nakamura, K., Koge, K., Onodera, T., Vainio, O., Toivanen, P., Hirota, Y., 2004. Preventive and therapeutic effects of sugar cane extract on

cyclophosphamide-induced immunosuppression in chickens. International Immunopharmacology 4, 983–990.
Feldman, K.S., Sahasrabudhe, K., Smith, R.S., Scheuchenzuber, W.J., 1999. Immunostimulation by plant polyphenols: a relationship between tumor necrosis factor-alpha production and tannin structure. Bioorganic and Medicinal Chemistry Letters 9, 985–990.
Hisada, S., Shiraishi, K., Inagaki, I., 1972. Phloroglucinol derivatives of dryopteris dickinsii and some related ferns. Phytochemistry 11, 2881.
Hoover, S.K., Barrett, S.K., Turk, T.M., Lee, T.C., Bear, H.D., 1990. Cyclophosphamide and abrogation of tumor-induced suppressor T cell activity. Cancer Immunology Immunotherapy 31, 121–127.
Ito, H., Muranaka, T., Mori, K., Jin, Z.X., Tokuda, H., Nishino, H., Yoshida, T., 2000. Ichthyotoxic phloroglucinol derivatives from *Dryopteris fragrans* and their antitumor promoting activity. Chemical and Pharmaceutical Bulletin 48 (8), 1190.
Iwashina, T., Kitajima, T., Matsumoto, S., 2006. Flavonoids in the species of *Cyrtomium* (Dryopteridaceae) and related genera. Biochemical Systematics and Ecology 34, 14–24.
Kapadia, G.J., Tokuda, H., Konoshima, T., Takasaki, M., Takayasu, J., Nishino, H., 1996. Anti-tumor promoting activity of *Dryopteris phlorophenone* derivatives. Cancer Letter 105 (2), 161.
Katsiari, C.G., Liossis, S.N., Sfikakis, P.P., 2010. The pathophysiologic role of monocytes and macrophages in systemic lupus erythematosus: a reappraisal. Seminars in Arthritis and Rheumatism 39, 491–503.
Klimp, A.H., de Vries, E.G., Scherphof, G.L., Daemen, T., 2002. A potential role of macrophage activation in the treatment of cancer. Critical Reviews in Oncology Hematology 44, 143–161.
Lee, E.J., Ko, E., Lee, J., Rho, S., Ko, S., Shin, M.K., Min, B.I., Hong, M.C., Kim, S.Y., Bae, H., 2004. Ginsenoside Rg1 enhances $CD_4^{(+)}$ T-cell activities and modulates Th1/Th2 differentiation. International Immunopharmacology 4, 235–244.
Liu, K.Q., Wu, C.J., Wan, J., Han, P., 2004. Pharmacognostical research on Guanzhong. Journal of Traditional Chinese Medicine 22, 1806–1871.
Lou, Z.Q., Qin, B., 2003. The Arrangement of Common Traditional Chinese Drugs and Quality Research, Northern ed. University Medicine press, Beijing, pp. 55–106.
Lopez-Posadas, R., Ballester, I., Abadia-Molina, A.C., Suarez, M.D., Zarzuelo, A., Martinez-Augustin, O., Sanchez de Medina, F., 2008. Effect of flavonoids on rat splenocytes, a structure-activity relationship study. Biochemical Pharmacology 76, 495–506.
Minato, K., Kawakami, S., Nomura, K., Tsuchida, H., Mizuno, M., 2004. An exo β-1,3-glucanase synthesized de novo degrades lentinan during storage of *Lentinule edodes* and diminishes immunomodulating activity of the mushroom. Carbohydrate Polymers 56, 279–286.
Nwabueze, T.U., 2007. Effect of process variables on trypsin inhibitor activity (TIA) phytic acid and tannin content of extruded African bread fruit-corn-soy mixtures: a response surface analysis. Food Science and Technology 40, 21–29.
Pearson, D., 1976. The Chemical Analysis of Foods. Churchill Livingstone, London.
Raymond, C.R., Wilkie, B.N., 2004. Th-1/Th-2 type cytokine profiles of pig T-cells cultured with antigen-treated monocyte-derived dendritic cells. Vaccine 22, 1016–1023.
Salgame, P., Abrams, J.S., Clayberger, C., Goldstein, H., Convit, J., Modlin, R.L., Bloom, B.R., 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. Science 254, 279–282.
Wu, Y.G., Song, L.R., 1996. Chinese Materia Medica Hand-picked Anthology. Shanghai Science and Technology press, Shanghai p. 219.
Wang, H., Wang, M., Chen, J., Tang, Y., Dou, J., Yu, J., Xi, T., Zhou, C., 2011. A polysaccharide from *Strongylocentrotus nudus* eggs protects against myelosuppression and immunosuppression in cyclophosphamide-treated mice. International Immunopharmacology 11, 1946–1953.
Wang, M., Meng, X.Y., Yang, R.L., Qin, T., Wang, X.Y., Zhang, K.Y., Fei, C.Z., Li, Y., Hu, Y. L., Xue, F.Q., 2012. *Cordyceps militaris* polysaccharides can enhance the immunity and antioxidation activity in immunosuppressed mice. Carbohydrate Polymers 89, 461–466.
Xiang, Y., Wang, H., Wen, Y.Y., 2002. Studies on the Chemical Constituents *Platycerium wallichii* Hook. Journal of Tropical and Subtropical Botany 10, 69–73.
Yang, C.Y., Li, C.D., 1993. Survey of sources and original plants of traditional Chinese herbal drugs guanzhong. Bulletin of the Academy of Military (Medical Sciences) 17 (3), 202–206.
Yuan, Y.V., Bone, D.E., Carrington, M.F., 2005. Antioxidant activity of dulse (*Palmaria palmata*) extract evaluated in vitro. Food Chemistry 91, 485–494.
Yuan, H., Song, J., Li, X., Li, N., Dai, J., 2006. Immunomodulation and antitumor activity of kappa-carrageenan oligosaccharides. Cancer Letter 243, 228–234.
Zhang, J., Li, S., Ji, B., He, J., 2008. Modulation effects of *Artemisia integrifolia* on immune function of mice. Food Science 5, 92.
Zhishen, J., Mengcheng, T., Jianming, W., 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chemistry 64, 555–559.
Zhou, X.R., Dai, X., Shang, J., Jiang, B., 2009. Resources of medicinal pteridophyta in Damu Mountain Nature Reserve Area. Lishizhen Medicine and Materia Medica Research 20 (10), 2563–2565.