

Original Research Article

Polysaccharides isolated from *Morinda officinalis* How roots inhibits cyclophosphamide-induced leukopenia in mice

Yaxin Zhao¹, Meng Wang², Yang Li^{3*} and Wanting Dong⁴

¹Department of Pharmacy, ²Medical Department, ³Chinese Medicine Department, Jining Traditional Chinese and Western Medicine Hospital, Jining 272000, Shandong, PR China.; ⁴Ministry of Education, Jining No. 1 People's Hospital, Jining 272000, Shandong, PR China

*For correspondence: **Email:** tcmliyang@126.com; **Tel:** +86-0537-3168581

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Abstract

Purpose: To investigate the optimum parameters for extracting polysaccharides from *Morinda officinalis* How (MOP), and explore their inhibitory effects on leukopenia in mice.

Methods: Orthogonal design was performed to investigate the optimum parameters for extracting MOP. A leukopenia mouse model was established by injection of cyclophosphamide (CTX) for three days. Thereafter, MOP (100, 200 and 400 mg/kg) was administered orally for 10 days. Furthermore, blood cells (leukocytes, neutrophil, lymphocyte and mononuclear cell) were analyzed, while serum IL-3 and IL-6 were determined by ELISA. The thymus and spleen of the mice were separated and weighed to determine viscera indices.

Results: Orthogonal design showed that the influence order of the four factors was extraction times (C) > ratio of water to raw material (RWM, D) > extraction time (B) > extraction temperature (A). The optimum extraction parameters for MOP were: extraction temperature (80 °C), extraction duration (2 h), no. of extractions (3), and ratio of water to raw material (30 mL/g). Furthermore, the results indicate that MOP (100, 200 and 400 mg/kg) elevated the levels of leukocyte ($p < 0.01$), neutrophil ($p < 0.01$), lymphocyte ($p < 0.01$) and mononuclear cell ($p < 0.01$) in leukopenia mice. Besides, MOP (100, 200 and 400 mg/kg) also increased thymus ($p < 0.01$) and spleen ($p < 0.05$) indices and serum levels of IL-3 ($p < 0.05$) and IL-6 ($p < 0.01$).

Conclusion: Orthogonal design is a good strategy for optimizing extraction parameters of MOP. Furthermore, MOP stimulated synthesis of leukocytes in CTX-induced leukopenia in mice. Thus, MOP is a potential adjunct for the treatment of tumors/cancers.

Keywords: *Morinda officinalis*, Polysaccharide, Orthogonal design, Leukopenia, Thymus index, Spleen index

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INTRODUCTION

In recent years, studies have demonstrated that malignant cancers have become one of the most common incurable diseases worldwide [1]. For the treatment of cancers, besides surgery, long-term chemotherapy is another commonly used

effective method [2]. However, chemotherapy could result in myelosuppression, leading to many serious side-effects, especially leukopenia [3]. Leukopenia could result in extremely low leukocyte levels in the body, causing hyp immunity. In this situation, severe inflammatory cascades reactions could be induced, leading to sepsis and even death [4].

Thus, it is important to prevent leukopenia in the process of chemotherapy for treating cancers.

Currently, existing drugs for elevating levels of leukocytes, such as vitamin B6, leucogen and granulocyte colony-stimulating factors (GCF), cannot enhance body immunity, but only increase the cell number of leukocytes [5]. Increasing investigations have demonstrated that traditional herbal medicines are tremendous resources for finding novel drugs for treating various diseases, especially some incurable diseases [6,7]. A previous report indicated that *Morinda officinalis* How, a folk traditional herbal medicine in China, possesses notable immunity enhancing effects and could be used for treating leukopenia induced by chemotherapeutic agent [8-10]. However, information on the details of the active substances in *M. officinalis* is lacking. Therefore, in this study, the isolation of the polysaccharide of *M. officinalis* (MOP) was optimized using orthogonal design, and the inhibition of MOP on leukopenia induced by cyclophosphamide (CTX) in mice was also investigated.

EXPERIMENTAL

Plant material

The roots of *Morinda officinalis* How were purchased from *Tongrentang* Chinese Medicine Co. Ltd. (Beijing, China) and authenticated by a taxonomist in the Chinese Medicine Department of Jining Traditional Chinese and Western Medicine Hospital (Jining, China). A voucher specimen (no. BJT2015-7-4H) was kept in the herbarium of the department for future reference.

Animals

BALB/c mice (20 ± 2 g) were purchased from the Experimental Animal Center of the Academy of Military Medical Sciences (Beijing, China). All animal protocols in this study were in accordance with the guidelines of National Institute of Health Guide for the Care and Use of Laboratory Animals [11], and were approved by the ethics committee of Traditional Chinese and Western Medicine Hospital (approval no. AER-2015-6-01).

Chemicals and reagents

Cyclophosphamide injection (CTX) was purchased from Jiangsu HengRui Medicine Co. Ltd. (Lianyungang, China). Pentobarbital sodium was purchased from Sigma-Aldrich Co. (Shanghai, China). Interleukin (IL)-3 and 9 ELISA

kits were purchased from BOSTER Co. (Wuhan, China). D-glucose standard agent was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other chemicals and reagents used in this study were all of analytical grade (AR).

Isolation of polysaccharides from roots of *M. officinalis*

The dried roots of *M. officinalis* were powdered and then extracted with 95 % ethanol for 8 h to remove monosaccharides, oligosaccharides and other small molecule agents. Then, the residues after filtration were extracted with distilled water by hot dipping. The extracts were cooled at room temperature, and subsequently concentrated. Then, ethanol was slowly added to a final concentration of 80 %. The precipitates were collected after leaving overnight at 4 °C in a refrigerator. Then the precipitates were washed three times with absolute ethanol and acetone, and subsequently dried by freeze drying as to obtain MOP.

Determination of polysaccharide yield

The polysaccharide contents were determined according to the previous described phenol-sulfuric method using D-glucose as standard reference [11-12]. The calibration curve between absorbance (A) and polysaccharide contents (C) in this study is $A = 0.0075C + 0.04$ ($r = 0.9986$). Polysaccharide yield (%) was calculated as in Eq 1.

$$\text{Yield (\%)} = (W_1/W_0)100 \dots\dots\dots (1)$$

where W_1 is weight (g) of polysaccharides, and W_0 is the weight (g) of dried herbal material.

Orthogonal design and statistical analysis

Based on previous investigations regarding polysaccharide isolation, four major influence factors were investigated namely extraction temperature (A), extraction time (B), extraction times (C), and ratio of water to raw material (RWM, D). To optimize the extraction of polysaccharide of *M. officinalis* (MOP), the orthogonal design was performed with L9 (3^4) experiment and the four factors were subjected into three levels (Table 1). All the tests were repeated three times, and the range analysis and variance analysis (ANOVA) were conducted to analyze the orthogonal design results.

Table 1: Influence factors and levels values in the orthogonal design

S/N	Factor			
	A: Temperature (°C)	B: Time (h)	C: Times (n)	D: RWM (mL/g)
1	80	1	1	10
2	90	2	2	20
3	100	3	3	30

RWM = ratio of water to raw material

Determination of effect of MOP on leukopenia mice induced by CTX

Fifty BALB/c mice were randomly divided into five groups (n=10): normal group, control group, three MOP treated groups (100, 200 and 400 mg/kg). Mice in control and MOP groups were intraperitoneally injected with CTX (80 mg/kg, ip) for three days to induce leukopenia. Then, mice in the normal, control and MOP treated groups were administrated orally with normal saline (20 mL/kg), normal saline (20 mL/kg) and MOP (100, 200 and 400 mg/kg) for 10 days, respectively. Blood samples were collected using an abdominal aortic blood sampling protocol under anesthesia with pentobarbital sodium (45 mg/kg, ip), and then mice were subsequently sacrificed by decapitation. The blood cells (leukocytes, neutrophil, lymphocyte and mononuclear cell) were analyzed using a full automatic blood analyzer (SYSMEX XE2100, Kobe, Japan). In addition, the serum IL-3 and IL-6 were determined using commercial ELISA kits according to the manufactures' instructions. Finally, thymus and spleen were separated and weighed to determine viscera index [4,14] using Eq 2.

$$\text{Viscera index} = (M_1/M_0)10 \dots\dots\dots (2)$$

where M₁ is the weight (mg) of thymus or spleen, and M₀ is the weight (g) of the mice.

Statistical analysis

One-way ANOVA following by Dunnett multiple

comparisons tests was used to analyze the differences between different groups with SPSS software (SPSS for Windows 15.0, SPSS Inc., USA). Data are expressed as mean ± SD, and differences were considered significant at *p* ≤ 0.05.

RESULTS

Optimized extraction by orthogonal design

As can be seen from the Table 2, the range analysis results of orthogonal design showed that among the four factors, extraction times (C) and RWM (D) are the dominating influence factors for the MOP extraction rate with an *R* value of 1.100 and 0.803 respectively, followed by the extraction time (B, *R* = 0.477) and extraction temperature (A, *R*=0.143). From the range analysis results showed in Table 2, the influence order of the four factors was C > D > B > A, and the optimum extraction was C₃D₃B₃A₁. Based on the range analysis results, ANOVA was also determined, and the results showed that the extraction times and RWM were significant (*F* < 0.05) (Table 3). However, no obvious significance was observed in the extraction temperature (A) and extraction time (B) (*F* > 0.05).

Collectively, combined with realistic conditions, the optimum extraction was obtained: extraction temperature of 80 °C, extraction time of 2 h, extraction times of 3, and ratio of water to raw material weight of 30 mL/g.

Table 2: Results of range analysis of orthogonal design

No.	A	B	C	D	Yield (%)
1	1	1	1	1	3.54
2	1	2	2	2	5.27
3	1	3	3	3	5.86
4	2	1	2	3	5.15
5	2	2	3	1	5.25
6	2	3	1	2	4.67
7	3	1	3	2	5.21
8	3	2	1	3	4.81
9	3	3	2	1	4.62
k1	4.890	4.633	4.340	4.470	
k2	5.023	5.110	5.013	5.050	
k3	4.880	5.050	5.440	5.273	
<i>R</i>	0.143	0.477	1.100	0.803	

Table 3: Results of variance analysis (ANOVA) of orthogonal design

Factor	Sum of square	Freedom	F-ratio	F _{0.05}	Significance
Temperature (°C) (Error group)	0.038	2	1.000	19.000	
Time (h)	0.404	2	10.632	19.000	
Times (n)	1.845	2	48.553	19.000	F < 0.05
RWM (mL/g)	1.032	2	27.158	19.000	F < 0.05

RWM = ratio of water to raw material

Table 4: Blood count in CTX-treated mice (10⁹/L)

Treatment	Leukocytes	Neutrophil	Lymphocyte	Mononuclear cell
Normal	10.13±1.85**	1.22±0.36**	9.13±1.46**	0.52±0.11**
Control	1.13±0.35	0.37±0.14	0.67±0.19	0.09±0.02
100 mg/kg	5.74±0.85**	0.62±0.22**	4.88±0.52**	0.28±0.05**
200 mg/kg	7.61±1.23**	0.83±0.35**	6.51±0.85**	0.33±0.08**
400 mg/kg	9.58±1.67**	0.93±0.41**	8.26±1.03**	0.44±0.12**

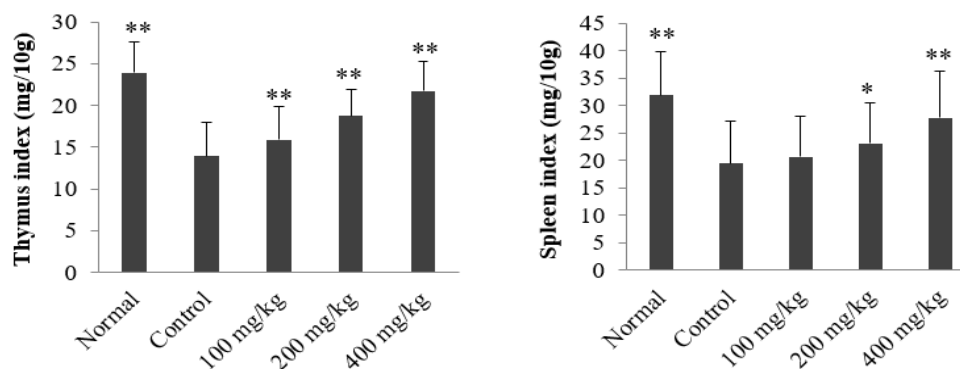
**P < 0.01, compared with control

MOP elevated levels of leukocyte, neutrophil, lymphocyte and mononuclear cell

From the results shown in Table 4, after the CTX injection (80 mg/kg), all of the four testing cells were sharply decreased ($p < 0.01$), suggesting that leukopenia was successfully established by CTX injection. Interestingly, the results also demonstrated that the MOP (100, 200 and 400 mg/kg) elevated the decreased levels of leukocyte ($p < 0.01$), neutrophil ($p < 0.01$), lymphocyte ($p < 0.01$) and mononuclear cell ($p < 0.01$) compared to control mice in a dose-dependent manner.

MOP increased thymus and spleen indices

As shown in Figure 1, both the thymus and spleen indices of control mice were decreased compared with normal mice ($p < 0.01$). After treatment with MOP (100, 200 and 400 mg/kg) for 10 days, the thymus indices were significantly increased compared to the control group ($p < 0.01$), in a dose-dependent manner. In addition, MOP at 200 ($p < 0.05$) and 400 mg/kg ($p < 0.01$) also increased the spleen indices compared to the control group.

**Figure 1:** Thymus and spleen indices of CTX-treated mice; * $p < 0.05$, ** $p < 0.01$, compared with control

MOP increased IL-6 and IL-3 in serum

In this investigation, levels of IL-6 and IL-3 in serum were also studied. As can be seen from Figure 2, similar to the thymus and spleen indices, IL-6 and IL-3 were decreased by injection of CTX (80 mg/kg, $p < 0.01$). Interestingly, compared with the control mice, IL-6 level in serum were increased by treatment with MOP (100, 200 and 400 mg/kg, $p < 0.01$) in a dose-dependent manner. Besides, IL-3 in serum was also increased by MOP (100, 200 and 400 mg/kg) compared to the control mice ($p < 0.05$, $p < 0.01$, $p < 0.01$).

DISCUSSION

It is reported that plant-derived polysaccharides possess multiple pharmacological activities and relatively low toxicity, and in particular several polysaccharides exhibit notable immune stimulating effects and could be used to treat tumors and leukopenia [11,14]. This study investigated the optimum extraction of polysaccharide of *Morinda officinalis* (MOP), and also indicated that MOP stimulated synthesis

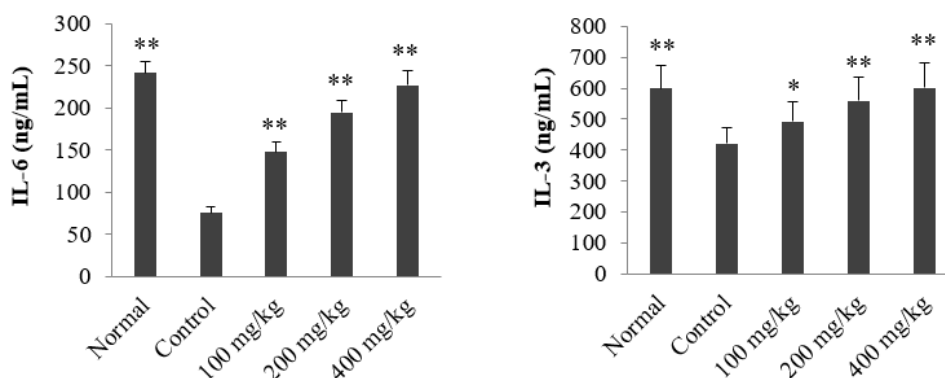


Figure 2: Serum IL-6 and IL-3 in CTX-treated mice; * $p < 0.05$, ** $p < 0.01$, compared with control

of leukocytes on leukopenia induced by CYT for first time.

Orthogonal design is one of the most popular experimented design methods to select representative parameters for replacing full factorial experiment with a less expensive, faster and partial factorial experiment [15,16]. Based on the range analysis, the factors' influence could be ordered, and ANOVA could estimate relative significance of each factor according to percentage contribution to the overall response [16]. In this study, based on the range analysis and ANOVA, the MOP extraction parameters were optimized: extraction temperature (80 °C), extraction time (2 h), extraction times (3), and ratio of water to raw material weight (30 mL/g).

Cyclophosphamide (CTX) is a commonly used anticancer drug in the clinic and could result in several side-effects, especially leukopenia [17,18]. Therefore, CTX induced leukopenia in mice model is one of the most used animal model for evaluation of leukocyte inducing drugs [4]. In the present study, leukopenic BALB/c mice were successfully prepared with CTX injection. Immune organs, such as thymus and spleen, are crucial for the growth and development of leukocytes. The results indicated that MOP could increase thymus and spleen indices, suggesting that MOP might be beneficial for the growth and development of leukocytes. Previous investigations indicated that IL-3 and IL-6 could promote the proliferation and differentiation of leukocytes [19,20]. Interestingly, results of this study also revealed that MOP could increase the serum levels of IL-3 and IL-6, which is also beneficial for the proliferation and differentiation of leukocytes.

CONCLUSION

For optimum extraction of polysaccharide of *M. officinalis* (MOP), the extraction parameters are extraction temperature (80 °C), extraction time (2

h), extraction times (3), and ratio of water to raw material weight (30 mL/g). Furthermore, the isolated polysaccharide also possesses notable leukocyte inducing-like effects and therefore is a potential adjunct therapeutic agent for the treatment of tumors/cancers.

DECLARATIONS

Acknowledgement

None.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Yaxin Zhao and Meng Wang contributed equally to this work.

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