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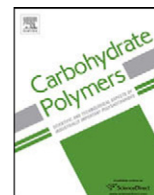

# A polysaccharide from the fungi of Huaier exhibits anti-tumor potential and immunomodulatory effects

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ABSTRACT

A neutral water-soluble polysaccharide (W-NTRP), with a molecular weight of $2.5 \times 10^4$ Da, was isolated from the fruit bodies of *Trametes robiniophila* (Huaier). Gas chromatography (GC) results indicated that W-NTRP was determined to be galactose (Gal), arabinose (Ara) and glucose (Glc), with a relative molar ratio of 4.2:2.5:0.7. Its antitumor and immunomodulatory activity were evaluated in vitro. W-NTRP showed remarkable inhibitory effect on three human cholangiocarcinoma cell lines (QBC939, Sk-ChA-1 and MZ-ChA-1), with respective $IC_{50}$ values of 47.8, 75.9, and 43.7 μg/mL, but had no cytotoxicity to L-929 normal cells. Furthermore, W-NTRP had proliferation promoting effect on mouse splenocytes with or without concanavalin A (ConA) or lipopolysaccharide (LPS) in a bell-shaped dose-response manner. In addition, W-NTRP could prominently stimulate macrophages to produce nitric oxide (NO) through the up-regulation of inducible NO synthase (iNOS) activity. These results suggest that W-NTRP could be explored as a potential antitumor agent for cholangiocarcinoma.

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## 1. Introduction

*Trametes robiniophila* Murr (Huaier) is a sandy beige mushroom found on the trucks of trees such as *Sophora japonic*, belonging to Hymenomycetes, Basidiomycotina (Anithworth, Sparrow, & Susman, 1973). This fungus has been applied in traditional Chinese Medicine (TCM) for approximately 1600 years (Li, Ye, Wang, & Tang, 2006), first recorded by Shi-Zhen Li, who was a famous Chinese practitioner in Ming Dynasty. Many, if not all, Basidiomycetes mushrooms contain biological active protein-bound polysaccharides or polysaccharides in fruit bodies, cultured mycelium or culture broth, which exert antitumor and immunomodulating activity (Wasser, 2002). In recent decades, Huaier has been found and used as a complementary agent for cancer therapy. The water extract from Huaier is mainly polysaccharide protein which has been proved to be the main active ingredient in the respect of anticancer effects and immunity-enhancing actions (Cui, Goh, Archer, & Singh, 2007). As an anti-carcinogen medicine, Huaier has been used for the treatment of liver cancer with satisfactory results. Accumulated evidences suggest that the anti-tumor mechanism of Huaier may be associated with inhibition of the proliferation of endothelial cells, interference with tumor angiogenesis (Xu, Jia, Ma, & Yu, 2003), system immune activation (Chen, Lu, Lu, & Li, 2004; Jia, Dong, Lu, Guo, & Wei, 2009), induction of tumor cell apoptosis and suppression of tumor cell proliferation (Zhu et al., 2008). Irrespective of its efficient treatment effect for liver cancer, so far, there is no information available about the anticancer effects of Huaier on cholangiocarcinoma, and its underlying mechanism of action is still largely unknown. As we know, cholangiocarcinoma is a malignant tumor arising from the bile duct epithelial cells or cholangiocytes of the intrahepatic and extrahepatic biliary system (de Groen, Gores, LaRusso, Gunderson, & Nagorney, 1999; Shaib & El-Serag, 2004), characterized by a poor prognosis, high recurrence rate and resistant to radiotherapy/chemotherapy due to its special biological characteristics. Therefore, cholangiocarcinoma is considered to be a multidrug and radio-resistant tumor and has a high mortality (Sirica, 2005). The increasing global incidence of this tumor requires the urgent need for novel and effective therapeutic agents for cholangiocarcinoma. Recently much attention has been focused on medicinal plants in an effort to discover novel anticancer agents that lack the toxic effects associated with current therapeutic agents (Schwartsmann et al., 2002). Since last century, our hospital (the Second Affiliated Hospital, Harbin Medical University) had succeeded in administering Huaier extract to patient suffered from cholangiocarcinoma to prolong the survival of patients or reduce the recurrence rate. With the purpose of developing this hospital pharmaceutical preparation to be an open access drug, we

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intend to make a series of experiment-based research to identify its efficiency on cholangiocarcinoma. Current study we will extract a water-soluble polysaccharide from this fungus and preliminarily test its immunomodulatory and tumor-inhibitory activities in vitro.

## 2. Materials and methods

### 2.1. Materials

The fruiting bodies of Huaier were purchased from Henan Limin Biotechnology Co., Ltd. Standard sugars, T-series dextran, bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trifluoroacetic acid (TFA), concanavalin A (ConA) and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DEAE-52 Cellulose, Sepharose CL-6B and Sephadex G-100 were purchased from Amersham (Sweden). All other chemical reagents used were analytical grade.

### 2.2. Isolation and purification of the polysaccharide

The fruit bodies of Huaier (0.5 kg) were extracted with 95% EtOH (3 L) at 100 °C for 1.5 h to remove lipid, and the residue was extracted with boiling water twice in a 1:15 (w/v) ratio 3 h each time. After the centrifugation, the supernatant was concentrated 10-fold and precipitated with ethanol (1:4, v/v) at 4 °C overnight. The precipitate collected by centrifugation was suspended in distilled water to remove the protein by the Sevage method (Staub, 1965). After that the polysaccharide was exhaustively dialyzed against water for 2 days, the concentrated dialysate was precipitated with 4 volumes of 95% EtOH, followed by washing with absolute ethanol, acetone and ether, respectively to obtain the crude polysaccharide (W-CTRP) of Huaier.

The crude polysaccharide (8 g) was redissolved in distilled water (100 mL) and applied to a DEAE-52 Cellulose chromatography column (2.0 × 40.0 cm). The column was first eluted with distilled water, followed by a 5-step gradient of 0–2 M sodium chloride (0.2, 0.5, 0.8, 1.0 and 2.0 M). Guided by the phenol–sulfuric acid method, the water eluting fraction with high content of sugar was collected, dialyzed, lyophilized, and purified by Sepharose CL-6B (2.6 × 100 cm) and Sephadex G-100 (2.6 × 100 cm) gel-permeation chromatography eluted with 0.15 M sodium chloride to afford a purified Huaier polysaccharide (W-NTRP).

### 2.3. Analysis of physico-chemical characteristics of polysaccharide

The total carbohydrate content was determined by the phenol–$H_2SO_4$ method, with glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein content was quantified according to the Bradford's method (Bradford, 1976). Total uronic acid contents were measured by m-hydroxydiphenyl method using galacturonic acid or glucuronic acid as the standard (Filisetti-Cozzi & Carpita, 1991).

### 2.4. High-performance gel-permeation chromatography (HPGPC) and monosaccharide component analysis

The homogeneity and molecular weight of W-NTRP were determined by high-performance gel-permeation chromatography (HPGPC), which was performed on a LC-10ATvp Plus HPLC system (SHIMADZU, Japan) fitted with a TSK-GEL G5000PWXL column (7 μm, 30 × 7.8 mm i.d.) and an evaporative light scattering detector (ELSD) (Zhu et al., 2010). The sample was diluted to a concentration of approximately 2 mg/mL by adding 0.2 M $Na_2SO_4$ solution and centrifuged (10,000 rpm, 3 min), and 20 μL of supernatant was injected for each run and eluted with 0.2 M $Na_2SO_4$ solution at a flow rate of 0.8 mL/min at 25 °C. The molecular mass was reported relative to the T-series dextrans of known MWs (T-10, T-40, T-70, T-500, T-2000 and blue dextran T-2000).

Gas chromatography (GC) was used for identification and quantification of the monosaccharides (Xie et al., 2010). W-NTRP (10 mg) was hydrolyzed with 2 M TFA at 100 °C for 2 h (Parikh & Madamwar, 2006). The monosaccharide was conventionally converted into the alditol acetate as described previously (Johnes & Albersheim, 1972; Oades, 1967) and was analyzed by GC on an Agilent 6280 instrument fitted with FID and equipped with a HP-5MS column (0.25 mm × 30 m × 0.25 μm). The temperature program used in the analyses was as follows: 130 °C for 10 min, 130 °C to 240 °C at 4 °C/min, and then kept for 5 min. The injector and detector heater temperatures were 280 °C and 300 °C, respectively. The rate of the $N_2$ carrier gas was 50 mL/min.

### 2.5. Cell lines and cell culture

The human cholangiocarcinoma cell lines QBC939, Sk-ChA-1 and MZ-ChA-1 and mouse fibroblast cell line L-929 were obtained from China Center for Type Culture Collection (Wuhan, People's Republic of China). The four cell lines were cultured in RPMI-1640 medium supplemented with 10% FCS, 100 μg/mL streptomycin and 100units/mL penicillin G at 37 °C in an atmosphere of 5% $CO_2$.

### 2.6. Cytotoxic assay

The inhibitory effects of the polysaccharides on the cells (QBC939, Sk-ChA-1 and MZ-ChA-1) were evaluated by the analysis of viable cells number determined with a MTT-based colorimetric assay (Jiao et al., 2009). Briefly, the cells were seeded in 96-well plates at a density of $1 \times 10^5$ cells/well in 100 μL culture medium. Following 24-h incubation and attachment, sterilized test samples (dissolved in PBS) were added to a 96-well plate. Phosphate buffer saline (PBS) was used as negative control. After cultivation for 48 h at 37 °C in a humidified 5% $CO_2$ incubator, 10 μL of MTT (5 mg/mL) was added four hours prior to completion of incubation. The percentage of viable cells was determined by MTT assay. Absorbance (OD) was measured at 570 nm using a Benchmark microplate reader (Bio-Rad, California). The $IC_{50}$ value was determined as the concentration that caused 50% inhibition of cell proliferation (Peng, Li, Liu, Zhang, & Duan, 2008).

### 2.7. Assay of immunobiological activity

#### 2.7.1. Animals

Male BALB/c mice (8–12 weeks old) were purchased from Pharmacology Experimental Center of Harbin Medical University. They received standard mouse chow and water ad libitum. The room conditions were maintained at 22 ± 2 °C with relative humidity of 50 ± 5% and 12 h light/dark cycle at least 9 days before experiment.

#### 2.7.2. Splenocyte proliferation assay

The BALB/c mice were sacrificed by cervical dislocation and spleens were collected under aseptic conditions in RPMI-1640. Spleen cells of mice were prepared by gently mincing and grinding the spleen fragment in RPMI-1640 medium on a fine steel mesh, and centrifuged at 1500 rpm/min at 4 °C for 10 min, and then removed the supernatant. The red blood cells were removed by hemolytic Gey's solution. After two washes the cells were resuspended in RPMI 1640 complete medium. The purity and viability of splenocytes as assessed by Trypan blue dye exclusion always exceeded 90% and the cell concentration was adjusted to $1 \times 10^6$ cell/mL. Then the cell suspension mixed with various concentrations of the

polysaccharides (25, 50, 100, 200, 400 μg/mL) were seeded into a 96-well culture plate in the presence of ConA (5.0 μg/mL), LPS (10.0 μg/mL), or PBS and incubated at 37 °C in a humidified 5% $CO_2$ incubator for 72 h. Subsequently the MTT solution (1 mg/mL of phosphate buffer saline) was added to 96-well plates and diluted to 5 μg/mL in every well, and then further incubated for another 4 h. After the supernatant was aspirated from the wells, 200 μL of DMSO was added and shaken for 20 min. The absorbance of each well was then read at 570 nm using an ELISA reader (Model 550, Bio-Rad Instruments).

#### 2.7.3. Phagocytosis of macrophage assay

The resident macrophages of male BALB/c mice were harvested by peritoneal lavage, and the cells were subsequently cultured in RPMI 1640 complete medium. The purity of macrophages was tested by adherence. 100 μL/well of macrophage suspension were incubated for 3 h at 37 °C in a humidified 5% $CO_2$ incubator. The adherent cells, which were peritoneal macrophages, were incubated along with various filter-sterilized samples (25, 50, 100, 200, 400 μg/mL) or LPS (5 μg/mL, positive control) for 48 h in a 48-well culture plate. The stimulated cells were washed twice by PBS, and 100 μL neutral red (0.1%, w/v) was added, and incubated for 4 h. After the removal of unphagocytized neutral red by PBS, 100 μL cell lysate (the volume ratio of acetic acid to ethanol was 1:1) was added in and kept for 2 h. The OD value of each well was read at 540 nm using an ELISA reader (Model 550, Bio-Rad Instruments).

#### 2.7.4. Assay of nitric oxide (NO) production and inducible NO synthase (iNOS) activity

Briefly, peritoneal macrophages ($1 \times 10^6$ cells/mL) were incubated in complete RPMI medium alone or medium containing various concentrations of sample (25, 50, 100, 200, 400 μg/mL) or LPS (5 μg/mL) as a positive control for 48 h. And then 100 μL of isolated supernatants were allowed to react with Griess reagent (1% sulfanilamide, 0.1% napthyl ethyl diamine dihydrochloride in 5% phosphoric acid) at room temperature for 10 min. The absorbance was read at 540 nm, and the concentrations of $NO_2^-$ (μmol/mL) were determined from a least squares linear regression analysis of a sodium nitrite standard curve.

To investigate whether NO production was caused by enhancement of iNOS activity, the activities of iNOS were measured using commercially available colorimetric method (Nanjing Jiancheng Bioengineering, Nanjing, China), and was calculated according to the manufacturer's instructions. One nanomole of NO generated per $1 \times 10^6$ cells/min was defined as one activity unit.

### 2.8. Statistical analysis

All results were compared using the unpaired Student's *t*-test and values expressed as means ± SD. A probability of $P < 0.05$ and $P < 0.01$ was considered as significant.

## 3. Results

### 3.1. Isolation, purification and composition of the polysaccharide

The water-soluble crude polysaccharide (W-CTRP) was obtained as a light-yellow powder from the fruit bodies of Huaier by hot-water extraction, ethanol precipitation, deproteinization and lyophilization, with a yield of 14.5% of the crude material. The crude polysaccharides portion was further fractionated on a DEAE-52 Cellulose column with water and different concentrations of stepwise NaCl solution elution (0.2, 0.5, 0.8, 1.0 and 2.0 M), leading to the isolation of three sub fractions W-CNTRP, W-CATRP1 and W-CATRP2. Among those fractions, W-CNTRP showed the relatively high content. The other components were in trace amount, and will be reported in the future. W-CNTRP eluted by water was further separated on Sepharose CL-6B (2.6 × 100 cm) and Sephadex G-100 (2.6 × 100 cm) gel-filtration columns, giving only one main fraction. The main fraction was collected, lyophilized and named as W-NTRP for further structure characterization and biological activity.

**Table 1**
Components of monosaccharide and properties of the polysaccharide W-NTRP from Huaier.

| Sample | W-NTRP |
|---|---|
| Carbohydrate (wt%) | 96.5 |
| Uronic acid (wt%) | 0 |
| Protein (wt%) | 0 |
| Proportion of monosaccharide (mole %) | |
| Galactose (Gal) | 4.2 |
| Arabinose (Ara) | 2.5 |
| Glucose (Glc) | 0.7 |
| Molecular weight (Da) | $2.5 \times 10^4$ |

Chemical composition of the purified polysaccharide was determined as shown in Table 1. The total carbohydrate content of W-NTRP was 96.5%, as determined by the phenol-sulfuric acid method. It had a negative response to the Bradford method, and no absorption was detected by the UV spectrum at 280 and 260 nm, indicating the absence of protein and nucleic acid. W-NTRP was hydrolyzed by TFA into individual monosaccharides, which were further reduced and acetylated for GC analysis. The results showed that W-NTRP was determined to be galactose (Gal), arabinose (Ara) and glucose (Glc), with a relative molar ratio of 4.2:2.5:0.7, based on the GC analysis. These results suggest that the W-NTRP is an arabinogalactan mainly consisting of galactose and arabinose. The HPGPC profile showed a single and symmetrically sharp peak, indicating that W-NTRP was a homogeneous polysaccharide (Fig. 1). According to the calibration curve with standard dextrans, the average molecular weight of W-NTRP was estimated to be about $2.5 \times 10^4$ Da.

### 3.2. Antiproliferative effects of the polysaccharide on human cholangiocarcinoma cell lines

The inhibitory effects of W-NTRP were tested on three human cholangiocarcinoma cell lines (QBC939, Sk-ChA-1 and MZ-ChA-1) under different dosages of 25, 50, 100, 200, and 400 μg/mL in vitro

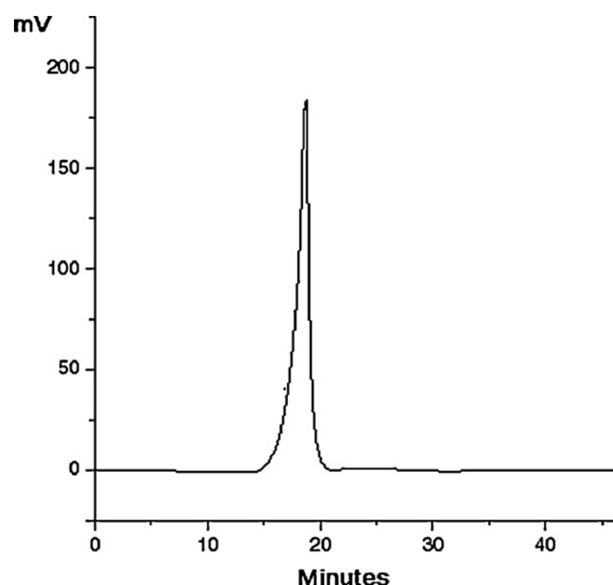


**Fig. 1.** High-performance gel-permeation chromatography (HPGPC) of the polysaccharide W-NTRP from Huaier.

**Table 2**
Cytotoxicity of the polysaccharide W-NTRP from Huaier against three human cholangiocarcinoma cell lines by MTT ($IC_{50}$ value, μg/mL) assay.

| Sample | $IC_{50}$ (μg/mL) | | |
|---|---|---|---|
| | QBC939 | Sk-ChA-1 | MZ-ChA-1 |
| W-NTRP | 47.8 | 75.9 | 43.7 |

by MTT assay. Based on the OD values determined, the $IC_{50}$ values of W-NTRP in human tumor cells were calculated and the results are shown in Table 2. W-NTRP showed strong tumor growth inhibitory activity against the cells tested, with $IC_{50}$ values of 47.8, 75.9 and 43.7 μg/mL for QBC939, Sk-ChA-1 and MZ-ChA-1, respectively. On the other hand, W-NTRP exhibited no cytotoxicity against normal cells (L-929). The results indicated that W-NTRP possessed a potent antitumor effect on three human cholangiocarcinoma cell lines, especially for MZ-ChA-1, but had no cytotoxicity to normal cells L-929.

### 3.3. *Effects of the polysaccharide on splenocyte proliferation*

Lymphocytes are the key effector cells of mammalian immune system and our studies showed that the different subpopulations of lymphocytes were activated by W-NTRP at varying levels. As shown in Table 3, the effects of W-NTRP on lymphocyte proliferation were observed using stimulant mitogen (ConA: T-cell mitogen and LPS: B-cell mitogen), and medium (Control). ConA- and LPS-induced splenocyte proliferation were significantly enhanced by W-NTRP at the concentrations of 25, 50, 100, 200, 400 μg/mL, exhibiting a bell-shaped dose-response curve. When the lymphocytes were incubated without mitogenic stimuli, W-NTRP still stimulated lymphocyte proliferation with the maximum effect at 100 μg/mL, which was significantly different from the control group ($P < 0.01$).

### 3.4. *Effects of the polysaccharide on macrophage phagocytosis*

One of the most distinguished features of macrophage activation would be an increase in pinocytic activity (Im, Kim, & Lee, 2006). The effect of W-NTRP on the phagocytosis of macrophages was examined by the uptake of neutral red (0.1%) and shown in Fig. 2. The results showed that W-NTRP at five doses could effectively enhance macrophage phagocytosis, especially at the dose of 100 μg/mL, and then the phagocytic activity decreased.

### 3.5. *Effects of the polysaccharide on NO production and iNOS activity*

The nitrite concentrations in the supernatant of the polysaccharide stimulated macrophages were determined as a reflection of the production of NO. As shown in Fig. 3, W-NTRP significantly enhanced NO production from peritoneal macrophages in a bell-shaped dose-response manner and went through a peak at a concentration of 100 μg/mL, and then decreased. The maximum amount of NO produced by W-NTRP at the concentration of 100 μg/mL was close to that elicited by 5 μg/mL LPS.

Based on the ability of W-NTRP polysaccharide fractions to induce NO release, we evaluated whether this response was associated with the up-regulation of iNOS activity. Supporting these findings, the iNOS activity of macrophages was significantly enhanced after treatment with W-NTRP (Fig. 4). Therefore, the augmentation of iNOS activity resulted in the increase of NO production in macrophages.

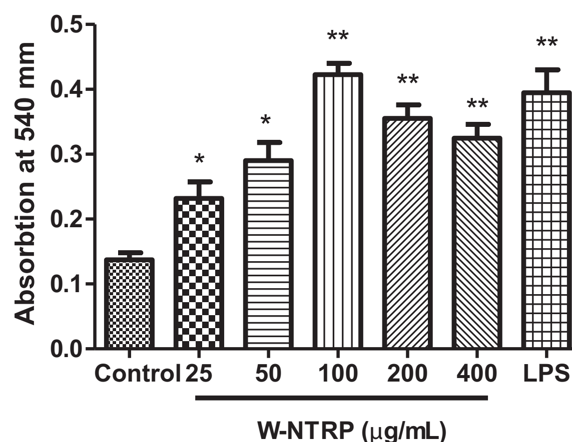


**Fig. 2.** Effects of the polysaccharide W-NTRP from Huaier on pinocytic activity of macrophages. Values represent mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

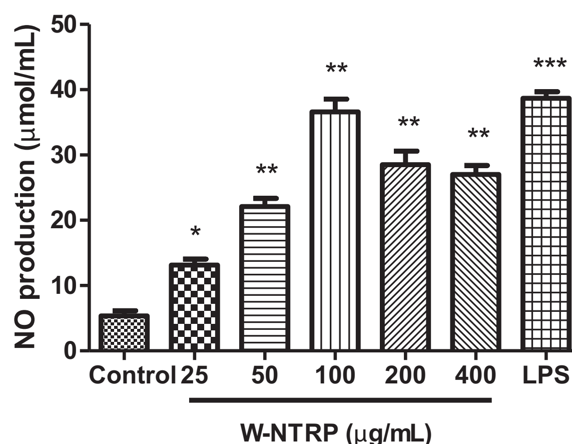


**Fig. 3.** Effects of the polysaccharide W-NTRP from Huaier on the production of NO by peritoneal macrophages in vitro. Values represent mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

**Table 3**
Effects of the polysaccharide W-NTRP from Huaier on lymphocyte proliferation. Proliferation activity was expressed at 570 nm. Values represent mean ± S.D. ($n = 3$).

| Sample | Concentrations (μg/mL) | PBS($A_{570}$) | ConA ($A_{570}$) | LPS($A_{570}$) |
|---|---|---|---|---|
| Control | – | 0.25 ± 0.02 | 0.35 ± 0.03 | 0.36 ± 0.04 |
| W-NTRP | 25 | 0.35 ± 0.04[a] | 0.42 ± 0.04[a] | 0.43 ± 0.04[a] |
| | 50 | 0.39 ± 0.05[b] | 0.48 ± 0.06[b] | 0.49 ± 0.08[b] |
| | 100 | 0.44 ± 0.07[b] | 0.54 ± 0.07[b] | 0.57 ± 0.06[b] |
| | 200 | 0.39 ± 0.07[b] | 0.51 ± 0.06[b] | 0.51 ± 0.05[b] |
| | 400 | 0.37 ± 0.06[b] | 0.50 ± 0.06[b] | 0.47 ± 0.05[b] |

[a] $P < 0.05$.
[b] $P < 0.01$ vs. control.

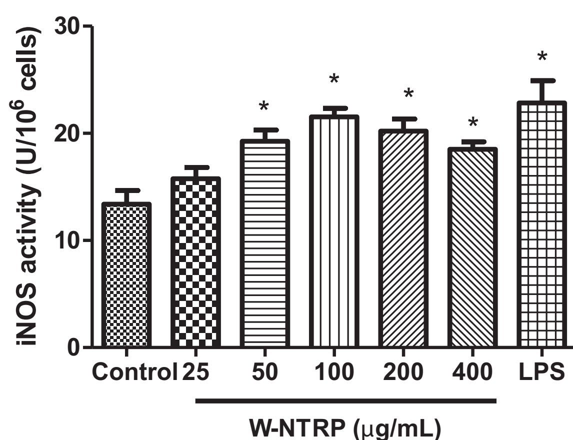


**Fig. 4.** Effects of the polysaccharide W-NTRP from Huaier on the iNOS activity of peritoneal macrophages in vitro. Values represent mean ± S.D. ($n$ = 3). *$P$ < 0.05 vs. control.

## 4. Discussion and conclusion

Cancer is one of the major causes of human death worldwide. There are many anti-cancer therapies available, including chemotherapy and anti-cancer drugs. The great majorities of them are known to be cytotoxic to cancer cells, but are also toxic to normal cells and harmful to the immune system (Yang, Guo, Zhang, & Wu, 2007). Thus discovery of new safe compound, capable of potentiating immune function, has become an important goal of research in the biomedical sciences. Recently, mushrooms have attracted much attention in the biochemical and medical fields due to their useful therapeutic effects (Balkwill, 2009). It is well documented that various mushroom-derived polysaccharides are used as remedies and prevention agents for cancer and immune disorders, and so on (Goodridge, Wolf, & Underhill, 2009; Kim, Choi, Lee, & Park, 2004). For instance, several polysaccharides isolated from *Lentinus edodes*, *Schizophyllium commune*, *Coriolus versicolor*, *Phellinus linteus*, etc. have been shown to possess immunostimulatory activities (Kim et al., 1996; Ooi & Liu, 2000; Zheng, Jie, Hanchuan, & Moucheng, 2005).

In this study, we prepared a homogeneous polysaccharide from the fruit bodies of Huaier by using DEAE-52 Cellulose column and Sepharose CL-6B/Sephadex G-100 column chromatography. Its chemical compositions were determined and the results showed that W-NTRP is an arabinogalactan with an average molecular weight of $2.5 \times 10^4$ Da. In vitro tumor growth inhibitory assay, three human cholangiocarcinoma cell lines (QBC939, Sk-ChA-1 and MZ-ChA-1) were sensitive to W-NTRP, with the $IC_{50}$ value of 47.8, 75.9, and 43.7 μg/mL, respectively. More importantly, W-NTRP exerted no cytotoxicity to L-929 normal cells, indicating its safety. Lymphocytes proliferation is an indicator of immunopotentiation. Lymphocytes proliferation induced by ConA or LPS may be used as a method to evaluate T or B lymphocyte activity. In vitro lymphocyte proliferation assay, W-NTRP had directly mitogenic effect on mouse splenocytes in a bell-shaped dose-response manner. Simultaneously, W-NTRP showed a significant comitogenic activity on ConA-, and LPS-stimulated lymphocytes. These data indicated W-NTRP was a potent immunomodulating and immunoenhancing agent. Macrophages activation is considered to be one of the important components of the host defense against tumor growth (Fidler & Kleinerman, 1993), or is another way to enhance immunological activity (Stuelp-Campelo et al., 2002). The present study showed that W-NTRP could augment the uptake of neutral red by polysaccharide-treated macrophages, especially at the dose of 100 μg/mL. Activated macrophages also released some cell factors, such as TNF-α, NO, reactive oxygen intermediates (ROI) and other substance to kill and inhibit tumor cell growth. As a major cell factor released from the activated macrophages, NO production and iNOS activity are regard to be central in the regulation of immune response against tumors (Jiao et al., 2009). The results showed that W-NTRP significantly enhanced NO production from peritoneal macrophages in a bell-shaped dose-response manner. Consistent with this finding, the iNOS activity was also significantly enhanced after treatment with W-NTRP. Taken together, these results suggest that this polysaccharide isolated from Huaier has immunoregulatory and anti-tumor activities in vitro. Further research on the structural features and mechanism of immunomodulatory activity of W-NTRP in vivo is suggested to be needed.

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## References

Anithworth, G. C., Sparrow, F. K., & Susman, A. S. (1973). *The fungi: An advanced treatise*. Academic Press: New York.
Balkwill, F. (2009). Tumour necrosis factor and cancer. *Nature Reviews Cancer*, *9*, 361–371.
Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein binding. *Analytical Biochemistry*, *72*, 248–254.
Chen, L., Lu, P., Lu, Z. X., & Li, D. C. (2004). Anticancer effect of PS-T on the experimental hepatocellular carcinoma. *Chinese-German Journal of Clinical Oncology*, *3*, 55–59.
Cui, J., Goh, K. K., Archer, R., & Singh, H. (2007). Characterisation and bioactivity of protein-bound polysaccharides from submerged culture fermentation of Coriolus versicolor Wr-74 and ATCC- 20545 strains. *Journal of Industrial Microbiology and Biotechnology*, *34*, 393–402.
de Groen, P. C., Gores, G. J., LaRusso, N. F., Gunderson, L. L., & Nagorney, D. M. (1999). Biliary tract cancers. *The New England Journal of Medicine*, *341*, 1368–1378.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.
Fidler, I. J., & Kleinerman, E. S. (1993). Therapy of cancer metastasis by systemic activation of macrophages: from the bench to the clinic. *Research in Immunology*, *144*, 284–287.
Filisetti-Cozzi, T. M. C. C., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry*, *197*, 157–162.
Goodridge, H. S., Wolf, A. J., & Underhill, D. M. (2009). Betaglucan recognition by the innate immune system. *Immunological Reviews*, *230*, 38–50.
Im, S. A., Kim, K., & Lee, C. K. (2006). Immunomodulatory activity of polysaccharides isolated from *Salicornia herbacea*. *International Immunopharmacology*, *6*, 1451–1458.
Jia, X. N., Dong, W., Lu, W. D., Guo, L. Z., & Wei, Y. X. (2009). In vivo immunostimulatory and tumor-inhibitory activities of polysaccharides isolated from solid-state-cultured *Trametes robiniophila* Murrill. *World Journal of Microbiology and Biotechnology*, *25*, 2057–2063.
Jiao, L., Li, X., Li, T., Jiang, P., Zhang, L., Wu, M., et al. (2009). Characterization and antitumor activity of alkali-extracted polysaccharide from *Enteromorpha intestinalis*. *International Immunopharmacology*, *9*, 324–329.
Johnes, T. M., & Albersheim, P. (1972). A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharide. *Plant Physiology*, *49*, 926–936.
Kim, G. Y., Choi, G. S., Lee, S. H., & Park, Y. M. (2004). Acidic polysaccharide isolated from *Phellinus linteus* enhances through the up-regulation of nitric oxide and tumor necrosis factor-alpha from peritoneal macrophages. *Journal of Ethnopharmacology*, *95*, 69–76.
Kim, H. M., Han, S. B., Oh, G. T., Kim, Y. H., Hong, D. H., Hong, N. D., et al. (1996). Stimulation of humoral and cell mediated immunity by polysaccharide from mushroom *Phellinus linteus*. *International Journal of Immunopharmacology*, *18*, 295–303.
Li, L., Ye, S., Wang, Y., & Tang, Z. (2006). Progress on experimental research and clinical application of *Trametes robiniophila*. *China Cancer*, *16*, 110–113.
Oades, J. M. (1967). Gas–liquid chromatography of alditol acetates and its application to the analysis of sugars in complex hydrolysates. *Journal of Chromatography*, *28*, 246–252.
Ooi, V. E., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. *Current Medicinal Chemistry*, *7*, 715–729.
Parikh, A., & Madamwar, D. (2006). Partial characterization of extracellular polysaccharides from cyanobacteria. *Bioresource Technology*, *97*, 1822–1827.

Peng, Y. R., Li, Y. B., Liu, X. D., Zhang, J. F., & Duan, J. A. (2008). Antitumor activity of C-21 steroidal glycosides from *Cynanchum auriculatum* Royle ex Wight. *Phytomedicine*, *15*, 1016–1020.

Schwartsmann, G., Ratain, M. J., Cragg, G. M., Wong, J. E., Saijo, N., Parkinson, D. R., et al. (2002). Anticancer drug discovery and development throughout the world. *Journal of Clinical Oncology*, *20*, 47S–59S.

Shaib, Y., & El-Serag, H. B. (2004). The epidemiology of cholangiocarcinoma. *Seminars in Liver Disease*, *24*, 115–125.

Sirica, A. E. (2005). Cholangiocarcinoma: Molecular targeting strategies for chemoprevention and therapy. *Hepatology*, *41*, 5–15.

Staub, A. M. (1965). Removal of protein-Sevag method. *Methods in Carbohydrate Chemistry*, *5*, 5–6.

Stuelp-Campelo, P. M., De Oliveira, M. B., Leão, A. M., Carbonero, E. R., Gorin, P. A., & Iacomini, M. (2002). Effect of a soluble a-d-glucan from the lichenized fungus *Ramalina celastri* on macrophage activity. *International Immunopharmacology*, *2*, 691–698.

Wasser, S. P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, *60*, 258–274.

Xie, Y., Jiang, S. P., Su, D. H., Pi, N. N., Ma, C., & Cao, P. (2010). Composition analysis and anti-hypoxia activity of polysaccharide from *Brassica rapa* L. *International Journal of Biological Macromolecules*, *47*, 528–533.

Xu, G. L., Jia, W. D., Ma, J. L., & Yu, J. H. (2003). Experimental study of extract of fungi of huaier on angiogenesis in vitro. *Chinese Pharmacological Bulletin*, *19*, 1410–1412.

Yang, X., Guo, D., Zhang, J., & Wu, M. (2007). Characterization and anti-tumor activity of pollen polysaccharide. *International Immunopharmacology*, *7*, 427–434.

Zheng, R., Jie, S., Hanchuan, D., & Moucheng, W. (2005). Characterization and immunomodulating activities of polysaccharide from *Lentinus edodes*. *International Immunopharmacology*, *5*, 811–820.

Zhu, B. W., Zhou, D. Y., Li, T., Yan, S., Yang, J. F., Li, D. M., et al. (2010). Chemical composition and free radical scavenging activities of a sulphated polysaccharide extracted from abalone gonad (*Haliotis Discus Hannai Ino*). *Food Chemistry*, *121*, 712–718.

Zhu, Z. C., Tong, Y. X., Yue, Y. P., Deng, Y., Yu, D. D., Xiao, W., et al. (2008). Apoptosis-inducing effect of Jinke on Molt-4 cells and its mechanism. *The Chinese-German Journal of Clinical Oncology*, *7*, 487–491.