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# Immunomodulatory activities of polysaccharides isolated from *Taxillus chinensis* and *Uncaria rhyncophylla*

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ABSTRACT

*Taxillus chinensis* and *Uncaria rhyncophylla* are the herbs used in traditional Chinese anticancer formulations. During the past decade, research on plant polysaccharides has gained importance due to their therapeutic value and minimum side effects. In this study, hot water extraction method was employed to isolate polysaccharides from the stems of *T. chinensis* and stems with hooks of *U. rhyncophylla*. Size-exclusion chromatography was then used for further fractionation. Separated fractions from *T. chinensis* were designated as TCP-1, TCP-2 and TCP-3 and those from *U. rhyncophylla* were termed UC-1 and UC-2. Their sugar compositions were estimated using gas chromatography that revealed the presence fructose, glucose, xylose, arbinose, and rhamnose. Amino acid analysis of these fractions has indicated that they are protein-bound polysaccharides. The antioxidant activities were investigated using DPPH and yeast assays. The ability of these polysaccharide fractions to stimulate mouse macrophages was measured using Griess reagent and ELISA test. The results revealed that some of the isolated fractions (TCP-2, TCP-3, UC-1 and UC-2) displayed significant antioxidant activities and were also found to be effective immunomodulators in a concentration-dependent manner. Outcomes of this research strongly indicate that *U. rhyncophylla* and *T. chinensis* have therapeutic potential to be used for the treatment of cancer.


## 1. Introduction

In recent years, many polysaccharides and protein-polysaccharide complexes isolated from botanical sources such as mushrooms, algae, lichens and medicinal herbs (Jeong et al., 2010; Jeong, Koyyalamudi, Jeong, Song, & Pang, 2012; Schepetkin & Quinn, 2006) have been shown to possess strong antioxidant activities and also to activate cells involved in innate immunity without harming the host (He, Niu, Li, Xu, & Lu, 2012; Jeong et al., 2010, 2012; Minato, 2010; Qi et al., 2005; Schepetkin & Quinn, 2006; Sun, Wang, Shi, & Ma, 2009; Tomida et al., 2009; Wang, Zhang, Zhang, Song, & Li, 2010; Xu et al., 2009; Zhang et al., 2012).

Oxidative stress induced by free radicals is known to cause serious illnesses including heart disease and cancer (Hollrnan, Hertogt, & Katan, 1985; Pham-Huy, He, & Pham-Huy, 2008). Consequently, the use of natural antioxidants has escalated rapidly over the past decade. However, most of the commonly used small organic antioxidants are suspected to have serious side effects (Wang, Zhang, & Cheng, 2011). Owing to their non-cytotoxic properties, natural polysaccharides have attracted a great deal of attention and are proving to be promising alternatives as antioxidants with many additional therapeutic benefits including anticancer and immunomodulatory properties (He et al., 2012; Lazareva et al., 2002; Minato, 2010; Schepetkin & Quinn, 2006; Wang et al., 2011). It is therefore of great interest to investigate plant based polysaccharides that represent an unlimited source for providing health benefits to humans, including treatment of dangerous diseases like cancer.

To the best of our knowledge, this is the first study of its kind involving pharmacological activities of polysaccharides of *Uncaria rhyncophylla and Taxillus chinensis*. These two plants are extremely important Chinese medicinal herbs used in traditional anticancer formulations (Huang et al., 2008). *U. rhyncophylla* (belongs to Rubiaceae family) is widely known as Gou-teng (Chinese) and Chotoko (Japanese) (Suk et al., 2002), which mainly contain indole alkaloid (Aisaka et al., 1985; Heitzman, Neto, Winiarz, Vaisberg, & Hammond, 2005; Yano et al., 1991). Traditionally, Gou-teng is used as sedative, hypotensive, antispasmodic, analgesic, anticonvulsive, antihypotensive, antiepileptic, and antiviral agent (Aisaka

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et al., 1985; Heitzman et al., 2005; Lee et al., 2003; Liu & Mori, 1992).

The stems of *T. chinensis* (belongs to Loranthaceae family) contain rutin, quercetin and phlorin (Huang et al., 2008) and have been used in traditional Chinese medicine for the treatment of obesity, inflammation and cancer (Huang et al., 2008; Zhang et al., 2011).

Preliminary studies carried out in authors' laboratory with water extracts of *U. rhyncophylla* and *T. chinensis* have demonstrated high antioxidant activities (Ravipati et al., 2012; Zhang et al., 2011). These herbs have also displayed high antiproliferative activities against several cancer cells (Ravipati, 2011; Zhang, 2012). Literature demonstrates that herbal extracts which display significant antioxidant activities, concurrently exhibit immunomodulatory effects (Bafna & Mishra, 2010; Mikhaeil, Badria, Maatooq, & Amer, 2004). It is important to note that immunomodulatory polysaccharides also exhibit anti-tumour properties (Jeong et al., 2012; Schepetkin & Quinn, 2006). Therefore, the discovery of potent immunomodulators is the critical step towards the invention of anticancer agents. Hence, this study aims to isolate polysaccharide fractions from *U. rhyncophylla* and *T. chinensis* and evaluate their antioxidant and immunomodulatory activities. DPPH and yeast assays were used for the measurement of antioxidant activities. Immunomodulatory properties of polysaccharide fractions have been evaluated by measuring their effect on mouse macrophages (J774A.1) to produce NO and TNF-α.

## 2. Experimental

### 2.1. Materials

500 g of dried stem of *U. rhyncophylla* and *T. chinensis* were obtained from BeiJing Tong Ren Tang Chinese Herbal Medicine shop, Sydney, Australia. A voucher specimen of the plant has been deposited in the laboratory. The plant materials were ground to a fine powder in a grinder before extraction.

Standard dextrans, glucose, bovine serum albumin (BSA), Folin-Ciocalteu (F-C) reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide ($H_2O_2$), ascorbic acid, cumene hydroperoxide (CHP) and linoleic acid hydroperoxide (LAH), naphthyl ethylenediamine, lipopolysaccharide (LPS: *Escherichia coli* serotype 0127:B8) and sulfanilamide were purchased from Sigma–Aldrich (Australia). 95% ethanol was obtained from Lomb Scientific Pty Ltd. (Australia). Antibiotics (streptomycin and penicillin), trypan blue, Dulbecco's modified eagle's medium (DMEM), foetal bovine serum (FBS) and glutamine were purchased from GIBCO. Tumour necrosis factor-α (TNF-α)-enzyme-linked immunosorbent assay (ELISA) kits were obtained from BD Biosciences (San Jose, CA, USA). Mouse monocyte macrophage cell line (J774A.1) (ATCC number TIB-71) was purchased from American type culture collection (ATCC).

### 2.2. Extraction and fractionation of polysaccharides from U. rhyncophylla and T. chinensis

The stem with hook of *U. rhyncophylla* and *T. chinensis* were powdered, and then homogenized. The sample was then autoclaved for 2 h at 121 °C, followed by cooling to room temperature before filtration. The supernatant was collected and precipitated with 95% ethanol (1:4 vol/vol) overnight at 4 °C. The precipitate containing the polysaccharides and proteins was pelleted by centrifugation at 10,000 rpm for 20 min and then the pellet was resuspended in distilled water. The supernatant was filtered through a filter paper (pore size, 0.45 μm) and lyophilized to obtain a crude polysaccharide extract with a yield of 8.64 g/kg and 8.9 g/kg (Jeong et al., 2004, 2006; Jeong, Yang, Jeong, Koyylalmudi, & Song, 2007).

The crude polysaccharide extract was dissolved in 3 mL of distilled water at a concentration of 10 mg/mL and was fractionated by size-exclusion chromatography using Sepharose CL-6B column (2.4 cm × 99 cm) equilibrated with distilled water and eluted with distilled water at a flow rate of 0.42 mL/min. The polysaccharide elution profile was determined by the phenol–sulfuric acid method and Lowry's method (Dubios, Gills, Hamilton, Rebers, & Smith, 1956; Jeong et al., 2006, 2012; Lowry, Rosebrough, Farr, & Randall, 1951). The relevant fractions were collected, pooled, and concentrated by freeze-drying.

### 2.3. Determination of molecular weights of polysaccharide fractions

Estimation of molecular weights of the purified polysaccharide fractions was done on the basis of the elution volume and the molecular weight using a standard dextran series that included T2000 (2000 kDa), T450 (450 kDa), T150 (150 kDa), T70 (70 kDa), T40 (40 kDa) T10 (10 kDa) and glucose at a concentration of 10 mg/mL each for calibration of the Sepharose CL-6B column. The linear regression of the data has provided the standard equation, $y = -0.2128x + 1.4771$ with a high correlation coefficient ($R^2 = 0.937$). This standard curve can be used to determine the molecular weights of extracted polysaccharide fractions (Jeong et al., 2004, 2012; Zhang et al., 2012).

### 2.4. Analysis of mono-saccharides

The total sugar content was determined by the phenol–sulfuric acid method (Dubios et al., 1956) using glucose as a reference standard. Total protein content of polysaccharides was determined by the method of Lowry et al. (1951). Bovine serum albumin (BSA) was used as a standard to measure the protein content (Jeong et al., 2004). The monosaccharide composition was analyzed by a Hewlett Packard 5890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame-ionization detector using a BP-20 capillary column (12 m long, 0.22 mm i.d., 0.25 μm film thickness; SGE Analytical Science Pty Ltd., Ringwood, VIC, Australia). Mono-sugars have been produced from polysaccharide fractions by hydrolysis and obtained sugars were acetylated (Jones & Albersheim, 1972). Fructose, glucose, xylose, arbinose, galactose, ribose, rhamnose and fucose sugars were used as standards.

### 2.5. Amino acid analysis

Amino acid analysis was carried out by hydrolyzing the samples with 6 N HCl in sealed ampoules in an oven at 110 °C for 22 h. Excess acid was removed by continuous flash evaporation at 45 °C under reduced pressure, dissolved in citrate buffer (pH 2.2) and aliquot of the sample was loaded into an automatic amino acid analyzer (Biochrom-30, Cambridge, UK). Cysteine and methionine were determined separately after performic acid oxidation to cysteic acid and methionine sulfone respectively (Moore, 1963). Tryptophan was determined after barytic hydrolysis according to the method described by Landry and Delhaye (Darragh, 2005; Landry & Delhaye, 1992). Each amino acid was identified and quantified using externally calibrated standards (50, 100 and 150 pmol/μL) and the results have been validated using authentic amino acid standard mixture (National Institute of Standards and Technology, SRM 2389).

### 2.6. Antioxidant activity

The DPPH free radical scavenging assay was conducted using the Blois method (Blois, 1958). Each fraction (50 μL) was added to a 150 μL of 62.5 μM DPPH. After 30 min of incubation, the

absorbance of the reaction mixtures was measured at 492 nm using a microplate reader (Multiskan 141 EX, Thermo Electron, USA). Ascorbate (vitamin C), an antioxidant, was used as a positive control. The blank control was distilled water instead of polysaccharides. The standard concentrations (0, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 μM) were prepared in 60% methanol. The free radical scavenging activities of plant-derived polysaccharides were calculated as the ascorbic acid equivalent against the calibration standard curve. The linear regression equation of $y = -0.0007x + 0.2544$ was obtained with a correlation coefficient ($R^2 = 0.9211$).

The antioxidant capacities of purified polysaccharides were measured *in vitro* using a *Saccharomyces cerevisiae* based high throughput assay (Wu et al., 2011). *S. cerevisiae* BY4743 was cultured overnight in a 50 mL volume by inoculation of a single colony. The culture was then diluted to an optical density at 600 nm (OD600) of 0.2 in media, and 180 μL of each strain was added into a well in a 96-well microtiter plate, where 10 μL per well of each fraction was also added in duplicate. Ten microliters of three different oxidants, namely, hydrogen peroxide ($H_2O_2$), cumene hydroperoxide (CHP) and linoleic acid hydroperoxide (LAH) were added to a final concentration of 2 mM, 150 μM and 75 μM, respectively. The initial OD600 reading was taken using a microplate reader (Multiskan EX, Thermo Electron, USA), and the plates were then incubated in a 30 °C incubator with shaking at 750 rpm. Yeast growth was monitored by reading OD600 at the end of 20 h. Ascorbic acid was used as a positive control. The effect of samples on the net growth of yeast cells was measured after inducing oxidative stress with three different oxidants, by using the following equation.

$$P_{\text{yeast growth}} = \frac{P_{\text{Sample}} - P_{\text{Control}}}{P_{\text{Control}}} \times 100\%$$

where, '$P_{\text{yeast growth}}$' is the net growth of $H_2O_2$, CHP and LAH induced yeast cells after treatment with polysaccharides, '$P_{\text{Sample}}$' is the optical density of yeast cells with the treatment of polysaccharides, '$P_{\text{Control}}$' is the optical density of yeast cells with the treatment of negative control (Oxidants).

### 2.7. Measurement of NO production

J774A.1 macrophages were incubated at 37 °C in an atmosphere of 5% $CO_2$ for 40 h in culture medium containing various doses of polysaccharide fractions (20 μL) or LPS (20 μL) as a positive control in wells of 96-well flat-bottom microtiter plates at a density of $4 \times 10^5$ cells per well. After incubation, 100 μL of the culture supernatant was removed and assayed for NO production using a colorimetric method with sodium nitrite as a standard. In brief, supernatants were mixed with equal volumes of Griess reagent (0.1% [w/v] naphthyl ethylenediamine and 1% [w/v] sulfanilamide in 5% [v/v] phosphoric acid) in wells of 96-well flat-bottom microtiter plates. After 5 min at room temperature, the absorbance was measured at 550 nm in a Bio-Rad (Hercules, CA, USA) microplate reader (Zhang et al., 2011).

The respective intercept and slope values were used in the measurement of NO production capacity of polysaccharides. The linear regression equation of $y = 0.0024x + 0.1941$ was obtained with a good correlation coefficient ($R^2 = 0.9666$).

### 2.8. Assay for the measurement of TNF-α production

J774A.1 cells were incubated for 40 h in culture medium with or without various doses of polysaccharide fractions (20 μL) or LPS as a positive control in 96-well flat-bottom microtiter plates at a density of $4 \times 10^5$ cells per well. TNF-α secreted in the culture supernatants were quantified using enzyme-linked immunosorbent assay kits (BD Biosciences, San Jose, CA, USA). Cytokine concentrations were determined by extrapolation from TNF-α standard curve, according to the manufacturer's protocol (Zhang et al., 2011).

The respective intercept and slope values were used in the measurement of TNF-α production capacity of polysaccharides. The linear regression equation of $y = 0.0014x + 0.0559$ was obtained with a significant correlation coefficient ($R^2 = 0.997$).

### 2.9. Determination of cell viability by Alamar Blue assay

Alamar Blue assay is a calorimetric assay involving the cellular reduction of resazurin to resorufin. 100 μL of Alamar Blue solution (10% Alamar Blue (Resazurin) in DMEM media) was added to each well and incubated at 37 °C for 1–2 h. Fluorescence was measured (excitation at 545 nm and emission at 595 nm) and expressed as a percentage of that in control cells after background fluorescence was subtracted.

### 2.10. Statistical analysis

Data are expressed as mean ± standard deviation (SD) values. All measurements are performed in triplicate. The group mean was compared using a one-way analysis of variance and Duncan's multiple range tests. The statistical difference was considered significant at $P < 0.05$. The analysis was performed using SPSS Version 20 Software (IBM SPSS, Chicago, IL, USA) and Excel.

## 3. Results and discussion

### 3.1. Extraction and fractionation of polysaccharides from U. rhyncophylla and T. chinensis

Polysaccharides were extracted from *U. rhyncophylla* and *T. chinensis* as by autoclaving (Section 2.2) and were separated by size-exclusion chromatography on a Sepharose CL-6B column. Five major fractions were collected on the basis of the total carbohydrate and protein elution profiles as described in a previous publication (Zhang et al., 2012). These crude polysaccharide fractions are designated as TCP-1, TCP-2, TCP-3, UC-1 and UC-2 (Fig. 1A and B). The molecular weights of the isolated polysaccharide fractions have been determined as described in Section 2.3. Average molecular masses of TCP-1 and UC-1 were found to be relatively large, with an estimated value of more than 500 kDa (Table 1). This was follow by TCP-2, TCP-3 and UC-2 which contained polysaccharides with average molecular masses of 18, 2 and 3 kDa, respectively.

### 3.2. Monosaccharide and amino acid compositions of the fractions

Monosaccharide and the amino acid compositions of isolated fractions were analyzed as described previously (Zhang et al., 2012) and these results are presented in Tables 2 and 3, respectively. As can be seen from these results, most of the fractions consisted mainly of glucose, fructose, xylose, arabinose and rhamnose. Aspartate, glutamate and glycine constituted 30–50% of the total amino acids. The total essential amino acid content was highest in UC-2 and lowest in UC-1. Different fractions had varying content of essential amino acids with lysine as the limiting amino acid in all the fractions. The results presented in Tables 2 and 3 indicate that the isolated fractions are protein-bound polysaccharides. All the polysaccharide fractions isolated from *U. rhyncophylla* and *T. chinensis* in this study contained higher proportions of glucose and average levels of xylose and arabinose (Table 2). Most of the fractions have also contained significant levels of fructose. These mono-sugars may be responsible for the observed antioxidant and immunomodulatory activities of TCP-2, TCP-3 and UC-1 (Sections 3.4 and 3.5). These findings are in agreement with the literature

**Table 1**
Antioxidant activities of polysaccharide fractions of *U. rhyncophylla* and *T. chinensis* along with their molecular weights.

| S.no. (1 mg/mL) | Molecular mass (kDa) | DPPH scavenging activity of water extracts (ascorbate equivalent μM)[a] | % Yeast cell growth (Water extracts)[b] | | |
|---|---|---|---|---|---|
| | | | CHP (150 μM) | $H_2O_2$ (2 mM) | LHA (75 μM) |
| TCP-1 | 541 | 207 ± 4 | 0 | 27.79 ± 2.11 | 23.02 ± 1.94 |
| TCP-2 | 18 | 447 ± 0 | 30 ± 3.54 | 39.7 ± 2.11 | 25.09 ± 1.46 |
| TCP-3 | 2 | 662 ± 0 | 37.5 ± 0 | 48.21 ± 2.11 | 32.65 ± 3.89 |
| UC-1 | 524 | 245 ± 0 | 45 ± 3.54 | 41.17 ± 4.21 | 22.68 ± 0.49 |
| UC-2 | 3 | 305 ± 0 | 77.5 ± 7.07 | 70.52 ± 2.11 | 25.09 ± 0.49 |

All the values are mean of triplicate determination ± standard deviation (SD).
[a] DPPH free radical scavenging activity was measured as equivalent of ascorbic acid.
[b] Yeast oxidative stress was measure on the basis of survival of yeast cells (yeast growth) after treatment with $H_2O_2$, CHP or LAH.

**Table 2**
Chemical composition (sugar contents) of polysaccharide fractions isolated from *U. rhyncophylla* and *T. chinensis.*

| | TCP-1 | TCP-2 | TCP-3 | UC-1 | UC-2 |
|---|---|---|---|---|---|
| Total carbohydrate (%) | 34.05 | 16.43 | 30.68 | 67.65 | 22.73 |
| Monosaccharide (% ratio) | | | | | |
| Rhamnose (%) | 11.69 | 12.41 | – | 8.85 | 6.04 |
| Fucose (%) | – | – | – | – | – |
| Ribose (%) | – | – | – | – | – |
| Arabinose (%) | 15.58 | 13.38 | 13.17 | 10.72 | 7.37 |
| Xylose (%) | 21.65 | 14.21 | 18.14 | 11.53 | 7.84 |
| Galactose (%) | – | – | 15.62 | – | 9.82 |
| Fructose (%) | – | 22.48 | 20.63 | 21.05 | 14.45 |
| Glucose (%) | 34.63 | 24.14 | 22.35 | 24.26 | 18.22 |
| Unknown (%) | 16.45 | 13.38 | 10.09 | 23.59 | 36.26 |

(Chandrashekar, Prashanth, & Venkatesh, 2011; Jeong et al., 2012; Schepetkin & Quinn, 2006; Wong, Lai & Cheung, 2011; Zhang et al., 2012).

### 3.3. Cell viability

Effect of various polysaccharide fractions derived from *U. rhyncophylla* and *T. chinensis* on the viability of mouse macrophage cells are given in Fig. 2. It is clear from these results that, most of the fractions from *T. chinensis* (except TCP-1) showed significant cell viabilities even at highest concentration (100 μg/mL) used in this study (Fig. 2A). Also the cell viability studies of polysaccharide fractions derived from *U. rhyncophylla* showed that they are non-toxic. These results are consistent with literature reports (Jeong et al., 2004, 2010, 2012; Schepetkin & Quinn, 2006) indicating that polysaccharides are non-toxic.

### 3.4. Radical scavenging and in vivo anti-oxidant activities of polysaccharide fractions

The results of free radical scavenging capacity of the crude polysaccharide fractions are presented in Table 1. All the

**Table 3**
Protein content and Amino acid analysis of the polysaccharide fractions isolated from *U. rhyncophylla* and *T. chinensis.*

| | TCP-1 | TCP-2 | TCP-3 | UC-1 | UC-2 |
|---|---|---|---|---|---|
| % of protein | 65.95 | 83.57 | 69.32 | 32.35 | 77.27 |
| Amino acids (g/100protein) | | | | | |
| Aspartic acid | 8.6 | 10.58 | 9.01 | 35.58 | 10.66 |
| Threonin[a] | 4.54 | 4.58 | 4.53 | 3.22 | 5.97 |
| Serine | 12.03 | 8.81 | 17.34 | 4.84 | 8.25 |
| Glutamic acid | 13.66 | 14.43 | 8.83 | 10.61 | 11.68 |
| Proline | 4.95 | 2.39 | 2.66 | 3.31 | 2.61 |
| Glycine | 10.18 | 10.56 | 11.81 | 5.91 | 7.65 |
| Alanine | 5.98 | 6.06 | 8.02 | 3.07 | 8.06 |
| Cystine | 9.37 | 8.04 | 4.56 | 3.02 | 2.59 |
| Valine[a] | 5.23 | 5.28 | 4.08 | 3.69 | 5.35 |
| Methionine[a] | 0.62 | 0.6 | 0.32 | 0.25 | 1.41 |
| Isolucine[a] | 1.62 | 2.4 | 2.11 | 2.2 | 3.05 |
| Leucine[a] | 2.81 | 4.07 | 3.04 | 2.3 | 5.32 |
| Tyrosine | 2.85 | 3.56 | 2.86 | 3.61 | 2.51 |
| Phenylalanine[a] | 1.64 | 0.88 | 3.34 | 2.84 | 7.19 |
| Histidine[a] | 1.36 | 1.91 | 1.74 | 2.31 | 1.75 |
| Lysine[a] | 1.9 | 1.56 | 2.51 | 4.45 | 6.16 |
| Arginine | 1.99 | 4.83 | 3.32 | 2.75 | 2.91 |
| Total amino acids | 89.31 | 90.53 | 90.07 | 93.95 | 93.12 |
| Total essential amino acids | 30.57 | 30.96 | 27.34 | 25.57 | 39.56 |
| Amino acid score | 33 | 27 | 43 | 35 | 81 |
| Limiting amino acid | LYS | LYS | LYS | LYS | LYS |

[a] Essential amino acids.

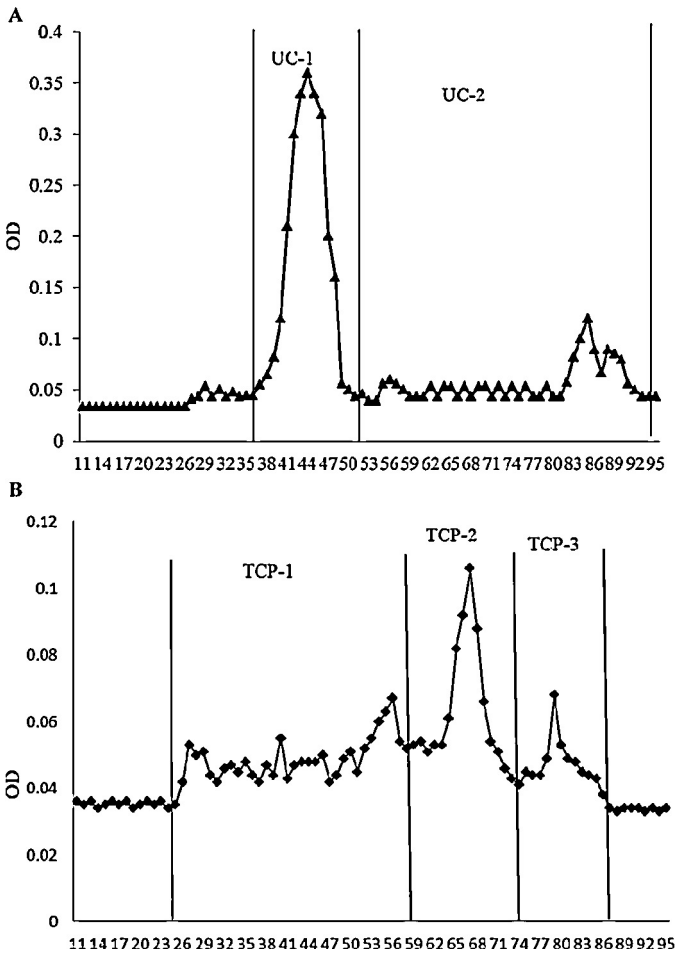


**Fig. 1.** Gel filtration chromatograms of polysaccharide fractions from *U. rhyncophylla* (A) and *T. chinensis* (B) (designated as TCP-1, TCP-2, TCP-3, UC-1 and UC-2).

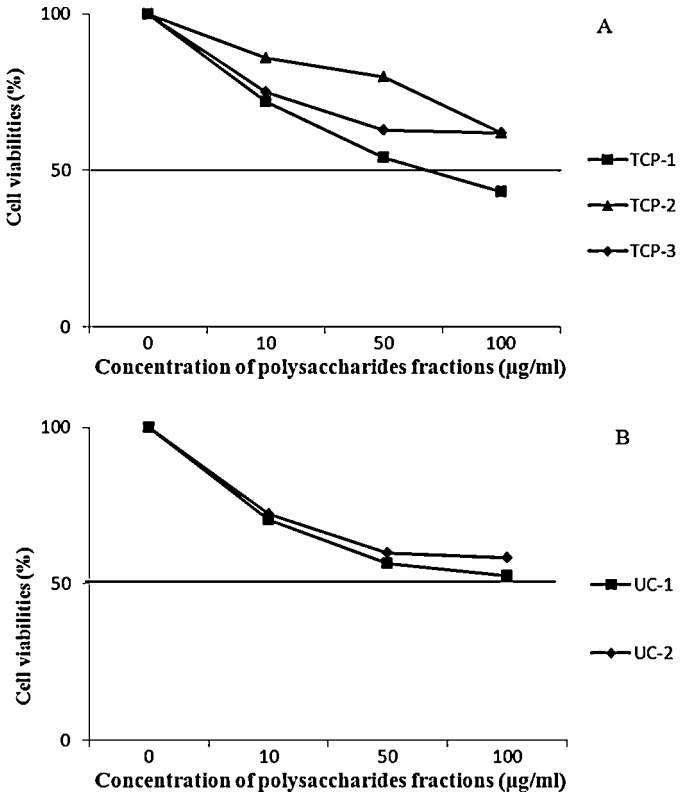


**Fig. 2.** Cell viabilities of isolated polysaccharide fractions from *T. chinensis* (A) and *U. rhyncophylla* (B) at different concentrations.

polysaccharide fractions exhibited significant scavenging capacity that ranged from 207 μM to 662 μM ascorbate equiv/g. High antioxidant activity was displayed by polysaccharide fractions TCP-2 and TCP-3, with scavenging capacities more than 400 μM ascorbate equiv/g (Table 1).

*In vivo* antioxidant activities of *U. rhyncophylla and T. chinensis* polysaccharides measured by using yeast model are also presented in Table 1. These results clearly demonstrate that the fractions TCP-2, TCP-3, UC-1 and UC-2 display significant *in vivo* antioxidant activities. It is interesting to note that these fractions, TCP-2, TCP-3, UC-1 and UC-2 showed high radical scavenging activity as well as in high *vivo* antioxidant activity.

An important conclusion that can be drawn from these results is that the antioxidant activities of the isolated polysaccharide fractions have displayed an inverse correlation with their average molecular weights (Table 1). For example, the antioxidant activities of TCP-2 and TCP-3 are stronger than that of TCP-1 which has highest average molecular weight (Table 1). Similarly, the activities of UC-2 are more significant than UC-1. This was true for the activities measured by both methods, namely, DPPH radical scavenging method and yeast oxidative stress model. Literature argues that, high molecular weight polysaccharides generally have high viscosity and poor water solubility and hence is difficult for these molecules to penetrate into the interior of the cell, which limits their biological activity (Jeong et al., 2012; Liu, Wang, Pang, Yao & Gao, 2010; Qi et al., 2005; Sun et al., 2009; Tomida et al., 2009; Wang et al., 2010; Zhang et al., 2012). TCP-1 showed toxic effects at the high concentration levels and did not display significant *in vivo* antioxidant activities against CHP. Hence, TCP-1 was not considered for further studies.

### 3.5. Immunomodulatory effects of polysaccharides derived from *U. rhyncophylla and T. chinensis*

Immunomodulatory properties of the isolated polysaccharide fractions were measured by treating J774A.1 cells polysaccharide fractions. These results revealed a dose-dependent enhancement in the production of TNF-α and NO (Figs. 3 and 4).

The results clearly demonstrate that the isolated polysaccharide fractions from *T. chinensis* (Fig. 3) have significant immunomodulatory activities as indicated by increased production of TNF-α in a dose-dependent manner (Fig. 3A and C). As can be seen from Fig. 3C, there is a sharp increase of immunomodulatory activity of TCP-2 in the concentration range of 10–100 μg/mL ($P < 0.05$). Most of the polysaccharide fractions of *T. chinensis* also activated macrophage NO production in a dose-dependent manner (Fig. 3B and D) ($P < 0.05$). These findings lead to the conclusion that TCP-2 is the most active *Taxillus* polysaccharide fraction.

The results of immunomodulatory activities of polysaccharide fractions from *U. rhyncophylla* are presented in Fig. 4. It can be seen from these results that the polysaccharide fractions of *U. rhyncophylla* display significant immunomodulatory activities as indicated by increased production of TNF-α as well as NO in a dose-dependent manner (Fig. 4). *Taxillus* polysaccharide fraction (TCP-2) is the most active one amongst all the fractions studied in this research.

Immunomodulatory effects of TCP-2 were highly significant in terms of TNF-α production with a sharp increase in the activity at concentrations greater than 10 μg/mL (Fig. 3C). Excellent immunomodulatory capacity has been observed for the polysaccharide fraction TCP-2 at 100 μg/mL that displayed nearly 3-fold increase in TNF-α production when compared with untreated

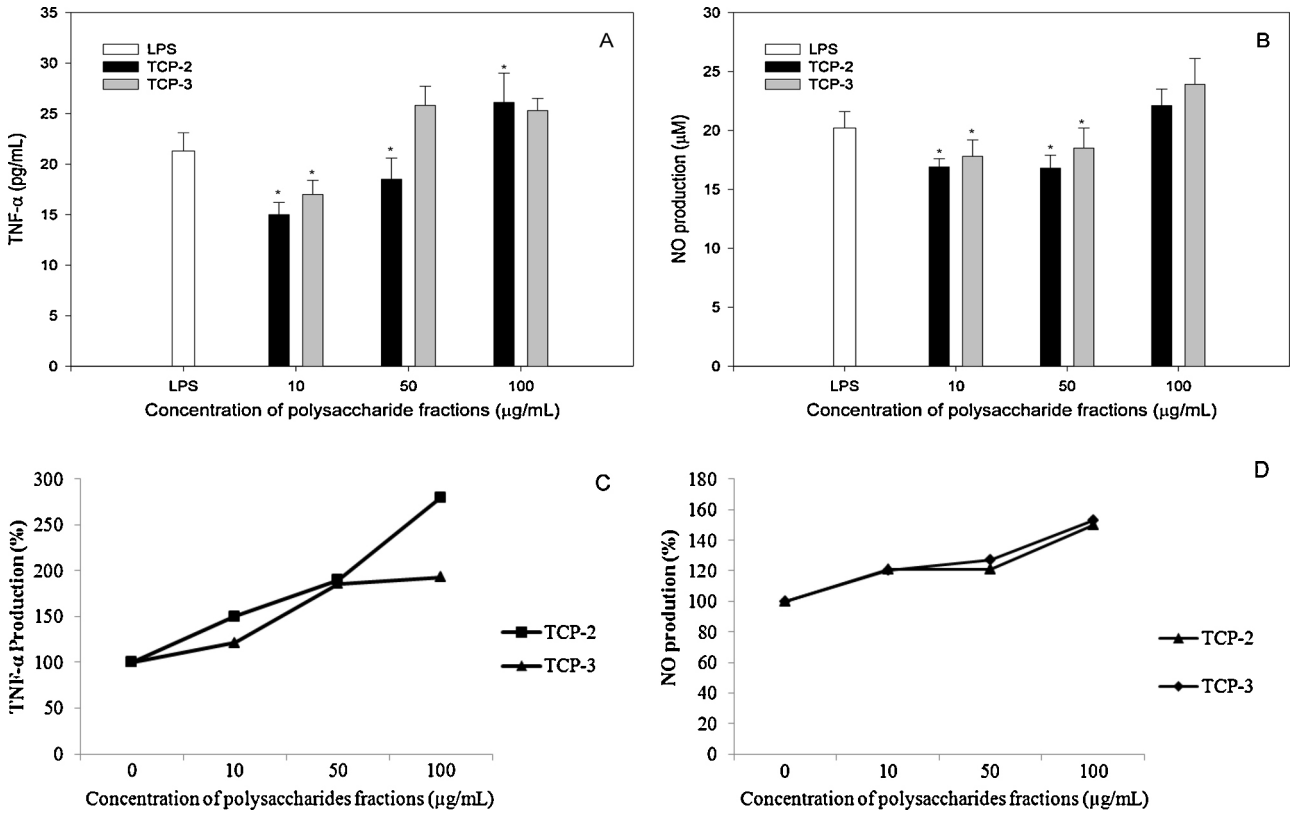


**Fig. 3.** Effects of *T. chinensis* polysaccharides on murine J774A.1 macrophages. (A and C) represent nitric oxide production, and (B and D) represent tumour necrosis factor-α (TNF-α) production. * Significant difference from control (LPS treated group), $n = 3$, $P < 0.05$.

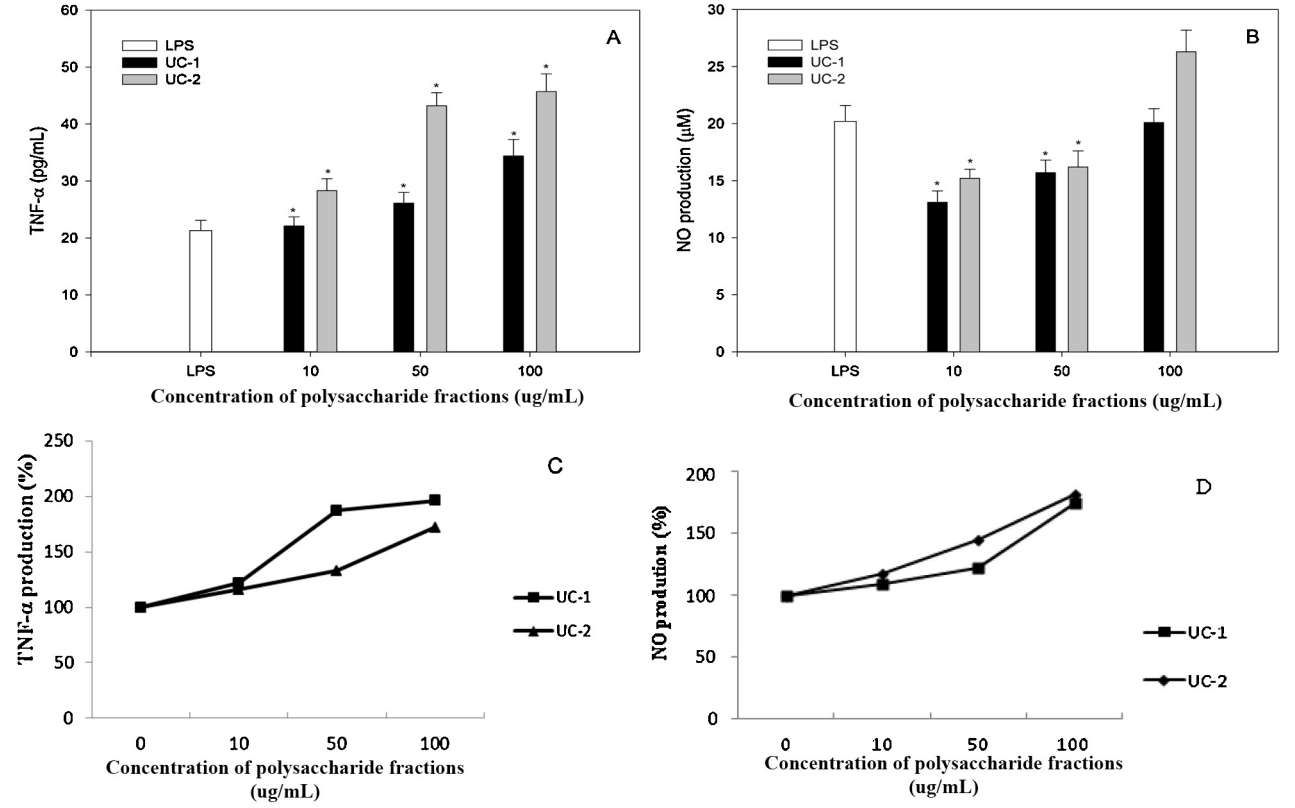


**Fig. 4.** Effects of *U. rhyncophylla* polysaccharides on murine J774A.1 macrophages. (A and C) represent nitric oxide production, and (B and D) represent tumour necrosis factor-α (TNF-α) production. *Significant difference from control (LPS treated group), $n = 3$, $P < 0.05$.

macrophages (Fig. 3C). Also, the induced NO production capacity of macrophage by polysaccharide fraction TCP-2 and TCP-3 were significant at the concentration of 100 μg/mL (Fig. 3B and D). These findings are very valuable and indicate that two of the polysaccharides isolated from *T. chinensis* are highly suitable candidates to modulate the macrophage function and stimulate the immune system. It is very interesting to note that the polysaccharide fraction UC-1 and UC-2 isolated from *U. rhyncophylla* also possesses strong potential for immunomodulatory activity (Fig. 4). It is important to note from the literature that (He et al., 2012; Jeong et al., 2006, 2007, 2012; Luo & Fang, 2008; Minato, 2010; Schepetkin & Quinn, 2006; Sun et al., 2012; Zhang et al., 2012) immunomodulatory activity plays a key role in anti-tumour activity. Hence, polysaccharide fractions isolated in this study from *U. rhyncophylla and T. chinensis* are likely to be potential candidates for anti-tumour activity. Preliminary studies on anti-proliferative activities of water extracts of *U. rhyncophylla and T. chinensis* carried out in the authors' laboratory support this hypothesis (Ravipati, 2011; Zhang, 2012). It is also very important to remark at this stage that both of these herbs are extremely favoured candidates in traditional anti-cancer formulations. The results of pharmacological investigations carried in this research strongly support their use in traditional Chinese medicine.

Recent studies revealed that certain structural characteristics of polysaccharides, such as β-(1→3)-D-glucan in their main chain are responsible for anti-tumour activity, and α-glucan has little bioactivity (Luo & Fang, 2008; Sun et al., 2012; Zhang, Cui, Cheung & Wang, 2007). Further studies involving purification and structural characterization of the immunomodulatory polysaccharides discovered in this research are required in order to understand the structure-activity relationship of these important classes of macromolecules. Research in this direction is in progress in our Laboratory.

## 4. Conclusion

Many plant-derived polysaccharides are known to possess immunomodulating, anti-tumour and other important activities (He et al., 2012; Jeong et al., 2004, 2006, 2007, 2010, 2012; Luo & Fang, 2008; Minato, 2010; Schepetkin & Quinn, 2006; Sun et al., 2012). In this study, fractionation of polysaccharides from *U. rhyncophylla and T. chinensis* was successfully carried out and their immunomodulatory activities were evaluated. The observed activities are found to be inversely related to the average molecular masses of the isolated fractions. Three of the polysaccharide fractions (TCP-2, TCP-3, UC-1 and UC-2) have shown highly significant immunomodulatory capacity at 100 μg/mL. These results suggest the potential of three of the isolated polysaccharides as natural anti-tumour agents.

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## References

Aisaka, K., Hattori, Y., Kihara, T., Ishihara, T., Endo, K., & Hikino, H. (1985). Hypotensive action of 3 alpha-dihydrocadambine, an indole alkaloid glycoside of *Uncaria* hooks. *Planta Medica*, *51*, 424–427.

Bafna, A., & Mishra, S. (2010). Antioxidant and immunomodulatory activity of the alkaloidal fraction of *Cissampelos pareira* Linn. *Scientia Pharmaceutica*, *78*, 21–31.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *181*, 1199–1200.

Chandrashekar, P. M., Prashanth, K. V. H., & Venkatesh, Y. P. (2011). Isolation, structural elucidation and immunomodulatory activity of fructans from aged garlic extract. *Phytochemistry*, *72*, 255–264.

Darragh, A. J. (2005). The effect of hydrolysis time on amino acid analysis. *Journal of AOAC International*, *88*, 888–893.

Dubios, M., Gills, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugar and related substance. *Analytical Chemistry*, *28*, 350–356.

He, X. J., Niu, X., Li, Y., Xu, J., & Lu, S. H. A. P. (2012). Immunomodulatory activities of five clinically used Chinese herbal polysaccharides. *Journal of Experimental and Integrative Medicine*, *2*, 15–27.

Heitzman, M. E., Neto, C. C., Winiarz, E., Vaisberg, A. J., & Hammond, G. B. (2005). Ethnobotany, phytochemistry and pharmacology of *Uncaria* (Rubiaceae). *Phytochemistry*, *66*, 5–29.

Hollrnan, P. C. H., Hertogt, M. G. L., & Katan, M. B. (1985). Role of dietary flavonoids in protection against cancer and coronary heart disease. *Food and Chemical Toxicology*, *23*, 659. Zhang al Int. J. Biological Macromolecules.

Huang, H. B., Li, K. X., Liu, T., Zeng, C. Q., Lin, J., & Qiu, M. (2008). *Kang Zhong Liu Zhong Yao Lin Chuang Ying Yong Yu Tu Pu (Clinical application of anti-tumor Chinese medicine)* (1st ed.). Guang Zhou: Guangdong Science and Technology Press.

Jeong, S. C., Jeong, Y. T., Yang, B. K., Islam, R., Koyyalamudi, S. R., Pang, G., et al. (2010). White button mushroom (*Agaricus bisporus*) lowers blood glucose and cholesterol levels in diabetic and hypercholesterolemic rats. *Nutrition Research*, *30*, 49–56.

Jeong, S. C., Koyyalamudi, S. R., Jeong, Y. T., Song, C. H., & Pang, G. (2012). Macrophage immunomodulating and antitumor activities of polysaccharides isolated from *Agaricus bisporus* white button mushrooms. *Journal of Medicinal Food*, *15*, 58–65.

Jeong, S. C., Yang, B. K., Jeong, Y. T., Koyyalamudi, S. R., & Song, C. H. (2007). Isolation and characterization of biopolymers extracted from the bark of *Acanthopanax sessiliflorus* and their anticomplement activity. *Journal of Microbiology and Biotechnology*, *17*, 21–28.

Jeong, S. C., Yang, B. K., Kim, G. N., Jeong, H., Wilson, M. A., Cho, Y., et al. (2006). Macrophage-stimulating activity of polysaccharides extracted from fruiting bodies of *Coriolus versicolor* (Turkey Tail Mushroom). *Journal of Medicinal Food*, *9*, 175–181.

Jeong, S. C., Yang, B. K., Ra, K. S., Wilson, M. A., Cho, K. Y., Gu, Y. A., et al. (2004). Characteristics of anti-complementary biopolymer extracted from *Coriolus versicolour*. *Carbohydrate Polymer*, *55*, 255–263.

Jones, T. M., & Albersheim, P. (1972). A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharides. *Plant Physiology*, *49*, 926–936.

Landry, J., & Delhaye, S. (1992). Simplified procedure for the determination of tryptophan of foods and feed stuffs from barytic hydrolysis. *Journal of Agricultural and Food Chemistry*, *40*, 776–779.

Lazareva, E., Spiridonova, T., Chernega, E., Plesskaia, L., Grunenkova, I., Smirnov, S., et al. (2002). Topical pectins for the treatment of burn wounds. *Antibiotic and Chemotherapy*, *47*, 9–13.

Lee, J. S., Son, D. W., Lee, P., Kim, J., Shin, D. K., Jang, M. C., et al. (2003). Protective effect of methanol extract of *Uncaria rhynchophylla* against excitotoxicity induced by N-methyl-D-aspartate in rat hippocampus. *Journal of Pharmacological Sciences*, *92*, 70–73.

Liu, J., & Mori, A. (1992). Antioxidant and free radical scavenging activities of *Gastrodia elata* Bl. and *Uncaria rhynchophylla* (Miq.) Jacks. *Neuropharmacology*, *31*, 1287–1298.

Liu, W., Wang, H. Y., Pang, X. B., Yao, W. B., & Gao, X. D. (2010). Characterization and antioxidant activity of two low-molecular-weight polysaccharides purified from the fruiting bodies of *Ganoderma lucidum*. *International Journal of Biological Macromolecules*, *46*, 451–457.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Luo, D. H., & Fang, B. S. (2008). Structural identification of ginseng polysaccharides and testing of their antioxidant activities. *Carbohydrate Polymers*, *72*, 376–381.

Mikhaeil, B. R., Badria, F. A., Maatooq, G. T., & Amer, M. M. A. (2004). Antioxidant and immunomodulatory constituents of henna leaves. *Zeitschrift fuer Naturforschung Section C: Journal of Biosciences*, *59*, 468–476.

Minato, K. (2010). Mushrooms: Immunomodulating activity and role in health promotion. In R. R. Watson, S. Zibadi, & V. R. Preedy (Eds.), *Dietary components and immune function* (pp. 529–539). New York: Humana press.

Moore, S. (1963). On the determination of cystine as cysteic acid. *Journal of Biological Chemistry*, *238*, 235–237.

Pham-Huy, L. A., He, H., & Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science*, *4*, 89–96.

Qi, H. M., Zhao, T. T., Zhang, Q. B., Li, Z. E., Zhao, Z. Q., & Xing, R. E. (2005). Antioxidant activity of different molecular weight sulfated polysaccharides from *Ulva pertusa* Kjellm (Chlorophyta). *Journal of Applied Psychology*, *17*, 527–534.

Ravipati, A. S. (2011). *Isolation and identification of biomarkers and cyclic peptides from medicinal herbs*. Australia: University of Western Sydney. Master of Science thesis.

Ravipati, A. S., Zhang, L., Koyyalamudi, S. R., Jeong, S. C., Reddy, N., Bartlett, J., et al. (2012). Antioxidant and anti-inflammatory activities of selected Chinese medicinal plants and their relation with antioxidant content. *BMC Complementary and Alternative Medicine*, *12*, 173.

Schepetkin, I. A., & Quinn, M. T. (2006). Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. *International Immunopharmacology*, *6*, 317–333.

Suk, K., Kim, S. Y., Leem, K., Kim, Y. O., Park, S. Y., Hur, J., et al. (2002). Neuroprotection by methanol extract of *Uncaria rhynchophylla* against global cerebral ischemia in rats. *Life Sciences*, *70*, 2467–2480.

Sun, L., Peng, X. X., Sun, P., Shi, J. H., Yuan, X. W., Zhu, J. J., et al. (2012). Structural characterization and immunostimulatory activity of a novel linear α-(1→6)-D-glucan isolated from *Panax ginseng* CA Meyer. *Glycoconjugate Journal*, 1–8.

Sun, L. Q., Wang, C., Shi, H., & Ma, Q. J. C. H. (2009). Preparation of different molecular weight polysaccharides from *Porphyridium cruentum* and their antioxidant activities. *International Journal of Biological Macromolecules*, *45*, 42–47.

Tomida, H., Fujii, T., Furutani, N., Michihara, A., Yasufuku, T., Akasaki, K., et al. (2009). Antioxidant properties of some different molecular weight chitosans. *Carbohydrate Research*, *344*, 1690–1696.

Wang, J., Zhang, Q. B., Zhang, Z. S., Song, H. F., & Li, P. C. (2010). Potential antioxidant and anticoagulant capacity of low molecular weight fucoidan fractions extracted from *Laminaria japonica*. *International Journal of Biological Macromolecules*, *46*, 6–12.

Wang, H., Zhang, X., & Cheng, F. S. (2011). Antioxidant activity of bioactive polysaccharides isolated from nature source. *Journal Animal Science Biotechnology*, *2*, 230–238.

Wong, K. H., Lai, C. K. M., & Cheung, P. C. K. (2011). Immunomodulatory activities of mushroom sclerotial polysaccharides. *Food Hydrocolloids*, *25*, 150–158.

Wu, M. J., O'Doherty, P. J., Fernandez, H. R., Lyons, V., Rogers, P. J., Dawes, I. W., et al. (2011). An antioxidant screening assay based on oxidant-induced growth arrest in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, *11*, 379–387.

Xu, J., Liu, W., Yao, W. B., Pang, X. B., Yin, D. K., & Gao, X. D. (2009). Carbompxymethylation of a polysaccharide extracted from *Ganoderma lucidum* enhances its antioxidant activities *in vitro*. *Carbohydrate Polymers*, *78*, 227–234.

Yano, S., Horiuchi, H., Horie, S., Aimi, N., Sakai, S., & Watanabe, K. (1991). $Ca^{2+}$ channel blocking effects of hirsutine, an indole alkaloid from *Uncaria* genus, in the isolated rat aorta. *Planta Medica*, *57*, 403–405.

Zhang, L. (March 2012). *Isolation and characterisation of biopolymer from anti-cancer medicinal herbs*. Australia: University of Western Sydney. Master of Science Thesis.

Zhang, L., Koyyalamudi, S. R., Jeong, S. C., Reddy, N., Smith, P. T., Ananthan, R., et al. (2012). Antioxidant and immunomodulatory activities of polysaccharides from the roots of *Sanguisorba officinalis*. *International Journal of Biological Macromolecules*, *51*, 1057–1062.

Zhang, L., Ravipati, A. S., Koyyalamudi, S. R., Jeong, S. C., Reddy, N., Smith, P. T., et al. (2011). Antioxidant and anti-inflammatory activities of selected medicinal plants containing phenolic and flavonoid compounds. *Journal of Agricultural and Food Chemistry*, *59*, 12361–12367.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: A review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science and Technology*, *18*, 4–19.