

Characterisation of antioxidant, anti-inflammatory, and immunomodulatory activities of polysaccharides derived from *Duchesnea indica* (Andrews) Focke

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Abstract

Duchesnea indica (Andrews) Focke is a traditional Chinese medicine used as antiviral, astringent, anti-inflammatory, and antitumor agent in various clinical settings. In the present work, *D. indica* polysaccharides (DIPs) were assessed following hot water extraction, ethanol precipitation, and deproteinisation. The molecular weight of DIPs was estimated by GPC as 3.018×10^5 Da. HPLC results indicated that DIPs were mainly composed of D-mannose, D-ribose, L-rhamnose, D-glucosamine, D-glucuronic acid, D-galacturonic acid, D-glucose, D-galactose, and D-arabinose, in a molar ratio of 1.54:0.50:3.83:1.56:1.33:3.39:23.69:15.26:8.86, respectively. With regard to antioxidant activities, DIPs had a strong radical scavenging capacity and protective effect against DNA damage. Furthermore, DIPs presented a significant anti-inflammatory ability *in vivo*, using both xylene-induced ear oedema and λ -carrageenan-induced paw oedema models. Lastly, DIPs significantly inhibited generation of NO and cytokines (TNF- α and IL-6) in LPS-induced RAW264.7 macrophages. These data strongly indicate that DIPs could be explored as potentially natural antioxidant, anti-inflammatory, and immunomodulatory agents.

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Introduction

Polysaccharides are comprised of macromolecular compounds that are widely found in living organisms. Within the last few decades, a representative number of studies have identified various polysaccharides from plants, animals, fungi, and algae that exhibited antitumor (Jin *et al.*, 2017), antioxidant (Jahanbin *et al.*, 2011), immunomodulatory (Meng *et al.*, 2014), and anti-inflammatory (Zheng *et al.*, 2003) activities. Importantly, these polysaccharides have been termed as impactful, non-toxic, and good biocompatible materials. Thus, polysaccharides have attracted increasing attention as adjuvants and/or main compounds in non-toxic natural foods and drugs.

Duchesnea indica (Andrews) Focke is a member of the Rosaceae family, which is extensively distributed in South Asia (Chen *et al.*, 2015). Used as folk medicine for a long time in China, *D. indica* extracts have shown diverse pharmacological activities, such as anti-inflammatory, antitumoral, antimutagenic, antiviral, and anticoagulant (Li *et al.*, 1996; Zhao *et al.*, 2008; Pawlaczyk *et al.*, 2009). Several small molecule components derived from *D. indica*, including flavonoids (Jiang *et al.*, 2017), triterpenoids (Ye and Yang, 1996), polyphenols (Zhu *et al.*, 2015), and

essential oils (Wang *et al.*, 2014) have been identified and further investigated, but limited work related to the characterisation and function of these macromolecules (e.g. polysaccharides) have been achieved so far. One exception regarding two acidic heteropolysaccharides (DIP30 and DIP60) have shown potent activity against the varicella-zoster virus (Jiang *et al.*, 2013). Thus, due to its potential therapeutic value, a detailed characterisation of the *D. indica* polysaccharides is warranted.

In the present work, we performed the extraction and further characterisation of crude polysaccharides from *D. indica*. Hence, we have presently annotated their basic physicochemical properties, consisting of molecular weight, monosaccharide composition, and infrared spectrum analysis. Functionally, their antioxidant activities were measured by two distinct *in vitro* assays, which were hydroxyl radical scavenging activity, and DNA damage assay. Potential anti-inflammatory properties were determined *in vivo* by means of xylene-induced ear oedema and λ -carrageenan-induced paw oedema in mice. Lastly, the immunomodulatory capacity of DIPs was examined by employing mouse RAW264.7 macrophages.

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Materials and methods

Plant materials and chemicals

D. indica was kindly identified by Prof. An-Cai Luo (Chongqing Normal University, China) in Bozhou City (Anhui province, China) in July 2017. The voucher specimen (No. 2017025) was stored at the College of Life Sciences, Chongqing Normal University, Chongqing, China. λ -carrageenan was obtained from Shanghai Yuanye Biotechnology Co. Ltd. (Shanghai, China). Lipopolysaccharides (LPS) from *Escherichia coli* O55:B5 was provided by Sigma Chemical Co. (St. Louis, MO, USA). Nitric oxide (NO) assay kit was obtained from Suoqiao Biotechnology Co. Ltd. (Shanghai, China), Interleukin-6 (IL-6) and Tumour Necrosis Factor- α (TNF- α) ELISA kits were acquired from NeoBioscience Technology Co. Ltd. (Shanghai, China) and Multi Sciences Biotechnology Co. Ltd. (Shanghai, China), respectively. Other chemicals and solvents of analytical grade were purchased from Chongqing Dongfang Chemical and Glass Co. Ltd. (Chongqing, China).

Extraction and preparation of D. indica polysaccharides

The polysaccharides of *D. indica* were extracted with hot distilled water following the original protocol (Wang *et al.*, 2017), including some minor modifications. Briefly, 100 g of the dried whole plant (*D. indica*) was crushed using a high-speed disintegrator (FW100, Tianjin Taisite Instrument Co. Ltd., Tianjin, China). Powdered plant was put into a volumetric flask with one litre of distilled water, and extracted three times for 3 h at 90°C. Supernatants were collected by centrifugation (8,000 g for 10 min) and concentrated to one-fifth of the initial volume, by using a vacuum rotary evaporator, at 65°C (0.9 kPa). The remaining solution was then mixed with absolute ethanol to a final concentration of 80% (v/v) and kept at 4°C for the whole day. After centrifugation and decreasing amounts of ethanol, acetone and ether were sequentially used to wash the extract precipitate and remove small molecules. The precipitate was deproteinated following the Sevag method (Jiang *et al.*, 2011). Deproteinated supernatants were further combined, concentrated, and dialysed by continuously flowing distilled water for 24 h, using a dialysis bag (diameter: 35 mm, molecular weight cut-off: 3500 Da). Dialysed solution was then lyophilised to obtain crude *D. indica* polysaccharides (DIP).

Physicochemical characterisation of DIPs

Measurement of polysaccharide content and molecular weight

Total sugar content was determined by phenol-sulfuric acid colorimetric method, employing glucose as a standard (Xu *et al.*, 2018). The uronic acid content was assayed by carbazole-sulphuric acid method, using D-glucuronic acid as a standard (Kintner and Van Buren, 1982). Reducing sugar content was analysed by the 3,5-dinitrosalicylic acid (DNS) method, using glucose as a standard (Dygert *et al.*, 1965). The DIPs' molecular weight was estimated by gel permeation chromatography (Wyatt, USA), equipped with OHpak SB-806 HQ (8.0 \times 300 mm), Waters 515, laser detector (LS), and a differential refractive index detector (DRI). For this, 500 μ L of solution (amount per sample) was loaded and eluted with 0.02% NaN₃ at a flow rate of 1 mL/min at 40°C. The respective molecular weight was calculated using a calibration curve (Sun *et al.*, 2014).

Analysis of monosaccharide composition

The DIPs' monosaccharide composition was analysed with a 1-phenyl-3-methyl-5-pyrazolone (PMP) derivative, based on previous report with minor modifications (Tang *et al.*, 2014). Briefly, 10 mg of DIPs were hydrolysed with 2 mL of 2 M TFA (trifluoroacetic acid), in a sealed ampule, at 110°C for 6 h. Following complete hydrolysis, the excess acid was removed by co-distillation with methyl alcohol, for several times, until neutralisation. The hydrolysed product was resolved in 1 mL of 0.3 M NaOH solution. Subsequently, 0.4 mL of hydrolysed sample was combined with 0.4 mL of PMP-methanol solution (0.5 M). The resulting mixture was then incubated at 70°C for 2 h, and then neutralised with 1 mL of 0.3 M HCl. The obtained solution was further extracted three times with 1.2 mL chloroform. The resulting aqueous layers were combined and purified by 0.45 μ m membrane filtration (Millipore, USA). Standard solutions of D-mannose, D-ribose, L-rhamnose, D-glucosamine, D-glucuronic acid, D-galacturonic acid, D-glucose, D-aminogalactose, D-galactose, D-xylose, D-arabinose, and L-fucose (0.4 mg/mL) were derivatised with PMP as previously indicated. The PMP-labelled samples of DIPs as well as the standard monosaccharide were evaluated accordingly, using an Agilent 1100 HPLC system (Agilent Technologies, USA) with a Zorbax Eclipse XDB-C₁₈ column (4.6 \times 250 mm, 5 μ m, Agilent Technologies, USA). Samples were eluted with a mixture of acetonitrile and 0.1 M phosphate buffer (20:80, pH 6.6) at a rate of 1.0 mL/min and a volume of 5 μ L per injection.

FT-IR spectroscopy

The Fourier transform infrared (FT-IR) spectrum of the DIPs was detected using a Nicolet 380 FT-IR

spectrometer supported with potassium bromide (KBr) discs (You *et al.*, 2014). For this, approximately 1.0 mg of DIPs was dried and then mixed with 200 mg of KBr. After grinding into fine powder, sample was pressed into a pellet, which was used for FT-IR spectral analysis in the range from 500 to 4000 cm^{-1} .

Determination of antioxidant activity in vitro

Hydroxyl radical (HO•) scavenging activity
The hydroxyl radical scavenging activity of DIPs was measured by means of Fenton reaction including some minor modifications (Liu *et al.*, 2007). The mixture system was composed of 1.0 mL sample at various concentrations (1, 2, 3, 4, and 5 mg/mL), 0.05 mL of 5 mM ferrous sulphate, 0.035 mL of 0.3% H_2O_2 (v/v), and 0.015 mL of 20 mM salicylic acid in ethanol solution. This mixture was fully mixed and then immersed in a 37°C water bath for 30 min. The solution's absorbance was detected at 510 nm. The scavenging activity of hydroxyl radical was computed using Eq. 1:

$$\text{Hydroxyl radical (HO) scavenging activity (\%)} = (A_0 - A_1 + A_2) / A_0 \times 100 \quad (\text{Eq. 1})$$

where, A_0 = absorbance of the control sample (deionised water replaced the sample solution for the blank), A_1 = absorbance in the presence of the mixture containing sample, and A_2 = absorbance of the sample only (distilled water substituted the ethanolic solution of salicylic acid as control).

Plasmid-based DNA nick assay

The protective effect of DIPs on DNA damage induced by H_2O_2 and UV treatment was measured as described by Bkhairia *et al.* (2018) with some changes. Briefly, 5 μL of PET-30a plasmid DNA in TAE buffer (1 mM EDTA and 10 mM Tris-HCl, pH 8.0) was mixed with 3 μL of DIP at different concentrations (0, 0.625, 1.25, 2.5, 5, and 10 mg/mL)/rutin (1 mg/mL), and 2 μL of H_2O_2 (0.3%, v/v). The mixture was then exposed to UV light (20 W) for 5 min at room temperature. Untreated plasmid DNA was used as a control. Subsequently, samples were resolved by 1% agarose gel electrophoresis. Gel was dyed with ethidium bromide and photographed with gel Doc EZ system (Bio-Rad, USA).

Determination of anti-inflammatory capacity in vivo Experimental model

Swiss-Kunming male mice (18 - 22 g) supplied by the Laboratory Animal Centre of Chongqing Medical University, Chongqing, China, were maintained under standard conditions (12-h light/dark

cycle at $22 \pm 1^\circ\text{C}$). Caged mice had free access to food and water. All animal procedures were approved by the Chongqing Municipal Public Health Bureau and conducted in accordance with Methods for the Management of Experimental Animals in Chongqing (No. 195).

Xylene-induced ear oedema model

Xylene-induced ear oedema in mice was carried out as previously (Lu *et al.*, 2006) with limited modifications. For this, 30 mice were divided randomly into five groups (six mice per group): saline (control group), dexamethasone positive group (10 mg/kg), and three DIP groups (62.5, 125, and 250 mg/kg). Each group were administered orally with designated doses. After 1 h of treatment, 20 μL xylene was applied into the inner and outer surfaces of the right ear of each mouse. The left ear of each animal was not treated with xylene and, therefore, used as control. All animals were sacrificed by cervical dislocation 1 h later, and the round ear samples (8 mm in diameter) were obtained by stainless steel punching and weighed on an electronic balance (Shanghai Youke instrument Co., Ltd.). To quantify the degree of induced oedema, the weight of the right ear sample was subtracted from that of left ear sample. The oedema rate was calculated using Eq. 2:

$$\text{Oedema rate (\%)} = [(\text{degree of oedema of blank control group} - \text{degree of oedema of treatment group}) / \text{degree of oedema of blank control group}] \times 100\% \quad (\text{Eq. 2})$$

λ -carrageenan-induced paw oedema model

In vivo assay was conducted in accordance with Liao *et al.* (2012) with a few modifications. Thirty mice were divided randomly into five groups (six mice per group). Each mice group was submitted to the oral administration of saline (0.9%, w/v), indomethacin (10 mg/kg) or DIPs (62.5, 125, or 250 mg/kg), respectively. One hour after treatment, animals were injected with 0.1 mL λ -carrageenan suspension (1%, w/v in saline) into the right hind paw. Mice were sacrificed by cervical dislocation after 5 h of injection, and paws were cut at the joint and weighed. The degree of paw oedema was evaluated by comparing the left paw weight (control) and right paw weight. The oedema rate was estimated using Eq. 3:

$$\text{Oedema rate (\%)} = [(\text{swelling degree of control group} - \text{swelling degree of administration group}) / \text{swelling degree of control group}] \times 100 \quad (\text{Eq. 3})$$

Evaluation of immunomodulatory activity in LPS-activated RAW264.7 macrophages

Cell culture

Mouse RAW264.7 macrophage cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM medium (Hyclone, GE Healthcare Life Sciences, USA), supplemented with 10% foetal bovine serum (Gibco, USA), 100 U/mL of penicillin, and 100 g/mL of streptomycin, in a CO₂ incubator (95% air and 5% CO₂) at 37°C.

Cell viability assay

The impact of DIP on the viability of RAW264.7 cells was analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-bromo diphenyltetrazolium (MTT) assay (Mosmann, 1983; Lin *et al.*, 2016). Accordingly, cells were first loaded into 96-well plate (1 × 10⁵ cells/mL) and incubated for 24 h, followed by the addition of increasing DIP concentrations (62.5, 125, 250, and 500 µg/mL). After incubation for additional 24 h, MTT solution was added to each well to a final concentration of 0.5 mg/mL, and culture plate were further stored in a dark bath at 37°C for 4 h. Afterwards, supernatants were removed and 500 µL DMSO (Dimethyl sulfoxide) were added for crystal solubilisation. Absorbance at 570 nm was measured by microplate reader (SP-Max 2300A2, Shanghai, China).

Measurement of nitric oxide (NO) and cytokine production

RAW264.7 cells (5 × 10⁵ cells/100 µL) were first induced with 1 µg/mL LPS (dissolved in DMEM) for 1 h. Subsequently, cells were treated with different concentrations of DIP (62.5, 125, 250, and 500 µg/mL) for 24 h. Cell supernatants were then collected and subjected to NO and cytokine assays. The levels of NO and cytokines were respectively determined following the manufacturer's instructions (NO, assay kit; TNF-α and IL-6, ELISA kits) (Cordeiro Caillot *et al.*, 2018).

Statistical analysis

All data were presented as mean ± standard deviation (SD). Statistical analysis was evaluated with one-way ANOVA and student's t-test, using SPSS software version 13.0 (SPSS, Chicago, IL, USA). p-values of 0.05 or less were considered as statistically significant.

Results and discussion

DIP characterisation

DIPs were extracted with a yield of 13.56% from the whole plant of *D. indica* using successive steps that included (i) hot water extraction, (ii) ethanol precipitation, (iii) deproteinisation, and (iv) lyophilisation. The content of total carbohydrate and reducing

sugar in DIP extract were 65.80 ± 1.87 and 24.55 ± 1.88%, respectively, while the content of uronic acid was 13.53 ± 2.08%. The DIPs' molecular weight was estimated to be 3.018 × 10⁵ Da. HPLC results showed that DIPs mainly consisted of D-mannose, D-ribose, L-rhamnose, D-glucosamine, D-glucuronic, D-galacturonic acid, D-glucose, D-galactose, and D-arabinose, in a molar ratio of 1.54:0.50:3.83:1.56:1.33:3.39:23.69:15.26:8.86, respectively (Figure 1). In terms of peak area, the main DIP-related monosaccharide was D-glucose, followed by D-galactose and D-arabinose.

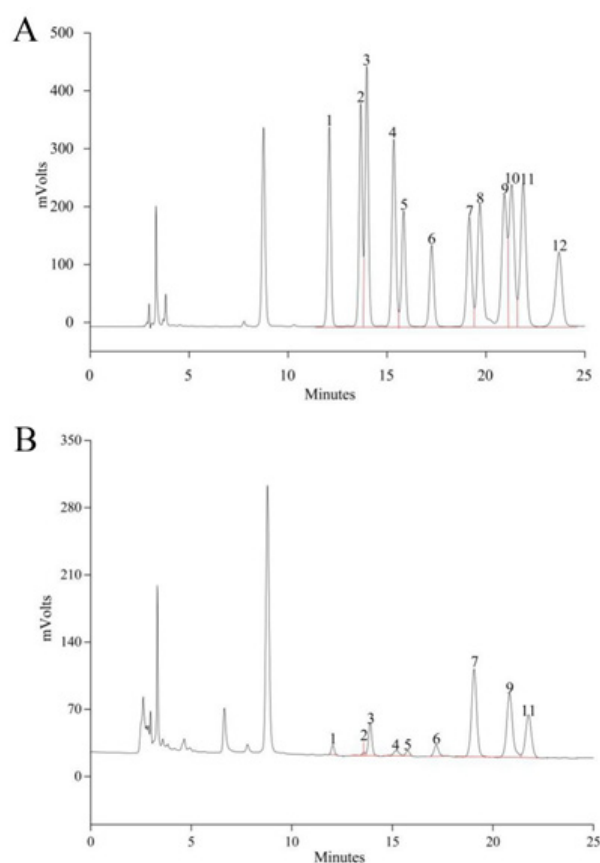


Figure 1. HPLC chromatograms of standard monosaccharides (A) and DIPs (B). Peaks: (1) D-mannose, (2) D-ribose, (3) L-rhamnose, (4) D-glucosamine, (5) D-glucuronic acid, (6) D-galacturonic acid, (7) D-glucose, (8) D-aminogalactose, (9) D-galactose, (10) D-xylose, (11) D-arabinose, and (12) L-fucose.

FT-IR spectrum analysis of DIPs is shown in Figure 2. The strong and intense absorption peak at 3414 cm⁻¹ was assigned to the O-H stretching vibration, while the peak at 2928 cm⁻¹ was assigned to the C-H stretching (Zhang *et al.*, 2010; Wang *et al.*, 2011). The peak at 1611 cm⁻¹ exhibited at C=O (carbon-oxygen double bond) asymmetric stretching vibration (Xu *et al.*, 2015b). In addition, the absorption peak at ~1403 cm⁻¹ demonstrated the existence of a symmetric

stretch vibration of -COO- related to uronic acid (Yang *et al.*, 2014), which is consistent with the detection of uronic acids in DIPs in the present work. The weak absorption peak at 1244.00 cm^{-1} appeared in the FT-IR spectrum due to asymmetrical stretching vibrations of S=O, therefore indicating that DIPs were composed of a sulphated polysaccharide (Percival and Wold, 1963). Moreover, several peaks on the region between 1000 to 1200 cm^{-1} could be observed in the spectrum. These peaks were mainly attributed to C-O-C stretching vibration as well as stretching vibrations of C-OH side groups (Barros *et al.*, 2002). These results indicate that DIPs possessed remarkable structural characteristics, based on the presence of glycosidic linkages, sulphates, and uronic acids.

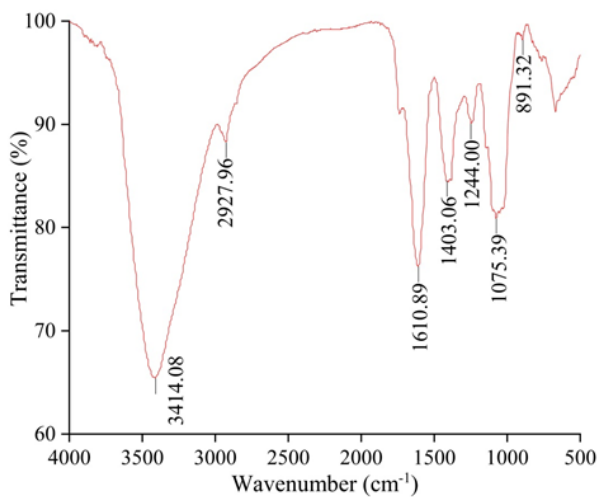


Figure 2. FT-IR spectrum of DIPs in KBr pellet, over the range of $4000 - 500\text{ cm}^{-1}$.

Antioxidant activity of DIPs *in vitro* Hydroxyl radical scavenging activity

As one of the most reactive free radicals, hydroxyl radicals have been universally recognised as

detrimental factors to most of the biomacromolecules in living cells (Xu *et al.*, 2015a). The scavenging capacity of hydroxyl radical can act as an indicator of the antioxidant activities for many reducing agents. As shown in Figure 3(A), the scavenging activity of hydroxyl radical from DIPs increased from 1 to 5 mg/mL, in a concentration-dependent manner. The scavenging impact of DIPs towards hydroxyl radicals was determined to be 82.41% at the highest test concentration (5 mg/mL). This percentage was lower than that of Vc (93.61%). These data indicate that DIPs had strong scavenging activities towards hydroxyl radicals, and therefore, could be considered as a putative natural antioxidant.

Protective effect of DIPs against DNA damage

Hydroxyl radicals have been considered as some of most potent mutagens produced in biological systems. These radicals can interact with the DNA, leading to strand breaks and formation of various base and ribose derivatives (Tepe *et al.*, 2011). The protective effect of DIPs against DNA damage was analysed by agarose gel electrophoresis (Figure 3(B)). An untreated PET-30a plasmid DNA was used as negative control and showed two bands, where the faster moving band corresponded to the native supercoiled circular DNA (Sc DNA), while the slower one represented the open circular form (Oc DNA) (lane 1) (Kumar and Chattopadhyay, 2007). Lane 6 indicates the DNA damage pattern induced by hydroxyl radical (produced by UV-photolysis of H_2O_2), revealing that the Sc DNA was cleaved to further generate the Oc DNA form. In the presence of 0.625, 1.25, 2.5, 5, and 10 mg/mL of DIPs (lanes 1 - 5), the damage on Sc DNA was significantly inhibited in a dose-dependent manner. These results suggest that DIPs possessed a remarkable protective effect towards hydroxyl radical-induced DNA damage.

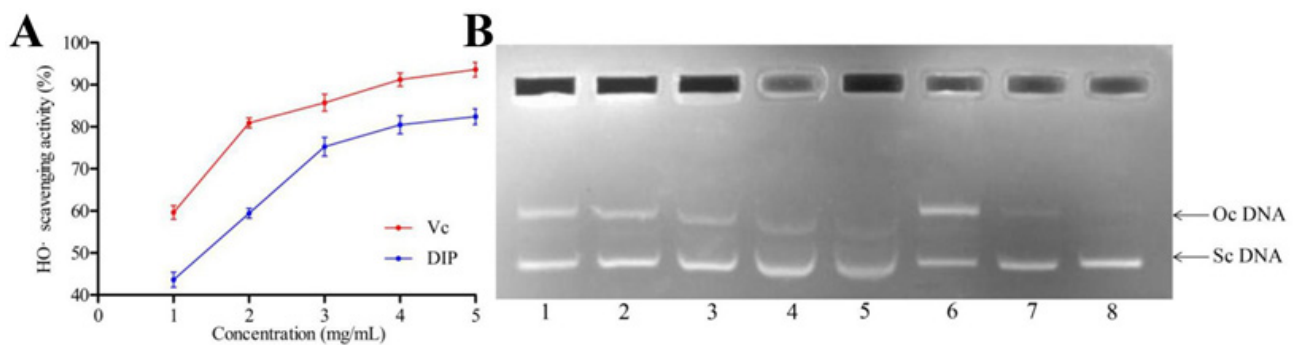


Figure 3. *In vitro* antioxidant activity of DIPs. (A) Hydroxyl radical scavenging activity of DIP and Vc at various concentrations (1, 2, 3, 4, and 5 mg/mL). (B) Protective effect of DIPs on plasmid DNA damage, induced by the photolysis of H_2O_2 . Lane 1 - 5 = H_2O_2 + UV treated with DIPs at different concentrations (0.625, 1.25, 2.5, 5, and 10 mg/mL); lane 6 = H_2O_2 + UV only; lane 7 = H_2O_2 + UV treated with rutin (positive control); lane 8 = untreated plasmid DNA (control); Sc DNA = supercoiled circular DNA; and Oc DNA = open circular DNA.

Anti-inflammatory potential of DIPs *in vivo*

Inhibitory effect of DIPs on xylene-induced mouse ear oedema

The xylene-induced ear oedema model, a well-established method used for the selection of anti-inflammatory agents *in vivo*, has been reported in vast number of studies (Yin *et al.*, 2018). The inhibitory effect of DIPs on ear oedema induced by xylene is illustrated on Figure 4(A). The results showed that DIPs can significantly attenuate ear oedema when compared with the control group, in a dose-dependent manner. Using doses of 125 and 250 mg/kg, the DIP treatment increased the inhibited rate to 79.89%, which was higher than that of indomethacin-treated mice (67.94%). These findings suggest that DIPs possessed a strong inhibitory role on xylene-induced ear oedema in mice, thus revealing a promising potential for the use of DIPs as an anti-inflammatory agent.

Inhibitory effect of DIPs on λ -carrageenan-induced mouse paw oedema

λ -carrageenan-induced paw oedema model is a commonly accepted *in vivo* approach that has been widely used to evaluate a series of anti-inflammatory drugs. At the same time, an increasing number of reports have illustrated that several plant polysaccharides display evident of anti-inflammatory activities (Ananthi *et al.*, 2010). Therefore, we further confirmed the probable DIPs' anti-inflammatory activity using a mouse paw oedema model (Figure 4(B-C)). As compared to control (untreated) animals, we observed that all tested DIP concentrations (62.5, 125, and 250 mg/kg) were able to significantly inhibit paw oedema in mice, after 5 h of λ -carrageenan injection ($p < 0.001$). Particularly, 250 mg/kg of DIPs was more effective to decrease local inflammation

(maximum efficacy of 51.16%) than the positive control indomethacin ($p < 0.05$). These data confirm that DIPs had a positive effect in alleviating λ -carrageenan-induced oedema.

Immunomodulatory activity of DIPs

Effect of DIPs on the viability of RAW264.7 cells

To evaluate the cytotoxic effect of DIPs *in vitro*, RAW264.7 macrophage cells were incubated at different concentrations of DIPs (62.5, 125, 250, and 500 $\mu\text{g/mL}$) for 24 h, and then cell viability was determined by MTT assay. As shown in Figure 5(A), DIPs did not significantly influence the viability of RAW264.7 cells when compared with the control cell group. These results suggest that DIP concentrations under 500 $\mu\text{g/mL}$ had no cytotoxic effect in RAW264.7 cells.

Effects of DIPs in cytokine and NO production

Macrophages are essential white blood cells that play a vital role in the human immune system. Upon exposure to outside noxious stimuli, such as pathogens, damaged cells, or irritants, macrophages are rapidly activated, leading to the release of NO and cytokines in the body (Mosser and Edwards, 2008). Lipopolysaccharides (LPS) are integral components of the cell wall of Gram-negative bacteria. LPS act as an inflammatory stimulus that induces macrophages to produce a variety of pro-inflammatory modulators, such as NO, TNF, IL-6, and others. An excessive release of cytokines can lead to severe harm to the human body (Liao *et al.*, 2015). Consequently, the effect of DIPs on the levels of NO, TNF- α , and IL-6 in LPS-activated RAW264.7 cells were further investigated. As shown in Figure 5(B-D), cells treated with LPS alone (1 $\mu\text{g/mL}$) exhibited higher levels of NO, TNF- α , and IL-6 as

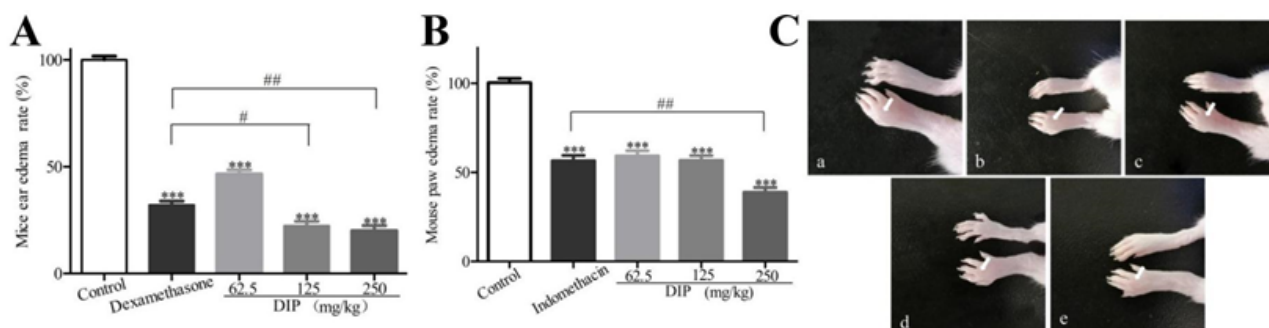


Figure 4. Xylene-induced ear oedema and λ -carrageenan-induced paw oedema in mice. (A) Solvent (control), dexamethasone, and DIPs-treated (62.5, 125, and 250 mg/kg) groups. (B) Solvent (control), indomethacin, and DIPs-treated (62.5, 125, and 250 mg/kg) groups. Data are means \pm SD of six mice ($n = 6$) per group. * = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$ as compared to the solvent (control) group. ## = $p < 0.01$ as compared to indomethacin group (one-way ANOVA). (C) Photomicrograph of mice induced with carrageenan and further treated with DIPs. Arrows indicate mice oedema. (a) Carrageenan-induced only (untreated), (b) indomethacin (10 mg/kg), (c) 62.5 mg/kg DIPs, (d) 125 mg/kg DIPs, and (e) 250 mg/kg DIPs.

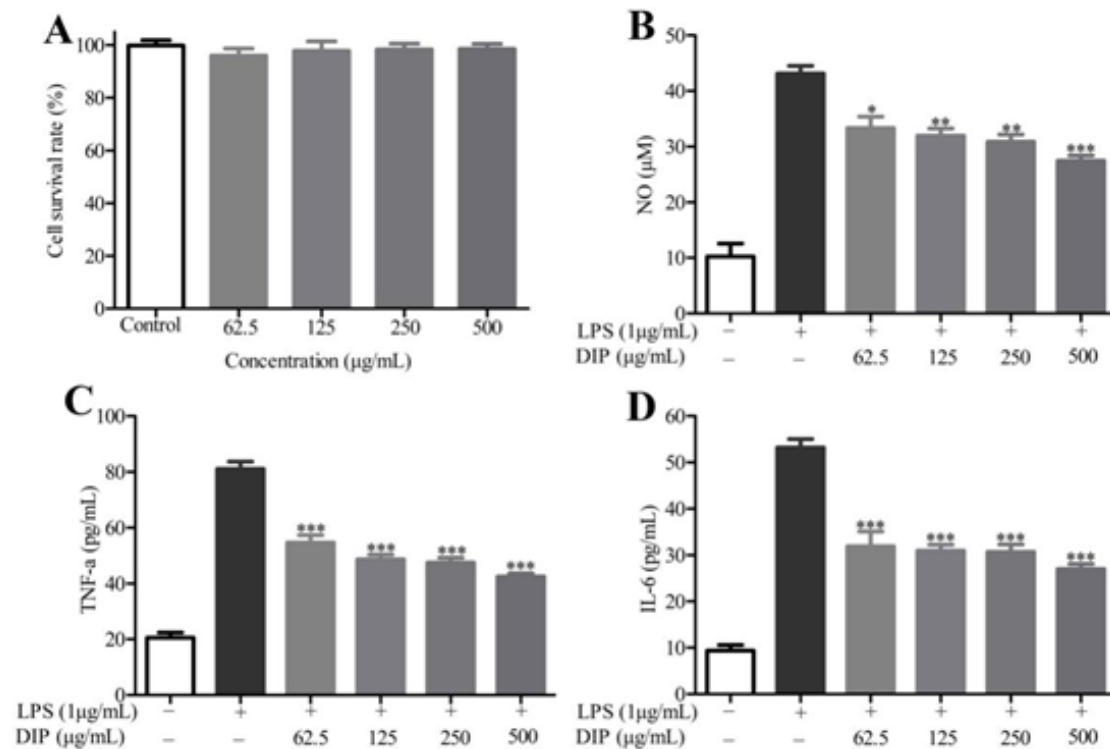


Figure 5. Evaluation of immunomodulatory activity in LPS-activated RAW264.7 macrophages. (A) Cell viability of polysaccharides present in DIPs on RAW264.7 cells in the presence of LPS. The viability of untreated cells (i.e. without DIPs and/or LPS treatment) was used as reference (100%, positive control). (B) NO concentration of culture supernatants was determined by Griess assay. (C) TNF- α concentration of culture supernatants was determined by ELISA, according to a standard curve. (D) IL-6 concentration of culture supernatants was determined by ELISA, according to a standard curve. Data are means \pm SD of three sets ($n = 3$) of independent experiments. * = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$ represent significant difference of DIPs-treated cells versus cells treated with LPS only.

compared to those from untreated cells, by increasing 4.22, 5.66, and 3.95 times, respectively. The addition of DIPs significantly decreased the production of NO, TNF- α , and IL-6 in LPS-induced cells, in a dose-dependent manner, according to the DIP concentrations presently tested ($p < 0.05$). At higher concentrations (500 $\mu\text{g/mL}$) of DIPs, NO, TNF- α , and IL-6 levels decreased by 36.70, 47.54, and 49.19% as compared to untreated LPS-induced cells, respectively. Consistently, recent studies have demonstrated that blackberry wine polysaccharides could significantly inhibit the production of NO and cytokines in LPS-treated RAW264.7 macrophages. Thus, our current findings strongly suggest that *D. indica* polysaccharides are promising candidates for the development of immunomodulatory agents.

Conclusion

In the present work, natural polysaccharides were extracted and characterised from *D. indica*. These DIPs had an apparent molecular weight of 3.018×10^5 Da, and mainly consisted of D-mannose, D-ribose, L-rhamnose, D-glucosamine, D-glucuronic acid, D-glucose, D-galactose, and D-arabinose, in a molar ratio of

1.54:0.50:3.83:1.56:1.33:3.39:23.69:15.26:8.86, respectively. *In vitro* assays to measure antioxidant activity indicated that DIPs exhibited strong radical scavenging capacities as well as a protective effect towards DNA damage. Moreover, DIPs possessed significant anti-inflammatory and immunomodulatory properties. These findings provide useful information for further applications of natural polysaccharides as potential antioxidant, anti-inflammatory, and immunomodulatory agents.

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