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Purification and characterization of a novel immunomodulatory lectin from *Artocarpus hypargyreus Hance*☆

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ABSTRACT

Lectins are proteins/glycoproteins of non-immune origin, which interact specifically and non-covalently with the carbohydrate moieties of respective receptors on the cell surface. In this study, a novel 65.2-kDa tetrameric lectin (AHL) was purified from *Artocarpus hypargyreus Hance* (*A. hypargyreus*) by affinity chromatography on a galactose-sepharose column. It is a glycoprotein with carbohydrate content of 6.91%. Its maximum haemagglutinating activity was maintained after incubation at a temperature range of 20–40 °C and pH range of 5.0–9.0. AHL-induced haemagglutination of erythrocytes was inhibited strongly by carbohydrates, such as methyl-galactose, methyl-mannose, and *N*-acetyl-D-galactosamine, indicating the existence of more than one carbohydrate binding sites in the AHL molecule. The AHL activity was gradually lost in the presence of urea and completely lost when being treated with ethylenediaminetetraacetic acid (EDTA). The immunomodulatory activity of AHL was assessed using human peripheral lymphocytes and rat peritoneal macrophages. AHL triggered proliferation and activation of human T lymphocytes and induced the release of Th1 cytokines, including IFN-γ, TNF-α and IL-6. Furthermore, AHL significantly stimulated the production of nitric oxide (NO) and pro-inflammatory cytokines, including TNF-α and IL-12, in rat peritoneal macrophages. However, AHL did not enhance proliferation of B cell-enriched rat splenocytes. Taken together, in this study, a novel immunomodulatory lectin was purified from *A. hypargyreus* and was found to be capable of inducing a Th1-type immune response, and thus, it may have potential immunoregulatory application in response to infections, immune diseases and cancer.

1. Introduction

Lectins are carbohydrate-binding proteins existing in many species. While these proteins are widely distributed in nature, plants are the most abundant source of lectins. Because of their carbohydrate-binding specificities, lectins are involved in important biological processes, such as tissue development, cell communication, embryogenesis, and pathogen recognition [1]. They show antiviral [2], insecticidal [3], antibacterial [4], antifungal [5], and anti-tumor activities [6]. In addition, some lectins also exert immunomodulatory effects [7–10], which are known to be initiated by their interaction with the glycan moieties present on the surface of lymphocytes and splenocytes, triggering signal transduction and resulting in cell proliferation, differentiation, migration, cytokine secretion, and immune responses against pathogen infection or tumor cells [11,12]. Thus lectins have been widely investigated for their mitogenic potential. As mitogens, binding of lectins to different glycoproteins on the cell surface could trigger multiple signal cascades, and induce T cell activation and proliferation [13,14]. Carbohydrate-binding specific mitogenic lectins are gaining much more interest and have been explored for various immunomodulatory and therapeutic applications [15–17].

The genus *Artocarpus* (*Moraceae*) comprises approximately 50 species, which are widely distributed in tropical and subtropical regions [18]. It is noted as an abundant source of flavonoids and other phenolic compounds with various biological activities [19]. It has been used as traditional folk medicine against inflammation, cirrhosis, hypertension, malarial fever, dysentery and tuberculosis and has been also used to control blood sugar level in diabetic patients [19,20]. *Artocarpus hypargyreus Hance*, (*A. hypargyreus*), an evergreen tree cultivated in South China, is a Chinese herb used for the treatment of headache, jaundice

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and rheumatism. However, to date, lectin activity in *A. hypargyreus* has not been evaluated and explored yet. Thus, the present investigation was carried out to purify and characterize novel lectin from *A. hypargyreus* (*Artocarpus hypargyreus* lectin, AHL) and to evaluate its mitogenic potential.

2. Materials and methods

2.1. Materials

The seeds of *A. hypargyreus* were collected and identified by Professor Liying Yu from Guangxi Botanical Garden of Medicinal Plants in August 2015. The voucher specimens were deposited in Guangxi Key Laboratory of Biological Molecular Medicine research

FITC labeled anti-CD3 and phycoerythrin (PE)-conjugated anti-CD25 were purchased from BD Biosciences (San Jose, CA, USA). Th1/Th2 cytokine ELISA kits were purchased from eBioscience (San Diego, CA, USA). Phytohaemagglutinin (PHA), lipopolysaccharide (LPS), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Sigma Aldrich, China). All the other chemicals and reagents used in this study were of analytical grade.

2.2. Purification of lectin from A. hypargyreus

The seeds of *A. hypargyreus* were grounded and extracted overnight at 4 °C in 0.01 M phosphate buffered saline (PBS)(pH 7.2), followed by centrifugation at 3000*g* and 4 °C for 10 min. Solid $(NH_4)_2SO_4$ was added to the obtained supernatant up to 60% saturation and the proteins were precipitated at 4 °C overnight. The precipitated proteins were centrifuged at 12,000*g* and 4 °C for 15 min, and then dissolved in PBS, followed by dialysis against PBS until free of NH_4^+ ions. The dialyzed product was loaded onto a 2.5 cm × 10 cm Galactose-Sepharose 6B column pre-equilibrated with 0.01 M PBS (pH 7.2) and washed with the same buffer. The column was eluted with PBS containing 0.2 M D-galactose at the flow rate of 1 ml/min. The absorbance was monitored at 280 nm (A_{280}). The fractions with higher hemagglutinating activity were collected, designated as AHL, dialyzed, and frozen for further study.

2.3. Determination of protein and carbohydrate contents

Protein concentration was measured by the standard Bradford assay [21] using bovine serum albumin as the control. Total neutral sugar content of AHL was measured using anthrone sulfuric acid assay with glucose as the reference according to a modified method [22].

2.4. Assay of hemagglutinating activity

Peripheral blood was obtained from healthy donors, and the study protocols were approved by Ethics Committee of Guangxi Medical University (Nanning, Guangxi, China). All the animal experiments were approved by the Animal Care and Use Committee of Guangxi Medical University. Hemagglutinating activity (HA) was determined using human, rat, mouse, cavy, duck, cattle, pig, goat and chicken erythrocytes. A serial 2-fold dilution of lectin (50 μl) was incubated with 2% (v/v) erythrocyte suspension (50 μl) at room temperature for 1 h. The unit of HA was defined as the highest dilution of lectin to promote the detectable agglutination of erythrocytes. Specific activity was defined as HA units per mg protein (mg/ml).

2.5. Gel filtration chromatography

AHL (480 μg) in 0.1 M PBS was loaded onto a Hiprep Sephacryl TM 16/60 S100 HR column (GE Healthcare, Sweden) with an ÄKTA prime system and the molecular weight of AHL was measured. The column was eluted with PBS at the flow rate of 1.0 ml/min, and monitored at 280 nm. Molecular mass standards, including phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were similarly chromatographed.

2.6. Polyacrylamide gel electrophoresis (PAGE)

The 15% (w/v) PAGE was carried out under acidic condition with the method from Reisfeld [23], followed by staining with Coomassie Blue R-250. The purity of fractions from affinity chromatography was determined by sodium dodecyl sulfate (SDS)-PAGE according to a modified method [24]. PAGE gels were stained with Coomassie Blue R-250.

2.7. Carbohydrate-binding specificity

In order to determine the specificity of AHL to carbohydrates, the inhibition of HA was performed with a modified method [25]. A serial 2-fold dilution of lectin (25 μl) was mixed with 25 μl of sugar solution (20 mmol/l) at room temperature for 30 min, and HA activity was tested as described above. Initial concentration of AHL was 0.4 mg/ml, and the HA was 128 U.

2.8. The pH and thermal stability

Lectin was mixed in buffers with different pH values ranging from 2 to 11 to evaluate its pH stability. The mixtures were kept at room temperature for 1 h and HA was then determined as mentioned above.

The temperature stability of AHL was analyzed by incubating lectin at different temperatures (20–100 °C) for 30 min, and HA was determined at room temperature as mentioned above. After incubation, the solutions were cooled to, and their hemagglutinating activity was determined.

2.9. Effects of urea and EDTA on lectin-induced hemagglutination activity

For detecting the effects of denaturants, AHL solution (0.4 mg/ml) in 0.1 M Tris-HCl buffer saline was incubated with 1–4 M of urea or 100 mM of EDTA at room temperature for 2 h. Then AHL solution was dialyzed against Tris–HCl buffer (pH 7.8) and subjected to hemagglutination assay as described above. AHL in the same buffer without denaturants was used as a control and its activity was considered to be 100%.

2.10. Cultivation of human peripheral T lymphocytes

Peripheral blood mononuclear cells were isolated from healthy donors by Ficoll density gradient centrifugation using standard procedures. Individuals were recruited during the period from June 2016 to February 2017. Selected individuals were informed about the study and invited to participate after consenting. Peripheral T lymphocytes were obtained by RosetteSep human T cell enrichment cocktail (Stemcell Technologies, Vancouver, Canada). The purified T cells (10^6 cells/ml) were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS (Gibco, Grand Island, N.Y., USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator. The protocol was approved by Ethics Committee of Guangxi Medical University.

2.11. Evaluation of cytotoxicity of AHL against T lymphocytes

T lymphocytes (1 × 10^6/ml) were seeded to 96-well plate and cultured in RPMI medium for 72 h in the absence and presence of AHL at various concentrations and phytohemagglutinin (PHA). Ten μl of 0.5 mg/ml MTT was added to each well and incubated for 4 h, and 100 μl of dimethyl sulfoxide (DMSO) was then added, followed by measuring the absorbance at 570 nm (A_{570}). In another experiment, the apoptosis of lymphocytes in response to AHL was also evaluated by fluorescence-activated cell sorting (FACS). T lymphocytes (2 × 10^6 cells/ml) were seeded into 6-well plates for 24 h in the presence of AHL at various concentrations, cells were collected and stained with Annexin V-FITC (BD Phamingen, San Jose, CA, USA) and propidium iodide (PI) at room temperature for 15 min. Percentages of apoptotic cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA).

2.12. Evaluation of T lymphocyte activation

T lymphocytes (1 × 10^6 cells/ml) were seeded into 6-well plates for 24 h in the presence of AHL and the control was cultured in presence of PBS. Cells were analyzed for two surface markers, i.e. CD3 and CD25, using anti-CD3-FITC andanti-CD25-PE. Flow cytometry data were analyzed by CellQuest-pro software on a FACS Calibur Becton-Dickinson flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.13. Measurement of levels of cytokines

Culture supernatants were collected from human T lymphocytes or rat peritoneal exudate cells stimulated with AHL or LPS for 24 h and the detected for secretion levels of IFN-γ, TNF-α, IL-12, IL-6, IL-10 and IL-4 using the corresponding ELISA kits according the manufacturer's instructions. All the experiments were performed in triplicate and were repeated three times.

2.14. Collection of rat peritoneal exudate cells (PECs)

Rat PECs were isolated from Wistar rats (male adult; 12 week-old; weighing 250–300 g) following the standard procedure [26]. The peritoneal macrophages (1 × 10^6 cells/ml) were cultured in the presence of AHL or LPS at various concentrations in a humidified incubator with 5% CO_2 at 37 °C for 24 h. All the animal experiments were approved by the Animal Care and Use Committee of Guangxi Medical University (Nanning, Guangxi, China).

2.15. Determination of nitric oxide (NO) release from rat PECs

NO is an important signaling molecule derived from L-arginine catalyzed by NO synthase. NO release can be used as an indicator of macrophage activation. The effect of AHL on the function of macrophages was assessed by measuring NO production. The amounts of NO released by rat PECs in culture supernatants in the presence of AHL or LPS were quantified by determining the stable end product, nitrite, by the Griess method [27]. 100 μl of cell culture supernatant and Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthylethyl)-enediamine in 2.5% H_3PO_4) were mixed well and incubated in a 96-well microtiter plate at 25 °C for 10 min. The absorbance at 540 nm (A_{540}) was measured in a microplate reader. NO concentration was determined using sodium nitrite as a standard.

2.16. Measurement of phagocytic activity

Phagocytosis assay was performed as described by Roy and Rai [28]. The collected rat PECs (1 × 10^6 cells/ml) were incubated with AHL at various concentrations on clean surgical slide for 90 min. Following activation, yeast cells with density of 1 × 10^8 cells/ml were added and kept for 90 min. Slides were then subjected to the differential Giemsa staining and observed under a microscope. At least 15 fields were counted for each slide. The phagocytic index was measured according to the average number of yeast cells engulfed or adhering to macrophages. The control phagocytic index was taken as 100% phagocytosis.

2.17. Effect of AHL on proliferation of rat splenocytes

All the animal experiments were approved by the Animal Care and Use Committee of Guangxi Medical University (Nanning, China). Wistar rats (250–300 g) were sacrificed by cervical dislocation. B cell-enriched rat splenocyte cell suspension was prepared and cell concentration was adjusted to 1 × 10^6 cells/ml in RPMI 1640 culture medium. A total of 100 μl of splenocyte suspension was seeded to 96-well plate along with the same amount of AHL or LPS at various concentrations. The cells were incubated at 37 °C in a humidified incubator with 5% CO_2 for 48 h. Then MTT reagent 20 μl (5 mg/ml) was added and incubated for 4 h. The MTT formazan crystals were dissolved in 100 μl of DMSO and A_{570} was measured with an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.18. Statistical analysis

All the data were analyzed with GraphPad Prism 3.0 and comparisons were analyzed by one-way or two-way ANOVA with Bonferroni's *post hoc* tests. $P < 0.05$ was considered to be of significant difference.

3. Results

3.1. Affinity purification of a lectin from A. hypargyreus

The crude extracts of *A. hypargyreus* seeds were divided into two fractions on a galactose-Sepharose affinity chromatography (Fig. 1). The extract was resolved into two peaks. Only fraction 2 with stronger

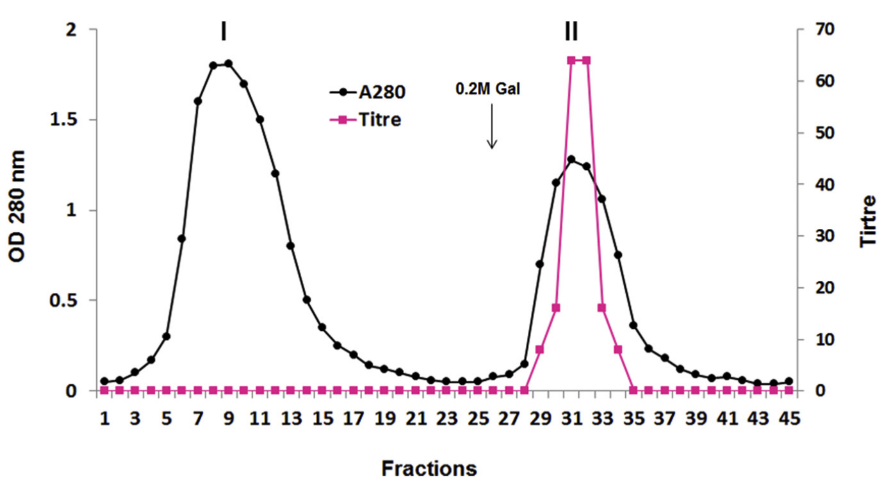

Fig. 1. Affinity chromatography of *AHL* on a galactose-Sepharose column. The arrow indicates the addition of 0.2 M D-galactose solution. Fraction II showed higher hemagglutinating activity. A total of 6.5 ml of fractions were collected and fractions 31–32 showed maximum haemagglutination titer. Each dot represents the average of three experiments.

Table 1
Purification of AHL and its hemagglutinating activity.

Fraction	Protein (mg)	Total activity (titer)	Specific activity (titer/mg)	Purification (fold)
Crude extract	1407	8510	6.05	1
60% $(NH_4)_2SO_4$	605	7280	12.03	1.98
Gal-Sepharose 6B	102	5280	51.76	8.56

The titer is the reciprocal of the highest dilution giving detectable agglutination of human O-type erythrocytes. Purification factor was based on the specific activity of the crude extract.

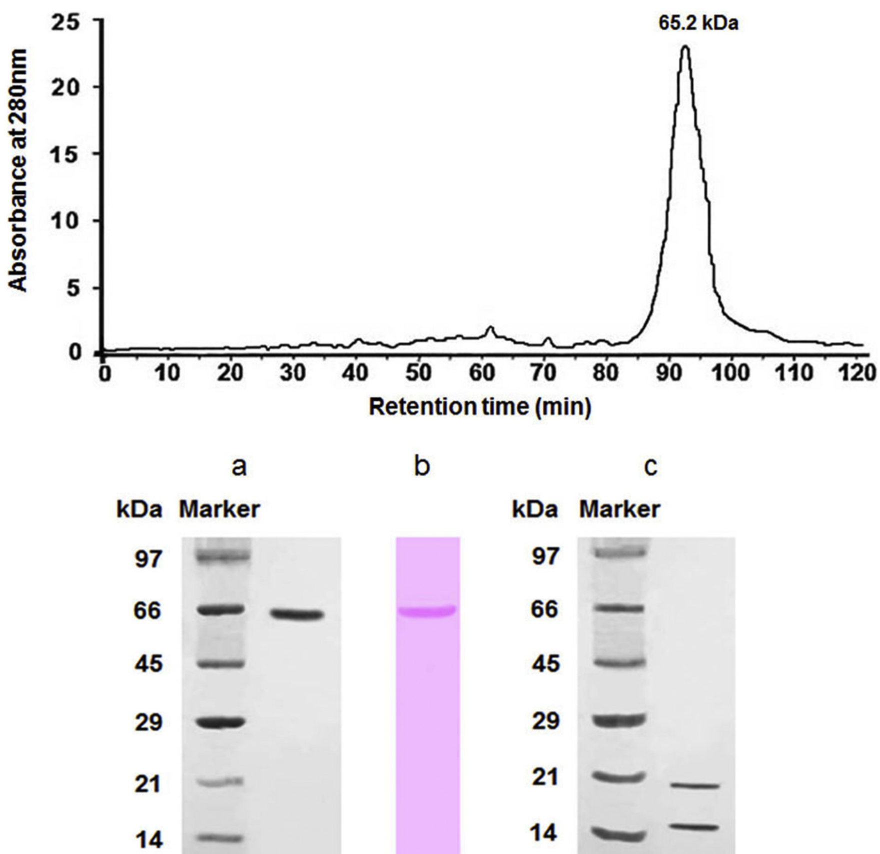

Fig. 2. Elution profile of AHL on gel filtration chromatography using a Hiprep 16/60 Sephacryl S-100HR column coupled to ÄKTA prime system. Total 480 μg of AHL was injected and eluted with 0.1 M PBS. The insets represent PAGE for native acidic proteins (a), stained by the periodic acid Schiff's reagent (b) and SDS-PAGE (c) of AHL.

HA was collected and dialyzed against 0.01 M PBS (pH 7.2), which was the purified lectin from *A. hypargyreus* seeds and designated as AHL. This process resulted in a purification factor of 8.56 (Table 1).

3.2. Structural characterization

AHL showed a single peak with a molecular weight of 65.2 kDa (Fig. 2). A single band was found under native acidic PAGE condition (Fig. 2a) without band under native basic PAGE condition. A brick-red band was observed after periodic acid-Schiff's staining, indicating that AHL is a glycoprotein (Fig. 2b). The sugar content of AHL was 6.91%, as measured by anthrone sulfuric acid method. SDS-PAGE of AHL showed two bands with molecular masses of 15 and 19 kDa, indicating that AHL is a tetramer with two different subunits (Fig. 2c).

3.3. Hemagglutination assay and carbohydrate specificity of AHL

AHL showed HA with most erythrocytes in the descending order of predilection as follows: human blood types B > A > O > AB = rat erythrocytes > mouse erythrocytes > cavy erythrocytes = duck erythrocytes = cattle erythrocytes > pig erythrocytes, but not against goat and chicken erythrocytes (Table 2).

When the concentration of carbohydrates was 20 mmol/L, the HA of AHL was partially inhibited by D-galactose, methyl-α-D-glucose, raffinose, D-mannose, and D-fructose, with inhibitory rates being 87.5%, 75%, 50%, 50% and 50%, respectively. HA was completely inhibited by methyl-alpha-D-galactose, methyl-alpha-D-mannose, and *N*-acetyl-D-galactosaminie. Inhibition by other carbohydrates was not found (Table 3), suggesting the complex sugar specificity of AHL.

Table 2
Hemagglutinating activity of AHL on different erythrocytes.

Types of erythrocytes	HA activity (titer/U)
Human blood types B	2^{12} (4096)
Human blood types A	2^{11} (2048)
Human blood types O	2^{10} (1024)
Human blood types AB	2^{9} (512)
Rat erythrocytes	2^{9} (512)
Mouse erythrocytes	2^{8} (256)
Cavy erythrocytes	2^{7} (128)
Duck erythrocytes	2^{7} (128)
Cattle erythrocytes	2^{7} (128)
Pig erythrocytes	2^{6} (64)
Goat erythrocytes	0
Chicken erythrocytes	0

Table 3
Effect of different carbohydrates on AHL (initial HA: 128 U).

Carbohydrate (20 mM)	HA activity (titer/U)
Methyl-α-D-galactose	0
Methyl-α-D-mannose	0
N-acetyl-D-galactosamine	0
D-galactose	16
Methyl-α-D-glucose	32
Raffinose	64
(D-Gal-α-(1 → 6)-D-GIc-β-(1 → 2)-D-Fru)	
D-mannose	64
D-fructose	64
D-glucose	128
N-acetyl-D-glucosamine	128
D-xylose	128
D-Fucose	128
L-Rhamnose	128
Arabinose	128
Lactose	128

Hemagglutinating activity (HA) was determined using cavy erythrocytes.

3.4. Effects of temperature, pH and denaturants on haemagglutination activity

The stability of AHL was evaluated at various pH values and temperatures by determining the remaining biological activity (hemagglutination). The HA of AHL was stable at temperature ranging from 0 to 40 °C. However, its activity was found to be decreased by 50% at 50 °C after 30 min and disappeared at and above 70 °C (Fig. 3a). As shown in Fig. 3b, optimal haemagglutination activity of AHL was observed at pH of 5–9. However, 32% and 64% activity was retained at pH 4 and pH 10, respectively. AHL lost above 95% of its activity in the presence of 1–4 M urea, and its activity was completely lost when being treated with EDTA.

3.5. AHL induces T lymphocytes proliferation and activation

Phytohemagglutinin (PHA), a lectin from *Phaseolus vulgaris*, was used as a T cell mitogen in vitro cell growth assays. To evaluate the effect of AHL on normal T lymphocytes, human peripheral T lymphocytes were isolated by RosetteSepAb cocktail, and the purified cells ($>$ 95% CD^{3+} T lymphocytes) were obtained. MTT assay showed that AHL promoted the significant proliferation of normal T lymphocytes in a concentration-dependent manner (Fig. 4a).

To further evaluate the cytotoxicity of AHL against normal T lymphocytes, the cells were incubated with AHL for 24 h, followed by analyzing PI and Annexin-V positive cells. As shown in Fig. 4b, there were no differences in the number of necrotic and apoptotic cells between the control and AHL-treated groups.

To determine whether AHL activates T lymphocytes, the expression of CD25 was assessed in T lymphocytes treated with AHL. As shown in Fig. 4c, the percentages of $CD3^+CD25^+$ T lymphocytes was increased significantly in response to AHL. CD25 expression rate in the group of AHL treatment (5 μg/ml), was 35%, and reached 68% in the group of 15 μg/ml of AHL treatment, indicating that AHL is capable of inducing T lymphocyte activation.

3.6. AHL induces secretion of Th1 cytokines

To explore AHL-mediated induction of cytokines in T lymphocytes, we analyzed the secretion levels of Th1/Th2 cytokines induced by AHL. IFN-γ, TNF-α, IL-6, IL-4 and IL-10 were analyzed by corresponding ELISA kits. As shown in Fig. 4d, IFN-γ, TNF-α and IL-6 were secreted at a higher level. In contrast, IL-4 and IL-10 were minimally secreted by T lymphocytes. These data suggested that AHL induces secretion of Th1 cytokines and stimulates a pro-inflammatory environment.

3.7. AHL activates rat peritoneal macrophages to release NO

NO is one of the important mediators in the inflammatory response. In this study, NO production levels by rat peritoneal macrophages incubated with AHL at different concentrations were shown in Fig. 5a. The released levels of NO in PECs treated with AHL at concentrations of 5 μg/ml and 15 μg/ml were significantly increased by 2.4–3.2-fold as compared to those of the untreated cells after 24 h. LPS also induced significant increase by 4.1-fold at 5 μg/ml concentration as compared to that of the control.

3.8. AHL promotes the secretion of IL-12 and TNF-α in rat PECs

The effect of AHL on the secretion of IL-12 and TNF-α in rat PECs is shown in Fig. 5b. AHL significantly increased the production of pro-inflammatory cytokines, showing an increase by 2.4–3.3-fold in IL-12 and an increase by 2.2–2.8-fold in TNF-α at concentrations of 5 μg/ml and 15 μg/ml compared to control. LPS, a known macrophage activator, also showed a similar effect on rat PECs at 5 μg/ml concentration, showing a 3.2-fold increase in IL-12 and 3.1-fold increase in TNF-α as compared to those of the control group.

3.9. AHL significantly stimulates the phagocytic activity of rat PECs

The effect of AHL on the phagocytic activity of rat PECs in engulfing yeast cells is shown in Fig. 5c. The phagocytosis of yeast cells by rat PECs was significantly increased by 2.1–2.5-fold ($p < 0.05$) in treatment with AHL as compared to that of the control group.

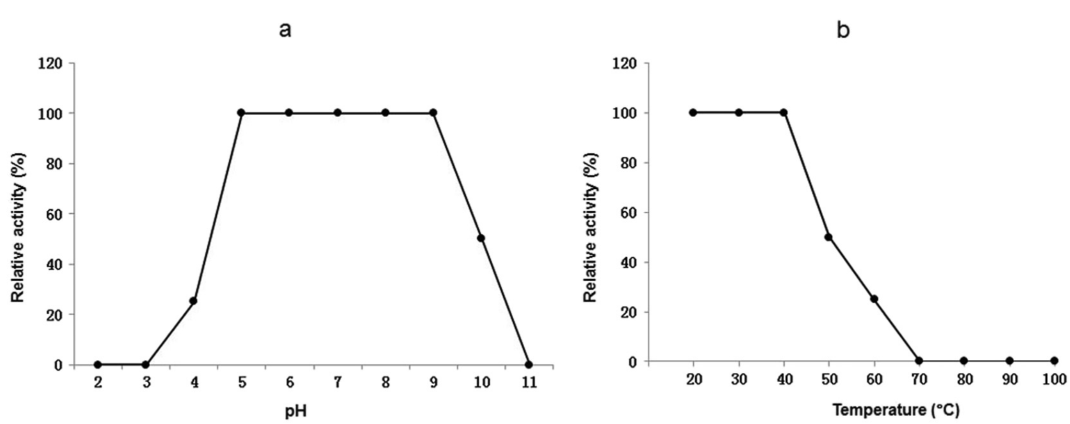

Fig. 3. Effects of pH (a) and temperature (b) on the hemagglutination activity of AHL. The figure shows the percentage activity in each case. Triplicate experiments have been performed, and the results were similar. The values represented one of the triplicate experiments.

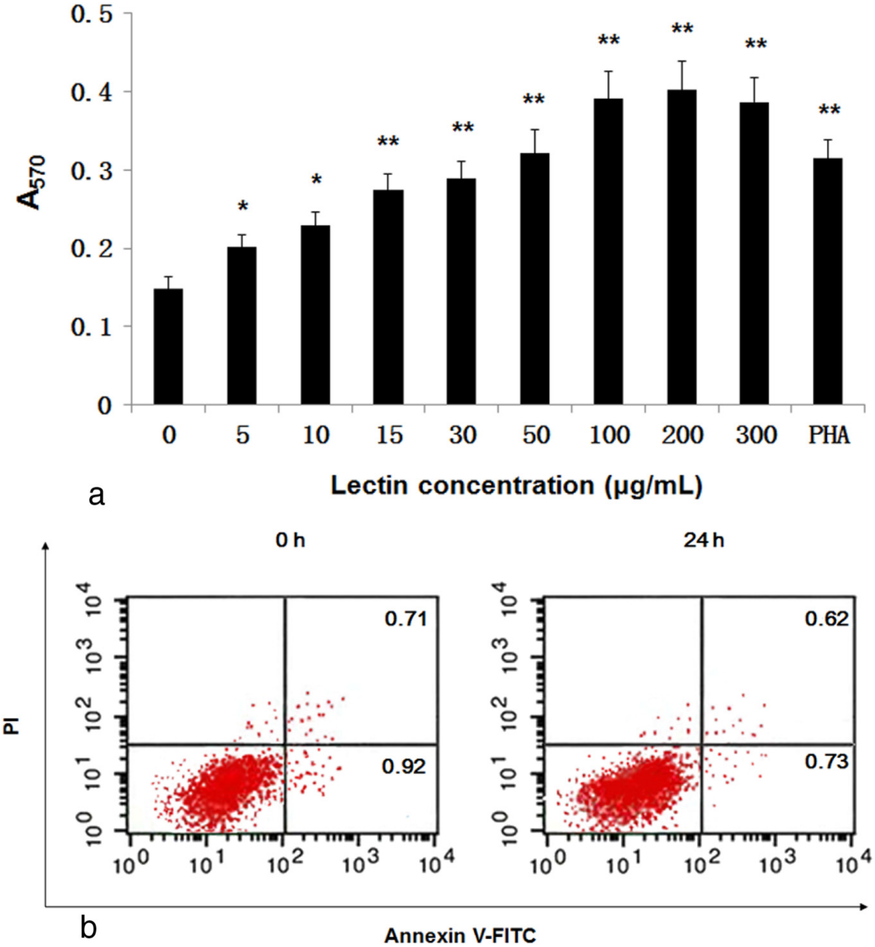

Fig. 4. AHL induces T lymphocytes proliferation and activation. A, Effects of AHL on proliferation of T lymphocytes. T lymphocytes were stimulated with AHL at designated concentrations and PHA (10 μg/ml) as described above for 72 h. The proliferation was measured by MTT assay. The OD_{570} values were expressed as mean ± standard deviation (M ± SD)($n = 3$). $*p < 0.05$ and $**p < 0.01$, vs. untreated cells. B, T lymphocytes were incubated with AHL (15 μg/ml) for 24 h, followed by flow cytometry analysis. C, Effect of AHL on CD25 expression in T lymphocytes. T lymphocytes were incubated in AHL at the indicated concentrations for 24 h. Expression of CD25 in $CD3^+$ T lymphocytes was analyzed by FACS. $**p < 0.01$, vs. untreated cells. D, AHL-induced secretion of Th1 cytokines. T lymphocytes were incubated with AHL at different concentrations for 24 h. Culture supernatants were analyzed for the levels of IFN-γ, TNF-α, IL-12, IL-4, and IL-10 using ELISA kit. The values are mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$, as compared with those of untreated cells.

3.10. Mitogenic activity of AHL in murine splenocytes

To examine the general effect of AHL on immune cells, we analyzed the proliferation of murine splenocytes in the presence of AHL at different concentrations. The effects of AHL and LPS on B cell-enriched rat splenocytes are shown in Fig. 6. LPS, a B cell mitogen, significantly induced the cell proliferation ($p < 0.05$) by 2-fold at 5 μg/ml. However, AHL showed only a slight increase in proliferation, which is not significant as compared to that of the control.

4. Discussion

Plant lectins are known for their significant potentials in medical and clinical applications [29]. This study presented a novel lectin in seeds of *A. hypargyreus*, a moraceae with important applications in traditional Chinese folk medicine. Most lectins are extracted using low ionic strength solutions [30]. Similarly, our results showed that AHL could be extracted in saline solution (0.01 M PBS) as well.

As the HA activity of the extract is inhibited by galactose, galactose was chosen to isolate AHL. The highest specific HA activity of AHL can be achieved after Galactose-Sepharose 6B chromatography when compared to the crude extract. The homogeneity of AHL was shown under the acidic PAGE condition.

The affinity chromatography provided a single peak with a molecular mass of 65.2 kDa. The result is comparable to other known lectins [14,31]. AHL revealed two bands as 19 kDa and 15 kDa on SDS-PAGE under reducing or non-reducing conditions. These results suggest that AHL is a tetrameric glycoprotein that is consisted of two subunits with molecular masses of 19 and 15 kDa, respectively. Almost all the plant lectins are multimeric proteins, and this heterodimeric subunit structural nature is in agreement with the previously reported lectins [14,32]. AHL is stable up to 40 °C and its activity is lost completely at 70 °C. This property is also similar to many reported lectins [14,33]. In addition, AHL is stable in solutions with pH ranging from 4.5 to 9.5. Highly stable lectins from *Lotus corniculatus*, *Phaseolus vulgaris*, and *Fusariumsolani* under extreme pH condition have been reported [23,34]. AHL was a glycoprotein containing 6.91% of neutral sugar. Many plant lectins usually contain around 5% of neutral sugars, although some exceptions are also reported. For instance, *Kaempferia rotunda* lectin and potato lectin were found to contain 31% and 50% sugar, respectively [35,36].

Lectins are a group of proteins with specific sugar-binding specificity. Their multiple functions are tightly correlated with carbohydrate-binding types, and their activities are usually suppressed by mono- and oligo-saccharides [37]. Although HA has been tested with several kinds of erythrocytes (A, B, O, AB, mouse, cavy, duck, cattle and pig), AHL

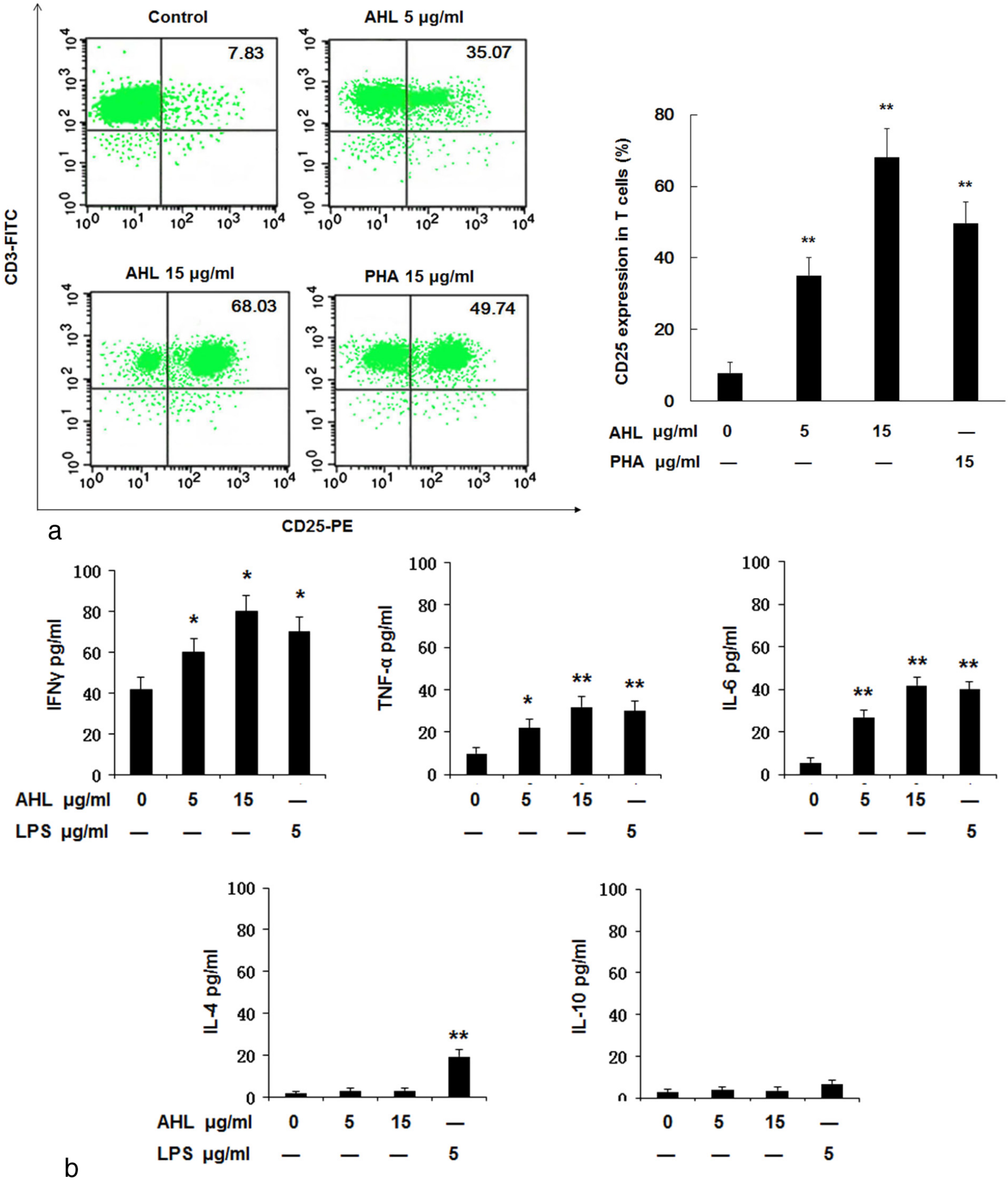

Fig. 4. (*continued*)

exhibits a preference for human B-type, possibly due to the discrepant oligosaccharides on surface of different blood cells. It is well known that hemagglutinating activity of lectins is correlated with the high binding specificity to oligosaccharides. Our study showed that the AHL activity could be suppressed by carbohydrates, such as methyl-alpha-D-galactose, methyl-alpha-D-mannose, *N*-acetyl-D-galactosamine, D-galactose, methyl-glucose, raffinose, D-mannose, and D-fructose, which possibly impede the binding of lectins with the plasma membrane. Owning to the first four kinds of sugar that showed the similar inhibition, we further clarified the inhibitory effects by using the lower concentration to test the agglutination and found that when the concentration of carbohydrates was 5 mmol/L, the HA of AHL was partially inhibited by methyl-alpha-D-galactose, *N*-acetylgalactosamine, methyl-alpha-D-mannose, with inhibitory rates being 87.5%, 75%, and 50%, respectively. However, the inhibition by D-galactose was not found.

Lymphocytes play a key role in cell defense and can be activated by binding to antigens or mitogens on receptors on their surface. Binding of lectins to different glycoproteins on cell surface can trigger multiple signaling cascades, and induce the activation, migration and apoptosis of T lymphocytes [38]. Immunomodulatory lectins, such as phytohaemagglutinin (PHA) from *Phaseolus vulgaris*, Concanavalin A (Con A) from *Canavalia ensiformis*, *Lens culinaris* agglutinin (LCA) from *Lens culinaris*, *Pisum sativum* lectin (PSA) from *Pisum sativum*, Antimicrobial potential of *Alpinia purpurata* lectin (ApuL) from *Alpinia purpurata* and

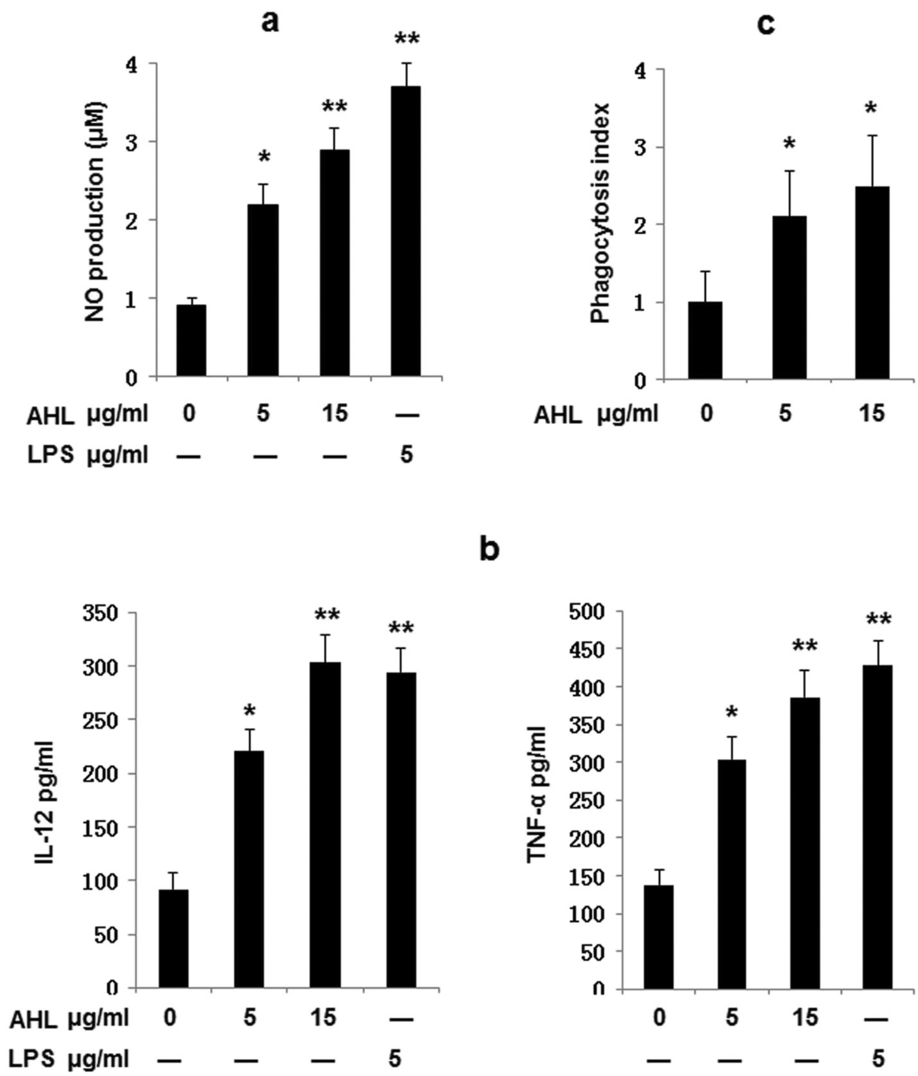

Fig. 5. AHL activates rat peritoneal macrophages. a, Effect of AHL on NO release by rat PECs in vitro at various concentrations for 24 h. NO production was determined using Griess reagent. Each value represents the mean ± S.D. * $p < 0.05$, ** $p < 0.01$ vs. control. b, the released levels of pro-inflammatory cytokines in rat PECs at various concentrations of AHL after 24 h incubation. Each value is represented as mean ± S.D. * $p < 0.05$, ** $p < 0.01$ vs. control. c, Effect of AHL on the phagocytic activity of rat PECs. The phagocytic index for control is taken as 1, which represents 100% phagocytosis. Each value represents the mean ± S.D. * $p < 0.05$ vs. control.

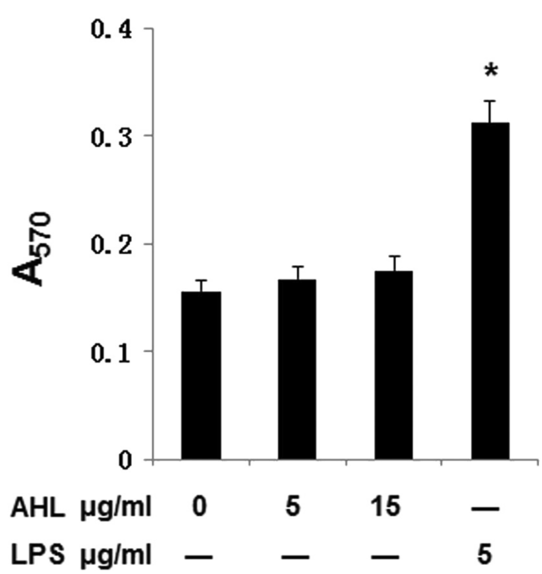

Fig. 6. Effect of AHL on the proliferation of B cell-enriched rat splenocytes after 24 h incubation. The proliferation was measured by MTT assay. The OD_{570} values were represented as mean ± S.D. ($n = 3$). * $p < 0.05$ vs. control.

Amaranthus caudatus agglutinin (ACA) from *Allium cepa* could induce Th1 immune response in murine splenocytes by promoting the secretion of IL-12 and IFN-γ [39–41]. Similar to these lectins, AHL could induce T cell activation and proliferation, and stimulate secretion of cytokines. AHL is capable of inducing Th1 immune response by increasing the levels of pro-inflammatory cytokines, including IFN-γ, TNF-α and IL-6. To ensure no LPS contamination in the AHL sample, we determined the LPS level with ELISA, which showed that LPS levels were very low (0.187 ± 0.028 ng/ml). IFN-γ is the main Th1 cytokine responsible for macrophage activation and differentiation as well as NO production [42]. Other lectins, such as *Canavalia brasiliensis* (ConBr) lectin from *Canavalia brasiliensis*, crab Charybdis feriatus lectin (CFL) from *Cratylia argentea*, and *Microgramma vacciniifolia* frond lectin (MvFL) from *Microgramma vacciniifolia* induced the production of IFN-γ and NO in peripheral blood mononuclear cells [7,10], while Cramoll from *Cratylia mollis* stimulated the secretion of IFN-γ in PECs [9] and mouse splenocytes [43]. In addition to IFN-γ, AHL inducd-Th1 response was reinforced by the stimulated secretion of TNF-α and IL-6. TNF-α exerts an important function in immunoregulatory response in activated macrophages, promoting NO production. IL-6 is a pleiotropic cytokine and functions in cell proliferation, survival, differentiation, migration, inflammation, and metabolism [44]. However, IL-6 may coordinate pro-inflammatory or anti-inflammatory responses based on the environmental conditions [45]. AHL did not induce a Th2 immune response without alteration of IL-4 and IL-10 production. AHL did not promote proliferation of B cell-enriched rat splenocytes and may not be a B-cell mitogen.

Jacalin, isolated from the seeds of jackfruit *Artocarpus integrifolia*, is a potent polyclonal activator for human lymphocytes and mostly T cells [46] but it does not induce B lymphocyte differentiation [47]. Both *Artocarpus integrifolia* and *Artocarpus hypargyreus Hance* are tropical and subtroical plants, belonging to *Moraceae Artocarpus.* However, their crown, fruit, the size and shape of seed are quite different. Comparing AHL with jacalin [48,49], there are similarities: (1) They both are glycoprotein and a tetramer with non-covalently bound subunits (AHL consists of 15 and 19 kDa subunits while jacalin consists of 15 and 18 kDa); (2) Both have similar molecular weight (AHL, 65.2 kDa, jacalin 65 kDa); (3) Their agglutination activities are inhibited by methyl-α-D-galactose, *N*-acetyl-D-galactosamine, methyl-α-D-mannose, D-galactose, and EDTA; and (4) both can activate T lymphocytes but have no effect on B lymphocytes. However, their difference is that AHL shows a relatively broad optimal pH range (5.0–9.0) as compared to that for jacalin (5.0–6.0).

Innate immunity is mediated by macrophages, dendritic cells, natural killer (NK) cells and others as the first line of host defense against microbial pathogens. Macrophages also act as the bridge between innate and adaptive immune responses. The activation of macrophages is usually evaluated by NO production and the increased phagocytic activity. NO is a cytotoxic effector against pathogens and tumor cells and plays a key role in immune modulation [50]. ArtinM from *Artocarpus heterophyllus* was found to activate various immune cells. Particularly, ArtinM activated macrophages through TLR-2 signal and induced Th1 immune response by stimulating IL-12 secretion [51]. ACA from *Allium cepa* activated rat peritoneal macrophages and enhanced production of NO and pro-inflammatory cytokines (TNF-α and IL-12) [42]. In this study, AHL activated macrophages in rat PECs as evident by promoting NO production and the secretion of IL-12 and TNF-α cytokines, indicating that AHL induces the macrophage proinflammatory responses, which eventually facilitates Th1 immune response in T lymphocytes. It would be interesting to further explore the immunomodulatory properties of AHL and the related molecular mechanism.

In conclusion, AHL isolated from *A. hypargyreus* is a novel tetrameric lectin with 65.2-kDa molecular weight. It shows lymphoproliferative activity and induces a Th1 immune response. However, it did not enhance the proliferation of B cell-enriched splenocytes. AHL induces the proinflammatory response and promotes phagocytosis in macrophages. Thus, AHL may serve as a potential immunomodulator to modulate the inflammatory response in immune dysfunctions.

Conflicts of interest

The authors have no conflicts of interest to disclose.

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