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A Lectin with Anti-HIV-1 Reverse Transcriptase, Antitumor, and Nitric Oxide Inducing Activities from Seeds of *Phaseolus vulgaris* cv. Extralong Autumn Purple Bean

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Lectins/hemagglutinins are a class of sugar-binding proteins which agglutinate cells and/or precipitate glycoconjugates. They occur widely in plants but manifest significant differences in activities, which means only a few of them own exploitable potentials. The objective of this study was to find and characterize a multifunctional plant lectin with high potential values in food chemistry and medicine. A 60-kDa lectin from *Phaseolus vulgaris* L. cv. Extralong Autumn Purple Bean (EAPL) was purified by liquid chromatography, and the sequence of its first 20 N-terminal amino acids was ANEIYFSFQRFNETNLILQR. It was galactose-specific and manifested hemagglutinating activity toward erythrocytes of rabbit, rat, mouse, and human ABO blood types. EAPL manifested anti-HIV-1-RT activity, and it could inhibit the proliferation of human tumor cells by inducing the production of apoptotic bodies. The nitric oxide-inducing activity of EAPL may find application in tumor therapy.

KEYWORDS: *Phaseolus vulgaris* cv. Extralong Autumn Purple Bean; purification; lectin; anti-HIV; antitumor; nasopharyngeal carcinoma; nitric oxide; apoptotic body

INTRODUCTION

More than a century ago, evidence started to accumulate for the presence of a class of proteins possessing erythrocyte-agglutinating and carbohydrate-specific binding characteristics, which were then named as hemagglutinins or lectins (*1*). Since the first lectin, ricin, was isolated from castor beans in 1888, a large number of lectins have been purified. Even until now, most of the lectins are still extracted from plants, and much fewer are from other origins, including animals and microorganisms (*2*). In addition to specific sugar-binding and cell-agglutinating activities, plant lectins (phytohemagglutinins) have been reported with many other functions, such as mitogenic, cytokine inducing, antihuman immunodeficiency virus (HIV)-1 reverse transcriptase (RT), antifungal, antitumor, and even overcoming self-incompatibility in plants such as *Petunia hybrida* and *Eruca sativa* (*1*−*4*). Furthermore, recent studies disclosed that some lectins induce secretion of nitric oxide (NO), which may partially contribute to their antitumor abilities (*5*, *6*).

Based on their specific sugar-binding characteristics, lectins are widely applied in biotechnological and medical regimens (*1*). A successful example is *Bauhinia purpurea* agglutinin (BPA), which is a multifunctional tool used to probe the structural and functional roles of cell surface sugars, to serve as a marker for identification of cell types and health conditions, and to serve as a tool for cell separations (*7*). Furthermore, *Amaranthus caudatus* lectin and peanut agglutinin are widely used as histochemical probes for their differential recognition mechanisms in tissue recognition (*8*). The biotechnological applications of lectins are also extended to glycoproteomic analysis of embryonic stem cells, clinical bone marrow transplantation, and other investigations (*9*, *10*). For medical therapy, a novel lectin actinohivin exhibits potent anti-HIV activity against various strains of T-tropic and M-tropic HIV-1 and HIV-2 (*11*). Recently, its anti-HIV mechanism has been elucidated. Actinohivin can block HIV binding and entry into susceptible cells due to multivalent interaction of the three sugar-binding pockets with three high-mannose type glycans of HIV envelop gp120 via the "cluster effect" of lectin (*11*).

Belonging to the same family of Fabaceae as soybean, the common bean (*Phaseolus vulgaris* L.) is an herbaceous annual plant grown widely all over the world and represents 50% of legumes for human consumption (*12*). As a world major producing center of common beans, China owns a cultivation history of over 400 years, and more than 200 landacres are planted in the country (*12*). *P. vulgaris* L. cv. Extralong Autumn Purple Bean is one of the popular cultivars. In this study, a 60-kDa dimeric lectin (EAPL) was isolated from this cultivar by liquid chromatography. The biochemical and functional activities of EAPL elucidated here unveiled the exploitable potentials in the fields of biotechnology and medicine.

MATERIALS AND METHODS

Materials and Reagents. Dried seeds of Extralong Autumn Purple Beans were purchased from a vendor in Mainland China and identified by

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Professor Shiu-Ying Hu, Honorary Professor of Chinese Medicine, The Chinese University of Hong Kong (CUHK) (*13*). Blue-Sepharose, Q-Sepharose column (Fast Flow), Mono Q5/50 GL column, and Superdex 75/300 GL column were obtained from GE Healthcare, China. BALB/c mice were provided by the Laboratory Animal Services Center of CUHK and handled following appropriate ethical recommendations from the CUHK Animal Research Ethics Committee. Human blood (A, B, O, and AB types) was donated by healthy volunteers, and the blood type was confirmed by clinical tests. All fungi used in this study were provided by Professor He-Xiang Wang from the Department of Microbiology, China Agricultural University, China. Lysis buffer was prepared by dissolving 8.29 g of NH_4Cl, 1.002 g of $NaHCO_3$, and 29.2 mg of EDTA in 1 L of deionized water. The solution was adjusted to pH 7.2 and sterilized by filtering through a 0.22-μm sterile filter. Griess reagent was composed of 1% (w/v) sulfanilamide and 0.1% (w/v) naphthalene-ethylenediamine dihydrochloride in 5% (v/v) H_3PO_4. All other reagents used were from Sigma (USA) unless otherwise mentioned.

Purification of Lectin from Extralong Autumn Purple Beans. First, 400 g dried seeds were soaked overnight in deionized water at 4 °C and then extracted thoroughly using a blender (10 mL of deionized water/g of sample), followed by centrifugation at 20000 *g* at 4 °C for 30 min. The supernatant was filtered through filter paper and then applied to a Blue-Sepharose column (18 cm × 5 cm) which had been pre-equilibrated with 20 mM Tris-HCl (pH 7.6) buffer. Unbound fraction was eluted with the same buffer. The bound fraction with hemagglutinating activity was then eluted from the column with 1 M NaCl in the same buffer. After exhaustive dialysis against deionized water at 4 °C, lyophilization, and dissolving in 20 mM NH_4HCO_3 buffer (pH 9.4), the Blue-Sepharose bound fraction was loaded on a Q-Sepharose column (18 cm × 5 cm) previously equilibrated with 20 mM NH_4HCO_3 buffer (pH 9.4). The column was washed with the equilibration buffer to elute the unbound fraction until OD_{280} reached the baseline. The adsorbed fraction was then eluted from the column with 1 M NaCl in 20 mM NH_4HCO_3 buffer (pH 9.4). After exhaustive dialysis against deionized water at 4 °C, lyophilization, and then dissolving in 20 mM NH_4HCO_3 buffer (pH 9.4), the Q-Sepharose bound fraction was applied to a Mono Q 5/5 GL column and eluted with two linear concentration gradients of NaCl (0−0.2 M and then 0.2−1 M). Fraction MQ-II eluted with the second gradient was then dialyzed, lyophilized, and chromatographed on a Superdex 75 10/300 GL column in 0.2 M NH_4HCO_3 buffer (pH 9.4). The purified lectin (second peak SUP-II) was dialyzed extensively, freeze-dried, and stored at −20 °C for further studies.

Purity and Molecular Mass Determination. Purity was ascertained by size exclusion chromatography of the isolated protein (2 mg/mL in 0.2 M NH_4HCO_3 buffer, pH 9.4) on a Superdex 75 10/300 GL column previously equilibrated and then eluted with the same buffer. For molecular mass determination, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE, 15% polyacrylamide gel) was carried out under reducing conditions. After electrophoresis (75 V for 150 min), the gel was stained with 0.1% Coomassie Brilliant Blue G for 30 min followed by destaining in 10% acetic acid. The low molecular mass calibration kit from GE Healthcare used is a mixture of six proteins, including phosphorylase b (97-kDa), bovine serum albumin (66-kDa), ovalbumin (45-kDa), carbonic anhydrase (30-kDa), soybean trypsin inhibitor (20-kDa), and α-lactalbumin (14.4-kDa). In addition, molecular mass was also determined by FPLC-gel filtration on a precalibrated Superdex 75 10/300 GL column using an AKTA Purifier (GE Healthcare).

Determination of N-Terminal Amino Acid Sequence. N-Terminal amino acid sequence analysis was performed using an HP 1000A Edman degradation unit and an HP 1000 HPLC system (Hewlett-Packard) after SDS-PAGE and membrane transfer procedures. Alignment work was done by using the software CluxtalX 1.83 and the on line BOXSHADE 3.21 server.

Assay of Hemagglutinating Activity and Inhibition by Sugars. A serial 2-fold dilution of the lectin solution (50 μL/well in 1 × PBS) in a Nunclon 96-well plate was mixed with 50 μL of 2% resuspended rabbit erythrocytes and incubated for 1 h at room temperature until the blank containing only red cells had fully sedimented. As previously defined (*14*), one hemagglutinating unit is the reciprocal of the highest dilution exhibiting hemagglutination activity, and specific activity is the number of units per milligram of lectin. The agglutination profiles of the lectin toward erythrocytes of other origins were similarly determined.

For the assay of the inhibitory activity of sugars on lectin-induced hemagglutination, 50 μL of a serial two dilution of a sugar (2-fold dilution to achieve a final concentration from 0.78 to 200 mM in PBS) was mixed with the same volume of the lectin solution (256 units/50 μL). After incubation for 30 min, the remaining hemagglutinating activity was measured. Similarly, to test the ability of various cations to restore hemagglutinating activity, the lectin solution (256 units/50 μL) was predialyzed at 4 °C overnight exhaustively against deionized water containing 10 mM ethylenediaminetetraacetic acid (EDTA) and the remaining hemagglutinating activity was tested. After 50 μL of each cation (final concentrations from 0.039 to 20 mM) had been added to the same volume of EDTA-treated lectin solution followed by incubation at room temperature for 30 min, hemagglutinating activity was determined (*5*). Minimum inhibitory concentrations of sugars and minimum recovery concentrations of cations were calculated.

Thermal and pH Stability. A 20 μL aliquot of a 1 mg/mL solution of the isolated lectin in 20 mM Tris-HCl buffer (pH 7.6) was exposed to various temperatures (0−100 °C) for 10 min by using a PCR machine and then cooled down to 0 °C before testing for residual hemagglutinating activity. To measure pH stability, lyophilized lectin was dissolved in buffer at a specified pH (from pH 2 to 12) to a final concentration of 1 mg/mL and left to stand at room temperature for 0.5 h. The buffers used included sodium citrate (pH 2−4), sodium acetate (pH 5), sodium phosphate (pH 6−7), Tris-HCl (pH 8), sodium bicarbonate (pH 9−10), and sodium hydroxide (pH 11−12). Remaining hemagglutinating activity was calculated as percent of the value at 30 °C or pH 7 for thermal stability or pH stability (*15*).

Assay of HIV-1 Reverse Transcriptase (HIV-1 RT) Inhibitory Activity. The assay was conducted using an HIV-1-RT ELISA kit, following the instructions of the manufacturer (Boehringer Mannheim, Germany). The assay is based on the mechanism that HIV-1 RT can synthesize DNA, commencing from the template/primer hybrid poly(A) oligo(dT). The inhibitory activity of the test protein was calculated as percentage inhibition compared with the control without any protein added (*3*). Pinto bean lectin with anti HIV-1-RT activity was chosen as a positive control (*16*).

Cell Lines and Cell Culture. The human nasopharyngeal carcinoma (NPC) cell lines CNE-1, CNE-2, and HNE-2 were purchased from the Sun Yat-sen University of Medical Sciences, Guang Zhou, China. Transformed human nasopharyngeal epithelial cell line NP 69 was generously provided by Prof. S. W. Tsao (Department of Anatomy, The University of Hong Kong). The human hepatocellular carcinoma Hep G2, human breast tumor MCF-7, and mouse leukemia L1210 cell lines were from the American Type Culture Collection, USA. All six tumor cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). NP 69 cell line was cultured in Keratinocyte-SFM medium (Gibco) plus supplements for Keratinocyte-SFM (1 × 2.5 μg of human recombinant epidermal growth factor and 1 × 25 mg of bovine pituitary extract, Gibco) (*17*). All cell lines were maintained at 37 °C in a humidified incubator under an atmosphere of 95% air and 5% CO_2.

Assay of Antiproliferative Activity. The MTT ([4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) test was used to monitor inhibition of cell growth as reported elsewhere (*3*). After the cells had grown to 90% confluence, the cells were trypsinized and seeded to 96-well flat-bottomed plates at a density of 5000 cells in 100 μL per well. After incubation for 8 h at 37 °C, the medium was carefully aspirated and cells were treated with a serial 4-fold dilution of the lectin at a final concentration from 0.25 μM to 1000 μM for 24 or 48 h. At the end of each incubation interval, the medium was removed, and 25.0 μL of fresh medium containing 5 mg/mL MTT solution was spiked into each well, followed by incubation for another 2 h at 37 °C. After careful removal of the medium, 150 μL of dimethyl sulfoxide was added to each well to solubilize the formazan product, followed by agitation on an orbital shaker for 5 min. The absorbance at 595 nm was measured by using a BIO-RAD microplate reader.

Assay of Apoptosis. Apoptosis was observed by chromatin staining with Hoechst 33342 (Sigma, USA), as described elsewhere (*18*). Tumor cells were first incubated with the lectin at different concentrations. After 24 h, the medium was discarded and cells were rinsed three times with PBS and exposed to Hoechst 33342 (1 μM in PBS) for 10 min at room temperature. After aspiration of the labeling medium, cells were rinsed

three times in PBS. Morphological changes of the cells were examined under ultraviolet illumination with a NIKON TE2000 microscope (Nikon, Japan).

Assay of Nitric Oxide (NO) Production. The assay was conducted as described before (*5*). Briefly, male BALB/c mice (25−35 g) were each given 1 mL of sterile 3% (w/v) aqueous thioglycolate by intraperitoneal injection to stimulate macrophage formation. The mice were sacrificed by cervical dislocation 3 days later. The peritoneal cavity was exposed, and peritoneal macrophages were collected by lavaging with PBS and then centrifugation at 1600 rpm for 3 min. After discarding the supernatant, lysis buffer was added to the cell pellet to destroy erythrocytes. The cells were washed with PBS and resuspended in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were seeded in a 96-well culture plate (2×10^5 cells/well) and left for adherence for 1 h before careful removal of the supernatant. Two hundred microliters of complete RPMI 1640 medium containing different concentrations of the lectin (0.13 to 4.12 μM final concentrations) were added to each well and incubated with the macrophages for 24 h. NO was determined by measuring the amount of nitrite in the cell culture supernatant. After brief centrifugation, a 100 μL aliquot of cell-free culture medium from each well was allowed to react with 50 μL of Griess reagent for 10 min. The absorbance at 540 nm was measured by using a BIO-RAD microplate reader, and the NO concentrations were calculated by using a standard calibration curve prepared by using different concentrations (1.5−200 μM) of sodium nitrite dissolved in the same medium. Lipopolysaccharide (LPS, final concentrations from 7.8 to 250 μg/mL) was used as a positive control. Polymyxin B sulfate was used as a specific LPS inhibitor (final concentration 10 units/mL). Dexamethasone (final concentration 10 μM) was used as a specific inhibitor of inducible nitric oxide synthase (iNOS).

Assay of Reverse Transcription Polymerase Chain Reaction (RT-PCR) for Detection of the mRNA Level of iNOS. First, mouse macrophages (2×10^5 cells/well) were treated with 0.1 μM EAPL, 2 μM EAPL, 1.25 μg/mL LPS, and 25 μg/mL LPS separately for 12 h, and total RNAs of different groups were then extracted using TRIzol Reagent (Invitrogen, USA). Second, 1 μg of total RNA was reverse transcribed with an oligo dT primer in a 10 mL reaction volume using a PrimeScript first strand cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's instructions. Third, PCR was conducted by using an AmpliTag Gold kit (Applied Biosystems Company). A PCR mixture was added to the RT product to make up a 50 μL reaction mixture containing Gold buffer, 2.5 mM $MgCl_2$, 200 μM dNTP, 0.5 μM forward and reverse primer (forward, 5′-CCG GCA AAC CCA AGG TCT AC-3′; reverse, 5′-CTC ACC ATT ATC TTT ACT CAG TG-3′), 1.25 U AmpliTag Gold. PCR was performed for 25 cycles, and a 354 bp PCR fragment was produced. A 452 bp GAPDH gene was used as a background control (forward, 5′-ACC ACA GTC CAT GCC ATC AC-3′; reverse, 5′-TCC ACC ACC CTG TTG CTG TA-3′). A 25 μL PCR product of each group was electrophoresed on a 2% agarose gel containing 0.1 μg/mL ethidium bromide. The gel picture was captured using a gel documentation system (Bio-Rad, USA). Results were quantitated using the software Alphaimager 2200 for semiquantitative analysis (*5*).

Assays of Other Activities. Assays of other activities, such as antifungal activity and ribonuclease activity, were carried out as described earlier (*3*, *19*). Fungi including *Mycosphaerella arachidicola*, *Fusarium oxysporum*, *Helminthosporium maydis*, *Valsa mali*, *Rhizoctonia solani*, *Alternaria solani*, *Setosphaeria turcica*, *Bipolaris maydis, Pythium aphanidermatum*, *Verticillium dahliae*, and *Fusarium solani* were used in this study.

Statistical Analysis. Results were collected from three independent experiments performed in triplicate, and data are expressed as means ± standard deviation. For between-group comparisons, Student's *t*-test or one-way ANOVA was used as appropriate, and differences are considered significant at $p < 0.05$.

RESULTS

Lectin Isolation from Extralong Autumn Purple Beans. The lectin in the seeds of Extralong Autumn Purple Bean was adsorbed on Blue Sepharose, Q-Sepharose, and Mono-Q columns. A crude extract of the seeds was first submitted to chromatography on Blue Sepharose. The Blue Sepharose-bound fraction eluted with 1 M NaCl was subsequently applied to a Q-Sepharose column followed by a Mono-Q column using an FPLC system, resulting in two peaks. Peak MQ-II from the Mono-Q column with hemagglutinating activity was in turn chromatographed on a Superdex 75 10/300 GL column, and the second peak (SUP-II) constituting purified lectin, hereinafter referred to as EAPL, was harvested (**Figure 1**). The purity of EAPL was confirmed by a single sharp absorbance peak after gel filtration on Superdex 75 (data not shown) and a single band in SDS-PAGE (**Figure 2**). The purification procedure is summarized in **Table 1**.

Biological and Biochemical Characterizations of EAPL. Its molecular mass was ascertained by SDS-PAGE and gel filtration (**Figure 2**). SDS-PAGE revealed that the lectin preparation was free of contaminants and appeared as a single 30-kDa band under reducing conditions. Gel filtration on Superdex 75 10/300 showed that the mass of EAPL was 60.6 kDa. Hence, the data suggest that EAPL was a dimeric lectin containing intramolecular disulfide bonds. The sequence of the first 20 N-terminal amino acids was ANEIYFSFQRFNETNLILQR. Sequence alignment between EAPL and other lectins from the same genus *Phaseolus* is presented in **Figure 3**. The results showed that EAPL was homologous with lectins from the same species (*P. vulgaris*) and shared similarity to some extent with lectins from other *Phaseolus* species, such as *P. coccineus* and *P. acutifolius*.

The specific hemagglutinating activity of EAPL toward rabbit erythrocytes was 10240 units/mg. In addition, EAPL agglutinated mouse, rat, and human A, B, AB, and O erythrocytes (**Table 2**). The hemagglutinating activity of EAPL was stable from 0 to 50 °C, but it started to decline above 60 °C ($p < 0.05$ compared with the value at 30 °C). This indicates that the hemagglutinating activity depends on the native protein conformation of the lectin. On the other hand, EAPL was stable at pH values ranging from 4 to 11, and the hemagglutinating activity decreased quickly when exposed to pH 3 and pH 12 ($p < 0.01$ versus the value at pH 7). Its hemagglutinating activity could be specifically inhibited by D-(+)-galactose at a concentration of 25 mM, and other sugars tested had no inhibition effect up to 200 mM. In addition, EDTA-induced loss of hemagglutinating activity could be recovered by adding Fe^{3+} ions and a number of divalent cations such as Ca^{2+}, Fe^{2+}, and Zn^{2+} mostly at the micromolar level (**Table 3**).

Anti-HIV-1 RT, Antitumor, and NO Inducing Activities. Results of our study showed that EAPL manifested significant HIV-1-RT inhibitory activity with an IC_{50} value of 1.8 μM (**Figure 4**). The IC_{50} value of the positive control (pinto bean lectin) was 3.9 μM. The *in vitro* MTT assay showed that EAPL demonstrated antitumor activity against a wide range of human tumor cells. **Table 4** shows that EAPL inhibited proliferation of human tumor cells in a dose- and time-dependent manner, including nasopharyngeal carcinoma cells (CNE-1, CNE-2, HNE-2), breast cancer cells (MCF-7), and liver cancer cells (Hep G2). As the most sensitive among tested tumor cells, Hep G2 cells were chosen for apoptosis analysis. In **Figure 5**, EAPL significantly reduced cell number and induced apoptosis at the doses of 35 μM and 100 μM, and typical apoptotic bodies were found (panels C-4 and D-4). The phenomenon could also be found in other tumor cell lines used in this study (data not shown).

EAPL could dose-dependently stimulate the production of nitric oxide in mouse macrophages (**Figure 6**). There was no significant difference in NO production between the EAPL group and the EAPL + polymyxin B sulfate group ($p > 0.05$), which revealed no significant LPS contamination in EAPL. In addition, the addition of dexamethasone could significantly lower the

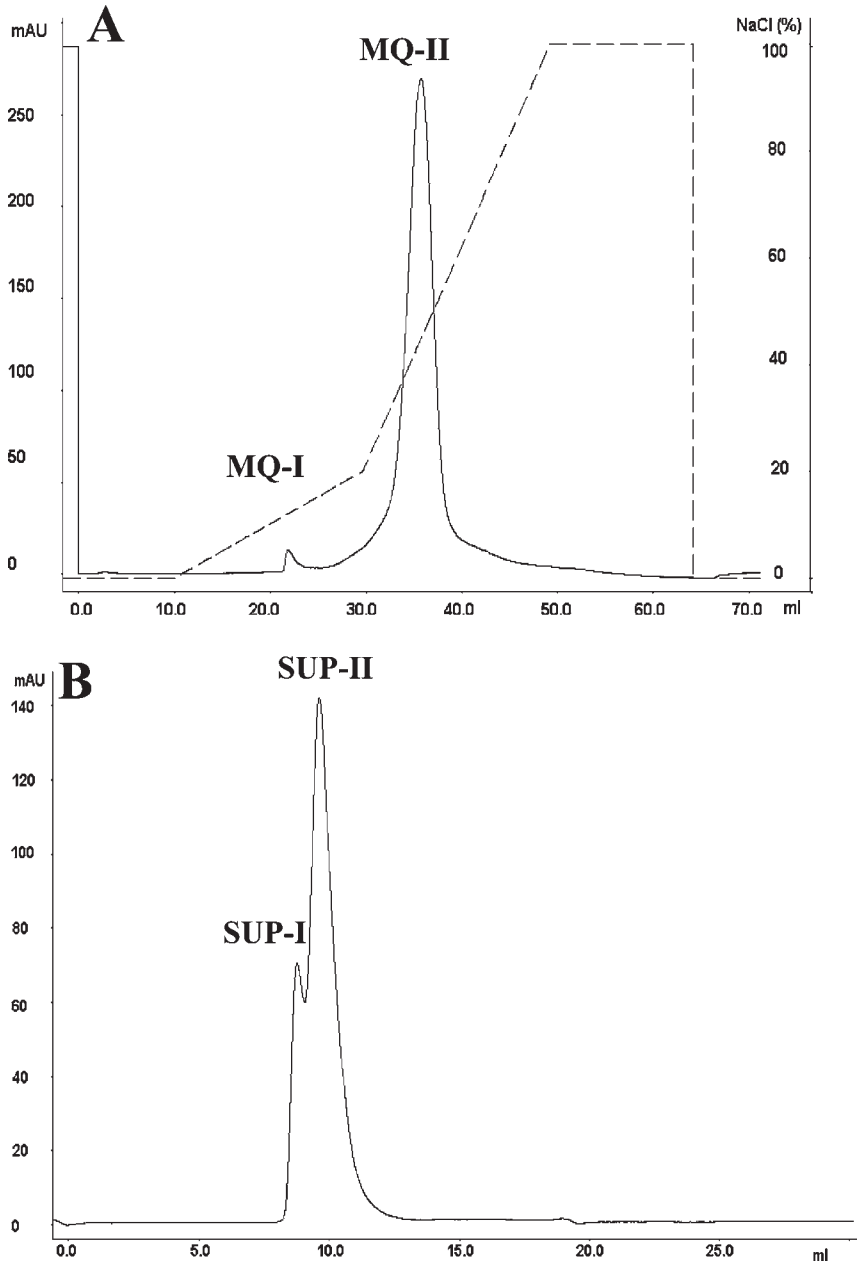

Figure 1. Purification of EAPL by chromatographic techniques. (**A**) The crude extract of Extralong Autumn Purple Beans was first applied to a Blue-Sepharose and then a Q-Sepharose column (data not shown). The fraction bound on Blue-Sepharose and subsequently on Q-Sepharose was then applied to a Mono Q 5/50 GL column. Only Peak MQ-II contained hemagglutinating activity. (**B**) Fraction MQ-II was subsequently loaded on a Superdex 75 10/300 GL column to yield purified lectin (SUP-II). The dashed line represents the concentration of NaCl employed to elute adsorbed proteins (100% NaCl equals 1 M NaCl). The *X*-axis represents elution volume (mL), and the *Y*-axis represents absorbance at 280 nm (milliabsorbance units, mAU).

concentration of NO to almost background level ($p < 0.01$), which disclosed the involvement of iNOS in EAPL-induced NO production. RT-PCR results showed that EAPL could dose-dependently induce the expression of iNOS mRNA (**Figure 7**).

EAPL was destitute of ribonuclease and antifungal activities when tested at concentrations up to 1 mM (not shown).

DISCUSSION

The common bean (*P. vulgaris* L) is a worldwide cultivated plant with seeds used as important food staples. Numerous investigations are targeted on this bean, encompassing genetic diversity, classification, breeding, purification, activity, and application associated research (*12*, *20*, *21*). Furthermore, many functional components have been isolated from different cultivars of the seeds of common bean and other *Phaseolus* species, such as antifungal defensin from *P. vulgaris* cv. Pérola (*21*) and lunatusin with anti-HIV-1 RT and antitumor activities from lima bean (*22*). We report herein a lectin EAPL with multiple activities isolated from a Chinese cultivar of the common bean, Extralong Autumn Purple Bean. EAPL is a dimeric lectin with a molecular weight of about 60-kDa, which was the same as those of other *P. vulgaris* lectins (*14*, *20*, *23*−*26*). The N-terminal amino acid sequence of EAPL is homologous to lectins from the other ten *Phaseolus* species listed in this study (**Figure 3**). This was in accordance with the similarity of some of their biological activities (*14*, *15*, *20*, *23*−*28*).

The most significant characteristics of lectins are their hemagglutinating and specific sugar-binding activities. Compared with other lectins, EAPL showed hemagglutinating activity that enabled it to strongly agglutinate native rabbit, rat, mouse, and

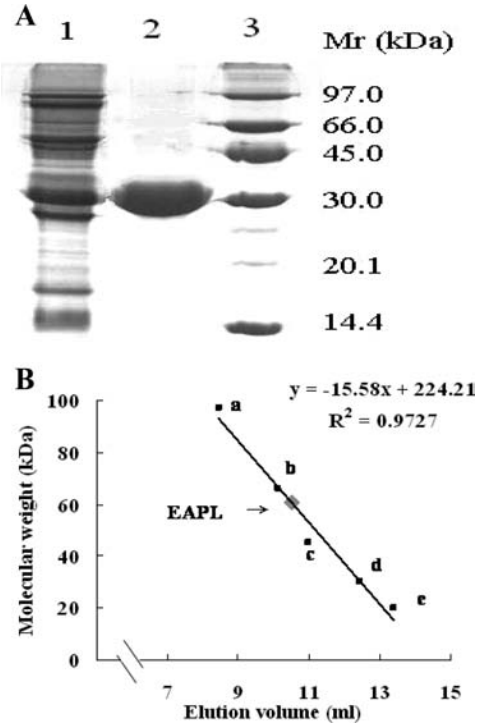

Figure 2. Determination of the molecular mass of EAPL by SDS-PAGE and gel filtration on Superdex 75 10/300. (**A**) SDS-PAGE (15% polyacrylamide gel) showing the purity and molecular mass of EAPL. Lane 1, crude extract of Extralong Autumn Purple Beans; Lane 2, purified lectin; Lane 3, molecular mass markers. (**B**) Calibration curve of a Superdex 75 column eluted with 0.2 M NH_4HCO_3 buffer at a flow rate of 0.5 mL/min. The markers included (a) phosphorylase b (97-kDa), (b) bovine serum albumin (66-kDa), (c) ovalbumin (45-kDa), (d) carbonic anhydrase (30-kDa), and (e) soybean trypsin inhibitor (20-kDa). The elution volume of EAPL was 10.50 mL, corresponding to a molecular mass of 60.6-kDa.

Table 1. Summary of Purification of EAPL from 400 g of Dried Seeds of Extralong Autumn Purple Bean

column	fraction with hemagglutinating activity	yield (mg)	specific hemagglutinating activity[a] (units/mg)	purification fold[b]
—	crude extract	5000	64	1
Blue-Sepharose	blue bound	1055	580	9.1
Q-Sepharose	Q bound	260	6120	96
Mono Q	MQ-II	220	7528	118
Superdex 75	SUP-II	140	10240	160

[a] Hemagglutinating activity toward rabbit erythrocytes was calculated as mentioned in the Materials and Methods. [b] Purification fold equals the value of the specific hemagglutinating activity of the chromatographic fraction divided by the value of the specific hemagglutinating activity of the crude extract.

human red blood cells. For instance, a galactose-specific *Bauhinia variegata* lectin can only agglutinate native human A and B erythrocytes but not red cells of the native O type (*29*). Another example is mushroom (*Pleurocybella porrigens*) lectin, which has no effect on native mouse and human A, B, and O red blood cells and needs Pronase pretreatment (*30*). An interesting phenomenon in our study was that human O erythrocytes were agglutinated at one dilution higher than A, B, and AB cells, the same as reported on red marine alga *Ptilota filicina* lectin (PFL) (*31*). The potential mechanism of this phenomenon may be associated with differences in surface glycoproteins among cells of different blood groups (*1*). This observation suggests that EAPL could be used as a potential tool for the study of the antigenic specificity on ABO and Lewis blood groups.

Unlike other plant lectins and marine lectins whose hemagglutinating activities can be inhibited by a number of simple sugars

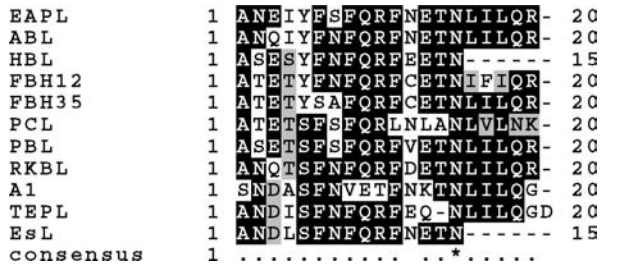

Figure 3. Alignment of the N-terminal amino acid sequences of EAPL from *P. vulgaris* in comparison with other *Phaseolus* lectins. Abbreviations are as follows: EAPL, *P. vulgaris* cv. Extralong Autumn Purple Bean lectin (this study); ABL, *P. vulgaris* cv. Anasazi Bean lectin (*14*); HBL, *P. vulgaris* cv. Haricot Bean lectin (*45*); FBH12, lectin from *P. vulgaris* cv. French Bean cultivar number 12 (*23*); FBH35, lectin from *P. vulgaris* cv. French Bean cultivar number 35 (*20*); PCL, *P. coccineus* lectin (*44*); PBL, *P. vulgaris* cv. Pinto Bean lectin (*24*); RKBL, *P. vulgaris* cv. Red Kidney Bean lectin (*25*); A1: a lectin arcelin-1 from *P. vulgaris* cv. RAZ-2 (*26*); TEPL, tepary lectin from *P. acutifolius* (*27*); EsL, lectin from *P. acutifolius* var. escumite (*28*). Conserved residues are shaded in black, and similar residues are shaded in gray. The asterisk (*) indicates identical amino acids throughout the lectins listed, and residues conserved in >50% are marked with black dots (●) underneath.

Table 2. Differential Specific Hemagglutinating Activity of EAPL toward Erythrocytes of Different Origins[a]

species	strain/line	hemagglutinating activity (units/mg)
Human		
A	Chinese	5120 ± 128
B	Chinese	5120 ± 128
AB	Chinese	5120 ± 128
O	Chinese	10240 ± 256
Animal		
rabbit	Cuniculus	10240 ± 128
mouse	BALB/c	5120 ± 128
rat	Sprague−Dawley	2560 ± 64

[a] Results represent the mean ± SD of three independent experiments.

Table 3. Effect of Salts on Hemagglutinating Activity of EAPL

salt	min recovery conc[a] (mM)
$Na_2S_2O_5$	—
KCl	—
$CaCl_2$	0.039 ± 0.01
$MgSO_4$	0.625 ± 0.04
$MgCl_2$	1.250 ± 0.12
$MnSO_4$	0.625 ± 0.04
$CuSO_4$	0.625 ± 0.04
$CuCl_2$	0.312 ± 0.02
$ZnSO_4$	0.312 ± 0.02
$Pb(CH_3COO)_2$	1.250 ± 0.12
$FeSO_4$	0.156 ± 0.01
$FeCl_3$	0.078 ± 0.01

[a] Min recovery conc = minimum concentration of cation capable of restoring 100% of 0.01 M EDTA-induced complete loss in hemagglutinating activity. —, recovery of 0.01 M EDTA-induced complete loss in hemagglutinating activity was undetectable at a final concentration of 5 mM. Results represent the mean ± SD of three independent experiments.

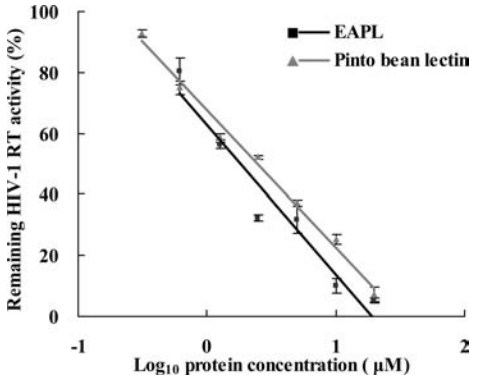

Figure 4. HIV-1 reverse transcriptase (RT) inhibitory activity of EAPL. A serial concentration of EAPL (0.62−20 μM) was chosen, and anti-HIV-1 RT activity was assayed by using a HIV-1-RT ELISA kit. Pinto bean lectin (0.31−20 μM) was used as a positive control. Results represent the mean ± SD of three independent experiments. The IC_{50} values of EAPL and pinto bean lectin were 1.8 μM and 3.9 μM, respectively.

Table 4. Antiproliferative Activity of EAPL on Different Tumor Cell Lines[a]

	IC_{50} (μM)	
cell lines	24 h	48 h
Tumor Cells		
CNE-1	725.0 ± 22.7	420.7 ± 19.2[b]
CNE-2	735.9 ± 35.0	253.2 ± 11.4[b]
HNE-2	396.1 ± 23.1	160.2 ± 7.9[b]
MCF-7	741.2 ± 50.4	171.6 ± 9.5[b]
Hep G2	83.0 ± 5.1	34.8 ± 2.3[b]
L 1210	—	—
Normal Cell		
NP69	—	—

[a] Results represent the mean ± SD of three independent experiments in the MTT assay. —, antiproliferative activity was undetectable or IC_{50} exceeded 1 mM. Statistical differences were determined by Student's *t* test between 24 and 48 h for each cell line. [b] $p < 0.01$ when IC_{50} (48 h) was compared with IC_{50} (24 h) of the same cell line.

and glycoproteins (*3*, *30*), the hemagglutinating activity of EAPL could only be specifically inhibited by D-galactose. Furthermore, the hemagglutinating activity of some lectins can be altered by EDTA-treatment and addition of metal cations (*9*) while others are not affected (*30*). In this study, EAPL requires the presence of metal ions for its hemagglutinating activity. Treatment of EAPL with 0.01 M EDTA diminished its hemagglutinating activity to almost indiscernible levels. Subsequent addition of Fe^{3+} ions and a number of divalent cations could totally reinstate its hemagglutinating activity. Of special notice is that 0.039 mM Ca^{2+} alone was sufficient to restore the hemagglutinating activity of EAPL, in accordance with reports on other lectins (*15*, *32*). The concentrations of other ions needed to restore the activity were higher, and this may reveal the Ca^{2+}-dependent carbohydrate recognition characteristic of EAPL, as in the C-type animal lectins (*33*). Besides the classical function in restoring the hemagglutinating activity lost due to the chelating action of EDTA, a recent study found that ions such as Ca^{2+} make phytohemagglutinins (PHAs) resistant to trypsin proteolysis due to stabilization of the PHA structure by the ions (*34*). This phenomenon was also noticed in EAPL (data not shown).

HIV infection has been a serious health threat worldwide for a long time, since the spread of the AIDS epidemic is fast and the existing therapies are facing the challenges of drug resistance (*35*). Currently, efforts have been devoted to discovery of natural components with potential for combating HIV. Various hemagglutinins have attracted attention as potential reagents for anti-HIV transmission (*3*). The IC_{50} of the anti-HIV-1 RT activity of EAPL was 1.8 μM, which was stronger than those of some other lectins (*24*). The IC_{50} value of the positive control pinto bean lectin agreed with the previously reported data (*24*). Since HIV-RT is the target of a large number of approved anti-HIV drugs, including nucleoside inhibitors and non-nucleosides, the anti-HIV potential of EAPL deserves further research (*36*).

Some lectins can selectively agglutinate tumor cells without any effect on their normal parental cells (*1*, *2*). This discovery captivated more interest in lectin-associated cancer investigations (*37*). The main mechanism of the antitumor activity of

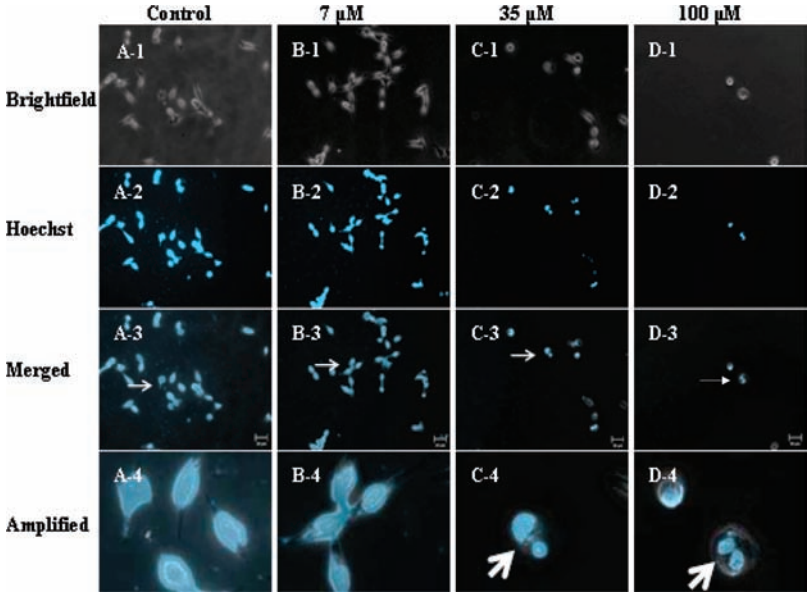

Figure 5. Dose response of EAPL induced nuclear morphological changes in human liver tumor Hep G2 cells. After treatment with different doses of EAPL including (**A**) 0 μM (control), (**B**) 7 μM, (**C**) 35 μM, and (**D**) 100 μM for 24 h, Hep G2 cells were stained with 1 μg/mL Hoechst 33342 for 10 min and the morphological changes of cells were examined by fluorescence microscopy. Row 1, bright-field images; row 2, fluorescent images; row 3, merged images between row 1 and row 2; row 4, 5-fold zoom local of merged images (row 3) as indicated by thin arrows. Typical apoptotic bodies in images C-4 and D-4 are indicated by bold arrows. Bar in merged image is 20 μm.

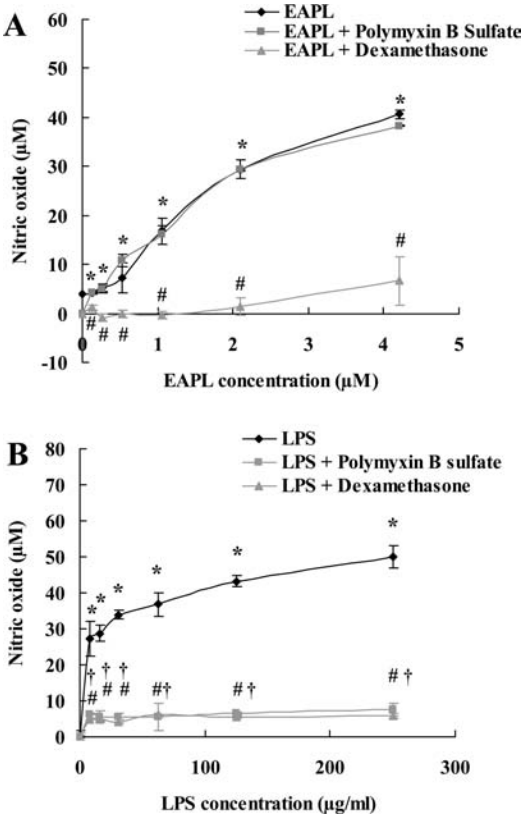

Figure 6. Nitric oxide (NO) production by mouse peritoneal macrophages after different treatments. Macrophages (2×10^5 cells/well) were treated with (**A**) EAPL (final concentrations from 0.13 to 4.2 μM) or EAPL + polymyxin B sulfate (final concentration 10 U/mL) or EAPL + dexamethasone (final concentration 10 μM) and (**B**) LPS (final concentrations from 7.8 to 250 μg/mL) or LPS + polymyxin B sulfate (final concentration 10 U/mL) or EAPL + dexamethasone (final concentration 10 μM). The value of each data point represents mean ± SD of three independent experiments. $^{*}p < 0.05$, EAPL (or LPS) group compared with control (no treatment); $^{\#}p < 0.05$, EAPL + dexamethasone (or LPS + dexamethasone) group compared with EAPL (or LPS) group at the same concentration level; $^{\dagger}p < 0.05$, LPS + polymyxin B sulfate compared with LPS group at the same concentration level.

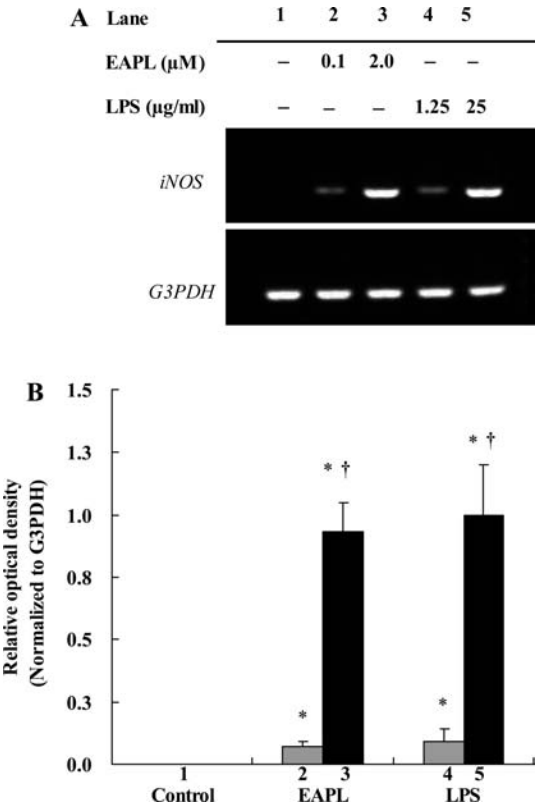

Figure 7. iNOS mRNA-inducing ability of EAPL. (**A**) Mouse macrophages were treated with 0.1 μM EAPL (lane 2), 2 μM EAPL (lane 3), 1.25 μg/mL LPS (lane 4), or 25 μg/mL LPS (lane 5) for 12 h, and lane 1 as control (no treatment). The mRNA levels of iNOS were then determined by RT-PCR, and one representative result was shown. (**B**) Semiquantitative RT-PCR results from three independent experiments. Ban intensity was measured using Alphaimager 2200, in which iNOS mRNA expression was normalized relative to the *G3PHD* expression levels. $^{*}p < 0.01$ compared with control, and $^{\dagger}p < 0.01$ compared with the smaller dose group of the same treatment.

lectins lies in their ability to induce apoptosis via different pathways (*37*). Our results unraveled that EAPL is a potential antitumor reagent, for it inhibited in a dose- and time-dependent way the proliferation of human tumor cells, including human breast cancer MCF-7cells and human liver cancer Hep G2 cells. The apoptosis assay disclosed that the potential mechanism of its antitumor ability on Hep G2 is that EAPL could attenuate tumor cell proliferation and induce chromatin condensation and even produce apoptotic bodies (**Figure 5**).

Of particular interest is that EAPL also inhibited the proliferation of human NPC cells, including CNE-1, CNE-2, and HNE-2 cells (**Table 4**). There was no significant inhibitory effect on normal nasopharyngeal NP 69 cells under the same treatments, which may be attributed to the selective tumor cell binding activity of EAPL. There is a potential of including EAPL in the diet to reduce symptoms or even mortality of NPC patients in Hong Kong and South Asia. Further clinical trials are needed.

NO has been implicated as an important signal molecule in plant iron bioavailavility and growth, development, and stress physiology (*38*). Recent *in vitro* and *in vivo* studies showed that plant lectins could trigger the formation of NO, which is a major effector molecule in cancer prevention (*39*). NO is synthesized from L-arginine and oxygen by four major isoforms of NO synthase (NOS), including neuronal NOS, endothelial NOS (eNOS), inducible NOS (iNOS), and mitochondrial NOS. eNOS is procarcinogenic, and iNOS is anticarcinogenic (*39*). In this study, an *in vitro* system was established to examine the NO inducing ability of EAPL. Considering that LPS is a powerful NO inducer (*40*) and its contamination has been a crucial problem in many types of studies, particularly in cultures of cells highly expressing LPS receptors such as macrophages (*41*), polymyxin B sulfate, which is a specific LPS inhibitor (*42*), was used in this investigation to eliminate the possibility of LPS contamination, which would give rise to false positive results. Furthermore, dexamethasone was used as a specific iNOS inhibitor (*42*). Our results (**Figure 6**) showed that no significant changes ($p > 0.05$) in NO production occurred between the EAPL group and the group treated with EAPL + polymyxin B sulfate, which revealed the absence of LPS contamination in EAPL. On the other hand, dexamethasone could significantly abrogate the production of NO to almost background level, indicating that EAPL induced NO production, probably through production of iNOS. This presumption was then confirmed by the RT-PCR results (**Figure 7**), in line with the literature (*5*). In addition, the iNOS-inducing ability of LPS (positive control) manifested in our system was also in accord with previous reports (*43*). This NO induction ability may contribute partially to the antitumor activity of EAPL against a wide range of human tumors cells. In this context, it deserves mention that only several plant lectins,

e.g., emperor banana lectin, have been shown to augment NO production by macrophages (*5*).

A protein, designated as bacisubin, was isolated from the bacterium *Bacillus subtilis* strain B-916. It exhibited hemagglutinating, ribonuclease, and antifungal activities (*19*). Several other lectins also manifested antifungal activities (*20*, *44*). However, EAPL was devoid of antifungal and ribonuclease activities, in agreement with the findings on the bulk of lectins (*3*, *14*, *15*, *23*).

In conclusion, we report a lectin EAPL isolated from a cultivar of the common bean, Extralong Autumn Purple Bean. It exhibited significant hemagglutinating, anti-HIV-1 RT, and antitumor activities. EAPL induced NO production in mouse peritoneal macrophages, probably through an increased expression of iNOS.

ABBREVIATIONS USED

anti-HIV-1-RT, antihuman immunodeficiency virus type 1 reverse transcriptase; EAPL, Extralong Autumn Purple Bean lectin; EDTA, ethylenediaminetetraacetic acid; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; NPC, nasopharyngeal carcinoma; RT-PCR, reverse transcription polymerase chain reaction.

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