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Research Article

Bioactive Compounds Extracted from the Pinto Bean Using Membrane Technology Inhibits Biofilm Formation of *Listeria monocytogenes*

¹Yong Jae Lee, ²Eunsoo Yoo, ²Dongin Kim and ³Seok Hoon Hong

¹Department of Nutrition and Food Science, Texas A and M University, College Station, TX, 77843, USA

²Department of Pharmaceutical Sciences, Rangel College of Pharmacy, Texas A and M University, College Station, TX, 77843, USA

³Department of Chemical and Biological Engineering, Illinois Institute of Technology, Chicago, IL 60616, USA

Abstract

Background and Objective: Pinto beans contain significant amounts of phytochemicals such as lectins and polyphenols. In this study, two bioactive-rich fractions from the pinto bean were extracted and isolated using acid treatment and membrane-based separation. Moreover, bioactive compounds from pinto bean were investigated to see whether it is effective in repressing biofilm formation of *Listeria monocytogenes* and ovarian cancer cell. **Materials and Methods:** The lectin- and polyphenol-rich fraction were extracted from the pinto bean using acid treatment and membrane technology, which are a simple, inexpensive and high yield promising method has not been reported previously. Two fractions were applied to inhibit the *Listeria* biofilm formation and ovarian cancer cell viability. **Results:** The lectin-rich fraction from ultrafiltration retentate (UFRT) was able to significantly reduce *L. monocytogenes* biofilm formation at 96.66% at 1,000 $\mu\text{g mL}^{-1}$ followed by 81.16% at 100 $\mu\text{g mL}^{-1}$ dosing concentrations. The polyphenol-rich fraction from nanofiltration retentate (NFRT) was not shown to significantly reduce the biofilm formation as well as the ovarian cancer cell viability which may be due to the low polyphenol activity and high oligosaccharides content. **Conclusion:** These results support that ultrafiltration is able to separate the lectin-rich fraction from pinto beans which can be used as a promising *Listerial* anti-biofilm agent to the food industry.

Key words: Lectin, polyphenol, pinto bean, membrane technology, *Listeria monocytogenes* biofilm, ovarian cancer

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Corresponding Author: Yong Jae Lee, Department of Nutrition and Food Science, Texas A and M University, College Station, TX, 77843, USA
Tel: (+1) 864 276 2363

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pinto beans (*Phaseolus vulgaris*) has been considered as an underutilized legume containing high amounts of phytochemicals such as lectins and polyphenols as well as high nutritional value¹. It has been reported that phytochemicals isolated from pinto beans possess anti-fungal, anti-bacterial, anti-diabetic and anti-tumor activities²⁻⁵. Biological activities of lectin from pinto beans have been studied to fight against bacteria, fungi, viruses and tumor during the last decades².

Lectins are natural substances that have at least one binding site without immunological characteristics or catalytic function. It is classified as a carbohydrate-binding protein which binds with mono and oligosaccharides^{5,6} and it is distinct from enzymes and antibodies⁷. Its anti-biofilm mechanism is not clearly identified but lectin can bind on the bacterial surface and inhibit initial attachment of bacteria on the solid surfaces since the cell wall of microbes contains abundant lipopolysaccharides and glycoproteins^{8,9}. The lectins from pinto beans are known as erythroagglutinin (PHA-E) and leucoagglutinin (PHA-L) which have molecular weights in the range^{5,6} of 31-34 kDa and are composed of 2.5-5.0% of pinto bean protein^{2,10}. Polyphenols (500-4,000 Da) are another major phytochemical in pinto beans that are largely found in natural materials and have an anti-oxidants effect involved in the prevention of cancer and metabolic syndromes^{11,12} as well as anti-diabetic, anti-obesity, anti-inflammatory and anti-microbial^{11,13}. Pinto beans contain plenty of polyphenols such as flavonoids, anthocyanins and tannins^{14,15} that are composed of about 11% of the whole pin to bean seed¹⁶.

Listeria monocytogenes is a food borne pathogen that is able to cause a severe illness called listeriosis^{17,18}. Biofilm formation of *L. monocytogenes* results in serious threats to food safety and economic loss in the related industry¹⁹. Moreover, *Listeria* cells embedded in biofilms are more resistant to antibiotics and disinfectants than the planktonic cells due to their robustness in structure and biofilm-specific physiology distinct from the planktonic ones²⁰⁻²². Therefore, it is important to explore alternative natural anti-bacterial agents that can effectively inhibit biofilm formation of *L. monocytogenes*.

The aim of this study was to extract and isolate two bioactive-rich fractions from the pinto bean using acid treatment and membrane-based separation method which has not been reported previously. This method has several advantages including a low-cost, an environmentally friendly approach and a scalable isolation of bioactive compounds, especially lectins. Moreover, extracted bioactive compounds

from pinto bean were investigated to see whether it is effective in repressing biofilm formation of *L. monocytogenes* and ovarian cancer cell.

MATERIALS AND METHODS

Materials: The pinto beans used in this study were obtained from a local store. It was ground using a commercial coffee grinder, then sieved with 40 mesh screens.

Extraction and separation of bioactive compounds from pinto bean powder bioactive compounds from pinto bean powder were produced with the acid treatment followed by membrane technology. The schematic procedure is shown in Fig. 1. The pH of pinto bean powder solutions (1:10 ratio) was adjusted and maintained to 4.3 using 85% phosphoric acid solutions at room temperature for 2 h. The acid treated slurries were centrifuged at 4,700 rpm for 30 min (Sorvall RC-5C Plus Centrifuge and SLA-1500 rotor, Kendro Laboratory Products, Asheville, NC, USA). The centrifuged wet cake was rinsed three times with acid solutions (85% phosphoric acid). The supernatants were used for further bioactive compounds separation with ultrafiltration and nanofiltration. Ultrafiltration retentate (UFRT) was prepared with filtration for the supernatant from the acid slurries with a spiral wound composite cross flow system (5 kDa MWCO, Synder, Vacaville, CA). The permeated liquid was filtered using a nanofiltration system (Synder, Vacaville, CA), which was 150-300 Da MWCO. The water of UFRT and NFRT solutions were evaporated using rotavapor and freeze-dried to obtain high purity bioactive compounds.

Proximate compositional analysis moisture was measured by oven hot air-drying method at 120°C for 24 h incubation. The fats and lipids were measured by Soxhlet extraction, ash was measured by muffle furnace at 550°C for 4 h and dietary fiber content was determined in triplicate by the AOAC²³ method. Protein content was analyzed by the high-temperature combustion process. The total carbohydrates and others were calculated by difference.

Biofilm formation assay using crystal violet staining For *Listeria* biofilm experiment, the freeze-dried UFRT and NFRT powder were dissolved in S30 buffer composed of 10 mM Tris-acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate to make a stock solution. The biofilm assay was conducted in 96-well polystyrene plates (Costar 3370, Corning Incorporated, Corning, NY) using 0.1% crystal violet staining²⁴. *Listeria monocytogenes* serotype 4b (ATCC 19115) was used for biofilm experiment. Bacterial cultures were grown overnight in Luria-Bertani (LB) at 220 rpm

Fig. 1: Bioactive compounds extraction from the pinto bean using acid treatment and membrane technology

at 37°C were adjusted to 0.05 of the optical density at 600 nm with M9 minimal medium supplemented with 0.4% glucose (M9G) (Cold Spring Harbor Laboratory). For biofilm formation, 300 mL of the diluted culture was added into the 96-well plate with UFRT and NFRT (final concentration 100 and 1,000 mg mL⁻¹). Biofilm plate was incubated at 37°C for 24 h without shaking. Cell growth (OD_{600nm}) was measured using Synergy HTX plate reader (Biotek, Winooski, VT) and biofilms were washed three times with dH₂O. Then 300 mL of 0.1% (w/v) crystal violet was added into the biofilm plate and incubated for 20 min at room temperature. The crystal violet dye stains both the air-liquid interface and bottom liquid-solid interface biofilm²⁵. Biofilm cells stained with crystal violet were resuspended in 95% ethanol. The total biofilm formation was measured at 540 nm. The *Listeria* biofilm level was normalized by dividing the total biofilms (OD_{540nm}) by cell growth (OD_{600nm}).

Ovarian cancer cell viability test A2780 cell at 5 × 10⁴ cells cm⁻² were seed in a 96 well plate and cultured

until 100% confluent under standard conditions at 37°C with 5% CO₂ and 100% humidity. The stock solutions of fractionated pinto bean samples were diluted to 0.001, 0.1, 1, 10, 100 and 1000 µg mL⁻¹ in cell medium (RPMI-1640). After 24 h incubation, 10 µL of the cell counting kit-8 (CCK-8; Enzo Life Science, Lausen, Switzerland) solution was added into appropriate wells. After 1 h incubation, the absorbance was measured at 450 nm using a SpectraMax M3 microplate reader (Molecular Devices Inc., Sunnyvale, CA).

Statistical Analysis: All treatments in this study were conducted in triplicate and a 95% confidence level significance was applied for data analysis. Measurements were analyzed by a one-way analysis of variance (ANOVA) using GLM (General Linear Model) procedure in SAS 9.1 software (SAS Institute INC., Cary, NC). The statistically significant difference between the averages in treatments was accessed by Duncan's multiple range tests. Differences were considered significant for p-values lower than 0.05.

RESULTS

Extraction and separation of bioactive compounds using an acid treatment and membrane filtration. Table 1 shows the mass balance of each fractionation during the process. The 99% of overall mass was recovered through the process. By acid treatment, 37% solid was solubilized from pinto beans which contain soluble carbohydrates, lectins and polyphenols while 62% solid was insoluble materials in acid condition (pH 4.3) such as aggregated proteins and fibers. About 47.22% of the lectin-rich fraction from UFRT and about 11.11% of the polyphenol-rich fraction from NFRT were produced from centrifuged supernatants. Table 2 shows the results of the proximate compositional analysis. Pinto bean powder contained 23.50% protein, 71.45% carbohydrates and others, which include simple sugars, dietary fibers and polyphenols.

With a centrifuge, the protein content increased to 31.75% in the centrifuged wet cake, while the dietary fiber content was increased and the simple sugar content was decreased from pinto bean powder. The UFRT contained 59.92% protein and 36.23% carbohydrates and others. The NFRT contained 2.12% protein and carbohydrates and others contents were significantly higher (94.32%) than others (protein, fat and ash). The NFPE contained 51.65% simple sugars.

The activity of UFRT and NFRT in inhibiting *L. monocytogenes* biofilm formation and the ovarian cancer cell. The *Listeria* anti-biofilm assay (Fig. 2) showed that lectin-rich fraction from UFRT was able to significantly reduce ($p < 0.05$) the biofilm formation by 100 and 1,000 $\mu\text{g mL}^{-1}$ dosing rate. The best results were found for *L. monocytogenes* biofilm, with a reduction of 96.66% in biofilm formation at 1,000 $\mu\text{g mL}^{-1}$ UFRT followed by 81.16% at 100 $\mu\text{g mL}^{-1}$ UFRT.

Table 1: Mass balance of each fractionation during the process

Description	Dry weight (g)	Dry weight/Initial solid weight (%)
Initial pinto bean powder	198 $\pm$ 4	100
Centrifuged wet cake	122 $\pm$ 3	62
Centrifuged supernatants	72 $\pm$ 2	37
UFRT	34 $\pm$ 2	17 (47.22)*
NFRT	8 $\pm$ 2	4 (11.11)*
NFPE	32 $\pm$ 3	16 (44.44)*

Solid output/input ratio = 0.99, Data are expressed as Mean $\pm$ SD. (n = 3). *Dry weight/centrifuged supernatants weight (%)

Table 2: Proximate compositional analysis of pinto bean fractions

Dry base (%)	Pinto bean powder	Centrifuged wet cake	UFRT	NFRT	NFPE
Protein	23.50 $\pm$ 0.07 ^a	31.75 $\pm$ 2.19 ^b	59.92 $\pm$ 2.81 ^c	2.12 $\pm$ 1.91 ^d	2.31 $\pm$ 1.43 ^d
Fat	3.24 $\pm$ 0.15 ^{ab}	3.92 $\pm$ 0.35 ^a	2.73 $\pm$ 1.32 ^b	2.32 $\pm$ 1.21 ^b	2.03 $\pm$ 0.45 ^b
Ash	1.81 $\pm$ 0.13 ^a	1.39 $\pm$ 1.04 ^b	1.12 $\pm$ 0.71 ^b	1.24 $\pm$ 1.01 ^b	38.34 $\pm$ 2.62 ^b
*Simple sugars	24.11 $\pm$ 2.71 ^a	11.99 $\pm$ 4.32 ^b	2.13 $\pm$ 0.35 ^c	8.21 $\pm$ 1.43 ^d	51.65 $\pm$ 5.39 ^e
*Carbohydrates and others	71.45 $\pm$ 2.71 ^a	62.94 $\pm$ 3.61 ^b	36.23 $\pm$ 5.21 ^c	94.32 $\pm$ 5.26 ^{cd}	57.32 $\pm$ 2.24 ^d
Dietary fiber	23.80 $\pm$ 1.12 ^a	32.30 $\pm$ 3.92 ^b	3.92 $\pm$ 1.21 ^c	2.09 $\pm$ 0.92 ^{cd}	1.98 $\pm$ 0.62 ^d

Data are expressed as mean $\pm$ S.D. (n = 3), *Means within a row with different letters are significantly different ($p < 0.05$), *Containing glucose, fructose, galactose, sucrose, maltose, #Including also the bioactive compounds such as polyphenols

Fig. 2: Biofilm formation of *Listeria monocytogenes* in the presence of pinto bean UFRT and NFRT. Biofilms of *L. monocytogenes* formed in the MG9 medium for 24 h at 37°C with/without pinto bean (PB) UFRT and NFRT (100 and 1000 $\mu\text{g mL}^{-1}$). Biofilm formation was normalized by dividing total biofilms ($\text{OD}_{540\text{nm}}$) by cell growth ($\text{OD}_{600\text{nm}}$)

The error bar indicates the standard deviation of six replicates from two independent cultures

Fig. 3: Ovarian A2780 cancer cell viability test

The polyphenol-rich fraction from NFRT was not shown to significantly reduce the biofilm formation. The ovarian A2780 cancer cell viability was not significantly affected ($p < 0.05$) by pinto bean UFRT and NFRT (0.001-1,000 $\mu\text{g mL}^{-1}$ dosing rate) treatment (Fig. 3). The slight reduction of cancer cell viability was observed in NFRT but was not significant.

DISCUSSION

Extraction of bioactive compounds. Lectins are found in many plants such as beans and reported various health benefits^{26,27}. Typically, lectins are isolated by precipitation methods using acids (e.g., acetic acid used by Naeem *et al.*²⁸), organic solvents (e.g., acetone used by Medeiros *et al.*²⁹) or salts (e.g., ammonium sulfate). Then it is purified with various chromatographic methods such as affinity chromatography, ionic exchange chromatography, hydrophobic interaction chromatography and gel filtration^{27,30}. Whereas, polyphenols traditionally are extracted with solvent-based treatment which gives a higher yield, however, it has limited human consumption due to the toxicity from harmful solvent³¹. Therefore, the conventional production methods of lectins and polyphenols are considered to have a high cost and is a hazardous process. In order to improve these backwards, lectin-rich and polyphenol-rich fraction were extracted through acid treatment followed by membrane filtration which was developed in this study as a safety, economic and scalable promising method for purifying lectins and polyphenols from the pinto bean. Membrane technology has been widely used to separate and concentrate from two liquids differ in their molecular size. This technology has become gradually attractive last decades because of its environmental friendly process, low energy consumption, high efficiency of separation and the improved final product quality³².

According to Tan *et al.*¹, typical pinto bean protein showed the minimum solubility into the pH range from 4.0-5.5 which is due to their isoelectric pH and the net charge will be little or zero. While pH values lower than 3 and higher than 6, the protein solubility was reached the almost 100%. Electrostatic repulsive forces among the proteins were reduced and led to protein precipitation with the pH³³ 4.0-5.5. However, lectins are proteins which possess at least at one non-catalytic domain that binds reversibly to specific carbohydrates and has a unique soluble characteristic compared to other normal proteins. On the contrary to typical proteins, lectins were able to be extracted with the pH³⁰ 4.3-4.6 and thus at this pH range, only lectins are able to become a soluble state among proteins which may bind to soluble mono or oligosaccharides and consequently lectins are able to be separated using membrane technology, not using a precipitation method. Moreover, the acid condition is able to increase the affinity of polyphenols which assistance to extraction. In this study, the pH 4.3 was adjusted to the pinto bean solution and filtered (UF and NF) according to their respective molecular sizes (lectin-rich fraction from UFRT, polyphenol-rich fraction from NFRT). Dietary fiber and aggregated proteins should be insolubilized with the acid condition.

According to Wong *et al.*³⁴, the molecular mass of the homodimeric lectin from pinto beans was 62 kDa and that of each of its subunits was 31 kDa which was stable with the pH 3-12 and 0-70°C. Therefore, the UF system (5K MWCO) was able to retentate all extracted lectin fraction. According Ye *et al.*², about 27.5 mg of purified lectins were produced from 100 g red kidney bean using Affi-gel blue gel and CM-Sephacrose. They obtained 5.29 g lectin-rich crude extracted protein fraction with Tris-HCL buffer treatment. Whereas about 10 g lectin-rich protein fraction was produced from 100 g pinto bean in this study. Moreover, it can be estimated by the significant amount of polyphenols extracted which was supported by the results of low content of simple sugars and dietary fiber in NFRT solution. Polyphenols molecular weight is typically between³⁵ 500-4,000 Da. Therefore, the NF system (150-300 Da MWCO) was able to retentate polyphenols. These results supported that the acid treatment and membrane technology could be promising methods to extract the lectins and polyphenols from the pinto bean.

The inhibition of *L. monocytogenes* biofilm formation and ovarian cancer cell. The lectin-rich fraction from UFRT was able to significantly reduce the biofilm formation. Pinto bean lectin was identified as a galactose specify and thermostable

up^{2,36} to 70°C. Typically, antibacterial activity of lectins is explained by interaction with peptidoglycan, lipopolysaccharides and other molecules present in the cell wall of micro-organisms, interfering with blocking interaction sites with host cells preventing cell growth and viability³⁷⁻³⁹. However, bacterial biofilms show the totally different mechanisms compared to individual bacteria. There are multicellular communities enclosed in a self-produced extracellular polymeric matrix which are unable to be deeply penetrated by antibacterial molecules into the biofilm. Furthermore, bacterial cells are able to express resistance mechanisms making them recalcitrant under antibiotic treatment condition⁴⁰. The polyphenol-rich fraction from NFRT was not shown to significantly reduce the biofilm formation which may be due to the low antimicrobial activity of pinto bean polyphenols and high oligosaccharides content. Polyphenols are secondary metabolites compounds in the plant which have important roles as defenses against plant pathogens. Its antimicrobial activity, especially flavan-3-ols, flavanols and tannins have been studied over the last decades and on several bacterial species, such as *Vibrio*, *Streptococcus*, *Campylobacter*, *Clostridium*, *Escherichia coli* and *Candida*. However, *L. monocytogenes* has not been significantly affected by pinto bean polyphenols. Some phenolic acids, non flavonoid compounds such as gallic, caffeic and ferulic acids showed the antimicrobial activity⁴¹, but not as significantly because of their low contents in pinto bean.

The ovarian cancer cell viability was not significantly affected by UFRT and NFRT. Typically, plant lectins have been considered alternative therapeutic agents due to their anticancer properties *in vivo* and *in vitro* clinical studies^{42,43}. For that reason, drug delivery systems strategies have been continuously increased to the bioavailability of antitumor lectins⁴⁴⁻⁴⁶. The anticancer mechanisms of lectins are generally initiated by interaction with specific receptors on the cancer cell membrane and then the lectins can be internalized through endocytosis which induces the activation of signaling pathways related to cell death^{47,48}. However, pinto bean lectin is interestingly devoid of antiproliferative activity to the leukemia cells^{34,49}. The antiproliferative activity should depend on the carbohydrate binding activity of lectins. The binding sites of pinto bean lectin, glucosamine-specific lectins, should be blocked by the sugar, thus hindering the interactions with the cells, resulting in the reduction of the anti-proliferative effect⁵⁰. The anti-cancer activities are also highly associated with the presence of phenolic compounds as well as lectins¹⁶. In this study, however, the polyphenol-rich fraction from NFRT was not significantly affected by cancer cell viability. It should be due to the low

polyphenols activity and high carbohydrates content which could become the nutrient sources for cell growth.

More research is needed to improve the polyphenols activity for inhibition of biofilm formation and ovarian cancer cell viability with further purification and concentration of NFRT in the future. The accurate measurements of lectin and polyphenol content in the fraction are also needed.

CONCLUSION

In this study, it applied a new approach to extract lectins and polyphenols rich fractions from the pinto bean using acid treatment and membrane technology. Based on the obtained results, it may be inferred that pinto bean lectin-rich fraction from UFRT is effective in significantly inhibiting biofilm development of *L. monocytogenes*. Therefore, pinto bean lectin may be able to serve as *listeria* anti-biofilm agents to the food industry.

SIGNIFICANCE STATEMENT

This study discovers the new approach to extract lectin rich fractions from the pinto bean using acid treatment and membrane technology that can be beneficial for inhibiting biofilm development of *L. monocytogenes*. These findings encourage further research to investigate the critical areas of *listerial* biofilm formation in food industry that many studies were not able to explore and understand.

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