Running title: Corn Bran Arabinoxylans and Immunomodulatory Activity

Characterization of Nitric Oxide Modulatory Activities of Alkaline-Extracted and Enzymatic-Modified Arabinoxylans from Corn Bran in Cultured Human Monocytes

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ABSTRACT

The ingestion of foods and food-derived substances that may mediate the immune system is widely studied. Evidence suggests cereal arabinoxylans (AXs) have immunomodulatory activities that may impart health benefits in terms of immune enhancement. This study extracted AXs from corn bran using alkali and developed a modification process using three endoxylanases to obtain fractions of lower molecular weight ranges. In vitro studies showed extracted and modified AXs significantly ($P < 0.05$) elevated nitric oxide (NO) synthesis by the human U937 monocytic cell line (ranging from 53.7±1.1 to 62.9±1.2 μM per million viable cells) at all concentrations tested (5-1000 μg/ml), indicative of immune enhancement compared to an untreated control (43.7±1.9 μM per million viable cells). The study suggested the dose range and Mw distribution of AXs are key determinants of immune-modulatory activity. AXs in the low Mw range (0.1KDa-10KDa) were the most effective at inducing NO secretion by U937 macrophages at low AX concentration ranges (5-50 μg/ml) with NO production peaking at 62.9±1.2 μM per million viable cells with 5 μg/ml of AX ($P = 0.0009$). In contrast, AXs in the high Mw range (100-794KDa) were most effective at inducing NO at high AX concentration ranges (500-1000 μg/ml) with NO production reaching a maximum of 62.7±1.3 μM per million viable cells at 1000 μg/ml of AX ($P = 0.0011$). The findings suggest that dietary AXs from corn bran may heighten innate immune responses in the absence of infection or disease.

Keywords: Arabinoxylan; Corn bran; Molecular weight (Mw); Immune-modulatory activity; in vitro

INTRODUCTION

The immune system plays a pivotal role in the protection and maintenance of human health [1]. Several factors including malnutrition and unhealthy lifestyles (e.g. poor diet) can disturb human immune functions [2-4]. Consequently, the ingestion of foods and food-derived substances that may enhance the immune system is widely studied. Dietary intervention is considered an efficient way of preventing a decline in immune functions and reducing the risk of infection or cancer [5,6]. Some dietary fiber-derived food substances (such as β-1,3-glucan, β-1,6-glucan and α-1,6-mannan) have been discovered that show immune stimulation activity [7,8]. Arabinoxylan (AX), an important hemicellulose found in the outer-layer and endosperm cell walls of cereals, is a dietary fiber. In vivo studies have reported that cereal AXs can modulate both the innate (typical of natural killer cells and monocytes/macrophages) and adaptive (typical of T and B cells) immune activities after oral administration in mice and chicken [9]. For example, dietary supplementation of AXs (at 100-200 mg/kg body weight each day for 15 days) has been shown to induce murine macrophage activation and phagocytosis [10, 11].

Although the exact mechanism of how dietary fibers, including AXs, modulate the immune response is not well understood, several studies have shown that dietary fibers can be taken up by microfold cells, macrophages and dendritic cells in the intestines and transported to lymph nodes, thus providing a mechanism for the distribution of AXs in the body. Moreover, direct interaction of fibers with colonic epithelial cells or innate immune cells (such as macrophages) can mediate cytokine production leading to inflammatory and

anti-tumor effects [8, 12, 13]. Macrophages are present in high numbers in the intestine and are frequently in close proximity with epithelium cells [14]. A previous study demonstrated that rice-derived and wheat-derived AXs are dietary fibers that are taken up by gastrointestinal macrophages and transported to the spleen, lymph nodes and bone marrow [12]. Furthermore, these studies also showed that dietary AXs can interact with murine intestinal macrophages and stimulate immune responses in vivo [12, 14]. Once transported to tissues such as the spleen, lymph tissues and bone marrow dietary-derived AXs can modulate inflammatory responses in non-intestinal, peripheral tissues by interacting with resident inflammatory cells (including tissue macrophages) or with bone marrow-derived leukocytes (e.g. monocytes) that may be subsequently recruited from circulation to sites of infection, injury or disease. Indeed, a human study involving 80 human participants showed that oral consumption of AX (3 g daily for 8 weeks) as a dietary supplement significantly increased interferon gamma (IFN-γ) production in circulating leukocytes of healthy adults [15], thus confirming non-intestinal inflammatory effects of AXs on peripheral blood mononuclear cells in vivo.

The structural properties of AXs are affected by the plant source, extraction processes, modification and purification methods. This results in AX extracts with varying molecular size and degree of branching [16]. Intestinal innate immune cells, such as macrophages, can interact with an array of AXs with differing structural properties typically found in the diet [12]. Thus, it is essential to investigate the relationship between structural details and biological activity. Recent in vivo and in vitro studies suggest the immunological properties of wheat AXs might be influenced by structural features including molecular characterization,

degree of branching and monosaccharide compositions [10]. However, there has been no clear consensus as to which structural properties of AXs modulate immune function. Thus, the precise structure-activity relationship of AXs remains to be elucidated. Corn bran is a by-product of corn starch processing, which has been identified to be a cost effective source of AXs (25-30% by content) for which extraction methods have been developed [17-19]. However, very little data exists on immunomodulatory properties of corn-derived AXs. Endoxylanases and cellulase are commonly used to extract and degrade AXs from cereal cell wall tissue [16]. Endo-β-1,4-xylanases (EC 3.2.1.8) cleave the β-glycosidic bond between two β-(1,4) D-xylopyranosyl units [20, 21]. Endo-β-xylanases are mainly classified into two glycoside hydrolase (GH) families, namely GH 10 and GH 11. Endoxylanases from the GH11 family are commonly used to hydrolyze AXs because of their higher substrate specificity compared to GH 10 endoxylanases [21, 22]. Three GH 11 endoxylanases from *Thermomyces lanuginosus*, *Neocallimastix patriciarum* and *Penicillium funiculosum* have been characterized and used in different studies for isolation and modification of cereal hemicellulose [23]. However, the effects of these three enzyme modification processes on the molecular structures of corn bran AXs have not been well documented. Thus, this investigation used chemical extraction and enzyme modification processes for modifying the chemical structures of AXs extracted from corn bran. Subsequently, corn-derived AXs were assessed for their immune-modulating effects on human macrophages in a monocytic (U937) cell line by measuring nitric oxide (NO) levels. NO mediates inflammation and host defenses by regulating signaling pathways and transcription factors, vascular responses, leukocyte adhesion and transmigration, cytokine expression as well

as proliferation and apoptosis [24]. The U937 cell line is frequently used to model human macrophages instead of using non-human (animal) macrophage cell lines such as RAW264.7 and has been previously used to investigate the effects of cereal AXs (at concentrations ranging from 1 to 500 μg/ml) on several immune activities in vitro, including NO production [25].

MATERIALS AND METHODS

Chemicals. The sample of dried corn bran (moisture content 4.5%) was kindly provided from the Chinese Academy of Agricultural Sciences (Beijing, China). There were three endo-1,4-β-xylanase products (EC 3.1.2.8) used in the modification of AXs, which were Pentopan Mono BG (P-BG, 2500 U/g) from *Thermomyces lanuginosus* (Novozyme, Bagsvaerd, Denmark), E-XYLNP (1000-1500 U/mg) from *Neocallimastix patriciarum* (Megazyme, Bray, Wicklow Ireland) and Optimash VR (O-VR, 3150 U/g) from *Penicillium funiculosum* (Genencor, Leiden, Netherland). The 1,4-α-$_D$-glucan glucanohydrolase (α-Amylase heat stable, ≥500 U/ml) from *Bacillus licheniformis* and proteinase (≥500 U/ml) from *Aspergillus melleus* were purchased from Sigma-Aldrich, Gillingham, United Kingdom. $_D$-(+)-xylose (≥ 99%) for AX extraction yield determination was purchased from Acros Organics (Loughborough, UK). Eight pullulan (without side chains) standards of varying molecular weights (5–800KDa) were purchased from Shodex (Shanghai, China). $_D$-glucose (≥ 99.5%), $_D$-xylose (≥ 99%), $_L$-arabinose (≥ 99%), and $_D$-galactose (≥ 99%) were purchased from Sigma–Aldrich (Gillingham, UK). U937 cells were purchased from the Public Health England Culture

97 Collections [26]. Lipopolysaccharide (LPS) (*E. coli* serotype O111:B4, 25mg) was purchased from Sigma
98 Aldrich, UK. RPMI-1640 cell culture medium with L-Glutamine was purchased from Lonza (Verviers,
99 Belgium). Foetal bovine serum (impurities $\leqslant$ 10 EU/mL endotoxin) and penicillin-streptomycin (10,000
100 units penicillin and 10mg streptomycin/ml) were purchased from Sigma-Aldrich (Gillingham, USA).

101 **Extraction and purification process of AXs from corn bran. The** alkaline extraction of AXs from
102 corn bran was based on Doner, et al. [19]. Milled corn bran (300g) was mixed in 2000g of distilled water (1.5:10,
103 w/w) using a hand blender (800W, WSB800U) for 45s. The mixture was adjusted to pH 7.0 before adding
104 780µl Termamyl α-amylase and placing in a 90°C shaking (100 rpm) water bath for 1h. The mixture was
105 then boiled for 15 min to inactivate the enzyme. Following subsequent centrifugation of the mixture (6000 x
106 g, 20min), the residue was recovered and oven-dried overnight at 45°C to obtain de-starched corn bran. De-
107 starched corn bran samples were mixed in distilled water (1:10, w/w). Appropriate amounts of NaOH were
108 added to make up 1%, 2%, 4% and 8% w/w NaOH concentrations. The mixtures were boiled for 1h with
109 stirring, followed by centrifugation (6000 x g, 20min). The supernatants were recovered and weighed for
110 further precipitation. The ethanol precipitation method was used from Li, et al. [27]. Briefly, 600ml AXs-
111 containing supernatant was mixed with 400µl proteinase at 60°C for 1h to remove protein. The solution was
112 then placed in boiling water bath for 15min to inactivate the proteinase followed by centrifugation (6000 x g,
113 20min). The supernatant was collected and mixed with 1.4L of 70% (v/v) ethanol. The mixture was kept at
114 4°C overnight followed by centrifugation (6000 x g, 20min). After centrifugation, the precipitate was washed

115 twice with 20ml aliquots of ethanol and re-suspended in 20ml acetone and washed for 1min. After washing,
116 the precipitate was dried in an oven overnight at 45°C to obtain the alkaline extracted AX samples (AEAXs).
117 The AEAXs were milled using an analytical mill (IKA A11 Basic, Guangzhou, China, 50/60Hz, 160W).
118 **Modification process.** A 1/25 (w/w) solution of AEAX was prepared by adding 3g AEAX (8% NaOH
119 extracted) to 72g distilled water. Three different endo-1,4-β-xylanase products (E-XYLNP, P-BG, O-VR)
120 were used to modify the AEAX sample (subsequently referred to as E-AEAX) using two different incubation
121 times of 24h and 48h. Briefly, to each AEAX solution, 0.03g of enzyme (corresponding to 400 ppm) was
122 added and mixed well. The optimum pH and temperature of each enzyme was provided according to studies
123 of Malunga and Beta [28] and Li, et al. [27], together with the manufacturer instructions. The conditions of each
124 enzyme treatment were set as pH 6.0, 50°C and 24h/48h for E-XYLNP treatment; pH 4.5, 50°C and 24h/48h
125 for P-BG treatment; and pH 4.0, 50°C and 24h/48h for O-VR treatment. After 24h/48h of enzymatic treatment,
126 the mixture was placed in a boiling water bath for 15min to inactivate the enzyme. The water content of the
127 E-AEAX sample was then removed by rotary vacuum evaporation followed by drying overnight in a 45°C
128 oven to form a dried residue that was milled with an analytical mill. Chemical reagents containing nitrate or
129 nitrite were avoided in the alkaline extraction or the enzyme modification process in order to prevent
130 contamination in the subsequent in vitro NO stimulation assay.
131 **Analysis of AX extraction yields.** A standard curve of xylose was constructed using the method
132 described by Douglas [29] for determination of the xylose content of corn bran and extraction supernatants. In

133 turn, these measurements were used to calculate the content of AX in supernatants based on their A/X ratio
134 as tested in monosaccharide composition analysis. A coefficient of 0.88 was used to correct for hydration
135 water [30] in the equation AX% = Xylose% × (1+A/X) × 0.88. The AX content in the extraction supernatant
136 was then used to calculate the extraction yield of AX from raw material based on the weight of the supernatant
137 and raw material according to the equation: AX extraction yield (%) = AX% × weight of supernatant(g) ÷
138 weight of raw material(g) × 100**.**

139 **Analysis of AX sugar compositions.** The monosaccharide compositions of AX samples were analyzed
140 by following a method developed from Li, et al. [31] whereby 1ml of H_2SO_4 (1M) was added to 20mg dried
141 AX sample. The mixture was hydrolyzed for 2h at 100°C in a glycerin bath followed by a 20-fold dilution
142 (1mg/ml) in HPLC grade water. The pH of the diluted solution was adjusted to 6.5-7.2 with 1M NaOH. The
143 solution was then filtered through a 0.45μm nylon membrane for high-pressure liquid chromatography
144 (HPLC) analysis. Isocratic elution using HPLC water as the mobile phase was conducted on a JASCO RI-
145 2031 Refractive Index (RI) detector and Phenomenex ThermaSphere TS-130 column. Temperature of
146 treatment was 85°C in all cases.

147 **Analysis of AX molecular weight distributions**. Size exclusion HPLC (SE-HPLC) test was used to
148 determine the molecular weights and size distribution of dried AXs samples, according to the methods
149 described by Li, et al. [31] and Stoklosa and Hodge [32]. The average degree of polymerization (avDP) was
150 calculated by dividing the apparent peak molecular mass by the molecular mass of anhydropentose sugars

(Mw=132Da) [33]. Mobile phase was prepared by dissolving 17g $NaNO_3$ and 0.65g NaN_3 in 2L of HPLC grade water. Eight pullulan standards with molecular weights in the range 5,000-800,000Da were used to construct a standard curve. The standard and dried AX samples were dissolved in the mobile phase to form 0.5 and 2 mg/ml solutions, respectively, and gently stirred overnight prior to filtering through a 0.45μm nylon membrane for SE-HPLC analysis. Isocratic elution was conducted on a JASCO RI-2031 refractive index (RI) detector (Jasco Corporation, Tokyo, Japan) together with BioSep-SEC-S 4000 and BioSep-SEC-S 3000 columns (Phenomenex, Macclesfield, UK). Two continuous columns were connected in series (starting with BioSep-SEC-S 4000) to improve the peak shape of the AXs samples. Running time was 60min per sample and flow rate was 0.6 ml/min throughout.

Human U937 Cell Culture. Complete cell culture medium was prepared from RPMI-1640 with L-glutamine (Lonza, Belgium), 10% foetal bovine serum (FBS) and 2% penicillin-streptomycin (P/S). The human U937 macrophage cell line was grown in complete culture medium using sterile tissue culture flasks under sterile conditions in an incubator at 37°C with 5% CO_2. The cells were sub-cultured every 2 days and only used for experiments if viability was ≥ 90%.

Polysaccharide medium preparation. The 10mg AEAX sample from the alkaline extraction (NaOH 8%) of corn bran, the 10mg E-AEAX sample from the enzymatic modification (O-VR 48h) and 1ml LPS (5mg/ml) were added to culture medium (RPMI-1640 with 5% FBS) to form a total volume of 10ml and solubilized for 24h at 37°C prior to filtration through a 0.45μm sterile filter. Solubilized samples were diluted

169 further in culture medium for subsequent cell culture treatments according to concentrations (5 μg/ml, 50
170 μg/ml, 500 μg/ml, 1000 μg/ml) used in similar published studies [25, 27].

171 **Cell viability and growth analysis.** Cell growth and viability of U937 cells following treatment with
172 AX samples and LPS were assessed by cell count and trypan blue uptake method [34]. U937 cells were
173 centrifuged (1000g for 10min) and re-suspended in culture medium (RPMI-1640 with 10% FBS) such that
174 the density of live (viable) cells was set at 1×10^6/ml. A 100 μl cell suspension was pipetted in each well of a
175 96-well microplate. Sterile AX and LPS samples prepared in culture medium at three high concentrations
176 (50, 500 and 1000 μg/ml) were pre-warmed to 37°C before adding 100μl to six appropriate wells containing
177 U937 cells and mixed thoroughly. After 24h incubation (37°C, 5% CO_2), 40μl of cell fluid was added to an
178 equal volume of trypan blue (Sigma-Aldrich, UK). The number of trypan blue stained (non-viable) and non-
179 stained (viable) cells in each sample were counted using a TC10 automated cell counter (Bio-Rad, UK). The
180 percentage (%) viability of cells was calculated as the number of viable cells divided by total (viable and
181 non-viable) cells multiplied by 100.

182 **Nitric oxide (NO) stimulation assay.** This assay was used to evaluate the ability of AX samples to
183 induce NO production in the cell line U937 using Griess reagent. Griess reagent was freshly prepared
184 immediately before use in the NO stimulation assay according to the method of Dawson and Dawson [35] and
185 Griess [36]. Griess reagent is made up by two components. Component A is 37.5mmol/L sulphanilamide with
186 deionized water in 6.5mol/L HCl by 1:1 (v/v). Component B is N-1-napthylethylenediamine dihydrochloride

187 (NEED) in deionized water at 12.5mmol/L. Both NEED and sulphanilamide were purchased from Sigma
188 Aldrich, UK. Various immune cell lines including macrophages have been shown to produce elevated levels
189 of NO in response to immune-stimulants such as LPS and cytokines [37]. Thus, LPS was used as an appropriate
190 positive (polysaccharide) control in this study. A 50µl suspension of U937 cells was added to an equal volume
191 (50µl) of sterile AX or LPS diluted in culture medium to give a series of increasing concentrations (5
192 µg/ml, 50 µg/ml, 500 µg/ml, 1000 µg/ml) in wells of a 96-well microplate prior to 24h incubation (37°C, 5%
193 CO_2) as described previously [25, 27]. Untreated controls were included on the microplate by adding 50µl culture
194 medium (in place of AX/LPS) to appropriate wells containing 50µl of viable U937 cells. Background levels
195 of nitrite and/or interference from nitrate present in AX samples were internally controlled for within the
196 assay by taking in account the direct activity of AX samples in wells containing 50µl culture medium and an
197 equal volume of diluted AX sample in the absence of U937 cells. All experimental samples were evaluated
198 in triplicate with appropriate adjustments for background levels as indicated above. After 24h incubation,
199 50µl of Component A was added to each well and mixed. The microplate was incubated at room temperature
200 for 10 minutes before adding 50µl of Component B to each well and thoroughly mixing. The microplate was
201 then incubated at 4°C for 20 minutes. The absorbance of each well was then measured at 540nm using a
202 microplate reader (Synergy HTX Multi-Mode Reader, Biotek, UK). A nitrite standard reference curve was
203 used to quantitatively determine the indicative concentration of NO in the experimental wells. Nitrite
204 standards were prepared using sodium nitrite (Sigma Aldrich, UK) diluted in medium (RPMI-1640 with 10%

205 FBS) at a range of concentrations (0, 0.1, 1.0, 10, 25, 50, 80 and 100μM).

206 **Statistics.** Experiments, unless otherwise stated, were performed in triplicate and analyzed by one-way
207 ANOVA followed by a post-hoc Tukey test. A value of $p<0.05$ was considered statistical significance in all
208 cases. Data were expressed as mean ± standard error of the mean (SEM) unless stated otherwise.

209 **RESULTS AND DISCUSSION**

210 **Extraction and modification recovery yield of AXs.** Alkaline extraction was used to investigate the
211 effect of NaOH concentration on corn bran AX extraction yields. Compared to water extraction, alkaline
212 extraction was highly efficient (Table 1). The AEAXs extraction yield increased from 2.58% up to 20.8%
213 with increasing NaOH concentration from 1% to 8%. The total AX content was determined as 26.0% of corn
214 bran (dry matter basis) using the method described by Douglas [29]. Thus, the AX recovery rate reached about
215 80% of the total AX using 8% NaOH. The increased AX yield achieved using alkaline treatment was likely
216 due to the ability of alkali (OH^-) to disrupt the hydrogen bonds between AXs and other components compared
217 to water treatment. Alkali may also disrupt some covalent bonds, such as ester linkages, thus loosening up
218 the cell wall matrix and solubilizing components including AXs from the cell wall of corn bran [38]. Following
219 extraction with 8% NaOH, the AEAX sample was modified with six types of endoxylanase treatments;
220 including P-BG, E-XYLNP and O-VR from *Thermomyces lanuginosus*, *Neocallimastix patriciarum* and
221 *Penicillium funiculosum* with 24h and 48h treatment time respectively. High recovery yields (≈88%) of AXs
222 from the AEAX sample (dry matter basis) was achieved from enzyme treatments (Table 1).

Effects of enzyme treatments on monosaccharide composition of AXs. AEAX and E-AEAX samples were mainly composed of arabinose (38-41%), xylose (45-47%) and galactose (14-15%). The various endoxylanase treatments had no significant effect ($p>0.05$) on the proportion of monosaccharide and A/X ratio when comparing the monosaccharide composition of AEAX with E-AEAXs. The AEAX sample extracted from corn bran using 8% NaOH had an A/X ratio of 0.82 (Table 2), which is in concordance with the results from a previous study [19] that also reported the A/X ratio for alkaline extracted AXs of corn bran to be 0.82. During the modification process, GH11 endoxylanase treatments did not show obvious effects on the degree of substitution of AXs (0.82-0.89). In addition, the monosaccharide composition of E-AEAX and AEAX showed a similar proportion of galactose content and no glucose component.

Effects of enzyme treatments on molecular weight (Mw) distributions of AXs. After 24h treatment with the three enzyme preparations, there was no difference between the Mw distribution of E-AEAXs compared with AEAX (Figure 1a). The Mw curve of E-AEAXs and AEAX following 24 hours of enzyme treatment contain a similar peak at large Mw (501KDa; $\log_{10}$Mw≈5.7; avDP≈3800). However, AEAXs subjected to longer enzymatic treatments (48h) demonstrated a change in Mw distribution (Figure 1b). The Mw distribution curve of the E-AEAXs following 48h of enzyme treatment contained two peaks, one in the large Mw range as found with 24h enzyme treatments and an additional peak in the small Mw range (3.98KDa; $\log_{10}$Mw≈3.6; avDP≈30). This longer exposure to enzymes reduced the proportion of large Mw AX in range 1 (100KDa to 794KDa) by approximately 20-32% and increased the proportion of small Mw AX in ranges

241 3 and 4 (0.1KDa to 10KDa) by 17-30% (Table 2). When comparing the three different enzymes, the E-AEAX
242 sample following O-VR treatment for 48 h (O-VR 48h) showed the largest increase in the proportion of AX
243 with small Mw (ranges 3 and 4) compared to AEAX (Table 2). These findings suggest that the duration of
244 enzyme treatment is a significant factor in the Mw modification of AEAX by endoxylanases. AXs have a
245 tendency to form macrostructures in aqueous solutions through chain aggregation and physical entanglements
246 [39]. The molecular size of AX is a key factor contributing to its behavior in solution. High Mw AXs can form
247 aggregations more easily and exhibit weakly elastic properties in solution compared to low molecular weight
248 fractions [40]. The Mw of the AEAX fraction (Table 2) consists largely (>80%) of high molecular weight
249 material (100KDa to 794KDa). Thus, some of AEAX may form macrostructures that inhibit the AX behavior
250 in the aqueous solution. Family GH 11 endoxylanases have a β-jelly roll structure and are considered able to
251 pass through the pores of the xylan network owing to their smaller molecular sizes [41]. When the network of
252 AEAX becomes more tightknit via physical entanglements, this would form a barrier to the endoxylanase,
253 thus preventing hydrolysis of the xylan chain of the AXs. In this case, a longer treatment time (48h) would
254 be necessary to degrade the molecules of entangled AXs. Thus, improving solubility and reducing the
255 influence of AEAX aggregation should be considered when developing the modification process of AEAXs
256 in future studies. In addition, Biely, et al. [42] reported that AXs with a low degree of branching are more
257 susceptible to endoxylanase action. In the present study, the A/X ratio of AEAX was 0.82, which is a high
258 degree of branching and may explain why the Mw modification process using enzyme treatments required a

long treatment time to demonstrate effect. Furthermore, comparison of the abilities of the three types of endoxylanases isolated from *Thermomyces lanuginosus, Neocallimastix patriciarum* and *Penicillium funiculosum* in modifying the Mw of AEAX from corn bran, showed that the most effective one to be O-VR from *Penicillium funiculosum*. From previous studies, it is important to note that due to the differences in substrate specificities, binding modules and enzyme production technology, different xylanases may have different activity in reducing the Mw of xylans [21, 43]. In conclusion, enzyme modification processes altered the Mw distribution of the AX extracts obtained following 48h treatment but did not appreciably change the observed A/X ratio or individual monosaccharide composition. Thus, the Mw distribution was identified as the major factor that differed between alkaline extracted AX and enzymatic modified AX.

Effect of AX treatments on growth and viability of U937 cells. Figures 2a and 2b demonstrate that the viable and total cell count of U937 cells treated with AEAX or E-AEAX (at concentrations of 50, 500, 1000μg/ml) were not significantly different to those of the untreated negative control ($p>0.05$). The viability of macrophages following AX treatments was over 90% in all cases. Polysaccharides extracted from plants have received considerable attention due to their wide immune-modulatory activities and low toxicity [44]. The present study indicated that AX samples have no significant detrimental effects on cell growth and viability of human U937 macrophages, even at a high AX concentration of 1000μg/ml. However, in direct contrast, cell viability and growth were significantly reduced after treatment with high concentrations (500 and 1000μg/ml) of LPS compared to the untreated negative control ($p<0.01$). This finding is in agreement with

277 previous studies that have also shown that LPS inhibits macrophage proliferation and survival in a time- and
278 dose-dependent manner [45, 46]. Stimulation of inflammatory cells with LPS acts as a useful model to investigate
279 immune activation following bacterial infection. Vadiveloo, et al. [47] found that bacterial-derived LPS impairs
280 cell proliferation by inhibiting the expression of cyclin D1, an essential protein for cell proliferation in many
281 cell types, including macrophages.

282 **Effect of AXs on NO production by U937 cells.** AEAX and E-AEAX were assessed in terms of their
283 ability to induce NO production by U937 cells. The relationship between structure and immunomodulation
284 was investigated in U937 macrophages by assessing changes in NO secretion following treatment with a
285 range of AX concentrations and Mw distributions. Treatment with AEAX, E-AEAX or LPS at all
286 concentrations (5 to 1000μg/ml) tested significantly increased NO production (Table 3; Figure 3a and 3b) in
287 U937 cells compared with the untreated control ($p<0.05$). This finding is consistent with previous studies
288 that found AXs from other cereal sources were able to stimulate NO production in rat and human
289 macrophages [48, 25]. At high concentrations (500-1000μg/ml) of LPS, the raw levels of NO produced by the
290 U937 cells significantly decreased ($p<0.05$) compared to levels generated by lower concentrations of LPS,
291 mirroring the substantial inhibitory effect of LPS on cell growth and viability (Figure 2c) at high
292 concentrations. Thus, when allowing for these changes in cell counts, LPS significantly increased NO
293 production per million viable cells in a dose-dependent manner (Figure 3b). NO production was significantly
294 ($P<0.05$) higher following E-AEAX treatment at low concentration ranges (5-50μg/ml) compared to AEAX

295 treatment at corresponding concentrations. NO production did not significantly increase in a dose-dependent
296 manner following E-AEAX treatment, suggesting the optimal dose range for E-AEAX was already reached
297 at the low AX concentration range (5-50μg/ml). In contrast, the AEAX treatment was most effective at
298 inducing NO production at higher concentration ranges (500-1000μg/ml) with the optimum predicted at some
299 value above 1000μg/ml by extrapolation of Figure 3a and 3b. The optimum dose ranges of AEAX and E-
300 AEAX were clearly different; AEAX induced significantly lower NO production compared to E-AEAX and
301 LPS at concentrations below 50μg/ml ($p<0.05$). Thus, this study suggests that E-AEAX is more effective
302 than AEAX as a stimulator of NO release at low (5-50μg/ml) AX concentrations. In contrast, AEAX needs
303 to be at high concentrations (>500μg/ml) to be more effective than E-AEAX at inducing NO production.
304 NO production by U937 cells was influenced by the Mw distribution of AXs. Depending on the
305 enzymatic modification process, AEAX and E-AEAX exhibited significantly different Mw distributions;
306 33.1% of E-AEAX had Mw ranging from 158Da to 10KDa (avDP=30) whereas AEAX only contained 2.82%
307 AX in this range. In addition, the branch degree (A/X) and monosaccharide composition of AEAX and E-
308 AEAX were similar. The results suggest the dose range and Mw distribution of AXs are key determinants
309 of immune-modulatory activity. Regardless of dose, corn bran AXs in both the low and high Mw range
310 significantly induced NO production compared to untreated macrophages. AXs in the low Mw range
311 (0.1KDa-10KDa) were the most effective at inducing NO secretion by U937 macrophages at low AX
312 concentration ranges (5-50 μg/ml) with NO production peaking at 62.9±1.2 μM per million viable cells with

5 μg/ml of AX (P = 0.0009). In contrast, AXs in the high Mw range (100-794KDa) were most effective at inducing NO at high AX concentration ranges (500-1000 μg/ml) with NO production reaching a maximum of 62.7±1.3 μM per million viable cells at 1000 μg/ml of AX (P = 0.0011). The molecular structure of AXs have already been reported to have an effect on some of their physicochemical properties in solution, including tertiary conformation of the AX chain in solution, viscosity of the solution and elastic properties of the solution [9, 49]. Therefore, future work should investigate the effect of molecular weight combined with relative physicochemical properties on the immune-modulating activities of AXs.

Rice bran AX has been shown to induce macrophage-derived nitric oxide (NO) production in vitro in a dose-dependent manner using both a murine macrophage cell line RAW264.7 and murine peritoneal macrophages [25]. Cereal-derived AXs have also been shown to induce phagocytosis in a dose-dependent manner both in the human U937 macrophage cell line and in murine peritoneal macrophages [25, 50]. The in vitro production of pro-inflammatory cytokines tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) are stimulated following treatment with rice bran AX in U937 human macrophages, RAW264.7 murine macrophages and murine peritoneal macrophages [25]. This investigation in a U937 cell line supports these published findings by showing AXs from corn bran are capable of directly stimulating NO production in human macrophages at all concentrations investigated (5-1000 μg/ml). Macrophage-derived NO can form a number of oxidation products such as NO_2, NO_2^- and N_2O_3 because of its highly reactive free radical structure [51], and these products come into play during macrophage-mediated immune defense against numerous

pathogens following infection [52]. Thus, this investigation indicates a potential nutritional/prophylactic benefit of dietary AXs to stimulate low (background) levels of innate immunity in the absence of infection or disease. An ongoing low level of immune activity generated through dietary intake of AXs may reduce the likelihood or severity of infection following subsequent exposure to pathogens. This is supported by the fact that oral administration of wheat AXs (at 2500 mg/kg, n = 10) induces anti-inflammatory effects in mice treated with bacterial LPS and reduces NO production in LPS-stimulated murine macrophages [50]. NO production by macrophages is also reported to mediate T cell responses, suppress anti-inflammatory effects and regulate of leukocyte recruitment [53]. Thus, dietary AXs may induce a wide range of immune system responses via NO pathways, thereby heightening natural immunity and maintaining human health at multiple levels.

Further investigations are now required to determine the precise signaling mechanism through which AXs induce NO production. In addition, in vivo studies are essential to determine whether the effects of dose range and Mw distribution on AX-induced NO production demonstrated in this study are mirrored in the body.

ABBREVIATIONS USED

AXs, arabinoxylans; AEAXs, alkaline extracted AXs; A/X, ratio of arabinose to xylose; DP, degree of polymerization; E-AEAXs, enzyme-modified AEAX; E-XYLNP, Endo-1,4-β-xylanase of megazyme; GH, glycoside hydrolase; LPS, lipopolysaccharide; Mw, molecular weight; NO, nitric oxide; O-VR,

OPTIMASH^{TM} VR enzyme; P-BG, pentopan mono BG enzyme.

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Figure Captions/Legends

Figure 1. The molecular weight distributions of E-AEAXs generated using three different enzyme (E-XYLNP, P-BG, O-VR) treatments for 24h and 48h treatment.

Figure 2. Effects of AXs and LPS on the growth & viability of U937 cells. *a-c*: U937 cells were treated with the LPS, AEAX and E-AEAX for 24hrs; The count of viable (live) and total (viable and non-viable) cells was confirmed using the trypan blue exclusion method. The solid line '—'and the dotted line '---' indicate the viable and total cell count of the control (that lacks treatment with AX or LPS) respectively. The viable/total cell counts after treatment with AX or LPS were compared with the corresponding viable/total cell counts of the control using the Student's t-test (*: p-value < 0.01). Cell counts are presented as the mean ± SEM of six sample replicates.

Figure 3. Effect of AEAX, E-AEAX and LPS treatments on NO production by U937 cells. Panel **a** shows the raw NO production measured following treatment with AX or LPS at increasing concentrations of 5-1000 μg/ml. Panel **b** indicates the corresponding NO production per million viable cells. The symbol * indicates that the NO production following treatment with AX or LPS was significantly ($P<0.05$) greater to that of the untreated control. The symbol # indicates NO production significantly changed ($p<0.05$) from previous sample dosage. The symbol @ indicates NO production with E-AEAX treatment was significantly ($p<0.05$) different to the corresponding AEAX treatment at the same AX concentration. The symbol $ indicates NO production with AEAX or E-AEAX treatment was significantly ($p<0.05$) different to the corresponding treatment with LPS at the same concentration.

TABLES

Table 1. AXs extract yields from corn bran and the recovery yield of modified AX using three types of endoxylanase

Alkaline extraction yield (%)	NaOH concentrations				Control	Total AX content [a]
	1%	2%	4%	8%	Water	
	2.58±0.07	3.85±0.05	9.71±0.05	20.8±0.1	0.71±0.04	26.0±0.3
Enzyme modification recovery yield (%)		Endoxylanases				
	Treatment time	P-BG		E-XYLNP	O-VR	
	24h	88.1±0.4		88.0±0.1	88.0±0.3	
	48h	88.1±0.3		88.1±0.1	88.0±0.1	

a: Total AX content indicates original AX content in corn bran (dry base). The yields are presented as mean ± SD. All experiments were conducted in triplicate.

Table 2. The monosaccharide compositions and Mw distribution of AEAXs and E-AEAXs

Samples	Monosaccharide compositions [a]					Mw distributions [b]			
	Ara (%)	Xyl (%)	Glc(%)	Gal (%)	A/X	Range 1: 1×10^{5}-$10^{5.9}$(Da)	Range 2: 1×10^{4}-10^{5}(Da)	Range 3: 1×10^{3}-10^{4}(Da)	Range 4: 1×10^{2}-10^{3}(Da)
AEAX [c]	38.3±0.5	47.0±0.6	/	14.7±0.4	0.82	83.5%	13.7%	2.82%	/
24 hour enzyme treatment (E-AEAXs 24h) [d]									
E-XYLNP	39.1±1.7	47.1±1.0	/	14.5±1.0	0.83	78.1%	19.2%	2.73%	/
P-BG	40.0±1.0	45.6±0.9	/	14.5±0.3	0.88	79.7%	17.5%	2.80%	/
O-VR	39.2±2.1	47.1±1.6	/	14.0±0.5	0.83	79.7%	17.3%	2.95%	/
48 hour enzyme treatment (E-AEAXs 48h) [d]									
E-XYLNP	41.2±1.3	45.3±0.7	/	14.5±1.0	0.89	63.7%	16.3%	17.8%	2.19%
P-BG	39.9±0.8	45.2±0.8	/	14.9±0.1	0.88	54.8%	14.4%	23.4%	7.36%
O-VR	38.8±2.3	47.3±1.6	/	13.9±0.9	0.82	51.2%	15.7%	24.5%	8.64%

Monosaccharide compositions and molecular weight (Mw) distribution of AEAXs and E-AEAXs (all experiments conducted in triplicate). *a*: The proportion of each monosaccharide in AX samples presented as mean ± standard deviation (SD); *b*: The overall Mw distribution of AXs ranged from 0.1KDa to 794KDa and was divided into four molecular weight ranges. Percentage of AXs in different Mw ranges were analyzed using the LC Data Analysis (SHIMADZU Corporation). *c*: The AEAX sample indicates AXs extracted using 8% NaOH treatment; *d*: The enzyme treatments indicate modified AEAXs using one of three different enzymes (E-XYLNP, P-BG, O-VR) for 24 hours or 48 hours.

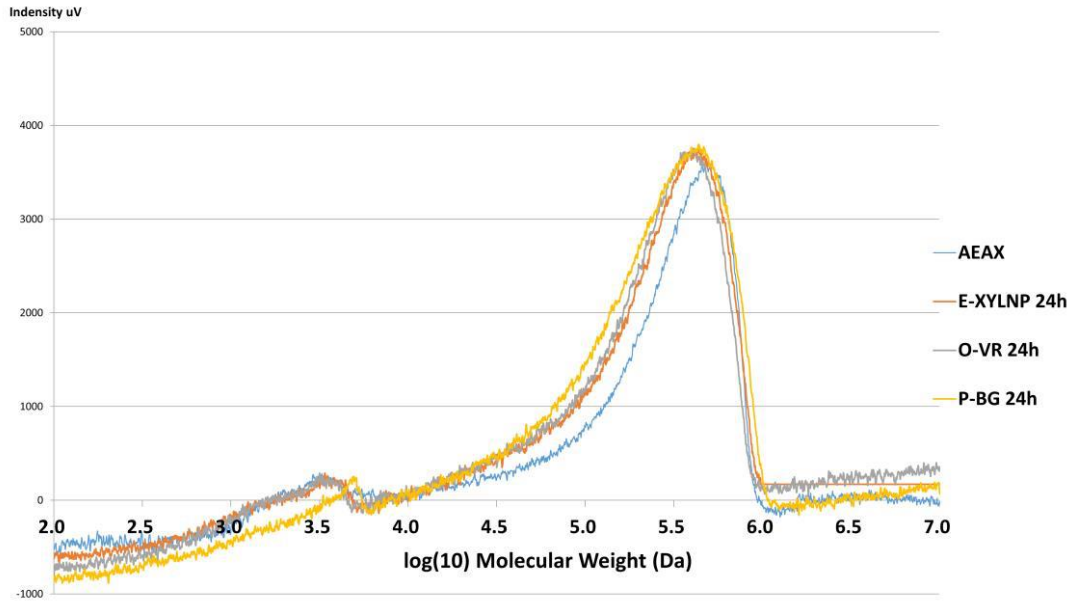
Table 3. NO production by U937 cells after 24h treatment with AEAX, E-AEAX and LPS

Sample	(a) Raw NO Production (μM) **(b) NO Production (μM) Per Million Viable Cells**				
	Concentration of AX or LPS (μg/ml)				
	0	5	50	500	1000
LPS					
(a)	-	70.4±1.3 *	71.8±2.3 *	64.6±1.1 * #	56.5±0.1 * #
(b)	-	**65.6±0.9 ***	**65.7±1.6 ***	**70.8±0.9 * #**	**72.2±0.0 * #**
E-AEAX					
(a)	-	67.3±1.7 * @	67.7±2.4 * @	64.8±2.5 *	63.3±1.3 * $
(b)	-	**62.9±1.2 * @**	**62.2±1.7* @**	**58.6±1.7* $**	**59.0±0.9* $**
	-				
AEAX					
(a)	-	57.6±1.5 * $	61.0±2.3 * $	65.5±2.0 * #	66.9±1.9 * # $
(b)	-	**53.7±1.1 * $**	**55.9±1.6 * $**	**60.2±1.4 * # $**	**62.7±1.3 * # $**
Untreated Control					
(a)	46.1±2.0				
(b)	**43.7±1.9**				

The NO_2^- concentration (μM) (mean ± SEM) is an indication of NO production in U937 cells; The symbol * indicates NO production significantly increased ($p<0.05$) compared to the untreated control; The symbol # indicates NO production significantly changed ($p<0.05$) from previous sample dosage; The symbol @ indicates NO production with E-AEAX treatment was significantly different to the corresponding AEAX treatment at the same concentration ($p<0.05$); The symbol $ indicates NO production with AEAX or E-AEAX treatment was significantly different to the corresponding treatment with LPS at the same concentration ($p<0.05$). The p-values indicated are those provided by a post-hoc Tukey test following an overall significant ($p<0.05$) difference measured by one-way ANOVA using SPSS.19. In all cases, experiments were conducted in triplicate.

Fig 1.

a.


Indensity uV
5000
4000
3000
2000
1000
0
-1000
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
log(10) Molecular Weight (Da)
AEAX
E-XYLNP 24h
O-VR 24h
P-BG 24h

b.

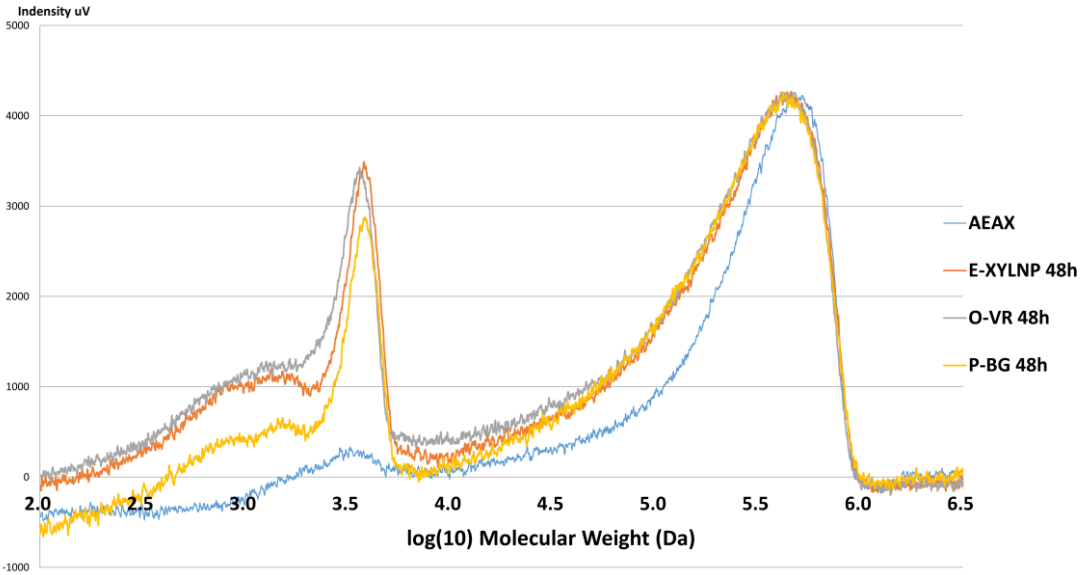
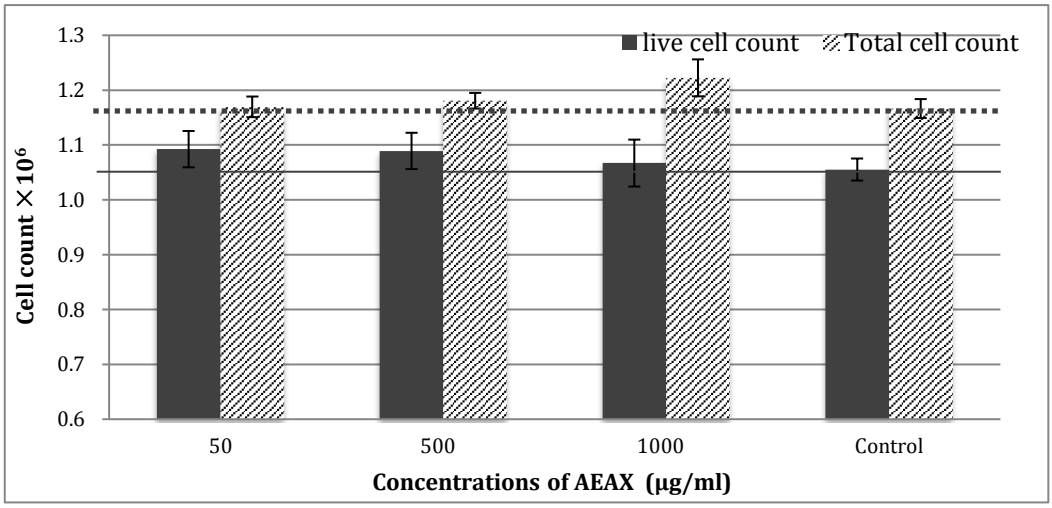
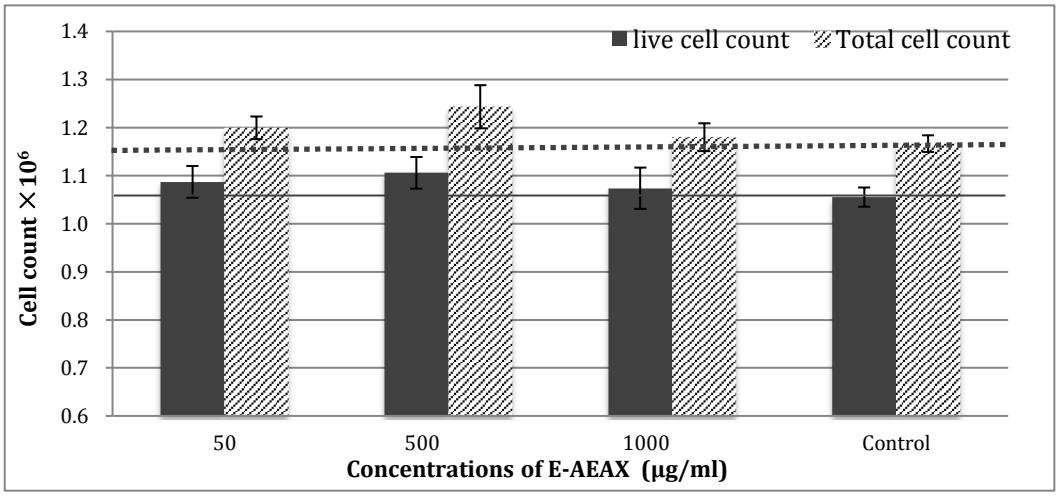

Indensity uV
5000
4000
3000
2000
1000
0
-1000
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
log(10) Molecular Weight (Da)
AEAX
E-XYLNP 48h
O-VR 48h
P-BG 48h

Fig.2

a.


live cell count
Total cell count
Cell count × 10⁶
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
50
500
1000
Control
Concentrations of AEAX (µg/ml)

b.


live cell count
Total cell count
Cell count × 10⁶
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
50
500
1000
Control
Concentrations of E-AEAX (µg/ml)

c.

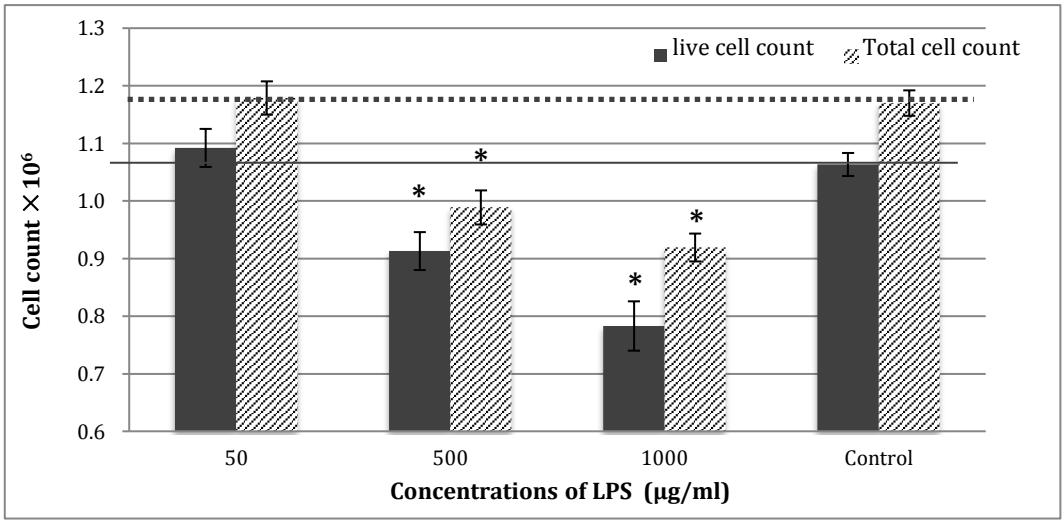
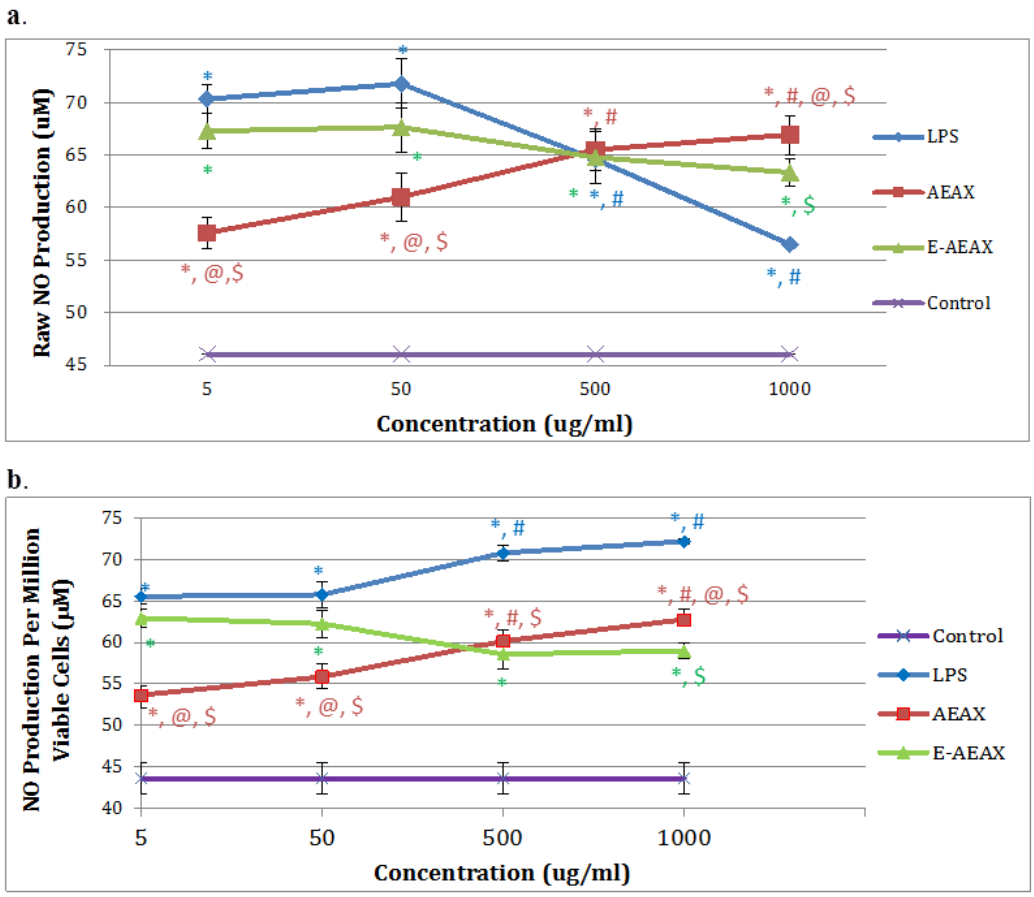
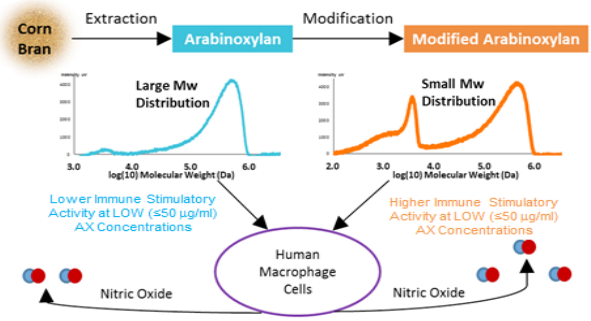

live cell count
Total cell count
Cell count × 10⁶
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
*
*
*
*
50
500
1000
Control
Concentrations of LPS (µg/ml)

Fig 3.


a.
Raw NO Production (uM)
75
70
65
60
55
50
45
5
50
500
1000
Concentration (ug/ml)
LPS
AEAX
E-AEAX
Control
b.
NO Production Per Million Viable Cells (μM)
75
70
65
60
55
50
45
40
5
50
500
1000
Concentration (ug/ml)
Control
LPS
AEAX
E-AEAX

TOC Graphic


Corn Bran
Extraction
Arabinoxylan
Modification
Modified Arabinoxylan
Large Mw Distribution
Small Mw Distribution
3.0
4.0
5.0
6.0
2.0
log(10) Molecular Weight (Da)
Lower Immune Stimulatory Activity at LOW (≤50 µg/ml) AX Concentrations
Higher Immune Stimulatory Activity at LOW (≤50 µg/ml) AX Concentrations
Human Macrophage Cells
Nitric Oxide
Nitric Oxide