Immunomodulatory Properties of Water Soluble Arabinoxylans from Extruded Rice Bran and Wheat Pentosan in an *In vitro* Model of Human Monocytes and Macrophages

A thesis submitted in partial fulfilment of the requirements of the Manchester Metropolitan University for the degree of Doctor of Philosophy

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Abstract

Arabinoxylans (AXs) are major components of non-starch polysaccharides (NSPs). Recently, AXs have attracted a great deal of attention, because of their possible antitumor and immunomodulation activities. These activities have been suggested to be related to the content of low molecular weight (Mw) AXs, in particular those with a Mw below 32 kDa.

Rice bran and wheat pentosan are rich sources of AXs. However, extraction of AXs is difficult and often gives low yield. Various methods have been used to increase the extraction yield of AXs with varying degrees of success, such as alkaline hydrolysis and enzymatic treatment. However, some of these treatments have been reported to be either expensive or produce hazardous wastes and non-environmentally friendly. Extrusion processing has been used to increase the solubility of cereal dietary fibre, however, these studies used alkaline or enzyme treatments with the extrusion to maximise the extraction yield.

The use of extrusion alone as a pre-treatment method to increase the extraction yield and reduce the molecular weight (Mw) of AXs from rice bran or wheat endosperm pentosan has not been investigated. Hence, the current study aimed to determine if extrusion alone could change the extraction yield and Mw of the AXs. Wheat endosperm pentosan and rice bran were extruded with a twin-screw extruder at screw speeds of 80 and 160 revolutions per minute (rpm). It was found that the extraction yield of AXs increased with an increase in screw speed and was accompanied by a decrease in the Mw of the AXs.

In vitro studies using immunoassays to measure proinflammatory markers showed that AXs from extruded rice bran and wheat pentosan significantly ($P<0.05$) increased nitric oxide (NO) and tumour necrosis factor α (TNFα) production from both U937 monocytes and macrophages in a concentration-dependent manner at (50, 500 and 1000 μg/ml), respectively. Moreover, the immunomodulatory activity of AXs was associated with the very low Mw of AXs. Moreover, inhibition of toll like receptor 4 (TLR4), which is known to be the receptor for bacterial lipopolysaccharides (LPS),

significantly inhibited the AXs-induced increase in NO and TNFα production in both U937 monocytes and macrophages (*P<0.05*), suggesting the actions of AXs may be mediated at least in part through TLR4.

The findings of this study indicate that AXs may compete with LPS for the same receptor TLR4, resulting in decreasing the inflammatory response that LPS produces during the infection. Thus AXs can produce a non-detrimental moderate increase in the inflammatory response.

Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of Manchester Metropolitan University or any other university.

List of publications and achievements derived from this project

- Research article (submission stage): (The effect of extrusion on water-extractable arabinoxylan extracted from wheat and rice bran). Targeted Journal (Carbohydrate Polymers).
- Research article (submission stage): (The effect of modified arabinoxylans extracted from wheat and rice bran on the immunomodulatory response of U937 cells monocytes and macrophages). Targeted Journal (Journal of Cereal Science).
- Li. W. Zhang S., Hu H., Fadel A. and Smith C. A Potential Immune-stimulating Health Benefit of Wheat Arabinoxylans for Foods. 17_{th} IUFoST World Congress, Montreal, Canada. August 17-21, 2014.
- Li. W. Zhang S., Hu H., Fadel A. and Smith C. Immune modulating properties of wheat endosperm arabinoxylans and potential health benefit for foods 2014 International Symposium on Bioactive Compounds in Cereal Grains and Foods ICC, Vienna. April 25, 2014.
- Poster publication at the fourth International Conference and Exhibition on Food Processing & Technology (August 10-12, 2015, organised by OMICS, London):
- The title of Poster (The effect of extrusion processing on solubility and molecular weight of water-soluble arabinoxylans).
- Poster presentation at the 2015 SCIENCE & ENGINEERING RESEARCH SYMPOSIUM (September 17, 2015, organised by MMU): The title of the poster (Extrusion and arabinoxylan extractability from wheat endosperm pentosan and rice bran).

Awards

- 3MT competition (3 minutes thesis competitions), Winner over Science and Engineering School Manchester Metropolitan University (Arabinoxylans and immune response) (May 2015).
- Winner of Young Research Forum, sponsored by Aggelakis S. A., Greece, obtained in at the fourth International Conference and Exhibition on Food Processing & Technology (August 10-12, 2015, organised by OMICS, London): (The effect of extrusion processing on solubility and molecular weight of water-soluble arabinoxylan).

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I could not find words to thank my family who believed in me and I hope to see them in the near future. Although I have not seen them for seven years, I am sure that I have been always in their prayers. Above all, I wish that my father were near when I submit this thesis to share the happiness of success as I always aspired to make him proud because he is the essence of my existence and the constant motivation of my work.

Last but not least, special thanks to my wife Nermin, who opened all the closed doors for me and believed in my potentials.

Dedication

To the good soul of my late beloved mother, who wished to see me a successful scholar,

To my beloved father, who strived to secure a good education for me,

To my dear brothers and sisters; Fatima, Amer, Samah, Abdulaliem and Abdulhamid,

And to my dear wife Nermin.

List of Abbreviations

AXs	Arabinoxylans
Ar/Xy	Arabinose /xylose ratio
CD14	Cluster of differentiation 14
DC	Dendritic cell
DF	Dietary fibre
FA	Ferulic acid
HPLC	High performance liquid chromatography
IFNγ	Interferon gamma
IL	Interleukin
kDa	Kilo dalton
LPS	Lipopolysaccharide
mRNA	Messenger RNA
Mw	Molecular weight
MyD88	Myeloid differentiation primary response gene 88
MΦ	Macrophage
NK	Natural killer
NO	Nitric oxide
P160	Pentosan with 160 rpm screw-speed
P80	Pentosan with 80 rpm screw-speed

PAMP	Pathogen associated molecular pattern
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
PW	Pentosan without extrusion
RB160	Rice bran with 160 rpm screw-speed
RB80	Rice bran with 80 rpm screw-speed
RBPS2a	Rice bran hetero-polysaccharide
RBW	Rice bran without extrusion
RNA	Ribonucleic acid
rpm	Revolution per minute
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
WEAX	Water-extractable arabinoxylan
WUAX	Water-unextractable arabinoxylan

Chapter 1 Introduction

Cereal grains contain variable amounts of non-starch polysaccharide (NSP, namely cell wall material). Cereal grains are composed of hemicelluloses, celluloses, and other materials such as lignins and pectins and collectively are known as dietary fibre (Mod *et al*., 1978; Lai *et al*., 2007).

Rice and wheat are a staple food for most of the world's population. A large amount of waste is produced in growing these materials, and the maximum benefit is not being obtained from it as it is often fed to animals rather than the valuable components being extracted for human use. This occurs because the material is difficult and expensive to breakdown. Hence, there is a need for improved extraction technologies that both improve the extraction yield and reduce the cost of processing (Mohan *et al*., 2010; Zhao *et al*., 2016).

Arabinoxylans (AXs) are the main non-starch polysaccharide constituents of many cereals and it is predominantly found in the outer layers (bran) and starchy endosperm (flour) (Zhou *et al*., 2010). Arabinoxylans (AXs) have been reported in many cereals such as maize, rye, barley, oats, sorghum, wheat and rice (Izydorczyk and Biliaderis, 1995). AXs constitute about (1.37-2.06 %) of the wheat endosperm and the water-extractable portion of this is between 0.54 and 0.8 % (Izydorczyk *et al*. 1991; Houben, 1997). In rice, it constitutes about (4.84-8.5 %) of the bran and the water-extractable portion of this is between (0.2-0.77 %) (Hashimoto *et al*. 1987a; Choct, 1997). Whilst only a small portion of the arabinoxylan is soluble, it is possible to apply chemical, enzymatic or physical treatments to increasing the extraction yield (Fry, 2004).

AXs are polysaccharides composed of backbone chains of β-(1-4)-linked D-xylopyranosyl residues to which α-l-arabinofuranose units are linked as side chains in the second and/or third carbon positions, so they are often named as pentosans (Courtin *et al*., 2000; Roubroeks *et al*., 2000; Zhou *et al*., 2010).

The degree of branching is an important factor in determining the physiochemical properties of AXs (Hopkins *et al*., 2003). A large proportion of arabinoxylan cannot be solubilised in water due to the formation of di-ferulic acid bridges and covalent ester bonding between carboxyl groups on individual arabinoxylan chains (Zhou *et al*., 2010). In order to solubilise and extract AXs, pre-treatments might be applied e.g. alkaline hydrolysis or enzymatic digestion. Each of these pre-treatments have different effects on the solubility and molecular weight of AXs (Vasanthan *et al*., 2002).

Alkaline treatment is an efficient way for extracting AXs from cell wall materials However, it changes the functional properties of the AXs by breaking down (hydrolysing) some functional groups of the AXs, such as ferulic acid, and hence tends to give high molecular weight AXs fractions (100- 200 kDa) (Zhou *et al*., 2010; Cao *et al*., 2011).

On the other hand, enzymatic treatments increase AXs solubility by attacking the AXs backbone in a different manner, producing lower molecular weight fractions to those produced by alkaline hydrolysis. The enzymatic treatment produces lower molecular weight fractions of AXs with a lower extraction yield (Swennen *et al*., 2006; Zhou *et al*., 2010). However, this is not as efficient as alkaline hydrolysis and gives rise to different functionality.

Physical pre-treatments have been applied to increase the solubility of dietary fibres such as extrusion (Vasanthan *et al*., 2002; Zhang *et al*., 2011). Extrusion is a process where the materials can be exposed to a combination of temperature, pressure and shear forces, which might lead to a variety of chemical reactions and molecular transformations (Liu *et al*., 2011). Recently, extrusion has been used to increase the solubility of water-extractable arabinoxylans (WEAX) in corn fibre (Jeon *et al*., 2014). Studies have suggested that low molecular weight AXs extracted from different cereals may have desirable biological effects (Li *et al*., 2015). Low molecular weight cornhusk AXs showed an increase in the activity of natural killer cells and an increase in cytokine production *in vitro* (Zhang *et al*., 2004). Other studies found that AXs

extracted from enzymatically modified rice bran (Biobran) with low molecular weight can stimulate both the adaptive and innate immune system by enhancing dendritic cell maturation, macrophage phagocytosis, and natural killer cell activity (Ghoneum and Matsuura, 2004). On the other hand, low molecular weight arabinoxylans extracted from rice bran, without any enzyme pre-treatment, have shown anti-complementary and anti-inflammatory activities *in vitro* (Wang *et al*., 2008; Hoshino *et al*., 2010). Whilst the effect of extrusion on molecular weight and solubility of AXs has been studied, no studies have tested the functionality of extruded fibre on human cell lines. In order to address this gap in knowledge, rice bran and wheat endosperm pentosan have been physically treated with a twin-screw extruder and the immunomodulatory response was tested on a human cell line.

Aims

The aim of this thesis was to extract and modify water-soluble AXs from extruded wheat endosperm pentosan and rice bran and to study the relationship between their structural characteristics and immune modulatory potential. The modification of AXs was achieved using a twin-screw extruder and the sugar composition and molecular weight of AXs were characterised using high performance liquid chromatography (HPLC). To study the immunomodulatory potential of AXs in relation to their structures, U937 human monocytes/macrophages were treated with arabinoxylans *in vitro* and assayed for any immune responses after AXs stimulation.

Objectives

1. To extrude wheat endosperm pentosan and rice bran using a twin-screw extruder.
2. To extract water-extractable AXs (WEAX) from the extruded samples.
3. To characterise the sugar composition and molecular weight of purified arabinoxylans using HPLC.
4. To study the immunomodulatory potential of purified AXs via measuring nitric oxide production and tumour necrosis factor alpha (TNFα) secretion.
5. To determine AXs mechanism of action by inhibiting TLR4 receptor and monitoring the TNFα and nitric oxide secretion from both monocytes and macrophages.

Chapter 2 Literature review

2.1 Rice production

Rice (*Oryza Sativa*) and wheat (*Triticumaestivum L*) are major crops cultivated globally. The production of rice is 495.2 million tons, which feeds nearly 50% of the world population (Food and Agriculture Organization, 2016). Rice bran is the by-product of rice milling and it is mainly used for animal feed. However, it has been under-utilised as human food. Rice bran is rich in antioxidants and phytochemicals such as ferulic acid and tocopherols, some of which have additional bioactive/immunomodulatory properties (Spears *et al*., 2004).

2.2 Rice structure

Rice belongs to the *Poaceae* family and it is a monocot (Chase and Reveal, 2009). The rice grain (Figure 2-1) comprises embryo and bran (8-12 %), starchy endosperm (70-72 %) and hull (20 %) (Gujral *et al*., 2012). Rice bran alone comprises approximately 5 % of the rice grain.

Rice bran is composed of aleurone, pericarp, testa, germ and some endosperm (Saunders, 1985). The macronutrient content of rice bran is typically 7-10 % ash, 15-20 % lipids, 7-11% fibre, 12-16% protein and 34-52% carbohydrate (McCaskill and Zhang, 1999).

Recently, rice bran has attracted a great deal of attention from the immunologists and chemists because it might possess immunomodulatory and anti-cancer properties. The bioactive polysaccharides of rice bran appear to evoke many physiological responses thought to prevent different diseases including, heart disease, hypercholesterolemia, kidney stones and cancer (Jariwalla, 2000; Wang *et al*., 2008). It has been reported that the structural features of polysaccharides from different sources have various immune-modulating activities (improve the host defence against invasive viruses, fungi and bacteria) *in vitro* (Wang *et al*., 2008).

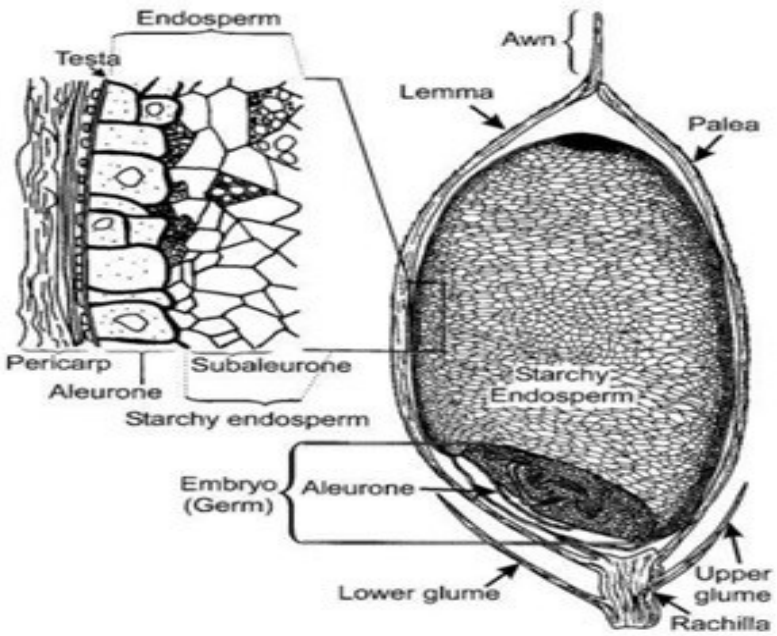

Figure 2-1 Rice grain structure
Source: (Champagne, 2004)

One of the most abundant classes of polysaccharides in plant cell walls are hemicelluloses. Hemicelluloses are usually attached to other cell wall materials such as pectin, lignin and cellulose (Peng *et al*., 2012). These cell wall polysaccharides are characterised by having β-(1 → 4)-linked bases of xylose, mannose, or glucose (Gabrielii *et al*., 2000). Hemicelluloses can be extracted by either water or, more frequently, aqueous alkali from plant tissue (Peng *et al*., 2012). It has been reported that Rice bran hemicelluloses have a notable effect in reducing thymus atrophy, improving the sensitivity of insulin, increasing the peripheral blood lymphocytes and improving immune function (Tzianabos, 2000; Peng *et al*., 2012).

2.3 Wheat production worldwide

Wheat (*Triticumaestivum L*) is an international staple food with a global production of 712.7 million tons (Food and Agriculture Organization, 2016). Wheat provides around 20% of the calories and 55% of the carbohydrate consumed globally (Safa and Samarasinghe, 2013).

2.4 Wheat structure

The wheat kernel (Figure 2-2) is composed of 80-85% endosperm, 2-3% germ and 13-17% bran (Šramková *et al*., 2009). The primary constituent of wheat is carbohydrate, which makes up about 72 % of the kernel by weight. The carbohydrate in the wheat kernel is composed of starch, cellulose and hemicellulose.

Starch is the most abundant carbohydrate found in cereals. There are two major structural forms of starch; amylopectin and amylose. Amylopectin is made of glucopyranose monomers connected through α-D-(1-4)-glycosidic bonds, which branch every 20 to 25 glucopyranose units to form α-D-(1-6)-glycosidic bonds. Conversely, amylose forms linear chains of glucopyranose units connected by the α-D-(1-4)-glycosidic bond (Izydorczyk and Biliaderis, 1995). There is another class of carbohydrates in addition to starch known as non-starch polysaccharides, which make up most of the cell wall material (CWM).

2.5 Non-starch polysaccharides

The non-starch polysaccharides are indigestible by human gut enzymes and are therefore referred to as dietary fibre. Non-starch polysaccharide makes up 75 % of the cell wall and are composed of glucomannan, (1-3) (1-4) β glucan, cellulose and arabinoxylans (pentosans) (JMares and Stone, 1973). Pentosans or arabinoxylans are the major hemicellulosic polysaccharides in cereals and they make up more than 80% of the NSP in wheat and 10% of rice bran (Malathi and Devegowda, 2001; Mansberger *et al*., 2014).

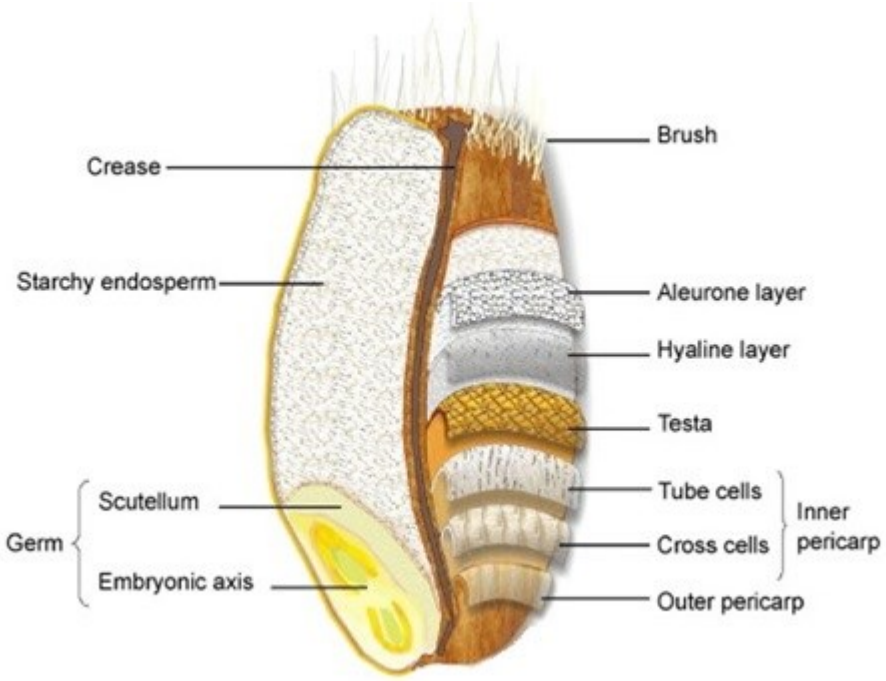

Figure 2-2 Schematic presentation of the wheat grain
Source: (Saulnier *et al*., 2007)

2.6 Arabinoxylans

Arabinoxylans are non-starch polysaccharides (Figure 2-3) composed of backbone chains of β-(1-4)-linked d-xylopyranosyl residues to which α-l-arabinofuranose units are linked as side chains in the second and/or third carbon-positions (Courtin *et al*., 2000; Roubroeks *et al*., 2000; Zhou *et al*., 2010).

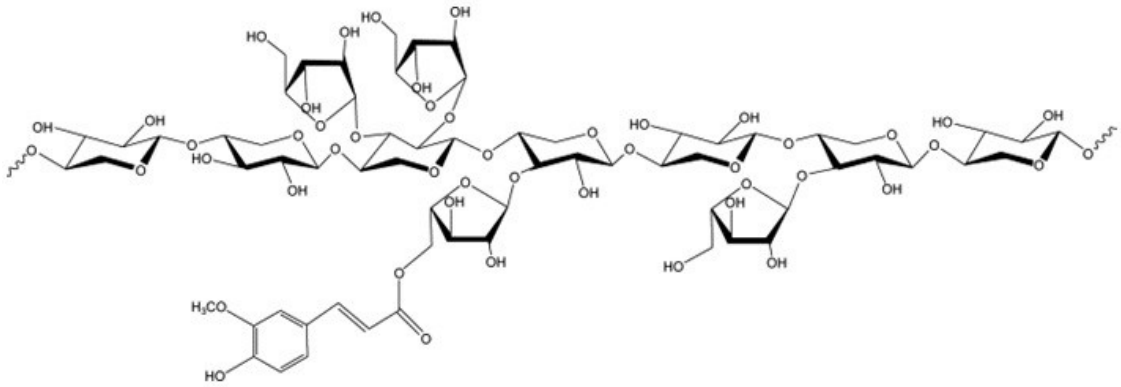

Figure 2-3 Arabinoxylans (AXs) structure
Source: (Garófalo *et al*., 2011)

AXs' structural characteristics are determined by the substitution of the xylopyranose linked xylan backbone. L-Arabinofuranose is the main substituent sugar and it can substitute xylopyranose residues at O-2 and/or O-3 via α-1, 2 and α- 1, 3 glycosidic linkages. This leads to three different forms namely, un-substituted xylopyranose, mono-substituted xylopyranose at O-2 or O-3 and di-substituted xylopyranose at O-2 and O-3 (Izdorczyk and Biliaderis, 1995; Saulnier *et al*., 2007; Brokaert *et al*., 2011). On the other hand, arabinofuranose substitutions can form short oligosaccharide side chains and comprise two or more arabinofuranose residues (Figure 2-4) (Izdorczyk and Dexter, 2008).

Brokaert *et al*. (2011) have reported that D-glucose, D-galactose, glucuronic acids and acetyl groups are substituted at O-2 and/or O-3 of the xylan backbone. This structural diversity of arabinoxylan can vary between cereals due to the complexity of tissue components within cereal grains (Dervilly-Pinel *et al*., 2001; Zhang *et al*., 2015). Ordaz-Ortiz and Sulnier (2005) have reported that arabinoxylan content is different between wheat endosperm, bran and husks with various arabinose to xylose ratios and the yield will depend on the method of extraction.

There are many techniques available for AXs extraction and different extraction methods will give different yields and range of degrees of branching, molecular weight distribution and tertiary conformation (Lu *et al*., 2005), i.e. hot water extraction (Izydorczyk *et al*., 1998; Cyran *et al*., 2003; Iqbal *et al*., 2011) and ultrasound-assisted enzymatic extraction (Wang *et al*., 2014).

AXs can be classified, according to their solubility in water, as either water-unextractable AXs (WUAX) or water-extractable AXs (WEAX) (Moers *et al*., 2005). The structure of WUAX is somewhat different from that of WEAX, WUAX will not solubilise in water, however, it will be solubilised in alkaline solutions (Gruppen *et al*., 1993).

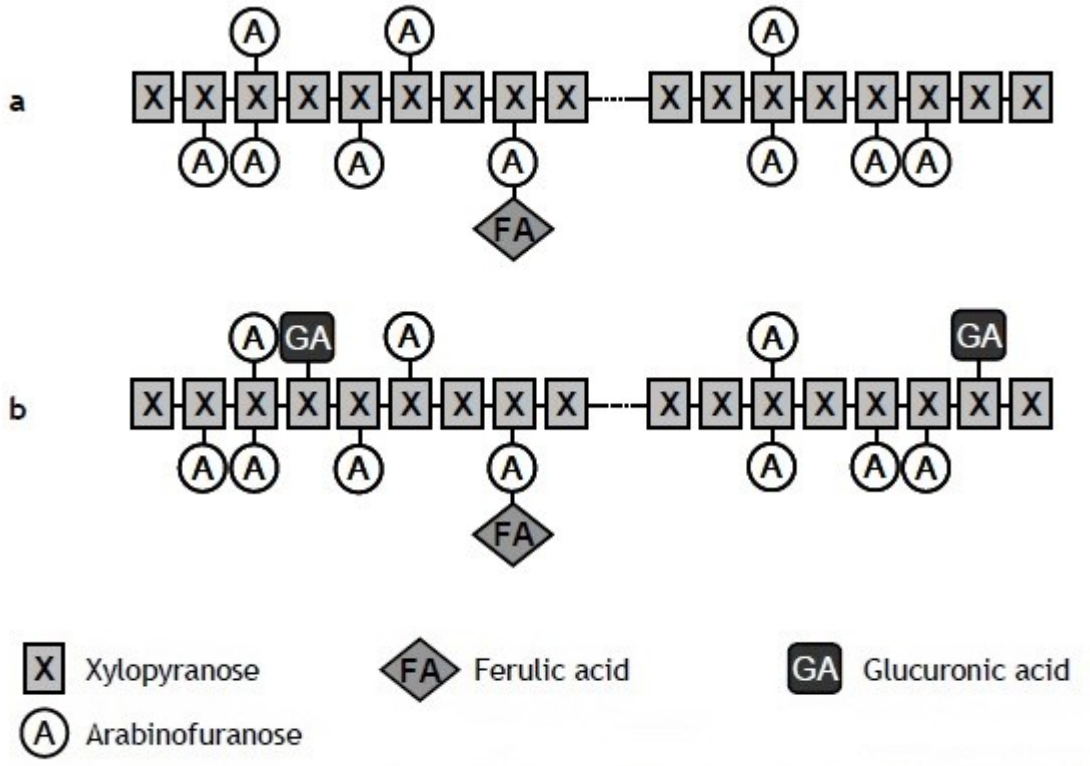

Figure 2-4 Simplified schematic representation of arabinoxylans AXs (a) Wheat flour and (b) Wheat bran
Substituents above and below the backbone represent C (O)-2 and C (O)-3 positions, respectively, based on data from (Izydorczyk and Biliaderis, 1995; Edwards *et al*., 2003).

2.7 Arabinoxylan solubility in cereals and cereal by-products

As mentioned previously, AXs in cereals and cereal by-products can be classified into water-extractable (WEAX) and water un-extractable (WUAX) (Moers *et al*., 2005). It has been reported that arabinoxylans in rye are part of the cell wall material and is bound covalently and non-covalently to other cell wall materials such as proteins, cellulose or lignin (Fengler and Marquardt, 1988). In contrast, AXs in wheat are loosely bound to the surface of the cell wall (JMares and Stone, 1973). Sasaki (2000) suggested that the difference in water extractabilty of AXs in cereals is due to the degree of cross-linking with other cell wall materials. These cross-links can be covalent ester bonds between the carboxylic acid group of uronic acids and AXs hydroxyl groups or diferulic acid bridges between adjacent AXs chains (Gruppen *et*

al., 1992; Fry, 2004). It was reported that wheat endosperm contains between (31-111 mg/100g) ferulic acid (Michniewcz *et al*., 1990; Hüseyin, 2015), whereas, rice bran contains 303 (mg/100g) (Jung *et al*., 2007). It also has been reported that ferulic acid side chains are esterified to some arabinose residues (Snelders *et al*., 2013). Moreover, these cross-links make extraction of arabinoxylans difficult and there is a need to use other treatments such as enzyme treatment, alkali solutions or mechanical treatments to effectively remove the arabinoxylans from what is a very stable network of covalent and non-covalent cross-links (Courtin and Delcour, 2001; Jacquemin *et al*., 2012). Moreover, the low solubility of AXs might be due to the close packing of the cell content which is proposed to be due to steric hindrance (Faulds *et al*., 2006).

Previous studies (Table 2.1) have shown that the percentage of WEAX is generally far lower than the WUAX in cereals or cereal by-products. Therefore, increasing and improving WUAX solubility has been very important for those who are interested in converting WUAX to WEAX. It has been reported that treating WUAX with alkali resulted in releasing WUAX from cell wall material due to the breaking up of bridges between the AXs and the covalent bonds and hydrogen atoms of the cell wall material (Gruppen *et al*., 1991). In a later study carried out by Courtin and Delcour (2001), the possibility of increasing the extractability of AXs from wheat using enzymes was investigated. WUAX treated with endoxylanases resulted in an increase in the solubility of AXs due to the degradation of the xylan backbone. Additionally, this resulted in a reduction in the molecular weight of the extracted AXs fraction (Li *et al*., 2013). There is a limit to the increase in solubility from treating with endoxylanase due to the branched sections, which are not affected by the endoxylanase treatment. On the other hand, several reports show that AXs' solubility depends on the AXs' degree of branching (Maes and Delcour, 2001; Mandalari *et al*., 2005). AXs with high arabinose substitution have higher solubility in water and vice versa.

Table 2.1 Water extractable and water-unextractable AXs in some cereals (dry weight basis) weight basis

WEAX and WUAX in some cereal grains and cereal by-products (dry weight basis)					
Cereal	Tissues	Total AXs %	WEAX %	WUAX %	References
Rice	Bran	4.84-5.11	0.35-0.77	4.34-4.49	Hashimoto *et al*. 1987 (b)
	Bran	8.5	0.2	8.3	Choct, 1997
	Hulls	8.36-9.24	0.11	8.25-9.13	Hashimoto *et al*. 1987 (b)
	Whole grain	2.64	0.06	2.58	Hashimoto *et al*. 1987 (b)
Wheat	Bran	25	1	24	Hollmann and Lindhauer 2005
	Bran	19.38	0.88	18.5	Hashimoto *et al*. 1987 (a)
	Flour	1.37-2.06	0.54-0.68	0.83-1.38	Izydorczyk *et al*. 1991
	Whole grain	5.77	0.59	5.18	Hashimoto *et al*. 1987 (a)
	Whole grain	8.1	1.8	6.3	Choct, 1997
	Pollard	21.8	1.1	20.8	Choct, 1997
Rye	Whole grain	8-12.1	2.6-4.1	5.4-8	Hansen *et al*. 2003
	Flour	3.2-3.64	2.2-2.65	0.99-1	Cyran *et al*. 2003
	Whole grain	8.9	3.4	5.5	Choct, 1997
Corn	Bran	29.86	0.28	29.58	Hashimoto *et al*. 1987 (b)

2.8 Arabinoxylan extraction

Most of the AXs in the intact cell walls of cereals are cross-linked with other cell wall materials to form a structural complex, which is not soluble in water. Therefore, there is a need to increase the extraction yields through improving the solubility of the WUAXs fraction (Gruppen *et al*., 1991). Several methods have been developed, investigated and reported for the extraction and purification of AXs (Courtin and Delcour, 2001). These include water extraction (Hollman and Lindhauer, 2005; Li *et al*., 2013) enzyme hydrolysis (Beaugrand *et al*., 2004; Li *et al*., 2013), acid and alkaline extraction (Gruppen *et al*., 1991; Maes and Delcour, 2001; Hollman and Lindhauer, 2005; Due *et al*., 2009; Zhou *et al*., 2010) and mechanically assisted treatment in the form of extrusion (Jeon *et al*., 2014; Zeitoun *et al*., 2010). The literature relating to water and mechanically assisted treatment in the form of extrusion is introduced below, with other alkaline and enzyme extraction methods outside the scope of the present work.

2.8.1 Water Extraction

Extraction of AXs using water is one of the most common methods used to isolate AXs from different cereals, followed by precipitation with 65% ethanol (Izydorczyk and Biliaderis, 2007; Ganguli and Turner, 2008). Using water alone to extract AXs has several advantages such as it is environmentally friendly, cheap, available and the product edible. It was reported that the extraction yield of WEAX from two barley cultivars varied depending on barley subfraction and varieties (Fleury *et al*., 1997). In (2003) Cyran *et al*. reported the extraction yields of WEAX in rye flour were 1.1-1.4 % at 4 °C, 0.17-0.33% at 40 °C and 0.41-0.51% at 100 °C. It has been reported that the yield of AXs achieved by the enzymatic and chemical methods is higher than that achieved by the water method. It is suggested that combining water with gentle conditions (i.e. a temperature below 100 °C) is not sufficient to break the cross-linkages between arabinoxylan and the cell wall matrix (Zhang *et al*., 2014). To counter this, water extraction combined with hydrothermal techniques have been developed, using high pressure (5-40 MPa) and high temperature at (200-600 °C), to increase the extraction yield of hemicelluloses to 65-90% (Mok and Antal, 1992; Garrote *et al*., 1999; Josefsson *et al*., 2002; Peterson *et al*., 2008). However, it has been indicated that although these techniques are environmentally friendly, they are degrading the hemicellulose structure, which may damage the structure of the arabinoxylan, which in turn may affect its functionality (Josefsson *et al*., 2002). As a significant amount of AXs remain after water extraction, some researchers have dismissed water extraction methods in preference to enzyme and alkali treatments (Hollman and Lindhauer, 2005).

2.8.2 Alkaline extraction

This method of extraction involves disrupting covalent and hydrogen bonds in the matrix of polysaccharides to liberate various polysaccharides from the cell wall (Zhang *et al*., 2014). Hydrogen bonds between hemicellulose and cellulose can be disrupted by hydroxyl ions, and hydrolysis of the ester linkages, which in turn solubilises part of the hemicellulose material (Cyran *et al*., 2003). On the other hand, under alkaline conditions, uronic acids change to their negatively charged form,

causing repulsion between different molecules, which results in an increase in the extractability the arabinoxylan present (Zhang *et al*., 2014).

There are several techniques developed to extract AXs using alkaline solutions. The first alkaline solution used to release WUAX from cereals was barium hydroxide, which was introduced by Gruppen *et al*. (1991); barium ions forms insoluble complexes with β glucans, resulting in release of 80% of the WUAX from wheat flour. Additionally, Bergmans *et al*. (1996) reported that 50% of WUAX was extracted from wheat bran using this technique.

On the other hand, dilute alkaline solutions such as hydrogen peroxide (H_2O_2) have been used to extract WUAX, resulting in yields of around 69%, of the total AXs content, from wheat bran (Maes and Declour, 2001).

Table 2.2shows the extraction yield of AXs and hemicelluloses using different alkaline solutions.

Although alkaline extraction yield is higher than the water extraction, alkaline extraction has been reported to affect the molecular structure of arabinoxylans due to the disruption of cross-linkages, resulting in different molecular structures in WUAX than that would occur naturally, which in turn result in different functional characteristics (Zheng *et al*., 2011).

Table 2.2 AXs and hemicelluloses extraction yield

Sources	Extraction	Yield% of AXs[a]/hemicellulose[b]	A/X	References
Wheat bran	0.5% H2O2 (0.15 M NaOH v/v)	18.5[a]	0.8	Zhou *et al*.,2010
Wheat bran	0.44 M NaOH (pH 12.5)	34.30[a]	0.54	Aguedo *et al*., 2014
Wheat endosperm	4.27 M KOH	56[a]	0.91	JMares and Stone, 1973
Rice straw	1% NaOH followed by 0.5% H2O2 (pH 11.5)	18.6[b]	/	Sun *et al*., 2005
Rice straw	0.25 M NaOH (1:25 w/v)	0.37[a]	0.36	Etanol, 2016

2.8.3 Enzymatic extraction

In order to extract AXs from cereals, enzymatic techniques are often used. The most common enzyme family used for isolating AXs are the GH 11 endo-β-(1,4)- xylanases (Beaugrand *et al*., 2004; Escarnot *et al*., 2012) Endoxylanases penetrate the cell wall, then cleave internal β-(1,4)- bonds thus solubilising the AXs (Andersson *et al*., 2003; Swennen *et al*., 2006; Li *et al*., 2013). Table 2.3 AXs extraction yield from some cereals and cereal by-products by enzyme extraction.

Table 2.3 AXs extraction yield from some cereals

Sources	Extraction	Yield% of AXs	Ar/Xy	References
De-starched wheat bran	Xylanase 150 units, lab-scale	12.4	0.56	Zhou *et al*., 2010
De-starched wheat bran	Pentopan mono BG 0.75% , lab-scale	15.28	na	Zhang *et al*., 2008 a
Rye flour	20 U β-glucosidase, 250 U (1-3,1-4)-β-glucan 4-glucano-hydrolase and 400 U amyloglucosidase, lab scale	1.08	0.5	Dervilly-Pinel *et al*., 2001

Comparing the extraction yields of enzyme treatments and alkaline treatments (Tables 2.2 and 2.3), shows lower extraction yields of AXs from the enzyme treatments, for example, the extraction yield from de-starched wheat bran extracted with xylanase is 12.4% (Table 2.3), lower than that achieved with alkaline sodium hydroxide at 34.3% (Table 2.2). Zhang *et al*. (2014) suggested that the low extraction yield might be due to the existence of enzyme inhibitors and the crystalline structure of lignocellulose might limit the hydrolysis.

Although, the extraction yield using enzyme hydrolysis is not as high as the alkaline extraction, the action of alkaline solutions is not environmentally friendly as it produces hazardous waste and it might release the ferulic acid due to the breaking of the ester bond between AXs and the ferulic acid side chain, resulting in loss of antioxidant functionality (Zhou *et al*., 2010). Ferulic acid is known for its low solubility in water (Anson *et al*., 2009). Zhou *et al*. (2010) indicated that AXs extracted with enzymes have higher ferulic acid content and it enhance the immune response more than AXs extracted with alkaline in an *in vivo* trial.

2.8.4 Mechanical extraction

There are several mechanical technologies that have been studied as pre-treatments to improve the extraction yield of dietary fibre in general and specifically AXs. These include techniques such as microwave irradiation, steam explosion and extrusion (Cara *et al*., 2006; Rose and Inglett, 2010; Zeitoun *et al*., 2010).

2.8.4.1 Microwave assisted extraction

Microwave irradiation has been investigated as a technique to improve the extractability of hemicelluloses. There are several advantages of using microwave irradiation in the extraction of AXs, which are the ability to reach high temperatures and shorter extraction times (Roos *et al*., 2009). Microwave irradiation causes vibration between molecules, which potentially ruptures bonds. Rose and Inglett (2010) optimised the processing conditions for arabinoxylan extraction from maize; it was found that 50% of the AXs could be extracted at 200 °C for 2 minutes or 180 °C for 10 minutes. It was also reported that a combined microwave/pressure treatment of corn pericarp increased the extraction yield of AX to 70.8% of total carbohydrates, consisting mainly of xylo-oligosaccharides. This high extraction rate can be achieved under pressurised water at 170.5 °C, solid:liquid ratio 1:20 (g/ml), 2 min to reach operating temperature and 16 minutes heating time, respectively (Yoshida *et al*., 2010).

2.8.4.2 Steam explosion treatment

This method has been studied to increase the extractability of arabinoxylans from cereal materials and it is also an effective pre-treatment method to break down the lignocellulosic structure and is environmentally friendly (Avellar and Glasser, 1998). The principle of this treatment is to put the sample under great pressure in a vessel in the presence of steam and the sample is then fired into a low-pressure area causing the explosive depolymerisation, which breaks the lignocellulosic structure making hemicelluloses more easily extracted (Cara *et al*., 2006). Krawczyk*et al*. (2008) isolated AXs from barley husks using steam explosion pre-treatment followed by ultrafiltration and diafiltration steps. AXs made up 35% of the polysaccharide fraction after steam explosion and ultrafiltration and this increased to 45% after diafiltration. Moreover, Sun *et al*. (2005) extracted 20.5-28.5% of the total hemicelluloses by pre-treating wheat straw with steam explosion. His technique included increasing the temperature to (220 °C at 22 bar for 3-8 min, or 200 °C at a pressure of 15 bar for 10-33 min).

2.8.4.3 Extrusion pre-treatment

Extrusion cooking is a valuable short-time, shear force, high-temperature, high pressure, processing technique, which has been used since the 1930s for the production of textured foods, ready-to-eat snacks, baby foods and breakfast cereals (Burtea, 2001; Kim *et al*., 2006; Masatcioglu *et al*., 2014). Extrusion cooking improves the bioavailability of nutrients and the digestibility of protein and starch in comparison to other conventional cooking techniques (Gu *et al*., 2008; Singh *et al*., 2010; Dehghan-Shoar *et al*., 2011).

Extrusion cooking technology has been used as a pre-treatment to extract hemicelluloses and increase the solubility of dietary fibre from wheat bran, pea hulls, lemon fibre, waxy barley and corn fibre (Ralet *et al*., 1990; Ralet *et al*., 1993; Ralet *et al*., 1994; Vasanthan *et al*., 2002; Singkhornart *et al*., 2013). Wang *et al*. (1993) have reported that there is a significant increase in soluble dietary fibre from 1.25% in raw

whole wheat grain to 2.19% in samples extruded at 400 rpm. Moreover, a higher increase in solubility was reported from 1.75% in raw samples to 2.47% in extruded wheat bran samples at 400 rpm. Zhang *et al*. (2011) have investigated the effect of temperature changes on dietary fibre solubility of oat bran. He reported that at a fixed screw-speed of 50 rpm and 10% feed moisture, the solubility of dietary fibre increased from 9.9% to 14.2% when the temperature increased from 100^{o}C to 140^{o}C.

However, there are conflicting findings on the effect of extrusion on the solubility of dietary fibre. Camire *et al*. (1993), reported that at a constant screw-speed of 300 rpm, the solubility of dietary fibre from potato peels decreased from 4.69 % (at 104^{o}C barrel temperature and 31% feed moisture) to 3.75% (at 143^{o}C barrel temperature and 36% feed moisture). In addition, Zeitoun *et al*. (2010) compared twin-screw extrusion and stirred reactor extraction for hemicellulose extraction from wheat bran. She reported that extrusion pre-processing decreased the soluble hemicellulose from 59%, using a stirred reactor, to 24%. There are conflicting findings about the extrusion effect on dietary fibre/AXs solubility, which are summarised in Table 2.4

Interestingly, extrusion not only has an effect on the solubility of dietary fibre, it also has an effect on molecular weight. The effect of extrusion on molecular weight has been investigated by Ralet *et al*. (1991), who found that extrusion cooking reduced the molecular weight (Mw) of hemicelluloses extracted from sugar beet pulp fibre resulting in an increase in water solubility. In another study, Margareta and Nyman (2003) reported that extrusion of vegetables reduces the dietary fibre Mw, which was suggested to be due to the breaking of dietary fibre glycosidic bonds, resulting in depolymerisation of the dietary fibre.

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Table 2.4 Main findings of the effect of extrusion of dietary fibre and AXs solubility

Food Source	Extrusion conditions	DF/ AXs change	References
Corn fibre	Twin-screw extruder, L/D ratio 24:1, 3.0 mm die diameter, moisture; 30,40 and 50%, fixed screw-speed 200 rpm and feed rate 9 kg/h	Soluble AX increased from 463 g/kg to 530 g/kg and 586.3 g/kg at 30 and 40% moisture	Singkhornart *et al*., 2013
Wheat straw and wheat bran	Twin-screw extruder, barrel length 1.6m, liquid/ solid ratio 7, feed rate 13.8 kg/h, screw-speed 150 rpm and fixed temperature at 50 °C	Hemicelluloses extraction yield decreased from 59% using stirred reactor to 24% using an extruder. Xylan % decreased from 76% using stirred reactor to 53% using extruder	Zeitoun *et al*., 2010
Rice bran, oat bran, and wheat bran	Twin-screw extruder, barrel diameter 62.2 mm, fixed temperature 160 °C, feed rate 68 kg/h for oat and rice bran and 51.25 kg/h for wheat bran, screw-speeds 50, 70 and 100 rpm	In oat bran, soluble DF increased from 3.45% WE to 5.46, 5.24 and 4.58% at 50, 70 and 100 rpm respectively. In rice bran, solubility increased from 2.0% to 2.5, 2.33 and 2.01% using screw-speeds at 50, 70 and 100 rpm. In wheat bran, increasing in solubility from 3.11% to 3.45 and 3.35% at 70 and 100 rpm while there was a decrease at 50 rpm to 2.97%	Gualberto *et al*., 1997
Whole wheat and germinated wheat	Twin-screw extruder with CO2 injection, L/D ratio 24:1, and die diameter 3.00 mm, extrusion carried out at 90 °C and 130 °C, screw-speeds were 150 and 200 rpm at constant moisture feed at 30%. CO_2 injection rate was 500 ml/min and inlet pressure 20 MPa	AXs solubility decreased from 2.37 in whole wheat to 1.91 and 1.65% at 150 and 200 rpm screw-speed respectively. In germinated wheat solubility decreased as well from 2.64 to 2.28 and 2.09% at 150 and 200 rpm respectively	Singkhornart *et al*., 2014
Wheat bran	Twin-screw extruder with a screw diameter of 57 mm, L/D ratio 24:1, moisture 20,25 and 30%, die temperature 165, 175 and 185 °C, screw-speeds at 180, 190 and 200 rpm	The solubility of dietary fibre at 175 °C temperature, 25% moisture and 200 rpm screw-speed was 11.75% in comparison with 2.54% for untreated wheat bran	Long *et al*., 2014
WE: without extrusion, DF: dietary fibre			

Reducing the molecular weight of the AXs not only increases their solubility in water but also increases their biological health benefits (Li *et al*., 2013). Recently, pronounced effects of low Mw arabinoxylans (66 kDa) have been observed to have a higher prebiotic stimulation in an *in vitro* study when compared to higher molecular weight (Mw) AXs (Hughes *et al*., 2007). Modification of the molecular characteristics of arabinoxylans such as Mw is important to achieve the optimum prebiotic, anti-tumour activities and immune stimulation (Li *et al*., 2013).

2.9 Immune system

AXs are of significant importance to human health because of their modulatory effect on the immune system (Li *et al*., 2013). The immune system of the human body consists of a complex network of molecules, cells and organs that interact and communicate together to respond to the invasion of pathogens and maintain the body's homeostasis. The immune system consists of innate immunity, which is a rapid and stereotyped response, and adaptive immunity, which is slower than the innate response and arises in response to a stimulus. The innate immune response operates in conjunction with an adaptive immune response through activation of signalling pathways. Figure 2-5 gives an overview of the interactions between the innate and adaptive immune systems (Cruvinel *et al*., 2010).

2.9.1 Innate immune system

The innate immune system is the first line of defence in the human body. It is composed of three stages; the first stage is the chemical and physical barriers while the second stage depends on cell-intrinsic mechanisms (Mogensen, 2009). The host cells can prevent viral infections by destroying the nucleic acid of the invading visruses and secrete digestive enzymes that destroy pathogenic invaders (Medzhitov and Janeway, 2000). The third stage of defence relies on recognising preserved pathogen features (pathogen-associated immune-stimulants) by the complement system and phagocytosis by immune cells such as natural killer cells, neutrophils and macrophages. Pathogen immune stimulants are referred to as pathogen-associated molecular patterns (PAMPs) and they include pathogen cell wall polysaccharides such as chitin and mannan from fungi, lipopolysaccharides (LPS) from gram-negative bacteria and peptidoglycan from gram-positive bacteria (Volman *et al*., 2008; Kawai and Akira , 2010; Wiersinga *et al*., 2014).

Interestingly, AXs may contain similar structures to PAMPs after modification using chemicals, enzymes or by extrusion cooking. PAMPs are well studied especially lipopolysaccharides (LPS). LPS has the ability to initiate the host defence through recognition of its bioactive component, lipid A, via co-receptor MD-2 and toll-like

receptor 4 (TLR4) (Saitoh *et al*., 2004; Ohto *et al*., 2012; Kang *et al*., 2016). The structure of lipid A is composed of six fatty acyl chains, 1 and 4 phosphate groups and a di-glucosamine backbone (Maeshima and Fernandez, 2013). AXs and di-glucosamine in lipid A share some structural similarities since both of them contain sugar monomers with 1-6 glycosidic linkages (Park *et al*., 2009).

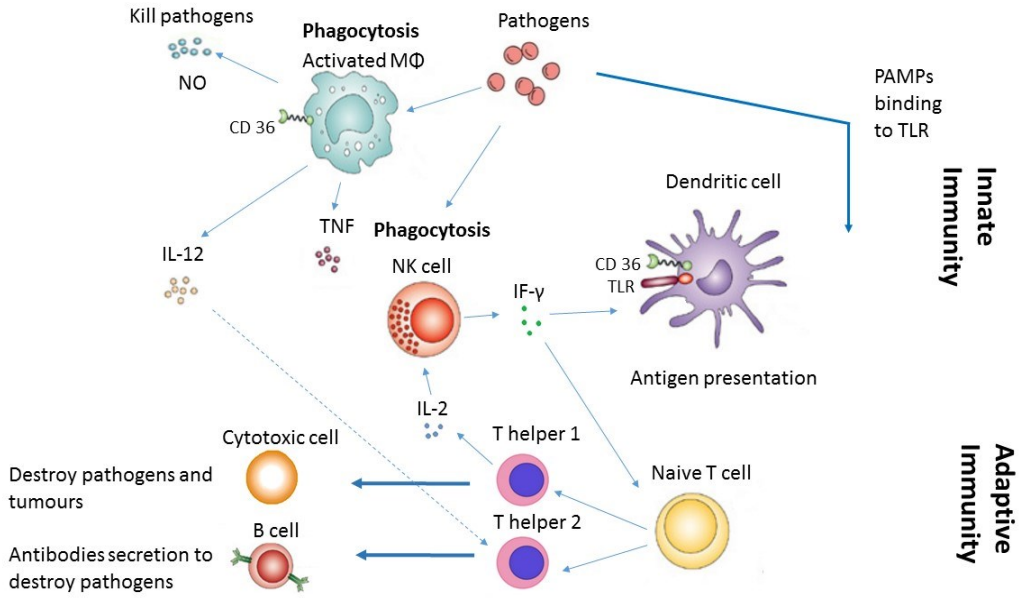

Figure 2-5 Simplified overview of the immune system
IL- interleukin, TNFα tumour necrosis factor α, IFγ- interferon γ. Adapted from (Dranoff, 2004; Stevenson and Riley, 2004).

The complement system is part of the innate immune system. It is responsible for enhancing the ability of phagocytic cells to clear damaged cells and microbes from the system. Three pathways have been identified (classical, alternative and lectin complement pathways) (Takahashi *et al*., 2008). All three pathways have the ability to activate the key component (1-3) referred as C3. This activation is very critical for the complement reaction as it triggers the inflammatory response, which in turn activates the rest of the components from (5 to 9) (Ali *et al*., 2012).

The activation of the lectin pathway is through binding fucose and mannose sugars of the bacterial cell wall with the serum protein lectin. This activation triggers a cascade of events that leads to the activation and recruitment of other innate

immune cells. The PAMPs from the invaders bind to PRRs (pattern recognition receptors) which are displayed on host immune cells (Takeuchi and Akira, 2010). An example of PRRs is the Toll-like receptors (TLRs) which are found on the surface of phagocytes (dendritic cells, neutrophils and macrophages). For example, Toll-like receptor-4 (TLR4) activates the innate immune response through recognition of lipopolysaccharide (LPS) from the cell wall of gram-negative bacteria. Hence, understanding the PAMPs' molecular structure, which is recognised by PRRs, is very important to understand the immunomodulatory response of arabinoxylans (AXs). Post activation, neutrophils, dendritic cells and macrophages secrete cytokines to communicate with other cells in the immune system and to stimulate the immune response. On the other hand, activation of the innate immune cells produces digestive enzymes and reactive oxygen radicals that destroy pathogens (Takahashi *et al*., 2008). Furthermore, dendritic cells play an important role in transferring ingested pathogens to the lymph nodes to activate T lymphocytes, thereby initiating a specific immune response that is part of the adaptive immune system (Kim *et al*., 2006).

2.9.2 Adaptive immune system

Adaptive immunity protects the human body from certain death by infection. Once the innate response is initiated, it calls the adaptive immune system into play, then both work together to eliminate pathogens (Warrington *et al*., 2011). Unlike the innate immune response, the adaptive immune response is slow but highly specific against pathogens and its protection is long lasting (Cooper and Alder, 2006). It is pointless to mount the adaptive immune response against harmless foreign molecules, otherwise the adaptive immune response might be deleterious. This is normally avoided because the adaptive immune response is triggered by the innate immune system only when the latter one recognises molecules of the attacking pathogens (PAMPs) (Bonneaud *et al*., 2003).

Dendritic cells, also known as pathogen-presenting cells, have a surface that is packed with PRRs, which bind to the PAMPs of foreign pathogens and initiate phagocytosis (Visintin *et al*., 2001). The dendritic cells with the ingested pathogens

then move to a peripheral lymphoid organ or to a nearby lymph node where the dendritic cells present the antigens of the ingested pathogens to T lymphocytes (Cravens and Lipsky, 2002). The cell surface of T cells are full of various receptors that recognise extraneous antigens, which can be foreign polysaccharides or large proteins (Caramalho *et al*., 2003). In addition, to complete the activation of the T cell, a co-stimulatory signal is sent from the dendritic cell, resulting in the proliferation of T cells with the same receptor, thereby inducing antigen-specific adaptive immune responses (Chen and Flies, 2013). To eliminate the pathogen at the infection site, T cells mature and differentiate into different types of effector T cells including cytotoxic, helper and regulatory T cells. Cytotoxic T cells have the ability to eliminate pathogens that proliferate inside the host cell. Helper T cells can release cytokines that guide dendritic cells to stay in their active form and they can stimulate antibody production from B cells that kill pathogens (Rissoan *et al*., 1999). Helper T cells can also express co-stimulatory proteins on their surface and release cytokines to activate more cytotoxic T cells and macrophages (Croft, 2003). The regulation and control of the activated immune cells is regulated by regulatory T cells, which can inhibit the activity of cytotoxic T cells, helper T cells, and dendritic cells to avoid autoimmune responses (Sakaguchi *et al*., 2008). Activated B cells secrete serum proteins and synthesize antibodies that bind directly to the pathogens to inactivate them. They also recruit innate immune cells to eliminate the invaders (Clark and Ledbetter, 1994).

2.9.3 Monocytes

Monocytes are leucocytes circulating in the blood. Monocytes can express CD11b and Toll-like receptor-4 (TLR4) associated with CD14, which are triggered by LPS from the cell wall of gram-negative bacteria (Taylor *et al*., 2005). Monocytes originate from haematopoietic stem cells in the bone marrow. Activation of these stem cells results in differentiation to common myeloid progenitors (CMPs) which differentiate further into macrophage and granulocyte progenitors (Ogawa, 1993). Prior to the transforming of haematopoietic stem cells into circulating monocytes, they undertake a series of divisions (Chow *et al*., 2011). Monocytes circulate in the blood

and have the potential to differentiate into dendritic cells or macrophages (Geissmann *et al*., 2010). Differentiation to macrophages requires the activation of runt-related transcription factor, which encodes the ETS family transcription factor PU.1 (Lawrence *et al*., 2011).

2.9.4 Macrophages MΦ

Monocytes circulate in the blood for up to two days, and then they migrate into tissues and differentiate into MΦ (Van Furth and Cohn, 1968; Geissmann *et al*., 2010). Macrophages and neutrophils are phagocytes and they have the ability to take up pathogens through a process known as phagocytosis which is engulfment of large particles > 0.5 μm into cells, through an actin-dependent mechanism (Silva, 2010). The phagocytosis recognition mechanisms in macrophages occur through PRRs that recognise PAMPs (Aderem and Underhill, 1999). Activation of MΦ results from PAMPs' recognition of gram-negative and gram-positive bacteria (Plüddemann *et al*., 2011). Macrophages have a range of PRRs including TLR, which are triggered by phagocytosis of the pathogens. This activation results in pro-inflammatory cytokine production including IL-23, IL-12, IL-6 and TNFα (Mosser and Edwards, 2008). Activated macrophages also express inducible nitric oxide synthase (iNOS), which is responsible for generating nitric oxide (NO), a key mediator for the killing of bacteria within macrophages (Adams *et al*., 1997).

2.9.5 PAMPs and PRRs

As mentioned previously, PAMPs are responsible for initiating the innate immune response through PRRs, of which the TLR family has been extensively investigated in recent years (Medzhitov, 2001; Akira *et al*., 2006). PAMPs share three characteristics, including being essential for pathogens, invariant in all pathogens and distinguishable from self (Mogensen, 2009). Upon recognition of PAMPs, PRRs at the cell surface send a signal to the host triggering antimicrobial and pro-inflammatory responses through activation of intracellular pathways including transcription factors and kinases (Akira and Takeda, 2004), resulting in activation of gene expression and the production of chemokines and cytokines which result in host response to infection.

2.9.6 Toll-like receptor family (TLRs)

The most investigated class of PRRs is the TLR family. Their name is derived from their homology to the Toll protein in *Drosophila melanogaster* (Medzhitov *et al*., 1997). The structure hallmark of all known TLRs is composed of a cysteine-rich domain in the extracellular part, leucine-rich motifs (LRR) and a cytoplasmic signalling Toll/IL 1 receptor (TIR) homology domain in the intracellular region (Gay and Keith, 1991; O'Neill and Bowie, 2007). The intracellular signal transduction is due to receptor oligomerisation that is induced by ligand binding to TLRs. To date, 10 TLRs have been identified in mammals and each of them recognises distinct PAMPs derived from bacteria, viruses, fungi and protozoa (Akira *et al*., 2006).

The TLRs include TLR1, TLR2, TLR4 and TLR6 that recognise lipoproteins such as Triacyl Lipopeptides and LPS, while TLR3, TLR7, TLR8 and TLR9 recognise nucleic acids such as dsRNA or ssRNA (Akira *et al*., 2006).

2.9.6.1 TLR4 structure and gene mapping

TLR4 is composed of two main domains; an intracellular 187-residue and an extracellular 608-residue domain (Medzhitov *et al*., 1997). However, it has been demonstrated that for ligand-induced activation, TLR4 transfection is not enough and TLR4 has to be associated with myeloid differentiation 2 (MD2) (Shimazu *et al*., 1999). Furthermore, CD14 and LPS binding protein assist the transfer of LPS monomers to TLR4 and MD2 (Zanoni *et al*., 2011). After LPS binding, adaptor proteins (TIR domains) in the cytoplasmic tail are recruited and the TIR interacts with the domains present on the adaptors. In general, TLR4 recognise LPS via the lipid A part (Poltorak *et al*., 1998a). Chromosomal mapping of TLR4 has been achieved for mouse and human in 1998; it was mapped to chromosome 9q32-33 in human and to chromosome 4 in mouse (Rock *et al*., 1998; Poltorak *et al*., 1998b). The LR4 mouse gene is 91.7 kb in size, while the human gene is only 19 kb, with the longer length of the mouse gene due to longer intronic sequences. Apart from that, they are structurally similar (Smirnova *et al*., 2000). The intracellular TIR domain is the signal-mediating fragment of TLR4, while the extracellular part has 9 N-linked glycosylation sites that are

essential to transport the protein to the cell surface (Rock *et al*., 1998; Da Silva Correia and Ulevitch, 2002).

2.9.6.2 TLR4 gene expression

TLR4 gene expression has been found in foetal ileum, foetal skin, heart, lung, epithelial cells, Kupffer cells, endothelial cells and many other cells and tissues (Medzhitov *et al*., 1997; Frantz *et al*., 1999; Su *et al*., 2000; Laflamme and Rivest, 2001;; Song *et al*., 2001; Holmlund *et al*., 2002). The regulation of TLR4 expression is crucial for the innate immune response; there are only up to 1000 TLR4 receptors on the cell surface and overexpression of the gene will lead to heart failure and hypersensitivity to LPS (Frantz *et al*., 1999). On the other hand, downregulation of the gene will lead to LPS tolerance (Medvedev *et al*., 2000).

2.9.6.3 TLR4 ligands

It has been suggested that LPS from gram-negative bacteria has the ability to mediate cytokine production, but it also works as a receptor for some ligands such as heat shock protein (hsp60) and it induces pro-inflammatory responses (Ohashi *et al*., 2000; Okamura *et al*., 2001; Smiley *et al*., 2001). In addition, TLR4 mediates maturation of murine β-D2- defensin and dendritic cells by LPS (Biragyn *et al*., 2002). TLR4 stimulators are illustrated in Table 2.5.

Table 2.5 Some exogenous and endogenous TLR4 stimulants

Stimulant	Source	References
Endogenous		
Surfactant protein A	Lung endothelium	Guillot *et al*., 2002
Hsp70	Cytoplasm	Asea *et al*., 2002
Fibrinogen	Serum	Smiley *et al*., 2001
Exogenous		
LPS	Cell wall of gram negative bacteria	Poltorak *et al*., 1998a
P fimbriae	Cell wall of gram negative bacteria	Frendéus *et al*.,2001
Glucuronoxylomannan	Yeast	Shoham *et al*., 2001

2.9.7 Lipopolysaccharides (LPS)

LPS is a major component of the cell wall of gram-negative bacteria (Guha *et al*., 2001), consisting of three parts; a polysaccharide side chain also known as O-antigen or O-chain, a non-repeating core polysaccharide and lipid A which is a hydrophobic part. The polysaccharide side chain and non-repeating core polysaccharide are projections from the surface while the hydrophobic domain is embedded in the outer membrane. The lipid A domain is a source of toxicity while the O-chains are easily detected by the host antibodies and to avoid detection they are often modified by bacteria (Lerouge and Vanderleyden, 2002; Miller *et al*., 2005). Lipopolysaccharide structure is illustrated in Figure 2-6. Low levels of LPS are sufficient to induce a substantial inflammatory response of the innate immune system. LPS binds to the LPS binding protein (LBP) in serum, before being transferred to CD14 then to MD2, which is associated with TLR4. The receptor complex then promotes the secretion of nitric oxide (NO) and pro-inflammatory cytokines such as TNFα and IL 8 in monocytes and macrophages (Johnson *et al*., 2002; Termeer *et al*., 2002; Miller *et al*., 2005).

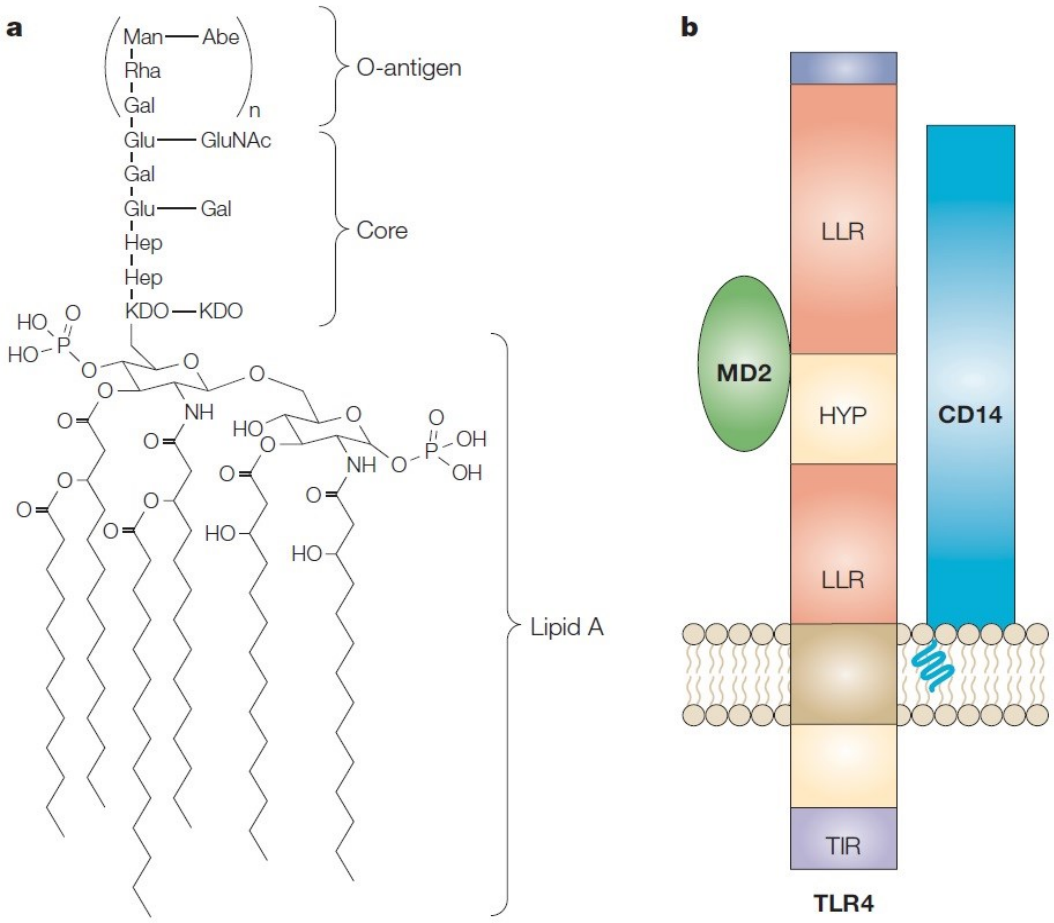

Figure 2-6 Lipopolysaccharide (LPS) structure
a. LPS lipid A, O-antigen and core oligosaccharide. b. TLR4-MD2-CD14 receptor complex (Miller *et al*., 2005).

2.9.8 Cytokines

Cytokines are small, soluble proteins that affect the function or growth of cells. Cytokines can act in a paracrine way (affect nearby cells) or an autocrine way (affect the same cell). However, some cytokines might have systemic effects such as IL-6, IL-8 and TNFα. The action of cytokines is on immune cells and in orchestrating immune system responses (Vilček and Feldmann, 2004).

2.9.8.1 Tumour necrosis factor alpha (TNFα)

TNFα is a pro-inflammatory cytokine and it has various biological effects. Local TNFα production is critical for the elimination of local infections (Motley *et al*., 2004). TNFα systemic release also plays a vital role in septic shock. Therefore, TNFα expression is

regulated on all levels; transcriptional, translational and post-translational. TNFα is released in macrophages and monocytes in response to foreign stimuli such as LPS from gram-negative bacteria. The secretion of TNFα from T-cells is initiated by activation of the T-cell receptor. In addition, natural killer cells and B-cells can produce TNFα (Eissner *et al*., 2000; Yu *et al*., 2009). There are two receptors that mediate the effects of TNFα, these are TNFR1 and TNFR2. The effect of TNFα on endothelial cells includes upregulation of leukocyte adhesion molecules that contribute to leukocyte recruitment. Moreover, TNFα is involved in apoptosis, differentiation and proliferation. It is also involved in inflammatory diseases such as rheumatoid arthritis (Motley *et al*., 2004).

2.9.8.2 Nitric oxide (NO)

Nitric oxide (NO) is a short-lived, gaseous, small molecule composed of one atom of oxygen and one atom of nitrogen, thus making it a free radical due to unpaired electrons (Pacher *et al*., 2007). In the human body, NO is defined as a product of macrophage activation by pro-inflammatory cytokines, microbial endotoxins such as LPS or both. NO is a product of l-arginine degradation, the reaction being catalysed by an enzyme called inducible nitric oxide synthase (iNOS) (Bogdan, 2001). This reaction requires several cofactors including calcium/calmodulin, flavin mononucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH4), oxygen and haem (Marletta, 1994; Baek *et al*., 1993). There are three known isoforms of NOS; their names come from where they were first found. Inducible NOS (iNOS) was found in macrophages, endothelial NOS (eNOS) found in endothelial cells and the neuronal NOS (nNOS) found in the brain. These isoforms are also known as NOS-2, NOS-3 and NOS-1 respectively (Alderton *et al*., 2001).

2.9.8.2.1 NO role in macrophages

NO production in macrophages depends on their activation in response to cytokine or bacterial endotoxin stimuli. Macrophages act as patrolling cells and produce low

levels of NO in quiescent conditions. However, once activated by stimuli, macrophages produce an excessive amount of NO, releasing NO_2- and NO_3-, which are important in scavenging pathogens (Marletta *et al*., 1988; Hibbs, 2002). Although NO production is critical for macrophage phagocytosis, an excessive amount of NO has been associated with high levels of necrosis and apoptosis (Sarih *et al*., 1993). In addition, it has also been reported that high levels of NO induce autoimmune reactions such as asthma and arthritis (Schmidt and Walter, 1994; Barnes and Liew, 1995). Therefore, it is crucial to modulate macrophage-produced NO.

2.10 Immunomodulating properties of food

The functionality of the immune system is crucial for protecting the body from the attacks of pathogens or cancer cell proliferation. Thus, it plays a vital role in health homeostasis. However, many factors disturb immune functions such as an unhealthy lifestyle, malnutrition, physical stress, disease and aging (Nahrendorf and Swirski, 2015). Evidence suggests ingestion of foods with immune-modulatory effects are able to prevent declining immune function or reduce the risk of infection (Kaminogawa and Nanno, 2004). There are many studies suggesting that food ingestion can improve depressed immune functions by moderating the severity of infectious diseases and reducing infection rates (El-Gamal *et al*., 2011). Thus, foods with the ability to improve immune responses, particularly in patients with impaired immunity such as cancer patients (Braga *et al*., 1996), are both clinically and commercially valuable. Cereals feed more than half of the world population; they are composed mainly of starch, protein, some minerals and non-starch polysaccharides that cannot be hydrolysed by human enzymes (Mohan *et al*., 2010). Arabinoxylans are the main non-starch polysaccharides of many cereals (Zhou *et al*., 2010). It has been reported that arabinoxylans possess immune-modulatory effects (Li *et al*., 2015).

2.11 Rice bran AXs (MGN-3/Biobran)

Biobran/MGN-3 is an arabinoxylan extracted from rice bran with modification by hydrolysing enzymes from shiitake mushrooms, and its structure is composed of a xylose backbone attached to arabinose monomers with a molecular weight of 30-50 kDa (Ghoneum *et al*., 2004; Pérez-Martínez *et al*., 2015). There are several *in vivo* and *in vitro* studies suggesting that MGN-3 is capable of enhancing the function of both innate and adaptive immune cells such as B cells, T cells, macrophages, natural killer cells and dendritic cells (Ghoneum and Brown, 1999; Ghoneum and Abedi, 2004; Ghoneum *et al*., 2004; Ghoneum *et al*., 2008; Cholujova *et al*., 2009). The mechanism of action of biobran/MGN-3 is not fully understood, however, it was suggested that modification of the long chain arabinoxylans is involved and that modifying them to low molecular weights is important in order for them to be taken up by M cells (microfold cells) in the Peyer's patches. In M cells, polysaccharides are transported to underlying immune cells (Samuelsen *et al*., 2011). Moreover, it was suggested that low molecular weight arabinoxylans can be transferred directly to the blood stream or can diffuse into the blood stream through the intestinal walls, and then transported to different immune cells residing in lymph nodes (Ghoneum and Jewett, 1999).

2.11.1 MGN-3 *in vitro* studies

Several studies have investigated the effect of MGN-3 on different cell lines. It has been reported that MGN-3 is able to transform human monocytes to immature dendritic cells in the presence of two cytokine solutions (LPS and IFNγ) and (IL-1β, TNFα and IL-6) (Cholujova *et al*., 2009). MGN-3 showed a substantial (190%) increase in phagocytosis by U937 macrophages. It has also been reported that MGN-3 is able to increase the IL-6 and TNFα in treated macrophages from U937 and RAW264.7 (murine macrophage cell line) (Ghoneum and Matsuura, 2004). MGN-3 effect on different cell lines is illustrated in Table 2.6.

Table 2.6 MGN-3 effect on different cell lines *in vitro*

Cell line type	*In vitro* assay	References
Peripheral blood monocytes	Dendritic cell differentiation	(Cholujova *et al*., 2009)
HUT78 Leukaemia cell line	Apoptosis assay, Bcl-2 and CD95 expression	(Ghoneum and Gollapudi, 2004)
RAW264.7 and P-MΦ cell lines	Nitric oxide production assay	(Ghoneum and Matsuura, 2004)
PBLs human peripheral blood lymphocytes	IFNγ ELISA assay and ^{51}Cr release assay	(Ghoneum, 1998)

2.11.2 MGN-3 *in vivo* studies

Several *in vivo* studies have investigated the functionality of MGN-3 on animal immune systems. Badr El-Din *et al*, (2008) investigated the effect of intra-tumoural and intra-peritoneal supplementation of MGN-3 on Ehrlich carcinoma bearing mice, reporting that at a concentration of 40 mg/kg body weight of MGN-3 is able to delay tumour growth. The inhibitory effect of MGN-3 treatment on tumour growth had positive effects from day 14 post-injection, with tumour weight and volume reducing by 45% and 63%, respectively in mice at day 35. Moreover, MGN-3 showed antitumor effects and an increase in IFNγ production of 154%, apoptotic activity of 76%, TNFα secretion of 11% and NK cell activity of 100%. These results suggested that the antitumor effect of MGN-3 is due to its ability to induce IFNγ and TNFα. Key studies that have investigated the effects of MGN-3 *in vivo* are listed in Table 2.7.

Table 2.7 MGN-3 effect on different animal models *in vivo*

Animal models	*In vivo* assay	References
Mice bearing Ehrlich ascites carcinoma (EAC)	Apoptotic assay	(Badr El-Din *et al*., 2016)
Rats gastric cancer	Apoptosis assay, tumour marker Ki-67 assay	(El-Din *et al*., 2016)
Mature female Swiss albino mice	Antioxidant assay, gene expression and anti- tumour activity assay	(Noaman *et al*., 2008)
Mature female Swiss albino mice	Tumour apoptosis, IFNγ, TNF α and IL 10 ELISA, 51Cr release assay	(Badr El-Din *et al*., 2008)

2.12 Immunomodulatory effects of rice bran polysaccharides

Many researchers have studied the effect of modified arabinoxylan (MGN-3) on the immune system *in vitro* and *in vivo*. However, there is limited research on non-modified arabinoxylans extracted from rice, including polysaccharides extracted from rice without enzyme modification. In 2008, Wang *et al*. studied the effect of RBPS2a (rice bran hetero-polysaccharide) extracted with hot water on anti-complementary activity. The study indicated that RBPS2a has the ability to induce *in vitro* red blood cell lysis and complement consumption through residual complement activity. Another study extracted another fraction of arabinoxylan from rice bran using carbohydrate-hydrolysing enzymes for a longer period. The extracted arabinoxylan had low molecular weight and structure similar to MGN-3 (Hoshino *et al*., 2010). Mast cells treated with the arabinoxylan (0.3 mg/ml) showed a remarkable depletion in β-hexosamindase secretion post-antigen stimulation. In addition, IL-4 and TNFα secretion were inhibited after treating the mast cells with the arabinoxylans, suggesting that arabinoxylans extracted from rice bran have the ability to suppress cytokine secretion and degranulation of mast cells (Hoshino *et al*., 2010).

Other rice bran polysaccharides (not AXs) have been suggested to exhibit antitumor and immunomodulatory activities. Oral administration of rice bran polysaccharide (α glucan) showed significant antitumor activity on gastrointestinal carcinogenesis in rats (Takeshita *et al*., 1992).

2.13 Immunomodulatory effects of wheat bran and wheat endosperm arabinoxylans

It has been reported that oral administration of arabinoxylans extracted from wheat bran using xylanases and alkali extraction have an immune-modulatory effect on both the innate and adaptive immune system (Cao *et al*., 2011; Zhou *et al*., 2010). Alkali extracted arabinoxylans from wheat bran showed an inhibitory effect on tumour growth and IL-2 production at 100-400 mg/kg on S 180 tumour bearing mice. The most significant results were at the highest concentration (400 mg/kg). Moreover, there was an increase in leukocyte count and stem cell proliferation was enhanced after oral administration of arabinoxylans (Cao *et al*., 2011).

Another study conducted by Zhou *et al*. (2010) indicated that (200 mg/kg) oral administration of enzyme extracted wheat bran AXs exhibits immunostimulatory effects on both innate and adaptive immunity. The enzyme-extracted arabinoxylans were reported to stimulate phagocytosis by macrophages and postpone hypersensitivity more than alkaline extracted arabinoxylans (Zhou *et al*., 2010).

Recently, Li *et al*. (2015) investigated the effect of enzyme-extracted arabinoxylans from wheat endosperm pentosan on U937 and Caco-2 cell lines. They reported that enzyme extracted arabinoxylans (AXE) generate higher nitric oxide (NO) levels than water extracted arabinoxylans (WEAX) and the increase in NO production was dose-dependent. It was reported that AXE is more effective than WEAX in stimulating IL-8 production (Li *et al*., 2015).

It is clear that there are several factors affecting the immunomodulatory potential of arabinoxylans including the method of extraction, enzyme/chemical treatments and botanical source.

2.14 Potential arabinoxylan receptors

Although receptors for AXs have not been identified, some potential receptors have been proposed. Previous studies suggested that AXs from different sources have

immunomodulatory potentials. Therefore, it is possible to speculate that their mechanism of action is by acting like PAMPs. Fascinatingly, extracted AXs from cornhusk and rice bran showed some similarities in terms of molecular weight and structure to lipopolysaccharide (LPS) from gram-negative bacteria (Ghoneum and Ogura, 1999; Ogawa *et al*., 2005). As an example, LPS has in its outer core hexoses such as glucose and galactose, which are found in arabinoxylans. Another similarity in the structure of LPS and AXs includes C-3 branched polysaccharides (Rietschel *et al*., 1994; Heinrichs *et al*., 1998). Moreover, enzyme-treated arabinoxylans from wheat endosperm pentosan showed low molecular weight (1-25 kDa) (Li *et al*., 2015) which is within the molecular weight range (10-20 kDa) of LPS (Sigma-Aldrich, 2016). Therefore, arabinoxylans might activate phagocytes by attaching to toll-like receptors expressed on the surface of phagocytes. Since LPS binds to TLR4 specifically, it suggests that TLR4 may also be a potential receptor for arabinoxylans. If true, arabinoxylans may compete with LPS for the TLR4 receptor in the presence of infection, thus mediating the LPS-induced immune response.

Other receptors besides TLRs may also act as receptors for arabinoxylans. These include the Dectin-1 receptor, which has been reported to be a β glucan receptor. However, recently Sahasrabudhe *et al*. (2016) have reported that arabinoxylan from wheat has the ability to stimulate Dectin-1 receptors and it enhanced IL-23, and IL-4 expression in Dectin-1 stimulated dendritic cells.

2.15 Structure-activity relationship

It has been suggested that the activity of arabinoxylans is dependent on their sugar composition, molecular weight and degree of branching (Zhou *et al*., 2010; Cao *et al*., 2011). The most investigated type of arabinoxylans (MGN-3) has a low molecular weight with a low arabinose to xylose ratio (0.5) (Zhang *et al*., 2015) which is similar to the enzyme extracted wheat bran AXs (Zhou *et al*., 2010). Both of the polysaccharides showed the ability to activate macrophages. However, MGN-3 appeared to be more effective which might be due to differences in the sugar composition since MGN-3 has more glucose and galactose side chains (Zhang *et al*.,

2015). In contrast, alkaline-extracted arabinoxylans from wheat bran and banana peel showed substantial immunomodulatory activity, despite the molecular weight of the arabinoxylans being large (288 and 352 kDa in banana peel and wheat bran respectively), suggesting several receptors may be involved (Zhang *et al*., 2008b; Zhou *et al*., 2010; Cao *et al*., 2011). Table 2.8 shows the relationship between the structural properties of AXs from different sources on immunomodulatory activities.

Table 2.8 Structural properties of AXs

Origin	Extraction method	Immunomodulatory activity	Mw (kDa)	Glu %	Gal %	Xyl %	Ara %	Ar/Xy	References
Wheat bran	alkaline	Tumour inhibition, Mϕ activation	352	7.7	na	50.2	41.8	0.83	Zhou *et al*., 2010
Wheat bran	enzyme	Mϕ activation	32.5	2.8	na	62.4	34.8	0.55	Zhou *et al*., 2010
Banana peel	alkaline	Mϕ activation	288	na	21.6	25.6	52.8	2.1	Zhang *et al*., 2004
Rice bran	na	Mϕ, DCs and NK activation	30-50	6	5-7	48-54	22-26	0.5	Zhang *et al*., 2015

Summary

Arabinoxylans are very important food additives due to several health benefits, especially their immunomodulatory function. Several extraction methods have been applied to increase the water-soluble AXs and to decrease its molecular weight from several sources. Furthermore, many *in vitro* and *in vivo* -studies have tested the immunomodulatory potential of AXs. However, there appears to be no research that has used extrusion cooking as a pre-treatment to increase water extractability and reduce the molecular weight of rice bran and wheat endosperm pentosan. Moreover, there is no research investigating the mechanism of action of AXs on human monocytes and induced macrophages. Therefore, this research study was divided into two stages; stage one studied the effect of extrusion cooking on molecular weight and water extractable AXs from rice bran and wheat endosperm pentosan. The second stage investigated the effect of extracts on NO production in both U937 monocytes and macrophages. Furthermore, the potential mechanism of action of AXs was investigated via pharmacological inhibition of the TLR4 receptor and testing the effect of AXs on downstream TNFα secretion from monocytes and, in particular, macrophages.

Chapter 3 The effect of extrusion screw-speed on water-extractable AXs from wheat endosperm and rice bran

3.1 Introduction

Extrusion cooking is an important food processing technology, especially for the production of cereal-based products (Vasanthan *et al*., 2002). It is a thermal processing that involves the application of high temperature, high shear forces and high pressure for a short time in which expansive, moistened food materials are cooked in a tube by a combination of mechanical shear, pressure, moisture and temperature, resulting in materials modification by physiochemical changes and molecular re-distribution (Riha *et al*., 1996; Castells *et al*., 2005; Thymi *et al*., 2005; Pawar *et al*., 2014).

Extrusion cooking has several advantages over other processing methods because of energy savings, versatility, low cost, productivity and speed (Zhang *et al*., 2011). The use of high temperature and high pressure in extrusion cooking results in the modification of structural, chemical and physical properties of the extruded products. The effects of extrusion on the nutritional profile depend on several factors, including extruder type, screw combination, temperature and moisture content (Björck and Asp, 1983).

The thermoplastic extrusion use in food processing is assisted by the type of the extruders, which are divided into single-screw and twin-screw extruders (Navale *et al*., 2015). Twin-screw extruders possess more advantages than single-screw extruders, including feeding, residence time distribution, mixing, heat transfer and pumping performance (Akdogan, 1996).

Several researchers have investigated the effects of extrusion on the composition of cereal-based products. Vasanthan *et al*. (2002) reported that extrusion could increase the soluble dietary fibre (SDF) in extruded barley flour. The increase of SDF content was from 5.62% in non-extruded barley flour to 7.24% in extruded barley flour under a barrel temperature of 140^{o}C, screw-speed of 50 rpm and moisture

content of 50%. The increase in SDF was explained by transglycosylation from non-dietary fibre components (i.e. starch) to SDF and transformation of insoluble dietary fibre (IDF) to SDF.

Recently, Daou and Zhang (2012) investigated the effect of extrusion cooking on rice bran dietary fibre solubility. A twin-screw extruder was used with a screw-speed of 90 rpm, barrel temperatures of 50-75-95-100^{o}C (feed end to die) and a moisture content of 18 %. They found that the content of soluble dietary fibre had increased from 5.9% in non-extruded samples to 6.8% in extruded samples. The possible explanation for the increase in dietary fibre solubility might have been due to the reduction in particle sizes of the extruded samples.

A previous study looked at the effects of extrusion on soluble/insoluble dietary fibre of rice, oats and wheat (Gualberto *et al*., 1997). Their extrusion conditions and soluble dietary fibre alteration are reported in Table 2.4. The results showed a significant decrease in IDF whereas SDF effectively increased in all samples. The decrease in IDF and increase in SDF could be explained by the breaking of chemical bonds in IDF due to the extruder shear force, which might have transformed the IDF macromolecules into smaller molecules, which are soluble. In addition, it can be noted that the highest amount of soluble dietary fibre was obtained at lower screw-speeds (50 and 70 rpm) which might generate high pressure inside the extruder resulting in the breaking up of the covalent/non-covalent bonds between the IDF macromolecules and transformation of IDF to SDF.

Recently, Jeon *et al.* (2014) investigated the effect of extrusion moisture content and screw-speed on the WEAX in corn fibre. The highest extraction yield of WEAX was obtained at 30%, which was 917g/kg in comparison to non-extruded corn fibre, which was 717g/kg. The screw-speeds used were 200, 250 and 300 rpm. The WEAX contents increased with increasing screw-speed from 717g/kg in non-extruded samples to 870, 917 and 1011 g/kg in samples extruded at screw-speeds of 200, 250 and 300 rpm, respectively.

AXs are major dietary fibres found in many cereals such as corn, wheat and rice (Broekaert *et al*., 2011). Only a few researchers have investigated the effect of extrusion cooking on AXs' solubility and molecular weight distribution. Santala *et al.* (2013) investigated the effect of extrusion cooking on WEAX levels and molecular weight. It was reported that the extrusion of wheat bran increased the solubility of AXs from 1.7% to 2.5% when the water content decreased from 92% to 42-60% respectively under a fixed screw-speed at 65 rpm and fixed barrel temperature at 50 ^{o}C. They reported that the increase in WEAX level might be due to the action of endogenous hydrolytic enzymes of the bran material (Dornez *et al*., 2009). Another possible explanation could be the reduction of enzyme inhibitors by the low water content (Jerkovic *et al*., 2010). The effect of extrusion on molecular weight was significantly reduced from 158 kDa in non-extruded samples to 131, 79 and 68 kDa with the water content of 37, 48 and 60%, respectively in wheat bran. It was suggested that the endogenous enzymes of bran material were not able to hydrolyse WEAX at 37% water content, whereas, at higher water contents of 48 and 60% the molecular weight was significantly lower, suggesting that these amounts of water content are the optimum for endogenous enzymes to hydrolyse the WEAX (Santala *et al*., 2013).

Low molecular weights of AXs between (30-50 kDa) have been reported to have immunomodulatory effects *in vivo* and *in vitro* (Ghoneum and Matsuura, 2004). Therefore, the target we were trying to achieve was to increase the solubility of AXs and reduce their molecular weights, which might be achieved with extrusion as a pre-treatment method.

3.2 Materials

3.2.1 Carbohydrates

Ener-G, General Dietary Ltd (Surrey, UK) kindly provided rice bran. Henan Lianhua Monosodium Glutamate Group Co. Ltd (Xiangcheng, China) kindly provided wheat endosperm pentosans.

3.2.2 Chemicals

D-(+)-Xylose, dextrose (D-glucose) anhydrous, acetic acid (glacial), hydrochloric acid, phloroglucinol and ethanol were purchased from Sigma-Aldrich Co, Brøndby, Denmark. These chemicals were used in the determination of xylose in wheat endosperm pentosan and rice bran. Eight pullulan (linear α-(1-4) glucans with no side chains) standards, of varying molecular weights (ranging from 5-708 kDa), were purchased from Shodex (Shanghai, China) and were used for SEC-HPLC to characterise the Mw of AXs. Sodium nitrate ($NaNO_3$) and sodium, azide (NaN_3) for the HPLC mobile phase were purchased from Sigma-Aldrich, Gillingham, UK.

3.2.3 Enzymes

Termamyl (α-amylase), type XII-A, *Bacillus licheniformis* A3403-1MU and proteinase, type XXIII, *Aspergillus melleus* P4032 were purchased from Sigma-Aldrich Co, Brøndby, Denmark.

3.3 Methods

A significant amount of time was spent developing the water extraction and purification methods.

3.3.1 Fat extraction

Wheat pentosan and rice bran fats were extracted prior to extrusion using the method provided by (Buchi, 2016). Briefly, 10 g of the food sample was weighed into an extraction thimble (Buchi, Switzerland), placed in a hot extraction beaker (HEB) followed by addition 40 ml of petroleum ether (Fisher Scientific, Loughborough), and transferred to the extraction unit E-812/E-816 HE (Buchi, Switzerland).

3.3.2 Extrusion pre-treatment

A Werner Pfleiderer Continua 37 co-rotating, self-wiping twin-screw extruder (Werner Pfleiderer, Stuttgart, Germany) was used for the extrusion pre-treatment of rice bran and wheat endosperm pentosan. The extruder used for this process had the

following characteristics: a length to diameter ratio (L/D) of 27:1, rice bran and pentosan were treated at two different screw-speeds (SS) 80 rpm and 160 rpm (revolution per minute) with a feed rate of 10 kg/h. The extruder was equipped with a twin circular die with a 4 mm diameter. The barrel temperature was controlled in two zones and was set at 80 to 140 °C (feed end and die end, respectively) and samples were extruded at a fixed moisture content of 30% (w/w wet weight basis). Extruded samples were dried at 60°C for 12 hours. After drying, the extrudates were then stored in food grade bags and vacuum-sealed using a Turbovac SB425 Vacuum Packer (Stockport, UK) and kept at 21^{o}C for further analysis. An IKA A 11 basic analytical mill (IKA: Oxford, UK) was used to grind all samples to a powder. The resulting powder was subsequently passed through an Endecotts 0.4 mm testing sieve and the fraction passing through this was used in all subsequent analyses. Table 3.1 summarises the extrusion conditions applied to the rice bran and wheat endosperm pentosan samples.

Table 3.1 Summary of extrusion process conditions

Sample	Throughput (Kg/h)	Moisture (%)	Screw-speed rpm	Barrel temperature	Torque %
Rice bran	10	27.4	160	80 to 140 ^{o}C	30
Pentosan	7	25.9	160	80 to 140 ^{o}C	25
Rice bran	10	27.4	80	80 to 140 ^{o}C	49
Pentosan	7.0	25.9	80	80 to 140 ^{o}C	30

3.3.3 Extraction of water-extractable AXs

The extraction method used in this research was adapted from Li *et al* (2013). The pentosan and rice bran samples (100 g) were added to 333 ml water and then incubated in a shaking water bath (Precision SWB 15, ThermoScientific, London, UK) for two hours at 40^{o}C. After incubation, the samples were then centrifuged for 40 minutes at 6000 x g. The supernatants were transferred to Erlenmeyer flasks and the pH of the solution was adjusted to 7 using (1M HCl) or (1M NaOH), then 400 ppm of thermostable α-amylase (500 Units/mg) was added, incubated in a water bath at 91^{o}C for 60 minutes and the samples were then cooled to room temperature. Protein degradation was achieved with the addition of 400 ppm of proteinase (3 Units/mg) and incubation at 50 °C for 14h. After incubation, the enzymes were deactivated by placing the sample in a boiling water bath for 10 minutes. The samples were allowed to cool to room temperature naturally and centrifuged at 4600 x g for 20 minutes. Ethanol solution (95%) was added to the supernatant in a ratio of 70:30 (v/v) and allowed to stand at 4°C overnight. The precipitate formed was then recovered by centrifugation (4600 x g for 20 minutes). The supernatant was discarded and the residue was retained and weighed. The residue was washed and vortexed twice with 20 ml ethanol (99%). Finally, acetone (20 ml) was added and the sample vortexed for one minute followed by centrifugation at 4600 x g for 20 minutes. The final precipitates were dried for 48 hours at 45^{o}C in a drying oven and then stored in food grade bags and vacuum-sealed using Turbovac SB425 Vacuum Packer (Stockport, UK) and kept at 21^{o}C for further analysis.

3.3.4 Water-extractable arabinoxylan (WEAX) determination

The percentages of xylose in the extracts were determined using a phloroglucinol colorimetric assay following the method provided by Douglas (Douglas, 1981). Absorbance's of the samples was measured using a ThermoScientific GENESYS 10S Bio Spectrophotometer (London, UK) at 552 nm and 510 nm.

A xylose standard curve (Appendix 1) was constructed to determine the xylose content of the rice bran and wheat pentosan samples, which was subsequently used to calculate the amount of AXs in the extracts.

3.3.5 Determination of sugar composition of purified extracts by HPLC

The sugar composition of the purified extracts was determined using a method adapted from (Zheng *et al*., 2011). In this method, 100 mg of each supernatant was transferred into boiling tube and 1ml of 1M H2SO4 was added for acid hydrolysis. All the tubes were incubated in 100^{o}C glycerine bath for 2 hours and cooled down using flow of cold water to room temperature. The samples then were transferred to 20ml volumetric flask and diluted using HPLC water grade. The pH was adjusted to 7 using 1M H2SO4 and 1M NaOH, and then transferred to 100 ml volumetric flask and diluted to 100 ml volumetric using HPLC water grade.

A 5 ml aliquot of each sample was transferred into small beaker and 5ml polypropylene syringe was used to filter the samples through a 0.45 µm nylon membrane. Samples were transferred to a 1 ml glass shell vial and 20 µl aliquots were analysed by HPLC system. A Shimadzu LC-20 AB HPLC system, (Shimadzu Corporation, Tokyo, Japan), equipped with a Refractive Index Detector (RID) 10A, SUPELGUARD Pb (5 cm × 4.6 mm) guard column (Phenomenex, Macclesfield, UK) and SUPELCOGEL Pb (30 cm × 7.8 mm) column (Ion exclusion separation mode) (Phenomenex, Macclesfield, UK) was used to determine the sugar content of the samples. The column temperature, mobile phase and flow rate were 80^{o}C, HPLC grade water and 0.5 ml/min respectively in an isocratic run. Different concentrations (0.25, 0.5, 0.75 and 1 mg/ml) of glucose, xylose, galactose and arabinose were prepared as standards to plot a series of calibration curves (Appendix 2) from which the amount of each of the sugars was calculated based upon the relative peak areas.

3.3.6 Molecular weight standard curve

Five pullulan standards ranging from 5-375 kDa were used to construct the standard curve, the molecular weights of the standards are shown in Table 3.2. The standards

were prepared with mobile phase at 0.5 mg/ml concentration and left overnight at 5^{o}C. All samples and standards were filtered through a 0.45 µm nylon membrane and then transferred to 1 ml glass shell vials. To get the pullulan standard curve, the pullulan molecular weights were converted to log molecular weights and then they were plotted versus the retention time (Appendix 3).

Table 3.2 Molecular weight of pullulan standards

Samples	Molecular weight (Dalton)
P-5	5,900
P-20	21,100
P-100	107,000
P-200	200,000
P-400	375,000

3.3.7 Determination of the molecular weight distribution of AXs by HPLC

The dry samples were prepared for analysis by dissolving 2 mg of each sample in 1 ml mobile phase and left overnight at 5^{o}C. The mobile phase was prepared by dissolving 0.65 g NaN_3 and 17g $NaNO_3$ in HPLC-grade water and made up to 2000 ml.

The molecular weight distribution of the arabinoxylans was determined using size exclusion chromatography. All samples were analysed using a Shimadzu LC-10 HPLC (Shimadzu Corporation, Kyoto, Japan), equipped with a JASCO RI-2031 Refractive Index (RI) Detector (Jasco Corporation, Tokyo, Japan), and BioSep-SEC-S 4000 and BioSep-SEC-S 3000 columns (Phenomenex, Macclesfield, UK). An isocratic run was used, with a flow rate of 0.6 ml/min (Li *et al*., 2013).

3.4 Statistics

Data were expressed as mean ± standard error of the mean. The significance between samples was determined by one-way analysis of variance ANOVA with Tukey's multiple comparison tests on SPSS 23 software. A *P* value of less than 0.05 was considered statistically significant.

3.5 Results

3.5.1 The effect of extrusion on extraction of AXs from rice bran and wheat endosperm pentosan

The extrusion had a positive effect on the extraction yields of arabinoxylans in both rice bran and wheat pentosan. The extraction yields represents the percentage of total weight of sample. The increase in the extrusion screw-speed showed an increase in the extraction yield. The total AXs was calculated using the xylose standard curve (Appendix 1) and the Ar/Xy ratio obtained using HPLC (Appendix 2).

In rice bran, extrusion significantly increased the water-extractable AXs percentage from 0.58±0.0 % in rice bran without extrusion to 1.22±0.0 % (P<0.05) in rice bran at 80 rpm screw-speed and it reached 1.62±0.0 % (P<0.05) at 160 rpm screw-speed. Table 3.3 shows the % of total weight of AX for rice bran samples at different screw-speeds and the percentage of arabinose with xylose over the total sugars percentage.

Table 3.3 Extraction yield of AXs from rice bran

Sample	Ar/Xyl	Total AX g/100g Unpurified sample	Ar+Xyl %/total sugars %
RBW	1.55±0.020	0.58±0.082	88.13
RB80	1.46±0.008	1.22±0.09	88.31
RB160	1.40±0.006	1.62±0.07	88.85

In wheat pentosan samples, the extrusion significantly increased the water-extractable arabinoxylans percentage from 8.95±0.1 % in wheat without extrusion to 11.07±0.2 % (P<0.05) in wheat pentosan at 80 rpm screw-speed. It reached 12.45±0.2 % (P<0.05) at 160 rpm screw-speed. Table 3.4 shows the extraction yield percentages for wheat pentosan samples at different screw-speeds and the percentage of arabinose with xylose over the total sugars percentage.

Table 3.4 Extraction yield of AXs from pentosan

Samples	Ar/Xyl	Total AX g/100g Unpurified sample	Ar+Xyl %/total sugars %
PW	0.76±0.001	8.95±0.17	86.86
P80	0.809±0.005	11.07±0.22	86.97
P160	0.80±0.003	12.45±0.22	87.34

3.5.2 The arabinose to xylose ratio (Ar/Xy) of the extracts

Glucose, arabinose, galactose and xylose monosaccharides were identified in the purified arabinoxylans samples from rice bran and wheat pentosan. Figure 3-1 shows monomer sugar content of the purified arabinoxylans from rice bran and wheat pentosan. Each of the monosaccharides has its own calibration standard curve. The standard curves for the four monosaccharides are shown in the Appendix 2.

Table 3.5 Shows the Ar/Xy ratios for all samples.

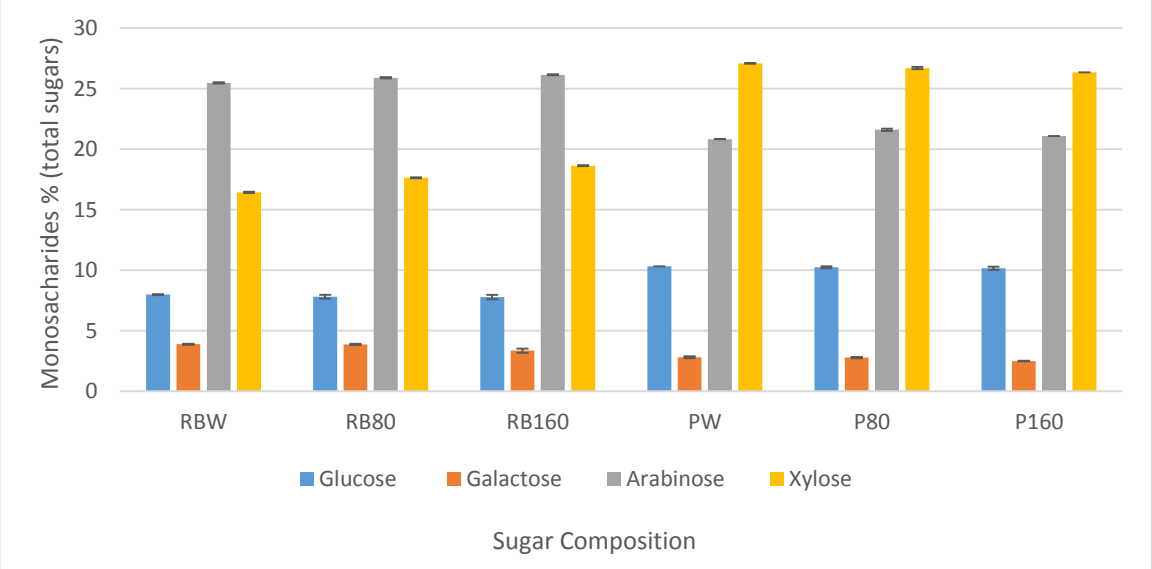

Figure 3-1 Sugar composition for purified AXs from rice bran and wheat pentosan

The ratio of arabinose to xylose (Ar/Xy) decreased in the rice bran samples with the increase in screw-speeds while in

wheat pentosan samples, it decreased with the increasing in screw-speeds

Table 3.5 Arabinose to xylose ratios (AR/Xy) in rice bran and wheat pentosan

	RBW	RB80	RB160	PW	P80	P160
Ar/Xy	1.55±0.02	1.47±0.008	1.40±0.006	0.77±0.001	0.81±0.005	0.80±0.003

3.5.3 Molecular weight analysis of AXs using HPSEC

3.5.3.1 Pullulan standard curve construction

The standard curve was constructed using five pullulan standards (P5, P20, P100, P200 and P400), as shown in Appendix 3. The standards were analysed using HPLC-SEC. The retention time and the molecular weight which were compared with the samples' molecular weight and retention time to analyse the molecular distribution of the AXs in the samples. Table 3.6 summarises the Mw of the five pullulan standards ranged between 5.9-375 kDa.

Table 3.6 Molecular weights of pullulan standards in relation to their retention times

Pullulan samples	Molecular weight (Da)	Retention time (Min)	Log Mw
P5	5,900	43.50	3.77
P20	21,100	38.40	4.32
P100	107,000	29.24	5.03
P200	200,000	26.61	5.30
P400	375,000	25.01	5.57

3.5.3.2 Molecular weight distribution of AXs

The molecular weight (Mw) distribution of the AXs of wheat pentosan and rice bran samples was characterized by HPLC-SEC.

Figure 3.2 and 3.3 illustrate the Mw distributions and peak areas. The chromatograms were divided into four distinct areas; each area has a distinct peak. Tables 3.7 and 3.8 illustrate the Mw ranges of AXs and their percentages. Results are presented as means ± SEM and all experiments were conducted in triplicate.

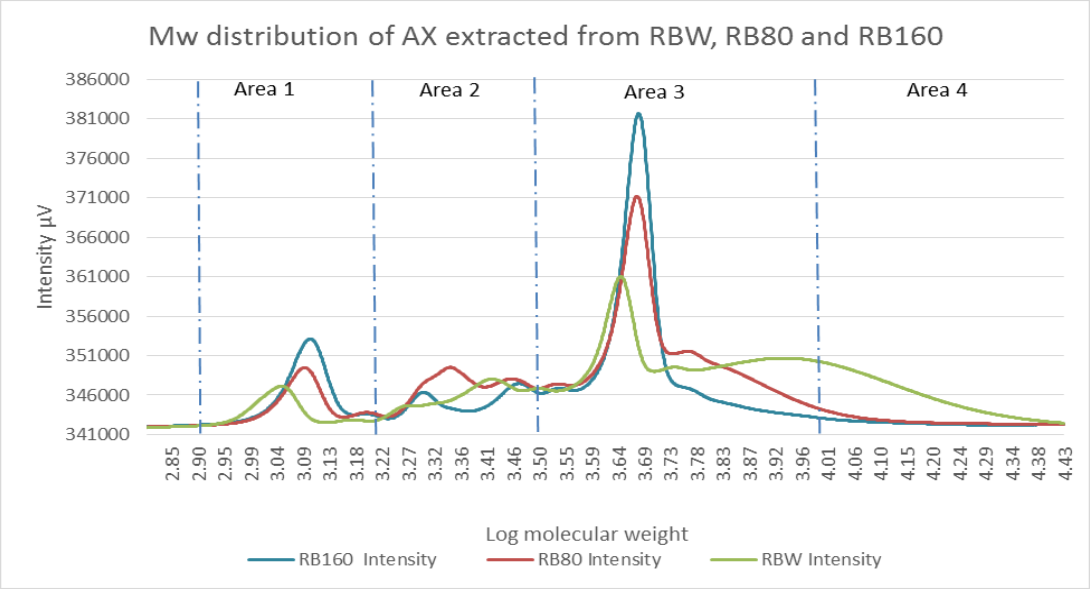

Figure 3-2 Mw distribution of RBW, RB80 and RB160 analysed by HPLC-SEC
The dashed lines separate the area under the curve into four areas, each of which represents a distinct Mw range.

Table 3.7 Mw distribution of AXs extracted from RBW, RB80 and RB160

Data are given as means ± SEM and $P< 0.05$ was calculated for all samples.

Areas	Log Mw	Mw range (kDa)	%		
			RB160	RB80	RBW
Area 1	2.90 - 3.2	0.79-1.58	18.24±0.006	14.15±0.08	11.42±0.0057
Area 2	3.2 - 3.5	1.58-3.16	11.52±0.08	16.13±0.01	13.15±0.01
Area 3	3.5 - 4	3.16-10	45.22±0.73	47.09±0.68	44.59±0.76
Area 4	4 - 4.4	10-25.11	25.05±0.027	22.63±0.008	30.84±0.01

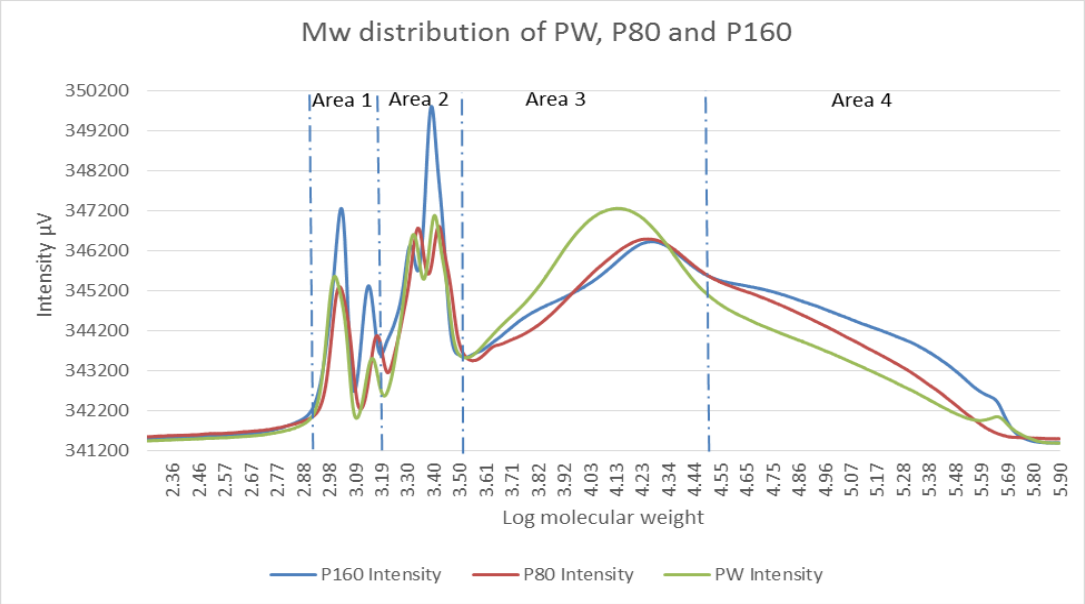

Figure 3-3 Mw distribution of PW, P80 and P160 analysed by HPLC-SEC

The dashed lines separate the area under the curve into four areas, each of which represents a distinct Mw range.

Table 3.8 Mw distribution of AXs extracted from PW, P80 and P160

Data are given as means ± SEM and $P< 0.05$ was calculated for all samples.

Areas	Log Mw	Mw range (kDa)	%		
			P160	P80	PW
Area 1	2.93-3.19	0.85-1.54	7.63±0.01	7.33±0.02	7.13±0.008
Area 2	3.19-3.5	1.54-3.16	15.58±0.03	14.58±0.005	13.95±0.003
Area 3	3.5-4.5	3.16-31.62	43.04±0.008	45.1±0.01	46.89±0.006
Area 4	4.5-5.9	31.62-794.3	35.38±0.005	33±0.01	30.4±0.02

From the results shown in Tables 3.7 and 3.8, it can be seen that RBW samples have higher percentages 11.42% of very low Mw (0.85-1.54 kDa) AXs whereas PW samples have only 7.13 %. On the other hand, PW showed a higher amount of AXs (84.42%) with a bigger molecular weight between 3.16-794.3 kDa, than RBW, which has only 74.43%.

Interestingly, the extruded samples from both rice bran and wheat pentosan showed significant increases in very low molecular weight fractions (between 0.78-1.58 kDa) which accompanied the increase in screw-speed from 80 rpm to 160 rpm. The

percentages of low molecular weight fractions of AXs increased from 11.42% in RBW to 14.15% and 18.24% in RB80 and RB160, respectively. Similarly, the percentages of low molecular weight fractions of AXs increased from 7.13% in PW to 7.33% and 7.63% in P80 and P160, respectively.

3.6 Discussion

3.6.1 Extraction rate of water-soluble AXs

In rice bran, WEAX is only around 0.9 % of the total AXs (Hashimoto *et al*., 1987b). Similarly, in the wheat endosperm, WEAX is only 25 % (Ordaz-Ortiz and Saulnier, 2005). The low extractability of AXs could be due to the large molecular weight of AXs (Saulnier *et al*., 2007; Beaugran *et al*., 2004) and to its ferulic acid content (0.31-0.56 mg/g) (Michniewicz *et al*., 1990). Ferulic acid (FA) side chains are esterified to some arabinose residues (Snelders *et al*., 2013), which form covalent/non-covalent bonds with the cell wall materials, thus, decreasing arabinoxylans' solubility in water. Jeon *et al*. (2014) state that using extrusion cooking, as a pre-treatment is an efficient, environmentally friendly and low-cost process to increase the level of water extractable arabinoxylans in corn fibre. The results of this study confirm those of Jeon *et al*. (2014), showing an increase in the WEAX content in the extruded rice bran and wheat pentosan with increasing screw-speed from 80 to 160 rpm. The increase in WEAX content in rice bran samples was by 1.1- and 1.8-fold at screw-speeds of 80 and 160 rpm, respectively. A less pronounced increase in WEAX was found in extruded pentosan samples with the extraction rate increasing by 0.23-fold and 0.4-fold in pentosan samples extruded at 80 and 160 rpm, respectively. There are several possible explanations for the increasing level of WEAX in the samples post extrusion including; the liberation of the ferulic acid side chains, softening of the lignin and reduction of Mw by high mechanical shear forces.

Holguín-Acuña *et al*. (2008) found that the ferulic acid content increased from 0.2 mg/g in non-extruded maize bran to 2.5 mg/g in extruded maize bran. The increase in ferulic acid content might be the reason for the increase in AXs' solubility as the more the ferulic acid is liberated from the side chains, the more the arabinoxylans are in contact with water. Moreover, the increase in screw-speeds from 80 to 160 rpm might soften the lignin (Yoo *et al*., 2012). AXs act as a glue between lignin and cellulose (Vermaas *et al*., 2015), therefore the high screw-speed will create high shear stress in the barrel, which might soften the lignin (Karunanithy *et al*., 2010; Yoo

et al., 2012) and consequently the AXs chains will be more exposed to water, resulting in an increase in their solubility in water.

3.6.2 Molecular weight distribution of AXs from extruded/non-extruded rice bran and wheat pentosan

Molecular weight determinations for wheat endosperm AXs were reported to be within the ranges 56-65 kDa using gel permeation chromatography (Girhammar and Nair, 1992) and 6-600 kDa using HPSEC (Li *et al*., 2013). Clearly, the method applied for Mw determination can affect the final result significantly. In this study, HPSEC was used to determine the Mw of AXs from extruded/non-extruded samples.

It is difficult to compare our AXs' Mw from rice bran with the literature since this has focused mainly on wheat and other cereals.

The findings from this research suggest that the molecular range of AXs from rice bran is between 0.79-25 kDa that is not comparable to any of the previously published work. Although, Rose *et al*. (2009) reported that AXs' Mw in rice bran are within the range of 0.6-500 kDa, however, their findings cannot be compared to ours because they used alkaline-hydrogen peroxide extraction which produces large Mw AXs (Kale *et al*., 2013). In addition, most of the research done on extrusion and its effect on rice bran was to study the effect of extrusion on fat and protein content of the rice bran but not on AXs (Rafe *et al*., 2016). On the other hand, wheat pentosan AXs' Mw have been reported to be within the range of 1-700 kDa in wheat pentosan (Li *et al*., 2015) which is within the range of our findings of 0.85-794 kDa.

Figures 3.2, 3.3 and Tables 3.7 and 3.8 demonstrate that AXs from extruded rice bran and wheat pentosan have higher percentages of low MW AXs when compared with AXs from non-extruded samples. The increases in the percentages of low Mw AXs in both samples might be due to the extrusion conditions, such as high shear forces and temperatures resulting in depolymerisation of the fibre (Svanberg *et al*., 1995). It is also possible that extrusion cooking breaks down the glycosidic bonds resulting in

depolymerisation of the cell wall material, thus reducing AXs' Mw (Margareta and Nyman, 2003).

From Tables 3.7 and 3.8, it can be concluded that the very low Mw (0.79-1.58 kDa) of AXs in both samples increased significantly. However, the increases of low Mw AXs in extruded rice bran was higher than that obtained from extruded wheat pentosan. This could be related to the differences in the xylan backbone between wheat pentosan and rice bran. In wheat pentosan, the xylan backbone facilitates the formation of inter-chain hydrogen bonds, whereas the rice bran xylan backbone carries more arabinose side chains (Annison *et al*., 1995), which might be esterified by ferulic acids. It has been reported that extrusion breaks up the ferulic acid side chains, thus reducing the Mw of AXs (Holguín-Acuña *et al*., 2008) from the extruded rice bran, more than that of wheat pentosan.

Chapter 4 Immunomodulatory potential of AXs from extruded rice bran and wheat pentosan

4.1 Introduction

The importance of dietary fibre consumption is well defined. Consumption of dietary fibre has been shown to have many health benefits such as anti-inflammatory and prebiotic activity (North *et al*., 2009; Li *et al*., 2013). Arabinoxylan is the major dietary fibre in cereal such as rice, corn and wheat. Arabinoxylan is composed of a xylose backbone with arabinofuranose bound to it through glycosidic linkages at the O-3 and/or the O-2 position (Li *et al*., 2015). Several methods have been reported to increase the extractability and modify the molecular weight of AXs such as an enzyme, alkali extraction and extrusion pre-treatment (Zhang *et al*., 2014). Extrusion is an environmentally friendly pre-treatment that has been reported to decrease the molecular weight of arabinoxylan from 158 to 68 kDa under specific conditions (Santala *et al*., 2013). Reducing the molecular weight of AXs not only increases their water solubility but it also enhances the immune response in both *in vitro* and *in vivo* assays (Li *et al*., 2013).

Studies have reported that (MGN-3), a modified rice bran AXs with a low molecular weight (between 30-100 kDa), stimulated the innate and the adaptive immunity both *in vitro* and *in vivo* (Ghoneum and Abedi, 2004; Li *et al*., 2015). Moreover, wheat AXs with low molecular weight (32.52 kDa) showed a greater immune response and antitumour activity than alkaline-extracted AXs with a high molecular weight (351.7 kDa) (Zhou *et al*., 2010; Cao *et al*., 2011).

Recently, Li *et al*. (2015) investigated the effect of the low Mw enzyme-extracted AXs from wheat pentosan on NO secretion by U937 monocytes. They reported that AXs with low molecular weight could significantly increase the NO secretion. The increase in NO secretion was dependent on molecular weight and concentration, increasing 26.7 fold at the highest concentration (1000 μg/ml) with a Mw (< 25 kDa) compared to untreated U937 monocytes (Li *et al*., 2015). NO is an important inflammatory

mediator produced in response to pathogens such as fungi and bacteria. It is produced from L-arginine by inducible nitric oxide synthase (iNOS) which is expressed in many cell types such as monocytes and macrophages (Förstermann and Kleinert, 1995; Li *et al*., 2015; Mendis *et al*., 2016). Cells express iNOS upon stimulation from bacterial cell wall LPS, viruses or cytokines. It has been reported that LPS stimulates NO production through binding to Toll-like receptor 4 (TLR4) on the surface of macrophages and monocytes (Chang *et al*., 2000). NO production is very important for its antibacterial properties and role in pathophysiological processes such as vasodilatation and neuronal communication (Kiemer *et al*., 2002; Joo *et al*., 2014). However, overproduction of NO is cytotoxic and can damage host cells (Wong and Billiar, 1995). Therefore, mediation of NO production may be effective in the treatment of inflammation accompanied with several chronic diseases such as cancer, diabetes and rheumatoid arthritis (Mendis *et al*., 2016).

Interestingly, AXs show similarities with LPS in terms of structure and molecular weight (Zhang *et al*., 2015). However, the mechanism of action of AXs needs to be investigated. Therefore, the aim of this work was first to study the effect of extruded WEAX extracted from wheat pentosan and rice bran on NO production in monocytes (typical of cells found in the circulation) and macrophages (typical of cells found in tissues following infection, injury or an inflammatory response). The second aim was to study the potential mechanism of action of AXs by inhibiting the TLR4 receptor in monocytes and macrophages and determining the effect of AXs on downstream TNFα secretion in comparison to the positive control for NO secretion, LPS.

4.2 Methods

4.2.1 Arabinoxylans

Extrusion, extraction and purification of water extractable AXs (WEAX) from the extruded/non-extruded rice bran and wheat endosperm pentosan was performed as previously described (Chapter 3).

4.2.2 Cell culture

The human monocyte cell line U937 (Sundström and Nilsson, 1976) (Health Protection Agency Culture Collections, Salisbury) was cultured at 37^{o}C at 5% CO_2 in complete culture medium, which contains RPMI-1640 medium with HEPES and L-Glutamine (Lonza, Slough) supplemented with 2% Penicillin-Streptomycin (Lonza, Slough) and 10% foetal bovine serum (FBS) (Sigma-Aldrich, Slough, UK).

4.2.3 Lipopolysaccharide (LPS)

Gel-filtrated, purified lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma-Aldrich, Dorset, UK) was used as a positive control.

4.2.4 Differentiation of U937 monocytes to macrophages

To differentiate U937 monocytes into macrophage-like cells, 10nM phorbol 12-myristate 13-acetate (PMA) (Applichem, Darmstadt, Germany) was added to the monocyte cell suspension for 72 hours.

4.2.5 Griess reagent

Griess reagent was used as a quantitative indicator of nitric oxide (NO) secretion by the active immune cells using published methodologies (Bredt and Synder, 1994). Griess reagent was prepared from two components A and B. Component A consisted of sulphanilamide reagent that is composed of 1% of sulphanilamide (Sigma-Aldrich, Gillingham, UK) in 0.1 mol/l hydrochloric acid (Fisher Scientific, Loughborough, UK). Component B was composed of 0.1% N-1-napthylethylenediamine dihydrochloride (NED) (Sigma-Aldrich, Gillingham, UK) in distilled water.

4.2.6 Sodium nitrite standard

Sodium nitrite ($NaNO_2$) (Sigma-Aldrich, Gillingham, UK) was prepared in cell culture medium (RPMI-1640 supplemented with 10% FBS and 2% penicillin-streptomycin).

4.2.7 TLR4-IN-C34

The TLR4 inhibitor TLR4-IN-C34 (Sigma-Aldrich, Gillingham, UK) was added to one set of sample treatments to assess whether NO production was mediated via TLR4 activation.

4.3 Methodology

4.3.1 Preparation of AXs treatments

The preparation of AXs treatments (Appendix 4) were adapted from Li *et al.* (2015). Purified AXs (20 mg) from rice bran and wheat pentosan were solubilised in 10 ml of cell culture medium (RPMI-1640 supplemented with 5% FBS) to obtain a final concentration of 2000 µg/ml. Concentrations of AXs prepared for treatments were 50, 500 and 1000 µg/ml (final concentration in each well). All treatments were filtered twice through 0.45 µm Minisart filters (Sigma-Aldrich, Gillingham, UK) to prevent microbial contamination. AXs treatments were then stored at 4^{o}C for subsequent use.

4.3.2 Preparation of LPS treatments

The preparation of LPS solutions (Appendix 5) were also adapted from Li *et al.* (2015). LPS was reconstituted in 1 ml of cell culture medium (RPMI-1640 with 5% FBS) to a final concentration of 5 mg/ml. The LPS solution was filtered twice through 0.45 µm Minisart filters (Sigma-Aldrich, Gillingham, UK) to prevent microbial contamination. Concentrations of 50, 500 and 1000 µg/ml LPS (final concentration in the well) were prepared to match the positive control concentrations used previously by (Li *et al*., 2015). LPS treatments were then stored at 4^{o}C for subsequent use.

4.3.3 Cell culture preparation

The 1ml frozen vials of U937 cells were thawed at 37^{o}C (Precision™ General Purpose Water Bath, ThermoFisher Scientific, London, UK) immediately upon receipt. Thawed cells were added to 9.0 ml of complete culture medium and centrifuged at 125 g for 7 minutes. The cell pellet was re-suspended in the complete culture medium, which contained RPMI-1640 medium with HEPES and L-Glutamine (Lonza, Slough, UK)

supplemented with 2% Penicillin-Streptomycin (Lonza, Slough, UK) and 10% foetal bovine serum (FBS) (Sigma-Aldrich, Slough, UK). The re-suspended cells were incubated in a Heracell™ VIOS 160i Incubator, (ThermoFisher Scientific, London, UK) at 37^{o}C in 5% CO_2 in air. Sub-culturing of the cells was maintained by centrifuging with subsequent re-suspension at 2x10^{5} viable cells/ml every three days. Cell growth and viability were measured using 0.4% (w/v) Trypan Blue solution (Sigma-Aldrich, Gillingham, UK) in a 1:1 ratio with cultured cells using a TC10 Automated Cell counter (Bio-Rad, Hempstead, UK).

4.3.4 Monocytes differentiation to macrophages

U937 monocytes were cultured to a density of one million viable cells/ml and a viability at or above 95% prior to supplementation with 10nM phorbol 12-myristate 13-acetate (PMA) for 72 hrs at 37^{o}C with 5 % CO_2 according to the method by Baek *et al*. (2009).

U937 monocytes were cultured to a density of one million viable cells/ml and a viability at or above 95%. Sextuplicate treatments were prepared by adding 100 µl of cultured cells to 100 µl of LPS so the final concentration is (50, 500 and 1000 µg/ml) or AXs sample (at 50, 500 and 1000 µg/ml) in appropriate wells of a 96-well plate. Negative control wells were prepared by adding 100 µl of cultured cells to 100 µl of complete medium. Treatments were incubated for 24 hrs at 37^{o}C with 5 % CO_2 prior to taking NO measurements. Background control wells were prepared by adding 100 µl of LPS (at 100, 1000 and 2000 µg/ml) or AXs sample (at 100, 1000 and 2000 µg/ml) to 100 µl of complete medium. Sample blank wells were prepared by adding just 200 µl of complete media to empty wells. The 96-well plate was incubated for 24 hrs at 37^{o}C with 5 % CO_2 prior to taking NO measurements.

4.3.6 Nitric oxide assay

NO is secreted from most immune cells in response to immune stimulants such as cytokines and LPS (Nussler *et al*., 1993). The NO levels produced after treatments with AXs were measured using an established methods called the Griess (NO) assay

(Chaea *et al*., 2004). Following incubation with the LPS or AXs treatments, 100 µl from each well of the 96 well-plate was transferred to the corresponding well in a new 96-well plate. Then, 50 µl of sulphanilamide solution was added to all wells in the new plate prior to incubation at room temperature for 5 mins in the dark to prevent exposure to light. Subsequently, 50 µl of NED solution was added to all experimental wells and incubated at room temperature for another 5 min in the dark. The absorbance was then promptly read at 540 nm on a Multiskan FC Microplate Photometer plate reader (ThermoScientific, London, UK).

4.3.7 Sodium nitrite standard curve construction

A nitrite standard curve was constructed by preparing five concentrations of sodium nitrite (1, 10, 20, 40, 60 and 80 µM) and reading their absorbance at 540 nm using the plate reader.

4.3.8 TLR4 inhibition in monocytes and macrophages

A 1 mM solution of the TLR4 inhibitor TLR4-IN-C34 was prepared in culture medium (RPMI-1640 supplemented with 5% FBS) and stored at -20^{o}C prior to use.

Replicate treatment plates were set up with sextuplicate wells containing 50 µl cultured cells (monocytes or macrophages), 50 µl of the 1mM TLR4-IN-C34 solution and 50 µl of LPS or AX at 2000 µg/ml.

4.3.9 Effects of cell growth and viability

The cell density was fixed at 1X10^{6} viable cells/ml prior to treatments with LPS or AXs. The change in cell growth and viability during the assay was likely to affect NO secretion. Therefore, cell counts and viability were measured as described in 4.3.3 following treatment with LPS or AXs from rice bran and wheat pentosan. In addition, the cell counts and viability were measured in the negative control (untreated monocytes) in a similar manner.

4.4 Statistics

All data are given as numerical mean ± standard error of the mean (SEM) and all results were analysed using one-way analysis of variance (ANOVA) followed by the post-hoc Tukey tests for comparison. A probability (*P*) value whereby $P \leq 0.05$ was considered to be statistically significant in all cases.

4.5 Results and Discussion

4.5.1 Nitric oxide production by AXs, LPS and culture media

Background NO levels produced by the AXs or LPS (background controls) were checked to make sure there was no direct interference with the NO assay. The absorbance values showed that there were no significant difference (ANOVA: P = 0.818; Tukey pairwise comparisons: P>0.05 in all cases) between AX or LPS samples in comparison to the sample blank (containing cell culture media only), thus confirming no interference with the NO assay (Appendix 6).

4.5.2 Effect of LPS and AXs concentrations on nitric oxide production by U937 monocytes

One-way ANOVA statistical analysis confirmed that the augmentation in NO production by U937 monocytes following stimulation with AXS (RBW, RB80, RB160, PW, P80, P160) or the positive control LPS was concentration- dependent.

The NO levels produced from U937 monocytes after stimulation with LPS, PW, P80, P160, RBW, RB80 and RB160 were compared to the negative control (Figure 4-1). NO produced following treatment with AX or LPS were significantly (*P<0.05) increased at all concentrations tested compared to the negative control (Figure 4-2). The elevation in NO secretion from monocytes was concentration-dependent across all treatments. The highest NO secretion was observed from monocytes treated with RB160 at 1000 µg/ml (11.8±0.3 µM) whereas the same concentration of PW produced lower levels of NO_2^- (7.5±0.1 µM). Furthermore, there was a gradual increase in NO secretion with increasing extrusion screw-speed, ranging from 10.1±0.2 µM for RBW at 1000 µg/ml to 11.1±0.2 µM and 11.8±0.3 µM for RB80 and RB160, respectively at 1000 µg/ml concentration. Moreover, NO secretion from monocytes increased from 7.5±0.1 µM for PW at 1000 µg/ml to 8.0±0.1 and 11.0±0.1 µM for P80 and P160 at 1000 µg/ml respectively.

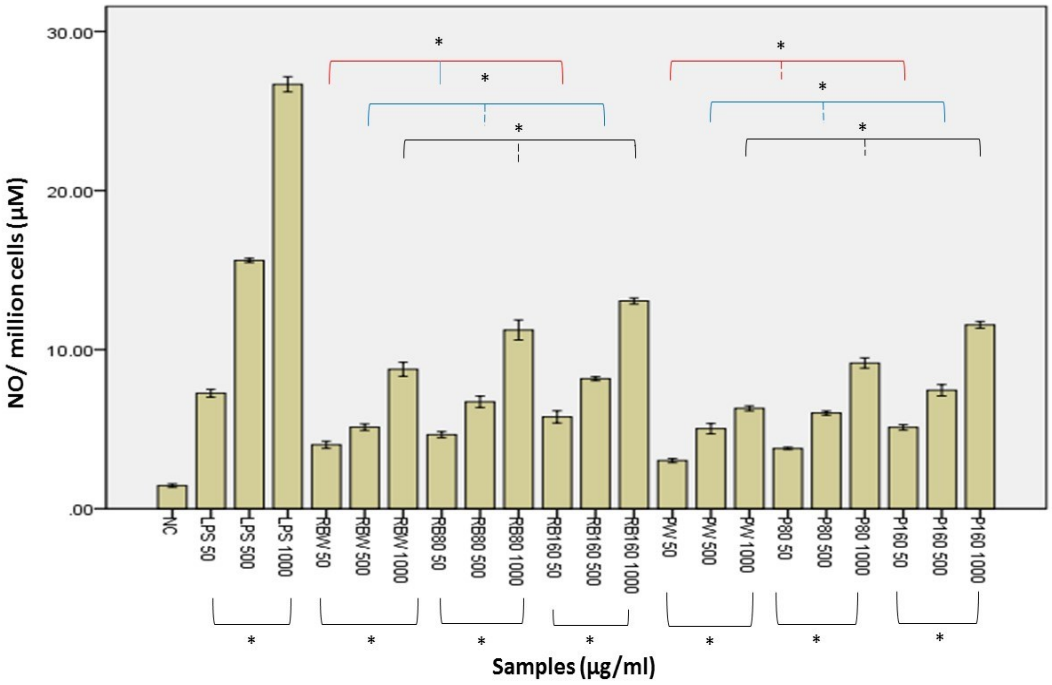

Figure 4-1 NO production by U937 monocytes after 24 hr treatment

Treatments with (LPS) or (AXs) samples in comparison to the negative control (NC). The amount of NO secreted from treated cells was calculated using the nitrite standard curve (Appendix 7). The concentration of NO, expressed as the mean ± standard error of the mean, was compared to the NC using one-way ANOVA; **P*<0.05, n = 6 in all cases. Brackets above bars indicate significant differences across groups, whereas brackets below bars indicates significant differences within groups.

4.5.3 Comparison of NO production from U937 monocytes in the presence and absence of the TLR4 inhibitor

Nitric oxide secretion was measured in U937 monocytes supplemented with the TLR4 inhibitor TLR4-IN-C34 after stimulation with LPS or AXs extracted from RBW, RB80, RB160, PW, P80 and P160. In this experiment, the U937 monocytes were treated with the highest concentration of AXs (1000 µg/ml). NO production from U937 cells in the absence of TLR4-IN-C34 and U937 cells in the presence of TLR4-IN-C34 after stimulation with 1 mg/ml of AXs from RBW, RB80, RB160, PW, P80, P160 and LPS were compared (Figure 4-2). The findings demonstrated a significant (*P*<0.05) decrease in NO production from U937 monocytes in the presence of the TLR4

inhibitor when compared to U937 monocytes in the absence of TLR4 inhibitor after treatments with 1 mg/ml AX from both rice bran and wheat pentosan. The highest decrease in NO secretion was observed in U937 monocytes supplemented with the TLR4 inhibitor following LPS treatment (1 mg/ml) when compared to the same LPS treatment in the absence of the inhibitor.

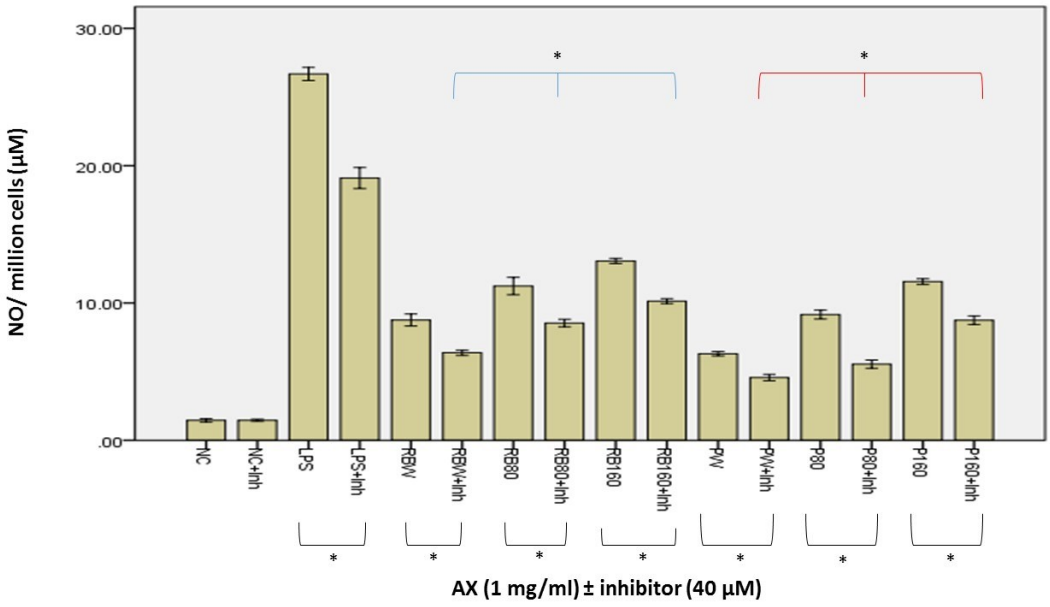

Figure 4-2 NO production from U937 monocytes supplemented with or without the TLR4 inhibitor (Inh)
Treatments with AXs or LPS (1mg/ml). One way ANOVA test showed significant differences between monocytes supplemented with the inhibitor and monocytes in the absent of inhibitor. Data represent the mean ± standard error of the mean, **P*<0.05, n = 6 in all cases. Brackets above bars indicate significant differences across groups, whereas brackets below bars indicate significant differences within groups.

4.5.4 The effect of AXs on cell growth and viability of U937 monocytes

Cell counts of the negative control (NC), positive control (LPS) at 50, 500 and 1000 µg/ml and AX treatments at 50, 500 and 1000 µg/ml extracted from rice bran and wheat pentosan samples were measured at the end of the NO assay. Higher concentrations of LPS or AX treatments resulted in significantly (*P*<0.05) lower cell

counts (Figure 4-3), confirming that both LPS and AXs had a negative effect on cell counts.

In general, AXs had a similar dose-dependent effect on monocyte cell growth and viability as LPS, with AXs just having less potency than LPS. Amongst the AXs, cell counts from RBW-treated monocytes showed the highest reduction in cell density as RBW concentration increased, from 1.6±0.0 x 10^6 viable cells/ml at 50 µg/ml RBW to 1.3±0.0 x 10^6 viable cells/ml at 1000 µg/ml RBW. In comparison, P80-treated cells showed a lower reduction in cell density as P80 concentration increased, from 1.7±0.0 x 10^6 viable cells/ml at 50 µg/ml P80 to 1.0±0.0 x 10^6 cells/ml at 1000 µg/ml P80.

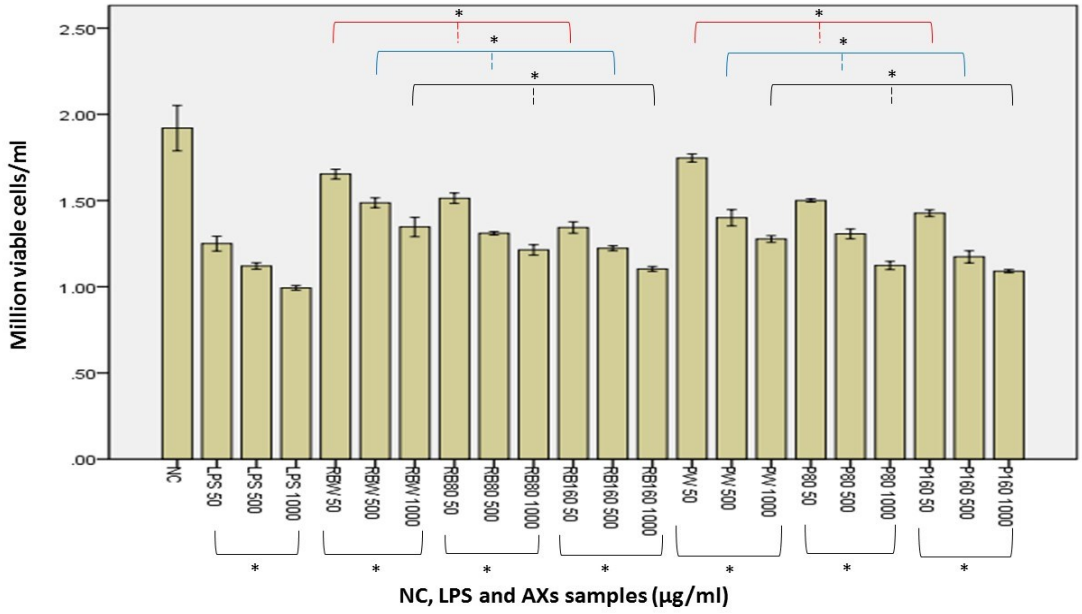

Figure 4-3 Effect of LPS and AXs concentrations on cell counts of monocytes

Bars indicate means ± standard error of the mean; n = 6 in all cases. Cell density in all treatment groups was significantly (P<0.05) lower than in the negative control (NC). Brackets above bars indicate significant differences across groups, whereas brackets below bars indicate significant differences within groups.

4.5.6 Effect of the TLR4 inhibitor on cell density of AXs-treated U937 monocytes

The effect of the TLR4 inhibitor TLR4-IN-C34 on viable cell density of monocytes treated with 1mg/ml LPS or 1mg/ml AXs was measured (Figure 4-4). There was a significant (*P*<0.05) increase in viable cell density in TLR4-IN-C34 treated monocytes compared to monocytes that were not supplemented with TLR4-IN-C34. TLR4-IN-C34 significantly (*P*<0.05) reversed the detrimental effect that AXs or LPS had on cell growth and viability in 4.5.4.

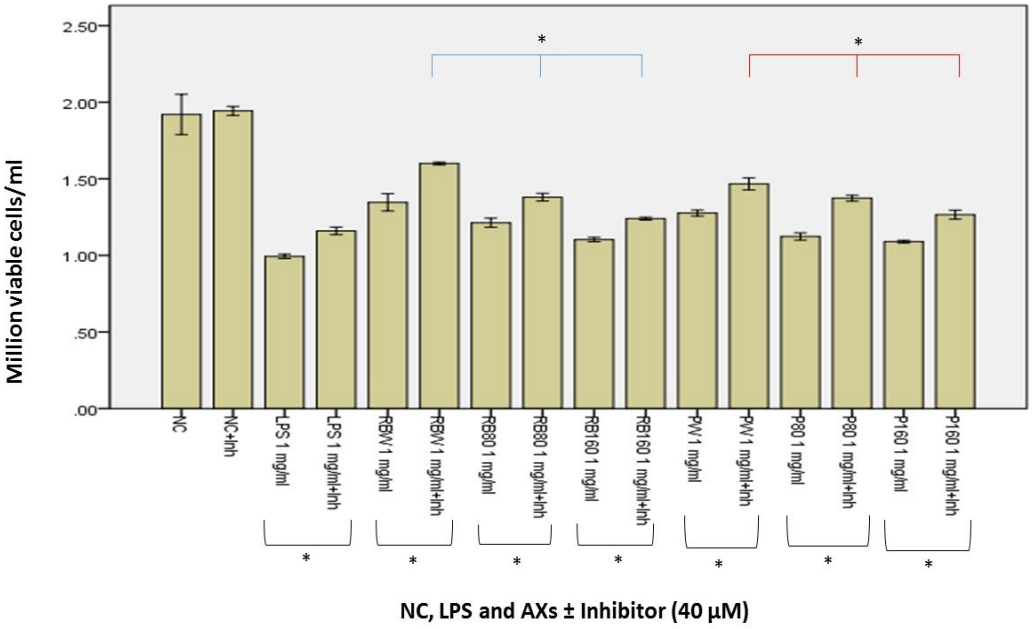

Figure 4-4 Effect of the TLR4 inhibitor (Inh) on cell density of monocytes following treatment with 1 mg/ml of LPS or 1 mg/ml AXs

Bars indicate mean ± standard error of the mean; n = 6 in all cases. Brackets below the graph indicate significant (**P*<0.05) differences between cell density in TLR4-inhibited monocytes compared to monocytes not supplemented with TLR4-IN-C34. Brackets above bars indicate significant (**P*<0.05) differences between cell density in TLR4-inhibited monocytes from different AXs treatments.

4.5.7 Effect of LPS and AXs on Nitric oxide production by U937 macrophages

NO secreted from U937 macrophages after stimulation with different concentrations of LPS and AXs from rice bran and wheat pentosan samples were investigated Figure

4-5. The findings confirm that the augmentation of NO secretion by macrophages stimulated with AXs and LPS is dose-dependent, with higher AX/LPS concentrations resulting in higher NO production. The results showed a significant (P<0.05) increase in NO secretion from macrophages following treatment with high concentrations of LPS or AXs. Among AXs, the highest NO secretion was from macrophages treated with RB160 at 1000 μg/ml (41.28±0.7 μM) whereas the same concentration of P160 resulted in substantially less NO secretion (28.5±0.8 μM). Moreover, the increase in NO secretion was related to an increase in screw-speed in all samples; NO increased from 20.23±0.4 μM with 1000 μg/ml RBW to 30.53±0.33 and 41.28±0.7 μM with 1000 μg/ml RB80 and 1000 μg/ml RB160, respectively. An increase in screw- speed had a positive effect on NO secretion by macrophages; NO was 12.53±0.02 μM with 1000 μg/ml PW but increased to 18.21±0.48 and 28.5±0.8 μM with 1000 μg/ml P80 and 1000 μg/ml P160 respectively.

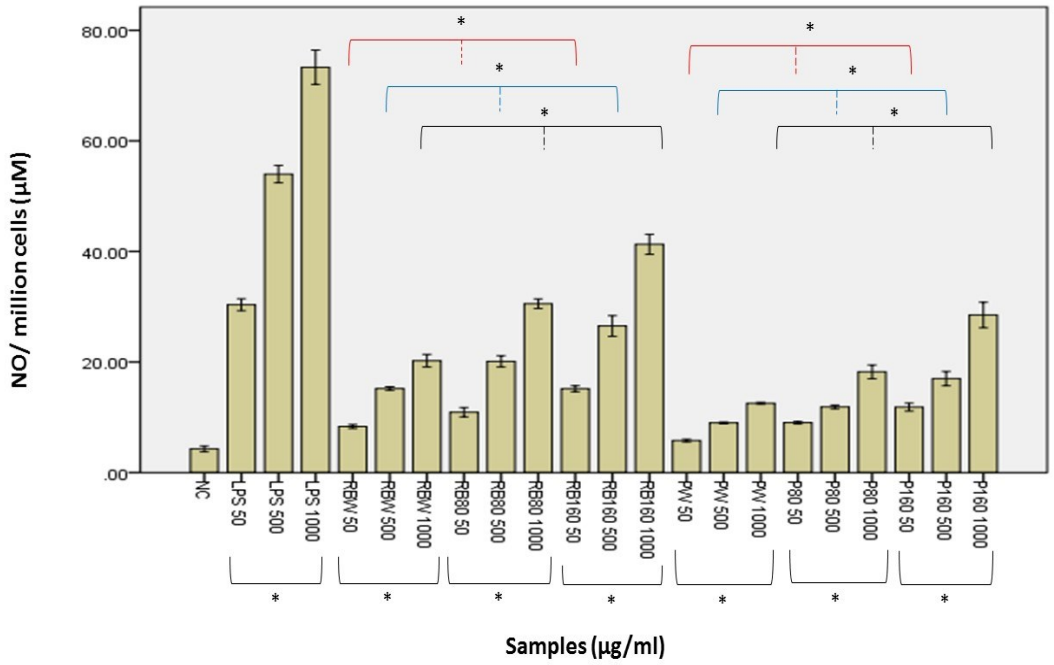

Figure 4-5 NO production by U937 macrophages after 24 hr treatment with LPS and AXs

The amount of NO secreted from treated cells was calculated using the nitrite standard curve (Appendix 7). The concentration of NO, expressed as the mean ± standard error of the mean, was compared to the NC using one-way ANOVA; **P*<0.05, n = 6 in all cases. Brackets above the bars indicate significant differences (**P*<0.05) between NO production in macrophages treated with the same concentration. Brackets below the bars indicate the significant differences across groups.

4.5.8 Comparison of NO production from U937 macrophages in the presence and absence of the TLR4 inhibitor

Nitric oxide secretion was measured in U937 macrophages supplemented with the TLR4 inhibitor after stimulation with LPS or AXs extracted from rice bran and wheat pentosan (RBW, RB80, RB160, PW, P80 and P160). In this experiment, the U937 macrophages were treated with the highest concentration of AXs (1 mg/ml). NO production from U937 cells in the absence and presence of the TLR4 inhibitor TLR4-IN-C34 after stimulation with 1mg/ml of AXs from RBW, RB80, RB160, PW, P80, P160 and LPS were compared (Figure 4-6). The findings demonstrated a significant (*P*<0.05) decrease in NO production in U937 macrophages supplemented with the

TLR4 inhibitor in comparison to U937 macrophages in the absence of TLR4-IN-C34 after treatment with 1 mg/ml of AXs from rice bran and wheat pentosan.

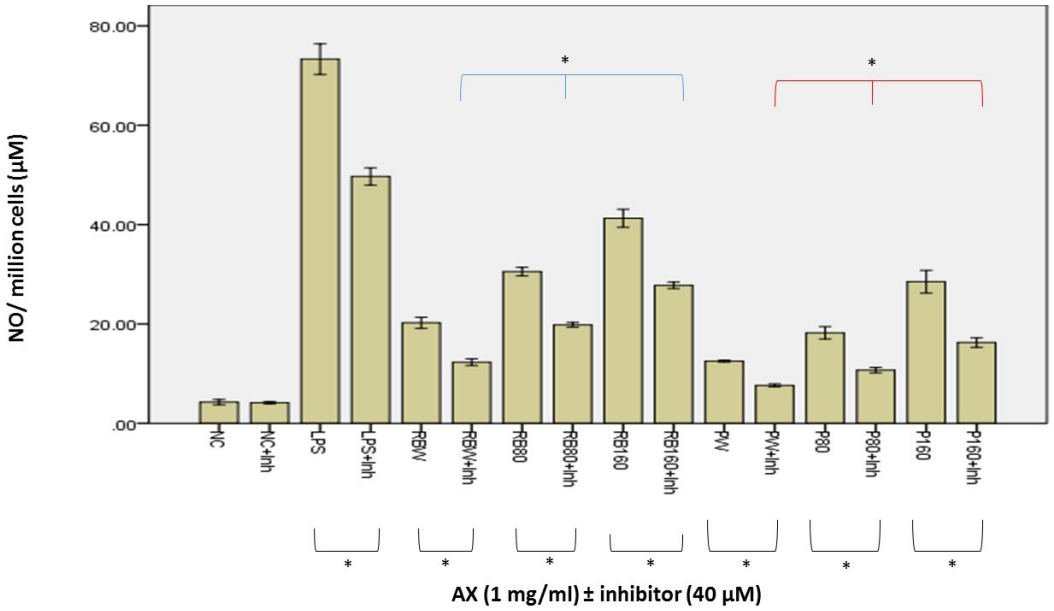

Figure 4-6 NO production from U937 macrophages supplemented with or without the TLR4 inhibitor (Inh)
Treatments with AXs or LPS (1mg/ml). One way ANOVA test showed significant differences between macrophages supplemented the inhibitor and macrophages in the absent of inhibitor. Data represent the mean ± standard error of the mean. **P*<0.05, n = 6 in all cases. Brackets below the graph indicate significant differences (**P*<0.05) between NO secretion in TLR4-inhibited macrophages compared to macrophages not supplemented with TLR4-IN-C34. Brackets above the bars indicate significant differences (**P*<0.05) between NO secretion in TLR4-inhibited macrophages from different AXs treatments.

4.5.9 The effect of AXs and LPS on cell growth and viability of U937 macrophages

Cell counts of the negative control (NC), positive control (LPS) at different concentrations (50, 500 and 1000 µg/ml) and AX treatments at different concentrations (50, 500 and 1000 µg/ml) extracted from rice bran and wheat pentosan samples were measured at the end of the NO assay. Higher concentrations of LPS or AXs treatment resulted in significantly (*P*<0.05) lower viable cell counts (Figure 4-7), confirming both LPS and AXs had a negative effect on viable cell count.

In general, AXs had a similar dose-dependent effect on macrophage cell growth and viability as LPS, with AXs just having less potency than LPS. Amongst the AXs, cell counts following treatment RBW significantly (P<0.05) decreased with increasing RBW concentration, from 0.77x10^6 viable cells/ml with 50 µg/ml to 0.6 and 0.5 x10^6viable cells/ml with 500 µg/ml RBW and 1000 µg/ml RBW respectively. The effect of screw-speed on the cell growth was investigated (Figure 4-7). There was a slight decrease in mean cell density from RBW-treated cells to RB80 and RB160, and from PW to P80 and P160 at 1 mg/ml concentration, but no significant differences were detected (P> 0.05 in all cases).

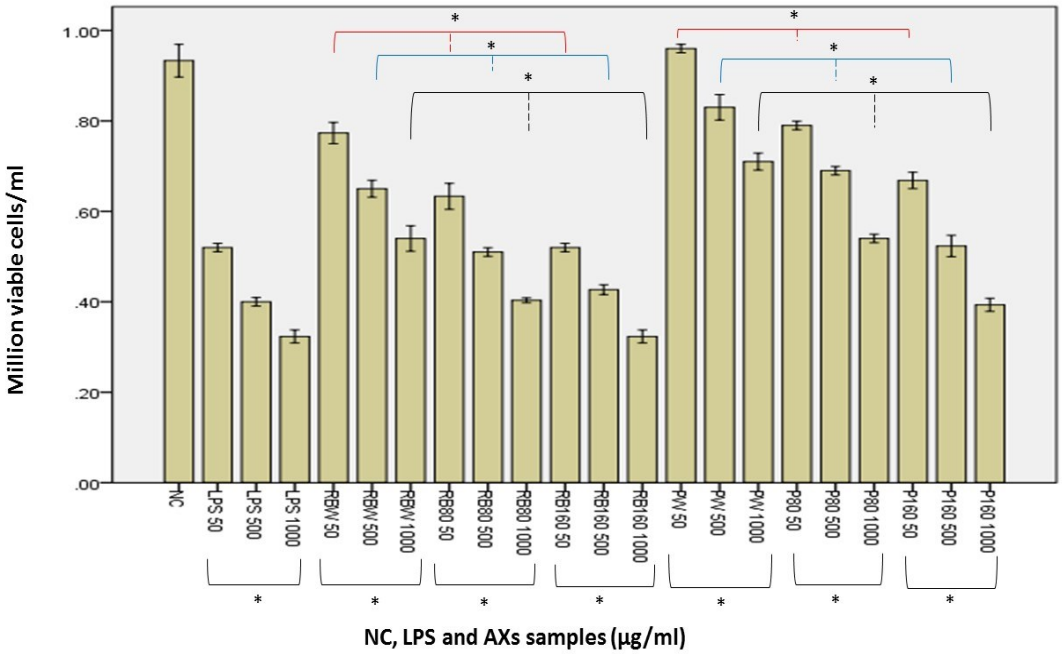

Figure 4-7 Effect of LPS and AXs concentrations on cell counts of macrophages

Bars indicate mean ± standard error of the mean; n = 6 in all cases. Cell density in all treatment groups was significantly (P<0.05) lower than in the negative control (NC). Cell density in all treatment groups was significantly (P<0.05) lower than in the negative control (NC). Brackets above bars indicate significant differences across groups whereas brackets below bars indicate significant differences within groups.

4.5.10 Effect of the TRL4 inhibitor on cell density of AX-treated U937 macrophages

The effect of the TLR4 inhibitor on the cell density of macrophages treated with 1 mg/ml LPS or 1mg/ml AXs was measured (Figure 4-8). There was a significant (P<0.05) increase in cell density in TLR4-inhibited macrophages compared to macrophages that were not supplemented with the TRL4 inhibitor. The TLR4 inhibitor significantly (P<0.05) reversed the detrimental effect that AXs or LPS had on cell growth and viability in Figure 4-7.

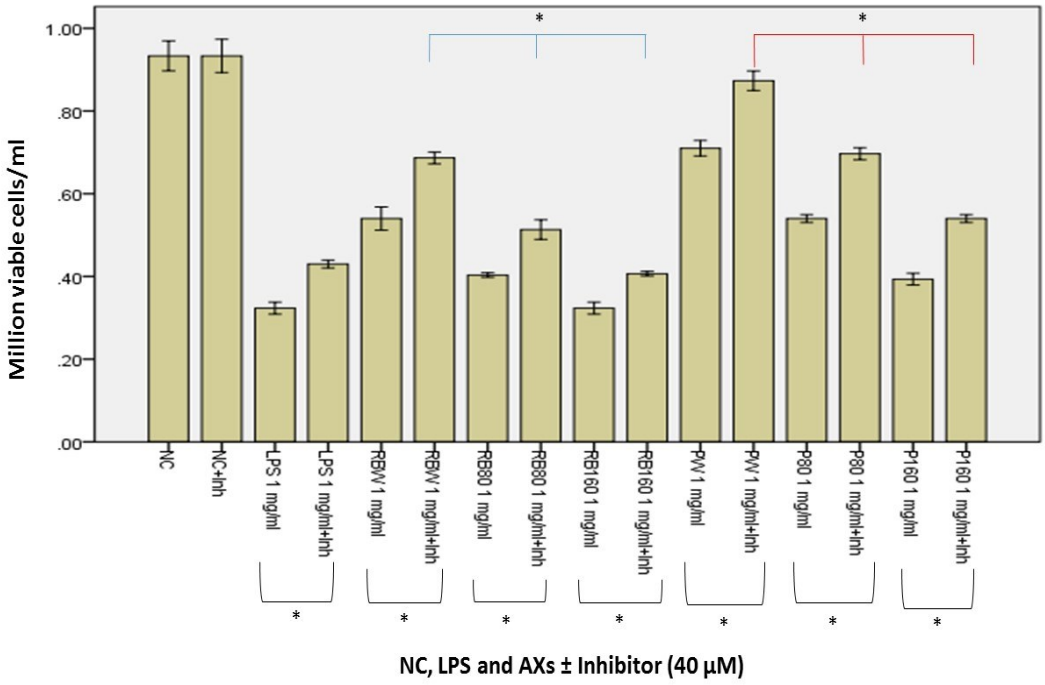

Figure 4-8 Effect of the TLR4 inhibitor (Inh) on cell density of macrophages following treatment with 1 mg/ml of LPS or 1 mg/ml AXs

Bars indicate mean ± standard error of the mean; n = 6 in all cases. Brackets below the graph indicate significant differences (*P<0.05) between cell density in TLR4-inhibited macrophages compared to macrophages not supplemented with TLR4-IN-C34, whereas brackets above bars indicate significant differences (*P<0.05) between cell density in TLR4-inhibited monocytes from different AXs treatments.

4.6 Discussion

4.6.1 Nitric oxide synthesis by U937 monocytes/macrophages

Nitric oxide (NO) is synthesised from the degradation of the amino acid L-arginine in the presence of oxygen by NO synthase. NO is a key free radical messenger for the innate immune defence (Bogdan, 2015). Several *in vivo* studies have reported that NO production accompanies diseases such as rheumatoid arthritis, inflammation and liver disease (Hibbs *et al*., 1992; Wang *et al*., 1997). *In vitro*, NO production by U937 monocytes/macrophages has been reported to be induced in response to infection from different bacteria such as *Mycobacterium bovis*, as well as gram-negative bacterial LPS (Nozaki *et al*., 1997; Bertholet *et al*., 1999). Hence, NO production by U937 monocytes/macrophages following treatment with (AXs) from extruded rice bran and wheat pentosan with/without inhibitor may reveal their immunomodulatory potential and provide evidence on potential receptor(s) through which the activities of AXs are mediated. Evidence suggests that LPS stimulates TLR4, which induces the secretion of pro-inflammatory cytokines, and NO (Lu *et al*., 2008).

In the current study, the TLR4 inhibitor TLR4-IN-C34 was used as a potential inhibitor for AXs and LPS. Treatment with 1 mg/ml LPS, RBW, RB80, RB160, PW, P80 or P160 significantly increased NO production in a dose-dependent manner in U937 monocytes/ macrophages, increasing 17.3, 5.0, 6.7, 8.0, 3.3, 5.3 and 7.0 fold, respectively in monocytes (Figure 4-5) and 16.1, 3.7, 6.1, 8.6, 1.9, 3.2 and 5.6 fold, respectively in macrophages (Figure 4-8), compared to the corresponding untreated negative control (NC).

The finding of this study currently closely with the AX levels in individual samples. However, at this stage without further detailed characterisation of the samples the effects of other bioactive components other than AX cannot be excluded.

4.6.2 Relationship between molecular weight and NO production

The results demonstrated that AXs extracted from rice bran and wheat pentosan significantly increased NO levels as AX concentration (50, 500 and 1000 µg/ml) increased. AXs from rice bran samples was the most effective stimulant of NO production in U937 monocytes/macrophages in comparison with AXs from wheat pentosan. In addition, the extrusion process showed that higher screw-speeds resulted in lower molecular weights of AXs. U937 monocytes/macrophages showed a higher level of NO stimulation after treatment with AXs from extruded samples from both rice bran and wheat pentosan. Interestingly, 75 and 77% of AXs in RB160 and RB80, respectively had Mw range between 0.79-10 kDa whereas 21.9 and 23.2 % of AXs in P80 and P160, respectively had Mw range between 0.85-3.16 kDa, with most of the AXs in pentosan samples within the molecular weight range between 3.16-31.62 kDa. Hence, the stimulatory effect of AXs from RB160, RB80, P160 and P80 on NO secretion by U937 monocytes/macrophages was associated with lower molecular weights (<10 kDa) rather than high molecular weight AXs. In addition, 0.79-1.58 kDa AXs from RB160 appear to be the most active immunomodulatory component compared with AXs from RB80, as those from RB160 have the highest percentage of 0.79-1.58 kDa AXs followed by AXs from P160, which correlates with their NO stimulation.

The findings suggest that low molecular weight AXs have greatest immunomodulatory effects on U937 monocytes/macrophages. It has been shown that the highest percentage of the low molecular weight AXs was obtained from RB160 that had the highest NO secretion by U937 monocytes/macrophages. Hence, the higher the screw-speed during extrusion, the lower the molecular weight of AXs and the higher the NO response.

4.6.3 Relationship between cell count and NO production

Bacterial cell wall components (i.e. LPS) have the ability to induce apoptosis in mammalian cells. LPS induces apoptosis through activation of Toll-like receptor 4 (TLR4) (Kuwabara and Imajoh-Ohmi, 2004). It has been reported that macrophages

have harmful effects under some circumstances such as the severe inflammatory response in septic shock, which is activated by the interaction of macrophages with LPS (Xaus *et al*., 2000). It has been reported that the NO and TNFα produced by macrophages upon treatment with LPS are the primary mediators for septic shock.

LPS and AXs from extruded/non-extruded rice bran and wheat pentosan showed a significant decrease in the proliferation of U937 monocytes/macrophages over 24 hours. Therefore, the augmentation in NO production was associated with the cell density (Figures 4.2 and 4.6).

The decrease in viable cell density was affected by LPS/AXs in a concentration-dependent manner, with higher concentrations of LPS/AXs causing the greatest inhibition in viable cell density but the highest NO secretion. According to Xaus *et al*. (2000), higher NO secretion is generally associated with reduced cell counts, in agreement with the findings of this study. In addition, the screw-speed had a significant effect on the molecular weight of AXs, which in turn increased the NO production and reduced the cell count.

In conclusion, LPS and AXs had a dose-dependent effect on cell count, significantly decreasing viable cell density as LPS/AX concentration increased. It can be concluded that the extrusion screw-speed had a significant effect on AX Mw (Chapter 3), nitric oxide production and viable cell count.

4.6.4 Possible receptor of AXs

It has been reported that there are some similarities between LPS and AXs in terms of structure and molecular weight, as both AXs and LPS have monosaccharides such as glucose and galactose (Ghoneum and Ogura, 1999; Ogawa *et al*., 2005; Zhang *et al*., 2015). The Mw of LPS is between 10-20 kDa (Sigma-Aldrich, 2016) and the AXs from extruded samples had a high percentage within the molecular range between (1-25 kDa) (Zhang *et al*., 2014, Mendis *et al*., 2016). Both LPS and AXs significantly increased NO secretion in monocytes and macrophages suggesting that both LPS and AXs are likely to be activating similar pathways and receptors.

Evidence suggests that LPS may mediate biological actions in part via TLR4 (Chang *et al*., 2000). Therefore, LPS/AX treatments were supplemented with a TLR4 inhibitor to assess whether NO activity is mediated, at least in part, through TLR4. The findings showed that the TLR4 inhibitor TLR4-IN-C34 could significantly reverse AXs/LPS-mediated increases in NO secretion by both monocytes and macrophages, thus suggesting that the NO production was at least in part acting through TLR4. Therefore, TLR4 may be a possible receptor for AXs. However, NO production was not completely blocked/reversed by the TLR4 inhibitor, suggesting either that the inhibitor concentration was insufficient for complete reversal or that one or more additional receptors may also mediate AX-responses. However, involvement of TLR4 has been demonstrated, indicating that AX-mediated NO secretion is mediated at least in part by TLR4. Other receptors that have been implicated in LPS activity include CD14 and myeloid differentiation protein-2 (MD-2) (Fujihara *et al*., 2003; Wright *et al*., 1990).

Chapter 5 The effect of AXs from extruded/non-extruded rice bran and wheat pentosan on TNFα secretion from U937 monocytes/macrophages

5.1 Introduction

Inflammation is the response of the immune system due to infection, injury or disease (Ferrero-Miliani *et al*., 2007). Upon inflammation, immune cells in the damaged area increase the blood flow and may cause an increase in pain, temperature and swelling (Abbas *et al*., 2012).

Cells of the innate immune system initiate the immune response. Monocytes and macrophages are major components of the innate immunity and they detect foreign antigens and microorganisms through pattern recognition receptors (PRRs) (Miller *et al*., 2005). Once PRRs are stimulated, they release cytokines, including TNFα. TNFα helps in communication with the innate immune cells by recruiting more macrophages and monocytes to the injured area (Kang *et al*., 2016).

TNFα facilitates the migration of the recruited cells via chemotaxis, stimulating cells to pass through tissues from the circulation. The recruitment of inflammatory cells leads to further TNFα secretion, generating a cycle of continued inflammation to protect the host until the infection is fully resolved (Mogensen, 2009). Activation of TNFα occurs via TNFα transcription, internal signalling and activation of cell surface receptors. Although there are two main receptors for TNFα, TNFR-1 and TNFR-2, it has been reported that Toll-like receptors, especially the TLR4 receptor, may mediate TNFα production.

It has been shown that TLR4 inhibition reduces TNFα and other pro-inflammatory cytokines such as IL-18 and IL-6 (Zhang *et al*., 2008b). TLR4 is a PRR that recognises specific molecules from pathogens called pathogen-associated molecular pattern molecules (PAMPs), like LPS from gram-negative bacteria (Molteni *et al*., 2016). Although inflammation has a protective function, in some diseases, an excessive inflammatory response might be detrimental. TLR4 activation has been involved in disease progression in both microbial (gram-negative infections) and non-microbial

etiology (neurological and neurodegenerative diseases) (Molteni *et al*., 2016). Furthermore, prolonged TLR4 activation and TNFα secretion have been associated with the pathogenesis of many diseases such as cancer, sepsis and arthritis (Chen and Goeddel, 2002).

Consequently, regulation of TNFα secretion is very important. Several suppressor cells secrete cytokines that inhibit TNFα secretion such as transforming growth factor β (TGFβ) and interleukin 10 (IL-10) (Cyktor and Turner, 2011). On the other hand, there are pharmaceutical TNFα inhibitors that can inhibit secretion of TNFα such as Vioxx and non-steroidal anti-inflammatory drugs (NSAIDS) (PAIOTTI *et al*., 2012). Although pharmaceutical chemicals can inhibit TNFα secretion, they also stimulate eicosanoid production, which blocks cyclooxygenase 1 and 2 enzymes (Ricciotti and FitzGerald, 2011). Eventually, prostaglandin synthesis will be inhibited, which is important for inflammatory mediation (Chang and Harris, 2005). The pharmaceutical industry invests a lot of money and time to design and develop drugs that inhibit TNFα via selective eicosanoids or by inflammatory mediator inhibition (Thun *et al*., 2002). Therefore, one proposed preventative action is to investigate the structure and mechanism of action of functional food components and plants that exert anti-inflammatory characteristics (Alissa and Ferns, 2012).

Non-starch polysaccharides are major constituents of many cereals such as rice and wheat (Lai *et al*., 2007). AXs are major non-starch polysaccharides that consist of xylopyranose backbone with arabinofuranose substitutions (Zhou *et al*., 2010). In addition, AX has been shown to contain hydroxycinnamic acid as ferulic acid, which has free radical scavenging activities (Yadav *et al*., 2007). Moreover, AX possess immunomodulatory effects such as cytokine modulation, activation of dendritic cells and apoptosis induction in cancer cells (Ghoneum and Gollapudi, 2004, Bermudez-Brito *et al*., 2015). Ghoneum and Matsuura (2004) have compared the effect of MGN-3, the enzymatic extract of arabinoxylan from rice bran, on TNFα secretion from three cell types; murine peritoneal macrophages, a human macrophage cell line and murine macrophage cell line. MGN-3 increased TNFα secretion in a dose dependent manner (1, 10, 100 µg/ml) in all the three cell types; however, the highest response

to MGN-3 among the three models was from the murine macrophage cell line at 100 µg/ml MGN-3 (Ghoneum and Matsuura, 2004). Furthermore, a recent study investigated the effect of MGN-3 on TNFα secretion from human macrophages, and showed a high concentration of MGN-3 (10 mg/ml) had the ability to induce TNFα production (132 pg/ml) but not to the same degree as LPS (10 ng/ml) that induced substantial TNFα secretion (7487 pg/ml) (Pérez-Martínez *et al*., 2015). A further study conducted by Badr El-Din *et al*. (2008) showed that MGN-3 significantly increased TNFα secretion by 11 % in mice bearing solid Ehrlich carcinoma (SEC) in comparison to the untreated mice.

Apart from MGN3, rice bran polysaccharide extract (RBP), which is composed of 22.58 % arabinose and 7.01 % xylose, showed a significant increase in TNFα production in macrophage RAW264.7 cells in a dose-dependent manner, with 500 µg/ml RBP increasing TNFα secretion 427.4-fold (Wang *et al*., 2008).

This study has shown that extrusion at different screw-speeds of wheat pentosan and rice bran increases the extraction rate and reduces the molecular weight of AXs significantly (Chapter 3). The aim of this chapter was to measure the effect of these purified AXs on TNFα secretion by U937 monocytes and macrophages, followed by supplementation with the TLR4 inhibitor to determine if TLR4 may be involved in the AX-mediated responses.

5.2 Materials

5.2.1 AXs

WEAX from extruded rice bran and wheat pentosan were extracted and purified as described in Chapter 3. AXs were prepared by dissolving in culture medium as described in Chapter 4.

5.2.2 Chemicals

All chemicals and culture medium preparation were as described in Chapter 3 and 4.

5.2.3 Cell line

U937 monocytes and macrophages were prepared as described in Chapter 4.

5.2.4 TNFα measurements

The TNFα secretion in the culture supernatant of monocytes and macrophages at 24 hours following stimulation was determined using a human TNFα sandwich ELISA Kit purchased from Abcam (Cambridge, USA).

5.3 Methods

5.3.1 AXs and LPS

AXs extracts were prepared at three concentrations (50, 500 and 1000 µg/ml). Lipopolysaccharides (LPS) was prepared at two concentrations (1 and 5 µg/ml) as described in Chapter 4.

5.3.2 Cell culture and TLR4 inhibitor

U937 monocytes and PMA-induced macrophages were prepared following the methodology described in Chapter 4. Monocytes and macropahges were treated with AXs samples and LPS for 24 hours in sextuplicate. The highest concentrations of LPS and AXs were also investigated using monocytes and macrophages supplemented with 1 mM TLR4 inhibitor for 24 hours. Untreated negative control wells were prepared by adding 100 µl of cultured cells to 100 µl of complete medium.

All samples were then centrifuged at 125 x g for 7 minutes and supernatants were collected and stored at -20^{o}C prior to TNFα quantification.

5.3.3 Human TNFα quantification

TNFα secretion by U937 monocytes and macrophages was measured following the protocol provided by the manufacturer. Briefly, replicate (n=6) wells containing TNFα antibody were supplemented with 100 µl of supernatants followed by 50 µl of anti-TNFα and incubated for 3 hours at room temperature. Streptavidin-HRP 100 µl was added to all wells followed by incubation for 30 minutes at room temperature. Chromogen TMB substrate (100 µl) was added to all the wells, which were then covered with aluminium foil and incubated for 15 minutes at room temperature. The stop reagent (100 µl) was added into each well. The ELISA plate was read within 30 minutes at two separate wavelengths (450 and 620 nm).

5.4 Statistics

Data were expressed as mean TNFα ± SEM. Significant differences between samples were determined by one-way analysis of variance (ANOVA) with multiple Tukey pairwise comparison tests on SPSS 23 software. A *P* value of less than 0.05 was considered statistically significant.

5.5 Results

5.5.1 The effect of LPS and AXs on TNFα secretion by U937 monocytes

U937 monocytes were stimulated with different concentrations of LPS or AXs extracted from rice bran and wheat pentosan at different screw-speeds (80 and 160 rpm) (Figure 5-1). The TNFα secretion significantly increased in a dose-dependent manner, with higher concentrations of AX or LPS resulting in higher levels of TNFα production.

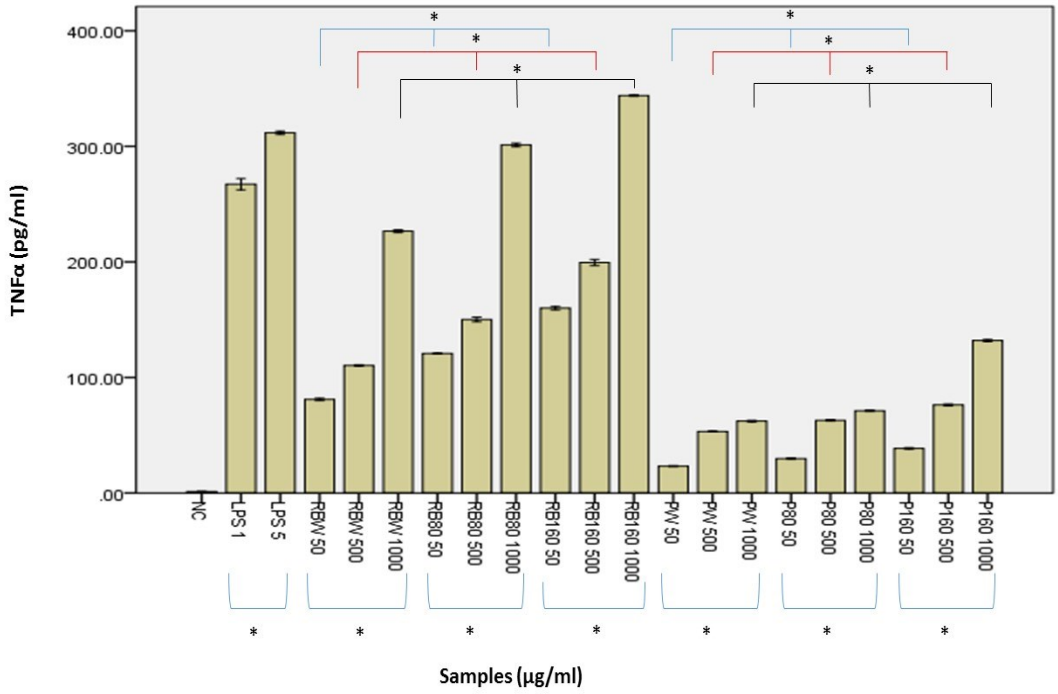

Figure 5-1 TNFα secretion by U937 monocytes after 24hr treatment with LPS and AXs

Bars represent mean TNFα ± standard error of the mean and n = 6 in all cases. Treatments were compared to the untreated negative control (NC) by one-way analysis of variance (ANOVA) with Tukey multiple pairwise comparison tests; *$P<$ 0.05 was considered significant. The brackets beneath the graph represent significant differences within the same sample, while the brackets above the graph represent significant differences between samples at the same concentration.

In monocytes treated with AXs extracted from pentosan, a significant increase in TNFα secretion was detected even at low concentrations of wheat pentosan, with 50

μg/ml pentosan resulting in 23.2 pg/ml TNFα in comparison to control cells, which secreted 1.1 pg/ml TNFα. There was a significant increase in TNFα secretion within the same sample as the concentration of AXs (or LPS) increased and significant increases in TNFα secretion as the screw-speed of extrusion increased from 80 rpm to 160 rpm (Figure 5-1). Within the same sample, TNFα secretion increased as the concentration of AX/LPS increased, with TNFα production in monocytes treated with AX from the non-extruded pentosan increasing from 23.2 to 53.2 and then to 62.1 pg/ml as the concentration of AX increased from 50 to 500 and then to 1000 μg/ml respectively. Similarly, in monocytes treated with AXs extracted from P80, the TNFα secretion increased from 29.7 to 62.8 and then to 71.1 pg/ml as the AX concentration increased from 50 to 500 and 1000 μg/ml respectively. In a similar way, TNFα secreted by monocytes treated with AXs extracted from pentosan extruded at the higher screw-speed of 160 rpm was also higher, with TNFα secretion increasing from 38.6 to 76.2 and then to 131.9 pg/ml as P160 concentration increased from 50 to 500 and then to 1000 μg/ml respectively.

TNFα secretion from monocytes treated with AXs from rice bran showed a dose-dependent increase (Figure 5-1), in a similar manner to that produced by AXs from wheat pentosan. However, the increase in TNFα secretion generated by AXs from rice bran was significantly higher than that produced by wheat pentosans. The production of TNFα was increased from 80.9 to 110.3 and then to 226.5 pg/ml as the concentration of non-extruded rice bran AXs increased from 50 to 500 and then to 1000 μg/ml. Furthermore, TNFα production significantly increased in monocytes treated with extruded rice bran at 80 rpm screw-speed, increasing from 120.7 to 150.1 and then to 301 pg/ml at 50 to 500 and then to 1000 μg/ml RB80 respectively. Similarly, the higher (160 rpm) screw-speed of extrusion of rice bran increased TNFα secretion in monocytes in a dose-dependent manner, increasing from 160.0 to 199.4 and then to 343.0 pg/ml as the concentration of RB160 increased from 50 to 500 and then to 1000 μg/ml respectively.

TNFα secretion not only increased in a concentration-dependent manner but it also significantly increased as the screw-speed increased. Thus, the higher the screw-

speed the higher the TNFα secretion. Moreover, AXs from rice bran had a significantly higher pro-inflammatory effect than AXs extracted from wheat pentosan, even in non-extruded samples.

5.5.2 Effect of the TLR4 inhibitor TLR4-IN-C34 on AXs-mediated secretion of TNFα by monocytes

The highest concentrations of LPS (5 μg/ml) and extruded/non extruded AXs (1 mg/ml) from rice bran and wheat pentosan were tested following supplementation of monocytes with the TLR4 inhibitor (TLR4-IN-C34) for 24 hours (Figure 5-2).

The TLR4 inhibitor had no significant (P>0.05) effect on TNFα production in the negative control (NC). On the other hand, a significant (P<0.05) decrease in TNFα secretion was observed from U937 monocytes treated with pentosan supplemented with the TLR4 inhibitor, decreasing from 53.2 pg/ml (without inhibitor) to 29.5 pg/ml (with inhibitor) in the non-extruded 1 mg/ml pentosan. Similarly, a significant (P<0.05) decrease in TNFα secretion was observed in U937 monocytes treated with 1 mg/ml pentosan extruded at 80 rpm (P80), decreasing from 71.1 pg/ml (without inhibitor) to 40 pg/ml (with inhibitor). In a similar way, TNFα secretion significantly decreased in U937 monocytes treated with pentosan extruded at 160 rpm (P160), decreasing from 131.9 pg/ml (without inhibitor) to 93.1 pg/ml (with inhibitor).

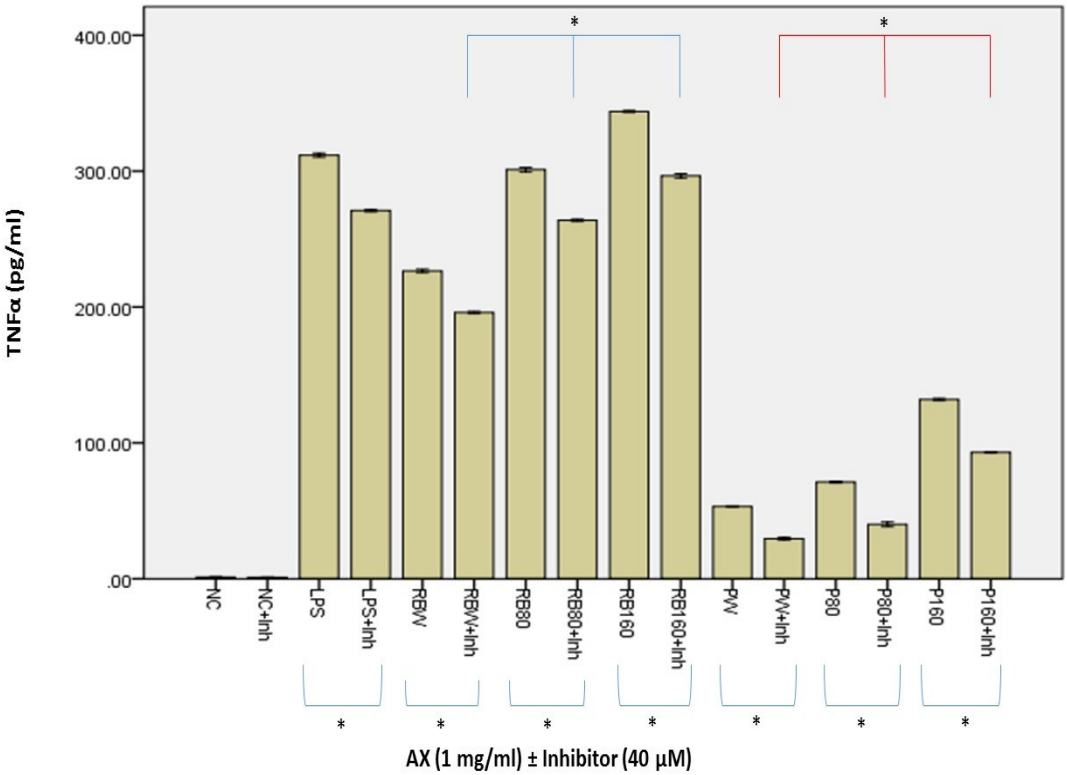

Figure 5-2 TNFα secretion by U937 monocytes after 24 hrs treatment with AXs with/without TLR4 inhibitor (TLR4-IN-C34)

P*<0.05 was considered significant; n= 6 in all cases. Bars represent the mean TNFα ± standard error of the mean of six replicates. Brackets below the graph indicate significant (P*<0.05) differences between TNFα secretion in TLR4-inhibited monocytes compared to monocytes not supplemented with the TLR4 inhibitor. Brackets above bars indicate significant (**P*<0.05) differences between NO secretion in TLR4-inhibited monocytes from different AXs treatments.

There was a significant (*P*<0.05) decrease in TNFα production in U937 monocytes treated with AXs from non-extruded rice bran following supplementation with the TLR4 inhibitor, decreasing from 226.5 pg/ml (without inhibitor) to 195.9 pg/ml (with inhibitor). In a similar fashion, TNFα production significantly (*P*<0.05) decreased from monocytes treated with AXs from rice bran extruded at 80 rpm (RB80) after supplementation with the TLR4 inhibitor, decreasing from 301.1 pg/ml (without inhibitor) to 296.5 pg/ml (with inhibitor). Similarly, TNFα production significantly (*P*<0.05) decreased in U937 monocytes after treatment with 1 mg/ml RB160 and

supplementation with the TLR4 inhibitor, decreasing from 343.9 pg/ml (without inhibitor) to 296.5 pg/ml (without inhibitor).

Overall, supplementation of the TLR4 inhibitor significantly (P<0.05) reversed all the AX-induced or LPS-induced elevations of TNFα secretion by monocytes, suggesting AXs and LPS may mediate TNFα levels, at least in part, through TLR4.

5.5.3 The effect of LPS and AXs on TNFα secretion by U937 macrophages

U937 macrophages were stimulated with different concentrations of LPS and AXs extracted from rice bran and wheat pentosan at different extrusion screw-speeds (80 and 160 rpm) (Figure 5-3). TNFα secretion significantly (P<0.05) increased in a dose dependent manner as AX/LPS concentration increased.

In U937 macrophages treated with AXs extracted from pentosan, TNFα secretion significantly (P<0.05) increased compared to the untreated negative control (NC). At the lowest concentration of pentosan (50 μg/ml), TNFα was 1.8 ng/ml in comparison to the negative control (NC) which had TNFα levels of only 0.8 ng/ml. There was a significant (P<0.05) increase in TNFα secretion with increasing concentration of AXs treatment and a significant (P<0.05) increase in TNFα production with extruded samples as the screw-speed increased from 80 rpm to 160 rpm (Figure 5-3).

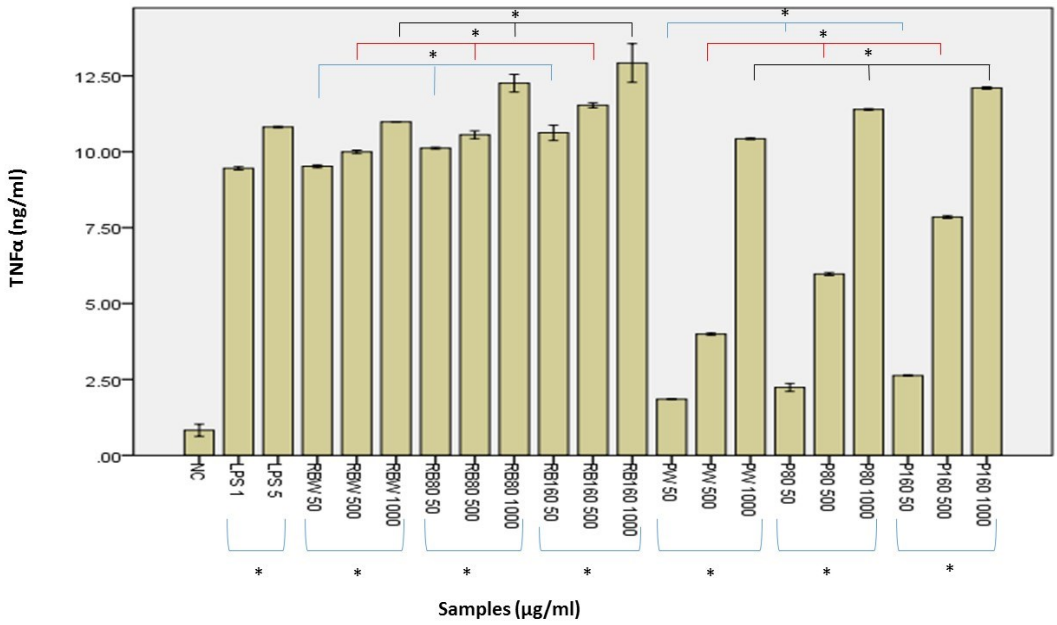

Figure 5-3 TNFα secretion by U937 macrophages after 24hr treatment with LPS and AXs

Bars represent mean TNFα ± standard error of the mean and n = 6 in all cases. Treatments were compared to the untreated negative control (NC) by one-way analysis of variance (ANOVA) with Tukey multiple pairwise comparison tests; **P*<0.05 was considered significant. The brackets beneath the graph represent significant differences within the same sample while the brackets above the graph represent significant differences between samples at the same concentration.

The TNFα secretion by macrophages significantly (*P*<0.05) increased in a dose-dependent manner, with higher concentrations of AXs or LPS resulting in higher levels of TNFα production. In macrophages treated with AXs from the non-extruded wheat pentosan, TNFα production increased from 1.8 to 3.9 and then to 10.4 ng/ml as the concentration of AX increased from 50 to 500 and then to 1000 µg/ml respectively. In a similar way, TNFα secretion from macrophages treated with AXs extracted from pentosan extruded with a screw-speed of 80rpm (P80) significantly (*P*<0.05) increased from 2.2 to 5.9 and then to 11.3 ng/ml as the AXs concentration increased from 50 to 500 and then to 1000 µg/ml respectively. Similarly, TNFα secreted by macrophages treated with AXs extracted from pentosan extruded at the higher screw-speed of 160 rpm (P160) was higher than that produced with the lower

screw-speed (P80), with TNFα secretion increasing from 2.6 to 7.8 and then to 12.0 ng/ml as the AXs concentration increased from 50 to 500 and then to 1000 μg/ml respectively.

TNFα secretion from macrophages treated with AXs from rice bran showed a dose-dependent increase as AXs concentration increased, similar to that shown with wheat pentosan. However, the increase in TNFα secretion following treatment with AXs from rice bran was significantly (P<0.05) higher than that generated with AXs from wheat pentosan. The production of TNFα by U937 macrophages increased from 9.5 to 9.9 and then to 10.9 ng/ml as the concentration of AXs extracted from the non-extruded rice bran increased from 50 to 500 and then to 1000 μg/ml. Furthermore, TNFα production increased in macrophages treated with rice bran extruded at a 80 rpm screw-speed compared to AXs from non-extruded rice bran. The increase in TNFα secretion was from 10.1 to 10.5 and then to 12.2 ng/ml as the AX concentration increased from 50 to 500 and then to 1000 μg/ml respectively. Similarly, TNFα secretion increased in macrophages treated with AXs extracted from rice bran at the higher screw-speed (160 rpm) in a dose-dependent manner, increasing from 10.6 to 11.5 and then to 12.9 ng/ml as the concentration increased from 50 to 500 and then to 1000 μg/ml respectively.

Overall, TNFα secretion not only increased in a concentration-dependent manner but it also increased as the extrusion screw-speed increased. Moreover, the increase in TNFα production from macrophages compared to monocytes was substantial, with pg/ml levels produced by monocytes compared to ng/ml levels produced by macrophages.

5.5.4 Effect of the TLR4 inhibitor (TLR4-IN-C34) on AXs-mediated secretion of TNFα by macrophages

LPS at 5 μg/ml and extruded/non-extruded AXs from rice bran and wheat pentosan at 1 mg/ml were tested following supplementation of macrophages with the TLR4 inhibitor for 24 hours (Figure 5-4). The TLR4 inhibitor had no significant (P>0.05) effect on TNFα secretion by macrophages in the negative control (NC). On the other

hand, a significant (P<0.05) decrease in TNFα level was observed in macrophages treated with 1 mg/ml AXs from non-extruded wheat pentosan following TLR4 supplementation compared to treatment in the absence of the inhibitor, with TNFα decreasing from 10.4 ng/ml (without inhibitor) to 6.5 ng/ml (with inhibitor). Similarly, a significant (P<0.05) decrease in TNFα secretion following TRL4 inhibitor supplementation was observed in the U937 macrophages treated with 1 mg/ml AXs from pentosan extruded at 80 rpm (P80), with TNFα decreasing from 11.3 ng/ml (without inhibitor) to 7.7 ng/ml (with inhibitor). Similarly, TNFα secretion decreased following TRL4 inhibitor supplementation in macrophages treated with AXs from pentosan extruded at 160 rpm (P160), with TNFα decreasing from 12 ng/ml (without inhibitor) to 8.3 ng/ml (with inhibitor).

In addition, there was a significant (P<0.05) decrease in TNFα production by macrophages treated with AXs extracted from non-extruded rice bran following supplementation with the TLR4 inhibitor, with TNFα decreasing from 10.9 ng/ml (without inhibitor) to 7.5 ng/ml (with inhibitor). In a similar fashion, TNFα production significantly (P<0.05) decreased in macrophages supplemented with the TLR4 inhibitor and treated with AXs from rice bran extruded at 80 rpm (RB80), with TNFα decreasing from 12.2 ng/ml (without inhibitor) to 8.1 ng/ml (with inhibitor). Moreover, the TLR4 inhibitor had a similar effect on TNFα production after treatment with 1 mg/ml AXs from rice bran extruded at 160 rpm (RB160), with TNFα decreasing from 12.9 ng/ml (without inhibitor) to 9.9 ng/ml (with inhibitor).

Overall, supplementation of the TLR4 inhibitor significantly (P<0.05) reversed all the AX-induced or LPS-induced elevations of TNFα secretion by macrophages, suggesting AXs and LPS may mediate TNFα levels, at least in part, through TLR4.

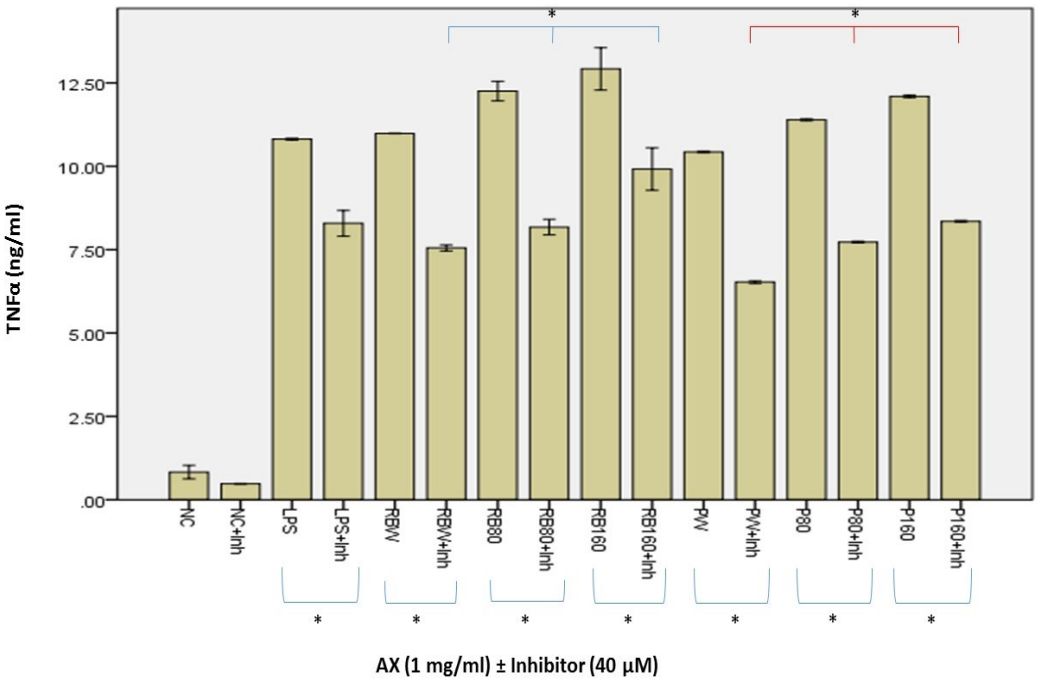

Figure 5-4 TNFα secretion by U937 macrophages after 24 hrs treatment with AXs with/without TLR4 inhibitor

P<0.05 was considered significant; n= 6 in all cases. Bars represent the mean TNFα ± standard error of the mean for six replicates. Brackets below the graph indicate significant (P<0.05) differences between TNFα secretion in TLR4-inhibited macrophages compared to macrophages not supplemented with the TLR4 inhibitor. Brackets above bars indicate significant (*P<0.05) differences between NO secretion in TLR4-inhibited macrophages from different AXs treatments.

5.6 Discussion

5.6.1 The effect of AXs on TNFα production by U937 monocytes/macrophages

Inflammation is the immune system response due to infection, injury or disease (Ferrero-Miliani *et al*., 2007). The inflammatory response is not necessarily detrimental; limited inflammation is essential to maintain good health. Pro-inflammatory cytokines produced upon inflammation are important in the phagocytosis of microbes (Vernaza *et al*., 2012).

The innate immune system cells, such as monocytes and macrophages, are important in detecting pathogens through pattern recognition receptors (PRRs), including the TLR4 receptor. Lipopolysaccharide (LPS) from the cell wall of gram-negative bacteria is an agonist of TLR4 in U937 cells (Johnson *et al*., 2002; Termeer *et al*., 2002; Miller *et al*., 2005). TNFα is a multifunctional pro-inflammatory cytokine that is released by macrophages in response to activation of the TLR4 receptor by LPS (Ghoneum and Mastuura, 2004; Zhang *et al*., 2008b). A similarity in the structure and molecular weight between AXs and LPS has been proposed previously in this study. Thus, confirming modulation of TNFα production in human inflammatory cells could have profound importance to human health.

It has been reported that MGN-3, a modified AXs with low molecular weight, significantly increases the levels of TNFα in a concentration-dependent manner (Ghoneum and Matsuura, 2004; Cholujova *et al*., 2009). MGN-3 has the potential to induce TNFα secretion from human lymphocytes and natural killer cells (NK cells) (Ghoneum and Jewett, 1999; Ghoneum and Mastuura, 2004).

In this study, the effect of AXs from extruded/non-extruded rice bran and wheat pentosan on TNFα production by U937 monocytes/macrophages was investigated. TNFα secretion by monocytes significantly increased following treatment with AXs from extruded/non-extruded samples and the response was concentration dependent, with TNFα secretion increasing as AX concentration increased. For rice bran, the highest TNFα secretion observed was from monocytes treated with AXs from rice bran extruded at 160 rpm (RB160) at 1 mg/ml followed by a lower

stimulatory effect in RB80 and RBW respectively. Similarly, for wheat pentosan, the highest TNFα secretion was from monocytes treated with AXs from pentosan extruded at 160 rpm (P160) followed by a lower amount of TNFα production from P80 and PW respectively. This confirmed that extruded samples with a higher concentration of the low Mw of AXs resulted in a higher immunostimulatory potential in terms of TNFα secretion.

Similarly, TNFα secretion by macrophages was significantly stimulated with AXs from extruded/non-extruded rice bran and wheat pentosan. For rice bran, the highest TNFα secretion was observed in macrophages treated with AXs from rice bran extruded at 160 rpm followed by a lower secretion of TNFα by RB80 and non-extruded RBW respectively. In macrophages treated with AXs from wheat pentosan, a similar effect on TNFα secretion was observed. The highest level of TNFα was secreted from macrophages stimulated with AXs from wheat pentosan extruded at 160 rpm followed by a lower secretion of TNFα by RB80 and non-extruded RBW respectively. This again confirmed that extruded samples, with a higher percentage of the low molecular weight of AXs, resulted in higher immunostimulatory potential in terms of TNFα secretion.

Furthermore, stimulation of macrophages with AXs produced substantially higher levels of TNFα in comparison with monocytes. Interestingly, it has been found that the capacity of monocytes to produce TNFα in response to LPS stimulation is less than that of macrophages (Mata *et al*., 1990; Wigmore *et al*., 1998; Takashiba *et al*., 1999), which concurs with the findings in this study.

5.6.2 TLR4 inhibition and TNFα secretion

It has been reported that TNFα secretion is stimulated through the activation of the TLR4 receptor by LPS (Ghoneum and Mastuura, 2004; Zhang *et al*., 2008b). Therefore, to investigate the effect of TLR4 inhibition on TNFα production by monocytes/ macrophages treated with AXs, cells were treated with a TLR4 inhibitor prior to stimulation with AXs from extruded/non-extruded rice bran and wheat pentosan. The TLR4 inhibitor significantly reversed the AX-mediated stimulation of TNFα

secretion from monocytes/macrophages treated with AXs from extruded/non-extruded rice bran or wheat pentosan (Figures 5.2 and 5.4). This demonstrated the AX-induced TNFα secretion by monocytes and macrophages was, at least in part, mediated via TLR4, thus suggesting that TLR4 is a receptor for AXs.

The concentration of AXs in samples can be estimated from the estimated purities together with the Mw ranges of AXs in the sample. Therefore, we can determine a concentration range for the low and high Mw AXs. The biological responses of AXs then be compared with the known concentration of LPS.

This study has confirmed that AXs mediate the immune response by human monocytes and macrophages, suggesting that dietary AXs may have important immunomodulatory activity in the human body. Moreover, these findings suggest that both LPS and AXs may be mediated (at least in part) by the same receptor, but that AXs may not activate the TLR4 receptor to the same degree as LPS. Thus, it could be speculated that in the presence of infection (LPS) in the human body, AXs from the diet may compete with LPS for the same receptor, thus potentially dampening the overall immune response via competitive inhibition of TLR4. In the absence of infection (LPS) in the human body, dietary AXs may moderately activate the TLR4 receptor, thus providing a low-level but heightened immune response in the absence of infection. Further work is required to investigate this theory and confirm these findings *in vivo* using animal models and conducting human trials on dietary AXs.

Chapter 6 General Discussion

Using extrusion to modify the solubility and Mw of AXs is an attractive proposition because of its low cost, high speed and its low environmental impact (Jeon *et al*., 2014).

Currently, most of the research carried out on extraction of AXs has applied very aggressive treatments (acid/alkaline hydrolysis, solvent extraction etc.) in order to obtain the maximum yield of AXs from the substrate. These processes can be hazardous and tend to produce effluents that are damaging to the environment and are costly to dispose of (Jeon *et al*., 2014). Extrusion is a cheap and environmentally friendly technology but it is not known if it would have the effect of increasing solubility and reducing Mw of AXs.

Rice bran and wheat endosperm pentosan was extruded at two screw speeds 80 and 160 rpm. The solubility of AXs from the non-extruded rice bran and wheat pentosan was significantly different with the amount of WEAX from rice bran being significantly (P<0.05) lower than that obtained from wheat pentosan (Tables 3.3 and 3.4).

The low solubility of AXs in the original samples was suggested to be due to the different amounts of ferulic acid in the rice bran and wheat pentosan samples. The ferulic acid content in rice bran was 303 mg/100g whereas in wheat pentosan it was between 31-111 mg/100g (Jung *et al*., 2007; Michniewicz *et al*., 2013; Hüseyin, 2015). Ferulic acid is known for its low solubility in water (Anson *et al*., 2009). It was also reported that ferulic acid has the ability to form diferulic acid bridges between adjacent AXs chains (Gruppen *et al*., 1992; Fry, 2004) and it can be esterified to some arabinose side chains, resulting in lower AXs solubility (Snelders *et al*., 2013). Moreover, the low solubility of AXs in the raw samples could be due to the close packing of the cell wall content, which might be due to steric hindrance (Faulds *et al*., 2006).

Studies by Vermaas *et al*., (2015); Karunanithy *et al*., (2010); Yoo *et al*., (2012) have suggested that increasing screw speed would increase the shear stress applied to the sample resulting in breaking of glycosidic bonds and depolymerisation of the AXs. This in turn may be expected to increase the soluble fraction. It can also be proposed that the high shear stress produced in the extruder barrel due to the increase in screw speeds from 80 to 160 rpm, might break the glycosidic bonds resulting in depolymerisation of AXs and explain the increase in solubility (Margareta and Nyman, 2003).

Studying the Mw distribution of AXs is important to understand their immunomodulatory potential. Li *et al* (2015) reported that low Mw AXs enhances the immune response both *in vitro* and *in vivo*. Mw distribution was analysed using HPSEC. The Mw range of rice bran and wheat pentosan samples was within the range of (0.79-25) and (0.85-794) kDa respectively. The Mw distribution of AXs from wheat pentosan was within the range reported by Li *et al* (2015).

The findings of this research suggested that extrusion has an effect on the Mw distribution of AXs from both rice bran and wheat pentosan. It was found that extruded rice bran and wheat pentosan have a higher amount of very low Mw of AXs when compared with AXs from non-extruded samples.

This reduction in Mw range is in agreement with the findings of Svanberg *et al*. (1995) and Margareta and Nyman (2003) and it is suggested to be a result of depolymerisation of the cell wall material due to the high shear and high temperature applied in the extrusion process (Svanberg *et al*., 1995). It can also be suggested that the high shear produced in the barrels might lead to break down of the glycosidic bonds (Margareta and Nyman, 2003).

Comparing the Mw distribution from rice bran and wheat pentosan showed a significant difference ($P<0.05$) in the quantity of very low Mw AXs obtained from extruded rice bran samples, which was higher than that obtained from wheat pentosan samples. The higher level of low Mw of AXs from rice bran may be due to

the fact that the rice bran xylan backbone carries more arabinose side chains than the xylan backbone of wheat pentosan. Thus, a higher degree of esterification would mean more bonds to be broken and therefore perhaps less extractable AX overall. However, it must be highlighted that only a concentration range is estimated for each AX sample in this study rather than the actual concentration.

Moreover, it can be postulated that the screw speed has an effect on the Mw range, as the higher the screw-speed the higher percentage of very low Mw AXs obtained from both rice bran and wheat pentosan samples (Figures 3-3 and 3-4). The high screw speed (160 rpm) produces a higher shear stress and a higher pressure in the barrels, which may break down the glycosidic bonds of the cell wall material.

It has been reported that the lower the Mw of AXs the higher the immune response and to study the immuno-modulatory potential of the extruded AXs, NO synthesis by monocytes and macrophages was determined following treatment with AXs.

The intestinal uptake of AXs and their subsequent transportation to body tissues such as the spleen, lymph and bone marrow might start when the intestinal macrophages are exposed to dietary AXs and they might be involved (at least in part) in the uptake and transportation of AXs to non-intestinal tissues.

The largest pool of tissue macrophages in the body are strategically located in the sub-epithelial lamina propria in the gastrointestinal mucosa. These macrophages are in close proximity to enormous numbers of antigenic stimuli and luminal bacteria. The gastrointestinal macrophages regulate inflammatory responses to antigens and bacteria that rupture the epithelium, protect the mucosa against harmful pathogens, and scavenge dead cells and foreign debris (Nagashima *et al*., 1996). Figure 6.1 shows mucosa exposure to pathogens and host response. There are four ways that microbes gain entry into the lamina propria through the mucosal epithelium: (1) through Microfold (M) cells, (2) between or through intact epithelial cells, (3) through disrupted or injured epithelium, and (4) by dendritic cells (Smith *et al*., 2011).

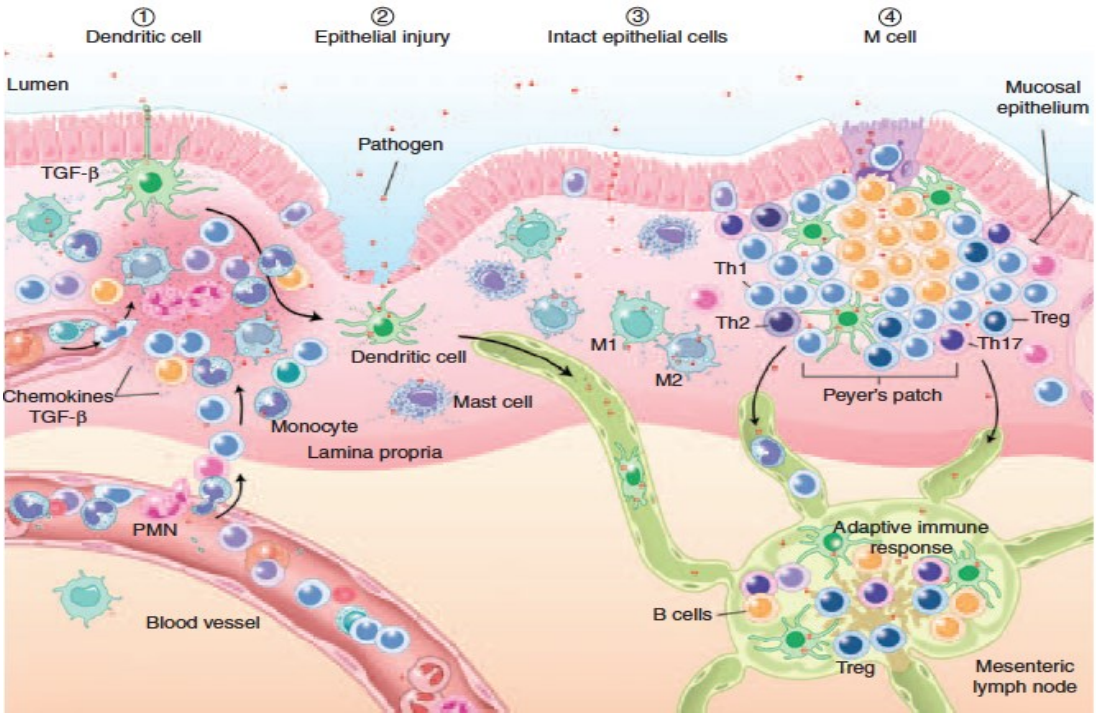

Figure 6-1 Mucosal exposure to pathogens and host response

Depending on the extracellular milieu, the monocyte/macrophage is activated directly by the binding of pathogen associated molecular patterns (PAMPs) from pathogens or alternatively activated by the binding of cytokines produced from other cells, and exhibits characteristics typical of M1 and / or M2 macrophages (Taken from Smith *et al*., 2011).

Oral administration of dietary fibres (e.g. β-glucans and AXs) come into contact with the mucosal immune system (Volman *et al*., 2008; Mendis *et al*., 2016). The intestinal epithelial cells together with the immune cells of the Peyer's patches play an important role in regulating immune responses. Previous studies showed oral administration of dietary fibres can modulate the mucosal immune response of the cells in Peyer's patches as well as intestinal intraepithelial lymphocytes in mice (Suzuki *et al*., 1990; Tsukada *et al*, 2003). Rice *et al*. (2005) isolated fluorescently labelled β-glucan from a subpopulation of the intestinal epithelial cells and Peyer's patches after oral administration, thus suggesting β-glucans with a high molecular mass (2×10^6 Da) can be taken up from the intestinal lumen by M-cells (specialized

epithelial cells for the transport of macromolecules in Peyer's patches) and transported to underlying lymphoid tissue. Sandvik *et al*. (2007) indicated that mucosal Dendritic cells (DCs) are also responsible for the uptake of polysaccharides, including dietary fibres. It is now well recognized that DCs sample and interact with gut contents locally via cellular projections that cross the epithelium (Rescigno *et al*., 2001). In addition, murine studies have shown that orally administered dietary fibres (400µg/day) are taken by gastrointestinal macrophages and then transported to the spleen, lymph, and bone marrow (Hong *et al*., 2004). Thus, the principal mechanisms through which dietary fibre is taken up from the diet appear to be via by M-cell, DC and/or macrophage transportation across the mucosal epithelium.

At present, levels of AXs in plasma and tissues are unknown, but dietary fibres have been detected within human tissues following administration (Rice *et al*., 2005). Once transported to 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 bone marrow-derived leukocytes (e.g. monocytes) that may be subsequently recruited from circulation to sites of infection, injury or disease.

Absorbed dietary fibre can interact with intestinal macrophage in the lamina propria including Peyer's patches, leading to increased cytokine production by macrophages and enhanced resistance to infection (Wismar *et al*., 2010; Mendis *et al*., 2016). Dietary fibre decreases the synthesis of some pro-inflammatory cytokines such as interleukin-18 (IL-18) (Esposito *et al*., 2003). Increasing dietary fibre intake has been show to decrease circulating levels of C-Reactive protein (CRP), a systemic marker of inflammation (Ma *et al*., 2006).

Moreover, a human study involving 80 human participants showed that oral consumption of AX as a dietary supplement significantly increased interferon gamma (IFN-γ) production in circulating leukocytes of healthy adults (Choi, 2014), thus confirming non-intestinal inflammatory effects of AXs on peripheral blood mononuclear cells.

The viable cell count of U937 monocytes/macrophages supplemented with LPS/AXs from extruded/non-extruded rice bran and wheat pentosan at 50, 500 and 1000 μg/ml were significantly lower than those of the untreated negative control (P<0.05).

The current study indicated that AXs/LPS have a concentration dependent effect on viable cell count. These findings are in agreement with previous studies that have shown that LPS induces apoptosis and inhibits cell growth (Xaus *et al*., 2004; Kuwabara and Imajoh-ohmi, 2004). AXs from extruded samples had a greater effect in reducing the viable cell density in comparison with AXs from non-extruded samples. Moreover, cell density was lower in monocytes/macrophages treated with AXs from extruded/non-extruded rice bran than from wheat pentosan, which suggests that the source, process and Mw of AXs play a key role in cell apoptosis and cell growth. In particular, it is postulated that the changes in cell density may be due to the higher amount of very low Mw AXs from rice bran compared to wheat pentosan. Furthermore, the screw speed of the treated cereal had a substantial effect on the AXs and cell density following treatment with AXs. Cells treated with AXs obtained from a high screw speed of 160 rpm had a far lower cell density than those treated at a lower screw speed of 80 rpm or the non-extruded samples. High amounts of very low Mw AXs were significantly associated with increased NO secretion and lowered cell density, in concordance with other studies showing higher NO secretion is generally associated with reduced cell count (Xaus *et al*., 2004). The NO secretion was investigated after 24 hrs treatment with LPS/AXs in both monocytes and macrophages. Our findings were in agreement with previous studies showing both AXs and LPS are able to induce NO secretion in human monocytes and macrophages (TUNÇTAN *et al*., 1998; Ghoneum and Matsuura, 2004; Shen *et al*., 2015).

Comparing the effect of LPS and AXs on NO secretion highlighted a difference in the response of monocytes and macrophages. The NO produced from monocytes was much lower than that produced by macrophages after supplementation with AXs/LPS. The substantial difference in NO secretion could be related to the fact that the expression of TLR4 in monocytes is lower than that found in macrophages (Vaure

and Liu, 2014). In addition, the maturation of macrophages by PMA (phorbol 12-myristate 13-acetate) was shown to increase the sensitivity of macrophages to LPS, which is correlates with increases in CD14, MD-2, TLR4 and MyD88 mRNA levels in macrophages (Zarember and Godowski, 2002).

Moreover, the AXs extracts are known to contain other sugar residues such as glucose. A high glucose concentration has previously been shown to induce TLR4 activation (Dasu *et al*., 2008; Garay-Malpartida *et al*., 2011). AXs and LPS are similar in that both of them have glucose and galactose within their structures (Zhang *et al*., 2015), and both of them are within the same Mw range (1-25 kDa). It could therefore be postulated that AXs and LPS may share the same receptor.

To investigate whether AXs and LPS activate the same receptor (TLR4), a TLR4 inhibitor was used. Monocytes/macrophages were supplemented with TLR4 inhibitor prior to induction by LPS and AXs. The cells supplemented with the inhibitor prior to treatment with AXs and LPS showed significantly reduced NO secretion and increased in cell density. This suggests that AXs and LPS are at least in part mediating cell responses via activation of TRL4.

It has been previously reported that LPS not only increases NO production by monocytes and macrophages, but also causes other inflammatory responses (Ghoneum and Mastuura, 2004; Zhang *et al*., 2008). However, such inflammatory responses are not necessarily harmful; limited inflammation is important to maintain a good health and fight infection. Pro-inflammatory cytokines such TNFα are important for phagocytosis (Vernaza *et al*., 2012). It has been reported that TNFα is released by monocytes and macrophages following TLR4 activation by LPS (Ghoneum and Mastuura, 2004; Zhang *et al*., 2008). Moreover, MGN-3, a modified AXs with low Mw, has been shown to significantly increase the level of TNFα production in macrophages (Ghoneum and Mastuura, 2004; Cholujova *et al*., 2009).

In this study, the TNFα secretion by U937 monocytes/macrophages was investigated after treatment with LPS and AXs from extruded/non-extruded rice bran and wheat

pentosan. The results suggest that both LPS and AXs are capable of inducing TNFα production from monocytes and macrophages. The increase in TNFα production was found to be dose-dependent with both the rice bran and wheat pentosane samples. As the dose of AX was increased, the production of TNFα also increased.

Additionally, it was found that the highest increase in TNFα production was obtained from monocytes/macrophages treated with AXs from extruded rice bran at a screw speed of 160 rpm (RB160) followed by a lower stimulatory effect in RB80 and RBW respectively.

In a similar fashion, the highest TNFα secretion was from monocytes/macrophages treated with AXs from extruded wheat pentosan at 160 rpm followed by a lower amount of TNFα secretion from P80 and PW respectively. These results are in agreement with previous findings that showed extrusion leads to a reduction the Mw of AXs, which then causes a higher immunostimulatory response than that induced by the non-extruded samples.

Interestingly, comparing the TNFα production by monocytes and macrophages it was found that monocytes stimulated with AXs and LPS secrete an appreciable amount of TNFα. This amount is however, much less than that produced by macrophages treated with AXs and LPS. This is in agreement with previous studies, which have found that monocytes have less capacity to produce TNFα in response to inflammation than macrophages (Mata *et al*., 1990; Wigmore *et al*., 1998). These responses could be related to the similarities that LPS and AXs share, as well as their activation of the same receptor (TLR4).

To investigate the effect of TLR4 inhibition on TNFα, cells were supplemented with a TLR4 inhibitor prior to treatment with LPS and AXs. The results showed that the TLR4 inhibitor significantly reduced production of TNFα from monocytes/macrophages treated with LPS or AXs from extruded/non-extruded rice bran and wheat pentosan. This demonstrated that TNFα induction was at least in part mediated via TLR4, thus

suggesting that TLR4 is a receptor for AXs. Therefore, this suggest AXs behave like an analogue of LPS, stimulating the same receptor.

LPS and AXs might share several characteristics in terms of structure and their ability to activate the same receptor. However, unlike LPS, which can cause septic shock when present in large amounts in the body, AXs do not show any detrimental effects on the body. It can be postulated that in the presence of infection and elevated LPS levels in the body, dietary AXs are likely to compete with LPS at the TLR4 receptor, dampening the effect of LPS and resulting in much reduced immune response than that with LPS stimulation alone. In contrast, regular inclusion of dietary AXs as part of daily diet in the absence of infection (LPS) is likely to elevate basal/background levels of TNFα and NO production, leading to an immune system that is in a continual state of readiness to fight infection by pathogens.

6.1 Conclusions

This research was conducted to study the effect of extrusion on solubility and Mw of WEAX from rice bran and wheat pentosan and to study the effect of the AXs on NO and TNFα. It was found that extrusion increases the solubility of AXs and reduces their Mw with increasing screw speed.

AXs from extruded/non-extruded rice bran and wheat pentosan have immunomodulatory potential by inducing the secretion of NO and TNFα by U937 monocytes and macrophages treated with AXs. The study also showed that AXs extracted from cereals extruded the highest screw speed had the highest immune response, possibly due to the higher amount of very low Mw AXs in samples extruded at the highest screw speed.

The study also showed that inhibition of the TLR4 receptor significantly reduced NO and TNFα secretion by monocytes and macrophages treated with LPS or AXs from extruded/non-extruded rice bran and wheat pentosan.

The study elucidated a potential mechanism of AXs action, suggesting that dietary AXs may compete for the same receptor as LPS (TRL4). The study proposes that dietary AXs may compete with LPS for the same receptor (TLR4), resulting in a damping of LPS-induced immune responses during infection but producing a non-detrimental moderate elevation of background inflammatory responses in the absence of infection. Thus, the study suggested that regular ingestion AXs might enhance (heighten) the natural immune response, thereby preparing the immune system for potential pathogen.

6.2 Future work

Investigations into the effect of extrusion screw speeds on Mw and solubility have provided an initial insight into its potential for both improving extractability of AXs from cereal foods and reducing the Mw of extracted AXs. This work forms the basis for further investigations, including the interaction between screw speed and barrel temperature or the addition of a heat/shear stable feruloyl esterase to the process. To develop this further, different extrusion conditions can be tested to investigate in more detail the effect extrusion may have on the structure and Mw of AXs. In addition, further investigations (beyond the scope of this current study) could be performed to characterise the AXs structure and Mw using technologies such as Nuclear Magnetic Resonance (NMR). Using NMR would give information on the substitution of AXs molecules.

This study has proposed a possible receptor that AXs may stimulate; however, further competition assays needs to performed using both AXs and LPS to investigate whether AXs will compete with LPS for the TLR4 receptor. Moreover, this study has investigated the involvement of only one receptor; however, it is likely that other receptors may also be involved in the inflammatory process induced by AXs. Therefore, further receptors (e.g. Dectin-1) can be tested to fully explore the mechanisms of action.

Ultimately, the AXs can be tested further on various *in vitro* such as RAW 264.7 (macrophage murine cell line) and CACO-2 (human colon cancer cell line) and various cytokines can be investigated such as IL-6, IL-12 and IL-23. Moreover, AXs can be tested *in vivo* using animal models to confirm *in vitro* findings before assessing their function in humans.

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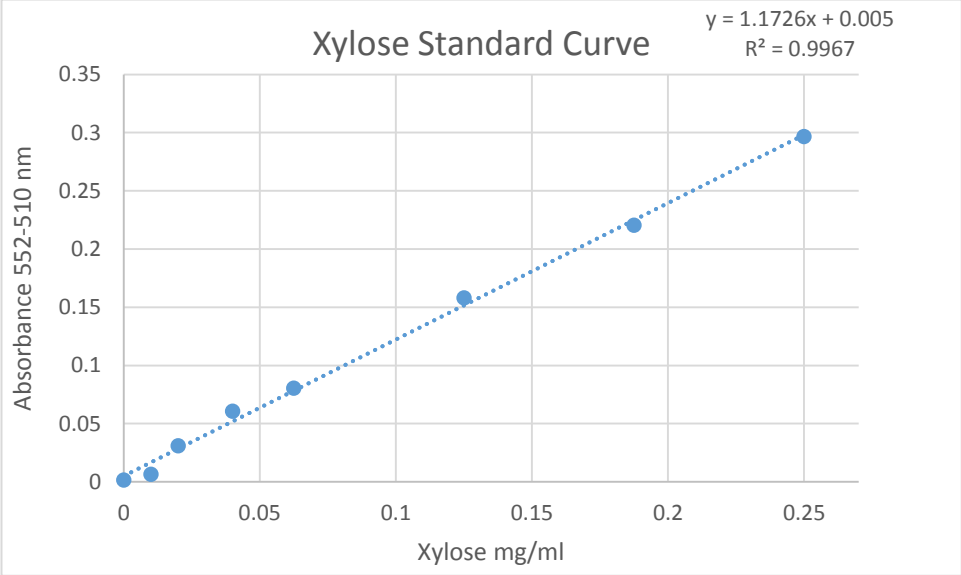
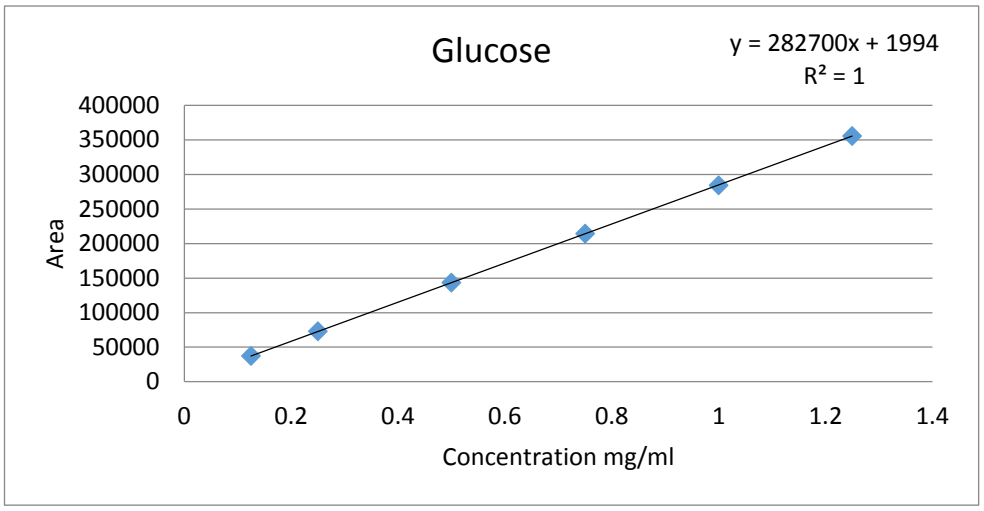
Appendices

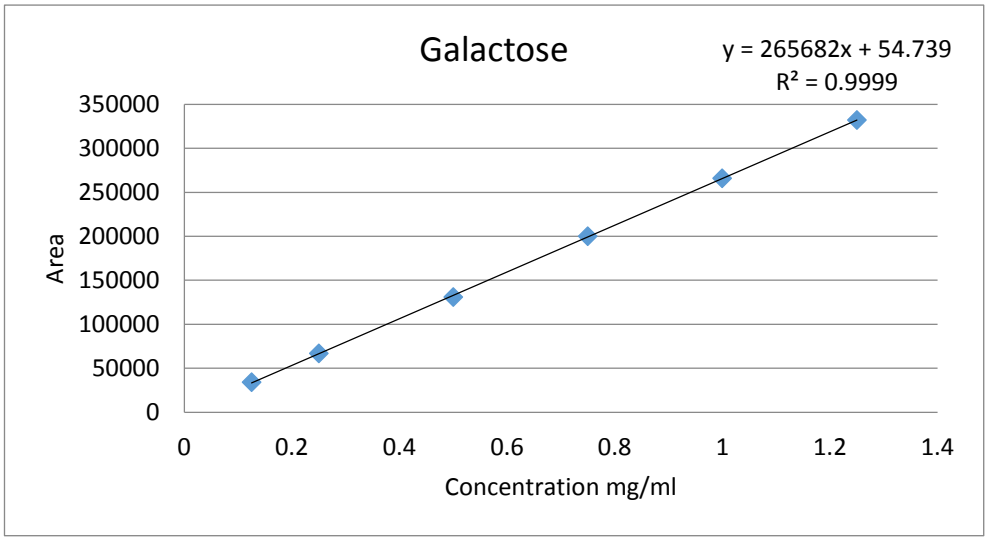
Appendix 1

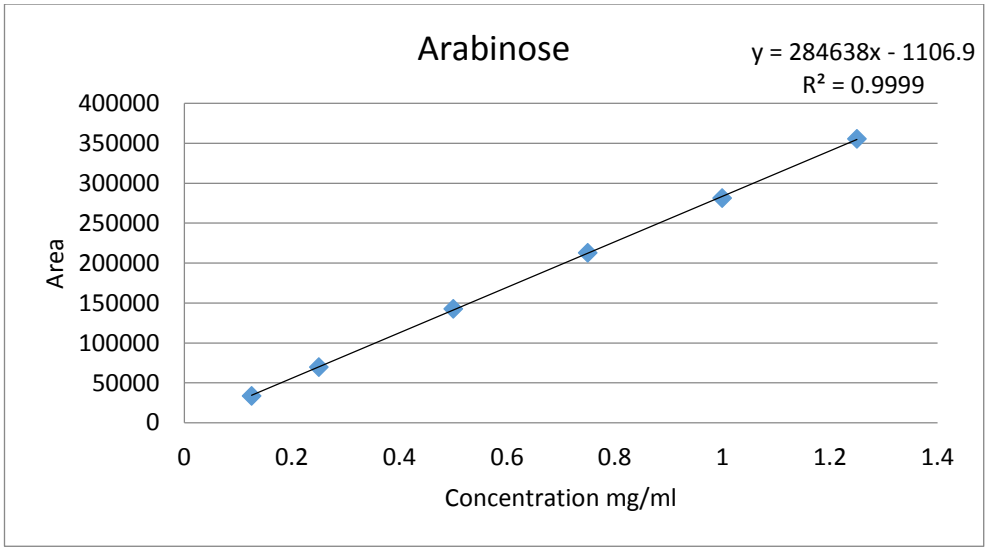


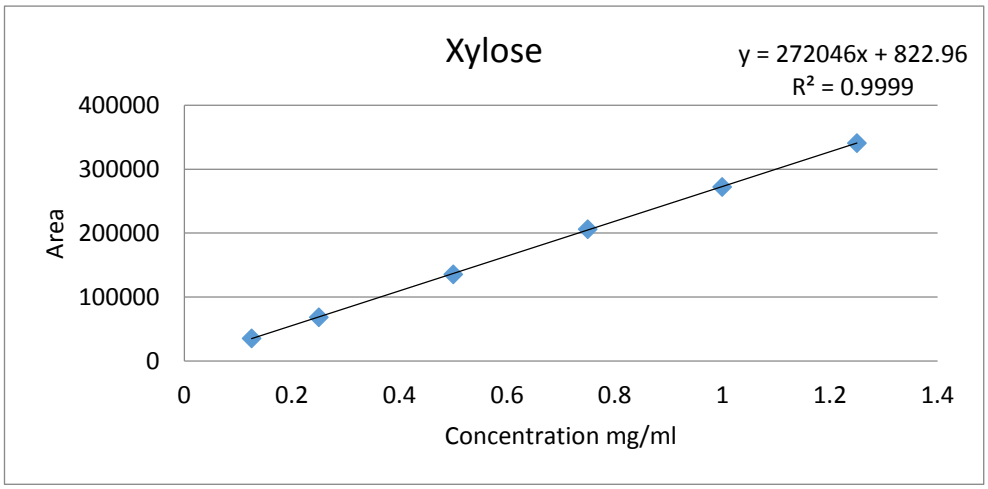
Xylose standard curve is used to calculate the xylose concentration in the extracts based on the average value. Values are shown as means ±SEM, n=6.

Appendix 2


Glucose
y = 282700x + 1994
R² = 1
400000
350000
300000
250000
200000
150000
100000
50000
0
Area
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Concentration mg/ml

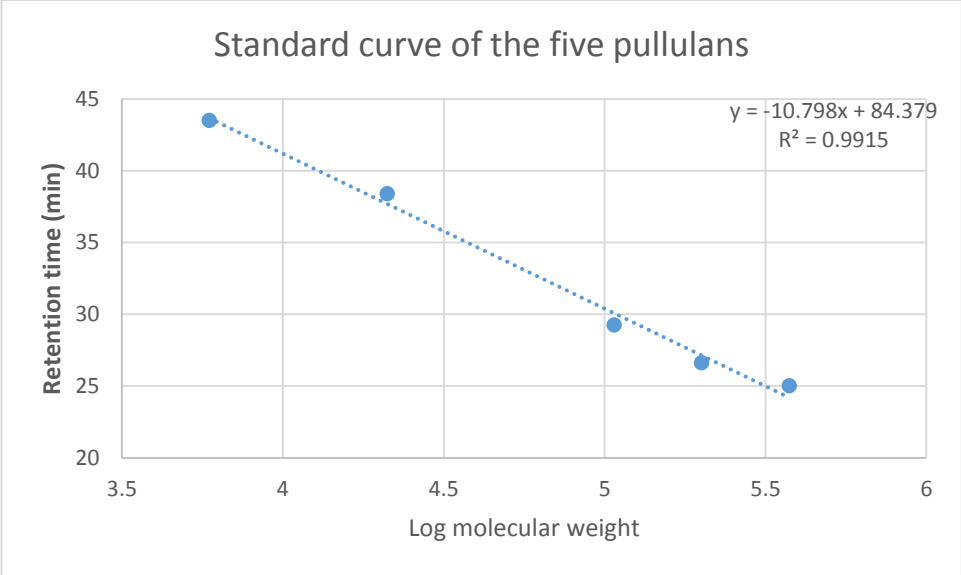

Galactose
y = 265682x + 54.739
R² = 0.9999
350000
300000
250000
200000
150000
100000
50000
0
Area
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Concentration mg/ml





Glucose, galactose, arabinose and xylose standard curves were used to calculate the sugar composition of the purified AXs extracts from extruded/non-extruded wheat pentosan and rice bran

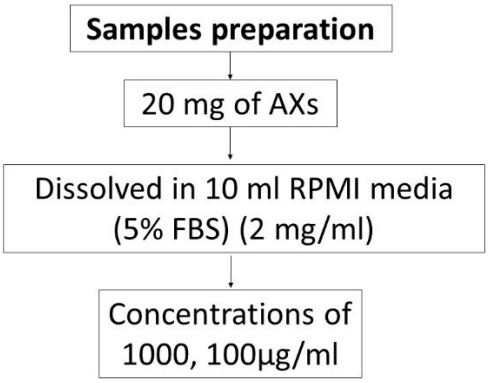
Appendix 3



The five pullulan standard curve used to characterise the Mw of RBW, RB80, RB0, PW, P80 and P160.

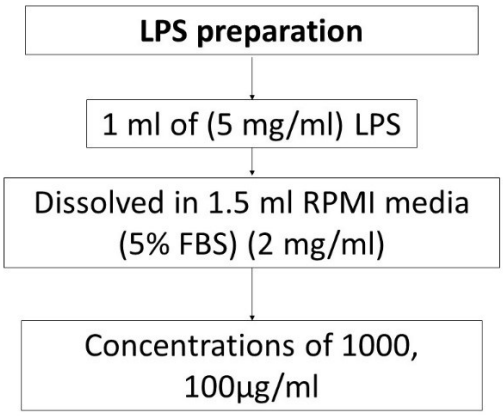
Appendix 4

Arabinoxylans sample preparation for cell line work



Appendix 5

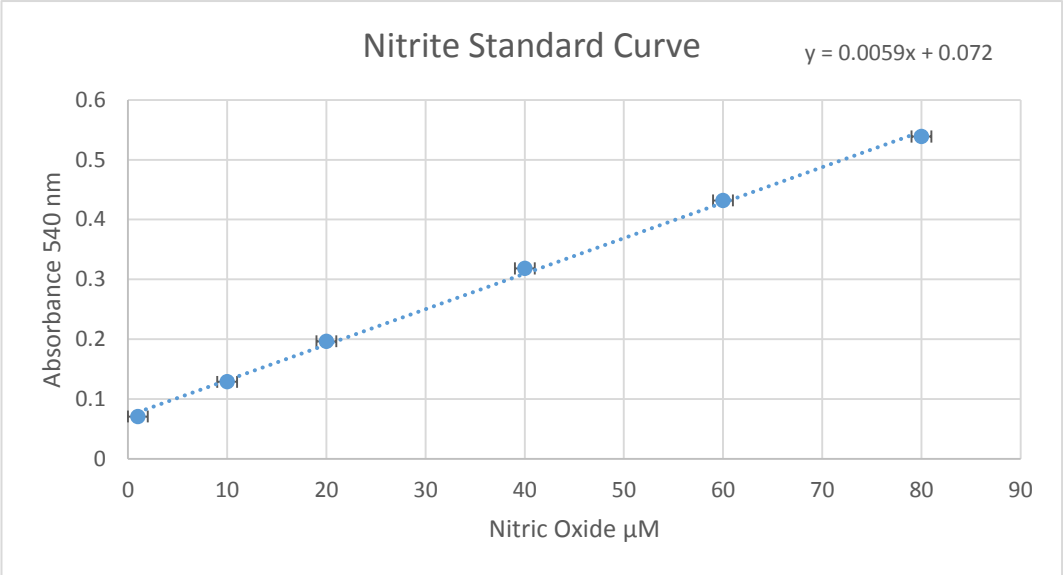
Lipopolysaccharides sample preparations for cell line work



Appendix 6

Conc. µg/ml	Absorbance		
	LPS	Rice bran	Pentosan
50	0.046±0.0003	0.047±0.0003	0.046±0.0003
500	0.046±0.0005	0.046±0.0005	0.047±0.0005
1000	0.047±0.0005	0.046±0.0005	0.046±0.0005
Culture medium	0.046±0.0003		

Appendix 7



Sodium nitrite standard curve was used to calculate the amount of nitric oxide production by monocytes/macrophages after supplementation with arabinoxylans or LPS.