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RESEARCH ARTICLE

DETERMINATION OF THE EFFECTS OF COMPLEX CARBOHYDRATES ON CYTOKINE STATUS OF MONOCYTES

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ABSTRACT

Objectives: To determine the immunomodulatory effects of xanthan, glycated albumin and mixture of both on the induction of monocytes cytokine synthesis and the cell viability.
Methods: Monocytes were cultured with xanthan, glycated albumin and mixture of xanthan/glycated albumin as treatments at different concentrations for 3 h. The quantitative determination of TNF-α, IL-6, IL-10 and IL-12 was performed in cultured monocytes by ELISA.
Results and Discussion: Results obtained demonstrated that the mixture of xanthan/glycated albumin induced pro-inflammatory cytokine production, in the noticeable degree TNF-α compared to treatment and with only xanthan and/or glycated albumin. IL-6, however which has both pro- and anti-inflammatory properties seems to show more of the former than the latter in this study. The result also shown that xanthan, glycated albumin and the mixture of xanthan/glycated albumin significantly increased IL-10 production compared to other cytokines produced by the effects of the same polysaccharides and at the same concentrations. This indicated that IL-10 that is well-known in suppressing the production of pro-inflammatory cytokines such as TNF-α, IL-12, IL-6 as shown by several studies.
Conclusion: It is safe to conclude that xanthan and glycated albumin does not possess cytotoxicity on monocytes. The data in this study also showed that monocytes cultured with both xanthan and glycated albumin are capable to convert soluble MTS into insoluble formazan even at higher concentration (10 µg/ml), compared to PMA which serve as our control. Therefore the result strongly indicated that both xanthan and glycated albumin protects cells surviving in vitro and had ability to keep the cell viability.

INTRODUCTION

In recent years, many polysaccharides and protein-polysaccharide complexes isolated from botanical sources such as mushrooms, algae, lichens and medicinal herbs have been shown to possess strong antioxidant activities and also to activate cells involved in innate immunity without harming the host (Karnjanapratum and You, 2011; Kim *et al.,* 2011; Tang *et al.,* 2012; Xia *et al.*, 2012; Zhang *et a*l., 2013). Hence, polysaccharides are becoming increasingly important as sources of pharmacotherapeutics, either as herbal drugs for the treatment of chronic diseases or as raw materials from which compounds with particular biological activities are isolated (Wang *et al*., 2013). Most polysaccharides are demonstrated to be activators of immune cells through their capacity to enhance production of immune mediators, such as reactive oxygen species, nitric oxide (NO), TNF-α, IFN-γ, IL-10 and also their immunomodulatory characteristics are mainly dependent on their unique molecular structures in activation of varied surface receptors(Jeurink *et al.,* 2008; Weng *et al.,* 2011). Several classes of polysaccharides, such as lectins are capable of interacting with the immune system to regulate specific aspects of the host response, hence are considered to have potent effects on the immune system (Stanilova *et al*., 2005). Among these compounds, plant derived anti-complementary, cell proliferation activating substances and macrophage-activating substances have been studied(E. Han, Ding, Jin, and Ju, 2011; Hu and Xu, 2011; Stanilova *et al.*, 2005). Some plant binding glycoproteins such as lectins mediate cell–cell interactions in numerous biological processes, such as blood coagulation, immune response, viral infection, pathological processes, inflammation, embryogenesis and cellular signal transfer (Han *et al.*, 2011; Hu and Xu, 2011), thus they are recognized as ligands for carbohydrate recognition proteins (Hu and Xu, 2011; Nakamura *et al*., 2013; Wu *et al*., 2006). The immunomodulatory properties of bioactive agents also include their ability to induce specific cytokine production by the activated target cell (Stanilova *et al*., 2005).

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Moreover, the extent to which polysaccharides exerts their bioactivities is closely related to their chemical composition, molecular weight, branching, chain conformation, and water solubility. The basic understanding of both the primary and secondary structures for the polysaccharides is essential for the successful interpretation of their bioactivities; hence the monosaccharide composition analysis of polysaccharides is the most important step for the further discovery of its physicochemical properties, structure and structure–bioactivity relationship (Huang *et al*., 2007; Xie *et al*., 2013). Xanthan is the industrial gum obtained by fermentation with the highest commercial production and is normally isolated from bacterial plant *Xanthomonascampestris* (Casas *et al*., 2000; Garcia-Ochoa *et al*., 2004). Xanthan gum is a natural microbial extracellular heteropolysaccharide whose main chain is based on a linear backbone of 1,4-linked β-d-glucose; at the C(3) position of every alternate glucose residue there is a charged trisaccharide side chain containing a glucuronic acid residue between two mannose units (i.e it contains repeating units of five monosaccharides formed by two d-glucose, two d-mannose and one d-glucuronic acid) (Casas *et al*., 2000; Han *et al*., 2012; Helmenstine, 2013). It is a non-toxic, biodegradable and biocompatible glycosaminoglycan. Furthermore, the experimental evidence has shown that it displayed no cytotoxicity and a protective effect on interleukin-1β induced rabbit chondrocytes (Han *et al*., 2012).

Glycated albumin (GA) is produced alongsideglycosylated hemoglobin (HbA1c) and fructosamine as a result of a in a non-enzymatic glycation of proteins. GA binds to specific receptors such as receptors for glycated albumin end products (RAGE) and induces cellular signaling pathways and secretion of the proinflammatory cytokines such as IL-1β and TNF-α (Brandt and Krantz, 2006; Zheng *et al*., 2012; Yamaguchi *et al*., 2005). Studies have shown that GA triggers inflammatory response by binding to RAGE there by mediating inflammation known as a key underlying cause in the development of vascular complications leading to an increased expression of cytokines, growth factors and adhesion molecules with mediate an immune response (Brandt and Krantz, 2006; Choi *et al.,* 2013; Hirose *et al.,* 2011; Yamaguchi *et al.*, 2005; Zheng *et al.*, 2012). Intracellularly, GAs that are formed inside and outside of cells induces a more unspecific chemical reaction, the production of ROS increases which causes cross-linking with other cellular proteins and generation of lipid peroxidation product (Kasper and Funk, 2001; Li *et al*., 2010). RAGE, found in most tissues, have potent immunomodulatory actions, promoting ROS production and inflammation (Vlassopoulos *et al*., 2013).

MATERIALS AND METHODS

Note: Unless stated otherwise, all the reagents were obtained from Sigma-Aldrich (UK)

PMA (positive control) preparation

10 µl of 1mg/ml of concentrated PMA was diluted with 990 µl phosphate buffered saline (PBS) giving rise to diluted PMA which is equivalent to 100 µg/ml concentration ready for use.

Xanthan preparation

A dried xanthan gum powder obtained from San-Ei Gen corporation (Japan) was weighed 0.01g and supplemented into a sterile bottle containing 10 ml of RPMI media (+10% FBS, antibiotic/antimycotic which gives the final concentration of 1000 µg/ml and then autoclaved by subjecting it to high pressure saturated steam at 121 °C for around 15–20 minutes.

Glycated albumin preparation

An amount of 25 mg of human glycated albumin was diluted with 5 ml of PBS to make a 5000 µg/ml concentration.

Cell culture

5 x10^5 cells/ml were maintained in a sterilized 24 wells plate containing RPMI media (10% FBS, and 1% antibiotic/antimycotic at 37^{o}c, 5% CO_2, humidity for 24h to allow the cells to attach.

Polysaccharide stimulation

The cultured cells were stimulated with either 1µg/ml PMA, 10 µg/ml each of xanthan and glycated albumin, 100 µg/ml each of xanthan and glycated albumin, 10 µg/ml combination of xanthan and glycated albumin, 100 µg/ml combination of xanthan and glycated albumin and 10 µg/ml. The stimulated and non-stimulated (control) cells were incubated at 37^{o}c, 5% CO_2, humidity for 3 h.

Cytokine determination

The quantity determination of TNF-α, IL-6, IL-12p40 and IL-10 after 3 h in culture was performed by ELISA using commercially available kits purchased from Bioscience, following the manufacturer's protocol. Briefly, each well of the corning costar 9018 ELISA microplate was coated with 100µl of re-constituted (as directed by eBioscience TDS protocol) human IL-6, IL-10 IL-12, and TNF-α ELISA capture antibodies, sealed and incubated overnight at 4 °C. The plates were washed 5 times with wash buffer and blot dried with absorbent paper to remove any residual buffer. One part of 5X concentrated assay diluents was diluted with four parts of distilled water in which 200 µl of the diluted portion was added into each well, sealed and incubated for 1 h. The plates were washed 5 times with wash buffer and blot dried with absorbent paper. Using the diluted assay diluents, the standards were diluted according to the manufacture's instruction (eBioscience TDS protocol) and then added 100 µl into appropriate wells with 100 µl of samples also added into appropriate wells, sealed and incubated overnight at 4 °C. The plates were washed 5 times with wash buffer and blot dried with absorbent paper and then 100 µl (re-constituted according to the manufacture's instruction (eBioscience TDS protocol) human IL-6, IL-10 IL-12, and TNF-α ELISA detection antibodies were added to each well, sealed and incubated at room temperature for 1h. After 5X washes, 100 µl of an HRP–avidin solution was added to the wells and incubated for 30 minutes at room temperature, then washed 7X, followed by the addition of 100 µl of TMB substrate

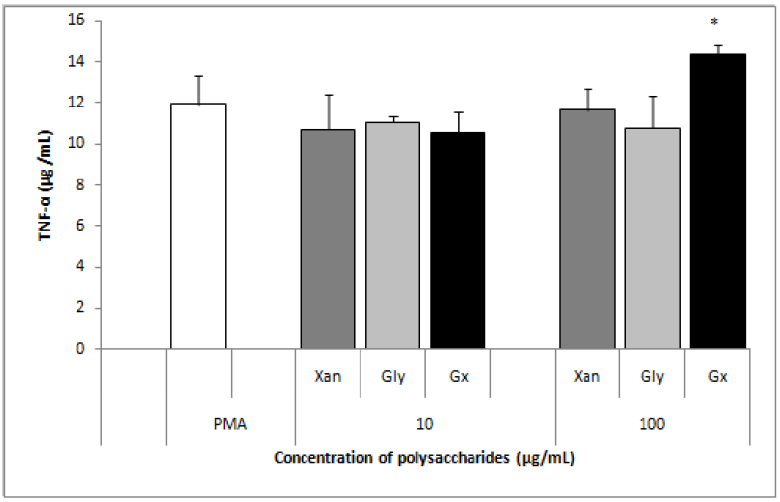

Figure 1. Effects of xanthan, glycated albumin and a mixture of both on monocytes. Representing the level of concentration of TNF-α. *Significantly (0.05) different from PMA and other treatments. The data presented as the mean ±SD (n=6)

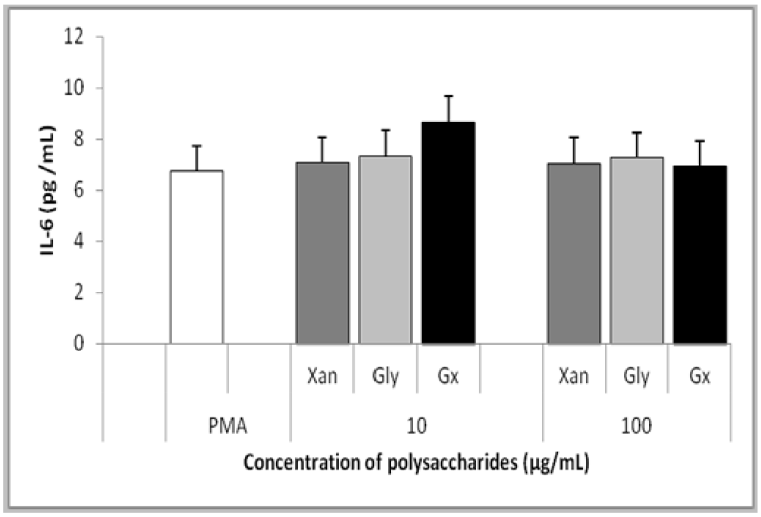

Figure 2. Effects of xanthan, glycated albumin and a mixture of both on monocytes. Representing the level of concentration of IL-6. *Significantly (p<0.05) higher than the control. The data presented as the mean ±SD (n=6)

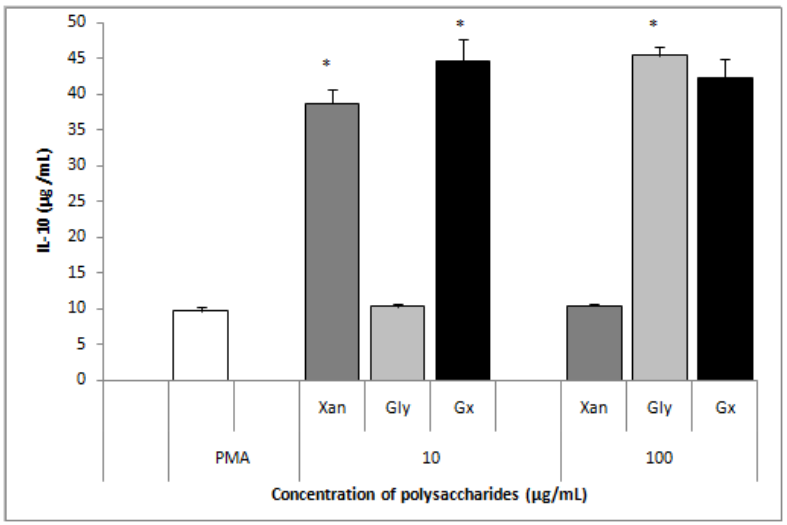

Figure 3. Effects of xanthan, glycated albumin and a mixture of both on monocytes. Representing the level of concentration of IL-10. *Significantly (p<0.05) higher than the control. The data presented as the mean ±SD (n=6)

solution to develop for 15 min. A 50 µl stop solution (1 M Phosphoric acid) was added to halt the reaction, and the absorbance was measured at 450 nm. The cytokine expression levels were calculated based on the linear portion of the standard curve.

Cell viability assay

Cell viability can be estimated by colorimetric analysis based on MTS (3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reduction assay by actively growing cells to a formazan product, which is then quantified by spectrophotometry. The CellTiter 96® AQueous one Solution Cell Proliferation Assay (Promega) was used according to the manufacturer's instructions. Reduction of the MTS compound to formazan is detected by colour development at 490 nm using a microplate reader.

Statistical analysis

The data are expressed as mean ± standard deviation (SD) values. The group mean was compared using a one-way analysis of variance. The statistical difference was considered significant at $P < 0.05$. The analysis was performed using SPSS Version 21 Software (IBM SPSS, Chicago, IL, USA) and Excel.

RESULTS

To study the influence of xanthan and glycated albumin on monocytes towards production of cytokines, PMA which is a lectin-like immunomodulator is used as a positive control for monocyte activation and compared it with these polysaccharides.

Mixture of xanthan and glycated albumin at higher concentration induced TNF-α production

The result presented in the Fig. 1, demonstrated a considerable and noticeable increase in TNF-α production when xanthan is mixed with glycated albumin at 100µg/ml compared to the control and other treatments. Although the level of the secreted TNF-α varied depending on the nature of the stimulating agent and dosage, but it is more noticeable in xanthan/glycated albumin mixture at 100 µg/ml level. The data indicated xanthan/glycated albumin mixture at 100 µg/ml increased significantly the TNF-α production compared to xanthan and glycated albumin at all the dosage level and of course the mixture of xanthan/glycated albumin at 10 µg/ml level. There were no significant differences ($p>0.05$) between treatment with xanthan and / or glycated albumin alone at all the dosage level when compared with the control.

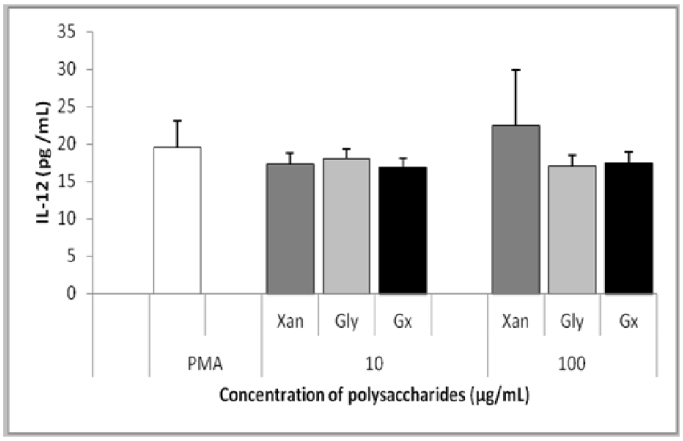

Figure 4. Effects of xanthan, glycated albumin and a mixture of both on monocytes, representing the level of concentration of IL-12. *Significantly ($p<0.05$) higher than the control. The data presented as the mean ±SD (n=6)

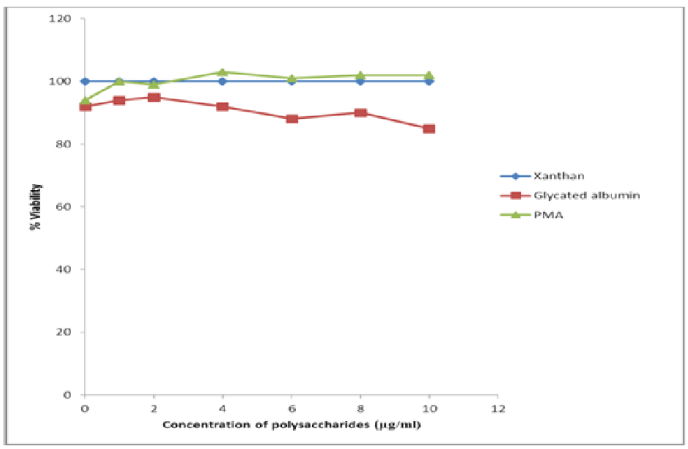

Figure 5. Cell viabilties of xanthan, glycated albumin and PMA at different concentrations. The cell viabilities were asssesed at 0, 1, 2, 4, 6 ,8 and 10 µg/ml concntrations. The results are presented as percentage viability (100%)

Mixure of xanthan and glycated albumin at 10 µg/ml dosage negligibly induced IL-6 production

The data presented in Fig. 2. shown that the mixture of xanthan and glycated albumin at 10 µg/ml stimulation induced a negligible increase in IL-6 production, there was no any significant ($p>0.05$) when compared with control and all other treatments.

Xanthan, glycated albumin and xanthan/glycated albumin mixture induced IL-10 higher quantity at different dosages compared to control

The quantity of IL-10 production also dependent on the dosage was the greatest after glycated albumin stimulation at 100 µg/ml dosage which is slightly or negligibly higher than mixture of xanthan/glycated albumin at 10 µg/ml dosage, followed by mixture of xanthan/glycated albumin at 100 µg/ml, then xanthan alone stimulation at 10 µg/ml dosage compared to control and xanthan at 100 µg/ml and glycated albumin at 10 µg/ml dosage Fig. 3. There was no significant different ($p> 0.05$) between control and xanthan at 100 µg/ml and glycated albumin at 10 µg/ml dosage. It is observed that xanthan, glycated albumin and mixture of xanthan/glycated albumin have different effects towards IL-10 production. There was a statistical significance ($p<0.05$) when all the stimulations at different dose dependent manner were compared with control, except xanthan at 100 µg/ml and glycated albumin at 10 µg/ml dosage.

Xanthan at 100 µg/ml induced noticeable increased in IL-12 production compare to control and all other stimulations at different dosage

It is observed in Fig. 4 that there was a considerable increased in IL-12 production when compared with control and all other stimulations at different dosage, but no any significant increased when stimulations other than xanthan at 100 µg/ml compared with control. There was no statistical significance ($p> 0.05$) when comparison was conducted between the groups at dose dependent manner.

Cell viability result

Effects of xanthan, glycated albumin and PMA on the monocytes in this study are given in Fig. 5. Both the polysaccharides had shown significant cell viabilities even at the higher level concentrations when compared with PMA.

DISCUSSION

Monocytes are produced by the bone marrow from hematopoietic stem cell precursors called monoblasts. Monocytes circulate in the bloodstream and differentiate into tissue resident macrophages or dendritic cells towards their role innate immune response andserving major functions in the immune system such as phagocytosis, antigen presentation and interaction with T-lymphocytes to modulate the adaptive immune response and cytokine production (Chen *et al*., 2010; Rogacev *et al*., 2010).Various stimuli may cause preferential stimulation in monocytes, since the cytokine production after stimulation is determined mainly by activated cells and the level of the released cytokines is a sign of the targeting cell population of stimulating agent.In this present study it is indicated that treatment with PMA, xanthan and glycated albumin tended to enhance the monocyte mitogenic reactivity, statistical significant ($p<0.05$) was observed when compared with the negative control. The treatments tended to significantly ($p<0.05$) show an increase in IL-10 Fig.4. And TNF-α at 100 µg/ml of the mixture of xanthan/glycated albumin, but shown no or negligible differences in all the treatments performed in dose dependent manner in the production of TNF-α, IL-6 and IL-12. Whilst this large increase in IL-10 was observed, it was associated with suppression of the pro-inflammatory cytokines TNF-α, IL-6 and IL-12. Taken together, these data indicate that both xanthan, glycated albumin in the treatment promotes an immunosuppressive cytokine phenotype, and are in line with previous reports indicating that IL-10 has a suppressive effects on the production of pro-inflammatory cytokines such as IL-12, IL-1β, IL-6 and TNF-α by monocytes–macrophages and down-regulates the expression of activating molecules on these cells and dendritic cells (Calvo *et al.,* 2005; Connor *et al.,* 2005; Kremlev and Palmer, 2005; Zediak and Hunter, 2003). Looking at Fig. 4 and of course upon combining both the two together, it is seen that xanthan was able to show synergistic effect at lower concentration (10 µg/ml) and antagonistic effect on higher concentration (100 µg/ml) level of treatments. However, the glycated albumin shown a synergistic effect at higher concentration (100µg/ml) and antagonistic effect on higher concentration (10µg/ml) level of treatments.

Minimal effects, limited sample size, marked individual variability in the variables measured and other unavoidable experimental errors might all contribute to the lack of appreciable statistical significances. The data in this study also tended to accept the idea of combining the xanthan/glycated albumin to treat monocytes especially in higher concentration (100 µg/ml) as it brought about significant production of TNF-α and the idea of mixing them was to find the synergism and antagonism among them, and this could in consistent with various studies that shown xanthan and glycated albumin induces secretion of the proinflammatory cytokines such as IL-1β and TNF-α (Brandt and Krantz, 2006; Han *et al.*, 2012; Yamaguchi *et al.,* 2005; Zheng *et al.,* 2012).The mononuclear phagocyte system (MPS) comprising monocytes, macrophages and dendritic cells participate actively in the innate immune response (Chen *et al*., 2010; Rogacev and Heine, 2010; Sheel and Engwerda, 2012) leading to the release of cytokines that plays a critical role in mediating signal transduction and stimulating the immune defense system (Chen *et al.,* 2008; Chen *et al.,* 2010; Schepetkin and Quinn, 2006; Stanilova *et al.,* 2005; Zha *et al.,* 2007). Cytokines regulate both cellular and humoral immune responses by affecting immune cell proliferation, differentiation and functions (Xu *et al.,* 2009). Pro- and anti-inflammatory cytokines are key mediators during immune responses which mediate defense mechanisms to antigen contact between cells and tissues, thus determining the quality (inflammatory or anti-inflammatory) and nature (humoral, cellular, and/or cytotoxic) of immune responses (Hofmann *et al.,* 2012). In response to innate immune, monocytes actively produces pro-inflammatory cytokines such as TNF-α, IL-12 with some delay ofanti-

inflammatory cytokines such as IL-10 (Hofmann *et al.,* 2012; Wu *et al*., 2005). IL10 which is known as an anti-inflammatory and pleiotropic Th_2 cytokine that plays a key role in the regulation of inflammatory responses and immune reactions, acting on both hematopoietic and non-hematopoietic cell, has been demonstrated to show a remarkable suppressive effects on the production of pro-inflammatory cytokines such as IL-12, IL-1β, IL-6 and TNF-α by monocytes–macrophages and down-regulates the expression of activating molecules on these cells and dendritic cells (Calvo *et al.,* 2005; Connor *et al.,* 2005; Kremlev and Palmer, 2005; Zediak and Hunter, 2003). IL-10 controls inflammatory processes by suppressing the expression of pro-inflammatory cytokines, chemokines, adhesion molecules, as well as antigen-presenting and co-stimulatory molecules in monocytes/macrophages, neutrophils, and T- lymphocytes. As all of these inflammatory proteins are transcriptionally controlled by nuclear factor-kB (NF-κB) it was suggested that IL-10 may exert a significant part of its anti-inflammatory properties by inhibiting this transcription factor (Calvo *et al.*, 2005; Hofmann *et al.,* 2012; Kremlev and Palmer, 2005; Wu *et al.,* 2005). In fact, a number of studies were able to demonstrate that IL-10 blocks nuclear translocation of the classic NF-κB p65/p50 heterodimer in monocytes/macrophages. It has been recently shown that IL-10 inhibits NF-κB activity through dual mechanisms. It blocks NF-κB nuclear translocation by inhibiting IκB kinase (IKK) activity. IL-10 blocks DNA-binding of NF-κB already present in the nucleus (Calvo *et al.,* 2005; Kremlev and Palmer, 2005; Wu *et al.,* 2005)

In this study however, it was interesting to notice that although other cytokines like TNF-α, IL-6 and IL-12 were produced on dose dependent manner, but only remarkable increase in the production of IL-10 were observed at concentrations 10 µg/ml of xanthan and mixture of xanthan/glycated albumin and at 100 µg/ml of glycated albumin and xanthan/glycated albumin mixture Fig. 4. IL-6, however which has both pro- and anti-inflammatory properties (Scheller *et al*., 2011)seems to show more of the former than the latter in this study. Considering the evidence from one study that xanthan has a protective effect on interleukin-1β (which is a pro-inflammatory cytokine) induced rabbit chondrocytes (Han *et al*., 2012), it is possible that in this study the pro-inflammatory such as TNF-α, IL-6 and IL-12 that failed to show noticeable increased were negatively inhibited back by the remarkably noticeable IL-10 production. This also vividly reminded that in this study the determination of IL-10 was unintentionally performed after about 2 weeks storage of the samples, while the other cytokines determination was performed almost less than a day after treatment. In view thereof, this might indicate that perhaps the pro-inflammatory cytokines were released, but later inhibited by the up- coming IL-10 during the two weeks storage of the samples. Thus, IL-10 has been shown to inhibit monocytes-macrophage production of IL-12, IL-1β, IL-6, and TNF-α (Zediakand Hunter, 2003). Although pro-inflammatory responses to pathogens leading to the release of pro-inflammatory cytokines such as IL-1 and TNF-α are essential for host defence and are of great importance in the innate immune response, uncontrolled inflammation results in immune pathology that can result in death. The involvement of IL-10 in inflammatory processes during infection and autoimmunity has been established in various models of infection and autoimmunity (Hofmann *et al.,*2012,Wu *et al*., 2005). These experimental results demonstrated both xanthan and glycated albumin induced the remarkable production of IL-10 which could alone involve in the in inflammatory processes during infection and autoimmunity even when itself suppresses the production of pro-inflammatory cytokines.Finally, it is detected in this study that xanthan and glycated albumin exhibited significant cell viabilities. Some other authors also reported that xanthan show significant cell viabilities (Han *et al*., 2012; Papagianni and Anastasiadou, 2009). Therefore, it is safe to conclude that both xanthan and glycated albumin does not possess cytotoxicity on monocytes. The data in this study also showed that monocytes cultured with both xanthan and glycatedalbumins are capable to convert soluble MTS into insoluble formazan at higher concentration (10 µg/ml). This result strongly indicated that both xanthan and glycated albumin protected cells surviving in vitro and had ability to keep the cell viability. Since both xanthan and glycated albumin contributed towards the production higher quantity of IL-10, it suggested that a part of their ability to protect cell viability might be determined by the higher level of IL-10 in the monocytes culture. Further studies are required to examine whether the extent to which polysaccharides exerts their bioactivities is closely related to their chemical composition, molecular weight, branching, chain conformation, and water solubility. The basic understanding of both the primary and secondary structures for the polysaccharides is essential for the successful interpretation of their bioactivities

Conclusion

Many plant-derived polysaccharides are demonstrated to be activators of immune cells through their capacity to enhance production of immune mediators and also their immunomodulatory characteristics are mainly dependent on their unique molecular structures in activation of varied surface receptors (Weng *et al*., 2011; Jeurink *et al*., 2008). Two polysaccharides and their mixture (xanthan, glycated albumin and xanthan/glycated albumin) were used. These polysaccharides have displayed high immunomodulatory activities. They have shown highly significant IL-10 production capacity at both 10 µg/ml and 100 µg/m, indicating that they could be suitable candidates for effectively stimulating the immune system. Recent studies revealed that certain structural characteristics of polysaccharides such as chemical composition, molecular weight, branching, chain conformation, and water solubility are responsible for their immunomodulatory activities (Huang *et al*., 2007;Xie *et al*., 2013).

Further studies involvingstructural characterisation of these immunomodulatory polysaccharides are therefore required in order to understand their biological activities.

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