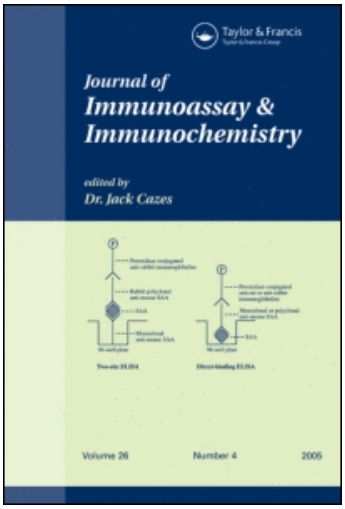

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THE EFFECTS OF *SACCHARUM OFFICINARIUM* (SUGAR CANE) MOLASSES ON CYTOKINE SECRETION BY HUMAN BLOOD CULTURES

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□ *This study investigated the effects of sugar cane molasses on the immune system, using cytokines as biomarkers. Whole blood cultures, stimulated in vitro with endotoxin or PHA, were incubated with various concentrations of molasses. No cell death occurred in whole blood cultures incubated with molasses samples. The addition of molasses (800 μg/mL) to unstimulated whole blood cultures resulted in increased levels of the biomarker of inflammation, Interleukin-6 ($P < 0.001$) and also the biomarker of humoral immunity, Interleukin-10 ($P < 0.001$). Molasses addition (800 μg/mL) to unstimulated whole blood cultures has no effect on the cell mediated immunity biomarker, Interferon gamma secretion. Molasses has no effect on Interleukin-6, Interleukin-10 and Interferon gamma secretion in stimulated whole blood cultures. Immunostimulation by molasses requires further investigation as it may have potential health impacts.*

Keywords cytokine, human blood cultures, immunotoxicity, molasses, sugar cane

INTRODUCTION

The immune system functions as an essential part in maintaining health and is a highly regulated organ network that requires the combination and communication of various immune cells, tissues and organs.[1,2] The immune system acts as a defence mechanism against possible invading infectious agents such as fungi and parasites, microorganisms (bacteria and viruses) and is also responsible for the elimination of non-self components.[1] The structural organization of the immune system enables it to respond efficiently to anything within the body that is not regarded as 'self'. The immune system functions by using diffusible substances that enable communication between the immune cells by relaying messages and instructions to these cells.[3] The immune system is categorised into

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two immune responses which include the innate and acquired immune pathways.

The innate immune system operates as a primary line of defense against invading pathogens and is as an inherent form of immune protection.[4] This form of immunity has mechanisms in place that are always alert and equipped towards fighting against infectious agents and pathogens. The cell mediated and humoral immune response makes up the acquired immune pathway. The cell mediated and humoral immune responses are regulated by T-helper (Th), T suppressor, T inducer and T cytotoxic (Tc) cells.[5]

Th1 and Th2 cells are involved at a critical level of functioning in the immune system. Th1 cells initiate the cellular or type 1 pathway which functions in eliminating viruses and other intracellular pathogens, cancerous cells and promote delayed–type hypersensitivity skin responses. Th2 cells stimulate the humoral or type 2 pathway that increases antibody production used in the defence against extracellular pathogens.[3]

T lymphocytes also produce cytokines that act in the modulation of B and T cell activity.[6] Cytokines are a group of protein molecules such as interleukins (IL's), interferons (IFN's), and numerous colony stimulating factors (CSF's).[3] IL-6 is a pro-inflammatory cytokine that initates the release of other cytokines. These cytokines are involved in the stimulation of hypothalamic-pituitary-adrenal (HPA) axis that produces steroid hormones. Pro-inflammatory hormones are in turn reduced by steroid hormones, thus creating a negative feedback loop. This regulates the process of inflammation.[7] T cell differentiation is maintained by the biomarkers IFNg and IL-10. IFNg directs the differentiation of naïve T cells (T0) into Th1 cells while IL-10 directs differentiation of Th2 cells.[8] This regulates both the cell mediated and humoral immune responses.

The complex nature of the immune system has made it a target for chemicals or any foreign agents also referred to as xenobiotics.[2] Therefore, a potential disturbance by a xenobiotic occurring at any level of the immune system may contribute to the immune system malfunctioning.[9] Various forms of toxicity may occur as a result of the potential effects of a xenobiotic (10). Bennett, as cited by Krzystyniak (1995) states that, "A chemical substance is considered immunotoxic when the chemical has the following effects: (1) a direct or indirect action of the xenobiotic on the immune system or (2) an immunologically based host response to the compound and its metabolite or when host antigens are changed by the substance or its metabolite."[1]

The effects of immunotoxicity include immunosuppression and immunopotentiation.[11] Immunosuppression can be defined as an inefficient immune response due to the direct effect on various component cells and organs of the immune system.[12] As a result, immunosuppression

can be associated with an increased susceptibility to certain infections and may contribute to the possibility of the progression of disease (10). Immunosuppression has also shown to contribute to the development of certain tumours.[11] On the other hand, directly induced stimulation of the immune system may lead to an exacerbated or lengthened immune response targeted towards an invading pathogen. Chemically-induced immune dysfunction and drugs may also stimulate an enhanced immune response that facilitates hypersensitivity reactions and autoimmunity.[13]

Research over the past decades has focused on the immunotoxicity of novel drugs as a result of the increasing awareness that pharmaceuticals may be causal factors for adverse immunological effects.[14] Research has shown that environmental contaminants such as heavy metals, organotin compounds, polychlorinated biphenyls (PCB's), tetrachlorodibenzofuran (TCDF) and insecticides display potential immunotoxic properties.[1] There has been a growing concern amongst the scientific and public communities that xenobiotics induce alterations in the immune system which may ultimately lead to life threatening diseases. Studies have shown that exposure to some xenobiotics may result in an increased susceptibility to various forms of diseases and this area of research has become well established. However, there has been a lack of evidence on the potential immunotoxic effect of common substances that are consumed on a regular basis and which forms an integral part of our daily diet. One such substance is sugar, which is a common ingredient in the human diet. The effects of sugar on the immune system have not been studied extensively. The immune system is an essential protective mechanism of the human body and therefore this should be an emphasised area of research. The aim of this study is to determine if commercially produced sugar cane molasses affects specific cytokines regulating the immune pathway. IL-6 was the biomarker used to detect an inflammatory response. IL-10 was used as the biomarker for the humoral response and IFNg as the biomarker of the cell mediated response.

Sugar cane molasses is the end product obtained from the refinement of sugar cane or beet into sugar. Molasses is thick syrup that ranges from light brown to dark in colour and is a good source of vitamins and minerals.[15] Dating back to the nineteenth century, molasses has been used widely in livestock and poultry feeds.[16] Today, molasses is increasingly being used as a flavour enhancer, has been substituted as a sweetener and also used as a preservative in jams and jellies.[17] Anecdotal reports also suggest that molasses may be used as a supplement in the human diet to improve conditions such as anemia, colds, coughs, ear aches, arthritis, ulcers, hair damage, eczema, high blood pressure, dermatitits, constipation, varicose veins, nerve damage, and bladder problems.[15,17,18]

Although molasses has been associated with various health benefits, there are also reports that suggest the inclusion of molasses in the diet of livestock may induce certain metabolic diseases. Such diseases include molasses toxicity, urea toxicity and bloat which may occur as a result of molasses being used as a supplement (vehicle for urea) or as the basis of livestock feed.[19] Molasses toxicity is defined as a condition affecting cattle or sheep fed high molasses diets with limited forage.[20] Affected animals suffer from symptoms similar to that of cerebro-cortical necrosis or polioencephalomalacia. Bloat is a condition characterised by the retention of gas in the rumen and occurs in most animal feeding systems. However, this disease appears to be most recurrent in diets consisting of carbohydrates supplied by unrefined sugar or maize grain that has little or no fibre, yet are easily digestible.[19]

There appears to be no direct evidence to connect sucrose intake with toxicity, however there is substantial evidence that suggest high sucrose intake may be a causal factor of numerous health problems. Progression of type II diabetes, cardiovascular diseases as well as obesity have all been associated with high dietary sugar consumption. Increased sugar intake has also been established as a potential risk factor for the development of dental caries.[21]

There are several reports in the literature which suggest that sugar intake has an effect on the body's ability to defend itself against invasions by microbes and cancer.[22,23] The process of neutrophilic phagocytosis is a vital part of the immune response and an important defense mechanism. Phagocytosis requires energy which is sourced from glucose. Therefore, an insufficient supply of glucose renders this process inefficient. However, there is also evidence that suggests excess glucose intake is associated with an inhibition of phagocytosis. Diabetic patients that are described by their high blood glucose levels, tend to be more susceptible to invading pathogens than non-diabetics. The high levels of blood glucose in diabetic patients have been associated with an inefficient process of neutrophilic phagocyctosis. The regulation of phagocytosis may be dependent on glucose intake as well as blood glucose levels.[22] Sugar consumption has also been associated with an increased risk to gastric cancer. This has been confirmed in a case study that investigated the effect of nutritional factors on gastric cancer. Foods containing simple sugars, calcium and saturated fat are risk factors of gastric cancer. This data has been supported by other research studies.[23] Michaud (2002) demonstrated that a hyperglycemic diet may increase the susceptibility to pancreatic cancer in sedentary and obese individuals or those who may already present with a resistance to insulin. The impairment of glucose metabolism may be a causal factor in the development of pancreatic cancer.[24]

The above mentioned evidence suggests that sugar cane molasses may have an adverse effect on the immune system and is a potential risk factor in the development of human as well as animal disease. The evaluation of sugar intake on human health is an area of research that is limited and requires further investigation. The current study was undertaken to provide information on the effects of molasses on cytokines that regulate the specific immune processes.

EXPERIMENTAL

The Effect of Molasses Samples on Endotoxin Stimulated Whole Blood Cultures

Blood samples were collected from healthy male donors by venous puncture into citrate–containing, sterile blood collecting tubes (Lasec, SA). Consent was obtained from all participants. Assays were conducted within 8 hours after blood collection and all methods were performed under sterile conditions. Stimulated whole blood cultures contain 1 volume of 10 ng/mL endotoxin in DMSO, 10 volumes of blood and 89 volumes of RPMI-1640 medium (Sigma, USA). Unstimulated blood contains 1 volume DMSO, 10 volumes of blood and 89 volumes of RPMI-1640. A dilution range of molasses (Health Connections Wholefoods, SA) in distilled water was dispensed at 3 µl/well in wells of 96 well plates (Nunc-Immuno plate, MaxiSorp). Endotoxin stimulated or unstimulated diluted blood (300 µl/well) was added to molasses samples and thereafter incubated at 37°C for 18 hours. At the end of the incubation period the cell supernatants were collected and assayed for LDH and IL-6.

The Effect of Molasses Samples on Phytoheamagglutinin (PHA) Stimulated Whole Blood Cultures

Blood samples were collected from healthy male donors by venous puncture into citrate–containing, sterile blood collecting tubes (Lasec, SA). Consent was attained from all participants. Whole blood cultures were conducted within an 8 hour period of blood collection and all methods were performed under sterile conditions. Stimulated whole blood cultures contains 10 volumes of blood and 89 volumes of RPMI-1640 medium (Sigma, USA) with PHA (Sigma, USA) in RPMI (Sigma, USA) at a final concentration of 16 ug/mL PHA. For unstimulated whole blood cultures, no additions were made to the diluted blood. A dilution range of molasses (Health Connections Wholefoods, SA) in distilled water was dispensed at 3 µl/well in wells of 96 well plates (Nunc-Immuno plate, MaxiSorp). PHA

stimulated or unstimulated diluted blood (300 μL/well) was added to molasses samples and thereafter incubated at 37°C for 48 hours. At the end of the incubation period the cell supernatants were collected and assayed for IFN and IL-10.

LDH Assay

Lactate dehydrogenase activity in cell culture supernatants was used to determine cytotoxicity of samples. LDH was measured using a Cytoxicity Detection kit (Biovision, USA). The kit contains all components required for the assay. Cells were lysed with cell lysis solution, was used to determine total cellular LDH. Cell culture supernatants were collected for the assay and the lysed cells were assayed on a 96 well plate (Nunc-Immuno plate, MaxiSorp). 100 μL of kit reaction mixture was added to each well and incubated for approximately 15 minutes. The absorbance of reaction mixtures were then measured at 492 nm using an ELISA reader.

Cytokine ELISA's

Whole blood culture supernatants were screened for IL-6, as a biomarker of inflammation, IFNg as a biomarker of cell mediated immunity and IL-10 as a biomarker for humoral immunity. Cytokine production by whole blood cultures were measured using ELISA kits (eBioscience, USA). The kits contain all reagents required for the assay. 96 well plates (Nunc-Immuno plate, MaxiSorp) were coated with 100 μL per/well of capturing antibody diluted appropriately in coating buffer and incubated overnight at 37°C. After 5 washings with wash buffer (autoclaved Phosphate buffered saline containing 0.05% Tween-20), non specific binding sites were blocked with assay diluent for 1 hour at room temperature. Cell culture supernatants were then added to the 96 well plate (Nunc-Immuno plate, MaxiSorp). Recombinant human cytokine standards were also included on each plate. The plate was sealed and incubated at room temperature for 2 hours. After 5 washings, 100 μL of detection antibody (Biotin–conjugated anti human cytokine) was added to each well. The plate was incubated for 1 hour at room temperature. The plate was washed again for 5 times and the biotineylated sandwich was detected by adding 100 μl of the Avidin-Horseradish peroxidase conjugate (HRP) to all wells. The plate was incubated for 30 minutes. After 7 washings the bound peroxidase was monitored by adding 100 μL of the substrate solution (Tetramethlybenzidine solution) to every well. The plate was incubated for approximately 15 minutes after which the reaction was stopped with the addition of

50 µl stop solution to all wells. Absorbance was read at 450 nm on an ELISA plate reader.

Statistical Analysis

Data was statistically analysed via one-way ANOVA using SigmaStat software (Systat Software Inc., USA).

RESULTS

The Cytotoxicity of Sugar Cane Molasses

The molasses samples were tested for cytotoxicity using LDH release from whole blood cultures as a biomarker. Upon exposure to toxic compounds, the cells die and due to leaching, release LDH into the medium. The standard curve of various dilutions of the total cellular LDH shows that there is a polynomial relationship between OD and LDH. The supernatants of both stimulated and unstimulated whole blood cultures incubated with the molasses samples, contains similar LDH levels to the control cultures incubated in the absence of molasses indicating that molasses is not cytotoxic (Data not shown).

The Inflammatory Activity of Molasses Samples

The synthesis of the pro-inflammatory cytokine, IL-6 by whole blood cultures was used as a biomarker to determine the inflammatory response induced by molasses. Exposure to an immunotoxic sample may produce an elevation or suppression in the levels of IL-6 generated. Standard curves obtained with the kit reagents show that there is a good correlation ($R^2 = 0.993$) between the absorbance and IL-6 concentration for the ELISA. The whole blood culture assay for molasses were repeated using four different donors. Results obtained for the donors were similar and Figure 1 depicts the average obtained for the whole blood cultures of four donors. Molasses has no effect on IL-6 synthesis by stimulated whole blood cultures ($P = 0.435$). Molasses samples do however have a major effect on IL-6 secretion by unstimulated whole blood cultures. The addition of molasses (800 µg/mL) samples to unstimulated whole blood cultures resulted in a significantly higher IL-6 secretion compared to the controls ($P < 0.001$). These results indicate that molasses samples stimulate inflammatory activity in vitro.

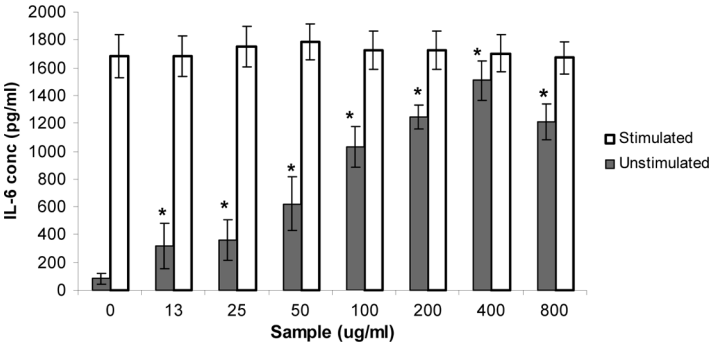

FIGURE 1 Induction of IL-6 (pg/mL) of whole blood cultures *in vitro* by LPS, in the presence of various concentrations of molasses samples and distilled water (control). An asterisk (*) designates the significant difference to control.

The Effect of Molasses on T Cell Differentiation

Cytokines, IFNg and IL-10 were used as biomarkers to determine the effect of the sugar cane samples on T cell activity. IFNg directs the differentiation of naïve T cells (T0) into Th1 cells while IL-10 directs differentiation of Th2 cells.[8] Exposure to an immunotoxic sample may produce an elevation or depression in the levels of IL-10 or IFNg produced.

Standard curves obtained with the IL-10 ELISA kit standards show that there is a good correlation ($R^2 = 0.984$) between the absorbance and IL-10 concentration for the ELISA. The whole blood culture assay for molasses were repeated using four different donors. Results obtained for the donors were similar and Figure 2 depicts the average obtained for the whole blood

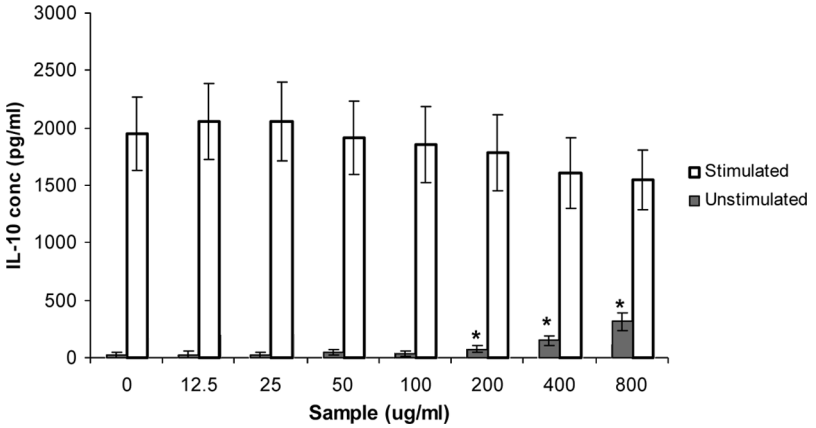

FIGURE 2 Induction of IL-10 (pg/mL) of whole blood cultures *in vitro* by PHA, in the presence of various concentrations of molasses samples and distilled water (control). An asterisk (*) designates the significant difference to control.

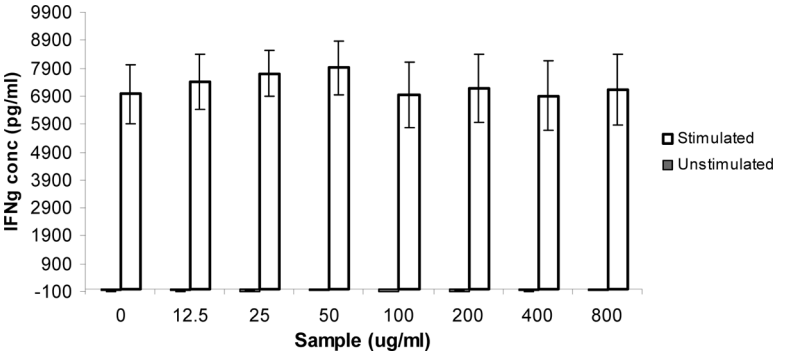

FIGURE 3 Induction of IFNg (pg/mL) of whole blood cultures *in vitro* by PHA, in the presence of various concentrations of molasses samples and distilled water (control). An asterisk (*) designates the significant difference to control.

cultures of the four donors. Molasses samples have a major effect on IL-10 secretion by unstimulated whole blood cultures. The addition of molasses (800 μg/mL) to unstimulated whole blood cultures resulted in a significantly higher IL-10 secretion compared to the controls ($P < 0.001$). These results indicate that molasses may have an immunostimulatory effect on the differentiation of Th0 cells to Th2 cells that are responsible for synthesising the cytokines required to mount an effective humoral mediated immune response.

Standards obtained with the IFNg ELISA kit standards show that there is a good correlation ($R^2 = 0.998$) between the absorbance and IFN concentration for the ELISA. The supernatants of whole blood cultures incubated with the molasses samples were screened using blood from four donors. Figure 3 depicts an average result of all donors, obtained for the whole blood cultures. Molasses has no effect on IFNg synthesis of both unstimulated and stimulated whole blood cultures ($P > 0.05$). These results indicate that molasses has no effect on the differentiation of Th0 cells to Th1 cells that functions in synthesising the cytokines required to mount an effective cell mediated immune response against intracellular pathogens.[25]

DISCUSSION

Results show that molasses increases the synthesis of cytokines, IL-6 and IL-10 under unstimulated conditions. IL-6 is a pleiotrophic, inflammatory cytokine that is produced by various cells such as mononuclear phagocytes, fibroblasts and endothelial cells.[26,27] IL-6 functions in both cellular and humoral responses. IL-6 also stimulates B cells to induce antibody production and hepatocytes to synthesis acute phase proteins.[27] IL-10 is

an anti-inflammatory cytokine produced by Th2 cells, which stimulates humoral immunity, i.e., B cell activation and maturation resulting in antibody production.[8,28,29] The current study shows molasses increases both IL-6 and IL-10 which are necessary requirements for B cells to synthesise antibodies. The data thus indicates that molasses may in fact upregulate humoral immunity.

The increase in the levels of both IL-6 and IL-10 by molasses can be associated with the upregulation of antibody production. Upregulation of antibody synthesis increase the defence against the occurrence of recurrent extracellular pathogens and their toxins.[4] Antibodies are important for opsonization that enhances phagocytosis and destruction of extracellular pathogens such as Pneumococcus.[26] Therefore, an efficient humoral response will act to eliminate such infective agents.

Studies have shown that numerous herbs have immunomodulatory activity and exhibit immunostimulatory effects in various ways.[30,31] Immunostimulants elevate specific immune responses by either increasing phagocytosis or the cell mediated and humoral response. IL-6 is a powerful inducer of B cell activation and many herb components such as aloeride (*Aloe vera*), polysaccharides consisting of glucopyranosyl (*Ganoderma lucidum*), angelan (*Angelica gigas*), ginsenosides (*Ginseng*) and gingerols (*Zingiber officinale*) induce IL-6 synthesis and enhance B cell activity. These herbal components are also capable of stimulating the cell mediated immune system.[30] In accordance with above study, the immunostimulatory synthesis of IL-6 by molasses may therefore be associated with a very efficient humoral response against extracellular pathogens.

The increase in levels of the inflammatory biomarker, IL-6 and anti-inflammatory biomarker, IL-10 also suggests that molasses has the potential to induce both an inflammatory and anti-inflammatory action on the healthy immune system. This is similar to the results of a study conducted on the herbal remedy, Sambucol. Therefore, molasses like Sambucol may have an immunostimulatory effect when administered to patients who suffer from a depressed immune system. These may include cancer patients on chemotherapy treatment or patients with AIDS.[28] Numerous cytostatic drugs such as, cyclophoshamide, cyclosporine A (CsA), prednisone and azathioprine cause immunosuppression in patients. This may unintentionally lead to numerous forms of cancers and an enhanced risk to bacterial infections.[1] Molasses may enhance the body's immune defense mechanisms by increasing B cell activity that may augment antibody synthesis and reduce the risk to these types of infections.

Anecdotal evidence suggests that molasses improves the health of patients with diseases such as rheumatoid arthritis, osteo-arthritis, nervous system dysfunction and various other disorders.[15] The current study shows that molasses has effects on some of the immune pathways. In vivo studies

must be conducted to show if these changes in the cytokine parameters affect defenses against specific infections.

CONCLUSION

Molasses has an impact on the cytokines regulating humoral immune system and has both inflammatory and anti-inflammatory potential. As a result, this compound may prove to be beneficial in promoting improved humoral responses. For this reason, we suggest that further investigations on molasses and its biological actions be conducted. Research investigating the in vivo effects of molasses on the immune system need to be conducted to determine if the upregulation of the humoral immune system biomarkers cause an increase in defence against extracellular pathogens.

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