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RESEARCH ARTICLE

Anti-Inflammatory Effect of Extracts of *Inonotus Obliquus* and Microalgae

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ABSTRACT
Chaga mushroom (*Inonotus obliquus*) and marine microalgae are two emerging natural products with many potential physiological health benefits. The aim of this study was to investigate the anti-inflammatory effects of two extracts prepared from Chaga mushroom and microalgae using lipopolysaccharide-stimulated RAW 264.7 murine macrophage cell model. The Chaga mushroom extract dose-dependently reduced the production of proinflammatory biomarkers of interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α). At a high concentration of 500 µg/L, Chaga mushroom extract significantly suppressed cyclooxygenase-2 levels. Similarly, the extract of microalgae suppressed the secretion of IL-6 and TNF-α by lipopolysaccharide-induced macrophages. Both extracts had no significant impact on the secretion of anti-inflammatory IL-4 production. These results suggest that extracts of Chaga mushroom and microalgae can be used in developing anti-inflammatory natural health products.

Keywords: Chaga, microalgae, inflammation, macrophage, cytokines, COX-2

1.0 Introduction

Inflammation is the body's defence response to harmful stimuli such as cellular damage, pathogens, chemical toxins, and irradiation. Acute inflammation is a necessary bodily process to resolve tissue injury and infections [1]. However, chronic inflammation is an etiological risk factor for many diseases, most notably, cancers, diabetes, cardiovascular, nonalcoholic fatty liver, kidney, and neurodegenerative diseases. Thus, novel strategies to prevent chronic inflammation are a primary element in reducing the burden of such diseases worldwide [2]. Currently, the use of natural compounds to develop anti-inflammatory drugs is of significant interest. Many natural compounds, including, polyphenols, alkaloids, terpenes, volatile oils, quinones, bioactive polysaccharides [3], and bioactive peptides [4] have been proven to possess anti-inflammatory properties.

Chaga mushroom (CM, *Inonotus obliquus*) is an important source of natural health products [5]. CM is a parasitic fungus that grows on hardwood trees, especially those of genus *Betula* such as birches. This fungus can also grow on other hardwood trees including, oak, poplar, alder, ash, and maple trees. CM can be found naturally in Canada, the United States, northern and eastern Europe, Ukraine, Kazakhstan, Siberia, Japan, South Korea, and China. CM has been used as a traditional medicine in these regions for several centuries [6]. The major bioactive compounds of CM include polysaccharides, triterpenoids, polyphenols, and lignin metabolites [5,7]. The beneficial health effects of CM, including anti-inflammatory activity, are considered to be primarily associated with polyphenols [8]. In addition to polyphenols, lanostane-type triterpenoids (lanostanoids) in CM have been proven to be beneficial for mitigating inflammation. A recent study was able to isolate and identify seven previously undescribed lanostanoids from CM and establish their anti-neuroinflammatory activity *in vitro* [9].

Marine algae (MA) are another well-known source for the extraction of bioactive compounds. The major bioactive compounds in MA are carbohydrates, proteins, fatty acids, minerals, polyphenols, and vitamins. MA also contains carotenoids, chlorophylls, carotene, xanthophyll, and phycobilins that can possess bioactive properties [10]. The amounts and types of anti-inflammatory molecules in MA vary between species of MA. The anti-inflammatory activities of bioactive compounds isolated from various MA have been demonstrated in many studies [11]. MA can be largely divided into two groups, the multicellular macroalgae (seaweed) and the unicellular microalgae [12]. Microalgae contains a higher concentration of bioactive compounds compared to macroalgae [13]. Therefore, utilization of microalgae can be beneficial over macroalgae for the production of natural health products. There are four main classes of microalgae; Cyanophyceae (blue-green algae), Chlorophyceae (green algae), Porphyridiophyceae (red algae), and Bacillariophyceae (diatoms) [14]. *Nannochloropsis gaditana*, *Tetraselmis chui*, and *Chlorella vulgaris* are green marine microalgae that can be valorized as food additives and dietary supplements and are excellent sources for the extraction of bioactive compounds [15–17].

The immune system regulates the body's inflammatory response through complex mechanisms involving immune cells (monocytes, macrophages, and lymphocytes), pro- and anti-inflammatory cytokines, and inflammatory signalling pathways [1]. Interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α are among the most studied proinflammatory cytokines while IL-4, IL-10, and IL-13 are recognized as anti-inflammatory cytokines [18]. Oxidative stress created by reactive oxygen species (ROS) can induce chronic inflammatory responses. Therefore, enzymes involved in the production of ROS such as xanthine oxidase and nicotinamide adenine dinucleotide phosphate oxidase (NOX) together with antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) play a vital role in maintaining inflammatory balance. Similar to ROS, prostaglandins (PGs) and leukotrienes (LTs) are capable of inducing inflammatory responses. Several enzymes, including phospholipase A2 (PLA2), cyclooxygenases (COX), and lipoxygenase (LOX) are well-known enzymes involved in the production of PGs and LTs [19].

Since both CM and MA are known to possess anti-inflammatory properties, we evaluated the potential of CM and MA extracts to reduce inflammation in lipopolysaccharide (LPS)-induced macrophages (RAW 264.7) model by measuring the production of pro- and anti-inflammatory cytokines and measuring the cellular levels of COX-2 enzyme. Bioactivities of CM and MA extracts differ significantly with extraction method and conditions [8,20]. The current study attempts to evaluate the efficacy of a supercritical extract of CM and an ethanol extract of MA for the reduction of inflammatory response in macrophages *in vitro*. The findings of this study will be beneficial for the production of CM- and MA-based products to aid in the mitigation of chronic inflammation.

2.0 Materials and methods

2.1 EXTRACTS

The CM extract was prepared using the supercritical extraction process previously described by Wagle and colleagues [21]. Briefly, 40 g of finely ground *I. obliquus* powder was processed with a pressure of 7,500 psi, a temperature of 50°C, and an extraction time of 1 h. Co-solvent of 10% ethanol was used for the supercritical fluid extract.

The MA extract was prepared using a mixture of microalgae comprised of 60% w/w *Nannochloropsis gaditana*, 15% w/w *Tetraselmis chui*, and 25% w/w *Chlorella vulgaris* [21]. First, 2.5 g of microalgae mixture was added to 1 L of extraction solvent (530 mL of EtOH, 300 mL of DI water, and 70 mL of acetonitrile) and sonicated for 20 min twice at room temperature. The sample was centrifuged at 3,000 × *g* for 10 min and filtered through P8 filters. The filtrate was collected into a separatory funnel and 100 mL n-hexane was added which was then vigorously shaken with degassing. The chlorophyll-free aqueous phase was concentrated by rotovaping and freeze-drying to generate a dry extract.

2.2 CELL LINE AND CULTURE CONDITIONS

RAW 264.7 (TIB-71™) macrophages were purchased from American Type Culture Collection (ATCC®), Manassas, VA, USA. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin (100 U/mL)-streptomycin (100 µg/mL). Cells were cultured at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. Cells were sub-cultured at 80% confluency.

2.3 ANTI-INFLAMMATORY ACTIVITY OF CHAGA MUSHROOM AND MICROALGAE EXTRACTS

The anti-inflammatory activity of CM and MA extracts was evaluated in RAW 264.7 macrophages stimulated with bacterial LPS [8]. Cells were seeded in 12-well plates at a density of 1×10^5 cells/well and incubated for 24 h under normal culture conditions. Cells were pre-treated with CM or MA at different concentrations (1 – 300 µg/mL) for 24 h. Subsequently, cells were exposed to 1 µg/mL of LPS from *Escherichia coli* O55:B5 (L6529, Millipore Sigma, Oakville, ON, Canada) for 24 h to induce inflammatory responses. The potential of CM and MA extracts to mitigate LPS-induced inflammation in macrophages was evaluated by measuring the production of the proinflammatory cytokine, IL-1*β*, IL-6, and TNF-*α*. Cellular levels of COX-2, an important modulator of the inflammatory response, were also measured. We further evaluated the ability of these extracts to upregulate the production of anti-inflammatory cytokines, IL-4 and IL-10. Production of the targeted cytokines by macrophages was quantified by measuring their concentrations in the phenol red-free cell culture medium.

2.3.1 Production of pro and anti-inflammatory cytokine by macrophages

Production of the targeted cytokines by macrophages was measured by using ELISA kits purchased from Mabtech, Inc., Cincinnati, OH, USA. Briefly, 96-well plates were coated with capture monoclonal antibodies (mAb) provided with the ELISA kits overnight at 4 °C. Subsequently, the plates were blocked with 0.1% bovine serum albumin (*w:v*) in phosphate-buffered saline (PBS) with 0.05% (*v:v*) Tween 20 for 1 h. The plates were washed (×5) with 0.05% (*v:v*) Tween 20 in PBS. Cell culture media (after treatments) were pipetted into the 96-well plates and incubated for 2 h at room temperature (RT). Again the plates were washed (×5) with 0.05% (*v:v*) Tween 20 in PBS and pipetted with detection mAb provided with the ELISA kits before incubating for 1 h at RT. Plates were washed as previously described, pipetted with Streptavidin-ALP and incubated for 1 h at RT. Plates were washed again as described above and pipetted with pNPP substrates. Plates were incubated with the pNPP substrate for 1 h at RT before measuring the absorbance at 405 nm wavelength. Concentrations of the targeted cytokines in cell culture media were determined by using standard concentration curves created for each of the targeted cytokines. Results were expressed as % cytokine level relative to the positive control.

2.3.2 Cellular levels of cyclooxygenase-2

Cellular levels of COX-2 in the macrophages were measured by using an ELISA test kit (ab210574) purchased from Abcam, Cambridge, MA, USA. Initially, the cell extract was prepared by using the cell extraction buffer provided with the test kit. Cells were harvested by using a cell scraper and separated from culture media by centrifuging at 1×10^3 *g* for 5 min. Cells were rinsed (×2) with PBS and incubated with the cell extraction buffer for 15 min on ice. Subsequently, the cell suspension was centrifuged at 18×10^3 *g* for 20 min at 4 °C to separate cell lysate from cell debris. The COX-2 standard provided with the test kit was diluted (0 – 30 ng/mL) with the cell extraction buffer to create a COX-2 standard concentration curve. Prepared cell lysate and diluted COX-2 standards were pipetted into 96-well plate strips with the antibody cocktail provided with the test kit. The plates were

sealed and incubated for 1 h at RT on a plate shaker. Plate wells were washed (×3) with wash buffer, added with TMB development solution and incubated for 10 min in the dark on a plate shaker. Then, the stop solution was pipetted into each well and absorbance was measured at 450 nm wavelength. Cellular levels of COX-2 were calculated by using the COX-2 standard concentration curve. Results were expressed as % COX-2 relative to the positive control.

2.4 STATISTICAL ANALYSIS

All experimental data were analyzed by the Minitab® statistical software (version 19.2020.1). Results were expressed as mean ± standard deviation of at least three individual experiments. Means were compared by the one-way analysis of variance (ANOVA) with Tukey's multiple mean comparisons at 95% confidence level.

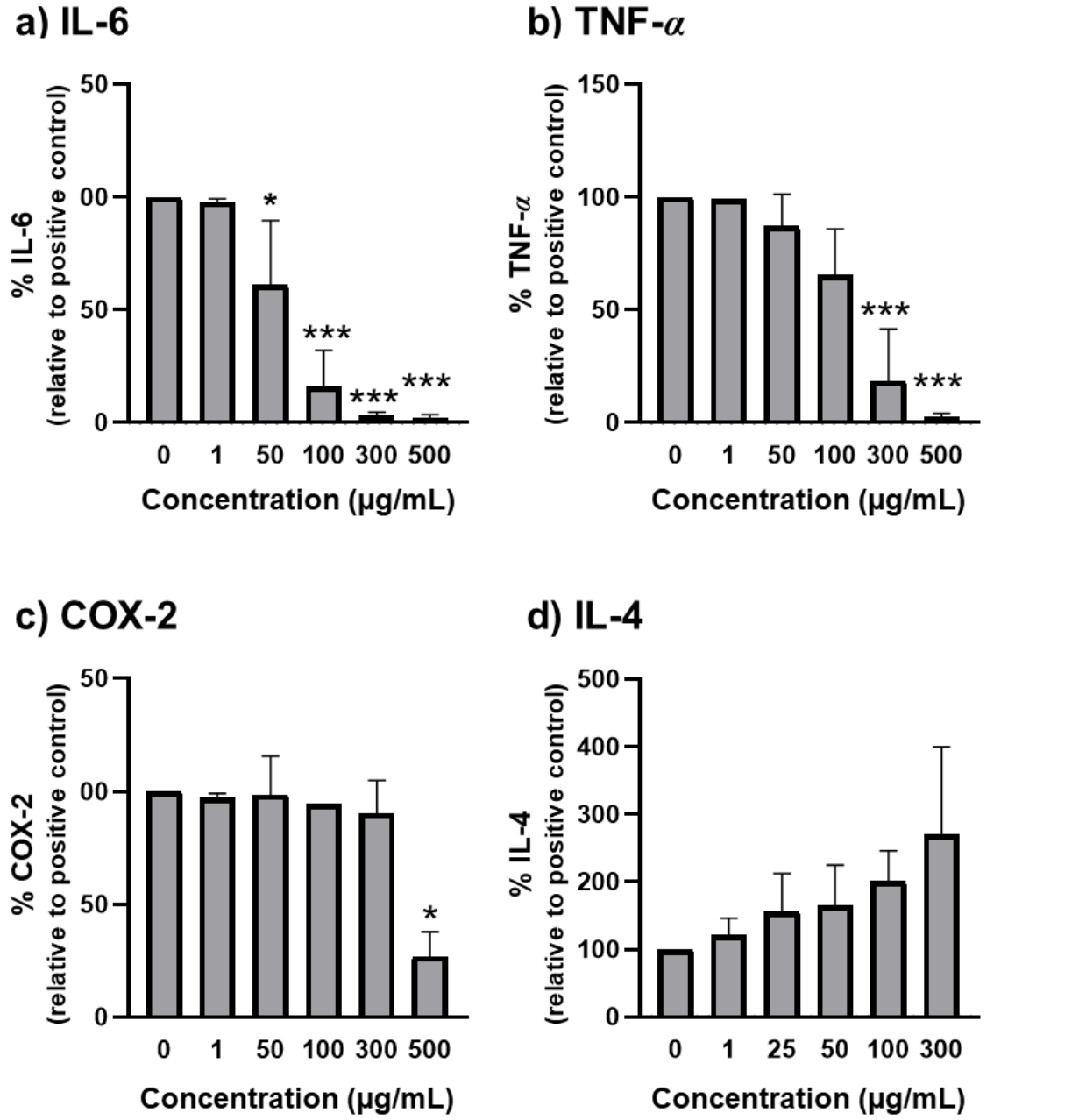

Fig 1: Effects of Chaga mushroom (CM) extract on the bacterial lipopolysaccharides (LPS)-induced inflammatory response in RAW 264.7 macrophage cells. Macrophages were pre-treated with CM extract (0 – 500 µg/mL) for 24 h and exposed to LPS (1 µg/mL) for 24 h to induce inflammation. Production of the proinflammatory cytokine, interleukin (IL)-6 (a) and tumour necrosis factor (TNF)-α (b) by the macrophages was studied by measuring their concentrations in the cell culture media. Cellular levels of cyclooxygenase-2 (COX-2) in macrophages were also measured (c). The potential of CM extract to promote the production of anti-inflammatory cytokine IL-4 was studied by measuring the IL-4 concentration in cell culture media (d).

3.0 Results

The anti-inflammatory activity of CM and MA extracts was studied in macrophage cells stimulated with LPS for inflammatory responses. CM extract significantly suppressed the production of proinflammatory cytokines, IL-6 and TNF-α by the macrophage cells stimulated with LPS (Fig 1 a and b). Suppression of the proinflammatory cytokine production by the CM extract was dose-dependent with higher concentrations drastically quenching the IL-6 and TNF-α production. The CM extract could significantly reduce the production of COX-2 in the macrophages stimulated with LPS. However, the extract was effective in COX-2 reduction only at the high 500 µg/mL level (Fig. 1 c). Interestingly, the macrophage cells depicted an uptrend for anti-inflammatory cytokine IL-4 production, when treated with the CM extract (Fig 1. d). However, this increase in IL-4 production was not statistically significant.

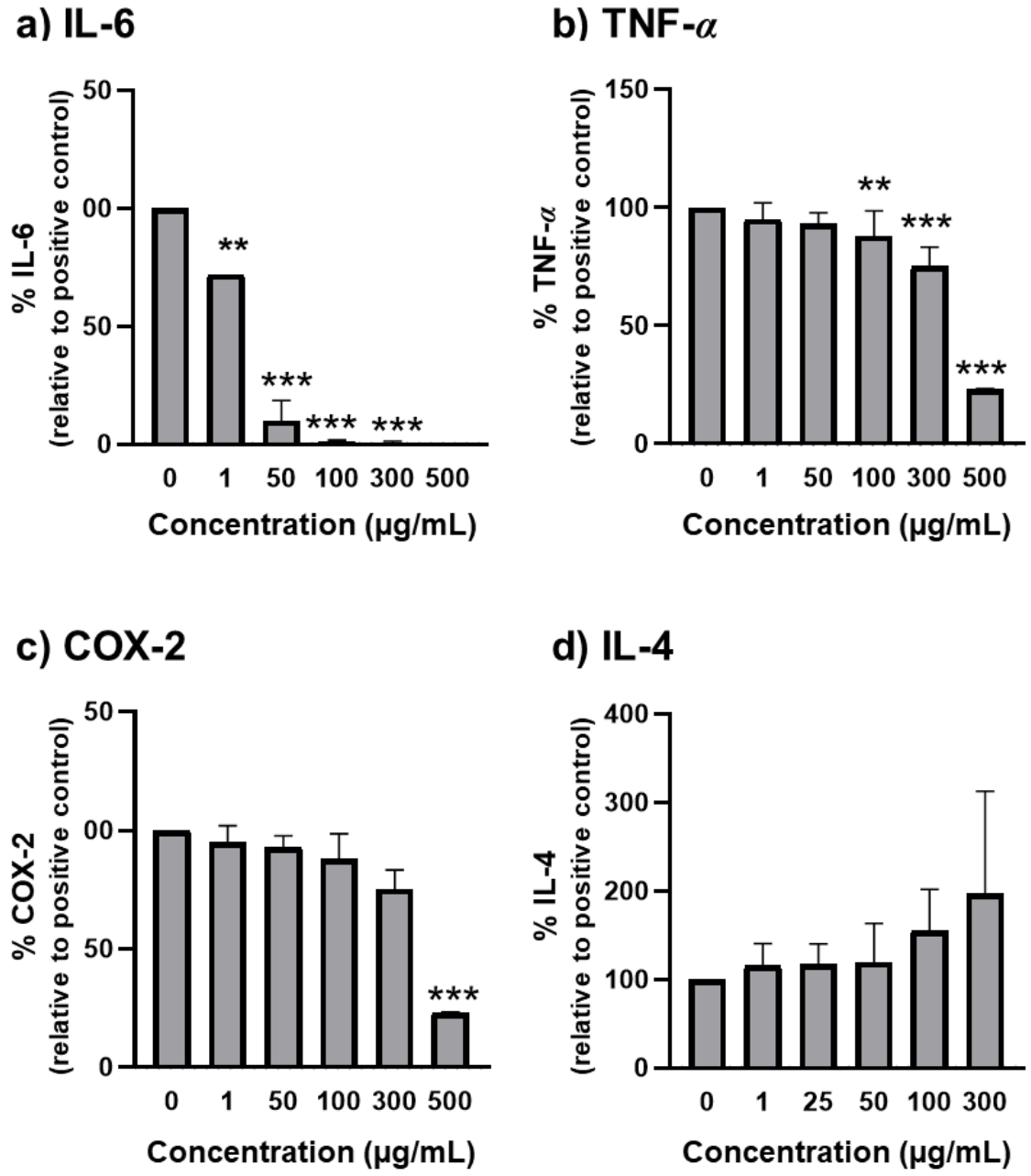

Fig 2: Effects of *microalgae* (MA) extract on the bacterial lipopolysaccharides (LPS)-induced inflammatory response in RAW 264.7 macrophage cells. Macrophages were pre-treated with MA extract (0 – 500 µg/mL) for 24 h and exposed to LPS (1 µg/mL) for 24 h to induce inflammation. Production of the proinflammatory cytokines, interleukin (IL)-6 (a) and tumour necrosis factor (TNF)-α (b) by the macrophages was studied by measuring their concentrations in the cell culture media. Cellular levels of cyclooxygenase-2 (COX-2) in macrophages were also measured (c). The potential of MA extract to promote the production of anti-inflammatory cytokine IL-4 was studied by measuring the IL-4 concentration in cell culture media (d).

MA extract demonstrated similar efficacy in the suppression of IL-6 and TNF-α production by the macrophage cells (Fig.2 a and b). Suppression of the IL-6 and TNF-α production was dose-dependent. The MA extract significantly reduced the IL-6 production at low concentrations (1 µg/mL) with concentrations at and over 50 µg/mL drastically suppressing the IL-6 production. Similar to the CM extract, COX-2 reduction by the microalgae extract was significant only at the high 500 µg/mL level (Fig. 2 c). The macrophage cells treated with MA extract demonstrated an increasing potency to produce the anti-inflammatory cytokine IL-4. However, increments in the production of IL-4 were not statistically significant. IL-1β and IL-10 cytokines were not detected in the current study. Both CM and MA extracts can significantly reduce the production of inflammation promoters, IL-6, TNF-α, and COX-2. These extracts may also have the potential to induce the production of anti-inflammatory cytokines such as IL-4.

4.0 Discussion

The LPS-mediated inflammatory responses in RAW 264.7 macrophages are primarily mediated by toll-like receptor 4 (TLR4) signalling [22]. LPS is a TLR4 agonist capable of inducing inflammatory responses in macrophages by activating (phosphorylating) nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPK), p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinases (ERK) [23]. Activation of the NF-κB and MAPK inflammatory signalling in the macrophages can significantly increase the production of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α together with key inflammatory modulators, nitric oxide (NO) and prostaglandin E2 (PGE2) [23]. In the current study, levels of IL-1β, IL-6, and TNF-α proinflammatory cytokines in cell culture media were directly measured. Cellular levels of the COX-2 enzyme were measured since NO activates the COX-2 enzyme and the conversion of arachidonic acid to prostaglandins such as PGE2 depends on the activity of the COX-2 enzyme [24]. Several studies have demonstrated the ability of CM extract to suppress LPS-induced inflammation in the RAW 264.7 macrophage cells [8]. CM can significantly reduce the production of IL-1β, IL-6, and TNF-α proinflammatory cytokines in the macrophages exposed to LPS. Also, the ability of CM extract to reduce the NO production in macrophages [8] aligns with the reduction of the COX-2 enzyme in the current study. CM is rich in bioactive phenolic acids such as syringic acid, *p*-hydroxy benzoic as well as and triterpenoids such as botulinic acid and trametenolic acid [21]. Syringic acid has shown anti-inflammatory effect through modulation of the NF-κB-inducible nitric oxide synthase (iNOS)-COX-2 and Janus kinases (JAK)-signal transducer and activator of transcription proteins (STAT) signaling pathways in methyl cellosolve-induced hepato-testicular inflammation in rats [25]. Triterpenoids such as inotodiol and butelin in CM are known to possess anti-inflammatory properties [26].

The extract of MA used in this study consisted mainly polyphenols such as dihydroferulic acid, syringic acid, cinnamic acid and protocatechuic acid [21]. The anti-inflammatory activity of MA extracts has been previously reported. Methanolic extracts of the MA are capable of ameliorating LPS-induced inflammation in macrophages by suppressing the NO production [27]. The phenolic constituent of the MA extract, dihydroferulic has been shown to protect hepatic cells in vitro from TNF-α-induced inflammation and oxidative stress [28]. Monogalactosyldiacylglycerols (MGDGs) in the MA extract are responsible for the suppression of NO production by the inhibition of the inducible NO synthase (iNOS) enzyme. Polyunsaturated fatty acids of the MGDGs may be the active moieties involved in suppressing NO production [27]. Both CM and MA extracts indicated some potential to promote IL-4 production in the macrophages. Studies demonstrating the ability of CM and MA to increase anti-inflammatory cytokine production by macrophages are limited. However, the potential of microalgae rich in long-chain polyunsaturated fatty acids (PUFA) to upregulate anti-inflammatory cytokines is previously demonstrated *in vivo* [28]. Supplementation with microalgae *Diacronema lutheri* could restore the anti-inflammatory cytokines, IL-4 and IL-10 in Wistar rats on a high-fat and high-fructose diet [28]. MA is considered a rich source of PUFA [29]. Therefore, MA may increase anti-inflammatory cytokines production in the macrophages.

We have demonstrated the synergistic cytotoxicity of the extracts of Chaga mushroom and microalgae against human and canine cancer cell lines (MCF-7, HepG2, HOS, D-17, and DH-82) [21]. Chronic inflammation caused by continued infections and metabolic disorders can increase the risk of carcinogenesis and development of many cancers [30,31]. Infections activate immune cells through interaction with receptors such as toll-like receptors (TLRs) and nucleotide-binding oligomerization-domain protein-like receptors (NLRs) [32]. As a result, these cells release pro-inflammatory cytokines and chemokines such as IL-1, IL-6 and IL-8, which in turn recruit and activate immune cells to produce

additional cytokines, chemokines as well as free radicals [33]. Therefore, the anti-inflammatory properties found in the extracts of Chaga mushroom and microalgae provides experimental evidence supporting the potential therapeutic application of these extracts and their constituents in cancer prevention. However, anti-inflammatory properties of natural extracts could contract with cancer therapies, especially immunotherapies. Therefore, more research is required to understand "double-edged sword effect" of natural anti-inflammatory compounds in cancer prevention and treatment.

5.0 Conclusions

This study shows that CM and MA extracts can significantly reduce the production of proinflammatory cytokines by the macrophages stimulated with LPS. These extracts can also reduce the cellular levels of COX-2 in the macrophages stimulated with LPS. Further studies are required to evaluate the potential of CM and MA extracts to promote the production of anti-inflammatory cytokines in vivo. Identification of the broad-spectrum natural anti-inflammatory compounds present in CM and MA is required.

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