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SUPPRESSION OF LPS-INDUCED INFLAMMATORY AND NF-KB RESPONSES BY OMEGA 3 FATTY ACIDS CONCENTRATE FROM MARINE MICROALGAE *DUNALIELLA SALINA*

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Abstract: The health benefits of omega-3 polyunsaturated fatty acids (ω-3 PUFA), mainly Eicosapentaenoic acid (EPA 20:5) and Docosahexaenoic acid (DHA, 22:6), have been long known. Still today, studies continue to demonstrate the health benefits of ω-3 PUFA; however, the mechanisms of action of ω-3 PUFA are not fully understood yet. The main commercial source for omega 3 fatty acids is marine fish oil, in this study we suggest an alternate source for their production and their effect in the regulation of production of various pro inflammatory markers by human peripheral blood mononuclear cells stimulated with Lipopolysaccharide (LPS). Treatment with omega 3 fatty acids resulted in significant down regulation of LPS-elicited production of TNF-α, IL-6 and COX-2. The cascade of molecular events in lipopolysaccharide signaling modulated by the inhibition of the translocation of transcription factor NF-kB to the nucleus upon Omega 3 fatty acids concentrate supplementation. The present study summarizes the role of the Omega 3 fatty acids concentrate from marine micro algae *Dunaliella salina* in the modulation of the expression of different inflammatory mediators and revealed that the inhibitory effects on cell signaling pathways are responsible for its anti-inflammatory activity.

Key words: *Dunaliella salina*, Omega 3 fatty acids concentrate, Inflammation, Lipopolysaccharide, Cyclooxygenase.

INTRODUCTION

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. Inflammation has been shown to be a multi-step process, mediated by activated inflammatory and immune cells including macrophages and monocytes. These cells have critical functions in the immune system, acting as regulators of homeostasis and as effecter cells in infection, wound healing and tumor growth. The nuclear factor-*k*B (NF-*k*B) is essential for host defense and inflammatory responses to microbial and to viral infections (Li 2002). In response to extracellular stimuli, such as bacterial lipopolysaccharide (LPS), tumor-necrosis factor-*a* (TNF-α), or other inflammatory mediators, the transcription factor NF-*k*B is often activated and subsequently facilitates the transcription of a number of genes involved in inflammation, such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and specific cytokines (Celec,2004). TNF-α and IL-6 secreted from macrophages and monocytes during infection play an important roles in immunity (Cross, 2003). Inflammatory cyclooxygenase-2 (COX-2),Tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) production need to be regulated by NF-kB because, these mediators contribute not only to the destruction of invading pathogens but also, if excessive, to harmful inflammatory responses such as those observed in chronic inflammatory diseases (Soren, 2004, Sarada, 2008).

Dietary omega 3 fatty acids are nutrients that can modulate a wide range of cell responses. There are several experimental and epidemiological data indicating that the consumption of omega -3 fatty acids decreases the risk of various diseases (Calder, 2002). The benefits of the high omega -3 fatty acids intake are attributed to their capacity to modulate cellular metabolic functions and gene expression (Calder, 2001). Omega-3 fatty acids, found primarily in fatty fish, consist of both Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), but fish cannot synthesize these; rather they are produced by single-celled marine algae that are consumed by the fish. Unicellular algae can be recognized as an important source

of omega 3 PUFAs especially omega 3 fatty acid series such Eicosapentaenoic acid (EPA, 20:5 ω-3) and Docosahexaenoic acid (DHA, 22: 6 ω-3). Several of unicellular algae, e.g., *Chlorella*, *Dunaliella* and *Spirullina* can accumulate appreciable amount of fat (up to 60% of dry weight basis) when grown under certain environmental conditions. *Dunaliella salina*, a unicellular green halophile microalgae and it is scientifically interesting for its capacity of producing â-carotene and glycerol. *D. salina* is known to increase its lipid production in response to halostress (Raja, 2007). Amount of total unsaturated fatty acids or the degree of unsaturation in *D. salina* was increased with salt concentration and it was around 35% (Griffiths, 2009).

In the present study we evaluated the in vitro mechanisms involved in the ability of omega 3 fatty acids concentrate from marine microalgae *Dunaliella salina* to modulate the production of TNF-α, IL-6, and COX-2 directly. We also tried to dissect the signaling pathways mediating these potential effects, by focusing on associated changes in nuclear factor-kB (NF-kB) translocation to the nucleus.

MATERIALS AND METHODS

Materials

The chemicals and reagents like 3-(4,5-dimethyl-thiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT), penicillin, streptomycin, lipopolysaccharide (LPS; from Escherichia coli strain 055:B5), Histopaque 1077 ,RPMI 1640,Antibiotic antimicotic solution were purchased from Sigma Chemicals Co, USA . Monoclonal antibodies from Sigma Aldrich, India. RT-PCR kit was purchased from Thermo Fisher Scientific Mumbai.

Culture and Isolation of omega 3 fatty acids concentrate from *Dunaliella salina*

Marine microalgae *Dunaliella salina* was obtained from the Culture Collection of Central marine fisheries research institute Cochin, Kerala. *Dunaliella salina* was cultured in a 2 liter flask with 1.5 L of F/2 culture medium (Guillard *et al.*, 1962) containing 8% NaCl and 5 mM nitrogen, pH 8. The cultures were continuously illuminated with cool white fluorescent lamps (Philips 40 W) for 12hr. The algal cell were harvested by centrifugation (6000 x g) at 4°C for 15 min and stored at -20°C. Lipids from *Dunaliella salina* was extracted by Bligh and Dyer method (Bligh *et al.*, 1959). A known amount of algal lipids (0.5 g) was saponified with potassium hydroxide and the unsaponifiables extracted with petroleum ether (b.p 40-60°C), washed several times with distilled water and dried over anhydrous sodium sulfate. The solvent was evaporated and the unsaponifiables matter was weighed. The soap solution was acidified with sulfuric acid (5 N), the liberated fatty acids were extracted with ether, washed several times with distilled water and methylated with Methanolic HCl solution. Fatty acid methyl esters were analyzed by gas chromatography (GC) coupled with mass spectroscopy (Farag *et al.,* 2007).

Cell Culture

Isolation of human peripheral blood mononuclear cells (hPBMCs)

Peripheral blood mononuclear cells were isolated from healthy human volunteers as described by Radhika *et al.* (2007). Briefly anticoagulated blood was layered over equal volume of Histopaque1077. After centrifugation at 400g for 30 minutes at room temperature, mononuclear cells were collected from interface and re suspended in 2ml PBS and mixed by gentle aspiration. Cells were sedimented by centrifugation at 250g for 10 minutes, washed and re suspended in 0.5 ml PBS-Tween.

Culture of Peripheral Blood Mononuclear Cells

The peripheral blood mononuclear cells isolated from anticoagulated blood were cultured in 35mm culture plates in RPMI medium supplemented with 5% homologous serum and were maintained by incubating the plates in a carbon dioxide incubator at 370 C in 95% air and 5% carbon dioxide atmosphere.

RNA preparation and Reverse Transc-riptase Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was isolated from PBMCs using RNA isolation kit (Thermo fisher Scientific) according to the manufacturer's instructions. The first strand of cDNA was synthesized using total RNA in Eppendorf thermal cycler. Then PCR analyses were performed on the aliquots of the cDNA

preparations to detect COX-2, TNF α, and GAPDH (as an internal standard) gene using two steps RT-PCR kit (Thermo Fisher Scientific Mumbai) according to the manufacturer's procedure. The primer pairs for human COX-2, TNF- á and GAPDH were as follows;

COX-2 (bp) Forward 52 -CAATCTGGCTGAG GGAACACAACA-32

Reverse 52 -ATCTGCCTGCTCTGGTCAATG GA-32

TNF-á (bp) Forward52 -GACCTCTCTCTAATC AGCCC-32

Reverse52 GCAATGATCCCAAAGTAGACCTG CCC-32

GAPDH (bp) Forward52 -GGAGTCAACGGA TTTGGTCGTAT-32

Reverse 52 AGGTCAGGTCCACCACTGAC-32 .

The primer sequences were purchased from Eppendorf India Ltd, Chennai. After amplification, products of the PCR reactions were electrophoresed through a 1.5% agarose gel, and the bands were visualized with Ethidium bromide and quantified by densitometry performed on the scanned images using BIO RAD gel Doc analyzer. The signals were expressed relative to the intensity of GADPH in each sample.

MTT assay

The viability of hPBMCs in culture on treatment with omega 3 fatty acids concentrate was assessed by performing MTT assay (Sladowski *et al.,* 1993). Brieûy hPBMCs were maintained in RPMI medium containing omega 3 fatty acids concentrate. Cells supplemented with ethanol served as control. At intervals of 24h the medium was removed and the cells were washed with Krebs Hanseleit buffer. The cells were then incubated with 0.5 mg/ml MTT in Krebs Hanseleit buffer for 4 h at 37°C in a CO_2 incubator, after that the solution was removed and the formazan dye formed in the cell was solubilized in DMSO. The absorbance at 570 nm was measured using a multiwell scanning spectrophotometer and the viability of the omega 3 fatty acid concentrate treated cells expressed as a percentage of the untreated control.

ELISA

Amount of TNF-k, IL-6 were quantitated by ELISA (Engvall et al., 1971). Cell culture medium pre-coated onto ELISA plates served as the antigen. O-dianisidine was used as the substrate. The concentrations of these antigens were estimated by measuring the absorbance of the colored HRP product spectro-photometrically at 405 nm.

Preparation of Cytosolic and Nuclear extracts

The cells were collected after culture and washed twice with cold PBS, resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM PMSF, 0.5 mM DTT, 10 µg/ml aportinin). After 15 min incubation on ice, the cells were lysed by the addition of 0.1% NP-40 and vigorous vortexing for 1 min. The nuclei were pelleted by centrifugation at 12,000 × g for 1 min at 4°C and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate). The cytosolic and nuclear extracts were stored in aliquots at -80°C.

Protein Assay

Protein was assayed by the method of Lowry et al (Lowry *et al.,* 1951).

Statistical Analysis

Statistical analyses were performed with Graph Pad Prism software (version 4.0). Differences were tested for statistical significance by 1-way ANOVA. Values are taken as mean of five experiments.

RESULTS

Effect of Omega 3 fatty acids concentrate on cell viability

hPBMCs were treated with various concentrations of omega 3 fatty acids concentrate isolated from *Dunaliella salina* (Fig. 1) and the cell viability tested by MTT assay (Fig. 2). Omega 3 fatty acids concentrate did not exhibit cytotoxicity at the range of 2µg to 20 ìg. Among these 20 ìg was used for the following experiments.

*** Fatty acid profile in the Sample 1- 590**
[GC MS study]

No.	RT	Name of the compound	Molecular formula	MW	Peak Area %
1	12.27	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	9.16
2	16.52	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	$C_{23}H_{34}O_2$	342	60.31
3	16.98	13-Docosenoic acid, methyl ester	$C_{23}H_{44}O_2$	352	10.69
4	19.06	5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)-	$C_{21}H_{32}O_2$	316	19.85

*Parameters tested are not covered under the scope of NABL accreditation

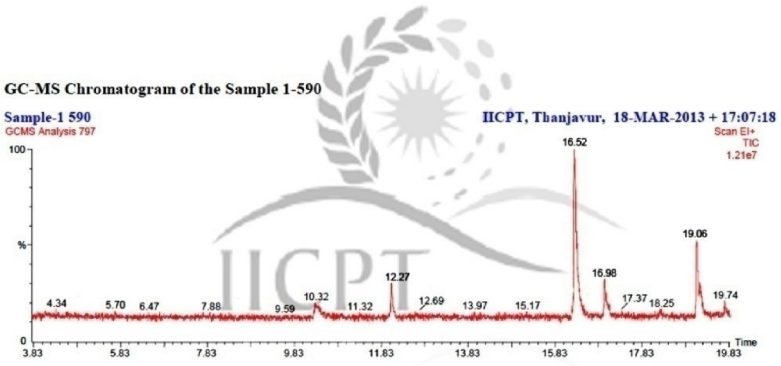

Fig. 1. FAME profile of polyunsaturated fatty acids from marine micro algae *Dunaliella salina*. Chromatographic conditions: Column- Elite-5MS (5% Diphenyl / 95% Dimethyl poly siloxane), 30 x 0.25mm x 0.25m df. Injector temperature 250 °C

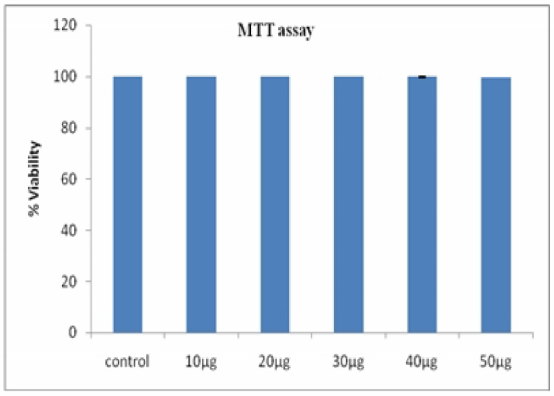

Fig. 2. MTT assay. The hPBMCs treated with Omega 3 fatty acids concentrate and controls were tested for their viability by MTT assay as described in the text. The amount of coloured dye formed was determined by measuring OD at 570 nm. Viability was expressed as percentage control and was calculated by dividing the absorbance per milligram protein of Omega 3 fatty acids concentrate treated samples by absorbance per milligram protein of the controls and expressed as percentage values and is expressed as a percentage of viability. Data are expressed as the mean±SD from five separate experiments

Effects of Omega 3 fatty acids concentrate on pro-inflammatory cytokines.

It was found that hPBMCs produce detectable amounts of IL-6 and TNF-α after stimulation with LPS.The in vitro anti-inflamma-tory activity of omega 3 fatty acids concentrate was monitored by evaluating the gene expression and protein levels of pro inflammatory cytokines (IL-6, and TNF-α) with ELISA and RT-PCR analysis (Fig. 3). Omega 3 fatty acids concentrate significantly suppressed the production of pro-inflammatory cytokines in LPS-stimulated monocytes.

Effect of Omega 3 fatty acids concentrate on COX-2 mRNA.

The expression of COX-2 mRNA in hPBMCs was monitored by PCR analysis. Cells were stimulated with LPS (1 µg/ml) and treated with omega 3 fatty acids concentrate for 24hr. LPS increased the COX-2 expression in gene level, which was inhibited by the treatment with Omega 3 fatty acids concentrate (Fig. 4). Hence, Omega 3 fatty acids concentrate was active in suppressing

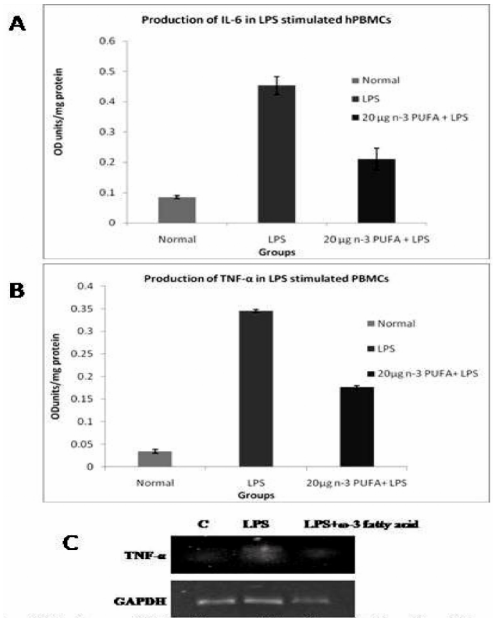

Fig. 3. Suppression effect of omega 3 fatty acids concentrate on the production of pro inflammatory cytokines IL-6 (A) and TNF-α (B). The mononuclear cells were stimulated with 1ug/ml of LPS only or LPS plus optimum concentration of omega 3 fatty acids concentrate for 24 hr. Cells were collected and TNF- α, IL-6 levels were determined in cell culture supernatant by ELISA.**C**: Reverse transcriptase PCR analysis of mononuclear cells for analyzing the inhibitory effect of omega 3fatty acids concentrate on TNF-α mRNA expression. The mRNA expression data were normalized to the GAPDH signal. Data are expressed as the mean± SD from five separate experiments.

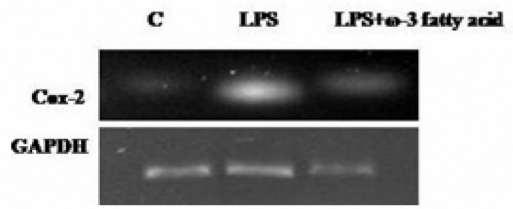

Fig. 4. Inhibition of LPS-induced COX-2 mRNA expression by omega 3 fatty acids concentrate in *in vitro* activated human PBMCs. Isolated LPS treated hPBMCs were maintained in culture in the presence of omega 3 fatty acids concentrate for 24 hr. The total RNA of untreated control, LPS, LPS+ Omega 3 fatty acids concentrate (20 µg) treated cells were isolated and RNA equivalent samples were used in RT-PCR.PCR products were electrophoressed and visualized by UV transillumination. GAPDH served as internal marker.

Effect of Omega 3 fatty acids concentrate on the activation of NF- kB

NF-kB proteins are present in the cytoplasm as inactive heterodimers composed of two subunits, P50 and P65, and are bound to the inhibitory protein IkB which prevents it from the translocation into the nucleus of the cell. Upon stimulation, IkBa is phosphorylated and proteolytically which facilitates NF-α B translocation into the nucleus and regulates gene transcription. To investigate whether could affect nuclear trans- location of NF-α B, western blot analysis of NF-kB p65 was carried out using hPBMC cell lysates. The amount of NF-kB p65 was markedly increased upon exposure to LPS alone, whereas omega 3 fatty acids concentrate inhibited it (Fig. 5). These results suggest that inhibition of IL-6, TNF-α, and COX-2 gene expression by omega 3 fatty acids concentrate may have been due to the down regulation of NF-kB activation.

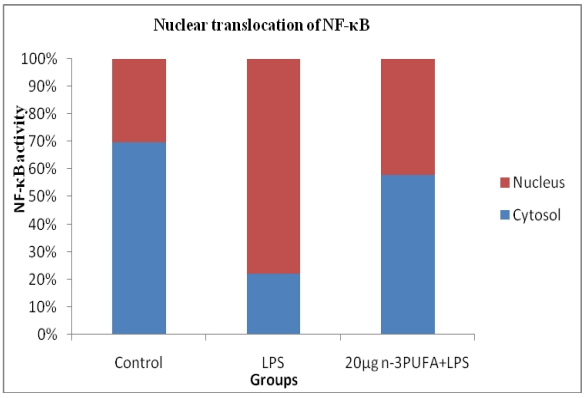

Fig. 5. Effect of omega 3 fatty acids concentrate on nuclear transcription factor activation in LPS treated hPBMCs. ELISA analysis of NFkB activation. LPS stimulated Cells were incubated with omega 3 fatty acids concentrate (20µg) for 24 h. Nuclear extract and cytosolic fraction were prepared and analysed by ELISA.

DISCUSSION

The current study was conducted to investigate the potential anti-inflammatory and anti-NF-kB responses of omega 3 fatty acids concentrate in LPS induced human peripheral blood mononuclear cells. LPS-induced gene expression is mediated by a series of signaling pathways, including NF-kB (Baeuerle *et al.*, 1996). Inflammation represents a highly coordinated set of events that allow tissues to

respond to injury, and it requires the participation of various cell types expressing and reacting to diverse mediators in a sequential manner. Although the inflammation may afflict different body compartments, one common characteristic of these conditions and diseases is excessive or inappropriate production of inflammatory mediators including eicosanoids and cytokines (Arend *et al.,* 2004).A prominent and characteristic feature of inflammatory diseases is the persistent production of pro-inflammatory cytokines by the inflamed synovium as well as by chondrocytes in the affected area (Westacott *et al.,* 1996). Therefore, the cytokine network constitutes an important target for the development of novel anti-inflammatory drugs (Alkharfy *et al.,* 2000). LPS-induced monocyte activation increased the production of pro-inflammatory cytokines, NO by iNOS and PGE2 by COX-2, which are the main cytotoxic and pro-apoptotic mechanisms participating in the innate response in many mammals .Therefore, LPS which stimulated macrophages can be effectively used as a model to study inflammation and potential anti-inflammatory mediators with their action mechanisms. In order to investigate the anti-inflammatory activity of omega 3 fatty acids concentrate in LPS-stimulated, several important infla-mmation mediators were examined.

Proinflammatory cytokines such as Il-6 and TNF-α are over produced in inflammatory conditions. In peripheral blood mononuclear cells, LPS induces transcription of several inflammatory mediators. Furthermore, LPS stimulation is well known to induce I*k*B proteolysis and NF-*k*B nuclear translocation (Freeman *et al.*, 2000). TNF-*α* elicits a number of physiological effects including septic shock, inflammation, cachexia and cytotoxicity (Aggarwal *et al.,* 1996). Moreover, a large body of evidence suggests that prostaglandins are involved in various pathophysiological processes, including inflammation and carcinogenesis, and inducible isoform of COX-2 is mainly responsible for the production of large amounts of these mediators (Simon, 1999). Based on this information, efforts have been made to reveal the anti-inflammatory activities of Omega 3 fatty acids concentrate on LPS-induced IL-6, TNF-*α* and COX-2 production in human peripheral blood mononuclear cells. In the present study, we showed that omega 3 fatty acids concentrate from marine micro algae *Dunaliella salina* clearly reduced the gene level expression of COX-2 in hPBMCs. Although the treatment with omega 3 fatty acids concentrate on LPS treated monocytes shows inhibition of IL-6 and TNF-k production.

The transcription factor NF-kB plays a significant role in several signal transduction pathways involved in chronic inflammatory diseases and various cancers .Furthermore, LPS stimulation is well known to induce I*k*B proteolysis and NF-*k*B nuclear translocation (Freeman *et al.,* 2000). Therefore, agents that are inhibiting NF-*k*B transcriptional regulation and modulate the inflammatory response could have therapeutic use and have a chemo-protective value. Recently, there has been a growing interest among researchers in the targeting of the NF-*k*B signaling pathway for the fight against diseases (Aggarwal *et al.,* 2006). Many inflammatory genes including COX-2 are known to be under the control of NF-kB, So we checked whether omega 3 fatty acids concentrate inhibit the NF-*k*B activity (Matthew *et al*., 2004). As, expected omega 3 fatty acids concentrate inhibited the activity of NF-*k*B, a common denominator of inflammatory stimulations. NF-*k*B is associated and tightly controlled by an inhibitory subunit, I*k*B, which is present in the cytoplasm in an inactive form. However, I*k*B is phosphorylated, which targets its proteolysis and allows NF-*k*B translocation to the nucleus, where it activates the transcription of NF-*k*B-responsible genes .We found that the translocation of NF-*k*B to the nucleus was inhibited in LPS treated hPBMCs by omega 3 fatty acids concentrate . So far, our results shows that ù-3 concentrate from marine microalgae *Dunaliella salina* exerts a potent in vitro anti-inflammatory effect which could be mediated through down-regulation of NF-kB pathway. In the present study, we found that Omega 3 fatty acids concentrate could inhibit the NF- kB nucleus translocation induced by Lipopolysaccharide. In summary, our data indicated that the naturally occurring omega 3 concentrate from marine micro algae *Dunaliella salina* was a potential target compound for the treatment of inflammatory diseases.

CONCLUSIONS

In summary this study demonstrated that omega 3 fatty acids concentrate could inhibit LPS-induced COX-2 and pro inflammatory

cytokines over production and these effects might be mediated through inhibition of NF-kB translocation to the nucleus.

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REFERENCES

Aggarwal, B.B., and Natarajan, K. 1996. Tumor necrosis factors: developments during the last decade. *Eur. Cytokine Netw*., 7: 93–124.

Aggarwal,B.B., and Shishodia, S. 2004. Suppression of the nuclear factor-êB activation pathway by spice-derived phytochemicals: reasoning for seasoning. *Ann. N. Y. Acad. Sci*., 1030: 434–441

Alkharfy,K.M., Kellum, J.A., and Matzke, G.R. 2000. Unintended immunomod-ulation: part II. Effects of pharmacological agents on cytokine activity. *Shock*., 13(5): 346–360.

Arend, W.P., and Gabay, C. 2004. Cytokines in the rheumatic diseases. Rev. Rheum. *Dis. Clin. North Am*., 30: 41–67

Baeuerle, P.A., and Baltimore, D. 1996. NF-kB: ten years after. *Cell.*, 87: 13–20.

Bligh, and Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. Can *J. Biochem Physiol.*, 37(8): 911–917.

Calder, P.C. 2001. Polyunsaturated fatty acids: inflam-mation and immunity. *Lipids.,* 36: 1007–1024.

Calder, P.C. 2002. Dietary modification of inflammation with lipids. Proc. Nutr. Soc., 61 (3): 345–358.

Celec, P. 2004.Nuclear factor kappa B-molecular biomedicine: the next generation. *Biomed Pharmacother.*, 58: 365-371.

Cross., R.K., and Wilson, K.T. 2003. Nitric oxide in inflammatory bowel disease. *Inflamm Bowel Dis*., 9: 179–89.

Engvall,E. and Perlman, P. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G.*Immunochemistry.*, 8(9): 871-4.

Farag, M.A., Huhman, D.V., Lei, Z., and Sumner, L.W. 2007. Metabolic prouling and systematic identification of ûavonoids and isoûavonoids in roots and cell suspension cultures of Medicago truncatula using HPLC-UV-ESI-MS and GC-MS. *Phyto-chemistry*., 68: 342– 354.

Freeman, B.D., and Natanson, C. 2000. Anti-inflammatory therapies and sepsis and septic shock. Expert Opin. Invest. Drugs., 9: 1651–1663.

Griffiths, M.J. and Harrison, S.T.L. 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. Journal of Applied Phycology., 21(5): 493-507.

Guillard, R.R.L., and Ryther, J.H. 1962. Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt and Detonula confervacea Cleve. *Can. J. Microbiol.*, 8: 229-239.

Li, Q., and Verma, I.M. 2002. NF-kappaB regulation in the immune system. *Nat. Rev. Immunol*., 2:725–734.

Lowry, O.H., Rosebrough, N. J., Farr, A.L. and Randall, R. J. 1951. Protein measurement with the Folins phenol reagent. *J. Biol. Chem.,* 193:265–275.

Matthew, S. Hayden and Sankar Ghosh. 2004. Signaling to NF-kB Genes Dev. 18: 2195-2224

Radhika, A., Jacob, S.S. and Sudhakaran, P.R. 2007. Influence of oxidatively modified LDL on monocyte–macrophage differentiation, *Cell Mol. Biol.*, 305: 133–143.

Raja, R. and Hemaiswarya, S. 2007. "Exploitation of Dunaliella for beta-carotene production." Applied Microbiology and Biotechnology 74(3): 517-523

Sarada, S.S., and Himadri, P., Mishra, C., Geetali P, Sai Ram, M. and Ilavazhagan G. 2008. Role of oxidative stress and NF-B in hypoxia induced pulmonary edema. *Exp Biol Med.,* 233: 1088-1098.

Simon, L.S. 1999. Role of regulation of cyclooxygenase-2 during inflam-mation. *Am. J. Med.,* 106: 37S–42S.

Sladowski, D., Steer, S.J., Clothier, R.H. and Balls, M. 1993. An improved MTT assay. *J Immunol Methods*, 157: 203-207.

Soren, B.and Steven, C.L.E. 2004. Functions of NF-kB1 and NF-kB2 in immune cell biology. *Biochem J*., 382: 393–409

Westacott, C.I. and Sharif. M. 1996. Cytokines in osteoarthritis: mediators or markers of joint destruction? S*eminars in Arthritis and Rheumatism*, 25(4): 254–272.