



## Research Article

## ANTIOXIDANT AND ANTI-INFLAMMATORY EFFECTS OF A POLYBOTANICAL MULTINUTRIENT FORMULA

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### ABSTRACT

The novel polybotanical multinutrient formula (PMF) IonShield was analyzed for its antioxidant and anti-inflammatory properties. PMF is a blend of botanical extracts and nutrients designed for cellular protection from free radical damage and oxidative stress such as from environmental, technological, occupational or medical related radiation exposure. This formula was shown to possess significant dose-dependent antioxidant activity over a broad range of concentrations. PMF was also shown to inhibit lipopolysaccharide (LPS)-induced TNF- $\alpha$  production in RAW 264.7 macrophages with complete inhibition at 2000  $\mu\text{g/mL}$  and inhibition of  $\text{H}_2\text{O}_2$ -induced lipid peroxidation with about 50% reduction at 500  $\mu\text{g/mL}$ . Additionally, both NF- $\kappa\text{B}$  activation and COX-II activity are also inhibited by PMF with about 40% reduction in COX-II activity at 1000  $\mu\text{g/mL}$ . Demonstration of antioxidant and anti-inflammatory properties suggest potential applications of PMF as a dietary supplement for the amelioration of effects related to oxidative stress and free radical damage.

**KEY WORDS:** Antioxidant, anti-inflammatory, TNF- $\alpha$ , lipid peroxidation, NF- $\kappa\text{B}$ , cyclooxygenase-II

### INTRODUCTION

Historically in traditional medicines and folklore, botanicals have been used alone and in combinations, and recent research has attributed their bioactivity to being rich in phytochemicals or secondary metabolites that possess a broad range of biological effects including antioxidant and anti-inflammatory activity.[1] Although a well-balanced diet may provide an adequate combination of antioxidants and prevent disorders associated with oxidative stress, the dietary supplementation with botanical and polybotanical formulations with known antioxidant/anti-inflammatory effects may represent an important strategy for disease prevention.[2,3] The increased reliance and use of dietary supplements for general health and chronic medical

conditions are an acknowledgment of their perceived benefit in managing oxidative and other stress beyond that provided by a well-balanced diet alone. It is thus essential that the development of dietary supplements for such applications be firmly established with evidence-based methodologies.

Many botanicals are rich sources of antioxidant molecules and specifically plant-derived phenols and polyphenols have received increasing interest.[4] The novel polybotanical multinutrient formula (PMF) IonShield contains several botanicals extracts, known for their antioxidant protective effects, such as green tea (*Camellia sinensis*), holy basil leaf (*Ocimum sanctum*), ashwagandha (*Withania somnifera*), astragalus root (*Astragalus*

*membranaceus*), frankincense (*Boswellia serrata*), cat's claw bark (*Uncaria tomentosa*), blueberry (*Cyanococcus vaccinium*), vitamins and minerals. Green tea extract containing the polyphenol epigallocatechin-3-gallate (EGCG), exerts antioxidant effects, cancer chemoprevention, improves cardiovascular health, protects skin from damage caused by ionizing radiation, and regulates multiple disease-specific molecular targets.[5] Flavonoids, epigallocatechin, and other polyphenols found in green tea confer radioprotection.[6] Treatment of splenocytes with EGCG two hours prior to gamma radiation protected cellular DNA against radiation induced strand breaks, inhibited cell death, decreased lipid peroxidation and membrane damage, and restored levels of glutathione.[7] The phytochemicals eugenol, rosmarinic acid, apigenin, myrethenal, luteolin,  $\beta$ -sitosterol, and carnosic acid from holy basil extract have prevented chemical-induced skin, liver, oral, and lung cancers by increasing antioxidant activity, altering gene expression, inducing apoptosis, and inhibiting metastasis and angiogenesis.[11] Holy basil extracts protected mice against gamma radiation-induced sickness and mortality, selectively protected normal tissues against the effects of radiation, and prevented radiation-induced DNA damage, and protected lymphocyte cells exposed to genotoxic substances from induced DNA strand breaks.[8,9] Traditionally, ashwagandha extracts are used in a wide range of medical conditions because of its adaptogenic, diuretic, anti-inflammatory, sedative/anxiolytic, cytotoxic and immunomodulatory effects. Extensive pharmacological research has identified multiple mechanisms of action across key inflammatory pathways.[10] Astragalus has demonstrated significant protection against induced oxidative damage, lipid peroxidation, protein oxidation and reactive oxygen species (ROS), and enhanced mitochondrial function.[11] Boswellic acids, found in frankincense gum extract, affected the cellular defense system by inhibiting activation of nuclear factor-kappaB (NF-kappaB), and down-regulating tumor necrosis factor-alpha (TNF- $\alpha$ ), with downstream reduction of pro-inflammatory cytokines IL-1, IL-2, IL-4, IL-6 and IFN- $\gamma$ , as well as reduced ROS formation.[12] The mechanisms of Cat's Claw effects were shown to be inhibition of TNF- $\alpha$  and induction of antioxidant activity.[13] Repair of DNA single and double strand breaks 3 hours after whole body irradiation was significantly improved in animals treated with Cat's Claw.[14] Polyphenols, extraordinarily rich in blueberries induced anti-inflammatory action by modulating the balance of pro-inflammatory cytokines through the suppression IL-1 $\beta$ , IL-6 and IL-12 gene expression.[15] The polysaccharide components found in Cordyceps (*Cordyceps sinensis*), Reishi (*Ganoderma lucidum*), and Oyster Mushroom (*Pleurotus ostreatus*), account for their demonstrated anti-inflammatory and antioxidant properties.[16-18] Also vitamins such as C and E, the stilbene resveratrol and diverse flavonoids are increasingly used as dietary supplements or therapeutics.[19-21]

In the inflammatory cascade, the role of TNF- $\alpha$  and NF- $\kappa$ B is well documented.[22,23] TNF- $\alpha$  has been identified as a major mediator of inflammatory processes, one of the most dramatic being gram-negative endotoxic shock.[24] This cytokine mediates early-stage responses of inflammation

by regulating the production of other cytokines, including interleukin-1 (IL-1) and IL-6. Because TNF- $\alpha$  is the main mediator for a number of inflammatory toxic responses to chemicals, it represents a promising target for the prevention of inflammatory toxicity. TNF- $\alpha$  has also been reported to induce NF- $\kappa$ B production and this protein is inhibited by the presence of antioxidants.[25,26] NF- $\kappa$ B is present in the cytoplasm as an inactivated dimer composed of p65 and p50 subunits. In response to inflammatory stimuli, I- $\kappa$ B is phosphorylated and degraded, and NF $\kappa$ B is released and translocated into the nucleus.[27] Because the expression of many inflammatory genes, including iNOS, COX-2, and TNF- $\alpha$ , are known to be modulated by the binding of NF- $\kappa$ B to its specific promoter regions, it represents a good target for suppressing NF- $\kappa$ B activation for the regulation of lipopolysaccharide (LPS)-induced inflammation.[28] In the present investigation, we have analyzed the anti-inflammatory and antioxidant properties as well as the mechanism of action of PMF in the mouse macrophage cell line.

## MATERIALS AND METHODS

### Polybotanical Multinutrient Formula (PMF)

The dietary supplement (IonShield<sup>®</sup>, Clinical Synergy Professional Formulas, Santa Rosa, CA) consists of the following ingredients in four capsules servings: vitamin C 125 mg, vitamin E 23 IU, folic acid 200  $\mu$ g, vitamin B12 25  $\mu$ g, magnesium 41 mg, zinc 10 mg, selenium 35  $\mu$ g, Immune Support Blend 725 mg [*Cordyceps sinensis* mushroom, *Ganoderma lucidum* mushroom, Oyster mushroom (*Pleurotus ostreatus*), *Astragalus membranaceus* root extract, cat's claw bark extract (*Uncaria tomentosa*)], Antioxidant and Cellular Support Blend 620 mg [trimethylglycine (TMG), alpha lipoic acid, boswellia resin extract (*Boswellia serrata*), methylsulfonylmethane (MSM), N-acetyl cysteine (NAC), blueberry extract (*Vaccinium angustifolium*), green tea leaf extract (*Camellia sinensis*)], and Energy Support Blend 400 mg [ashwagandha root extract (*Withania somnifera*), taurine, acetyl-L-carnitine, holy basil leaf extract (*Ocimum sanctum*), mixed tocopherol/tocotrienol complex 22 mg, bioperine<sup>®</sup>[Black pepper (*Piper nigrum*) fruit extract] 1 mg. Stock and serial dilutions of PMF were prepared in sterile phosphate buffered saline.

### Cell Line and Culture

Mouse macrophage cell line RAW 264.7 (ATCC<sup>®</sup> TIB-71<sup>™</sup>) was obtained from American Type Culture Collection (Manassas, VA). These cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin in a 5% CO<sub>2</sub> incubator maintained at 37°C.

### Antioxidant Activity

The principle of the antioxidant assay is based on the formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which oxidizes the 2,2'-azino-

bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to produce a radical cation,  $ABTS^+$ , a soluble chromogen that is green in color, can be measured spectrophotometrically at 405 nm. Antioxidants suppress the production of the radical cation in a concentration dependent manner and the color intensity decreases proportionally. Trolox, a water soluble vitamin E analog, was used as a control antioxidant for analyzing the antioxidant activity of PMF. In a 96-well plate, the assay was set up with 10  $\mu$ l of increasing concentrations of PMF (0-500  $\mu$ g/mL) and 20  $\mu$ l of myoglobin working solution according to the protocol described in the Antioxidant assay kit (Sigma-Aldrich, St. Louis, MO). Afterward, 150  $\mu$ l of ABTS working solution containing 0.0075%  $H_2O_2$  was added and incubated at room temperature for 5 min. The reaction was stopped by adding 100  $\mu$ l stop solution and absorbance was measured at 405 nm in a Bio-Rad Benchtop microplate reader. The decrease in absorbance indicated the antioxidant activity of PMF equivalent to the Trolox standard which was plotted against PMF concentrations.[29]

#### Lipopolysaccharide-Induced TNF- $\alpha$ Synthesis

Mouse macrophages ( $0.5 \times 10^6$ /mL) were plated in 24-well plates and starved overnight by growing in minimal essential medium containing 0.5% fetal bovine serum and antibiotics. On the following day, the plates were replaced with fresh starving medium and treated with increasing concentrations of PMF in the presence and absence of LPS. PMF was added initially and after incubation for 2 h at 37°C, 20 ng/mL LPS was added to induce an inflammatory response. The plate was incubated for an additional 4 h and culture medium was collected, centrifuged and stored at -80°C. TNF- $\alpha$  produced and secreted into the medium by the cells was analyzed by ELISA protocol using the mouse TNF- $\alpha$  Quantakine ELISA kit (R&D systems, Minneapolis, MN) according to manufacturer's instructions.[30]

#### Lipid Peroxidation

RAW 264.7 mouse macrophages ( $3 \times 10^6$  cells/5 mL) were treated with increasing concentrations of PMF (0-2000  $\mu$ g/mL) at 37°C for 72 h before they were challenged with 20  $\mu$ M  $H_2O_2$  overnight. The cell lysate was prepared according to the lipid peroxidation assay protocol (Sigma-Aldrich, St. Louis, MO) and protein concentrations were determined. The cell lysate (200  $\mu$ l) was analyzed for inhibition of lipid peroxidation according to manufacturer's protocol. Lipid peroxidation is determined by the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a colorimetric (532 nm) product proportional to the MDA present. To form the MDA-TBA adduct, 600  $\mu$ l of TBA solution was added into the 200  $\mu$ l of lysate and incubated at 95°C for 1 h. The reaction mix was cooled in an ice bath for 10 min and absorbance recorded at 532 nm in a Beckman spectrophotometer. MDA standards (0-2 nmoles) reacted with TBA was used as the standard for the calculation of lipid peroxidation activity. The reaction mix attains pink color and decrease in absorbance indicated the inhibition of adduct formation. The percentage of inhibition was calculated based on untreated cells and plotted against PMF concentrations.[29]

#### Nitric Oxide Synthesis

RAW 264.7 macrophages ( $1 \times 10^6$  cells/mL) were seeded in starving phenol free minimum essential medium (MEM) containing 0.5% FBS and antibiotics overnight in 24-well plates. On the following day, the starving medium was replaced with fresh medium and cells were treated with increasing concentrations of PMF for 2 h followed by NO stimulation with LPS (20 ng/mL) for a total of 24 h. Supernatants were collected after centrifugation and used for analysis of nitrite and nitrate levels using the nitric oxide quantitation kit (Active Motif, Carlsbad, CA).[30]

#### NF- $\kappa$ B Activation

RAW 264.7 cells ( $3 \times 10^6$ /5 mL) were incubated with increasing concentrations of PMF for 48 h and nuclear proteins were extracted with TransAM NF- $\kappa$ B p65 activation ELISA kit (Active Motif, Carlsbad, CA) according to manufacturer's instructions. The protein concentration of the nuclear lysate was determined and lysate equivalent to 20  $\mu$ g protein was analyzed for NF- $\kappa$ B activation using the TransAM NF- $\kappa$ B p65 kit.<sup>24</sup> In the TransAM kit, the NF- $\kappa$ B consensus site (5'-GGGACTTTC-3') is immobilized on the ELISA plate and the active form of NF- $\kappa$ B contained in the nuclear extract will specifically bind to the nucleotide. The complex can be detected with NF- $\kappa$ B primary and secondary antibody reactions followed by substrate color reactions. The ELISA plates were read at 450 nm with a reference wavelength of 655 nm in a microplate reader. The decrease in the absorbance compared to untreated sample indicated the inhibition of NF- $\kappa$ B activation (%) which was plotted against PMF concentrations.<sup>31</sup>

#### Cyclooxygenase-II Activity

Cyclooxygenase (COX) activity assay kit from Abcam (Cambridge, MA) and COX-II enzyme from Sigma-Aldrich (St. Louis, MO) were used for analyzing the effect of PMF on COX-II activity. The assay kit uses a chemiluminescent substrate to detect the peroxidative activity of the COX- II enzyme. After inhibition with NSAIDs (Ibuprofen) or increasing concentrations of PMF, the residual activity of COX-II is measured by addition of a luminescent substrate and arachidonic acid. Light emission will start immediately and is directly proportional to the COX-II activity in the sample which is measured quickly by using a Veritas Luminometer (Turner Biosystems, Sunnyvale, CA) equipped with injectors for both substrate and arachidonic acid. The relative light units (RLU) recorded by the luminometer were used to calculate the percent inhibition of COX-II activity by PMF according to the formula: Percent inhibition (1-Average Net inhibitor RLU/Average Net RLU for uninhibited) x 100. The inhibition percentage was plotted against PMF concentrations.

#### Statistical Analysis

Mean and standard deviation estimates were calculated using Excel software. The data were analyzed statistically by 1-way ANOVA with Dunnett's multiple comparison



test using GraphPad Prism software (La Jolla, CA) and *p* values were used to determine the significant difference between untreated and PMF concentrations.

## RESULTS

### Antioxidant Activity of PMF

The results of antioxidant assays are presented in Fig. 1. PMF showed a dose-dependent elevation of antioxidant activity in the lower doses up to 50 µg/mL and a plateauing effect towards higher doses above 100 µg/mL. PMF at 50 µg/mL has an activity equivalent to 1 mM dose of Trolox (vitamin E analog) compared to little activity with no PMF in the reaction mix. The antioxidant activity is induced by PMF at all concentrations which remained almost similar from 100 to 500 µg/mL PMF doses.

### Inhibition of LPS-Induced TNF- $\alpha$ Production by PMF

The effect of PMF on LPS-induced TNF- $\alpha$  is shown in Fig. 2. LPS treatment of RAW 264.7 cells induced TNF- $\alpha$  significantly (875.35 pg/mL) and a dose-dependent inhibition was noticed with increasing concentrations of PMF. Even though PMF concentrations from 0-500 µg/mL failed to inhibit LP-induced TNF- $\alpha$  synthesis significantly, 1000 µg/mL concentration showed significant inhibition of TNF- $\alpha$  (90.86 pg/mL) and complete inhibition is noticed at >2000 µg/mL.

### Inhibition of Lipid Peroxidation

Lipid peroxidation is the degradation of lipid that occurs as a result of oxidative damage and is a useful marker for oxidative stress. Polyunsaturated lipids are susceptible to an oxidative attack, typically by reactive oxygen species, resulting in a well-defined chain reaction with the production of end products like malondialdehyde (MDA). PMF has significant inhibitory effect on H<sub>2</sub>O<sub>2</sub> induced lipid peroxidation (Fig. 3). The level of MDA formation reduced with increasing concentrations of PMF up to 500 µg/mL and remained plateau between 500 to 2000 µg/mL doses. PMF at 500 µg/mL has a 50% inhibitory effect. However, no significant difference in lipid peroxidation was evident between from 200 to 2000 µg/ml PMF concentrations.

### Effect of PMF on Nitric Oxide Synthesis

Data presented in Fig. 4 shows that PMF induced a small activation of nitric oxide at 2000 and 5000 µg/mL as measured by nitrite + nitrate radicals in the culture medium of PMF-treated cells. This is mainly because of the elevation of nitrate radicals and not nitrite radicals (data not presented).

### Inhibition of NF- $\kappa$ B Activation

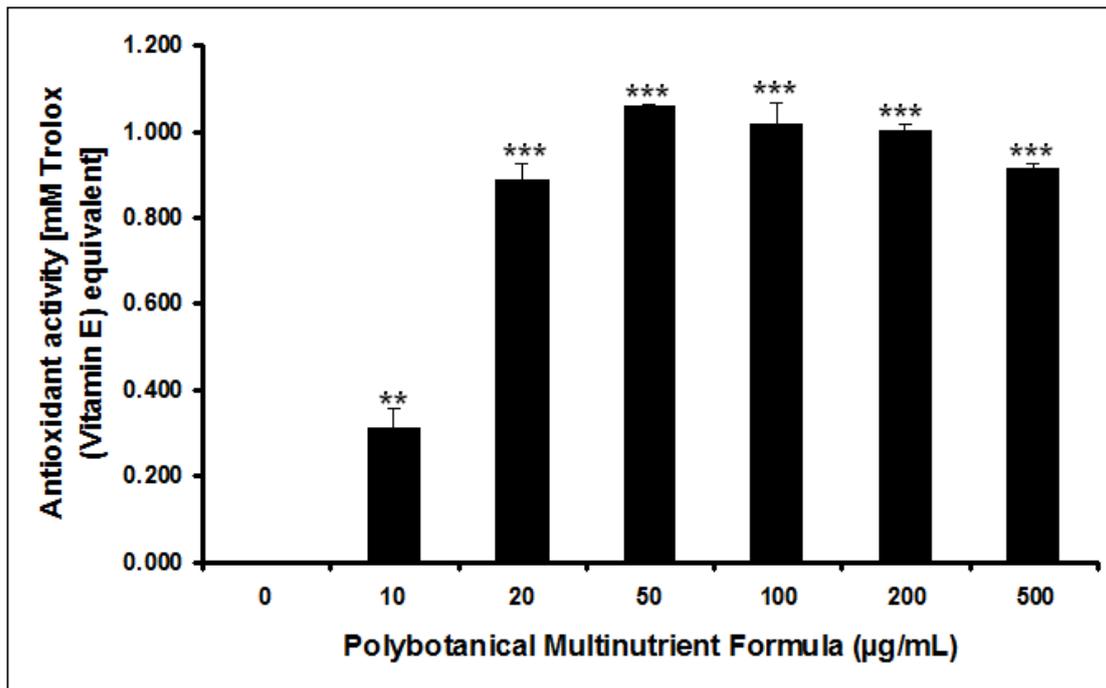
The transcription factor NF- $\kappa$ B is a key component of the inducible expression of a wide variety of cellular and viral genes that code for mediation of the immune and inflammatory response. Therefore, accurate monitoring of

NF- $\kappa$ B activation is crucial for drug development and signal transduction pathway studies. Treatment of RAW 264.7 cells with PMF showed a significant inhibitory effect on NF- $\kappa$ B activation (Fig. 5). The activity decreased to 40% at 1000 µg/mL PMF as compared to the untreated control. Even though, the inhibitory effect appeared to be dose-dependent up to 1000 µg/mL concentration, NF- $\kappa$ B activation (%) remained almost same from 500 to 2000 µg/ml PMF concentrations.

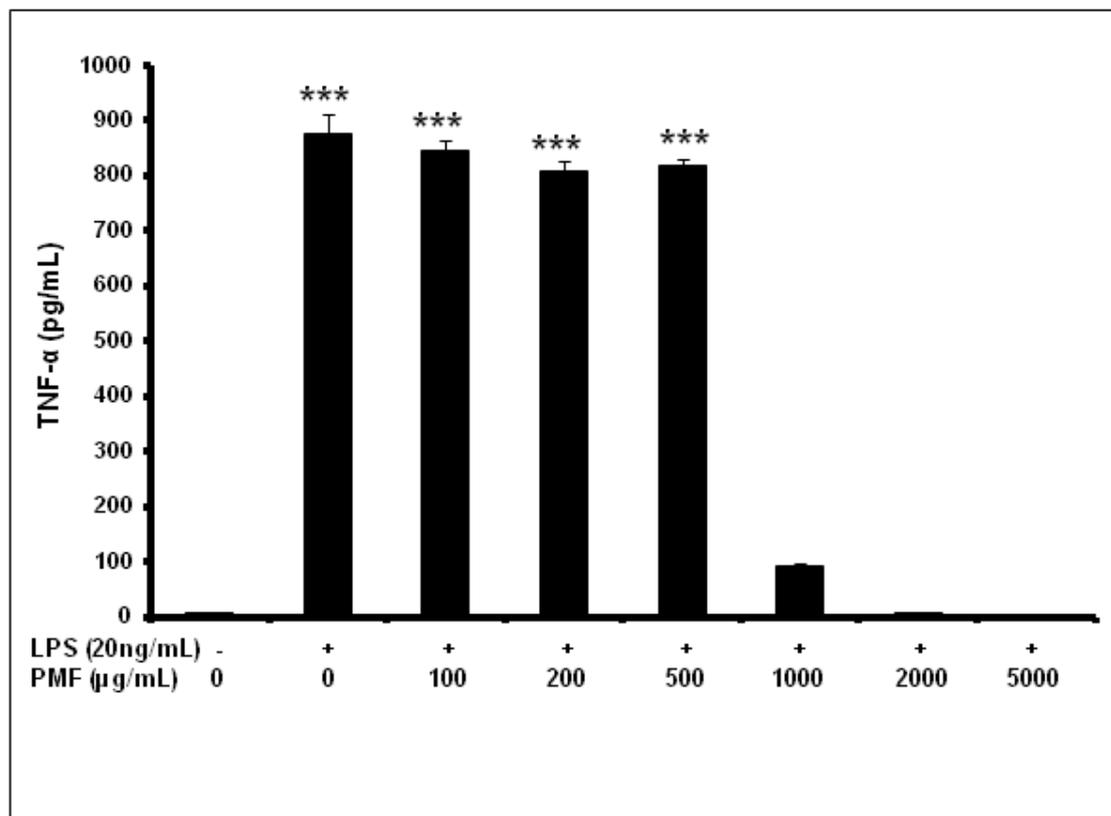
### Inhibition of Cyclooxygenase II activity

Inflammation mediators such as cytokines, growth factors, and endotoxin have been shown to induce COX-II activity. The effect of PMF on COX-II activity obtained by the cell-free assay is presented in Fig. 6. PMF has significant inhibitory effect on COX-II activity and >90% inhibition is noticed at 10 µg/mL of PMF. The inhibitory effect is more profound at lower concentrations up to 10 µg/mL and the rate of inhibition at higher doses is almost very similar and appeared to be plateaued.

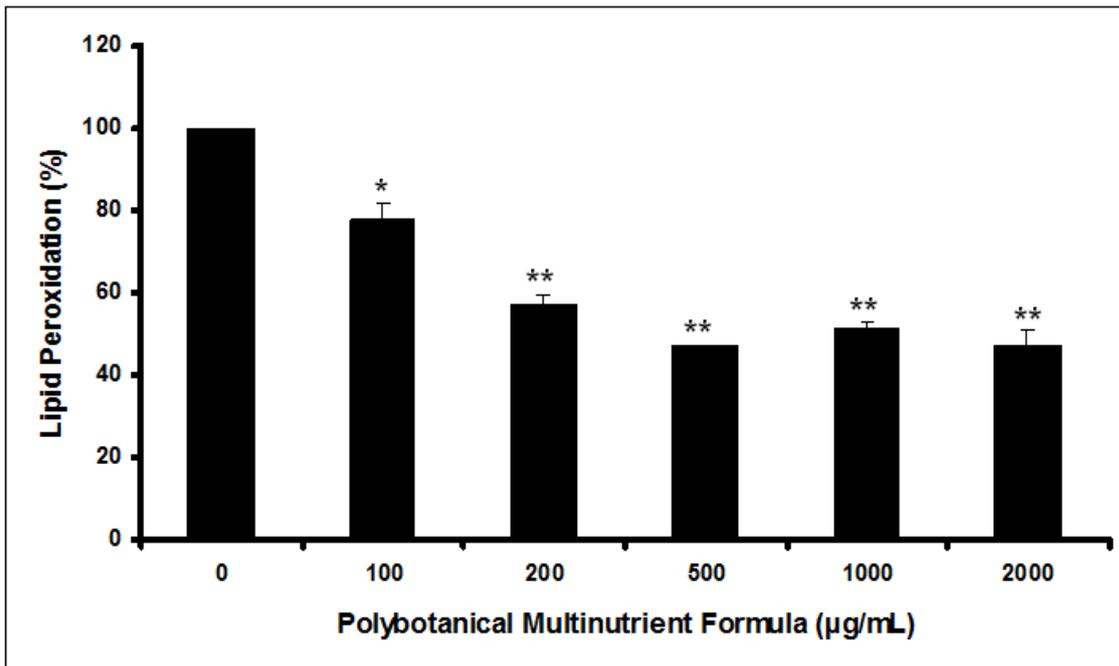
**Figure 1:** Antioxidant activity of polybotanical multinutrient formula. The activity equivalent to mM Trolox (Vitamin E analog) was analyzed using antioxidant assay kit and plotted against PMF concentrations (\*\*p < 0.01, \*\*\*p < 0.001 by ANOVA and compared with untreated control sample using Dunnett's multiple comparison test).



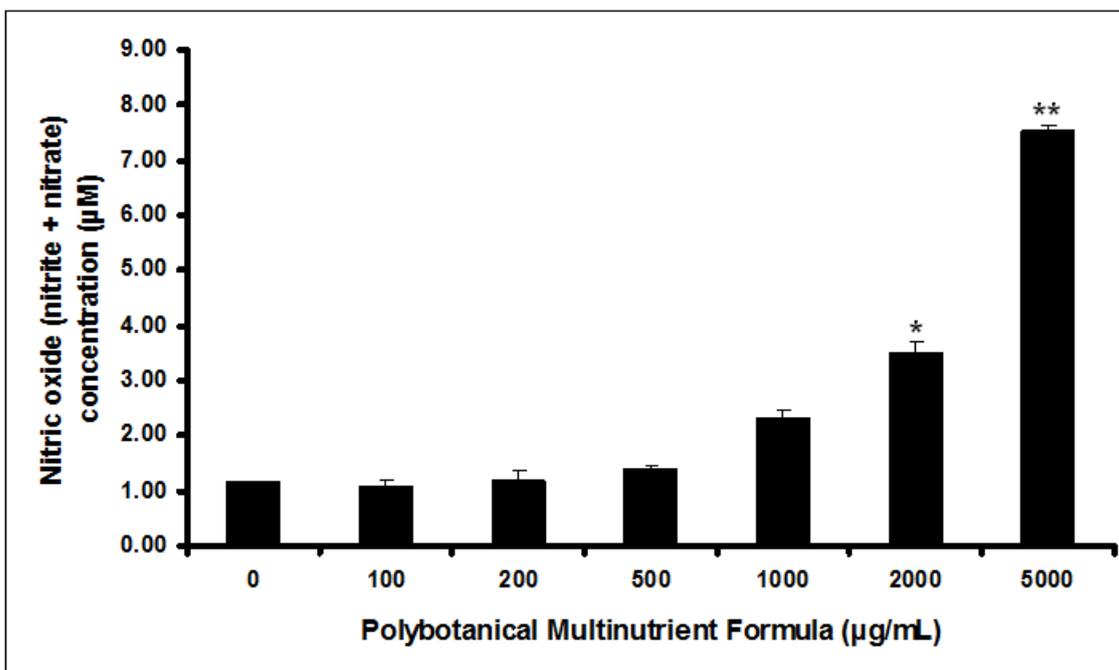
**Figure 2:** Inhibition of LPS-induced TNF- $\alpha$  (pg/ml) production by polybotanical multinutrient formula in RAW 264.7 mouse macrophage cell line. The cells were treated with polybotanical multinutrient formula and/or 20 ng/mL LPS in starvation medium and TNF- $\alpha$  analyzed by ELISA (\*\*p < 0.01, \*\*\*p < 0.001 by ANOVA and compared with untreated control sample using Dunnett's multiple comparison test).



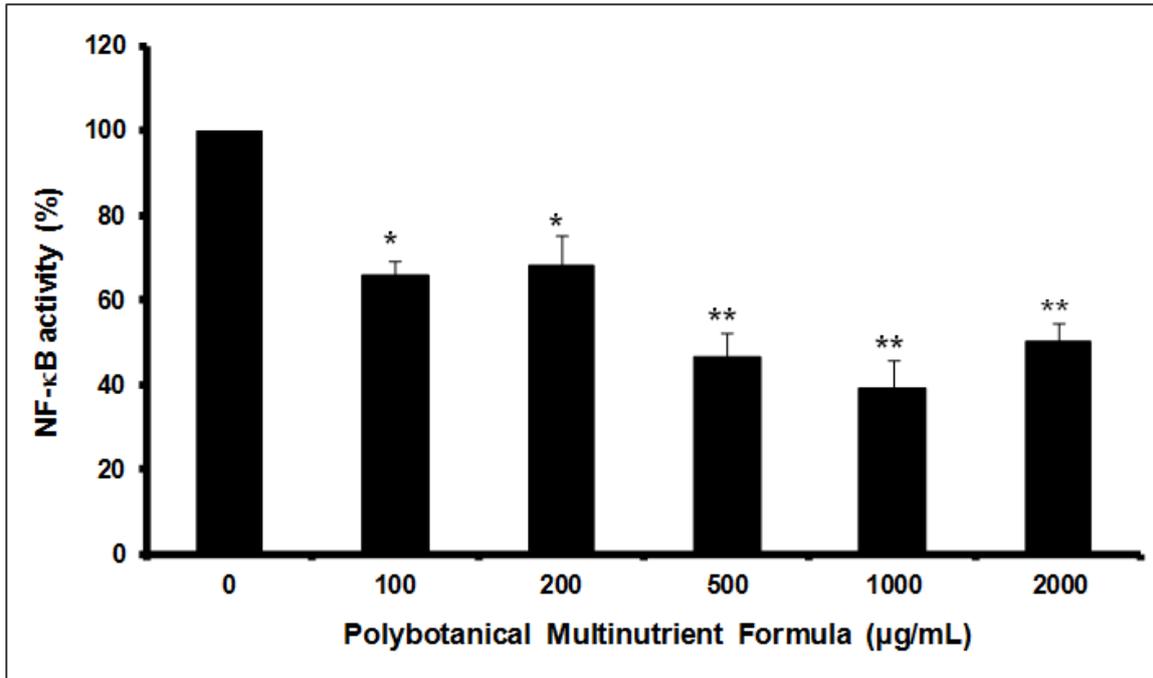
**Figure 3:** Inhibition of lipid peroxidation by polybotanical multinutrient formula in RAW 264.7 mouse macrophage cell line. Lipid peroxidation was significantly inhibited by PMF treatment of macrophages (\* $p < 0.05$ , \*\* $p < 0.01$  by ANOVA and compared with untreated control sample using Dunnett's multiple comparison test).



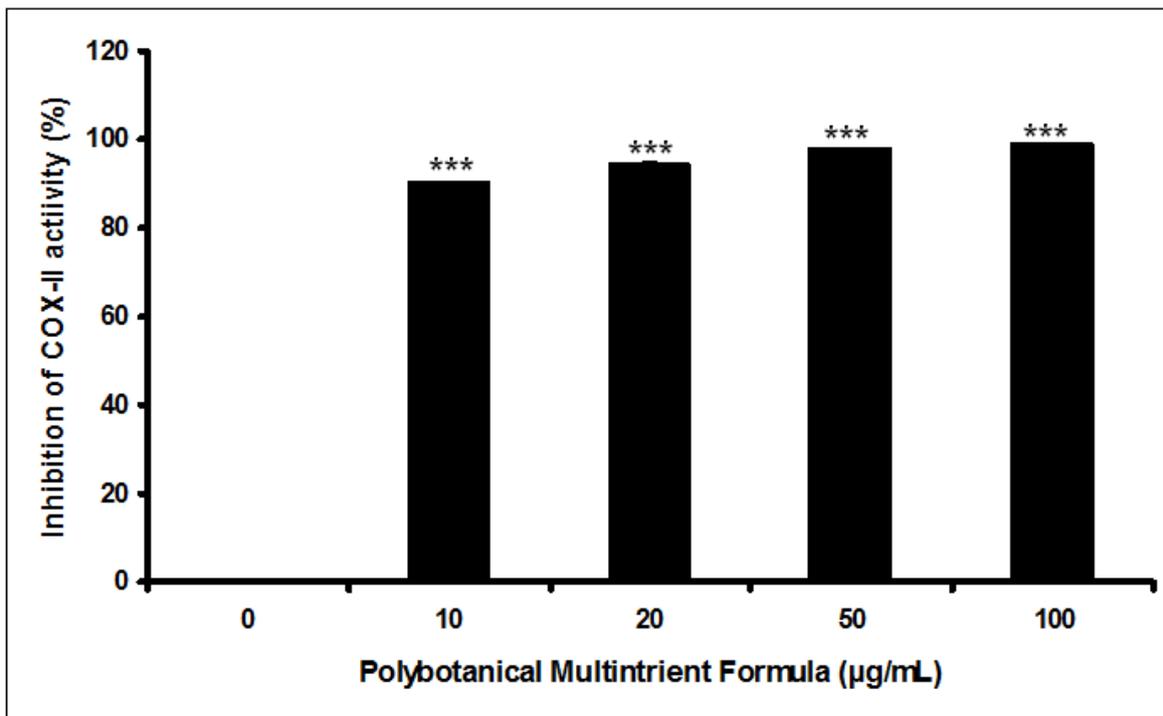
**Figure 4:** Effect of polybotanical multinutrient formula on nitric oxide synthesis in RAW 264.7 mouse macrophage cell line. Nitrate, and not nitrite levels, increased slightly with PMF treatment of macrophage cells (\* $p < 0.05$ , \*\* $p < 0.01$  by ANOVA and compared with untreated control using Dunnett's multiple comparison test).



**Figure 5:** Inhibition of NF-κB (p65) activation by polybotanical multinutrient formula in RAW264.7 macrophage cell line. NF-κB activation is inhibited significantly by polybotanical multinutrient formula treatment of macrophage cells (\*p < 0.05, \*\*p < 0.01 by ANOVA and compared with untreated control using Dunnett's multiple comparison test).



**Figure 6:** Inhibition of COX-II activity by polybotanical multinutrient formula. COX-II activity is very significantly inhibited by polybotanical multinutrient formula (\*\*\*p < 0.001 by ANOVA and compared with untreated control sample using Dunnett's multiple comparison test)



## DISCUSSION

PMF (IonShield®) was formulated by Clinical Synergy Professional Formulas, Santa Rosa, CA after extensive review of literature on each active ingredient for their medicinal properties in order to develop a dietary supplement that will prevent free radical damage and oxidative stress. The ingredients included a judicious blend of medicinal herbs, mushrooms, vitamins and minerals having immune support, energy support, antioxidative or cellular support properties. The medicinal herbs such as green tea (*Camellia sinensis*), holy basil leaf (*Ocimum sanctum*), aswagandha (*Withania somnifera*), astragalus root (*Astragalus membranaceus*), frankincense (*Boswellia serrata*), cat's claw bark (*Uncaria tomentosa*), blueberry (*Cyanococcus vaccinium*), and pepper fruit (*Piper nigrum*) were included into the formulation due to their reported chemopreventive, hepatoprotective, anti-inflammatory, anti-stress, antioxidant and immunomodulatory effects.[8,11-14,15-18,32,33] Furthermore, medicinal mushrooms such as Cordyceps (*Cordyceps sinensis*), Reishi (*Ganoderma lucidum*) Oyster mushroom (*Pleurotus ostreatus*), vitamins and minerals with demonstrated immunomodulatory, neuroprotective and antioxidant properties were also included in the PMF.[19-21,34-36]

Oxidative stress develops as a result of an imbalance between the production of reactive species and the body's ability to manage those using antioxidants.[37] Although small amounts of reactive oxygen species are essential for normal physiology and demonstrate a protective effect, overproduction can contribute to the immediate development of the inflammatory process.[38] Moreover, reactive oxygen species exert adverse effects by oxidizing biologically essential molecules and inducing the oxidative damage of cellular membranes, tissues, and enzymes. Oxidative stress is associated with pathologies such as cancer, aging, cardiovascular and neurodegenerative diseases. It has been suggested that a diet rich in antioxidants may be beneficial to human health and therefore, analysis of antioxidant properties of natural products is an important area of research. In the present investigation, PMF showed a significant dose-dependent antioxidant activity in the *in vitro* assay. In addition, PMF exhibited significant anti-lipid peroxidation activity in H<sub>2</sub>O<sub>2</sub>-treated mouse macrophages with about 50% inhibition at 500 µg/mL concentration. Since the formula contains multiple antioxidants including lipoic acid, blueberry, and green tea leaf extracts, such significant antioxidant effect can be expected. The antioxidant activity of nutraceuticals have been found to be highly indicative of the total phenolic content especially flavonoids. Furthermore, natural plant-derived antioxidants prevent reactive oxygen species (ROS) to initiate or speed up many conditions where inflammatory mediators are implicated.[39]

Inflammation is manifested by the activation of immunocytes such as monocytes and macrophages, and the secretion of inflammatory mediators such as nitric

oxide (NO), prostaglandin E2 (PGE2), and TNF- $\alpha$ . PGE2, which is derived from cyclooxygenase-II (COX-II) by inflammatory stimulants, is also thought to be involved in the pathogenesis of some inflammatory diseases.[40,41] Additionally, TNF- $\alpha$  is highly expressed in macrophages involved in the inflammatory process and causes severe tissue damage, septic shock, atherosclerosis, and systemic inflammatory response syndrome.[42,43] Therefore, the inhibition of these inflammatory mediators would be an effective therapeutic approach for regulating LPS-induced septic shock. In the present investigation, PMF treatment of macrophages has inhibited the LPS-induced TNF- $\alpha$  synthesis and NF- $\kappa$ B activation in macrophages. Moreover, PMF showed a significant inhibitory effect on COX-II activity directly. In a cell-free assay, PMF inhibited COX-II activity by 90% at a lower dose of 10 µg/mL. PMF almost nullifies the LPS-induced TNF- $\alpha$  completely at 2000 µg/mL concentration. Similarly, NF- $\kappa$ B activation is inhibited 50% by PMF at 1000 µg/mL concentration. Since these biomarkers are directly connected with the inflammatory process, anti-inflammatory properties can be demonstrated with the PMF treatment.

NF- $\kappa$ B is primarily composed of proteins with molecular mass of 50 (p50) and 65 kDa (p65), and is retained in the cytoplasm by I- $\kappa$ B. From its unstimulated form, NF- $\kappa$ B is activated by a wide variety of inflammatory stimuli. Most of these agents induce the phosphorylation-dependent degradation of I- $\kappa$ B proteins, allowing activated NF- $\kappa$ B to translocate into the nucleus, where it regulates gene expression including those encoding pro-inflammatory cytokines.[44] NF- $\kappa$ B comprises a family of inducible transcription factors that serve as important regulators of the host immune and inflammatory response. Activation of the NF- $\kappa$ B transcription family, *via* the nuclear translocation of cytoplasmic complexes, plays a central role in inflammation through its ability to induce transcription of pro-inflammatory genes such as TNF- $\alpha$ , IL-1, IL-6, and COX-II.[42] Activation of NF- $\kappa$ B by oxidative stress requires that NF- $\kappa$ B be released from its cytoplasmic retention by I- $\kappa$ B and inhibition of NF- $\kappa$ B by PMF would block the whole inflammatory cascade. It has been shown that several natural antioxidant compounds directly inhibit the expression of the NF- $\kappa$ B-dependent cytokines and COX-II, thus reducing inflammation[45] and the activation of the NF- $\kappa$ B complex is related to the cellular redox state.[46] Mushroom components, especially the polysaccharides in the PMF may also promote the synthesis and timing of anti-inflammatory cytokines such as IL-10 in addition to the inhibition of pro-inflammatory cytokines like TNF- $\alpha$ , thereby increasing the ratio of anti-versus pro-inflammatory cytokine production which will ultimately prevent inflammatory cascade. Therefore, further studies on those lines will be needed to address those issues.

In conclusion, this investigation has demonstrated the mechanism underlying the antioxidant and anti-inflammatory properties of PMF. The inhibition of NF- $\kappa$ B, TNF- $\alpha$ , and COX-II can occur at transcriptional, translational or post-translational levels. The signaling

pathways associated these genes can also be impacted by the formula. Therefore, further studies on these lines may be necessary to address these pathways, which may contribute more to the understanding of the PMF action. The demonstration of antioxidant and anti-inflammatory properties suggest potential applications of this novel dietary supplement for the amelioration of effects related to oxidative stress and free radical damage.

#### ACKNOWLEDGEMENTS

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#### CONFLICT OF INTEREST

The author BJW discloses employment at a dietary supplement company. IE discloses ownership of a dietary supplement company. CR and SJM disclose not having any conflict of interest in the research presented.

#### ETHICAL APPROVAL

This investigation did not involve human subjects or animal species and therefore, approvals from the institutional committees were not required. The investigation was performed adhering to the rules and regulations.

#### AUTHOR CONTRIBUTIONS

CR, BJW, SJM and IE were responsible for study design, experimentation and manuscript preparation. BJW and IE formulated polybotanical multinutrient formula for this investigation.

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