Inflammation is a normal physiological process; however, dysregulation of this process may contribute to inflammatory-based chronic disorders and diseases in animals and humans. Therefore, the antioxidant and anti-inflammatory properties of natural products, often recognized in traditional medicine systems, represent therapeutic modalities to reduce or prevent uncontrolled inflammatory processes which in turn potentially ameliorate or prevent sequelae of inflammatory-based symptoms of chronic diseases. We have investigated the antioxidant and anti-inflammatory effects of honokiol (HNK) and modified citrus pectin (MCP) in vitro and examined whether the MCP:HNK combination has synergistic effects on antioxidant and anti-inflammatory properties. Although both HNK and MCP induced a dose-dependent increase in antioxidant activity, the latter has a consistently higher antioxidant effect. The MCP:HNK (9:1) combination induced a synergistic effect on antioxidant activity suggesting that the combination is significantly more efficacious than individual compounds. In mouse monocytes, the lipopolysaccharide-(LPS-)induced tumor necrosis-factor-α (TNF-α) synthesis was significantly inhibited by HNK and the MCP:HNK combination in a dose-dependent manner and synergistic effects were clearly demonstrated with the combination on TNF-α inhibition. This combination effect was also evident on inhibition of nuclear factor-kappa B activity, cyclooxygenase-II activity, and lipid peroxidation in mouse monocytes. Further research into the combination is warranted.

1. Introduction

Dysregulated inflammation is often implicated as a pathophysiological phenomenon underlying many chronic diseases in humans and animals. Biomarkers associated with the responses are those that are often involved in the mediation of inflammation: proinflammatory cytokines, nitric oxide, and lipid mediators including cyclooxygenase enzymes and NF-κB factors produced by inflammatory cells [1, 2]. Inflammation is in part characterized by the activation of the subsets of the innate immune system, such as monocytes and macrophages, and the secretion of inflammatory mediators like tumor necrosis factor-α (TNF-α), prostaglandin E2 (derived from cyclooxygenase-II), and nitric oxide [3]. Plant-derived natural products with antioxidant and anti-inflammatory properties are thus potentially beneficial for prevention and treatment of inflammation-associated chronic diseases.

Honokiol (HNK) is a biphenolic neolignan from magnolia bark (Magnolia officinalis) possessing multiple biological activities including antioxidant, anti-inflammatory, anti-inflammatory anxiolytic, antidepressant, and neuroprotective properties [4–7]. It is widely used in Traditional Chinese Medicine and has already been shown in preclinical studies to be an effective multifunctional antioxidant, used for a wide variety of conditions including dermatological disorders [8], cancer prevention and therapeutics [4], neuromodulation [9], and cardiovascular conditions [10]. HNK at high concentrations is also reported to have anticancer and antiangiogenic properties whereas, at low doses (<10 μM), it is a potent scavenger of super oxide and peroxyl radicals [11]. It contains two phenolic groups which can exhibit antioxidant properties
like vitamin E. HNK inhibits freed radical-induced lipid peroxidation [12] and prevents oxidative modification of low-density lipoprotein (LDL), reducing the oxLDL-induced cytotoxicity, apoptotic features, and expression of adhesion molecules in endothelial cells [13].

Modified citrus pectin (MCP) is a well-investigated compound characterized from citrus peels that is recommended as a therapeutic agent for immune support [14], cancer [15–21], heavy metal toxicity [22–24], and fibrotic diseases [25–33]. The mechanism of action of MCP in inflammation, fibrosis, and cancer progression is that it works as a competitive inhibitor of extracellular galectin-3 (Gal-3). MCP blocks Gal-3 activity that drives fibrosis by reducing macrophage activity, proinflammatory cytokine expression, and apoptosis, as well as lowering inflammatory markers, thereby reducing tissue fibrosis. At elevated levels in the circulation, Gal-3 is involved in myofibroblast proliferation, inflammation and fibrogenesis, tissue repair, and ventricular and tissue remodeling [34, 35]. An elevated blood level of Gal-3 is associated with higher risk of death in acute decompensated heart failure and chronic heart failure patients [36] and also associated with the progression of autoimmune disease in studies of rheumatoid arthritis [37].

In the inflammatory cascade, the role of TNF-α and NF-κB activity is well understood [1, 38]. The cytokine, TNF-α, mediates early-stage responses of inflammation by regulating the production of other cytokines, including interleukin-1 (IL-1) and IL-6. Because TNF-α is the main mediator of several inflammatory toxic responses to chemicals, it represents a promising target for the prevention of uncontrolled inflammation. TNF-α has also been reported to induce NF-κB production and this protein is inhibited by the presence of antioxidants [39, 40]. Because the expression of many inflammation-associated genes, including iNOS, COX-2, and TNF-α, are known to be modulated by the binding of NF-κB to its specific promoter regions, it represents a good target for suppressing NF-κB activity for the regulation of lipopolysaccharide- (LPS-) induced inflammation [41]. In the present investigation, we have analyzed the anti-inflammatory activity of HNK, MCP, and MCP:HNK (9:1). In a 96-well plate, the assay was set up with 10 μl of increasing concentrations of compounds (0–500 μg/ml) and 20 μl of myoglobin working solution per the protocol described in Antioxidant Assay Kit (Sigma-Aldrich, St. Louis, MO). Afterward, 150 μl of ABTS working solution containing 0.0075% H₂O₂ was added and incubated at room temperature for 5 min. The reaction was stopped by adding 100 μl 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 HNK, MCP, and MCP:HNK (9:1) equivalent to the Trolox standard, which was plotted against concentrations of compounds [42].

2.3. Lipopolysaccharide-Induced TNF-α Synthesis. Mouse monocytes (0.5 × 10⁶/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 HNK, MCP, and MCP:HNK (9:1) in the presence and absence of LPS. The compound 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-α produced and secreted into the medium by the cells was analyzed by ELISA protocol using the mouse TNF-α Quantikine ELISA kit (R&D systems, Minneapolis, MN) as per manufacturer’s instructions [43].

2.4. Lipid Peroxidation. RAW 264.7 mouse monocytes (3 × 10⁶ cells/5 ml) were treated with increasing concentrations of MCP, HNK, and MCP:HNK (9:1) (0–2000 μg/ml) at 37°C for 72 h before they were challenged with 20 μM H₂O₂ overnight. The cell lysate was prepared per the Lipid Peroxidation Assay protocol (Sigma-Aldrich, St. Louis, MO) and protein concentrations were determined. The cell lysate (200 μl) was analyzed for inhibition of lipid peroxidation per manufacturer’s protocol. Lipid peroxidation is determined by the reaction of malondialdehyde (MDA) with thiorbarbituric acid (TBA) to form a calorimetric (532 nm) product dissolved is calculated based on the weight of undissolved residue separated by centrifugation. The solubility of MCP and HNK in PBS was 76.4% and 100%, respectively. The volume was adjusted to get accurate amount of the compound for treatment based on solubility factor.
proportional to the MDA present. To form the MDA-TBA adduct 600 μl of TBA solution was added to the 200 μl 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 were used as a 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 concentrations of compounds [42].

2.5. Nitric Oxide Synthesis. RAW 264.7 monocytes (1 × 10⁶ 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 compounds 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) [43].

2.6. NF-κB Activation. RAW 264.7 cells (3 × 10⁶/5 ml) were incubated with increasing concentrations of HNK, MCP, and MCP: HNK (9:1) for 48 h and nuclear proteins were extracted with TransAM NF-κB p65 activity ELISA kit (Active Motif, Carlsbad, CA) per manufacturer’s instructions. Protein concentration of the nuclear lysate was determined and lysate equivalent to 20 μg protein was analyzed for NF-κB activity using the TransAM NF-κB p65 activity kit. In the TransAM kit, the NF-κB consensus site (5’-GGGACTTTCC-3’) is immobilized on the ELISA plate and the active form of NF-κB contained in the nuclear extract will specifically bind to the nucleotide. The complex can be detected with NF-κB 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-κB activity (%) which was plotted against concentrations of compounds [44].

2.7. 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 MCH, HNK, and MCH: HNK (9:1) on COX-II activity. The assay kit uses a chemiluminescent substrate to detect the peroxidative activity of the COX-II enzyme. Ibuprofen (nonsteroidal anti-inflammatory drug, NSAID) is used as a positive control in the assay. After inhibition with increasing concentrations of MCP, HNK, or MCP: HNK (9:1), 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 MCH, HNK, or MCH: HNK (9:1) according to the following formula: percent inhibition (1 – average net inhibitor RLU/average net RLU for uninhibited) × 100. The inhibition percentage was plotted against PMF concentrations of compounds.

2.8. CompuSyn Analysis. To determine the synergistic/additive/antagonistic effects between MCP and HNK, data on various biochemical parameters were analyzed further using CompuSyn software (CompuSyn Inc., Paramus, NJ). This program is based on Chou and Talalay’s multiple drug effect equations [45] and it defines synergism as a more- than-expected additive effect and antagonism as a less-than-expected additive effect. The combination index (CI) was calculated by the Chou-Talalay equations for multiple drug effect equations [45] and it defines synergism as a more-than-expected additive effect and antagonism as a less-than-expected additive effect. The combination index (CI) was calculated by the Chou-Talalay equations for multiple drug effects, which consider both potency (inhibitory concentration values) and shape (slope, m) of the dose-effect curve [45, 46].

2.9. Statistical Analysis. Mean and standard deviation estimates were calculated using Excel software. The data were analyzed statistically by paired t test (GraphPad Prism software, La Jolla, CA) and p values were used to determine the significant difference between treatment groups.

3. Results

3.1. Antioxidant Activity. The results of antioxidant assay presented in Figure 1 showed that both HNK and MCP induced a dose-dependent increase in the antioxidant activity, the latter showing a consistently higher response than the former. MCP: HNK (9:1) mix showed significantly higher
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Figure 2: Inhibition of LPS-induced TNF-α (pg/ml) production by HNK, MCP, and MCP:HNK (9:1) in RAW 264.7 mouse monocyte cell line. The cells were treated with compounds and/or LPS in starvation medium and TNF-α analyzed by ELISA. Inhibition curves were analyzed by paired t test; *p < 0.05 for HNK versus MCP, and HNK versus MCP:HNK (9:1); S, synergism between MCP and HNK.

![Figure 2](image)

antioxidant activity than the single agents alone, especially at 200 and 500 µg/ml concentrations.

3.2. Inhibition of LPS-Induced TNF-α Production. When RAW 264.7 mouse monocyte cells were treated with LPS (20 ng/ml), proinflammatory cytokine TNF-α is induced (875.35 pg/ml) and secreted into the medium. Treatment of mouse monocytes with increasing concentrations of HNK inhibited the LPS-induced TNF-α synthesis significantly in a dose-dependent manner (Figure 2). As compared to HNK, MCP showed essentially no inhibitory effect on LPS-induced TNF-α synthesis by monocytes. However, the MCP:HNK (9:1) combination showed significantly better inhibitory effect than HNK alone. The highest dose of MCP:HNK (9:1) (5000 µg/ml) has almost completely inhibited the LPS-induced TNF-α synthesis in monocytes.

3.3. Inhibition of Lipid Peroxidation. The effect of HNK, MCP, and MCP:HNK (9:1) on H2O2-induced lipid peroxidation is shown in Figure 3. HNK has significant inhibitory effect on lipid peroxidation with 58% inhibition at 500 µg/ml concentration. Higher concentrations of HNK beyond 500 µg/ml dose do not inhibit the lipid peroxidation further. MCP and MCP:HNK (9:1) mix inhibit lipid peroxidation slightly with 20% reduction at 500 µg/ml dose. MCP demonstrated a steady inhibition of lipid peroxidation up to 200 µg/ml dose which remained stable with increase in dosage.

3.4. Inhibition of NF-κB Activity. The results of NF-κB activity assay presented in Figure 4 showed that HNK treatment of RAW 264.7 mouse monocytes has no significant inhibitory effect on NF-κB activity. However, MCP inhibited the NF-κB activity significantly with about 35% and 40% inhibition at 500 µg/ml and 2000 µg/ml doses, respectively. On the other hand, MCP:HNK (9:1) combination demonstrated the highest inhibitory effect at every concentration, with about 60% inhibition at 500 µg/ml and 2000 µg/ml doses.

3.5. Inhibition of COX-II Activity. HNK, MCP, and MCP:HNK (9:1) inhibit COX-II activity (Figure 5). The inhibition level plateaued between 10 and 100 µg/ml and then further increased at a concentration >200 µg/ml in all treatment groups. MCP alone demonstrates greater inhi-
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3.7. Synergism between MCP and HNK. The combination index (CI) estimates demonstrated synergism between MCP and HNK for inhibition of NF-κB activity whereas HNK alone showed little inhibition of NF-κB activity. Furthermore, by using CompuSyn analysis we showed that the combination of MCP and HNK has synergistic effect on the antioxidant activity of monocytes. The CI values of the MCP : HNK (9 : 1) combination for antioxidant activity are < 1 at ED<sub>50</sub>, ED<sub>75</sub>, or ED<sub>90</sub> levels, thereby indicating the strong synergism between MCP and HNK for antioxidant activity. Such synergistic effects for antioxidant activity have been observed for combinations of extracts from spices (anise, cardamom, clove, and cinnamon) [51] and also between black tea and curcumin [52], which may form the basis for a therapeutic that has superior effects compared to single agents.

4. Discussion

Overproduction of inflammatory mediators such as NF-κB, TNF-α, COX-II, and NO produced by macrophages, neutrophils, and other immune cells is very much involved in the pathogenesis of chronic diseases, for example, atherosclerosis, arthritis, type 2 diabetes, and cancer [47–49]. Therefore, identification of natural product-derived extracts or compounds that controls the production of inflammatory mediators is an extremely attractive therapeutic or preventative modality for these diseases [50]. In the present investigation, both HNK and MCP treatment have enhanced the antioxidant activity in monocytes. Furthermore, we have shown that both HNK and MCP treatment have enhanced the antioxidative modalities for these diseases [50].

The dysregulation of the redox system is implicated in numerous human disorders including neoplasia, inflammation, degenerative diseases, and environmental exposures [53, 54]. HNK has been shown to reduce intracellular superoxide 2-fold and therefore free radical scavenging activity is implicated as an important attribute of HNK [11]. Further, HNK inhibits copper-induced oxidative modification of LDL [12] and iron-stimulated lipid peroxidation [13]. It is also known that oxygen overproduction stimulates lipid peroxidation and the present investigation showed that HNK inhibits lipid peroxidation which may involve both initiation and propagation of peroxidation process [12].

NF-κB regulates the transcription of several genes, including iNOS, COX-II, TNF-α, and IL-6 and is thus important for the development of inflammation-associated diseases [1, 50, 55]. NF-κB is primarily composed of proteins with molecular mass of 50 (p50) and 65kDa (p65) and is retained in the cytoplasm by inhibitor of κB (I-κB). From its unstimulated form, NF-κB is activated by a wide variety of inflammatory stimuli like LPS. Most of these inflammatory mediators induce the phosphorylation-dependent degradation of I-κB proteins, allowing active NF-κB to translocate into the nucleus, where it regulates several genes including iNOS, COX-II, TNF-α, and IL-6 [55].

In the present investigation, we have shown that both MCP and MCP : HNK (9 : 1) combination have significant inhibitory effect on NF-κB activity whereas HNK alone showed little inhibition of NF-κB activity. Furthermore, we have shown that both MCP and MCP : HNK (9 : 1) combination have significant inhibitory effect on NF-κB activity whereas HNK alone showed little inhibition of NF-κB activity. Furthermore, we have shown that both MCP and MCP : HNK (9 : 1) combination have significant inhibitory effect on NF-κB activity whereas HNK alone showed little inhibition of NF-κB activity. Furthermore, very strong synergism between MCP and HNK for inhibition of NF-κB activity and also the DRI values (Table 1) may facilitate the reduction of MCP and HNK amounts in the combination to achieve the synergistic effect.
Figure 6: Effect of HNK, MCP, and MCP: HNK (9:1) on nitric oxide synthesis in RAW 264.7 mouse monocyte cell line. (a) Effect on nitrite concentration in cells treated with increasing concentrations of compounds. No significant difference was noticed among HNK, MCP, and MCP: HNK (9:1) treated monocytes. (b) Effect on nitrate concentration in cells treated with increasing concentrations of HNK, MCP, and MCP: HNK (9:1). Treatment groups were compared using paired $t$ test; $^\ast p< 0.05$ for HNK versus MCP and HNK versus MCP: HNK (9:1). No significant difference between MCP and MCP: HNK (9:1) treatment groups. (c) Effect on nitrite + nitrate concentration in cells treated with increasing concentrations of HNK, MCP, and MCP: HNK (9:1). Treatment groups were compared using paired $t$ test; $^\ast p< 0.05$ for HNK versus MCP and HNK versus MCP: HNK (9:1). No significant difference is noticed between MCP and MCP: HNK (9:1).

Table 1: Dose-effect relationship between modified citrus pectin (MCP) and honokiol (HNK).

| Biochemical traits                              | CI at ED$_{50}$ | CI at ED$_{75}$ | CI at ED$_{90}$ | DRI-MCP ED$_{50}$ | DRI-HNK ED$_{50}$ |
|------------------------------------------------|-----------------|-----------------|-----------------|-------------------|-------------------|
| Inhibition of LPS-induced TNF-$\alpha$          | 0.549           | 0.515           | 0.510           | 1.825             | 706.268           |
| Antioxidant activity                            | 0.670           | 0.546           | 0.446           | 33.682            | 1.562             |
| Inhibition of lipid peroxidation                | 1.070           | 1.765           | 1.189           | —                 | —                 |
| Nitric oxide synthesis ($\text{NO}_2^+\text{NO}_3^-$) | 2.932           | 4.675           | 5.684           | —                 | —                 |
| Inhibition of NFkB activity                     | 0.068           | 0.131           | 0.254           | 14.749            | 917.283           |
| Inhibition of COX-II activity                   | 2.248           | 3.080           | 1.870           | —                 | —                 |

CI, combination index; DRI, drug reduction index; ED, effective dose; CI values by CompuSyn analysis: $<0.1$: very strong synergism; $0.1–0.3$: strong synergism; $0.3–0.7$: synergism; $0.7–0.85$: moderate synergism; $0.85–0.90$: slight synergism; $0.90–1.10$: nearly additive; $1.10–1.20$: slight antagonism; $1.20–1.45$: moderate antagonism; $1.45–3.3$: antagonism; $3.3–10$: strong antagonism; $>10$: very strong antagonism.
defined therapeutic effect. The inhibition profile of TNF-α by these compounds also follows a similar pattern as NF-κB activity. HNK demonstrated the strongest inhibitory effect whereas MCP failed to show any inhibition of TNF-α unlike NF-κB. However, Compusyn analysis showed the synergism between MCP and HNK for TNF-α inhibition with CI values of <1 at ED_{50}, ED_{75}, and ED_{90} levels which were similar to NF-κB inhibition. Furthermore, all three compounds including the MCP: HNK (9:1) combination inhibited COX-II activity. The MCP: HNK combination does show an additive effect for inhibition of lipid peroxidation at ED_{50} level. In summary, MCP: HNK combination appears to be superior to individual agents due to the synergism for enhanced antioxidant activity, inhibition of NF-κB activity, and LPS-induced TNF-α synthesis.

5. Conclusion

The current investigation demonstrated the antioxidant and anti-inflammatory effects of MCP and HNK and the MCP: HNK in vitro. The MCP: HNK combination in general demonstrated significantly better effects than the single compounds due to the synergistic effects on antioxidant activity, inhibition of LPS-induced TNF-alpha production, and NF-κB activity. This demonstration of synergistic antioxidant and anti-inflammatory properties of MCP and HNK suggests a potential application for the amelioration of effects due to oxidative stress and free radical damage.

Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| ABTS | 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) |
| CI | Combination index |
| COX-II | Cyclooxygenase-II |
| DRI | Drug reduction index |
| ELISA | Enzyme linked immunosorbent assay |
| ED | Effective dose |
| Gal-3 | Galectin-3 |
| H_{2}O_{2} | Hydrogen peroxide |
| HNK | Honokiol |
| I-κB | Inhibitor of κB |
| IL-1 | Interleukin-1 |
| IL-6 | Interleukin-6 |
| iNOS | Inducible nitric oxide synthase |
| LDL | Low-density lipoprotein |
| LPS | Lipopolysaccharide |
| MCP | Modified citrus pectin |
| MDA | Malondialdehyde |
| MEM | Minimum essential medium |
| NF-κB | Nuclear factor-kappa B |
| NSAID | Nonsteroidal anti-inflammatory drug |
| PBS | Phosphate buffered saline |
| TBA | Thiobarbituric acid |

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.

Conflicts of Interest

The author Barry Wilk discloses employment at a dietary supplement company. Isaac Eliaz discloses ownership of a dietary supplement company. Cheppail Ramachandran and Steven J. Melnick do not have any conflicts of interest in the research presented.

Authors’ Contributions

Cheppail Ramachandran, Barry Wilk, Steven J. Melnick, and Isaac Eliaz were responsible for study design, experimentation, and manuscript preparation.

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