

The micronutrient combination with immune-enhancing effects

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Abstract

Infectious diseases have become a growing health problem worldwide. Thus, the search for novel non-toxic and effective approaches with immune system-enhancing effects, so it can resist and fight these infections is still sought after. It has been firmly established that both nutrition and the availability of specific micronutrients are important for achieving optimum immunity. In this study, we investigated the effects of natural compounds in enhancing the cellular response of immune cells to various challenges. The results showed that some vitamins, minerals, and plant extracts had significant enhancing effects on specific immune responses tested in this study. However, a combination of these natural compounds had superior efficacy compared to any of the individual substances in increasing phagocytic activity against specific bacteria and yeast. This micronutrient combination also increased the efficacy of NK cells in eliminating abnormal (cancer) cells as well as inhibiting the release of pro-inflammatory IL-6 by RAW macrophages. This composition of nutrients offers significant pleiotropic benefits in supporting the immune system function.

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Introduction

Optimum immunity involves the coordinated function of cells, tissues, and organs, as well as proteins and numerous biological factors to protect the body against microbial invasions and effectively search and destroy abnormal cells and foreign agents. Every stage of the immune response depends on the presence of various micronutrients, which interact in synergistic ways based on their complementary mode of action.

In this regard, vitamins, minerals, and many other natural components, including plant secondary metabolites, have been researched for their immune-enhancing or anti-microbial efficacy. The vast majority of these studies were conducted on individual agents and focused on their mechanisms of action. Since many of these compounds are available as nutritional supplements and are consumed not only as single nutrients but also combined with other natural products, it is important to know their overall effects when taken together. In general, the effectiveness of combined nutrients in supplement form is still not routinely subjected to efficacy testing and thus remains largely unknown.

Our scientific work has pioneered a new approach in the pleiotropic control of biological mechanisms involved in various pathologies such as cardiovascular disease, cancer as well as viral and bacterial infections, by channeling the complexity of micronutrient interactions towards defined specific metabolic targets.¹ The superior efficacy of such micronutrient combinations—called micronutrient synergies—has been documented in our numerous *in vivo*, *in vitro*, and clinical studies.¹

In this study we tested the efficacy of selected vitamins, minerals, and plant extracts, both individually and in combination, on several aspects relevant to healthy cellular immune response. These vitamins and minerals included:

Vitamin C is essential for general health including immune cell activity.² The body does not produce vitamin C on its own and the requirements for this

vitamin are affected by various health conditions and age. Vitamin C is essential to produce and repair collagen in epithelial tissues, which form the body's natural anti-infection barriers. Among many other functions, this antioxidant vitamin supports healthy cell growth, healthy circulation, and detoxifies the cells of the entire body.³ Vitamin C is also the most important blood antioxidant and high levels of vitamin C in neutrophils are necessary to counteract elevated oxidative stress.⁴ Although mean plasma vitamin C concentration typically oscillates around 50 $\mu\text{mol/L}$, neutrophils accumulate millimolar vitamin C concentrations against a concentration gradient, which indicates an important role for this vitamin during immune stimulation.⁵ Thus, the neutrophils' low vitamin C status during infectious episodes could potentially compromise their function.⁶ Meta-analyses have indicated that vitamin C intakes of at least 200 mg/day can decrease the risk of acquiring respiratory infections;^{7,8} however, due to increased demands for the vitamin during ongoing infection, intakes of gram doses of vitamin C are required.^{9,10}

Vitamin E decreases the production of prostaglandin E2 (which has immunosuppressive activity), and vitamin C modulates cytokine production and decreases histamine levels.^{4,2}

Zinc has been shown to be important for the structural and functional integrity of skin and mucosal cells.¹¹ Zinc together with vitamin C and iron may also play a role in the production of interferons (IFNs) involved in preventing viral replication.^{11,2} Zinc is also essential for the development, differentiation, and activation of T lymphocytes,^{4,12} while iron, copper, and selenium are important for their differentiation and proliferation.^{13,14} Also, it has been shown that zinc enhances the phagocytic activity of peritoneal macrophages for *Escherichia coli* and *Staphylococcus aureus*.¹⁵

Selenium is essential for the function of selenoproteins, which act as redox regulators and cellular antioxidants and are thus important for the function of leukocytes and NK cells.¹⁴ It has been shown that selenium

supplementation of adults (50 or 100 µg per day for 15 weeks) increases the production of IFN-γ, while vitamin A downregulates it.¹⁶

Some micronutrients have been also investigated for their combined effects, including:

Vitamins C and E, lipoic acid, and zinc and selenium are important cellular antioxidants and components of antioxidant enzymes and crucial for the effective protection of cells against oxidative damage.

Vitamins C and B12 facilitate the production of T cells, particularly cytotoxic T cells.^{17,14}

Vitamins B6 and B12, folate and ginger affect the composition of intestinal microbiota (i.e. the balance between commensal and pathogenic microorganisms) important for optimum immunity.^{18,19}

Vitamins B6, B12, C and E, folate, and zinc maintain or enhance NK cells' cytotoxic activity.^{13,4,20} These cells are activated if pathogens bypass the antimicrobial defenses, and they target and attack any host cells that display an abnormal or unusual protein pattern on the plasma membrane, killing these cells using cytotoxins.

Numerous plants display antimicrobial and antioxidant properties as a result of containing various phytochemicals such as flavonoids, alkaloids, tannins, and terpenoids synthesized in the secondary metabolism of the plant.^{21,22} These properties have been widely researched and applied in traditional medicine. Several phytotherapy manuals refer to various medicinal plants used in treating infectious diseases such as urinary tract infections, gastrointestinal disorders, respiratory disease, and skin infections.

In this study we evaluated the efficacy of several plant extracts both tested individually and combined with other micronutrients for their antimicrobial and immune-enhancing activities. The test compounds included:

Fucoidan which is a sulfated polysaccharide from brown

algae with a variety of immune-modulatory effects, including activation of various immune system cells and enhancement of antiviral and antitumor responses. It has been reported that fucoidan is involved in immune activities, such as those of macrophages, NK cells, and cytokines.^{23,24}

Lychee fruit extract is rich in a variety of antioxidants, including flavonoids and anthocyanins, such as gallic acid, chrysanthem, antirrhin, and oenin that may benefit the immune system. It is also rich in ascorbic acid, an essential vitamin for immune and other body functions. It has been investigated for its ability to reduce tumor cell viability in *in vitro* and *in vivo* models.²⁵

Aronia berries (chokeberries) native to North America have a total antioxidant potential more than three times that of blueberries and outscore blueberries for anthocyanins more than four times over.

Tart cherries are a rich source of polyphenols and vitamin C. Their antioxidant and anti-inflammatory properties have been demonstrated in various animal and cell culture studies as well as in human studies.²⁶

White mulberry fruits contain a plethora of phytonutrients that may exert immune-modulatory properties and they also support sugar and fat metabolism, which are important for overall well-being.

Ginger root has been used worldwide for thousands of years in folk medicine to various ends, including to promote healthy circulation and normal production of inflammatory markers, as well as to aid a healthy immune respiratory response. In addition, it has demonstrated effectiveness against human respiratory syncytial virus (HRSV)-induced plaque formation on airway epithelium by blocking viral attachment and internalization. It was also reported that it could stimulate mucosal cells to secrete IFN-β that may contribute to counteracting viral infection.²⁷ Its bioactive compounds may enhance immune system function through antioxidant and other properties.

Materials and methods

Test compounds. The following compounds, with a purity of 90%-98% according to the manufacturer, were obtained from Sigma (St. Louis, MO): vitamin C, vitamin B complex, selenium, fucoïdan, lipoic acid, and ginger extract. The compounds such as vitamin E, and zinc, with a purity of 90%-98% according to the manufacturer, were purchased from Powder City (York, PA).

Aronia melanocarpa extract, white mulberry extract, lychee fruit extract, and sour (tart) cherry fruit extract, with a purity of 97%-99% according to the manufacturer, were from Monterey Bay Spice (Watsonville, CA). All primary cells and cell lines were from ATCC (Manassas, VA). The list and concentrations of individual compounds tested in this study are listed in Table 1.

Table 1. Concentrations of individual compounds in the mixture applied in specific tests.

ID #	Test compound	Concentration (µg/mL)	Concentration (µg/mL)	Concentration (µg/mL)
1	Vitamin C	5	5	5
2	Vitamin B complex	5	5	5
3	Vitamin E	5	5	5
4	<i>Aronia melanocarpa</i>	20	5	5
5	Fucoïdan	5	15	5
6	Ginger root	5	5	5
7	Zinc	5	5	5
8	Selenium	5	5	5
9	Lipoic acid	5	5	5
10	White mulberry	5	5	5
11	Lychee fruit	5	10	5
12	Tart cherry fruit	5	5	20
	Total	75 µg/mL	75 µg/mL	75 µg/mL
	Test conducted	Phagocytosis (Fig.1)	NK cells (Fig.2)	IL-6 (Fig.3)

1. In vitro phagocytic assay (nitroblue tetrazolium (NBT) reduction test)

NBT reduction assay was carried out according to the method of Rainard et al.²⁸

A) RAW264.7 macrophages (5×10^6 cells/well of a 96-well plate) were cultured with different micronutrients and plant extracts (5-20 $\mu\text{g}/\text{mL}$) for 24 h at 37°C. Thereafter, 20 μl *Candida albicans* or *Streptococcus mutans* (5×10^7 cells/mL in PBS) suspension, and 20 μl nitroblue tetrazolium NBT (1.5 mg/ml in PBS) were added to each well. Wells that received PBS+DMSO were used as control. Next, cells were incubated for 3 h at 37°C, the supernatant was removed, and the adherent macrophages were rinsed with RPMI 1640. The cells were air dried before 80 μl of 2M KOH and 100 μl DMSO were added to each well. The absorbance was measured at 570 nm using the microplate reader. Percentage of NBT reduction (reflecting phagocytic activity) was calculated as the following equation: Phagocytosis = $[(\text{OD}_{\text{sample}} - \text{OD}_{\text{control}})/\text{OD}_{\text{control}}] \times 100$.

B) Human peripheral blood mononuclear cells PBMC (5×10^6 cells/well of a 96-well plate) were cultured with different micronutrients and plant extracts (5-20 $\mu\text{g}/\text{mL}$) for 24 h at 37°C. Thereafter, 20 μl *Candida albicans* or *Streptococcus mutans* (5×10^7 cells/ml in PBS) suspension, and 20 μl nitroblue tetrazolium (1.5 mg/mL of NBT in PBS) were added to each well. Wells that received PBS+DMSO were used as control. Next, cells were incubated for 3 h at 37°C and the cells received 50 μl of 2M KOH and 50 μl DMSO. The absorbance was measured at 570 nm using the ELISA plate reader. Percentage of NBT reduction (reflecting phagocytic activity) was calculated as the following equation: Phagocytosis = $[(\text{OD}_{\text{sample}} - \text{OD}_{\text{control}})/\text{OD}_{\text{control}}] \times 100$.

2. Cytotoxicity assay of Natural Killer (NK) cells

Splenic natural killer (as the effector cells) activity assay was carried out as described by Yi et al.²⁹

The splenocytes/splenic NK cells were plated into 96-

well plates at a density of 1×10^7 cells/mL per well in a 50 μl volume, and stimulated with different micronutrients and plant extracts (5-15 $\mu\text{g}/\text{mL}$) for 24 h at 37°C. Then, YAC-1 cells (1×10^6 cells/mL) were added into all experimental wells (group) and complete medium only was placed in the other wells as the effector control. At the same time, complete medium (100 μl), containing only YAC-1 cells (1×10^6 cells/mL), was added into empty wells as the target control. Next, the plates were incubated for 4 h, followed by another 3 h with MTT (5 mg/mL). Then, acidified isopropyl alcohol (100 μl) was added to each well followed by 1 h incubation. The absorbance was measured at 570 nm using a microplate reader. Cytotoxicity of NK cells was expressed as the following equation: Cytotoxicity (percent lysis of target cells) = $[\text{ODT} - (\text{OD}_{\text{exp.}} - \text{ODE})]/\text{ODT} \times 100$, where ODT represents OD value of the target control, whereas OD exp. and ODE represent OD value of the experimental group and effector control, respectively.

3. Pro-inflammatory IL-6 secretion

Pro-inflammatory IL-6 secretion by RAW264.7 macrophages was assessed using the IL-6 Quantikine ELISA assay kit (R&D, Minneapolis, MN) according to the manufacturer's protocol. Briefly, 2×10^6 of cells were plated in 6-well plates and allowed to settle for 2-4 h, followed by incubation with different micronutrients and plant extracts (5-20 $\mu\text{g}/\text{mL}$) for 24 h at 37°C in 5% CO₂ atmosphere. After the incubation period, cells were stimulated with LPS (10 ng/mL) for an additional 24 h and all supernatants were collected and subjected to ELISA assay according to manufacturer's protocol. As a positive control, LPS-stimulated cells without treatment were used. All experiments were done in triplicate.

Statistical analysis. Means and standard deviations were determined for all experiments and Student's t test analysis was used to determine significant differences. Statistical analysis was performed by paired two-sample t-test using GraphPad statistical software.

Results

The effect of natural compounds and their combination on phagocytosis of *Candida albicans* and *Streptococcus mutans*

The “professional phagocytes”, i.e., monocytes and macrophages, are essential components of the innate immune system. Their critical function involves destroying invading pathogens and clearing aging

and abnormal cells in the process of phagocytosis, as illustrated in Figure 1.

We evaluated the efficacy of natural compounds both individually and in combination on the phagocytic activity of rat macrophages and human peripheral blood mononuclear cells in ingesting live bacteria *Streptococcus mutans* and yeast *Candida albicans*.

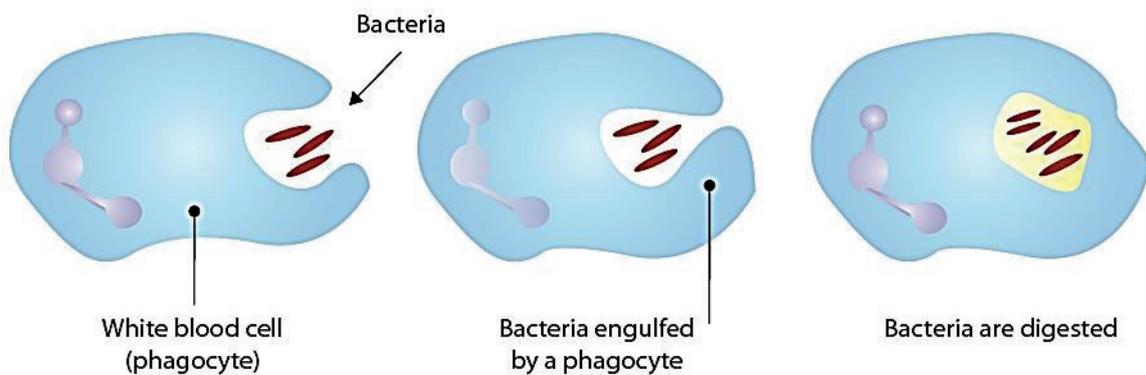


Figure 1: The principle of phagocytosis.

The test compounds are listed in Table 1 and the results of phagocytic activity are presented in Figure 2. The functional ability of phagocytes to ingest pathogens is

indicated by the amount of reduced formazan salt that occurs during the process of phagocytosis. The results are expressed as change in phagocytosis in %.

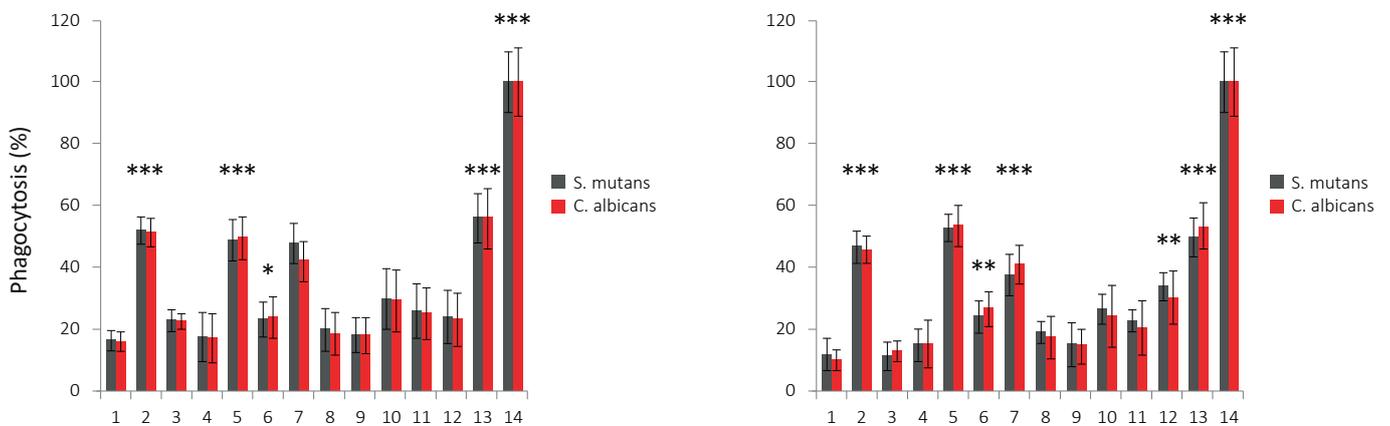


Figure 2. In vitro phagocytic assay on nitroblue tetrazolium (NBT) reduction test of RAW264.7 macrophage cell line (left panel) and human PBMC (right panel) treated with different micronutrients and plant extracts (5-20 µg/mL) for 24 h at 37 °C. Values shown are mean ± standard deviation (n = 4). Value significantly different from corresponding control at * p<0.05, ** p<0.01, *** p<0.001. Tested ingredients: 1-Control, 2-Vitamin C, 3-Vitamin B complex, 4-Vitamin E, 5-Aronia melanocarpa extract, 6-Fucoidan, 7-Ginger root extract, 8-Zinc, 9-Selenium, 10-Lipoic acid, 11-White mulberry extract, 12- Lychee fruit extract, 13-Sour cherry fruit extract, 14-Mix.

The results show that in the presence of vitamin C, plant extracts from *Aronia melanocarpa*, ginger root, and sour cherry, the phagocytosis for each of these compounds increased by approximately 30% compared to control. An even more dramatic increase in phagocytic activity, i.e., about 80% higher than in control, was achieved with the combination of all 12 components. This increase was similar for *S. mutans* and *C. albicans*, with highest statistical significance level of $p < 0.001$.

The effect of test compounds and their mixture on cytotoxic activity of splenic NK cells against T-lymphoma cells (YAK-1)

Natural killer (NK) cells are large granular lymphocytes with cytotoxic activity critical for the function of the innate immune system. Their role is similar to the cytotoxic T lymphocytes in the adaptive immune response. However, in contrast to T lymphocytes, NK cells do not require activation to kill cells with missing

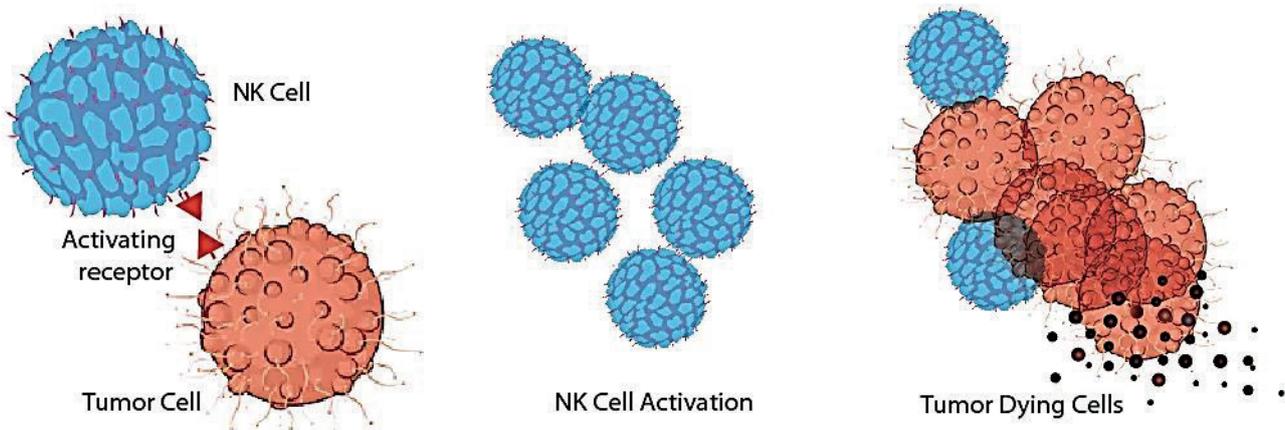


Figure 3: The principle of tumor cell elimination by NK cells.

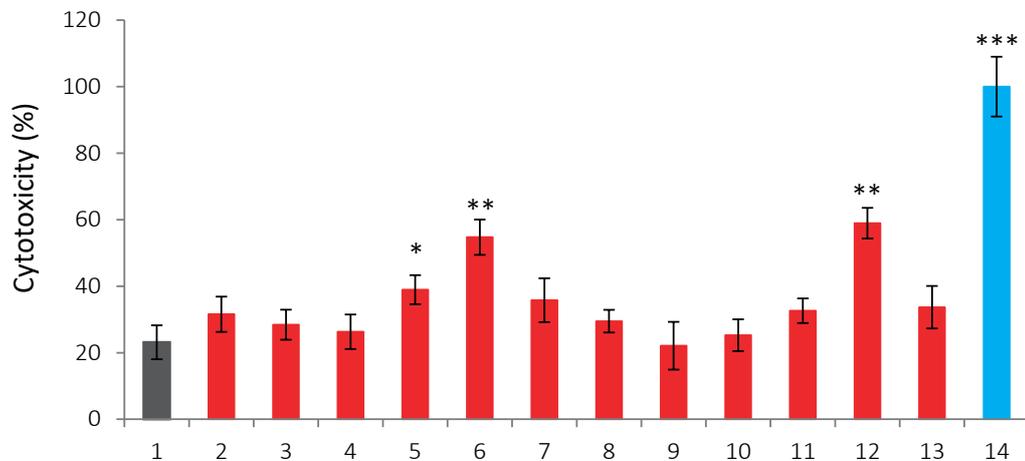


Figure 4. Cytotoxicity of NK cells stimulated by different micronutrients and plant extracts (5-15 µg/mL) for 24 h at 37 °C toward target YAC-1 cells. Cytotoxicity of NK cells was assessed by the MTT assay and expressed as the mean ± standard deviation (n = 4). Value significantly different from corresponding control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Tested ingredients: 1-Control, 2-Vitamin C, 3-Vitamin B complex, 4-Vitamin E, 5-Aronia melanocarpa extract, 6-Fucoidan, 7-Ginger root extract, 8-Zinc, 9-Selenium, 10-Lipoic acid, 11-White mulberry extract, 12- Lychee fruit extract, 13-Sour cherry fruit extract, 14-Mix.

“self” markers of MHC class I. Therefore, NK cells provide rapid and direct killing responses to virus-infected cells, cancer or abnormal cells, and bacteria by disrupting their cell walls (Fig. 3).³⁰

The results in Figure 4 present the efficacy of splenocytes/splenic NK cells in killing lymphoma YAC-1 cells which is expressed as a percentage of their cytotoxic activity.

The results show that NK cells incubated in the presence of fucoidan and lychee fruit extract eliminated about 50% of T-lymphoma cells respectively, compared to only 20% of cells eliminated by non-treated control NK cells. Furthermore, approximately 40% of T-lymphoma cells

were eliminated by NK cells in the presence of *Aronia melanocarpa* extract. However, in the presence of a combination of all 12 components the NK cells showed highest activity and could eliminate almost 100% of T-lymphoma cells.

Anti-inflammatory effect of individual natural compounds and their admixture

Upon exposure to stimulants displaying foreign antigens on their surface, e.g., bacterial and viral antigens, cellular debris, cancer cells, and others, the macrophages rapidly engulf and ingest them, a process which is accompanied by the secretion of a wide range of inflammatory mediators. One of them is IL-6, known for its pleiotropic

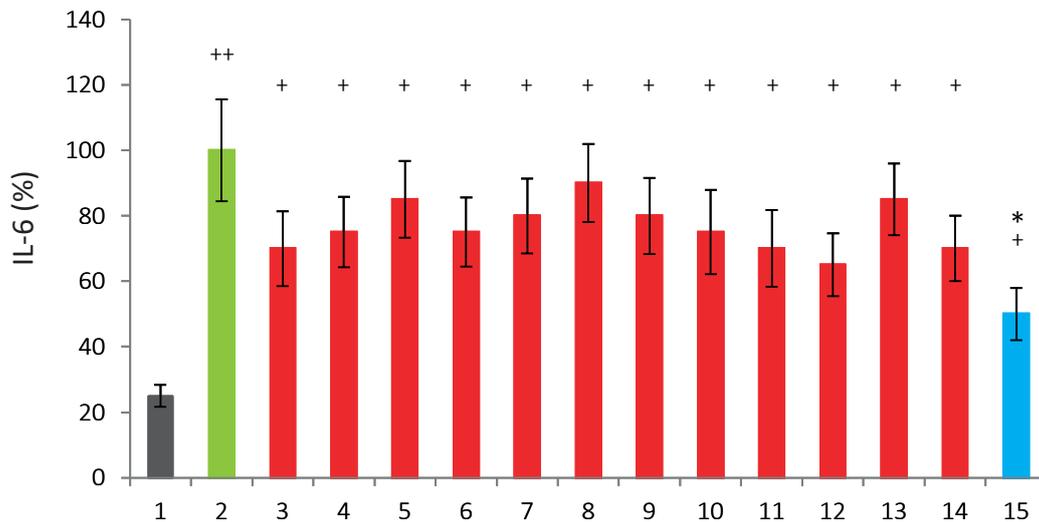


Figure 5. The effect of naturally derived compounds on pro-inflammatory IL-6 release by RAW264.7 macrophage cell line. Macrophages were pre-incubated with different micronutrients and plant extracts (5-20 µg/mL) followed by stimulation with LPS. Each column represents the mean ± standard deviation (n = 4). Value significantly different at * p<0.05 compared to control with LPS, + p<0.05 compared to control w/o LPS, ++ p<0.001 compared to control w/o LPS. Tested ingredients: 1-Control without LPS, 2-Control with LPS, 3-Vitamin C, 4-Vitamin B complex, 5-Vitamin E, 6-Aronia melanocarpa extract, 7-Fucoidan, 8-Ginger root extract, 9-Zinc, 10-Selenium, 11-Lipoic acid, 12-White mulberry extract, 13- Lychee fruit extract, 14-Sour cherry fruit extract, 15-Mix.

activity. IL-6 is produced in response to infection and tissue injury and contributes to host defense through the stimulation of acute phase responses.

As presented in Figure 5, the exposure of macrophages to lipopolysaccharide (LPS), a pro-inflammatory endotoxin from *E. coli*, resulted in approximately 75% increase in IL-6 secretion (column 2). However, pre-incubation of macrophages with different micronutrients or the combination thereof before subsequent LPS exposure,

attenuated IL-6 secretion to varying degrees. Among all 12 compounds tested individually, the vitamin C, B vitamins, lipoic acid, white mulberry extract, and sour cherry extract displayed the highest inhibitory effects on IL-6 secretion. In the presence of these compounds the IL-6 secretion by activated macrophages decreased by 25-30%. However, the combination of all 12 compounds was more effective than any of the individual compounds, resulting in about 50% inhibition of IL-6 secretion when compared to control cells.

Discussion

Immunity, infection, and malnutrition have been always interlinked and immune competence can be regarded as a measure of adequate nutrition. Within the normal healthy population micronutrient status varies due to age, genetics, socioeconomic situation, diet, stress, exercise, smoking habits, exposure to pollutants, and many other factors, all of which are known to have an impact on the immune function and response to pathogens. Micronutrient status is also compromised in people with chronic disease and those using pharmaceutical drugs, as they impair micronutrient absorption and utilization, causing nutritional imbalances that affect the immune system function and increase susceptibility to infections.

It has been established that various nutrients are required to support the normal functioning of the immune system. Their role and cellular mechanisms have been mostly investigated as single components. Many of these compounds, namely vitamin C, zinc, and various herbal extracts are available as nutritional supplements commonly used to support immunity and available over the counter. As such, vitamin consumers use a variety of supplements and often combine and consume them randomly without understanding their collective effects. Research on the cellular and health effects of micronutrients used in complex mixtures is very limited.

Our earlier *in vitro* and *in vivo* studies documented multiple benefits of specific combinations of vitamins, minerals, amino acids, and plant compounds in various aspects associated with viral infections of human influenza (H1N1),^{31,32} bird flu (H5N1, H9N2),³³⁻³⁵ and HIV.³⁶ Specific micronutrient complexes were also effective in controlling all morphological forms of *Borrelia spp.*^{37,38} and demonstrated *in vivo* and clinical benefits in Lyme disease patients.³⁹ We have also shown that TB (tuberculosis) patients undergoing conventional treatments benefited from supplementation with a specific micronutrient complex by achieving faster recovery, complete elimination of bacteria, and experiencing fewer side effects of conventional drug therapy.⁴⁰ Our approach of using synergy-based

combinations of nutrients as a means of achieving enhanced physiological effects has been increasingly applied by others.⁴¹

In this study we investigated the effects of natural compounds and plant extracts with known immune benefits, both individually and combined, on the phagocytosis of pathogens (bacteria, yeast) and cancer cells, and on the release of pro-inflammatory IL-6 by RAW macrophages. All compounds were used at non-toxic concentrations as determined by earlier screening (results not shown). Vitamin C in the mixtures was used at 5 µg/ml, which corresponds to the lower spectrum of human plasma concentration estimated as 5-15 µg/ml.⁴² Total concentration of the ingredients in the final mixture was 75 µg/ml.

Each of the natural compounds displayed differing efficacy in selected tests. For instance, exposure of macrophages to vitamin B complex resulted in a statistically significant decrease of IL-6 secretion. However, B vitamins did not enhance *S. mutans* and *C. albicans* phagocytosis by rat and human macrophages and did not increase NK cell activity against lymphoma cells. Similarly, zinc and selenium did not affect phagocytic and NK cell activity, but they had a lowering effect on IL-6 secretion by LPS-stimulated macrophages.

The most versatile properties in all tested aspects were shown by *Aronia melanocarpa extract*, which had strong statistically significant effects in increasing phagocytic activity against *S. mutans* and *C. albicans*, enhancing the activity of NK cells against lymphoma cells, and at the same time proved effective in lowering IL-6 secretion. Sour cherry extract was effective in stimulating phagocytosis and decreasing IL-6 secretion, and while ginger root extract showed statistically significant effects in stimulating phagocytosis, it had only a moderate effect on enhancing NK activity, while no effect on IL-6 secretion. Nonetheless, it has been shown that gingerol, shogaol, and other structurally related substances in ginger can inhibit prostaglandin and leukotriene biosynthesis through suppression of 5-lipoxygenase or prostaglandin synthetase. Additionally, they can also inhibit synthesis of pro-inflammatory cytokines such

as IL-1, TNF- α , and IL-8.⁴³ It is important to note that while none of the test compounds was consistently effective in all tested immunity aspects, the admixture thereof maintained uniform superior beneficial effects in increasing phagocytosis, NK activity, and anti-inflammatory effects (IL-6 secretion).

Presented results show that a combination of specific natural compounds with different cellular mechanisms of action can have a pleiotropic effect on various aspects of immune cell activity. Enhanced efficacy of this mixture compared to its individual components may result from its ability to affect numerous metabolic targets at once. The results imply that a balanced mix of vitamins, minerals, and select plant components can be beneficial and support healthy function of the immune system.

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