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Cellular effects of fruit extracts and their active components applied individually and in combinations

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Abstract
Scope: Regular consumption of fruits and vegetables rich in vitamins, minerals and bioflavonoids is essential for general health. Since many people fail to meet those consumption targets, there is a huge industry marketing fruit juices, extracts and blends, all touting various health benefits. However, the efficacy of these blends is rarely reported or studied.
Methods and results: In our study, we scientifically evaluated some cellular effects of a mix of select fruit powders and nutritional components for their likely physiological health benefits and to gain a better understanding of the cellular mechanisms behind those benefits.

Keywords: citrus fruits extracts, oxidative stress, phytochemicals, antioxidants, bioflavonoids

Introduction

A diet rich in fruits and vegetables forms the foundation of health. Increased intake of fruits and juices is traditionally recommended to people suffering from conditions ranging from colds to cancer prevention. [1, 2, 3] Based on the results of our previous studies, we assembled a mixture of whole fruit and vegetable extracts and other natural ingredients and tested this combination for specific health benefits at the cellular level. We also evaluated cellular efficacy of a smaller set of these mix ingredients designated as a Core.

The components of the test mixture include ascorbic acid, vitamin B complex, citrus bioflavonoids: mangiferin and naringin, whole citrus fruit powder i.e. mangosteen, and a cruciferous vegetable i.e. watercress. We included vitamin B complex because both mangosteen and watercress have low amounts of most B vitamins and lack Vitamin B12.

Humans unlike most animal species have lost the ability to synthesize ascorbic acid. Citrus fruits are all rich in ascorbic acid, which has protective effects on the vascular and neurological systems and efficacy against infectious diseases and pulmonary conditions like asthma, among others.[4, 5, 6] Citrus fruits are rich not only in ascorbic acid, but also various bioflavonoids however, it is not clear which mechanisms are involved in their specific physiological benefits. Therefore, we used whole fruit extract of mangosteen as well as individual flavonoids mangiferin and naringin in our test mix. Based on our earlier study we selected watercress extract with its high antioxidant and other benefits and an essential amino acid Lysine, for its benefits in maintaining healthy connective tissue,[7] bioenergy [8] and its antiviral properties, including against SARS-CoV. [9]

Our results indicate that our tested Mix can provide important health benefits by improving glucose utilization by the cells, protecting cells from oxidative stress and through its anti-inflammatory properties.

Materials and Methods

Ingredients Tested

Table 1 represents the list and sources of compounds tested in this study. Five ingredients in the Core Mix are highlighted. Each individual compound and fruit powders (indicated in Table 1) were solubilized in DMSO and their stock solutions aliquoted and frozen at -20°C. The samples were diluted in cell growth media or assay buffer provided in the test kit and filter sterilized via a 2-micron filter before testing. All ingredients in the mixes were present in equal amounts.

Table 1: Sources and their compounds tested in this study. Five ingredients in the Core Mix are highlighted.

Ingredient	Part of Core Mix	Commercial Source
L-Ascorbic Acid	Yes	Sigma. Darmstadt, Germany
Mangiferin	Yes	Skin Actives. Arizona, USA
Naringin	Yes	Bulk Supplements. Nevada, USA
Mangosteen Powder (whole fruit) – *Garcina mangosteen*	Yes	Z natural Foods. Florida, USA
Watercress Herb Powder- *Nasturtium officinale*	Yes	Jalpur Millers Pvt. Ltd. Leicester, UK
L-Lysine HCl	No	Bulk Supplements. Nevada, USA
Vitamin B Complex	No	Dr. Rath Health Programs B.V. Herleen, The Netherlands

Cell Lines tested

Human Lung Microvascular Endothelial cells (HMVEC-L) from Lonza (Basel, Switzerland); Rat Myoblast (L6) from ATCC (VA, USA); Human Aortic Smooth Muscle cells (AoSMC) from Lonza (Basel, Switzerland).

Cell lines L6 and AoSMC were maintained in Dulbecco's modified Eagle's medium (DMEM) from Thermofisher (MA, USA) supplemented with 10% Fetal Bovine Serum (FBS) from Thermofisher (MA, USA). Human Lung Microvascular Endothelial cells (HMVEC-L) were maintained and treated in EBMTM-2 Basal Medium (CC-

3156), supplemented with EGMTM-2 MV Microvascular Endothelial Cell Growth Medium SingleQuotsTM supplements (CC-4147) both from Lonza (Basel, Switzerland).

Ferrous equivalent (FRAP). This antioxidant evaluation test was performed using Ferric Reducing Antioxidant Power Assay from Abcam (Cambridge, UK). It is based on a colorimetric reaction which measures the test compound's ability to reduce Fe3+ ion to Fe2+ at low pH. Following the reduction of the ferric iron, a blue color develops and optical density is measured at 594nm. Antioxidant potential of samples is determined using a ferrous iron standard curve and results are expressed as Fe2+ equivalents (nanomoles).

Cell protection against H2O2

HMVEC-L cells were grown to confluency and pretreated with test compounds for 24 hours at 37°C. Media was removed and cells were exposed to Hydrogen Peroxide for 1 hour at 37°C. After one hour, H2O2 was removed and cells were incubated in DMEM supplemented with 1% BSA for a further 24 hours at 37°C to allow them to stabilize. Subsequently, cell viability was assesses using Alamar Blue Cell Viability Reagent from Thermofisher (MA, USA). Alamar Blue Reagent is an oxidized form of redox indicator that is blue in color. When incubated with viable cells, the reagent changes color from blue to red and can be measured by absorbance at 570nm.

IL-6 secretion

AoSMC's were grown to confluency and treated with 1ng Lipopolysaccharides from Escherichia coli (LPS) from Sigma (Darmstadt, Germany) with and without Citrus Mix. Supernatant was collected and centrifuged at 2000 g for 10 minutes to remove debris. Supernatant was assayed using Human IL-6 Quantikine ELISA Kit from RnD Systems (MN, USA).

L6 cells were grown to confluency and treated with 2ng Lipopolysaccharides from Escherichia coli (LPS) from Sigma (Darmstadt, Germany) with and without Citrus Mix. Supernatant was collected and centrifuged at 2000 g for 10 minutes to remove debris. Supernatant was assayed for Il-6 presence using Rat IL-6 ELISA Kit from Thermofisher (MA, USA).

Akt Phosphorylatio

L6 cells were grown to confluency in 96 well plates and treated with Citrus Mix for 3 days. AKT (Phospho) [pS473] ELISA Kit from Thermofisher (MA, USA) was used according to manufacturer's instructions.

Results

Anti-oxidant potential of individual components and their mixtures evaluated by FRAP

Antioxidant potential of select individual ingredients and their combinations evaluated by measuring Ferric Reducing Antioxidant Power (FRAP) is presented on Fig. 1. The results show that the combinations of two or three ingredients have higher antioxidant potential compared to their individual components. The highest antioxidant potential was shown by ascorbic acid which at 0.5 mg/ml concentration had FRAP values 5 times higher than control. Magniferin, mangosteen and naringin were applied at 5 mg/ml. The highest antioxidant potential was noted for magniferin (44 nmol FE) and the lowest for naringin (3 nmol FE). Watercress also showed antioxidant efficacy, but only when applied at a higher dose (35 mcg/ml). Sets consisting of two of these compounds showed enhanced antioxidant potential regardless of individual ingredients and adding a 3rd compound to the mix did not further increase the FE values, however the mix of these compounds had higher FE values than ascorbic acid alone (61.4 vs 51.3 nmol FE) respectively. We used this and similar experiments conducted by us earlier together with relevant available data to formulate our Mix and Core compositions.

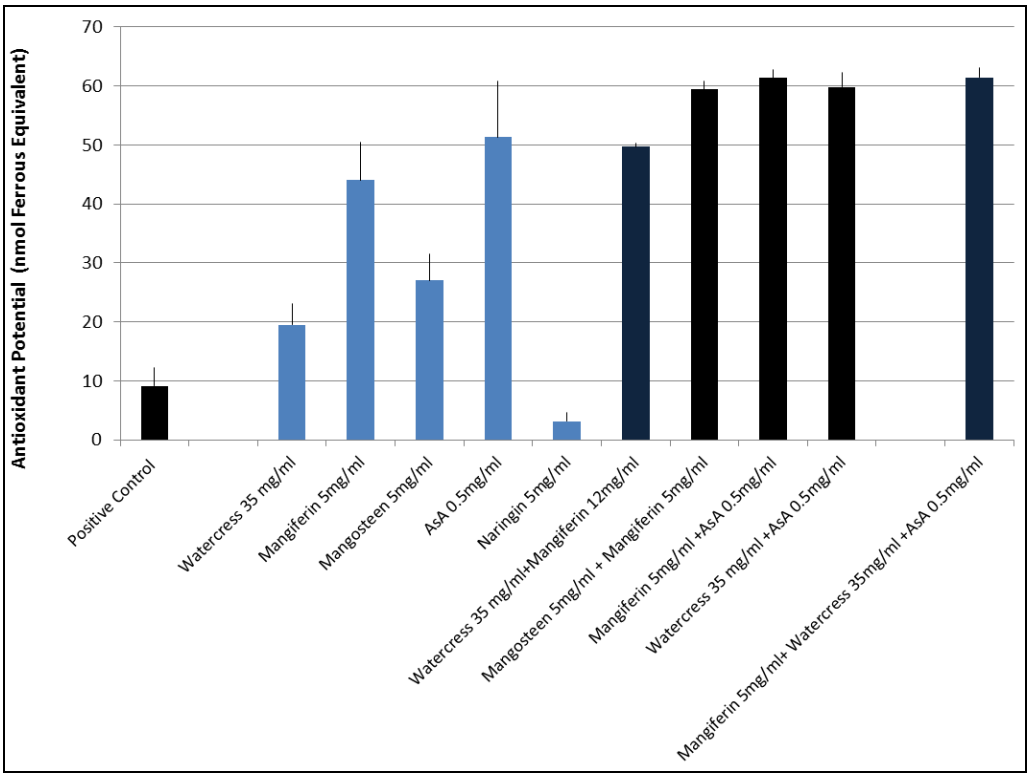

Fig 1: Antioxidant capacity based on FRAP assay as presented in Material and Methods

Cell Protection against H2O2 damage

We tested the protective effect of our Mix composition against oxidative damage by H2O2 in human lung microvascular cells. As presented in Fig. 2 the exposure of lung microvascular cells to the Mix resulted in dose dependent cell protection from oxidative damage. The Mix applied up to 0.49 µg/ml which equals to 0.07 µg/ml per ingredient protected 68% cells from H2O2 damage. At higher doses of the Mix no additional protection was observed. However, it still remained higher than the control level.

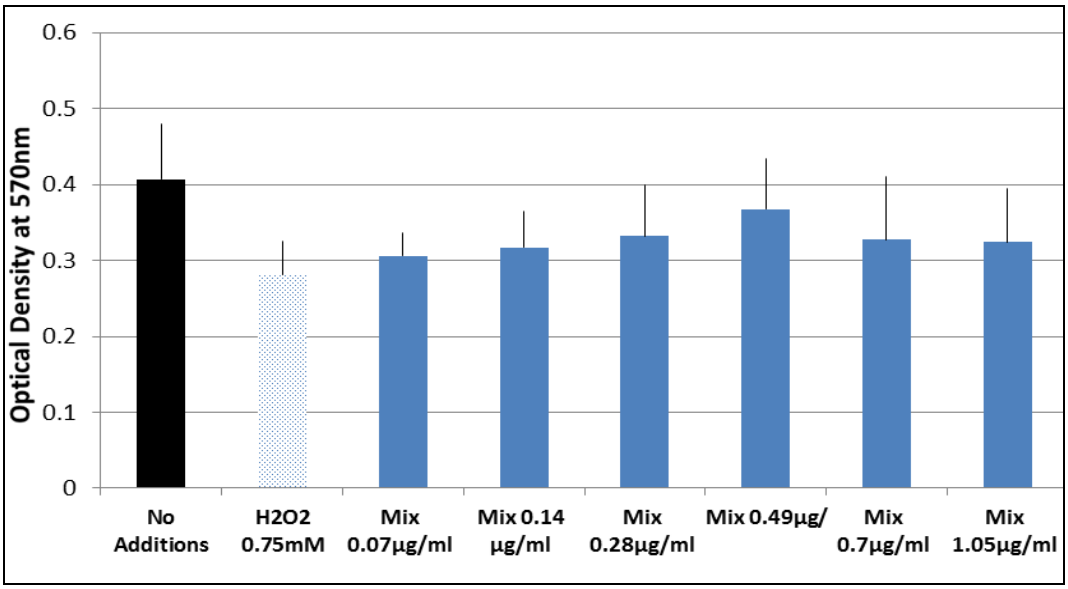

Fig 2: Protection of Lung Microvascular cells (HUMVEC-L) from oxidative stress using Alamar Blue assay as presented in Material and Methods

Anti-inflammatory effect of the test Mix

The effects of the Mix on the secretion of a proinflammatory cytokine, Interleukin-6 (IL-6) in the LPS stimulated human aortic smooth muscle cells and rat skeletal muscle cells are presented on Fig. 3A and 3B, respectively. Exposure of human aortic smooth muscle cells to LPS stimulated IL-6 secretion by 136% compared to control. In the presence of LPS and increasing concentrations of the Mix the secretion of IL-6 gradually decreased reaching 52% of the unprotected (LPS only) level when the Mix was applied at the dose of 1.4 micrograms/ml which corresponds to 0.2 micrograms/ml per ingredient. Fig. 3B shows that the IL-6 lowering effect of the Mix was also present in rat skeletal muscle cells where the Mix applied at 1.4 µg/ml reduced IL6 levels to 42% of the unprotected (LPS) level.

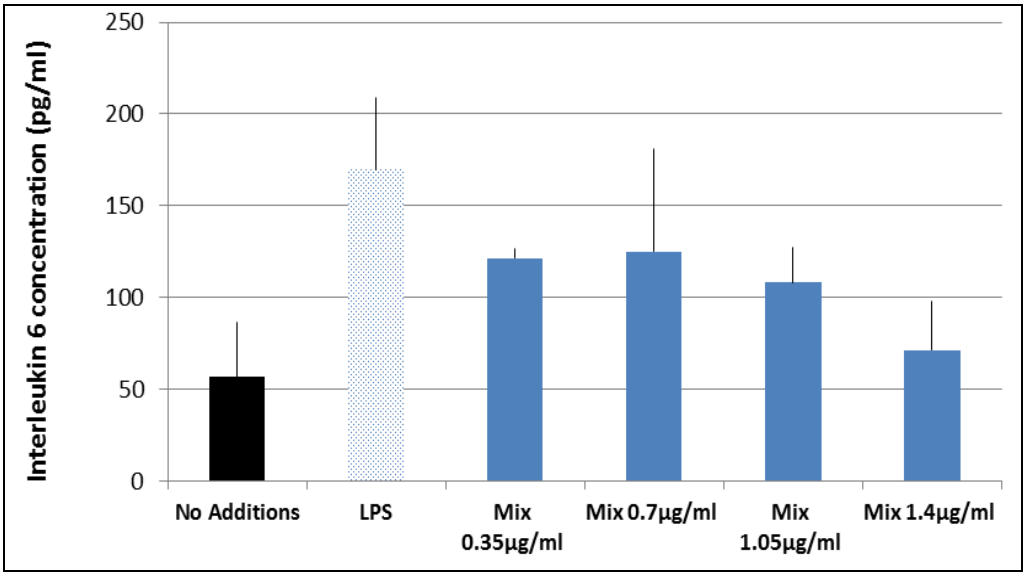

Fig 3: Reduction Interleukin 6 secretion by human AoSMC's stimulated by LPS (1ng/ml) when applied together with the Mix. Interleukin 6 ELISA assay described in Materials and Methods

Effect of the Mix and Core on Akt Phosphorylation

The phosphorylated form of Akt protein kinase mediates insulin action by relocating glucose transporters (GLUT1) towards the cell membrane. Impaired Akt phosphorylation has been implicated in diabetes and metabolic syndrome. [10]

The effects of the Mix and its Core composed of fewer ingredients on Akt phosphorylation in L6 cells are presented in Fig. 4A and 4B, respectively. The efficacy of these two mixes was compared based on the concentrations of individual components in these compositions.

The results on Fig. 4A show that the Mix applied at 1.05 µg/ml (corresponding to 0.15 µg/ml per ingredient), resulted in a 34-fold (3400%) increase in Akt phosphorylation compared to control. In the presence of the Core ingredients (Fig. 4B) the Akt phosphorylation was also stimulated, but to a lesser extent - a 4 times increase that of Control. These results indicate that this enhanced efficacy may result from synergistic interactions of the Mix ingredients.

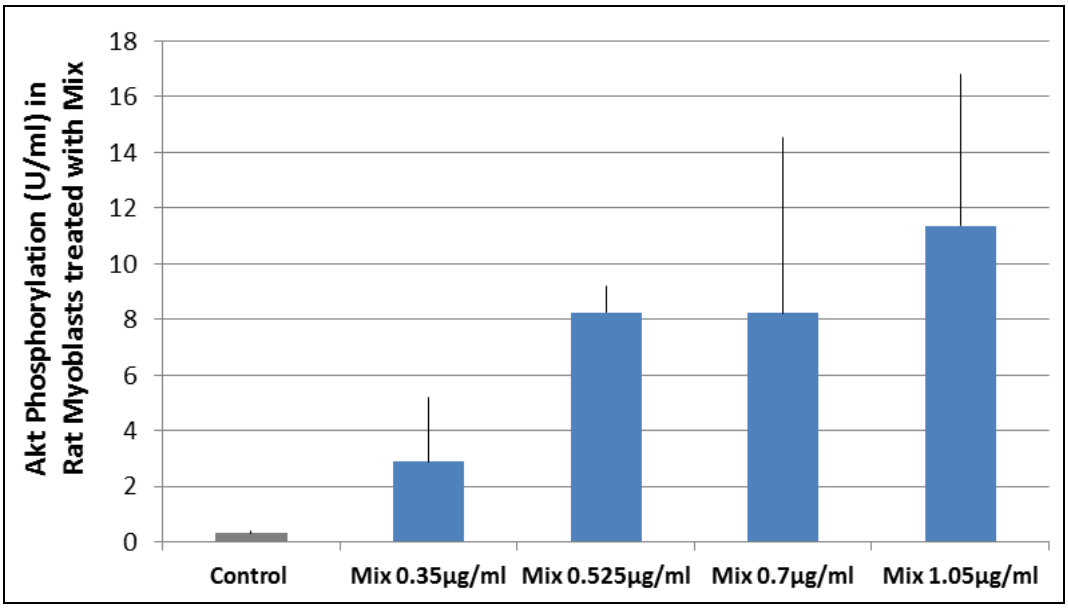

Fig 4A: Stimulation of Akt phosphorylation in rat myoblasts stimulated by Mix. AKT (Phospho) [pS473] ELISA assay performed as described in Materials and Methods

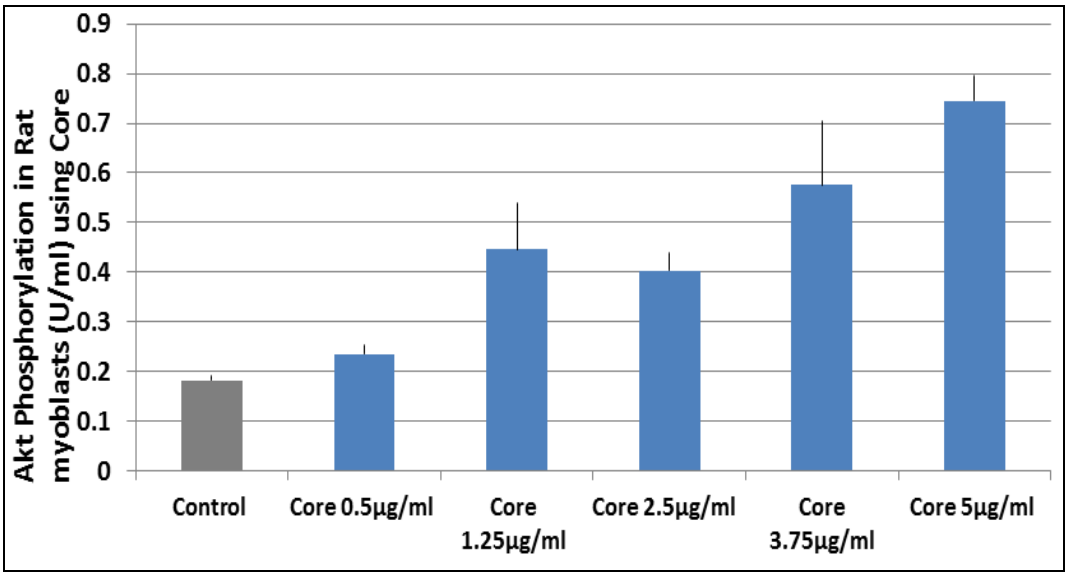

Fig 4B: Stimulation of Akt phosphorylation in rat myoblasts stimulated by Core Mix. AKT (Phospho) [pS473] ELISA assay performed as described in Materials and Methods

Discussion

Despite great advances in medical science, a healthy diet remains the first step to wellness and the strongest safeguard against chronic and infectious diseases.[11] All dietary guidelines stress the importance of plant-based diet with the USDA recommending an intake of 2 cups of fruit and 2.5 cups of vegetables per day.[12] In reality, these recommendations are rarely followed in a population due to various practical reasons. Therefore, the use different nutritional supplements or whole fruit and vegetables powders has become increasingly popular as a practical mean to meet health requirements for micronutrients. However, most commercially available formulations are not tested for their efficacy in specific health aspects and contain randomly selected and combined ingredients. Here we aimed at developing a blend of ingredients with demonstrated benefits in addressing critical metabolic aspects of health at the cellular level. These aspects included antioxidant potential of the mix and its cell protective effect against oxidative damage, bioenergy generation as well as anti-inflammatory efficacy associated with various pathologies.

Our Core Mix included ascorbic acid (vitamin C) and five individual Citrus flavonoids and Citrus fruit powders as the basic ingredients frequently applied in health drinks. Among whole fruit components we used mangiferin, which is known for anti-inflammatory and antioxidant effects. [13, 14] Naringin is shown to have antiviral and anti-cancer properties. [15] Watercress, rich in isothiocyanates, polyphenols and other compounds, has been used for its

benefits in glucose and lipid metabolism, hypertension, bronchitis and other health issues. [16] The regular intake of vitamin C is important as humans cannot produce it internally but due to its involvement in numerous cellular mechanisms this vitamin remains essential in assuring health and sustaining life. Our full Mix also includes the essential amino acid Lysine with its multiple health benefits, including connective tissue stability, bioenergy generation and immune enhancing properties as well as B-complex vitamins which together with vitamin C are essential in bioenergy metabolism.

The composition of the Mix was based on the results of our earlier studies [17] and cellular metabolic targets selected. Based on the FRAP assay we observed that the nutrients in the Mix act synergistically in increasing total antioxidant capacity and also show protective effects on cells in the organs subjected to a high level of oxidative stress, such as the lung cells.

The anti-inflammatory efficacy of the Mix was evaluated using two cell types: myoblasts (L6) and aortic smooth muscle cells (AoSMC). Muscle tissue is a common target of inflammation due to both injury and extensive physical stress. Inflammation processes in aortic smooth muscle cells may result in aneurysm.[18, 19] Our results showed that the Mix was effective in reducing the secretion of important pro-inflammatory cytokine - Interleukin 6 - in LPS stimulated AoSMC as well as myoblasts thereby exerting a protective effect on the cells composing these important organs. [20]

The important health aspect tested in our study involved metabolic effects of the Mix on glucose utilization. Cells are dependent on insulin to utilize glucose as a source of energy. Insulin lowers blood glucose levels and supports cellular bioenergy production by facilitating glucose uptake by the cells. This action of insulin is mediated by the phosphorylation of Akt. Phosphorylated Akt activates cellular Insulin Receptors and translocate GLUTs to the cell membrane, thus allowing for glucose uptake. [21] Deletion of Akt or damage to Akt phosphorylation is implicated in insulin resistance. Both the complete Mix as well as the Core stimulated Akt Phosphorylation in L6 cells where glucose utilization is important for bioenergy metabolism. As expected, the Core, lacking in B vitamins and Lysine was less potent than the complete Mix. We needed to apply the Core at five times higher concentration than the Mix to get 11% of the Mix's effect. It appears that that the combination of nutrients in the Mix may facilitate synergistic response resulting in higher cellular efficacy with lower doses of individual ingredients. This may also have practical implications since the intake of micronutrients at their lowest effective doses could reduce any potential for their overdosing in people who take multiple nutritional supplements.

Our data show that a combination of specific fruit components enriched with B-vitamins and Lysine can provide a range of physiological benefits in different tissues. These combinations can form the basis of targeted micronutrient supplementation addressing various physiological needs. The majority of supplement manufacturers claim various health benefits of their products based on the individual ingredients, without efficacy testing of the entire formulation. However, our results indicate that cellular efficacy of the mixture is often not a result of additive effect of its ingredients, but an outcome of nutrient synergy. Since nutritional deficiencies may persist despite people assuming consumption of a healthy diet, properly targeted supplementation should be the basis of health and wellness.

Conclusion

Results of these and many other studies confirm that nutritional supplements can fulfill a vital role in maintaining health and disease prevention provided they are selected according to scientific principles and tested rigorously. We used commercially available components in our studies; therefore, some variations are expected between the batches and manufacturers of these raw materials. However, our results show that a carefully selected mix of natural ingredients can have numerous health benefits. Further studies are pending.

Authors' contribution

AN, MR conceived, designed, supervised, and validated; MC, AG performed the experiments, analyzed data, wrote the manuscript; MC, AG, AN, MR reviewed the paper. All authors had full access to all data in the study, they have read and approved the final manuscript and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Declarations

Conflict of interest

Authors report no conflict of interest.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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