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Cellular effects of citrus compounds

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Abstract
Scope: Consumption of fruits and vegetables has been inversely associated with morbidity and mortality from degenerative diseases. As different citrus compounds and fruit extracts provide a variety of physiological benefits and they are widely used in nutritional supplements and food products. However, there are no systematic studies comparing their efficacy at a cellular level.
Methods and results: In this study, we evaluated the efficacy of select citrus fruit extracts and their active compounds for their antioxidant potential and protection against damage from oxidative stress in different types of human cells. Our results show the beneficial effects of different citrus compounds as well as whole fruit extracts in a quantitative and comparative manner. Using a variety of tests, we show citrus compounds and fruit extracts may work via different mechanisms.
Conclusion: Our work demonstrates the beneficial effects of these phytochemicals and whole fruits at the cellular level.

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

Introduction

The consumption of fruits and vegetables has been inversely associated with morbidity and mortality from degenerative diseases.[1, 2] While it is not well known which dietary constituents are responsible for this association, their antioxidant effects appear to play a major role in health protective effect of plant foods [3, 4, 5].

Most popular among fruits are citrus fruits which belong to the Citrus genus in the family Rutaceae. They are cultivated worldwide for food and medicinal uses as they are rich in ascorbic acid and various bioflavonoids, including hesperidin, rutin, and naringin. Known health benefits of citrus bioflavonoids include antioxidant capacity, protection against obesity and free radical cell damage.[6, 7, 8] It has been shown that deficiencies in the bioflavonoid hesperidin found in the rinds of orange, lemon, and lime can be linked to negative health effects.[9] Various bioflavonoids and other phytochemicals along with ascorbic acid in citrus fruits have been used for a long time in traditional medicine and are widely sold as nutritional supplements. Among other fruits with documented health benefits are mangosteen, which is a rich source of xanthones were shown to have anti-cancer effects [10]. Pineapple rich in bromelain has been shown to have anti-cancer, anti-inflammatory and anti-thrombotic properties [11].

Due to the chemical diversity of antioxidant compounds present in foods, complete databases on antioxidant content in fruit extracts or powders are not yet available. In addition, levels of single antioxidants in a juice or fruit do not necessarily reflect their total antioxidant capacity which may be influenced by synergistic redox interactions of multiple compounds.

Because different antioxidant compounds may act *In vivo* through different mechanisms, no single method can fully evaluate the antioxidant potential of a fruit powder. Therefore, our evaluation included different assays measuring antioxidant potential of individual compounds as well as fruit powders. We also tested the efficacy of these ingredients in protecting entire cells against oxidative damage. We tested individual compounds found in citrus fruits like mangiferin, rutin, hesperidin, ascorbic acid and naringin as well as extracts of whole citrus fruits like mangosteen, pomegranate, pineapple, grapefruit, lemon and lime.

Our evaluation included Trolox equivalent antioxidant capacity (TEAC) to assess the ability of individual compounds and a whole fruit to quench a radical cation (ABTS·+) in both lipophilic and hydrophilic environments [12], and ferric reducing-antioxidant power (FRAP) [13], which evaluates reducing power of the sample. In addition, we tested cellular effects of these compounds in protecting various human cells against damage by H_2O_2 and how they affect catalase activity – the main enzyme involved in cellular H_2O_2 degradation. These tests allow us to determine if the physiological effects are caused by the action of one or several bioflavonoids.

Materials and Methods

Ingredients Tested

Individual citrus compounds and fruit powders (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 kit and filter sterilized via a 2 micron filter before testing.

Table 1: The sources and stock concentrations of natural compounds tested in this study.

Batch No.	Name	Source
SA131112-B3436	Mangiferin	Skin Actives, AZ, USA
1803804	Rutin	Bulk Supplements, NV, USA
1804505	Hesperidin	Bulk Supplements, NV, USA
1808007	Naringin	Bulk Supplements, NV, USA

1906506	Grape Seed Extract- *Vitis labrusca*	Bulk Supplements, NV, USA
1806202	Lemon Balm extract- *Melissa officinalis*	Bulk Supplements, NV, USA
1016028	Grapefruit Powder- *Citrus × paradisi*	Manitou Trading Company, IL, USA
1901601	Pomegranate Juice Powder- *Punica granatum*	Microingredients, CA, USA
ZOMAGTP3055	Mangosteen Powder (whole fruit) – *Garcina mangosteen*	Z natural Foods, FL, USA
REC/OCT/033/2019	Orange Fruit Powder- *Citrus X Sinensis*	Indus Farms, IL, USA
19052701	Lime Powder- *Citrus aurantiifolia*	Microingredients, CA, USA
19040301	Pineapple Powder- *Ananas comosus*	Microingredients, CA, USA
1806512	L-Lysine HCl	Bulk Supplements, NV, USA
0202020	Watercress Herb Powder-*Nasturtium officinale*	Jalpur Millers Pvt. Ltd, Leicester, UK

Cell cultures

Human Skeletal Muscle Cells (HSkMC) from ATCC (VA, USA), Human Lung Microvascular Endothelial cells (HMVEC-L) from Lonza (Basel, Switzerland), Human Red Blood cells from Rockland (PA, USA).

Human Skeletal Muscle Cells were maintained in Skeletal Muscle Cell Growth Medium kit from Promocell (Heidelberg, Germany). For experiments, cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% 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 Single Quots TM supplements (CC-4147) both from Lonza (Basel, Switzerland).

Red blood cells were provided in a buffered suspension and used out of the original container.

Antioxidant efficacy

Trolox equivalent (TEAC): This assay was performed using Trolox Antioxidant Assay Kit from Sigma (Darmstadt, Germany). The method is based on the ability of antioxidant molecules to quench the long-lived ABTS·+, a blue-green chromophore with characteristic absorption at 734 nm, compared with that of Trolox, a water-soluble vitamin E analog. Results are expressed as Trolox equivalents.

Ferrous equivalent (FRAP): This 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 (nmoles).

Catalase activity

Catalase activity was measured in human red blood cells using Catalase Colorimetric Activity Kit from Thermofisher (MA, USA). Standard curve was generated using a bovine catalase standard provided in the kit. 10% washed pooled red blood cells provided in suspension were diluted 20 times in Assay Buffer and incubated with Hydrogen Peroxide and test ingredients for 24 hours at 37°C. The supplied Colorimetric Detection Reagent is then added, followed by diluted horseradish peroxidase (HRP), and incubated at room temperature for 15 minutes. The HRP reacts with the substrate in the presence of hydrogen peroxide to convert the colorless substrate into a pink-colored product. Using the linear part of the standard curve, the optical density of the samples is converted into Catalase Units or U/ml.

Cell protection against H2O2

Human skeletal muscle and human lung microvascular endothelial 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 570 nm.

Results

Antioxidant Potential

FRAP

Antioxidant potential of fruit juices and their active compounds evaluated by measuring Ferric Reducing Antioxidant Power (FRAP) is presented on Fig. 1.

Among all test compounds, the FRAP assay indicated the lowest antioxidant potential for orange, lime and pineapple powders, with values below control level. All other fruit powders and their active ingredients showed significantly higher levels of Fe2+ reduction compared to control. Among single compounds tested, rutin, and mangiferin displayed highest capacity of Fe+2 reduction which was comparable with grape seed and Lemon balm extract, grapefruit and mangosteen powders. Among individual compounds the lowest antioxidant potential was detected for naringin.

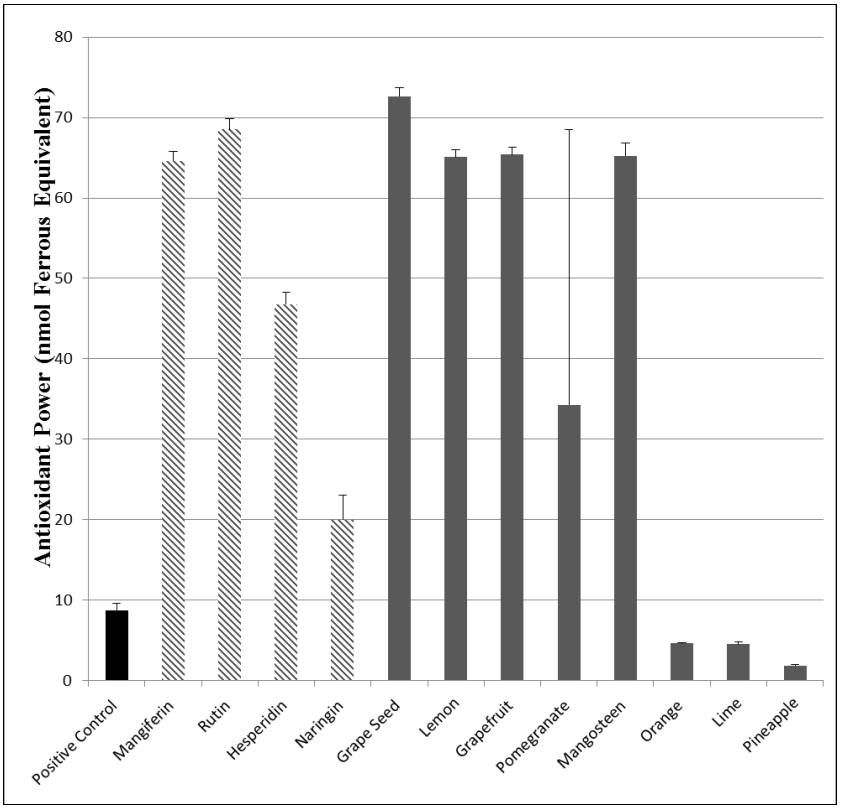

Fig 1: Antioxidant capacity of fruit powders and other natural ingredients evaluated by FRAP assay as presented in Materials and Methods. Each ingredient was tested at 35.5 mg/ml

Trolox

Antioxidant capacity of test ingredients evaluated by Trolox Antioxidant Assay is presented in Fig. 2.

The results show that naringin and lime, pineapple and pomegranate powders had higher antioxidant capacity compared to control. Trolox units of mangosteen, orange powders and lemon balm extract were comparable to control while that of the grapefruit powder was below control values.

This result confirms the importance of using different test analytes even for a similar process to capture broad metabolic effects of food substances. Trolox, being a Vitamin E analog, may work better with fat-soluble compounds or extracts that contain fat-soluble compounds.

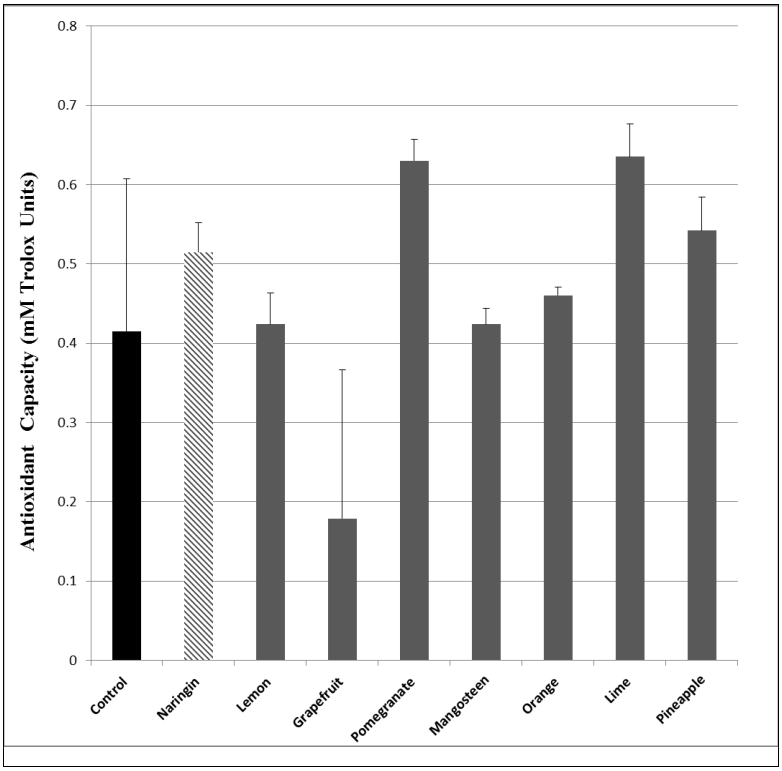

Fig 2: Fruit powders and individual natural ingredients tested for antioxidant capacity measures in Trolox units as described in Materials and Methods. Each ingredient was applied at 35.5 mg/ml.

Catalase Activity

Catalase is one of the most powerful antioxidant enzymes to decompose hydrogen peroxide and free radicals generated by cellular metabolism. We tested the effects of fruit powders, their active compounds, watercress, and an amino acid L-Lysine on catalase activity in human red blood cells.

The results presented on Fig. 3 show that catalase activity in human erythrocytes increased after 24 hour incubation with test compounds. As positive controls, we included the amino acid lysine and watercress extract. Lysine is known to couple with catalase in eggshell as lysyl oxidase[14] while watercress is known for its antioxidant properties.[15] As expected, both lysine and watercress extract were most effective at enhancing catalase activity, however all test ingredients stimulated catalase activity compared to control level.

The highest stimulation by about 37% was obtained in the presence of grapefruit and mangosteen, while the lowest increase in catalase activity of 14% was observed in the presence of lime.

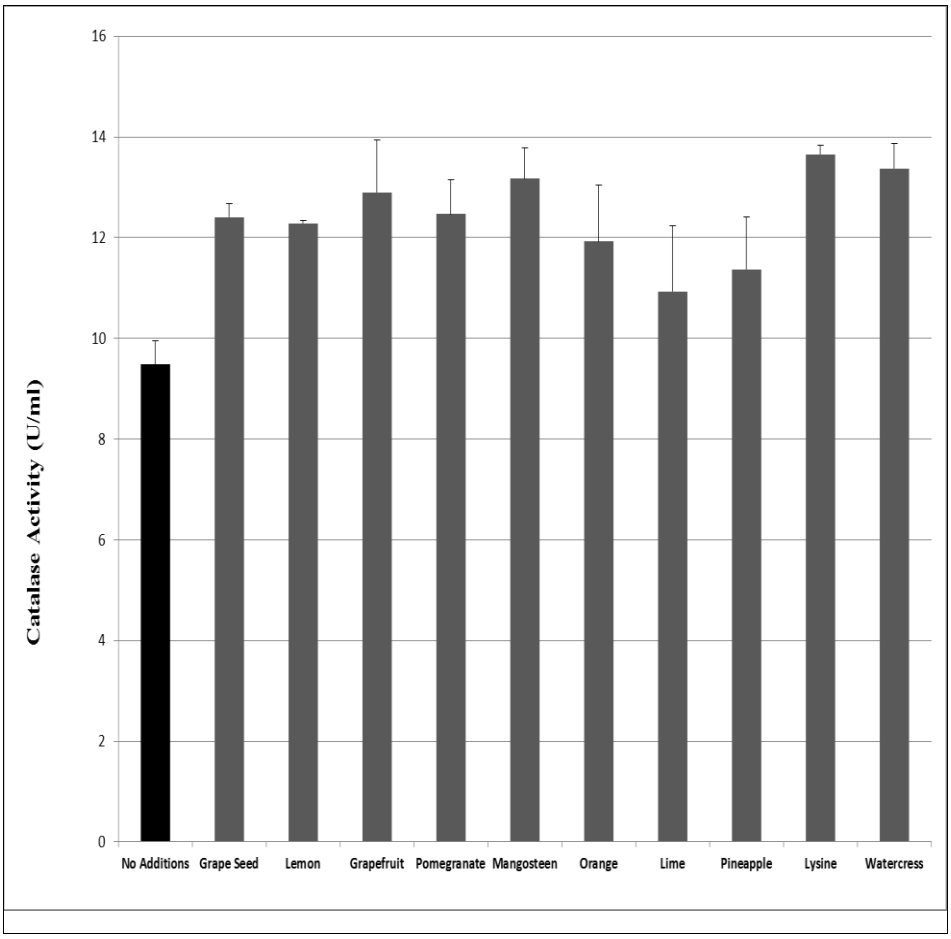

Fig 3: The effect of fruit powders and other compounds on catalase enzyme activity in human red blood cells as described in Material and Methods. Each ingredient was used at 5 µg/ml.

Cell Protection against H_2O_2 damage

The biochemical and enzymatic data are important in evaluation antioxidant potential of individual compounds but their antioxidant efficacy in cells may differ as it reflects their interactions in different metabolic pathways in a whole cell metabolism. Therefore, we analyzed efficacy of fruit powders and ascorbic acid on protecting cells from oxidative damage induced by H_2O_2. The tests were conducted in skeletal muscles cells where high metabolic activity routinely exposes them to oxidative stress and in lung microvascular endothelial cells which are subjected to oxidative stress associated with pulmonary function.

The results shown in Fig. 4 indicate that exposure of skeletal muscle cells to mangosteen, pomegranate, grapefruit or lemon balm extracts before inducing oxidative stress by hydrogen peroxide results in cell protection against damage. The greatest protection was seen by mangosteen with 67% cells protected and lowest with orange powder resulting in 5% cells protected. We could see that the whole fruit extracts were, by and large, more protective to cells than Ascorbic Acid which only provided 10% protection. Interestingly, mangosteen applied at 50 µg/ml concentration was more effective than ascorbic acid, also applied at 50 µg/ml, in protecting these muscle cells against damage by hydrogen peroxide.

The effects of naringin, mangosteen, and ascorbic acid on protecting human lung microvascular endothelial cells against H_2O_2 induced oxidative stress is presented on Fig.5. In this assay, we drastically reduced the doses of naringin and mangosteen to 2 µg/ml, to find a minimal effective dose. All three test compounds showed protective effects on these cells with 84% cell protection by naringin and 55% by mangosteen. Ascorbic Acid at 50 µg/ml was more effective in protecting vascular endothelial cells than skeletal muscle cells against H_2O_2 damage. Both naringin and mangosteen powder were used at low 2 µg/ml concentration had cell protective effects comparable to obtained with ascorbic acid applied at 50 µg/ml.

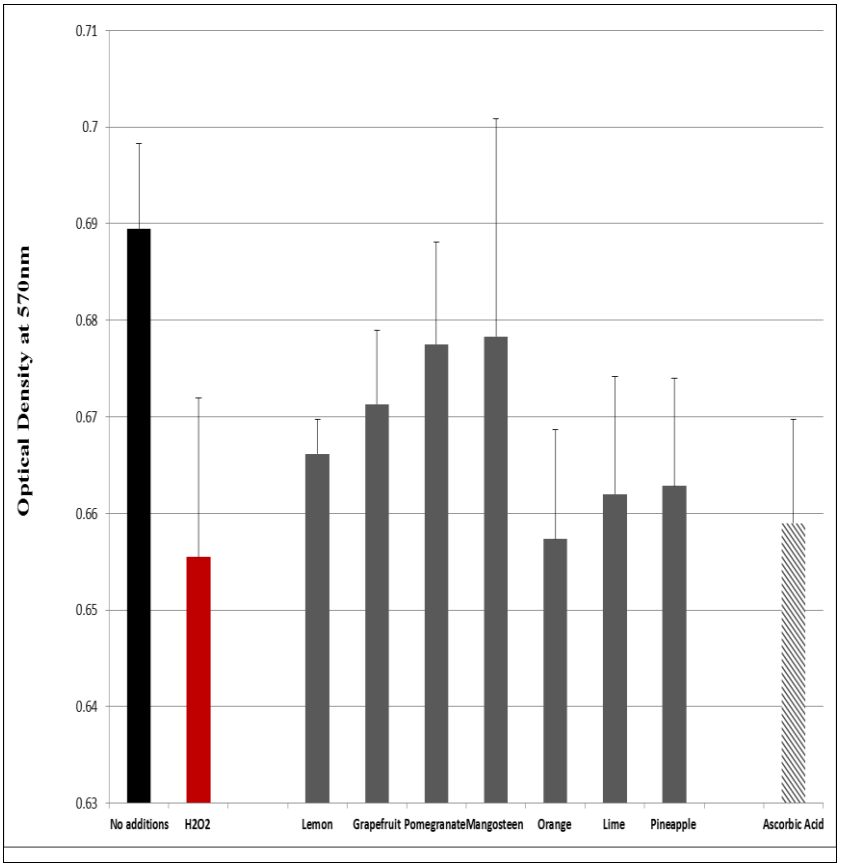

Fig 4: Viability of Human Skeletal Muscle cells pretreated with various fruit powders and ascorbate for 24 hours, followed by their exposure to Hydrogen Peroxide for 1 hour and stabilization in DMEM/ 1 % BSA for a further 24 hours. H2O2 at 1.5 mM was used to induce cell damage by oxidative stress (negative control). Lemon, grapefruit, pomegranate, orange, lime, and pineapple were used at 100 µg/ml. Mangosteen and ascorbic acid were used at 50 µg/ml.

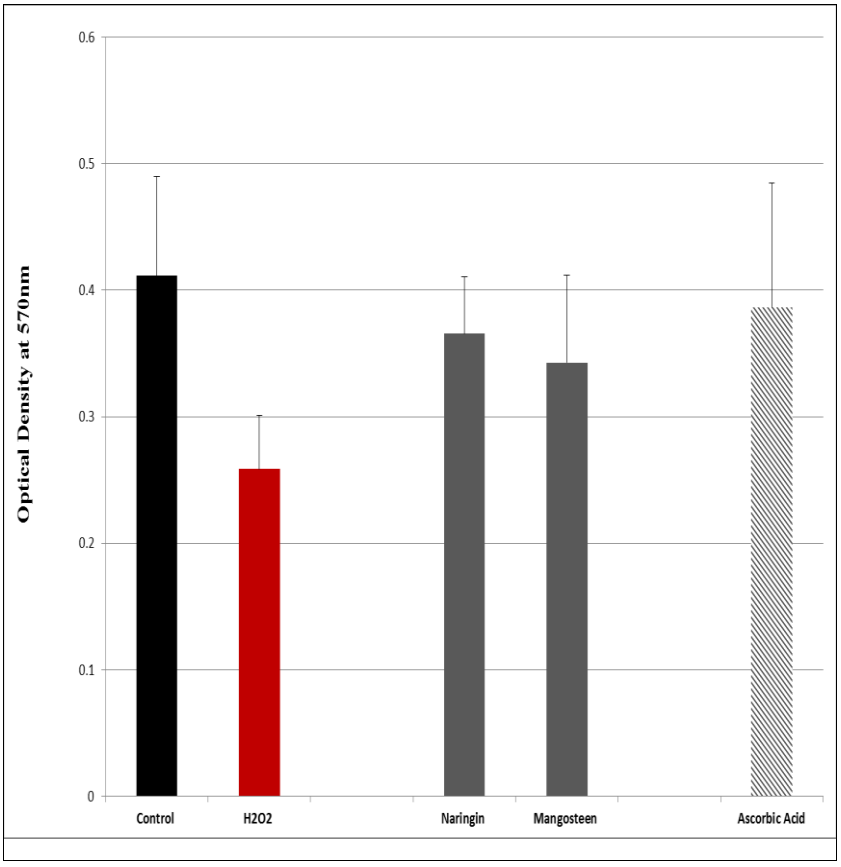

Fig 5: Viability assay on Human lung Microvascular cells after pre-treatment with citrus compounds for 24 hours, followed by exposure to Hydrogen Peroxide for 1 hour and stabilization in DMEM supplemented with 1% BSA for a further 24 hours. Both naringin and mangosteen powder used at 2 µg/ml, and ascorbic acid was used at 50 µg/ml.

Nutrient Synergy

Since the final efficacy of any natural compound is a result of their complex interactions with other components, we evaluated whether antioxidant potential of individual fruit powders can be affected when they are used in combination. The results in Fig. 6 show that FRAP evaluated antioxidant potential of two fruit powders: grapefruit and mangosteen applied each at 5 µg/ml was higher when these fruits are combined compared to their individual values.

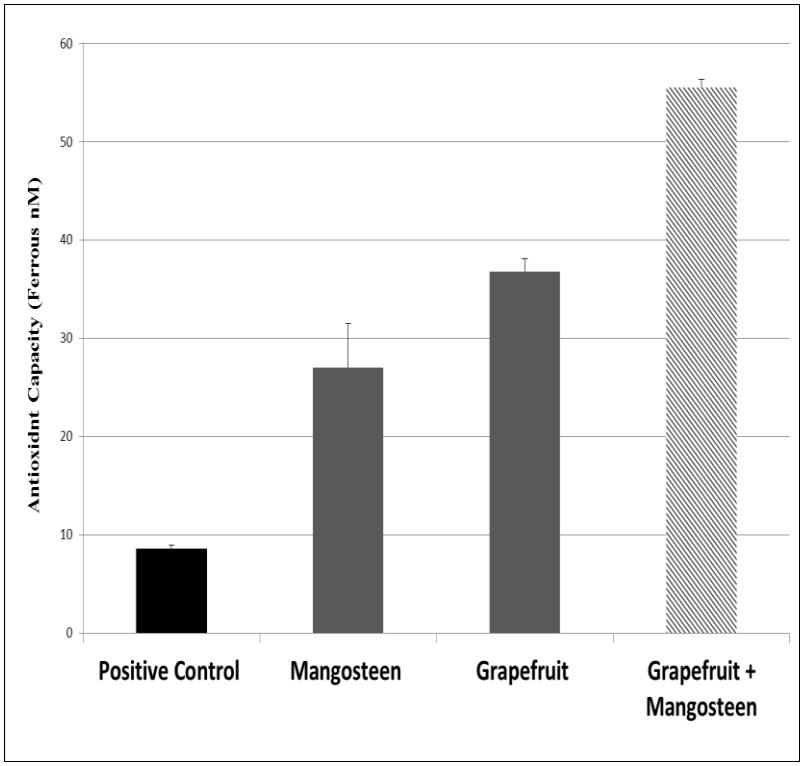

Fig 6: Antioxidant potential (FRAP) of mangosteen and grapefruit powders individually at 5 µg/ml and in combination.

Discussion

Oxidative stress is implicated in many diseases, including cardiovascular disease, neurodegenerative conditions like Alzheimer's disease, Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis[16] as well as metabolic diseases like diabetes.[17] Health professionals regularly stress the importance of consuming fruits and vegetables for health for their multiple health benefits. Here we evaluated the antioxidant effects of citrus fruits and individual natural compounds at the biochemical and cellular levels.

Our experiments show that citrus fruit compounds as well as whole citrus fruit powders have potent antioxidant effects. At the biochemical level, we measured antioxidant potential of all compounds using Trolox based and Ferrous based antioxidant assays. The results showed differences in antioxidant efficacy, which in case of pineapple powder may relate to different solubility in the respective assay buffers. Trolox, being a Vitamin E analog, is more suited to detect the antioxidant potential of fat-soluble compounds.

Catalase enzyme is found in the peroxisomes of mammalian cells and catalyzes the conversion of hydrogen peroxide to water and molecular oxygen. Low catalase levels in blood are associated with diabetes.[18] Catalase deletion in mice makes them prone to prediabetes.[19] Pineapple, lime and orange which did not show much antioxidant activity in FRAP assay, were effective at the enzymatic level by enhancing catalase activity in red blood cells. This shows that in addition to antioxidant potential citrus fruits and their compounds can modulate cellular redox status also by affecting H_2O_2 scavenging ability at the enzymatic level.

We tested comprehensive cellular benefits of these compounds on protecting cells against damage by H_2O_2. Interestingly, pineapple, orange and lime which had low antioxidant potential in the FRAP biochemical assay also were not very effective in improving viability of cells exposed to H_2O_2. Protection of skeletal muscle cells against this oxidative insult may involve more mechanisms than catalase activity. However, mangosteen, pomegranate, pineapple, and grapefruit extracts could enhance catalase activity in red blood cells and showed high protective effects against oxidative damage by H_2O_2 at the cellular level. In addition, grapefruit, pomegranate, and mangosteen showed effectiveness in both cell based and biochemical antioxidant assays.

In the FRAP assay we could see that like antioxidant potency of citrus compounds, mangiferin and rutin were comparable to the entire fruit powders. A similar effect was observed for naringin which protective effect towards human lung microvascular cells against oxidative damage was comparable to mangosteen or ascorbic acid alone. Since citrus fruits contain multiple antioxidant compounds, these assays help us to identify the most potent and efficacious for specific metabolic tasks.

We used far lower concentrations of test compounds in cell based and enzymatic assays compared to biochemical assays which may explain the different results in cellular versus biochemical tests. Based on varied environments and functions, we expect that different cell types will respond differently to citrus compounds. Even ascorbic acid was more protective towards human lung microvascular cells compared to human skeletal muscle cells.

Conclusion

Our work demonstrates beneficial effects of different citrus compounds as well as whole fruit extracts in a quantitative and comparative manner. This study indicates that specific combinations based on their synergistic metabolic effects can enhance the metabolic effects of individual natural compounds. Our earlier work utilized this principle in defining most effective micronutrient combination for addressing various human pathologies and achieving beneficial health effects.[20, 21, 22] Overall, our experiments establish the need to evaluate fruits and their ingredients using different test parameters. By investigating more cell types and applying wide range of experimental tests, we will achieve a better understanding of the basis of their vital beneficial effects in different health aspects.

Various bioflavonoid blends are already sold commercially, without any scientific tests for efficacy. This scientific approach should be also applied in commercial formulations of different blends, or mixtures, of natural compounds for optimal health benefits. We aim to address and promote scientific aspects of these phytochemicals so both the food industry and individuals are better informed.

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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