Bioactive Compounds in Nutritional Bar Based on Dry Orange Flakes Improve Lipid Profile and Increase Antioxidant Capacity in Hypercholesterolemic Patients

ANAT SOLOMON*,†, HAIM AVIV† AND GIDEON EARON‡

†Herbamed Ltd., Science Park, 7 Oppenheimer St. Rehovot 76701, Israel

‡21 Bartenura St., Tel Aviv 62282, Israel

Running title: Nutritional bar for hypercholesterolemic patients

***Corresponding author: Anat Solomon**

Herbamed Ltd., Science Park, 7 Oppenheimer St.

Rehovot 76701, Israel

TEL: +972-8-9409648/9

FAX: +972-8-9407460

E-mail: anats@herbamed.co.il

ABSTRACT

Herbamed has developed a new nutrition bar based on dried orange flakes. In this study, dry orange flakes were evaluated for fiber content, bioactive compounds, antioxidant capacity and their effect on hypercholesterolemia. Dry orange flakes were found to be high in polyphenols, flavonoids and antioxidant potential. The main flavanoid was found to be hesperidin. The antioxidant capacity was measured by ORAC, FRAP and $ABTS^{\cdot+}$ assays. The antioxidant capacities of hesperidin was represented by a high linear correlation (R^2=0.9842) and by T inhibition (T_{inh}) regarding the ORAC assay. Total cholesterol levels and HDL to LDL ratios were measured in hypercholesterolemic patients. Diets supplemented with dried orange flakes or in a bar dose form daily for 4 months, represent a dramatic shift of the study population to reduced ranges of total blood cholesterol and HDL to LDL ratios both in males and females without any apparent side effects.

KEYWORDS: orange flakes; hypercholesterolemia; total cholesterol; low density lipoprotein (LDL), high density lipoprotein (HDL); antioxidant; hesperidin

INTRODUCTION

Atherosclerosis and related diseases have emerged as the leading cause of morbidity and mortality in the western world and, therefore are a major public health concern *(1)*. The role of diet and lifestyle in the development of atherosclerosis and coronary heart disease is well known from animal, clinical and epidemiological studies. The influence of dietary factors is realized through their impact on body mass, lipids and blood pressure *(2)*. Understanding the effects of diet on chronic diseases may greatly aid in the prevention of cardiovascular and coronary heart diseases. Innovative nutritional strategies to reduce the main risk factors have been developed including both dietary changes and consumption of specifically targeted functional foods and dietary supplements.

Functional foods are foods or dietary components that may provide a health benefit beyond basic nutrition. These foods have the appearance of normal foods, but contain specific components whose activity on at least one measurable risk factor has been scientifically demonstrated *(3, 4)*. These nutraceutical products may also provide an alternative to lipid lowering, antihypertensive, and diabetic drugs *(3)*.

Dietary supplements, having formulations similar to drugs, allow the delivery of a bioactive ingredient in dosages that exceed those obtainable from normal food products *(3)*. Extensive research and epidemiologic data have shown that dietary fiber has an inverse relationship to coronary heart disease (CHD) risk, and thus dietary fiber is notes as a functional ingredient *(5)*. There are two types of dietary fiber, soluble and insoluble. Research shows that insoluble fiber has little to no effect on lowering cholesterol, however, soluble fibers including psyllium, beta-glucan, pectin, and guar gum have a positive effect in lowering cholesterol. According to these studies, the viscosity of soluble fiber is the mechanism behind its ability to lower

cholesterol and decrease dietary fat absorption in the intestines by altering the composition of the bile acid pool *(6)*. In addition, soluble fiber seems to alter GI transit time and delay gastric emptying, which leads to a feeling of satiety more quickly and for longer periods of time *(7, 8)*.

A major class of phytochemicals found commonly in fruits and vegetables are the flavonoids. Several mechanisms were advanced as to how flavonoids may protect against coronary vascular diseases (CVD). These include antioxidant, anti-platelet and anti-inflammatory effects, as well as possibly increasing HDL levels, lowering blood pressure, and improving endothelial functions *(9)*. Central to the pathogenesis of atherosclerosis is the oxidation of low-density lipoprotein (LDL). The chemical structure of flavonoids allows it to have both free radical scavenging ability, and the ability to chelate metals, which mean that flavonoids should have antioxidant effects *(9)*.

A Finnish study of approximately 10,000 people examined flavonoid intake and coronary mortality. The results of this study showed that total flavonoid intake was significantly associated with decreased coronary mortality in women *(10)*. The relationship between flavonoids and the risk of coronary heart disease was also examined as part of the Zutphen elderly study *(11)*. Flavonoid intake was strongly correlated with a decrease in the mortality from heart disease in elderly men and also negatively correlated with myocardial infarctions.

Current investigations continue to explore this in depth and are also examining potential synergies between dietary fiber and other phytochemicals such as flavonoids that may lower cholesterol. These studies, along with recent analyses of ongoing prospective cohort studies, have provided new insights into the probable protective role of dietary substances in the arresting of the progression of coronary heart disease

and other cardiovascular diseases *(8)*. Oranges, especially the peel, contain dietary fiber and an array of potent antioxidants including flavonoids (hesperitin and naringenin predominantly as glycosides), carotenoids (xanthophylls, cryptoxanthins, carotenes), and vitamin C in addition to other beneficial phytochemicals such as folate. All of these are believed to significantly contribute to the preventative effects of fruits and vegetables against cancer and heart disease *(12)*.

The objective of this research was to study the effect of dietary dry orange flakes taken in a bar dose form on patients suffering from hypercholesterolemia. We examined the influence of the snack or the powder on the cholesterol levels and the HDL to LDL ratio. In addition, the contents of dietary fiber, polyphenols, flavonoids and hesperidin, the major flavonoid in orange, were measured.

MATERIALS AND METHODS

Materials. Folin-ciocalteu reagent, sodium carbonate, sodium hydroxide, Aluminum chloride, ferric chloride, sodium nitrite, gallic acid, (+)-catechin, hesperidin, 6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid (trolox), fluorescein (FL), 2,2'-azobis-2-methyl-propanimidamide (AAPH), potassium per-sulfate ($K_2O_8S_2$), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), were purchased from Sigma-Aldrich (Rehovot, Israel). Acetonitrile (HPLC grade) was purchased from Bio-lab (Jerusalem, Israel). Redistilled water was filtered through a 0.45 μm membrane before use.

Obtaining of orange flakes. Total orange crush (Valencia variety) was purchased from the Prigat industry (Kibbutz Givat-Haim, Israel). The ground crush was put into drums and dehydrated under specific temperature and time conditions

required for needed dryness (110^{o} C, 1 min, final water content was below 10%) in Tavlinei Ha-Galil (Afula, Israel). This is how orange flakes were obtained.

In vitro assays

Methanolic extract. 500 mg of dried orange flakes were ground in liquid nitrogen to a fine frozen powder. The fine powder was put into a centrifuge tube. Forty-five ml of 70% methanol was added and the tube was placed into an ultrasonic bath for 30 min (42-45 W, 23-25 min, 31-34 ^{o}C). The sample was centrifuged (1500 rpm for 10 min) and the clear supernatant was collected. The procedure was repeated 4 times. Supernatants were combined and evaporated to dryness *(13)*. Extracts by ultrasonic treatment were filtered through a 0.45 μm microporous membrane. The extract was sent for analysis for flavone glycosides and an evaluation of antioxidant capacity. For comparison fresh liquid peels before drying were extracted by this method for flavone glycosides and an evaluation of antioxidant capacity.

Determination of total polyphenols content. Total content of polyphenols was determined by the Folin- ciocalteu colorimetric methods *(14)*. The appropriate dilution of extracts was oxidized with the Folin-Ciocalteu reagent, and the reaction was neutralized with sodium carbonate solution. Absorbance was measured at 750 nm against the prepared blank. Total orange polyphenol extract was expressed as fresh or dry weight as mg of gallic acid equivalents/ 100 g fresh or dry weight.

Determination of total flavonoids content. Total flavonoid content was determined calorimetrically as described previously *(15)*. Briefly, appropriate dilutions of sample extracts were reacted with sodium nitrite solution, followed by reaction with aluminum chloride to form a flavonoid-aluminum complex. Subsequently, sodium hydroxide solution was added to the mixture. Absorbance was

measured against a prepared blank at 510 nm. The flavonoid content was determined by a (+)-catechin standard curve and expressed as the mean of mg (+)-catechin equivalents/100 g fresh or dry weight.

Determination of hesperidin content. Reversed-phase liquid chromatography (RP-LC) was used to determine the content of hesperidin, the major flavonoid in orange peel **(Figure 1).** The HPLC system (Thermo separation products, Riviera Beach, FL) consisted of an auto-sampler (AS3000), an injector (100 µl), a column oven (30^{o} C), a pump (P3000) and a diode-array detector (UV6000). A reversed-phase (RP) C_{18} column (250 mm×3.2 mm, Phenomeniex, spherisorb 5u 00S-2), was used. Elution was performed by a linear gradient with the mobile phase consisting of 1.5% (v/v) acetic acid (solvent A) and Acetonitrile (solvent B) at a solvent rate of 0.8 ml/min. The solvents were mixed using a linear gradient composition: 90% A and 10% B for 5 min, then 5% A 95% B for 20 min, followed by 10% A and 90% B for 10 min. Hesperidin was monitored at 280 nm. The injection volume was 20 µL. Hesperidin was identified using a spectra derived from a photodiode array detection between 200-750 nm and compared with authentic standards and to the spiking pattern generated with a standard of hesperidin.

Quantification of hesperidin was determined by RP-LC using hesperidin equilibration curve and expressed as the mean of hesperidin in units of mg/100 g fresh or dry weight **(Figure 2)**. All samples were filtered through a 0.45 µm syringe filter.

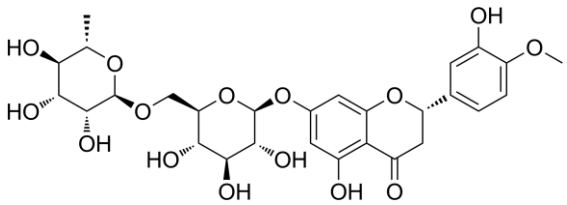

Figure 1. Chemical structure of hesperidin

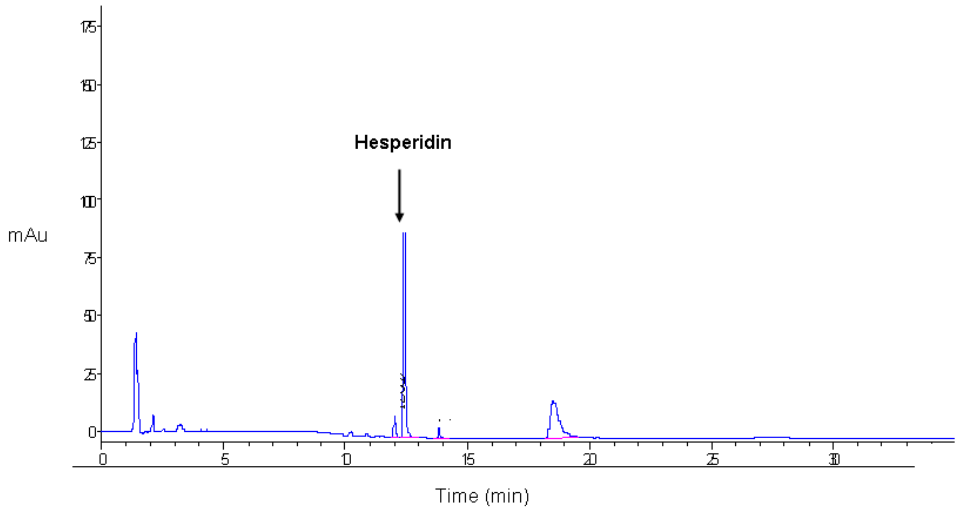

Figure 2. RP-LC chromatograph of the flavonoid fraction of Valencia dried orange flakes. Absorbance at 285 nm is shown. Hesperidin, the major flavonoid in dried orange flakes is shown at t_R = 12.395 min.

ORAC assay. An ORAC assay for fresh crushed orange and dry flakes extracts was carried out using a modified procedure of the method previously described by Prior et al. *(16).* This assay measures the ability of antioxidant components in test materials to inhibit the decline in disodium fluorescein (FL) fluorescence that is induced by the peroxyl radical generator 2',2'-Azobis (2-amidinopropane) dihydrocheloride (AAPH). The reaction took place on a fluorescent plate reader (Synergy HT, Bio-Tek Instruments, Winooski, VT). The Mixture contained in the final assay mixture 200 µL of total volume, FL, 120 µL, 70 µM final concentration and AAPH, 60 µL 32 mM final concentration.

FRAP assay. The FRAP assay was done according to the method of Benzie and Strain with some modifications *(17).* The stock solution included 300 mM acetate buffer (3.1 g $CH_3COONa\times3H_20$ and 16 ml CH_3COOH) pH 3.6, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_3\times6H_20$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer,

2.5 ml TPTZ solution, and 2.5 ml ferric cheloride solution, warmed to 37^{o} C before using. Fruit extracts (150 µl) were reacted with 2850 µl of the FRAP solution for 30 min. in the dark. Readings of the colored product (ferrous tripyridyltriazine complex) were read at 593 nm. Results are expressed in µM TE/100 g fresh fruit or dry flakes.

Trolox equivalent antioxidant capacity (TEAC). TEAC assay for fresh crushed orange and dry flakes extracts was carried out following the procedures previously described by Arnao et al. with some modifications *(18)*. 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\cdot+}$) was dissolved in water to a concentration of 7mM. The $ABTS^{\cdot+}$ radical cation was produced by reacting the ABTS stock solution with 2.45 mM potassium- persulphate (final concentration) and allowing the mixture to stand for 12-16 hr at room temperature in the dark before use. The solution was then diluted by mixing 1 ml $ABTS^{\cdot+}$ solution with 60 ml ethanol to obtain an absorbance of 0.7 ± 0.02 at 734 nm. Fruit extracts (150 µl) were allowed to react with 2850 µl of $ABTS^{\cdot+}$ solution for 6 min in dark conditions. The absorbance was then taken at 734 nm. Results are expressed in µM trolox equivalents (TE)/100 g fresh or dry weight.

Determination of dietary fiber. Samples were analyzed for soluble and insoluble dietary fiber fractions according to the Association of Official Agricultural Chemists (AOAC) method 991.43. The method is based on an enzymatic-gravimetric procedure. Briefly, samples containing a high content of sugar were extracted with 85% ethanol to remove most of the sugars. Residues were suspended with MES-TRIS buffer and digested sequentially with heat stable ά-amylase at 95-100^{o} C, protease at 60^{o} C and amyloglucosidase at 60^{o} C. Enzyme digestants were filtered through tarred fritted glass crucibles. Crucibles containing insoluble dietary fiber were rinsed with dilute alcohol followed by acetone, and dried in the oven overnight at 105^{o} C. Filtrates

were mixed with 4×volume of 95% ethanol to precipitate materials that were soluble in the digestates. After one hour, precipitates were filtered through tarred fritted glass crucibles. One of each set of duplicate insoluble fiber residues and soluble fiber residues was washed in a muffle furnace at 525^{o} C for 5h. Another set of residues was used to determine protein as total kjeldahl nitrogen×6.25. Soluble or insoluble dietary fiber residues (% original sample weight) minus % ash and minus % crude protein found in the residues were taken to be the values for the respectively dietary fiber fraction. Total dietary fiber was calculated as the sum of soluble and insoluble dietary fiber *(19)*.

Patients and treatments

Patients. A total of 113 hypercholesterolemic patients known to have unbalanced hypercholesterolemia participated in this study. 62 were women and 51 men, age range 40-74 years old.

Dietary Supplements. Herbamed. Ltd (Israel) developed a new snack bar based on dried orange flakes. The other ingredients within the bar are: fructose, resins and puffed rice. Each snack contains 2.5 g of dried orange flakes. The participants were divided into randomly to two groups. Patients belong to the first group consumed two snack bars daily for four months and the others consumed two portion of 2.5 g dried orange flakes daily for the same period. The patients did not change their lifestyle or eating habits or medications other than adding the citrus bar or dried orange flakes to their daily nutrition.

Determination of total blood cholesterol levels HDL and LDL. Blood cholesterol levels were taken from all trial participants at time 0, then every two weeks. Each patient was asked to fast 12-14 h prior to blood sampling, which is the accepted procedure.

Fasting total serum cholesterol was measured by an automatic biochemistry analyzer (Roche Diagnostics). Briefly, the Diazyme Total Cholesterol assay is based on enzymatic reactions (COD method) including cholesterol esterase (CE), cholesterol oxidase (CO) and hydrogen peroxidase (POD). Cholesterol esters are hydrolyzed by CE to produce cholesterol which is then oxidased by CO to produce hydrogen peroxide (H_2O_2). H_2O_2 is quantified by Trinder reaction using 4-aminoantipyrine, 4-chlorophenol and POD. The reaction is monitored at 546 nm in a 2 point end manner. High-density lipoprotein cholesterol (HDL-C) was measured directly by an enzymatic in vitro assay that uses poly-ethylen-glycol-modified enzymes in the presence of magnesium sulfate and dextran sulfate to get the selective catalytic activities of lipoprotein fractions (Roche Diagnostics). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation: LDL-C = total cholesterol − (HDL+ triglycerides/5) *(20)*. The HDL to LDL ratio was calculated from the HDL and LDL-C concentration for each subject.

Statistical analysis. All determinations were based on at least three independent replicate samples. Results were analyzed by JMP IN statistical discovery software using one-way variance analysis (ANOVA). When a significant differences was obtained ($p< 0.05$), the Tukey-Kramer HSD test was used to compare each pair of means.

RESULTS

In vitro assays

Determination of bioactive compounds in fresh orange crush and in dry Orange flakes. The results of the bioactive compounds are given in **Table 1**. The total polyphenols, flavonoids and hesperidin contents in dry orange flakes were 6,920 ± 418, 4,120 ± 342 and 3,500 ± 240 mg /100 g DW respectively. The total polyphenols, flavonoids and hesperidin contents in fresh orange crush were 672 ± 49 mg /100 g FW of gallic acid equivalents, 397 ± 30 mg /100 g FW and 342 ± 25 mg /100 g FW respectively. Total polyphenols were obtained on the basis of gallic acid equivalents. Total flavonoids were obtained on the basis of (+)-catechin equivalents.

Table 1. Total content of polyphenols, flavonoids and hesperidin (mg/100 g DW or FW) in orange fractions before and after drying

Fraction	Total polyphenols (mg of GAE/100 g)	Total flavonoids (mg of (+) catechin/100 g)	Hesperidin (mg/100 g)
Dried orange flakes [a]	6,920 ± 418	4,120 ± 342	3500 ± 240
Fresh orange crush [b]	672 ± 49	397 ± 30	342 ± 25

[a] mg/100 g DW (dry weight); [b]mg/100 g FW (fresh weight)

Determinations of antioxidant activity. The results of the measurement of ORAC, FRAP and $ABTS^{\cdot+}$ are summarized in **Table 2**. According to this table, the ORAC value for the dry flakes was 5,408.6 ± 252 μmole of TE/100 g DW and for fresh crush 518.8 ± 36 μmole of TE/100 g FW. The FRAP value for the dry flakes was 230.6 ± 21.2 μmole of TE/100 g DW and 21.9 ± 1.8 for the fresh crush. The TEAC value for the flakes and for the fresh crush were 2,419.7 ± 234.7 TE /100 g DW and 224.2 ± 20.8 TE /100 g FW respectively.

Table 2. Antoxidant potency of hesperidin and 70% methanol extraction fraction in orange flakes and in orange crush

Fraction	ORAC [a] (µmole of TE/100 g)	FRAP [b] (µmole of TE/100 g)	TEAC[c] (µmoleTE/100 g)
Dried orange flakes [d]	5,408.6 ± 252.3	230.6 ± 21.2	2,419.7 ± 234.7
Fresh orange crush [e]	518.8 ± 36.4	21.9 ± 1.8	224.2 ± 20.8

[a]ORAC, oxygen radical absorbing capacity. [b]FRAP, ferric reducing antioxidant capacity. [c]TEAC, Trolox equivalent antioxidant capacity; [d]mg/100 g DW; [e]mg/100 g FW.

Antioxidant activity of hesperidin compared to trolox. In both cases the measured inhibition time was directly proportional to the concentration of the antioxidant. In the tested concentration range 2.5, 5.0, 10.0 µM of trolox the T_{inh} were 14.6, 20.9, and 25.5 respectively. For comparison T_{inh} for 2.5, 5.0, 10.0 µM of hesperidin **(Figure 1)** were 26.8, 45.0 and 62.5 min respectively. **Figure 3** demonstrates the antioxidant capacity of hesperidin compared to trolox by the inhibition time (T_{inh}). In the ORAC assay T_{inh} measures the ability of antioxidant components in test materials to inhibit the decline in disodium fluorescein (FL) fluorescence that is induced by the peroxyl radical generator 2',2'-Azobis (2-amidinopropane) dihydrocheloride (AAPH). T_{inh} for 10 µM of hesperidin and 10 µM of trolox were 62.5 and 23.5 min respectively. From these results we can assume that hesperidin can scavenge more peroxyl radicals, indicated by a longer inhibition period than trolox.

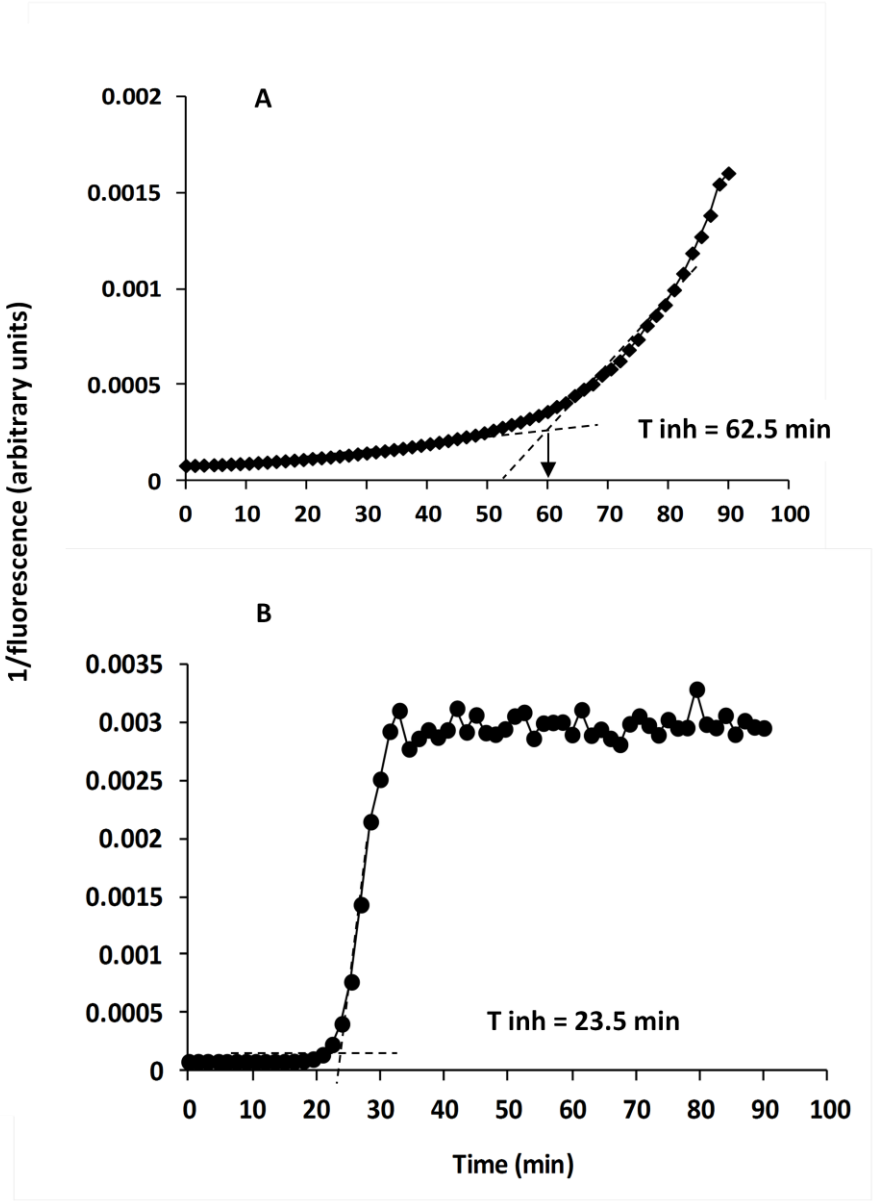

Figure 3. Antioxidant capacity of 10 µM of hesperidin (A) compared to 10 µM of trolox (B). T inh was estimated with Microsoft excel software as the point of intersection between the tangents to the inhibition and propagation phase curve.

Correlation between antioxidant capacity and phytochemical contents. A high correlation was found between the methanol extracted hesperidin contents and its antioxidant capacity with $R^2 = 0.8997$ and $R^2 = 0.9842$, respectively **(Figure 4A, B).**

Data suggests a high correlation between antioxidant capacity and hesperidin concentration.

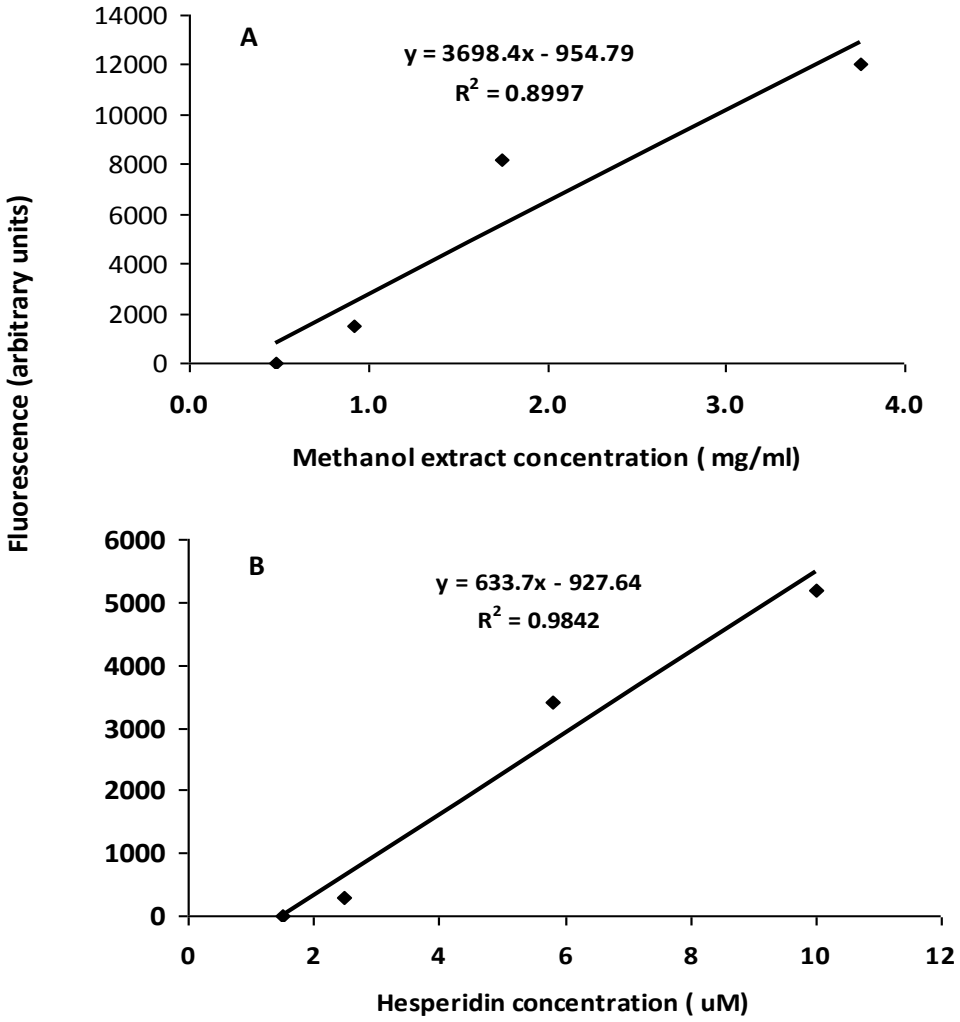

Figure 4. Correlation between antioxidant activity as expressed in ORAC assay vs. total methanol extract (A) and hesperidin content (B). The correlations were calculated by linear regression analysis. Higher R^2 coefficient was observed for hesperidin as compared to the all methanol extract.

Determination of dietary fibers in dried orange flakes. The content of total, soluble and insoluble dietary fibers in dry orange flakes were 53.1 ± 4.9, 21.9 ± 2.4 and 31.2 ± 3.1 g/100 g DW respectively.

Studies in vivo

Determination of blood cholesterol levels in hypercholesterolemic patients. Desirable blood cholesterol levels are less than 200 mg/dL. None of our patients had this desirable concentration at the beginning of our study. 108 of 113 subjects (96%) began the treatment with blood cholesterol levels that were higher than 240 mg cholesterol/dL. At the end of treatment only 45% had blood cholesterol levels higher than 240 mg/dL (a reduction of 51%). 4% of the patients had blood cholesterol levels between 200-239 mg/dL at the beginning of treatment and at the end of the treatment 33% of the patients had this range of levels (increasing of 29%). Furthermore, none of our patients had total blood cholesterol level below 200 mg/dL, while at the end of the clinical trial 22% of the patients had those desirable levels. The results presented here show that there was a dramatic shift of the study population to reduced ranges of total blood cholesterol as a function of consuming the functional bar daily for 4 months **(Figure 5)**. In addition, gender did not affect the impressive reduction in total blood cholesterol levels **(Figure 6)**.

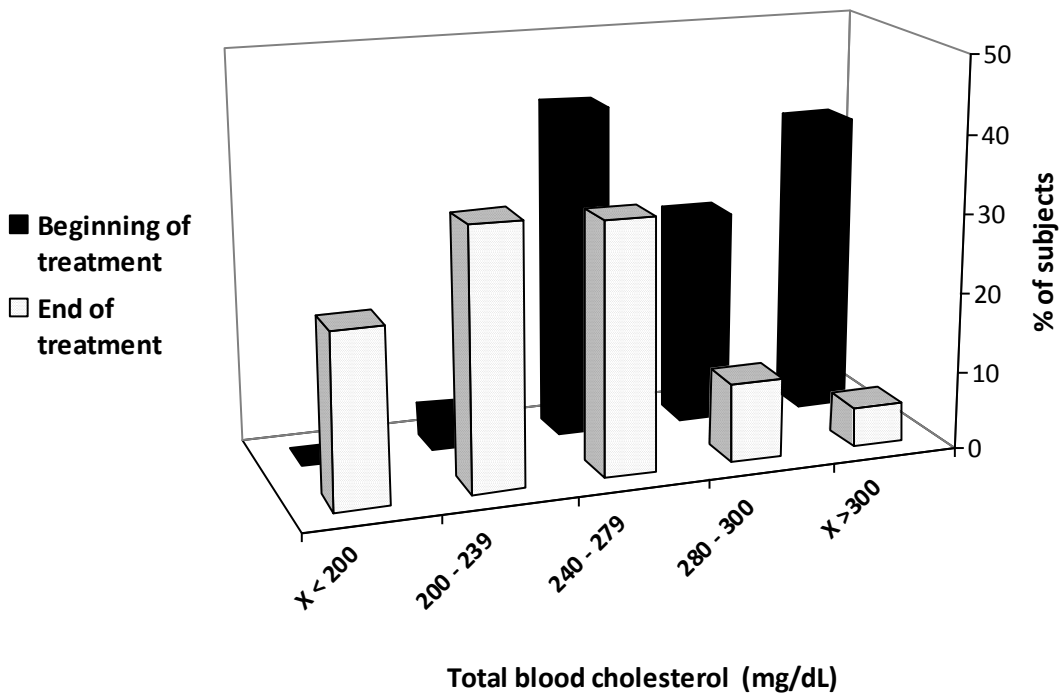

Figure 5. Changes in total blood cholesterol levels in 113 subjects who suffered from hypercholesterolemia. The results presented here show that there was a dramatic shift of the study population to reduced ranges of total blood cholesterol as a function of consuming the functional bar daily for 4 months. At the end of treatment only 45% had blood cholesterol levels higher than 240 mg/dL (a reduction of 51%). Additionally, none of our patients had total blood cholesterol level below 200 mg/dL, while at the end of the clinical trial 22% of the patients had those desirable levels.

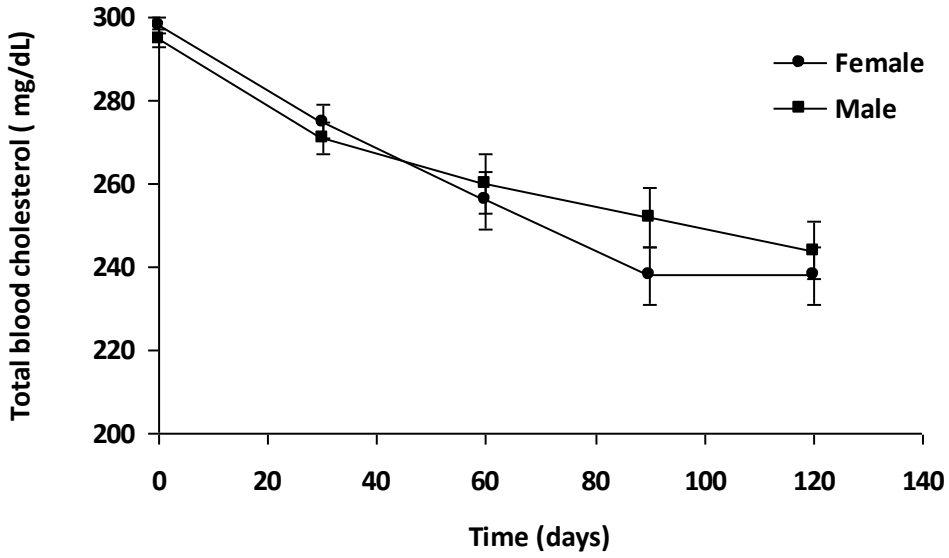

Figure 6. Changes in blood cholesterol levels of the 51 male and 62 female patients at the beginning and at the end of the clinical trial. All participants consumed two dried orange flakes nutrition bars daily for 4 months. Gender did not affect the reduction in total blood cholesterol levels. The reduction was observed both in male and female.

Determination of HDL to LDL ratios. HDL to LDL ratio is considered a key factor in assessing the risk of having a myocardial infarction (MI) due to high serum cholesterol levels. The optimal HDL to LDL ratio is lower than 1:3.2 with the ideal being lower than 1:2.5. 50% of the patients had higher HDL to LDL ratio than 1:4.5 at the beginning of the trial. Only 15% of the patients had such HDL to LDL serum ratio in the end of the trial **(Figure 7)**. At the beginning and at the end of the trial 18% and 21% respectively of the patients had HDL to LDL serum ratio in the range of 1:3.2 to 1:4.5. In addition, an impressive increase in number of people was detected in the serum range of 1:2.5-1.3.2. At the beginning only 24% of the patients had serum ratio between1:2.5 to 1:3.2 and at the end 42% of the participants had this range (increase of 18%). Furthermore, at the beginning 9% of the patient had the healthy HDL to LDL serum ratio lower than 1:2.5. At the end 24% of the participants had this serum ratio (elevation of 13%). At the beginning of treatment 33% of the patients had a ratio that was 1:3.2 or better which is considered as a very good ratio. At the end of

the study the number of the people with ratio of 1:3.2 or better doubled to 66%. The HDL to LDL ratio was elevated because of significant reduction in the LDL content. In addition, significant effect on the HDL content was not observed.

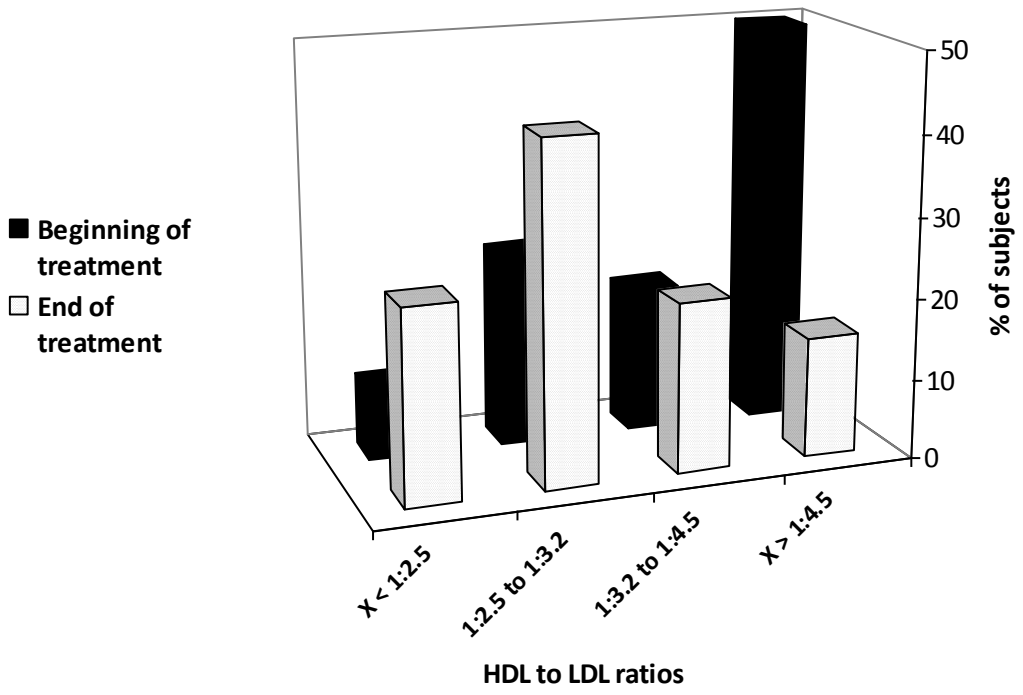

Figure 7. Changes in HDL to LDL ratios in 113 subjects who suffered from hypercholesterolemia. HDL to LDL ratios changed during the clinical trial period after consuming nutrition bars based on dried orange flakes. Each person consumed two nutrition bars daily for 4 month. The optimal HDL to LDL ratio is lower than 1:3.2 with the ideal being lower than 1:2.5. 50% of the patients had higher HDL to LDL ratio than 1:4.5 at the beginning of the trial. Only 15% of the patients had such HDL to LDL serum ratio in the end of the trial. At the beginning 9% of the patient had the healthy HDL to LDL serum ratio lower than 1:2.5. At the end 24% of the participants had this serum ratio (elevation of 13%).

DISCUSSION

Cardiovascular diseases (CVD) remain the top cause of death in the United States. Among the CVD risk factors, saturated fat and trans-fat intake, serum cholesterol, and obesity are considered major risk factors. According to the American heart association guidelines, an option that should be considered for treating or preventing hypercholesterolemia, is the use of functional foods *(21)*. Functional foods are foods that promote optimal health and reduce the risk of disease *(21)*. In addition to its nutritional values, the orange offers various health benefits in various chronic conditions such as heart diseases and insulin resistance *(22, 23)*.

In this study we monitored, for the first time, the influence of nutrition bars based on dried orange flakes on hypercholestrolemic patients. A fixed dose of 2 bars containing 5.7 g of citrus flakes were given daily to the subjects for 4 months. None of the patients changed their daily diet or lifestyle besides adding the bars to their daily food intake. A clear and significant reduction in serum cholesterol levels was observed in the treated patients **(Figure 5)**. The results presented here show that there was a dramatic shift of the study population to reduced ranges of total blood cholesterol as a function of consuming the functional bar daily for 4 months. 96% of the patients began the treatment with blood cholesterol levels that were higher than 240 mg cholesterol/dL. At the end of treatment only 45% had blood cholesterol levels higher than 240 mg/dL (a reduction of 51%). 4% of the patients had blood cholesterol levels between 200-239 mg/dL at the beginning of treatment and at the end of the treatment 33% of the patients had this range of levels (increasing of 29%). Additionally, none of our patients had total blood cholesterol level below 200 mg/dL, while at the end of the clinical trial 22% of the patients had those desirable levels. Patients who began the study with cholesterol levels over 300, in most cases showed a

very significant reduction in blood cholesterol levels. Subjects who started with cholesterol levels of approximately 250 mg/dL succeeded in lowering their levels to below 200 mg/dL. In addition, similar results were obtained in control patients who took only 2.5 gr of dried orange flakes twice a day (data not shown). However, there is a clear preference to taking the dried orange flakes as food bars and not as pills. HDL to LDL ratio is considered a key factor in assessing the risk of having a myocardial infarction (MI) due to high serum cholesterol levels *(24)*. HDL takes up excess cholesterol from the cells and returns it to the liver, making the cells more receptive to the LDL cholesterol particles. This prevents the LDL particles from staying in the arteries and oxidized by oxidants. When that happens, an artery-clogging plaque may form *(25)*. Only 33% of our patients had a ratio that was 1:3 or lower. This ratio is considered a good ratio for the prevention of heart disease. After treatment the number of people with this favorable HDL to LDL ratio doubled. If we take into account that these patients taking the orange flakes bar did not change their medications, eating habits or go on a special diet, the results are even more impressive and promising. A similar improvement in the HDL to LDL ratio was seen in the group who took dried orange flakes not in a bar form (data not shown). The HDL to LDL ratio was increase because of significant reduction in the LDL content.

Statins are a class of drugs that lower the levels of cholesterol in the blood by reducing the production of cholesterol in the liver *(26)*. There is no conclusive evidence showing that statins are beneficial for the prevention of vascular diseases in women, particularly in women without established CHD *(27)*. In addition, statins may have side effects such as headaches, muscle pain, diarrhea, weakness, liver failure and rhabdomyolysis *(28)*. Our data suggests that the bar based on dried orange flakes

lowered serum cholesterol levels in both male and female patients **(Figure 6)** without side effects.

Dried orange flakes include two fractions of dietary fiber, soluble and insoluble. The content of total, soluble and insoluble dietary fibers in dried orange flakes by our results were 53.1 ± 4.9, 21.9 ± 2.4 and 31.2 ± 3.1 g/100 g DW respectively. In another study the content of total and soluble fiber in dried orange peel (Valencia variety) were 61-69 g/100 g DW for total fiber content and 19–22 g/100 g DW for soluble fiber *(29)*. The differences may be due to the geographical region in which it has been grown, the procedure used for the extraction, the solvent used or the duration and the temperature of the extraction *(30)*.

It has been proposed that a protective effect of soluble dietary fiber against CVD is mediated through its direct or indirect effects on serum lipids. The reduction of cholesterol is possibly a sum of several effects. It is commonly accepted however that the majority of the soluble fiber's effect in reducing cholesterol is due to decreased absorption of bile acids. This causes a removal of steroids from the body by fecal excretion resulting in increased catabolism of cholesterol, an increase in the secretion of bile acids, a decrease in lipoprotein cholesterol secretion, and a reduction in the total body pool of cholesterol *(31)*. In addition, dietary fibers have shown to improve the lipid profile by lowering total and LDL cholesterol *(32)*. Chau et al. showed in their research that orange peels (*Citrus sinensis* L.) contain fiber rich fractions which were mainly composed of pectic substances and cellulose but also contained pectic polysaccharide-rich soluble dietary fibers. The physicochemical properties of the orange fiber such as water-holding capacities, oil-holding capacities, and swelling properties may explain the effects of the orange fiber in body weight management and in holding lipid-soluble substance such as cholesterol *(33)*.

Beside dietary fibers, the orange fruit contains polyphenols and flavonoids as bioactive compounds. In 1930, Szent-Gyorgyi isolated a new substance from oranges and classified it as vitamin P but later, it become clear that this substance was actually a flavonoid. Flavonoids are a sub-class of phenolic compounds and constitute one of the main classes of secondary metabolites *(34)*.

Flavonoids drew greater attention from researchers with the discovery of the French paradox *(35)*. Flavonoids may act as potent antioxidants and metal chelators. They have long been recognized to posse anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiatherosclerotic, antiviral, and anticarcinogenic activities *(35)*. Flavonoids may delay LDL oxidation by their antioxidant properties. This is due to their high propensity to transfer electrons, chelate ferrous ions, and scavenge reactive oxygen or nitrogen species (ROS/NOS) by acting as chain breaking antioxidants *(36)*. Furthermore, a Japanese study reported an inverse correlation between flavonoid intake and total plasma cholesterol concentrations *(37)*.

John A. Manthey is known for his work on citrus flavonoids in peel byproducts. Manthey's research has shown that the polymethoxyflavons (PFMs) decrease blood serum levels of apoprotein B, the structural protein of LDL cholesterol *(38, 39)*. The results of our analysis on orange flakes showed that total polyphenol and flavonoid content within the dry flakes were 6,920 ± 418 and 4,120 ± 342 mg/100 g DW respectively. In addition hesperidin content was 3,500 ± 240 mg/100 g DW **(Table 1, Figure 1)** 85% of the total flavonoids fraction **(Figure 2)**. Our data also suggests that the drying process used did not affect the activity of the bioactive compounds.

Due to the complexity of the antioxidant defense system and the involvement of many different types of free radicals and reactive species, several assays were used

in our research to study the antioxidant capacity of citrus flakes in an effort to establish the potential beneficial effects. The results of the measurement of ORAC, FRAP and $ABTS^{\cdot+}$ assays are summarized in **Table 2**. According to this table, the ORAC value in the dry flakes was 5,408.6 ± 252, the FRAP value was 230.6 ± 21.2 and the TEAC value was 2,419.7 ± 234.7 μmole TE /100 g DW.

In another study the researchers measured the total antioxidant capacity of fruits using the ORAC assay and found that there was indeed differences in the different fruits antioxidant capacity: strawberry > plum > orange > grapefruit > grape > banana > apple > tomato > pear *(40)*. Fruit and vegetables rich in anthocyanins (e.g. strawberry, raspberry and red plum) demonstrated the highest antioxidant activities, followed by those rich in flavanones (e.g. orange and grapefruit), while the hydroxycinnamate-rich fruit (e.g. apple, tomato, pear) consistently elicited the lower antioxidant activities *(41).* The antioxidant capacity of orange extract was lower than those observed for berry extracts since flavanones have consistently lower antioxidant efficiency in terms of H-donating capacity, with respect to anthocyanins and flavanols when evaluated using an *in vitr*o system *(42).*

Among the naturally occurring citrus flavonoids, hesperidin may be associated with potential health benefits such as prevention of arteriosclerosis progression *(43).*

The antioxidant capacity of hesperidin was measured compared to trolox. The antioxidant capacity was represented by T inhibition (T_{inh}). T_{inh} for 10 μM hesperidin or trolox using the ORAC assay were 62.5 and 23.5 min respectively **(Figure 2)**. From these results we can assume that hesperidin can scavenge more peroxyl radicals indicating a longer inhibition period than trolox. Our results are in agreement with the study of Kalpana and co-workers. These researchers showed that hesperidin has a

higher antioxidant capacity than trolox in pBR322 DNA and RBC cellular membrane systems where oxidative damage was induced by H_2O_2 *(44)*.

Beside the antioxidant capacity we examined the correlation between the antioxidant capacity and hesperidin concentration. A high linear correlation was observed with R^2 = 0.8997 and R^2 = 0.9842, respectively **(Figure 4A, B)**. Data suggests an excellent linear relationship between the antioxidant capacity and hesperidin's concentration within the flakes.

Gorenstein and co-workers found a lower linear correlation than what we found for hesperidin. The differences can be from the material used, the extraction procedure, geographical location, variety of the fruit used and the antioxidant assay itself *(45)*.

Cirico and others showed that hesperidin has an inhibiting effect on LDL oxidation *(43)*. Oxidized LDL is an important marker for the prevention of atherosclerosis *(46)*. Hesperidin's protective effect may be explained by its direct antioxidant effect as a radical scavenger and by its indirect effect on the endogenous antioxidative system. Hesperidin has been shown to increase the activity of the endogenous antioxidative enzymes such as catalase (CAT) and superoxide dismutase (SOD) *(47)***.** Moreover, Crespo and others showed that hesperidin was able to increase significantly glutathione levels in colitic animals *(48)*. Hesperidin has been shown to regulate hepatic cholesterol synthesis by inhibiting the activity of HMG-CoA reductase in rats and has a lowering effect on serum triglycerides, cholesterol and LDL. Moreover, hesperidin significantly increased HDL levels in hypercholesterolemic rats and elevated the HDL to LDL ratio *(49)*. In addition, citrus-based polyphenolic dietary supplements and hesperidin may help preventing obesity by decreasing Body Mass Index (BMI) and elevating leptin levels *(50, 51)*.

In conclusion, dried orange flakes contain high concentrations of antioxidant compounds and as a consequence possess a high antioxidant potential. Incorporation of a nutrition bar based on citrus flakes into a balanced diet represents a practical dietary strategy in the management of healthy blood lipid profiles, prevents LDL oxidation and may be beneficial in the prevention of atherosclerosis, mainly in hypercholesterolemic patients of either gender without side effects. Data pointed out that there was a shift of numbers of people to reduced ranges of serum ratios as a function of consuming our snack-bar daily for 4 months. Additionally, citrus flakes may act as appetite suppressants. In the near future we intend to perform a double-blind trail and to test the ability of this snack bar to elevate satiety.

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