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Nutritional Supplement-5 with a Combination of Proteasome Inhibitors (Resveratrol, Quercetin, δ-Tocotrienol) Modulate Age-Associated Biomarkers and Cardiovascular Lipid Parameters in Human Subjects

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Abstract

Background: Age-associated altered redox imbalances and dysregulated immune function, contribute to the development of a variety of age associated diseases. Inflammatory markers and lipid profiles are useful prognostic indicators of a variety of age-associated and cardiovascular diseases. We have previously studied the impact of several proteasome inhibitors on several markers of inflammation and lipid profiles *in vitro, in vivo*, in cell lines, animal models, and in human subjects. The current study represents an extension of this work. Our main hypothesis is that a combination of various naturally-occurring proteasome inhibitors, which inhibits nitric oxide (NO), and C-reactive protein (CRP) mediated inflammation, will have better efficacy in the prevention and treatment of age-associated disorders including cardiovascular disease.

Methods: Two double blind, randomized, placebo-controlled cross-over trials were conducted to determine the impact of a mixture of NS-5 (resveratrol, pterostilbene, quercetin, δ-tocotrienol, nicotinic acid) on serum NO, CRP, γ-glutamyl-transferase (γ-GT) activity, total antioxidant status (TAS), total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides levels. Healthy seniors (Group-1; *n* = 32) free-living (A, B; 16/group), and hypercholesterolemic (Group-2; *n* = 64) subjects on AHA-Step-1-diet were divided into two groups (C, D; 32/group). Baseline levels were established for parameters as mentioned above. Groups A, C were administered 4-capsules/d of NS-5 and groups B, D, placebo (starch) for 6-weeks. Groups were crossed-over, followed by a 2-week wash-out period. Groups A, C were given 4-capsules/d of placebo and groups B, D, 4-capsules/d of NS-5 for 6-weeks. Groups C, D were continued on AHA-Step-1-diet.

Results: All the subjects completed each phase in both studies without any complaints. There were significant (***P*** < 0.01 – 0.05) decreases in the serum levels of NO (30%, 26%), CRP (29%, 21%), γ-GT activity (14%, 17%), and blood pressure (systolic/diastolic, 3/6%, 3/3%) of Groups A and B, respectively, of free-living healthy seniors without affecting the total, HDL-, LDL-cholesterol or triglycerides compared to their respective baseline values. However, serum levels of NO (36%, 43%), CRP (31%, 48%), γ-GT (17%, 20%), total cholesterol (19%, 15%), LDL-cholesterol (28%, 20%), triglycerides (11%, 18%) of Groups C and D were significantly (***P*** < 0.01-0.05) decreased with NS-5 treatment of hypercholesterolemic subjects compared to baseline values, without affecting the serum HDL-cholesterol levels. The serum levels of total antioxidant status (TAS) were increased (10%, 14%; ***P*** < 0.05) in Groups A and B, increased (19%, 24%; ***P*** < 0.02), and blood pressure (systolic/diastolic, 5/6%, 3/5%) in Groups C and D with NS-5 treatment, compared to respective baseline values.

Conclusions: The consumption of NS-5 mixture decreased significantly serum NO, CRP and γ-GT levels, improved TAS and lipid profiles at risk cardiovascular and hold promise for delaying onset of age-associated diseases.

Keywords: Anti-inflammatory and anti-ageing agents; Resveratrol; Quercetin; δ-tocotrienol; Nitric oxide (NO); C-reactive protein (CRP); γ-glutamyl-transferase (γ-GT); Total antioxidant status (TAS)

Abbreviations: TNF-α: Tumor Necrosis Factor-α; IL-1β: Interleukin-1β; IL-6: Interleukin-6; NO: Nitric Oxide; CRP: C-Reactive Protein; iNOS: Inducible Nitric Oxide Synthase; PAMPs: Pathogen-associated Molecular Patterns; AP-1: Activator Protein-1; COX-2: Cyclooxygenase-2; HMG-CoA: β-hydroxy-β-methylglutaryl-coenzyme A; ICAM: Intracellular Adhesion Molecule-1; IκB: Inhibitory Kappa B; LPS: Lipopolysaccharide; MCP-1: Macrophages Chemoattractant Protein-1; MIP-1α; Macrophage Inflammatory Protein-1α ;NF-κB: Nuclear Factor-kappa B

Introduction

As humans age, they are at increased risk for a variety of age-associated diseases including, arthritis, diabetes, obesity, dementia, cancer, atherosclerosis and cardiovascular disease [1,2]. Over the past decade it has become increasingly appreciated that dysregulated immune function, leading to chronic inflammation, contributes

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to the pathogenesis of several of these age-associated diseases [2]. Inflammatory cytokines and other enzymes and adhesion molecules associated with inflammation (TNF-α, IL-1β, IL-6, COX-2, iNOS, PAMS, VCAM-1, ICAM-1, CRP) have all been shown to be up-regulated during the ageing process, and high plasma levels of CRP and IL-6 have been correlated with disability, morbidity, and mortality in elderly humans [2,3-7]. Moreover, the production of NO increases several-fold during ageing [8,9]. Gene expression for many of these inflammatory markers is modulated by transcription factor NF-κB, which may be a master regulator of the inflammatory process, and can be activated by oxidative stimuli [10] and the proteasome [11]. Over the last several years, our research has focused on the role of the proteasome in inflammatory responses [11]. We have identified a number of compounds that have the capacity to inhibit cellular proteasomes, and demonstrated that these compounds have anti-inflammatory properties, largely through their capacity to inhibit NF-κB activation [12-16]. Age-associated altered redox imbalances can activate the immune system through increased production of superoxide (O_2^-), hydroxyl radical (*OH), hydrogen peroxide (H_2O_2), reactive nitric oxide (NO), peroxynitrite ($ONOO^-$), and reactive lipid aldehydes [2].

We have been particularly interested in age-associated increases in NO, which plays an important role in many physiological activities, such as relaxation of smooth muscle, lysis of tumor cells, and destruction of microorganisms [17,18]. We recently reported that NO levels increase with age, that NO levels are significantly higher in hypercholesterolemic, as compared to humans with normal cholesterol levels, and that plasma NO levels are decreased by dietary supplementation with a combination of resveratrol, pterostilbene, quercetin, δ-tocotrienol, riboflavin, morin hydrate, and nicotinic acid (NS-7, or NS-6) [12]. Levels of other markers of cardiovascular risk (CRP, γ-glutamyl-transferase {γ-GT} activity and uric acid) were also reduced via these dietary supplementations [12]. Dietary supplementation with NS-7 or NS-6 did not have an effect on total cholesterol, LDL-cholesterol, or triglyceride levels of adults (~65 years old) with normal cholesterol levels. With hypercholesterolemic subjects, however, combined intervention with dietary supplementations and restriction to the AHA Step-1 diet reduced modestly serum total, LDL-cholesterol, and triglycerides levels [12].

The main differences between our recently published study and the present one are as follows: The only complaint during our most recent clinical trial was recorded by female participants, whose urine color became deep yellow due to riboflavin in the NS-6 or NS-7 mixtures [12]. Several participants failed to complete the trial due to concerns about their urine color. Therefore, riboflavin was eliminated from the NS-5 in the current study. Moreover, the reductions in serum total cholesterol, LDL-cholesterol and triglycerides levels in our earlier trials were not very pronounced as compared to our previous findings with tocotrienols [19,20], which may have been due to the use of low doses (2 capsules/d) of NS-6 or NS-7 dietary supplements. Therefore dosages of the NS-5 (resveratrol, pterostilbene, quercetin, δ-tocotrienol, and nicotinic acid; Figure 1) were increased from two capsules/d to four capsules/d (400 mg/capsule), as compared to our earlier study [12]. Furthermore, after screening, the hypercholesterolemic human subjects were not restricted to AHA Step-1 diet prior to the administration of two capsules of NS-6 or NS-7 [12]. The results would have been much better if all of the hypercholesterolemic subjects were transferred to AHA Step-1 diet for at least 4-weeks prior to treatment with NS-6 or NS-7 to establish the impact of dietary restriction. The current double blind, randomized, placebo cross-over trials were therefore conducted

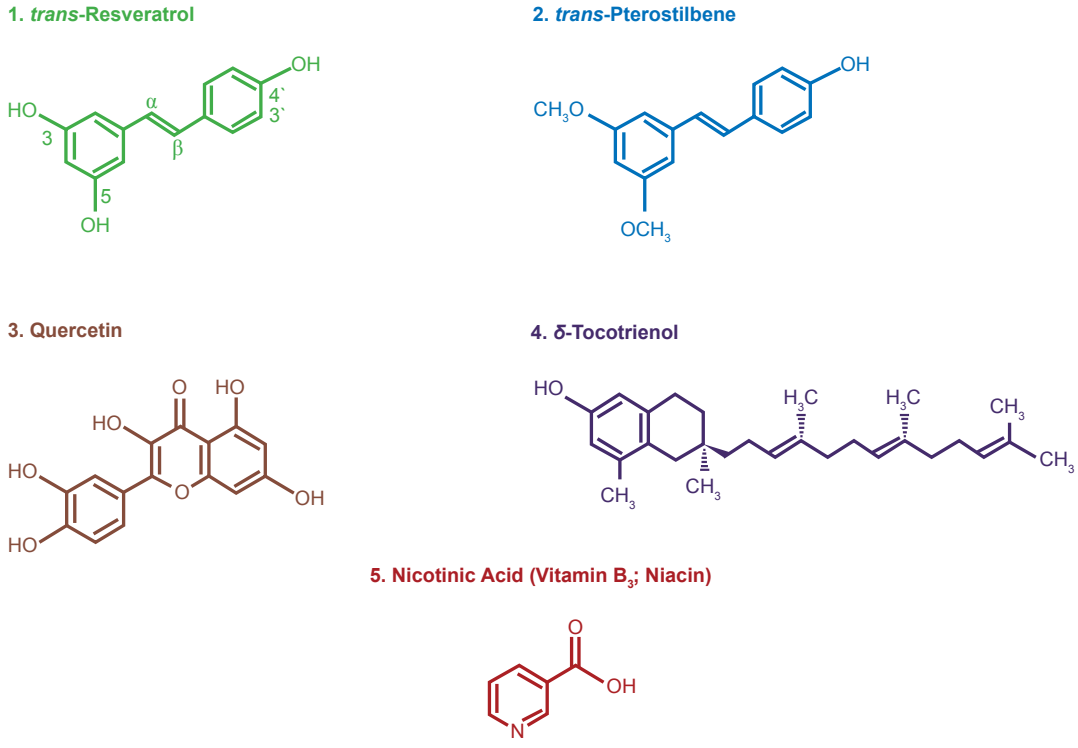


Figure 1: Chemical structures of nutritional supplements used in this study.

with free-living healthy seniors (> 65 years old) as well as with hypercholesterolemic (50 years old – 75 yr) subjects who were restricted to the AHA Step-1 diet for 4 weeks, prior to treatment with NS-5 capsule. The present study was designed to avoid the aforementioned confounding factors and it demonstrates a greater impact of nutritional supplement-5 (NS-5) on serum levels of NO, CRP, γ-GT activity, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides and antioxidant status. The overreaching goal of this research is to decrease the impact of age-associated diseases through dietary supplementation. Specifically, we have been focusing on FDA approved naturally-occurring compounds that are safe for human consumption and those that suppress inflammation via inhibition of the proteasome. The study was carried out under FDA approved IND number 036906.

Materials and Methods

Materials

The 50% purified δ-tocotrienol fraction from annatto seeds was purchased from American River (Boston, MA, USA), and purified to 98% δ-tocotrienol from the 50% δ-tocotrienol fraction by flash chromatography in house as described previously [16]. The purity of δ-tocotrienol was established by high pressure liquid chromatography (HPLC) against its standard as reported earlier [16]. *trans*-Resveratrol from ("Mega Resveratrol", 60 Newtown Road # 32, Danbury CT, USA), *trans*-pterostilbene (Shanxi Yong Yuan, Biotechnology Co, Ltd. China), and nicotinic acid (niacin, vitamin B_3) were purchased from (VOIGT Global Distribution Inc, P. O. Box. 1130, Lawrence, Kansas, USA). Quercetin was purchased from Alfa Aesar (Johnson Matthey Co. Lancastor, UK).

Composition of nutritional supplement-5 (NS-5)

Each 400 mg capsule of NS-5 contains δ-tocotrienol (25 mg from annatto seeds) + quercetin (50 mg) + resveratrol, pterostilbene, nicotinic acid (25 mg of each) + corn starch (250 mg). The placebo contained 400 mg/capsule of corn starch. The capsulation of mixture of NS-5, and placebo, and packing (80 capsules/bottle) was carried out at Kabco Inc. New Jersey, USA. The bottles were labeled as AMR-1, and AMR-2, respectively.

Experimental design

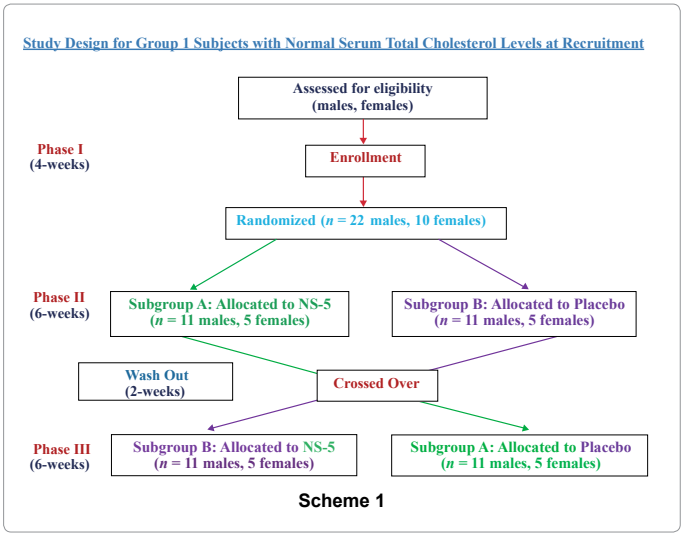
The present studies were carried out as double blind crossed over randomized control trial of dietary nutritional supplement (NS-5) in free-living healthy senior (> 65 years old) and in hypercholesterolemic citizens of Wah Cant, Pakistan, as outlined below. The study protocol was registered at the government agency (National University of Science and Technology, Pakistan), and approved by institutional review committee (IRC) of the Army Medical College, Abid Majeed Road, Rawalpindi, 64000, Pakistan. All participants signed an informed-consent statement, which was approved by the institutional review committee. The study was also carried out under FDA approved "Investigating New Drug" (IND) number 036906. The protocols, methods, results, and conclusions of the study were reported to FDA every year as Progress Reports since the very outset.

Initial screening and exclusion criteria

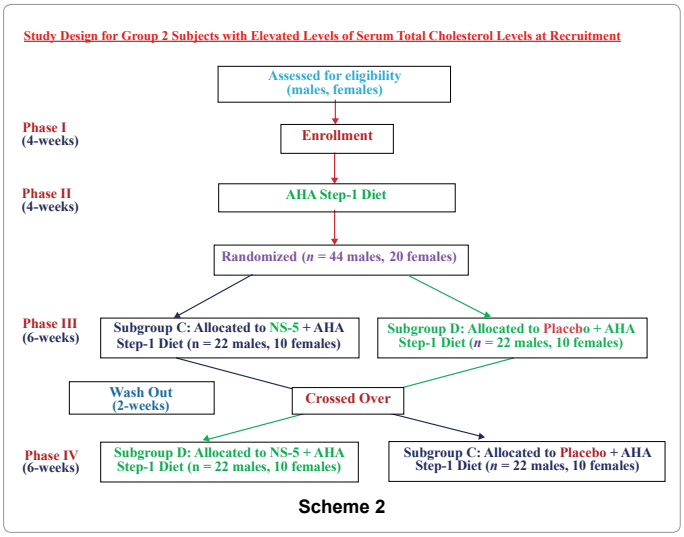
Two groups of subjects were included in the study. Study group 1 had free-living normal serum total cholesterol levels and Study Group 2 had elevated serum total cholesterol levels at the time of recruitment. Clinical history and physical examination were carried out for each participant. Initial measurements included the participants' height, weight, and systolic, diastolic blood pressure at rest (pre-treatment), and fasting serum glucose level. Also, subjects were queried on their history of significant diseases, medications taken (nitrates, calcium antagonist, angiotension-converting enzyme (ACE) inhibitors, and diuretics) and smoking habits. The participants' height and weight were measured while wearing light-weight clothing and no shoes. Body mass index (BMI, kg/m^2) was used as a measure of relative body weight. Weights were recorded daily. Venous blood samples (12 h fast, 7–9 am) were drawn at screening, to determine potential eligibility for Study Groups 1 or 2. Liver function, thyroid stimulating hormone (TSH), serum urea and fasting plasma glucose were analyzed to exclude any liver, thyroid, renal disorders and diabetes mellitus, respectively. Exclusion criteria included weight (> 125% of Metropolitan Life relative weight), an elevated serum glutamate-pyruvate or glutamate-oxaloacetate transaminase activity, an elevated blood urea nitrogen or fasting glucose value, or hypertensive disease.

Study group 1: The subjects were recruited from a free-living healthy population (age>65 yr and serum total cholesterol levels <5.0 mmol/L) from the "Senior Citizen Community Centre" in Wah Cant, Pakistan.

Prospective participants were grouped according to serum total



Scheme 1



Scheme 2

cholesterol levels (> median <, middle or average values) and sub-grouped by sex. There were a total of 32 subjects in Study Group 1; 22 males and 10 females. All subjects from Study Group 1 completed all 3 phases of the study as outlined in scheme below.

Phase I: Baseline serum levels for various inflammatory markers and lipid parameters were established for all Study Group 1 subjects during the first four weeks of the study (phase I). Venous blood samples (5 mL) (12 h fast, 7:00 – 9:00 am) were drawn at recruitment and at the end of each phase. Three-day diet records were taken prior to the start of phase I, and this recording was sustained each week throughout all 3 phases of the study, and during the washout period, in order to monitor the dietary intake of each subject. At the end of phase I, the members of Study Group 1 were randomly divided into two subgroups A and B, using a random number. There were 16 subjects in subgroup A (11 males and 5 females), and 16 subjects in subgroup B (11 males and 5 females) as reported in scheme below.

Phase II: Phase II lasted 6 weeks. During this 6 week period, subjects of subgroup A were given 4 capsules/d of NS-5 (400 mg/capsule; 2 capsules after breakfast and 2 capsules after dinner), and subjects of subgroup B were given 4 capsules/d of placebo (2 capsules after breakfast and 2 capsules after dinner).

Washout: After phase II was completed, all subjects in both subgroups stopped taking NS-5 or placebo capsules for two weeks. Dietary intake was monitored during this two week washout period.

Phase III: Phase III lasted 6 weeks. During this 6 week period, the subjects were crossed-over. Subjects of subgroup A, who took NS-5 capsules in phase II, were administered 4 capsules/d of placebo. Subjects of subgroup B, who took placebo during phase II, were given 4 capsules of NS-5 for 6 weeks.

Study group 2: Study Group 2 subjects were also recruited from the "Senior Citizen Community Centre" in Wah cant, Pakistan. In order to be eligible for study Group 2, subjects had to be 55 years of age and have serum total cholesterol levels > 6.4 mmol/L. There were a total of 64 subjects in Study Group 2; 44 males and 20 females. All subjects from Study Group 2 completed all 4 phases of the study as outlined in scheme below.

Phase I: Baseline serum levels for various inflammatory markers and lipid parameters were established for all Study Group 2 subjects during the first four weeks of the study (phase I). Venous blood samples (5 mL) (12 h fast, 7:00 – 9:00 am) were drawn at recruitment and at the end of each phase. Study Group 2 subjects met individually and in small group sessions with counselors, where the individual sessions focused on 24-h food consumption recall, and the group sessions provided instruction for keeping 3-day records of food intake (2 weekdays + 1 weekend day). All subjects were encouraged to follow their typical dietary pattern during phase I, and were instructed to keep food records for the terminal 3 days of this and remaining phases. Subjects also received an unanticipated telephone call for a 24-h recall of food intake after every two weeks. Diet records and 24-h recalls were analyzed; if required, a participant was individually counseled to modify food intake to maintain weight.

Phase II: Phase II lasted 4 weeks. During this 4 week period all of Study Group 2 subjects were limited to an American Heart Association (AHA) Step-1 diet, which limited their intake of cholesterol to 300 mg/day and only 30% energy from fat (by cutting out whole milk, ice cream, cheese, eggs, butter, and using skimmed milk for 4 weeks). Subjects met in small groups to discuss the relationship between diet and cardiovascular risk factors and for instruction on following the AHA Step-1 diet. Each subject received a copy of the 1988 AHA Step-1 diet, Patient Manual, and the telephone number of a staff contact person. To ensure subject-adherence to the AHA Step-1 diet, counseling continued for the duration of the study. At the end of phase II, the members of Study Group 2 were randomly divided into two subgroups C and D, using a random number. There were 32 subjects in subgroup C (22 males and 10 females), and 32 subjects in subgroup D (22 males and 10 females), as outlined in above scheme.

Phase III: Phase III lasted 6 weeks. During this 6 week period, subjects of subgroup C were given 4 capsules/d of NS-5 (400 mg/capsule; 2 capsules after breakfast and 2 capsules after dinner), and subjects of subgroup D were given 4 capsules/d of placebo (2 capsules after breakfast and 2 capsules after dinner). All patients were maintained on the AHA Step-1 diet, and dietary intake was monitored throughout this phase.

Washout: After phase III was completed, all subjects in both subgroups stopped taking NS-5 or placebo capsules for two weeks. All subjects were maintained on the AHA Step-1 diet, and dietary intake was monitored during this two week washout period.

Phase IV: Phase IV lasted 6 weeks. During this 6 week period, the subjects were crossed-over. Subjects of subgroup C, who took NS-5 capsules in phase III, were administered 4 capsules/d of placebo. Subjects of subgroup D, who took placebo during phase II, were given 4 capsules of NS-5 for 6 weeks, and maintained the AHA Step-1 diet for 6 weeks. All patients were maintained on the AHA Step-1 diet, and dietary intake was monitored throughout this phase.

Biochemical Analyses: All laboratory analyses were performed at the Department of Chemical Pathology, Army Medical College, NUST, Abid Majeed Road, Rawalpindi, 64000, Pakistan according to validated standard procedures of the lab as previously described [12,14].

Blood sample collection

Venous blood samples (5 mL) were drawn from each subject after an overnight-fast (12 h fast, 9 pm to 9 am) at screening, and at the conclusion of each phase, including systolic, diastolic blood pressure at rest (post-treatment). Processed serum samples were coded and held at -72°C. After initial screening, serum samples collected from Study Group 1 and 2 subjects were stored and held for analysis until all phases of the study were completed. The serum levels of NO, CRP, total antioxidant status, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, and γ-GT activity were determined as previously described in detail [12]. Data were reported as means ± SE in Table and Figures.

Estimation of serum levels of nitric oxide (NO)

In short, serum nitrate was carried out by colorimetric assay based on Griess reagent, using standard kit procedure (Cayman kit, Ann Arbor, MI.) at 540 nm on ELISA reader (Diamate 710, UK). Serum nitrate was measured as nitrite after enzymatic reduction by incubating with nitrate reductase and NADPH. After incubation, the reaction mixture was deproteinized and Griess reagent was added. After 10 min of color development (deep purple) at room temperature, the absorbance was measured by micro-plate reader at 540 nm. Values obtained by this procedure represent the sum of nitrate and nitrite. CV of the method was 4.1%.

Estimation of serum levels of total antioxidant status (TAS)

Serum total antioxidant status (TAS) was estimated by a kinetic

colorimetric assay kit (Randox, Crumlin, UK), on the automated clinical chemistry analyser, Selectra E (Vita Lab, Netherland) by following the previously reported method [21]. Serum TAS present in sample causes the decrease of 2,2'-Azino-di-[3-ethylbenthiazoline sulphonate] (ABTS) formation (radical cation) which form a relatively stable blue green color when incubated with peroxidase (metmyoglobin) and H_20_2. The decrease in color formation of ABTS is proportional to the concentration of TAS, which is measured at 600 nm.

#	Characteristics	Free-living healthy senior subjects		Hypercholesterolemic subjects	
		Group-A	**Group-B**	**Group-C**	**Group-D**
1	***Subjects***				
	Males (*n*)	11	11	22	22
	Females (*n*)	5	5	10	10
	Total (males + females) *n* =	**16**	**16**	**32**	**32**
2	***Age (years)***				
	Males	66.82 ± 2.33*	65.73 ± 1.89*	58.50 ± 0.66*	57.68 ± 1.15*
	Females	68.00 ± 4.44	68.00 ± 1.10	57.80 ± 0.87	56.60 ± 1.50
	Total (males + females)	65.41 ± 3.39	66.86 ± 1.50	58.15 ± 0.77	57.14 ± 1.33
3	***Body weight (kg)***				
	Males	79.36 ± 1.50	81.45 ± 0.97	72.32 ± 0.87	72.64 ± 0.65
	Females	78..00 ± 1.87	79.60 ± 1.21	73.00 ± 1.26	75.00 ± 1.67
	Total (males + females)	78.68 ± 1.69	80.53 ± 1.09	72.66 ± 1.07	73.82 ± 1.16
4	***Body mass index (kg/m²)***				
	Males	24.82 ± 0.38	26.07 ± 0.37	23.04 ± 0.29	24.16 ± 0.32
	Females	25.67 ± 0.92	31.08 ± 0.81	27.07 ± 0.75	27.26 ± 0.68
	Total (males + females)	25.25 ± 0.65	28.57 ± 2.51	25.05 ± 2.20	25.71 ± 0.50
5	***Initial height (m)***				
	Males	1.79 ± 0.01	1.77 ± 0.01	1.77 ± 0.01	1.74 ± 0.01
	Females	1.75 ± 0.04	1.60 ± 0.01	1.65 ± 0.03	1.66 ± 0.03
	Total (males + females)	1.77 ± 0.03	1.69 ± 0.01	1.71 ± 0.02	1.70 ± 0.02
6	***Blood pressure (mmHg)***				
	Males				
	Pre-treatment **(systolic/diastolic)**	134.55 ± 1.42/87.73 ± 1.04	137.73 ± 1.56/88.64 ± 1.92	140.45 ± 1.14/90.68 ± 1.24	138.64 ± 1.00/91.59 ± 0.83
	Post-treatment	130.00 ± 0.95/81.82 ± 0.76	135.00 ± 1.51/85.91 ± 1.13	135.00 ± 0.99/84.09 ± 0.91	135.23 ± 0.96/87.50 ± 1.17
	Females				
	Pre-treatment	138.00 ± 2.00/88.00 ± 3.00	138.00 ± 2.00/91.00 ± 2.92	143.00 ± 1.70/92.50 ± 2.01	139.00 ± 1.63/90.00 ± 1.97
	Post-treatment	133.00 ± 2.00/83.00 ± 1.22	133.00 ± 2.00/88.00 ± 1.22	135.50 ± 1.89/86.00 ± 1.45	134.00 ± 1.45/85.50 ± 1.74
	Total (males + females)				
	Pre-treatment	136.00 ± 1.70/87.90 ± 2.02	137.86 ± 1.78/89.82 ± 2.42	141.73 ± 1.42/91.59 ± 1.63	138.82 ± 1.32/90.80 ± 1.40
	Post-treatment	131.50 ± 1.50/82.40 ± 0.99	134.00 ± 1.76/86.95 ± 1.18	135.25 ± 1.44/85.05 ± 1.18	134.61 ± 1.21/86.50 ± 1.46
7	***Initial total cholesterol (mmol/L)***				
	Males	4.46 ± 0.13	4.03 ± 0.20	5.91 ± 0.08	6.06 ± 0.12
	Females	4.51 ± 0.24	4.20 ± 0.35	6.11 ± 0.14	6.52 ± 0.17
	Total (males + females)	4.48 ± 0.19	4.12 ± 0.28	6.01 ± 0.11	6.29 ± 0.15
8	***Initial triglycerides (mmol/L)***				
	Males	1.37 ± 0.13	1.16 ± 0.07	1.74 ± 0.14	2.16 ± 0.13
	Females	1.68 ± 0.32	1.34 ± 0.19	1.66 ± 0.17	2.19 ± 0.19
	Total (males + females)	1.52 ± 0.23	1.25 ± 0.13	1.70 ± 0.16	2.17 ± 0.16

[1]The subjects were fasted for 12 h, and the Physical Examination was done at 0800 h
*Data expressed as means ± SE (Standard Error)

Table 1: Characteristics of the study populations of free-living healthy senior and hypercholesterolemic human subjects[1].

Estimation of serum levels of lipid parameters, CRP and γ-glutamyl-transferase activity (γ-GT)

Serum total cholesterol was measured by a cholesterol oxidase method (CHOD-POD), and serum triglycerides by a GPO-POD colorimetric standard method using Pioneer Diagnostics Kit (USA, a Lot No. 804-FV). Serum HDL-cholesterol, and LDL-cholesterol were also analyzed. Serum γ-glutamy-ltransferase (γ-GT) activity was analyzed at 37°C by using glutamyl-3-carboxy-4-nitroanilide as substrate according to the protocol of Human kit (Germany) [22]. All the above mentioned assays were carried out on an automatic Chemistry analyser Selectra E (Vita Scientific, Netherland). Serum hS-CRP was analyzed by a two-site sequential chemiluminescent immunometric assay kit (Seimen, LA, California, USA) on Immulite 1000 (Immulite, Diagnostic Product Corporation, USA) with reagent used according to manufacturer directions. The analytical sensitivity was 0.1 mg/L. Elevated CRP was defined for values higher than > 4.0 mg/L.

Statistical analysis

Statistical analysis was performed using SPSS 16 (SPSS Inc, Chicago). Continuous, normally distributed variables were summarized as means ± SE, and percent differences were calculated from baseline values of each inflammatory marker or lipid parameter analysis of two-way variance was used to test whether changes in serum, CRP, γ-GT activity, oxidative stress and lipid parameters, occur in the course of supplementation, and whether there were between- and within-subject differences; because all observations were required, available degrees of freedom were reduced by this statistical approach. Paired Student's t-test was applied for normally distributed variables. A two tailed ***P*** value < 0.05 was considered significant.

Results

Four capsules of dietary Nutritional Supplement-5 (NS-5) or Placebo (corn starch) to each subject were administered for 6-weeks. Data were reported as means ± SE (Standard Error) in text, Table and Figures.

Characteristics of the study populations

The main characteristics (number of subjects, age, height, body weights and body mass index) of the study population were broken down by study group and subgroup. The subgroups of different of study groups were well matched for the markers or parameters determined. The detailed values were reported for male, female and combined male + female subjects for each marker or parameter in Table 1. In summary, there were 16 subjects in group A (11 males and 5 females; 65.41 ± 3.39 yrs of age, 78.68 ± 1.69 kg weight, 25.25 ± 0.65 body mass index, blood pressure (systolic/diastolic, mmHg) pre-dose 136.00 ± 1.70/87.90 ± 2.02, post-dose 131.50 ± 1.50/82.40 ± 0.60, and average baseline levels of serum total cholesterol 4.09 ± 0.20 mmol/L, triglycerides 1.52 ± 0.23 mmol/L, LDL-cholesterol 2.79 ± 0.10 mmol/L). The 16 subjects in group B were also 11 males and 5 females; 66.86 ± 1.50 yrs of age, 80.53 ± 1.09 kg weight, 28.57 ± 2.51 body mass index, blood pressure (systolic/diastolic, mmHg) pre-dose 137.86 ± 1.78/89.82 ± 2.42, post-dose 134.00 ± 1.76/86.95 ± 1.18, and average baseline levels of serum total cholesterol 4.12 ± 0.28 mmol/L, triglycerides 1.25 ± 0.13 mmol/L, LDL-cholesterol 2.65 ± 0.13 mmol/L).

Hypercholesterolemic participants of group C were 22 males and 10 females (58.15 ± 0.77 yrs of age, 72.66 ± 1.07 kg weight, 25.05 ± 2.20 body mass index, blood pressure (systolic/diastolic, mmHg) pre-dose 141.73 ± 1.42/91.59 ± 0.1.63, post-dose 135.25 ± 1.44/85.05 ± 1.18, and average baseline levels of serum total cholesterol 6.01 ± 0.11 mmol/L, triglycerides 1.70 ± 0.16 mmol/L, LDL-cholesterol 4.05 ± 0.07 mmol/L). The subjects of group D were also 22 males and 10 females (57.14 ± 1.33 yrs of age, 73.82 ± 1.16 kg weight, 25.71 ± 0.50 body mass index, blood pressure (systolic/diastolic, mmHg) pre-dose 138.82 ± 1.32/90.80 ± 1.40, post-dose 134.61 ± 1.21/86.50 ± 1.46, and average baseline levels of serum total cholesterol 6.29 ± 0.15 mmol/L, triglycerides 2.17 ± 0.16 mmol/L, LDL-cholesterol 3.99 ± 0.10 mmol/L). There was no change in the body weight, body mass index, and height of the participants during the treatment period in study # 1 (groups A and B) or study # 2 (groups C and D), and there were moderate decreases in blood pressure and glucose levels after consuming 4 capsules/d of NS-5 or 4 placebo capsules (groups A, B and groups C, D) for 6 weeks in free-living healthy seniors and hypercholesterolemic humans, indicating that components of NS-5 are safe, without any adverse effect and might be useful for human consumption.

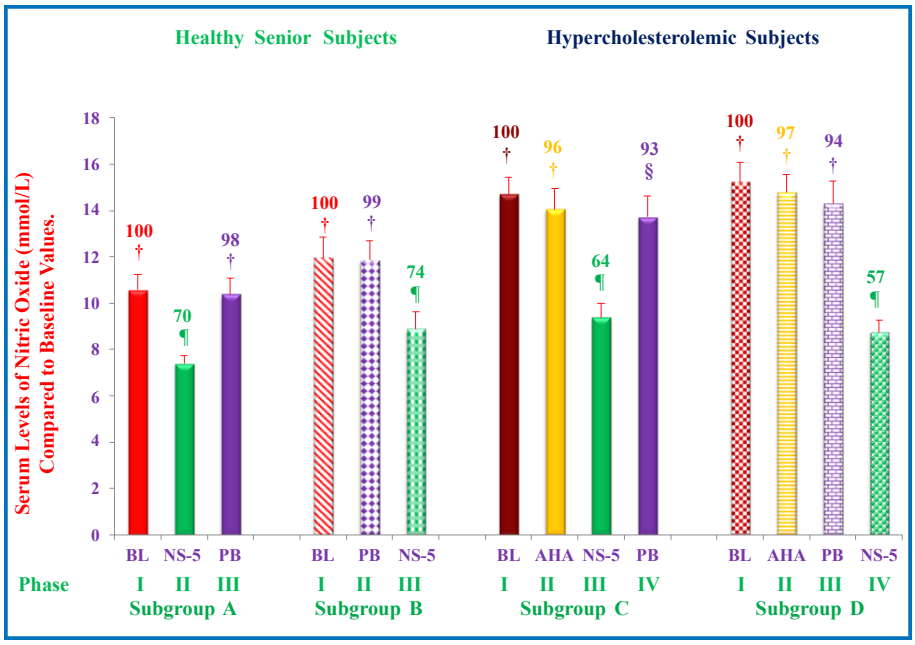
Consumption of NS-5 mixture reduces serum NO, CRP, and γ-GT levels (Figures 2-4)

We previously demonstrated that NO production was suppressed by naturally-occurring proteasome inhibitors in endotoxin stimulated murine macrophages, and that combinations of these proteasome inhibitors can enhance this suppressive effect [13]. We also demonstrated that serum NO, CRP, and γ-GT levels were reduced in humans whose diets were supplemented with combinations of proteasome inhibitors [12]. In that latter study, however, there was a significant dropout rate due to urine discoloration associated with riboflavin in the diet supplement. NS-5, the diet supplement used in the current study contained δ-tocotrienol, quercetin, resveratrol, pterostilbene, and nicotinic acid, but did not contain riboflavin. Deleting riboflavin from the supplement appears to have had its intended effect, as all subjects who entered the study completed all phases of the study. No significant adverse events were associated with ingesting this supplement.

Consumption of NS-5 mixture reduces serum levels of nitric oxide (NO) in all the subjects of study groups 1 and 2

The subjects of Study Group 1 consisted of free-living healthy seniors with normal serum total cholesterol levels were selected at entry level during phase I. Serum NO levels were significantly reduced in subjects receiving NS-5 of subgroup A compared to baseline levels (30% ***P*** < 0.02) at the end of phase II (6 week duration), as shown in Figure 2. Serum NO levels were not changed compared to baseline and at the end of phase II for subjects receiving placebo capsules (subgroup B). After a 2 week washout period, the subjects were crossed over for phase III, so that subgroup A now received placebo, while subgroup B received NS-5 capsules for 6 weeks. At the end of phase III, serum NO levels were also significantly reduced in subjects receiving NS-5 capsules of subgroup B compared to respective baseline levels (26% <0.02; Figure 2). Serum NO levels for subgroup A subjects, who received placebo capsules during the 6 weeks of phase III, returned to near baseline levels (Figure 2).

The subjects with elevated serum total cholesterol levels (hypercholesterolemic) were selected in Study Group 2 at entry level during phase I. During the 4 weeks of phase II, all Study Group 2 subjects were instructed to adhere to an AHA Step-1 diet and their diet was monitored weekly. No noticeable changes were seen between baseline levels (measured at the end or phase I) of NO and levels measured after 4 weeks of dietary restriction (end of phase II). After phase II, all



Figures 2: Serum levels nitric oxide (NO) are decreased with NS-5 consumption. The treatments 1 – 14 columns in the figure correspond to: Group A, *n* = 16; 1 = BL (Baseline); 2 = NS-5 capsule (Nutrition Supplement-5); 3 = PB capsule (Placebo-starch); Group B, *n* = 16; 4 = BL; 5 = PB capsule; 6 = NS-5 capsule; Group C, *n* = 32; 7 = BL; 8 = AHA (American Heart Association Step-1 diet); 9 = AHA + NS-5 capsule; 10 = AHA + PB capsule; Group D, *n* = 32; 11 = BL; 12 = AHA; 13 = AHA + PB capsule; 14 = AHA + NS-5 capsule. The baseline serum NO levels of free-living healthy seniors of subgroups A and B were established during phase I. Subjects were then randomized to receive NS-5 capsule (subgroup A) or placebo capsule (subgroup B) for 6 weeks (Phase II). After a 2 week washout (no treatment), the subgroups were crossed over so that subgroups A and B received placebo capsule and NS-5 capsule, respectively, for 6 weeks (phase III). Similarly, the baseline serum NO levels of hypercholesterolemic subjects of subgroups C and D were established during phase I. All the subjects of (C,D) were then placed on an AHA Step-1 diet for 4-weeks, and NO levels measured again after 4 weeks (Phase II). They were then randomized to receive NS-5 capsule (subgroup C) or placebo capsule (subgroup D) for 6 weeks (Phase III). After a 2 week washout, the subgroups were crossed over so that subgroups C and D received placebo capsule and NS-5 capsule, respectively, for 6 weeks (Phase IV). The subjects of subgroup C and D were remained on the AHA Step-1 diet during phases III and IV and during the washout. The experimental details for Figures 3 to 9 were exactly same as described above. Data are means ± SE. Values in a column not sharing a common symbol are significantly different of various groups at A-¶ $P < 0.02$, B-¶ $P < 0.02$, C-¶ $P < 0.02$, D-¶ $P < 0.01$. Percentages of each treatment compared to baseline values are above the column.

subjects of Study Group 2 remained on the AHA Step-1 diet, but were randomized to receive NS-5 capsules (subgroup C) or placebo capsules (subgroup D) for 6 weeks (phase III). At the end of phase III, serum NO levels were significantly reduced in subjects on the AHA Step-1 diet receiving NS-5 capsules (subgroup C) compared to baseline levels (36%, $P<0.02$), and compared to levels after 4 weeks of the AHA Step-1 diet alone (32%, $P<0.02$) levels at the end of phase III (Figure 2). Serum NO levels did not change between baseline and the end of phase III for subjects receiving placebo (subgroup D) 4 capsules/d.

After a 2 week washout period, the subjects were crossed over for phase IV, so that subgroup C now received placebo capsules, while subgroup D received NS-5 capsules for 6 weeks; both subgroups were maintained on the AHA Step-1 diet (Figure 2). At the end of phase IV serum NO levels were significantly reduced in subjects receiving NS-5 capsules (subgroup D) compared to baseline level for this group (43%, $P < 0.01$) and compared to levels measured at the end of phases II and III. Serum NO levels for subgroup C subjects, who received placebo capsules during the 6 weeks of phase IV, returned to baseline, or near baseline levels (Figure 2)

Consumption of NS-5 mixture reduces serum levels of C-reactive protein (CRP) in all the subjects of study groups 1 and 2

The free-living healthy senior subjects with normal serum total cholesterol levels of Study Group 1 were selected at entry level during phase I. Serum CRP was significantly decreased at the end of phase II in subjects receiving NS-5 capsules (subgroup A) compared to baseline levels (29%, $P < 0.03$; Figure 3). Serum CRP did not change between baseline and the end of phase II for subjects receiving placebo capsules (subgroups A or B). After a 2 week washout period, the subjects were crossed over for phase III, so that subgroup A now received placebo capsules, while subgroup B received NS-5 capsules for 6 weeks. At the end of phase III serum CRP levels were also significantly decreased in subjects receiving NS-5 capsules (subgroup B) compared to baseline levels (21% increased; $P < 0.04$). Serum CRP levels for subgroup A subjects, who received placebo capsules during the 6 weeks of phase III, returned to baseline levels (Figure 3).

The hypercholesterolemic subjects with elevated serum total cholesterol levels of Study Group 2 were selected during phase I at entry level. The serum CRP levels were not decreased, compared to baseline, by adherence to the AHA Step-1 diet alone of subgroups C or D for 4 weeks (end of phase II). Dietary supplementation with NS-5 capsules for an additional 6 weeks in conjunction with continuation of the AHA Step 1 diet (subgroup C; phase III) resulted in a significant decrease in CRP as compared to baseline levels (31%, $P < 0.02$). Serum CRP levels changed only 2% between baseline and the end of phase III for subjects receiving placebo capsules (subgroup D; Figure 3). After a 2 week washout period, the subjects were crossed over for phase IV, so that subgroup C now received placebo capsules, while subgroup D received NS-5 capsules for 6 weeks; both subgroups were maintained on the AHA Step-1 diet. At the end of phase IV, serum CRP levels were also significantly decreased in subjects on the AHA Step-1 diet

plus NS-5 capsules (subgroup D) compared to baseline levels (48% $P < 0.01$, Figure 3). Serum CRP levels of subgroup C subjects, who received placebo capsules during the 6 weeks of phase IV, decreased 6% compared to baseline levels (Figure 3).

Consumption of NS-5 mixture reduces serum levels of γ-glutamyl-transferase (γ-GT) in all the subjects of study groups 1 and 2

The serum levels of γ-GT activity decreased significantly in subjects of subgroups A and B (14% and 16%, $P < 0.05$) in Study Group 1 and in Study Group 2, and subgroups C and D (17%, $P < 0.05$ and 20%, $P < 0.02$), respectively, after consuming NS-5 capsules for 6 weeks as compared to respective baseline values (Figure 4) under identical conditions as described in the legend for Figures 2 and 3.

The effects of placebo capsules and AHA Step-1 diet on γ-GT were also similar as observed for serum levels of NO and CRP (Figures 2 and 3).

Consumption of NS-5 mixture improves serum total antioxidant status (TAS; Figure 5)

Age-associated altered redox imbalances can activate the immune system through excessive production of reactive oxygen species, and TAS has been used as a measure of the body's capacity to counteract the potentially detrimental effects of these reactive oxygen species [2,21]. We previously demonstrated that dietary supplementation with a combination of naturally-occurring proteasome inhibitors enhances serum TAS [12]. In the current study, using dietary supplementation with a different combination of proteasome inhibitors, we also observed significant improvements in TAS.

Study group 1 subjects with normal total serum cholesterol levels at entry, serum TAS was significantly increased at the end of phase II in subjects receiving NS-5 (subgroup A) compared to baseline levels (10%; $P < 0.05$ increased; Figure 5). Serum TAS did not change significantly (between baseline and the end of phase II for subjects receiving placebo (subgroup B). After a 2 week washout, the subjects were crossed over for phase III, so that subgroup A now received placebo, while subgroup B received NS-5 for 6 weeks. At the end of phase III serum TAS was also significantly increased in subjects receiving NS-5 (subgroup B) compared to baseline (14%, $P < 0.05$ increased). Serum TAS levels for subgroup A subjects, who received placebo during the 6 weeks of phase III, returned to baseline levels (Figure 5).

Study group 2 subjects with elevated total serum cholesterol levels at entry, serum TAS levels were not significantly increased, compared to baseline, by adherence to the AHA Step-1 diet alone for 4 weeks (end of phase II). Dietary supplementation with NS-5 for an additional 6 weeks in conjunction with continuation of the AHA Step 1 diet (subgroup C; phase III) resulted in a significant increase in TAS compared to baseline levels (19%, $P < 0.04$ increased). Serum TAS levels also changed significantly, but much more modestly (6% increased, $P < 0.05$) between baseline and the end of phase III for subjects receiving placebo (subgroup D; Figure 5). After a 2 week washout, the subjects were crossed over for phase IV, so that subgroup C now received placebo, while subgroup D received NS-5 for 6 weeks; both subgroups were maintained on the AHA Step-1 diet. At the end of phase IV, serum TAS was also significantly increased in subjects on the AHA Step-1 diet receiving NS-5 (subgroup D) compared to baseline (24%, $P < 0.02$ increased; Figure 5). Serum TAS levels for subgroup C subjects, who received placebo during the 6 weeks of phase IV, decreased significantly, but still remained significantly above (9%, $P < 0.05$) baseline levels (Figure 5).

Consumption of NS-5 mixture reduces serum total cholesterol, LDL-cholesterol, and triglycerides levels in subjects with elevated baseline serum total cholesterol levels.

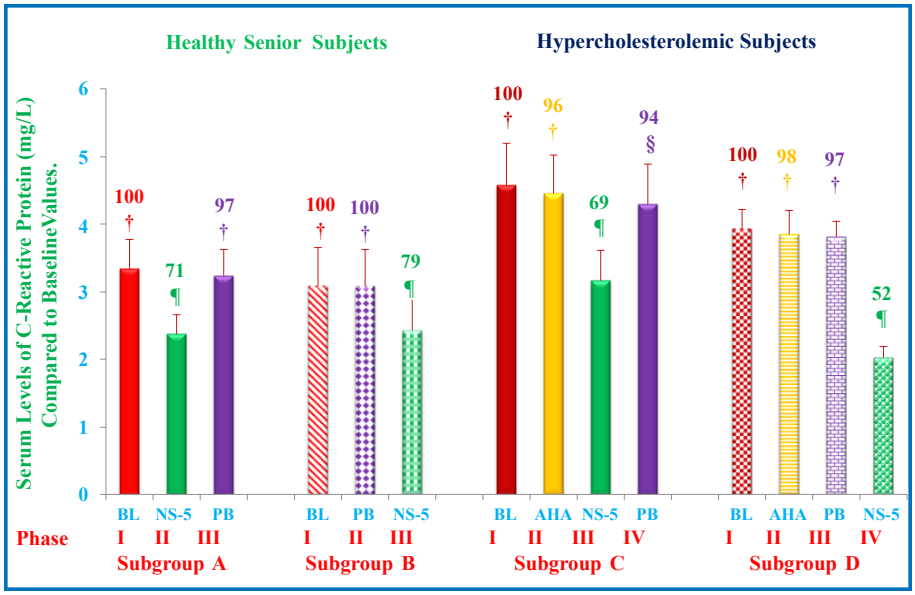


Figure 3: Serum C-reactive protein (CRP) levels are decreased with NS-5 Consumption. The treatments 1 – 14 columns in the figure correspond to: Group A, *n* = 16; 1 = BL (Baseline); 2 = NS-5 capsule (Nutrition Supplement-5); 3 = PB capsule (Placebo-starch); Group B, *n* = 16; 4 = BL; 5 = PB capsule; 6 = NS-5 capsule; Group C, *n* = 32; 7 = BL; 8 = AHA (American Heart Association Step-1 diet); 9 = AHA + NS-5 capsule; 10 = AHA + PB capsule; Group D, *n* = 32; 11 = BL; 12 = AHA; 13 = AHA + PB capsule; 14 = AHA + NS-5 capsule. Data are means ± SE. Values in a column not sharing a common symbol are significantly different of various groups at A-¶ $P<0.03$, B-¶ $P<0.04$, C-¶ $P<0.02$, D-¶ $P<0.01$. Percentages of each treatment compared to baseline values are above the column.

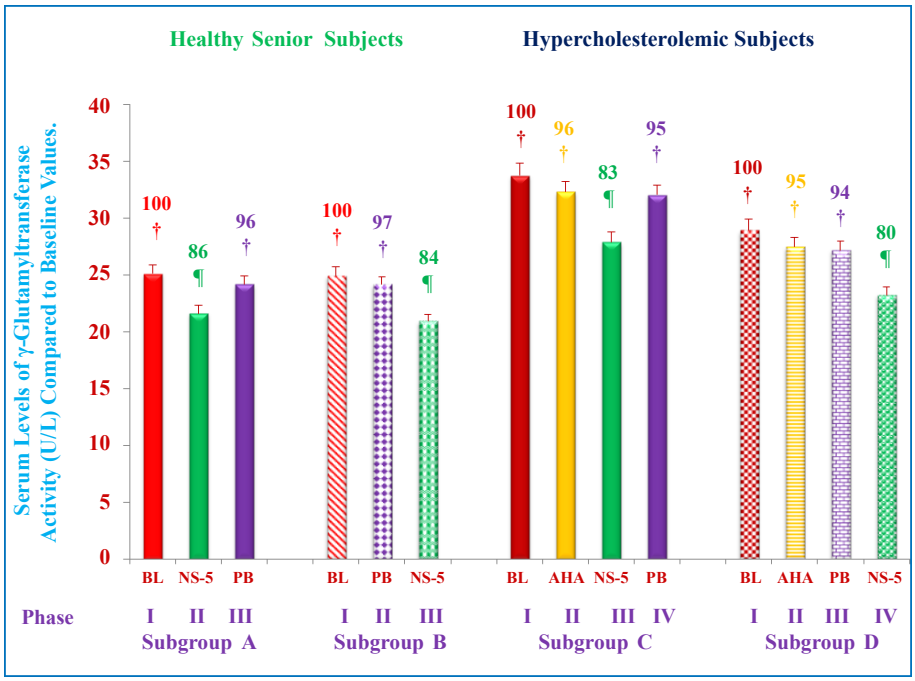


Figure 4: Serum γ-glutamyl-transferase (γ-GT) levels are decreased with NS-5 consumption. The treatments 1 – 14 columns in the figure correspond to: Group A, *n* = 16; 1 = BL (Baseline); 2 = NS-5 capsule (Nutrition Supplement-5); 3 = PB capsule (Placebo-starch); Group B, *n* = 16; 4 = BL; 5 = PB capsule; 6 = NS-5 capsule; Group C, *n* = 32; 7 = BL; 8 = AHA (American Heart Association Step-1 diet); 9 = AHA + NS-5 capsule; 10 = AHA + PB capsule; Group D, *n* = 32; 11 = BL; 12 = AHA; 13 = AHA + PB capsule; 14 = AHA + NS-5 capsule. Data are means ± SE. Values in a column not sharing a common symbol are significantly different of various groups at A-¶ ***P***<0.05, B-¶ ***P***<0.05, C-¶ ***P***<0.05, D-¶ ***P***<0.02. Percentages of each treatment compared to baseline values are above the column.

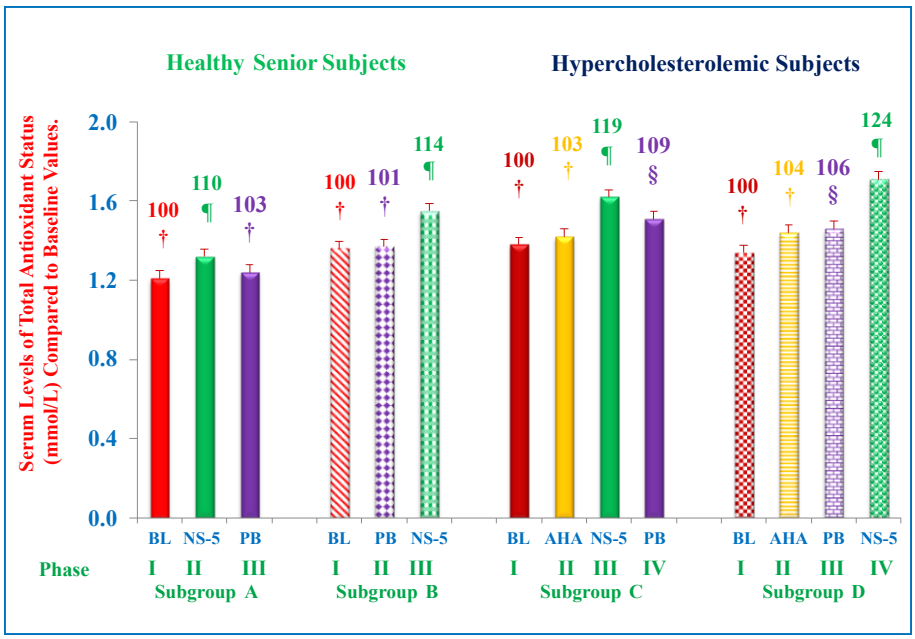


Figure 5: Serum total antioxidant status (TAS) levels are increased with NS-5 consumption. The treatments 1 – 14 columns in the figure correspond to: Group A, *n* = 16; 1 = BL (Baseline); 2 = NS-5 capsule (Nutrition Supplement-5); 3 = PB capsule (Placebo-starch); Group B, *n* = 16; 4 = BL; 5 = PB capsule; 6 = NS-5 capsule; Group C, *n* = 32; 7 = BL; 8 = AHA (American Heart Association Step-1 diet); 9 = AHA + NS-5 capsule; 10 = AHA + PB capsule; Group D, *n* = 32; 11 = BL; 12 = AHA; 13 = AHA + PB capsule; 14 = AHA + NS-5 capsule. Data are means ± SE. Values in a column not sharing a common symbol are significantly different of various groups at A-¶ ***P***<0.05, B-¶ ***P***<0.05, C-¶ ***P***<0.04; § ***P***<0.05, D-¶ ***P***<0.02; § ***P***<0.05 . Percentages of each treatment compared to baseline values are above the column.

This effect is not observed in subjects with normal baseline serum total cholesterol levels (Figures 6-8)

Elevated total cholesterol, LDL-cholesterol, and triglycerides levels are important lipid parameters for cardiovascular disease [12].

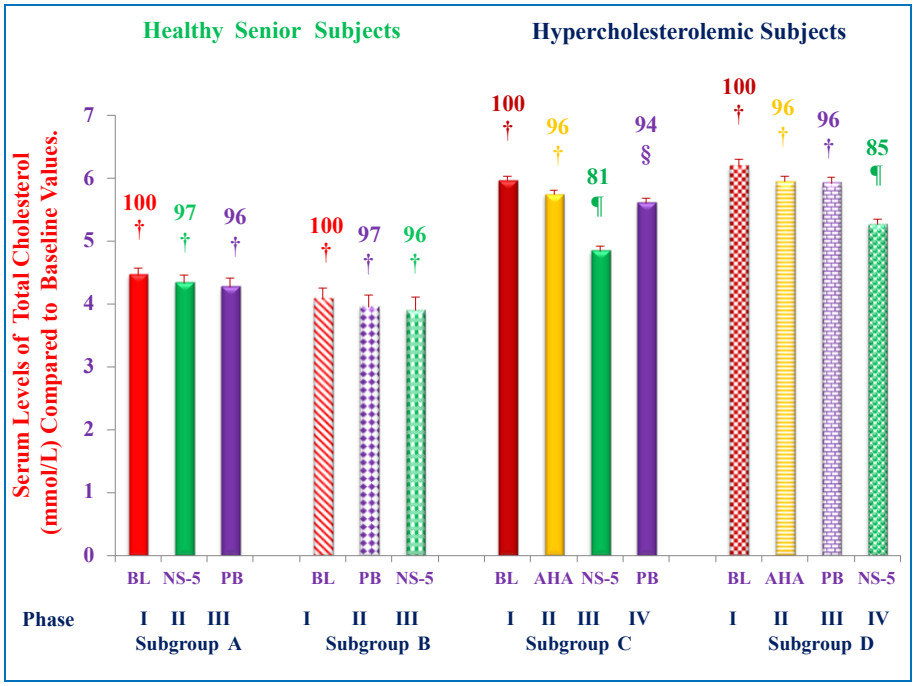


Figure 6: Serum total cholesterol levels are decreased with NS-5 consumption only in hypercholesterolemic subjects. The treatments 1 – 14 columns in the figure correspond to: Group A, *n* = 16; 1 = BL (Baseline); 2 = NS-5 capsule (Nutrition Supplement-5); 3 = PB capsule (Placebo-starch); Group B, *n* = 16; 4 = BL; 5 = PB capsule; 6 = NS-5 capsule; Group C, *n* = 32; 7 = BL; 8 = AHA (American Heart Association Step-1 diet); 9 = AHA + NS-5 capsule; 10 = AHA + PB capsule; Group D, *n* = 32; 11 = BL; 12 = AHA; 13 = AHA + PB capsule; 14 = AHA + NS-5 capsule. Data are means ± SE. Values in a column not sharing a common symbol are significantly different of various groups at C-¶ ***P***<0.05, D-¶ ***P***<0.05. Percentages of each treatment compared to baseline values are above the column.

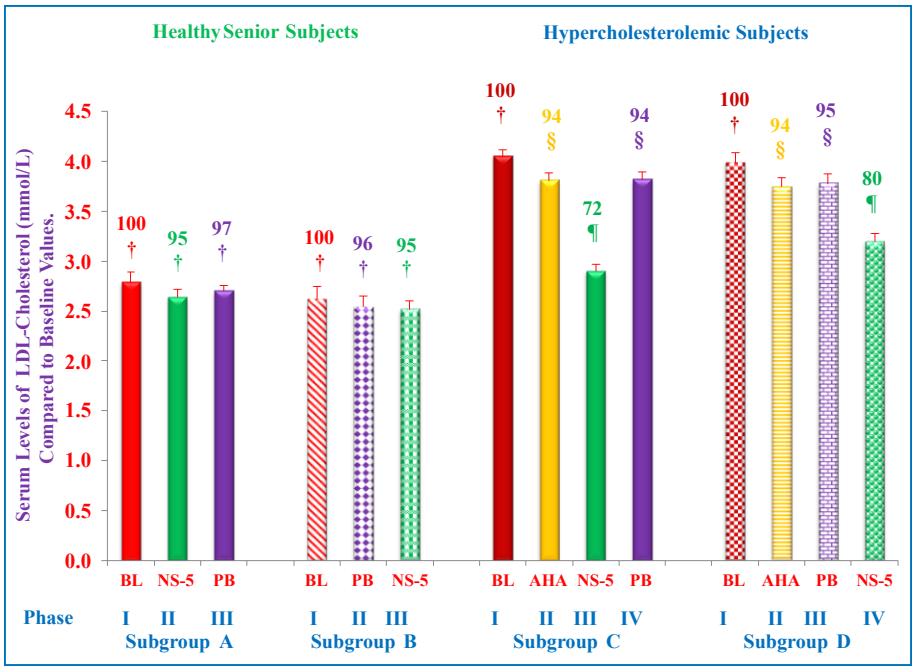


Figure 7: Serum LDL-cholesterol levels are decreased with NS-5 consumption only in hypercholesterolemic subjects. The treatments 1 – 14 columns in the figure correspond to: Group A, *n* = 16; 1 = BL (Baseline); 2 = NS-5 capsule (Nutrition Supplement-5); 3 = PB capsule (Placebo-starch); Group B, *n* = 16; 4 = BL; 5 = PB capsule; 6 = NS-5 capsule; Group C, *n* = 32; 7 = BL; 8 = AHA (American Heart Association Step-1 diet); 9 = AHA + NS-5 capsule; 10 = AHA + PB capsule; Group D, *n* = 32; 11 = BL; 12 = AHA; 13 = AHA + PB capsule; 14 = AHA + NS-5 capsule. Data are means ± SE. Values in a column not sharing a common symbol are significantly different of various groups at C-¶ ***P***<0.02, D-¶ ***P***<0.05. Percentages of each treatment compared to baseline values are above the column.

Although, statin drugs are widely used to improve serum lipid profiles and reduce cardiovascular risk, but statin drugs have potentially serious side effects, such as muscle damage, nausea, stomach cramps, headaches, loss of memory, and increased glucose levels [22]. Therefore, it was decided to try a mixture of naturally-occurring components of NS-5 to improve lipid profiles with fewer or no adverse effects.

Consumption of NS-5 mixture reduces serum total cholesterol levels only in hypercholesterolemic subjects. This effect is not observed in free-living healthy seniors with normal baseline serum total cholesterol levels

The free-living healthy seniors with normal serum total cholesterol levels of Study Group 1 showed no reductions in serum levels of total cholesterol, LDL-cholesterol, and HDL-cholesterol with any of the interventions used for subgroups A and B (Figure 6).

The hypercholesterolemic subjects with elevated total serum cholesterol levels of Study Group 2 were selected at entry level were instructed to adhere to an AHA Step-1 diet and their diet was monitored weekly during phase II of the study (4 weeks). No differences in serum total cholesterol levels were seen between baseline and levels measured after 4 weeks of dietary restriction (end of phase II). After phase II, all subjects of Study Group 2 remained on the AHA Step-1 diet, but were randomized to receive NS-5 capsules (subgroup C) or placebo capsules (subgroup D) for 6 weeks (Phase III). At the end of phase III, serum total cholesterol levels were significantly reduced in subjects on the AHA Step-1 diet plus NS-5 capsules (subgroup C) compared to baseline levels (19% $P < 0.05$), and compared to levels after 4 weeks of the AHA Step-1 diet alone (15% $P < 0.05$), respectively as shown in Figure 6.

Consumption of NS-5 mixture reduces serum LDL-cholesterol levels only in hypercholesterolemic subjects, but not in healthy seniors

Similar results as observed with serum total cholesterol levels were also observed for serum LDL-cholesterol levels for subgroups A, B, C, D of Study Group 1 and Study Group 2, after consuming NS-5 capsules for 6 weeks (Figure 7). There was a very modest reduction of 5% in serum levels of LDL-cholesterol observed of subjects of subgroups A and B (Figure 7). However, participants of subgroups C and D after consuming NS-5 capsules for 6 weeks plus AHA Step-1 diet showed significant decreases in the serum levels of LDL-cholesterol (28%, $P < 0.02$ and 20%, $P < 0.05$), respectively compared to baseline levels (Figure 7). When the participants of all the subgroups were kept on placebo capsules (corn starch) for 6 weeks either prior to or after NS-5 capsules treatment, the results did not indicate further reduction in LDL-cholesterol levels (Figure 7).

Consumption of NS-5 mixture reduces serum triglycerides levels only in hypercholesterolemic subjects, but not in healthy seniors

The serum levels of triglycerides of subjects of subgroups A and B of Study Group 1 were not changed by consuming NS-5 capsules for 6 weeks, compared to baseline values (Figure 8). However, serum triglycerides levels were significantly decreased in subjects of subgroup C (11%, $P < 0.05$) and D (18%, $P < 0.03$) of Study Group 2, after consuming NS-5 capsules plus AHA Step-1 diet for 6 weeks as compared to their respective baseline values (Figure 8). The serum levels of triglycerides for subgroups C or D subjects, who had received placebo capsules during phase IV or III, did not differ from levels measured at the end of phase II (Figure 8).

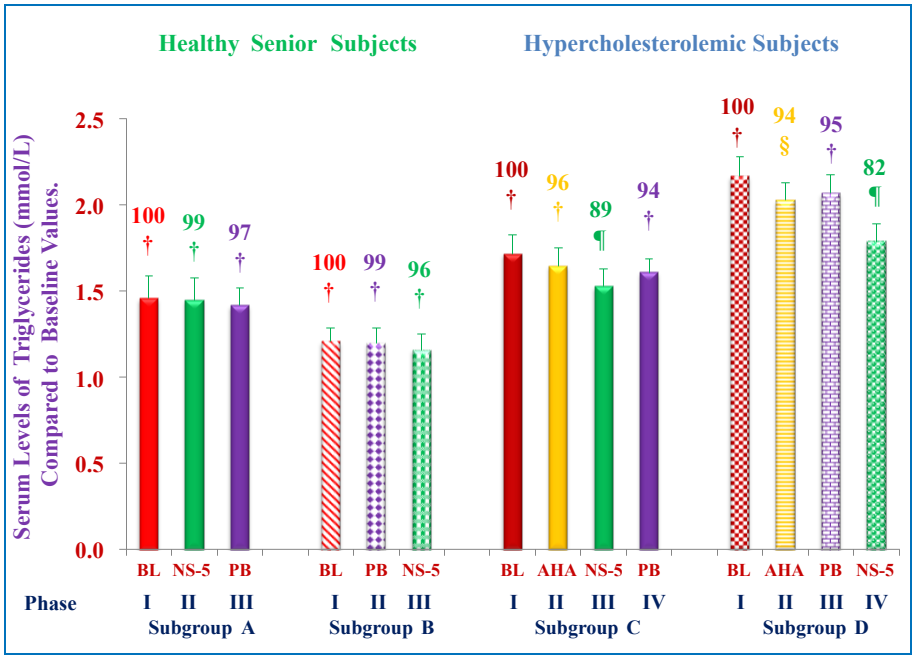


Figure 8: Serum triglycerides levels are decreased with NS-5 consumption in hypercholesterolemic subjects. The treatments 1 – 14 columns in the figure correspond to: Group A, *n* = 16; 1 = BL (Baseline); 2 = NS-5 capsule (Nutrition Supplement-5); 3 = PB capsule (Placebo-starch); Group B, *n* = 16; 4 = BL; 5 PB capsule; 6 = NS-5 capsule; Group C, *n* = 32; 7 = BL; 8 = AHA (American Heart Association Step-1 diet); 9 = AHA + NS-5 capsule; 10 = AHA + PB capsule; Group D, *n* = 32; 11 = BL; 12 = AHA; 13 = AHA + PB capsule; 14 = AHA + NS-5 capsule. Data are means ± SE. Values in a column not sharing a common symbol are significantly different of various groups at C-¶ $P < 0.05$, D-¶ $P < 0.03$. Percentages of each treatment compared to baseline values are above the column.

Consumption of NS-5 mixture has essentially no effect on Serum HDL-cholesterol levels

HDL-cholesterol is generally accepted to have beneficial effects, so we were hopeful that dietary supplementation with NS-5 would increase serum HDL cholesterol levels. No significant changes in HDL-cholesterol levels were observed with any of the interventions used in Study Group 1 (Figure 9). Similarly, the only significant effect observed with Study Group 2 was a modest increase in HDL cholesterol levels during phase III in subgroup C subjects who were restricted to the AHA Step-1 diet and whose diets were supplemented with NS-5 (6% increases compared to baseline, $P < 0.05$); a comparable change was not observed with subgroup D subjects (Figure 9).

In summary, all these results clearly demonstrate a reduction or improvement in serum levels of lipid parameters and risk factors of cardiovascular disease after the daily consumption of NS-5 by free-living healthy seniors and also in hypercholesterolemic subjects with restricted intake of fat and cholesterol (AHA Step-1 diet) for 6-weeks, which may provide pharmacological control relevant to the ageing process and onset of cardiovascular disease.

Discussion

In the current randomized, placebo controlled study, we found that serum NO, CRP and γ-GT levels were significantly decreased (Figures 2-4), and that TAS levels were significantly increased (Figure 5) in human subjects whose diets were supplemented for 6 weeks with NS-5, a mixture of polyphenols (resveratrol, pterostilbene, and quercetin) and vitamins (δ-tocotrienol and nicotinic acid). These changes were observed with subjects who entered the study with elevated total serum cholesterol levels as well as with subjects with normal total serum cholesterol levels. We also demonstrated that serum total cholesterol, LDL-cholesterol, and triglyceride levels were significantly decreased by diet supplementation with NS-5 in human subjects with elevated total serum cholesterol levels (> 6.4 mmol/L) levels; these effects were not detected in subjects with normal total serum cholesterol levels (Figures 6-8). Finally, we demonstrated that diet supplementation with NS-5 showed moderate decrease in blood pressure, and had essentially no effect on serum HDL-cholesterol levels (Figure 9).

These results of the current study are highly consistent with those of our previous study in which we studied the effects of dietary supplementation with a combination of resveratrol, pterostilbene, morin hydrate, quercetin, δ-tocotrienol, riboflavin, and nicotinic acid [12]. In the previous study, we achieved reductions in NO, CRP, γ-GT, total cholesterol, LDL-cholesterol, and triglycerides levels comparable to those achieved in the current study. One primary advantage of the current study is that we used NS-5 capsules, which did not include riboflavin. Riboflavin, used in the earlier study, produces significant urine discoloration, and this was cited by several subjects as their rationale for discontinuing the diet supplement [12]. In the current study, all subjects initially enrolled in the study completed all phases of the study, and no adverse effects were noted by any of the enrolled subjects. The collective results of this clinical trial support the conclusion that diet supplementation with these compounds reduces serum levels of a variety of markers of chronic inflammation, increases TAS, and decreases serum total cholesterol, LDL-cholesterol, and triglycerides levels. The significant reduction in the level of serum triglycerides in the present study was possible due to presence of nicotinic acid in the mixture of NS-5 capsule. It is well established that nicotinic acid is used to treat hyperlipidemia in humans by reducing very low density lipoprotein (VLDL), a precursor of low density lipoprotein (LDL). Nicotinic acid blocks the breakdown of fats, resulting in decreases in free fatty acids levels in the blood, and as a consequence, this leads to a

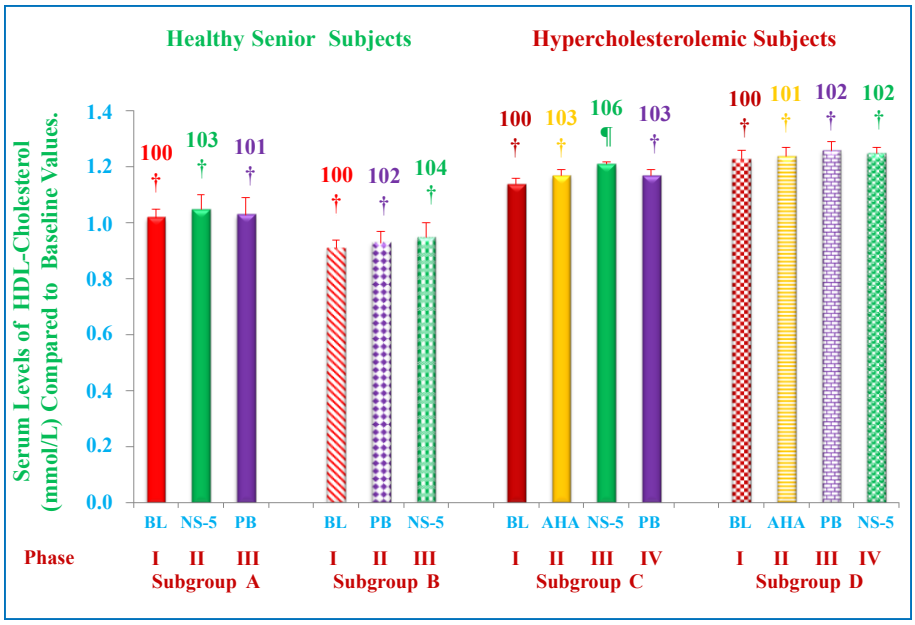


Figure 9: Serum HDL-cholesterol levels are essentially unchanged with NS-5 consumption. The treatments 1 – 14 columns in the figure correspond to: Group A, *n* = 16; 1 = BL (Baseline); 2 = NS-5 capsule (Nutrition Supplement-5); 3 = PB capsule (Placebo-starch); Group B, *n* = 16; 4 = BL; 5 = PB capsule; 6 = NS-5 capsule; Group C, *n* = 32; 7 = BL; 8 = AHA (American Heart Association Step-1 diet); 9 = AHA + NS-5 capsule; 10 = AHA + PB capsule; Group D, *n* = 32; 11 = BL; 12 = AHA; 13 = AHA + PB capsule; 14 = AHA + NS-5 capsule. Data are means ± SE. Values in a column not sharing a common symbol are significantly different of various groups at C-¶ P < 0.05. Percentages of each treatment compared to baseline values are above the column.

decrease secretion of VLDL and cholesterol, by the liver [23].

The results clearly indicate that consumption of NS-5 capsules reduced levels of these lipids and markers of inflammation, and increased, TAS levels, are associated with improved clinical outcomes, it is reasonable to speculate that these diet supplements could have beneficial health effects. All of the active ingredients included in the nutritional supplements used in these two clinical trials have been approved by the FDA for human consumption, and have been used for many years without any significant adverse effects [12-15]. Consequently, the potentially beneficial effects of diet supplementation with these compounds demonstrated in these studies can be achieved with relatively few regulatory obstacles.

As far as mechanisms by which these compounds work are concerned, our studies have revealed that NS-5 contained proteasome inhibitors which are known to affect several different signaling pathways and transcription factors, involved in inflammation and induction of IL-6, TNF-α, IL-1β, iNOS are important inflammatory markers [12-14]. We have previously shown that the proteasomes possess three different protease activities, the chymotrypsin-like, trypsin-like and the post-acidic activity that are crucial in modulating the inflammatory process. Quercetin and resveratrol are potent proteasome protease inhibitors that have different Ki's for blocking these protease-sites as discussed previously [14,24]. Blocking these proteasome's proteases has a pivotal effect on most inflammatory pathways [25,26] and on the gene and protein expression of cytokines and iNOS, and thus the proteasome inhibitors lead to a decrease in inflammation. We have shown that quercetin and resveratrol found in plants, fruits and vegetables inhibit expression IL-6 and other cytokines; and iNOS by direct inhibition of inflammatory signal transduction pathways [8,14]. Moreover, β-hydroxy-β-methylglutaryl coenzymeA (HMG-CoA) reductase, cholesterol-7α-hydroxylase and other regulatory proteins are ubiqitinated and degraded by the proteasome [11,16]. Proteasome inhibitors function by binding to crucial protease sites and by blocking the various activities of the proteasome and causes an accumulation of IαB and other proteins that are not degraded by the proteasome, thus affecting inflammation [14,24-26].

Apart from the explanation of the mechanisms for the inhibition of inflammatory markers or lipid metabolism of these compounds described above, there are several possible pathways by which these compounds might regulate the production of NO and inhibit the pro-inflammatory cytokines involved in normal and ageing processes [27-37]. We have discussed in detail two possible pathways, which involve NF-κB and toll-like receptors that are dependent on the proteasome's action in our earlier communication [13,14]. However, there are other pathways modulated by the proteasome which could be playing important roles in the inhibition of several pro-inflammatory cytokines involved in ageing process and atherosclerosis [29-34]. We have also shown that quercetin, resveratrol, and pterostilbene exert a cardio-protective action as a result of inhibition of the induction of iNOS and NO in murine macrophages and because of their anti-oxidant activity [12-14,38].

These effects are also related to modulation of proteasome's protease activities by these compounds present in NS-5 capsules. Long-term, moderate consumption of these specific compounds could play an important role in their cardio-protective actions [39].

Conclusions

All of the above discussed mechanisms indicate the importance of lowering the elevated levels of NO and CRP in order to improve the quality of life during ageing process and cardiovascular disease. The key findings of the present study are that serum NO, CRP and γ-GT levels were significantly decreased, and TAS levels significantly increased in human subjects after consuming NS-5 mixture, regardless of initial total serum cholesterol levels. Serum levels of total cholesterol, LDL-cholesterol, and triglycerides were significantly decreased with NS-5 in human subjects with elevated total serum cholesterol levels, but not in subjects with normal total serum cholesterol levels. The decreases in serum levels of NO, CRP, γ-GT activity, total and LDL- cholesterol, triglycerides, and blood pressure by NS-5 (resveratrol, pterostilbene, quercetin, δ-tocotrienol, and nicotinic acid; NS-5) capsules may be of clinical significance with regards to host defense mechanisms and treatment of inflammatory diseases.

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Competing Interests

The authors declare that they have no competing interests.

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