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Comparative Evaluation of NS-5 Mixture and its Components on Superoxide Production in HUVEC, and Inflammatory Biomarkers in Humans

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Abstract

Background: Inhibitory effects of NS-5 mixture of resveratrol, quercetin, δ-tocotrienol, nicotinic acid on several inflammatory and cardiovascular risk factors have been reported in normal cholesterolemic and hypercholesterolemic humans. The hypothesis was that combination of cholesterol-lowering and inflammatory-reducing properties of NS-5 mixture would be more effective than its individual components in reducing the serum levels of several biomarkers of cardiovascular disease in humans. However, effects of NS-5 mixture and its components on cytokines, gene expression, and microRNAs were not reported in previous publication. As this area is gaining importance in the understanding of various transcriptional factors and signal pathways, which regulate several biomarkers in various diseases.

Aims: Modulation of NS-5 mixture, and its components were evaluated on superoxide production in HUVEC *in vitro*, and on serum levels of total cholesterol, NO, CRP, TAS, plasma cytokines, gene expression, miRNAs *in vivo* in normal cholesterolemic and hypercholesterolemic humans.

Study design: Study was carried out as double-blind randomized, trial of NS-5 mixture, resveratrol, quercetin, and δ-tocotrienol in free-living healthy and hypercholesterolemic humans.

Results: The NS-5 mixture, resveratrol, quercetin, δ-tocotrienol, or nicotinic acid treatments caused reduction in superoxide production (11% to 24%; $P<0.01$) in HUVEC. These reductions were more pronounced with LPS-stimulated HUVEC (26% to 40%; $P<0.01$) compared to predose values. These findings were further supported by decreases ($P<0.01$) in serum total cholesterol levels of NS-5 treated group (24%) versus resveratrol (18%), quercetin (20%), and δ-tocotrienol (22%) in hypercholesterolemic humans, followed by reduction of NO, CRP and increases in TAS in normal cholesterolemic and hypercholesterolemic humans. There was significant ($P<0.001$) down-regulation in pro-inflammatory cytokines and gene expression of resistin, IL-2α, IL-6, IL-12, IL-18, TNF-α, and others, that are normally involved in pathogenesis of atherosclerosis, diabetes, and aging processes. The plasma inflammatory miRNAs (miR-101a, miR-125a, miR-155, miR-223) were down-regulated as compared to predose values. The elevated levels of miRNA-146a during senescence were down-regulated after treatment with these compounds.

Conclusions: This is the first report that describes the effects of NS-5 mixture, its components on proteomics, gene expression and levels of miRNAs in normal cholesterolemic and hypercholesterolemic humans. Results suggest that NS-5 mixture and its components are potent agents in the reduction of superoxide production, cardiovascular risk factors and inflammatory biomarkers, which are modulated by NF-κB. Maximum inhibition in superoxide production and other risk factors was observed with NS-5 mixture as compared with its individual components, thus supporting our hypothesis.

Keywords: NS-5 mixture; Resveratrol; Quercetin, δ-tocotrienol; Inflammatory biomarkers; Serum total Cholesterol; NO; hsCRP; Total antioxidant status; Plasma cytokines; Circulatory miRNAs

Abbreviations: AHA Step-1 diet: American Heart Association Step-1 diet; NO: Nitric Oxide; CRP: C-Reactive Protein; TAS: Total Antioxidant Status; miRNAs: microRNAs; mRNAs: messenger Ribonucleic Acids

Introduction

We have recently reported that serum NO levels increase with age, and are significantly higher in hypercholesterolemic humans as compared with normal cholesterolemic humans [1]. The serum NO levels decrease by dietary supplementation with a mixture of trans-resveratrol, trans-pterostilbene, quercetin, δ-tocotrienol, and nicotinic acid (NS-5) plus AHA Step-1 diet. Levels of other biomarkers of cardiovascular risk factors, C-reactive protein (CRP), and γ-glutamyl-transferase (γ-GT) activity were also reduced via this dietary supplementation in these two groups. The NS-5 mixture treatment had no significant effect on serum total cholesterol, LDL-cholesterol, or triglyceride levels in normal cholesterolemic humans. However, NS-5 mixture plus AHA Step-1 diet, when fed to hypercholesterolemic humans, reduced serum total cholesterol, LDL-cholesterol, triglyceride

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levels, several inflammatory biomarker levels, and increased the total antioxidant status [2]. These results were based on our hypothesis that combining the cholesterol-lowering and inflammation-reducing properties of naturally-occurring compounds including, resveratrol, pterostilbene, quercetin, δ-tocotrienol, and nicotinic acid mixture (NS-5) would greatly increase their inhibitory effect on the serum production of NO and the consequential beneficial effect in reducing cardiovascular risk factors in humans.

This previous study lacked information regarding the effects of NS-5 mixture on various cytokines/proteins, their gene expression, and circulatory microRNAs [2], an area is gaining importance in the understanding of various transcriptional factors and signaling pathways, known to regulate molecules involved in cardiovascular and other diseases [3,4]. In order to verify our hypothesis, first comparative effects of NS-5 mixture versus each of its component *in vitro* were carried out, followed by *in vivo* studies in humans. Human umbilical vein endothelial cells (HUVEC) were selected for the *in vitro* study to examine the relative inhibitory effects of NS-5 mixture, and its main components (resveratrol, quercetin, δ-tocotrienol, and nicotinic acid; (Figure 1) on superoxide production with and without LPS-stimulation. The LPS HUVEC (resulting in increased levels of nitric oxide) were included to imitate the impact of these compounds as observed in hypercholesterolemic and normal cholesterolemic humans. Pterostilbene was not included, because it is a dimethoxy ester of phenolic (hydroxyl) groups of resveratrol, and has similar positive, and physiological effects as resveratrol [5].

In our earlier publications, the important role played by endothelial vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in atherogenesis has been described [2]. Several histochemical studies have indicated that adhesion molecules are expressed in human atherosclerotic plaques, and have also suggested a role for adhesion molecules in atherosclerosis and risk of future myocardial infarction [6,7]. These studies have provided the basis to consider anti-adhesion therapy as a new method of reducing the risk of developing cardiovascular disease. Hence, attempts to lower the production of adhesion molecules have received wide attention. As a result, several *in vitro* studies have indicated that polyphenols and several other naturally-occurring antioxidants, such as flavonoids, vitamins, and tocotrienols represent potential inhibitors of adhesion molecule expression [8,9]. Expression of these adhesion molecules has been shown to depend much on the transcriptional activation of NF-κB, an antioxidant-sensitive transcription factor [10]. The naturally-occurring compounds that inhibit endothelial adhesion molecule expression have been of great interest in view of the role that adhesion molecules play in atherogenesis.

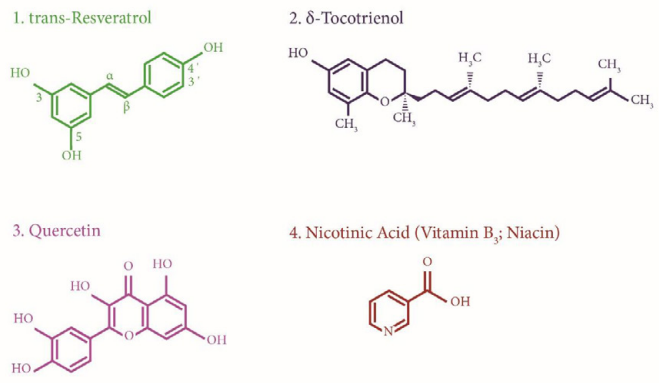

Figure 1: Chemical structures of dietary nutritional supplements used in the study.

Over the last several years, our research has focused on roles of several naturally occurring compounds in inflammatory response [11]. We have reported several compounds that have the ability to inhibit inflammatory biomarkers, largely through their capacity to inhibit NF-κB activation [5,11]. As mentioned above, NO level decreased by dietary supplementation of NS-5 mixture of resveratrol, pterostilbene, quercetin, δ-tocotrienol and nicotinic acid (vitamin B3) [1,2]. In addition, the consumption of NS-5 mixture caused significant reductions in the levels of serum CRP, and γ-GT activity (risk factors), as well as increased the serum levels of TAS in normal cholesterolemic and hypercholesterolemic humans [2].

Hypercholesterolemia is a disorder of elevated serum total cholesterol, resulting in progressive loss of arterial flexibility and formation of atheromatous plaque, leading to the narrowing of blood vessels, particularly arteries of the heart [12]. The major risk factors for atherosclerosis include elevated levels of serum total cholesterol, LDL-cholesterol and inflammation of coronary arteries caused in part, by increased levels of C-reactive protein (CRP), which is now considered the best indicator for the risk of coronary heart disease [13-15]. However, physiological and genetic factors also contribute to the progression of heart disease [16].

Circulating miRNAs are novel biomarkers for diverse cardiovascular diseases, including acute myocardial infarction, heart failure, coronary artery disease, diabetes, stroke, and hypertension [17-19]. Recently, a comprehensive review has reported the relationship between various cytokines and miRNAs, describing in detail how miRNAs mediate some of the known functions of these cytokines [19].

The present study evaluated the effects of NS-5 mixture, and its individual components on the production of superoxide *in vitro* in HUVECs, in the presence or absence of LPS. *In vivo* effects on serum levels of total cholesterol, NO, CRP, TAS, plasma cytokines, gene expression, and plasma microRNAs associated with aging process, cardiovascular, and other diseases were evaluated in normal cholesterolemic and hypercholesterolemic humans.

Materials and Methods

The study was carried out in the Department of Chemical Pathology & Endocrinology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan in collaboration with the Department of Basic Medical Sciences, University of Missouri-Kansas City, MO, USA The study protocol was registered and approved by the Institutional Review Board of AFIP, Rawalpindi, Pakistan. The study was carried out under FDA approved IND number 36906.

Materials

Primary cultures of HUVEC and endothelial cell culture media (EGM) were purchased from American Type Culture Collection (Rockville, MD) and Clonetics, BioWhittaker (Walkersville, MD). Reflection™ autoradiography films were purchased from NEN Life Science Research Products (Boston, MA). Capsules (250 mg/capsule) of *trans*-Resveratrol were a gift from "Mega Resveratrol", 60 Newtown Road # 32 (Danbury CT, USA). Quercetin was obtained from Alfa Aesar (Johnson Matthey Co. Lancastor, UK), and nicotinic acid (niacin, vitamin B3) was purchased from VOIGT Global Distribution Inc. P.O.Box. 1130 (Lawrence, Kansas, USA). Delta-Gold 125 mg

soft gels from annatto seeds (consisting of 90% δ-tocotrienol + 10% g-tocotrienol) were supplied by American River Nutrition, Inc. (Hadley, MA. USA). Serum total cholesterol levels were estimated by using reagent kits from Sigma Chemical Co. (St. Louis, USA). Pure total RNA was obtained from the EDTA treated fresh whole blood by using "total RNA purification kit # 17200 (NORGEN Bioteck Corporation, Thorold, ON, Canada). The various plasma cytokines, cDNA, and miRNA were estimated by using Signosis, Inc. (1700 Wyatt Drive Suite 10-12. Santa Clara, CA, 95054). Human Cytokine Elisa Plate Array I (chemiluminescence), Catalog number EA-4001, and for gene expression analyses and customized Human cDNA Plate Array for messenger ribonucleic acid (mRNA), Catalog Number AP-UM000416, were used (Signosis, Inc.). The estimation of circulating microRNAs (miRNAs) was carried out using customized miRNA Direct Hybridization Plate Array chemiluminescence; Catalog Number Inv-00465 according to the manufacturer's instructions (Signosis, Inc.).

Measurement of intracellular superoxide production by various compounds in HUVEC

Cell Culture: The stock solutions of each compound were prepared (1 mg/mL) in 95% ethanol and stored at -20°C. The working solutions (10 μM) of NS-5 mixture and its components were prepared by diluting the appropriate volume of each stock solution in culture medium to give the final ethanol concentration of 0.1% (v/v). Monolayers of HUVEC were maintained in culture dishes pre-coated with collagen type IV at 37°C, 5% CO_2 and 95% air in EGM supplemented with 2% fetal bovine serum (FBS), 10 ng/mL human epidermal growth factor, 1 μg/mL hydrocortisone, and 12 μg/mL bovine brain extract. Cells between the second and sixth passage were used for experimentation. Upon reaching confluency, cells were pretreated with NS-5 mixture and its components for 60 min and then treated second set of 1-6 with LPS (100 ng/mL) for 30 min followed by incubation with dihydroethidium dye (5 μM) specific for superoxide detection, and images were captured by epifluorescence microscope under constant exposure time and gain. The control cells received only 0.1% ethanol (v/v) 95% in culture medium.

Composition of nutritional supplement NS-5 mixture

Each 250 mg capsule of NS-5 mixture contained resveratrol+quercetin+δ-tocotrienol+nicotinic acid (50 mg of each)+corn starch (50 mg). The resveratrol, quercetin, δ-tocotrienol, nicotinic acid were purchased, as described in the Materials. The 250 mg/capsule of corn starch was used as placebo group. The capsulation of starch (placebo) and packing (30 capsules / bottle) was carried out at "Kabco Inc." (New Jersey, USA).

The present study was carried out as double blind randomized, placebo control trial of dietary nutritional supplements (NS-5 mixture), and its main components (resveratrol, quercetin, δ-tocotrienol) in free-living healthy adults and hypercholesterolemic humans of Wah Cantt, Pakistan. Nicotinic acid (vitamin B3) treatment was not included in current investigation, as its' pharmacological effects as an effective hypolipidemic agent in human studies have been well documented [20]. All participants signed an informed-consent form, which was approved by Institutional Review Board of the Medical College. The study protocol was approved by institutional review committee of Army Medical College (Rawalpindi, Pakistan). The study was carried out under a FDA approved IND number 36906.

Experimental design

Study group # (1): The participants (n=25; 15 males/10 females) were recruited from free-living healthy population (age>50 years and serum cholesterol levels 4.61 ± 0.07 mmol/L) from the "Senior Citizen's Community Centre" at Wah Cantt, Pakistan. Prospective participants were grouped according to cholesterol level (>median<) and sub-grouped by sex, and randomly divided into five groups of placebo, NS-5 mixture, resveratrol, quercetin, and δ-tocotrienol for treatments.

Study group # (2): The hypercholesterolemic participants (age >50 years and serum cholesterol levels 6.57 ± 0.14 mmol/L) also consisted of (n=25 subjects; 15 males/10 females. They were also randomly divided into five groups of the same treatments plus AHA Step-1 diet as for Study group # 1.

Inclusion criteria

Physical examination was carried out for each participant, which included the participant's height, weight, serum lipid parameters, glucose, alanine aminotransferase (ALT), systolic and diastolic blood pressure at rest, history of significant diseases, medications (any statin drugs, nitrates, calcium antagonist, angiotension-converting enzyme [ACE] inhibitors, and diuretics) and smoking. Height and body mass index (BMI, kg/m^2) was used as a measure of relative body weight. Venous blood samples were drawn at screening after overnight fast (12 h; 8 pm-8 am). At screening the participants were counseled to follow their normal dietary intake of study group # 1 or AHA step-1 diet (intake of <30% fat and <300 mg/d cholesterol) of study group # 2. Initial screening was accomplished during baseline phase I, ranging from three to four weeks. Venous blood samples were drawn at the termination of the baseline phase, and at week 6 after the treatments phase. Processed samples were coded and held at -72°C until analyses were carried out following the completion of treatment phases. All relevant investigations were carried out in the department of pathology, Army Medical College, NUST, Rawalpindi, Pakistan, 64000.

Exclusion criteria

Body weight should be >125% of Metropolitan Life relative weight, and participants having elevated levels of serum glutamate-pyruvate or glutamate-oxaloacetate transaminase activity, blood urea nitrogen or fasting glucose, or hypertensive disease were excluded. Liver function tests, thyroid stimulating hormone (TSH), serum urea and fasting plasma glucose were analyzed to exclude a liver, renal, and thyroid disorders and diabetes mellitus.

Each participant in study group # 1 was individually counselled to take his or her normal daily diet and for participants in study group # 2, AHA Step-1 diet was recommended. Participants of study group # 2 were also advised to stop using cholesterol-lowering drugs or antioxidants and were counselled individually to modify food intake to meet the goals of the AHA Step-1 diet. Human subjects were asked to stop the intake of whole milk, butter, cheese, eggs, animal fat and ice cream. In order to ascertain full compliance of dietary recommendation and intake of nutritional supplements, participants were contacted by telephone during each phase.

Study group # 1 (Free-living normal cholesterolemic humans)

The study consisted of two phases (phase I and II). All the human subjects were screened at baseline during the first four weeks (phase I). Three-day diet records were taken prior to start, and every week during each phase of the study to monitor dietary intake of each subject. The human subjects were then randomized into five groups 1-4. Human subjects of group 1 were given one capsule (250 mg) of placebo (starch), group 1 (NS-5 mixture), 2 (resveratrol), 3 (quercetin), and two capsules of (δ-tocotrienol; 125 mg/capsule) daily for four weeks.

Study group # 2 (hypercholesterolemic humans)

This experiment also consisted of two phases (I and II); the first phase, free choice of diet. Phase (baseline) was followed by a second phase, during which all participants were counselled to follow the American Heart Association Step-1 diet (AHA Step-1 diet), and all human subjects were divided into five groups. During phase II, subjects of group 2 were administered 1 capsule of placebo (starch), 5 (NS-5 mixture), 6 (resveratrol), 7 (quercetin), 8 (δ-tocotrienol; two 125 mg/capsule) plus AHA Step-1 diet for four weeks.

Blood samples collection

From the subjects of both studies, two sets of venous blood (10 mL of each) were drawn: one set in ethylene diamine tetra acetate (EDTA) glazed tube to get plasma for the purification of messenger RNAs, and a second serum samples set after overnight fast at the end of feeding period. Processed samples were coded and held at -72°C until analyses were carried for all the treatments.

Biochemical analysis

Estimation of serum levels of total cholesterol and C-reactive protein (CRP): All laboratory analyses were performed at the department of chemical pathology, Army medical college, according to validated standard procedures of the laboratory. Serum levels of total cholesterol were measured by cholesterol oxidase method (CHOD. POD). Serum hS-CRP was analyzed by two-site sequential chemiluminescent immunometric assay kit (Seimen, LA, California, USA) on Immulite 1000 (Immulite, Diagnostic Product Corporation, USA) according to the manufacturer's directions. The analytical sensitivity was 0.1 mg/L. Elevated CRP was defined for values greater than 4.0 mg/L.

Estimation of serum levels of nitric oxide (NO): Serum nitrate was carried out by colorimetric assay based on Griess reagent, using a 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 with a micro-plate reader at 540 nm. Values obtained by this procedure represented 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 analyzer, Selectra E (Vita Lab, Netherland), following the previously reported method [21]. Serum TAS present in the sample causes a decrease in 2,2'-Azino-di-[3-ethylbenthiazoline sulphonate] (ABTS) formation (radical cation) which develop a relatively stable blue green color when incubated with peroxidase (metmyoglobin) and H_2O_2. The decrease in color formation of ABTS is proportional to the concentration of TAS, which is measured at 600 nm.

Analyses of total RNA from EDTA treated whole blood after feeding NS-5 mixture and its components of normal cholesterolemic and hypercholesterolemic humans: The pure total RNA was extracted from EDTA treated fresh whole blood of human subjects fed NS-5 or it components (250 mg/d) for four weeks, by using total RNA purification kit # 17200 (NORGEN Bioteck Corporation, Thorold, ON, Canada). The purity of total RNA was carried out by measuring the absorption at several wavelengths using a Thermo Scientific NanoDrop 1000 Spectrophotometer. The purity of total RNA was determined by the ratio of 260/280 (2.02-2.04). The plasma miRNAs were also purified from plasma of participants of both study groups by using Plasma/Serum Circulating miRNA Purification Mini Kit (Slurry Format) Product # 51000 (NORGEN Bioteck Corporation, Thorold, ON, Canada).

Estimation of human plasma cytokines, cDNA, and miRNA: The various plasma cytokines/proteins were estimated by using Human Cytokine Elisa Plate Array I (chemiluminescence), Catalog number EA-4001 (Signosis, Inc., Santa Clara, CA, 95054). The gene expression was carried out by extracting mRNA from each sample, then converted to cDNA and plated on a cytokine cDNA array plate Customized Human cDNA Plate Array, Catalog Number AP-UM000416 (Signosis, Inc.). Assays for estimating the plasma cytokines/protein and gene expression of messenger RNAs were carried out according to the protocols provided by Signosis, Inc. The incubation of each assay mixture at various temperatures was carried out by using Enviro-Genie Shaker/incubator (Enviro-Genie Industries, Bohemia, NY). The intensity of chemiluminescence was detected using a Microplate Luminometer (GloMax Promega, Madison, WI) at 500 nm, and luminescence was monitored over 20 min period. Estimation of circulating miRNAs was carried out using "Customized miRNA Direct Hybridization Plate Array", chemiluminescence; Catalog Number Inv-00465 (Signosis, Inc., 1700 Wyatt Drive Suite 10-12. Santa Clara, CA, 95054).

Statistical analysis

Statistical analysis was performed using SPSS 16 (SPSS Inc, Chicago). Continuous, normally distributed variables were summarized as means ± SD, and percent differences were calculated from baseline values of each inflammatory marker or lipid parameter analysis of one-way variance was used to test whether changes in serum total cholesterol, NO, CRP, and TAS occur during the course of supplementation, and whether there were between- and within-subject differences. As 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 for percentage values of cytokines/proteins, and their gene expression and miRNAs. A two tailed P value <0.05 was considered significant. Data are reported as mean ± SD (Standard Deviation).

Results

The data presented was an average of three to four estimations of each sample. The values of cytokines, gene expression and miRNAs were based on percentages for comparative purposes. The physical characteristics of all the participants of pre-dose values of normal cholesterolemic and hypercholesterolemic humans are reported in Table 1. There were no changes in the body weight, height, body mass index, serum creatinine, serum glucose, serum alanine aminotransferase, systolic and diastolic blood pressure at the end of post-dose treatment of normal cholesterolemic and hypercholesterolemic humans. The results were based on pre-dose versus post-dose of respective groups rather than values compared with placebo group. There was no change in the values of the placebo group before and after treatment in normal cholesterolemic humans. However, there was a 4% decrease after treatment+AHA Step-1 diet of placebo group in hypercholesterolemic humans (data not shown).

Dietary supplement of NS-5 mixture and its components inhibit superoxide production with and without LPS-stimulated HUVEC

The superoxide productions were significantly inhibited by treatments with NS-5 mixture components in human umbilical vein

#	Parameters	Normal cholesterolemic (Means ± SD)	Hypercholesterolemic (Means ± SD)
1	Age	61.51 ± 7.46	60.82 ± 6.06
2	Males/Females (*n*)	3/2	3/2
3	Height (meter)	1.71 ± 0.09	1.72 ± 0.08
4	Weight (kg)	72.97 ± 9.49	77.88 ± 8.95
5	BMI (kg/m^2)	24.79 ± 2.05	26.43 ± 3.10
6	Systolic BP (mmHg)	136.43 ± 6.81	136.76 ± 7.67
7	Diastolic BP (mmHg)	84.71 ± 5.41	87 ± 6.96
8	Serum Creatinine (μmol/L)	90.77 ± 10.30	96.73 ± 12.47
9	Serum ALT (U/L)	32.14 ± 9.72	35.09 ± 5.73
10	Serum Total Cholesterol (mmol/L)	4.61 ± 0.07	6.57 ± 0.14
12	Serum Triglyceride (mmol/L)	1.47 ± 0.67	1.51 ± 0.48
13	Serum Glucose (mmol/L)	4.10 ± 0.22	4.91 ± 0.21
14	Serum NO (μmol/L)	6.58 ± 0.16	12.95 ± 0.24
15	Serum CRP (mg/L)	2.22 ± 0 .09	4.96 ± 0.17
16	Serum TAS (mmol/L)	1.39 ± 0.06	1.44 ± 0.06

Table 1: Baseline characteristics of normal cholesterolemic and hypercholesterolemic humans.

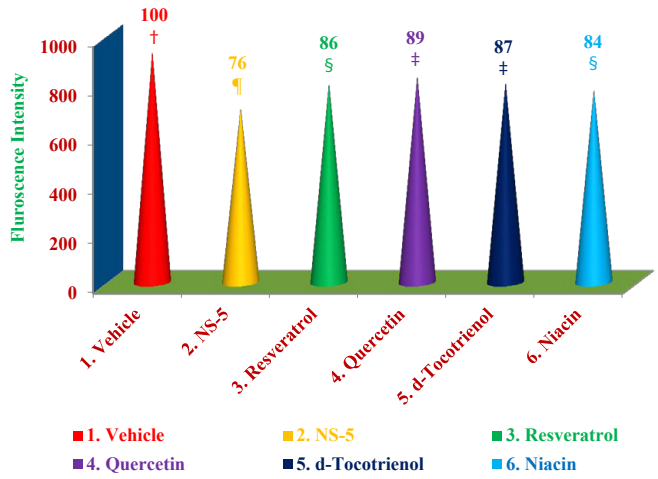

Figure 2: Dietary supplement of NS-5 mixture and its main components inhibit intracellular superoxide production in human umbilical vein endothelial cells (HUVEC): The working solutions (10 μM) of NS-5 mixture and its components were prepared by diluting the appropriate volume of each stock solution in culture medium to give the final ethanol concentration of 0.1% (v/v). Monolayers of HUVEC were maintained in culture dishes pre- coated with collagen type IV at 37ºC, 5% CO_2 and 95% air in EGM supplemented with 2% fetal bovine serum (FBS), 10 ng/ml human epidermal growth factor, 1 μg/ml hydrocortisone, and 12 μg/ml bovine brain extract. Cells between second and six passages were used for experimentation. Cells were pretreated with NS-5 mixture or its components (set of 1-6) for 60 min, followed by incubation with dihydroethidium dye (5 μM) specific for superoxide detection, and images were captured by epifluorescence microscope under constant exposure time and gain. The control cells received only 0.1% ethanol (v/v) 95% in culture medium. Data are mean fluorescence intensity of the cells (***n***=10-15) ± SD (standard deviation). Percentages of each treatment compared to control group (vehicle) of each treatment is above the column. Values in a column not sharing a common symbol are significantly different of various groups at ¶ ***P*** < 0.001; §, ‡ ***P*** < 0.01..

endothelial cells (HUVEC). The maximum reduction was observed with NS-5 mixture (24%; *P<0.*001) as compared to resveratrol (14%), quercetin (11%), δ-tocotrienol (13%) and nicotinic acid (16%) at *P<0.*01, respectively (Figure 2). In order to confirm these findings, the same treatments were repeated with and without LPS-stimulated HUVEC for the production of superoxide. As expected, the LPS-stimulation resulted in a significant increase in superoxide production (954 vs 1250), and there were maximum decreases of 40% (*P<0.*01) with NS-5 mixture, resveratrol (26%; *P<0.*01), quercetin (33%; *P<0.*01), δ-tocotrienol (38%; *P<0.*01), and nicotinic acid (27%; *P<0.*01) treatments compared to vehicle+LPS (Figure 3). The maximum inhibition in the superoxide production again was observed with NS-5 mixture treatment compared to its individual components. It is also interesting to note that inhibition of superoxide productions was much more pronounced in LPS-stimulated HUVEC (Figures 2 and 3), which also imitated the results as observed with normal cholesterolemic versus hypercholesterolemic humans [1,2]. The images captured by epifluorescence microscopy of all these treatments confirm these results as shown in Figure 4.

Effects of NS-5 mixture and its components on cardiovascular risk factors in normal cholesterolemic and hypercholesterolemic humans

Although, recent publications [1,2] described the effects of dietary supplementation of NS-5 mixture on several cardiovascular risk factors, including lipid parameters, now the comparative effects of NS-5 mixture versus resveratrol, quercetin, and δ-tocotrienol were compared only on selected four risk factors including serum levels of total cholesterol, nitric oxide, CRP, and TAS in normal cholesterolemic and hypercholesteromic humans. Serum total cholesterol levels were not changed with the consumption of NS-5 mixture or any of its components in normal cholesterolemic humans, however, there were significant (*P<0.*01) reduction in hypercholesterolemic humans with

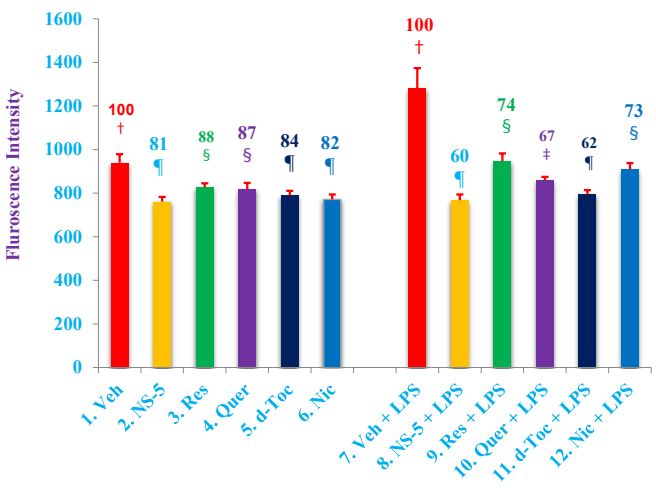

Figure 3: Dietary supplement of NS-5 mixture and its main components inhibit intracellular superoxide production in LPS-stimulated HUVEC: The working solutions (10 μM) of NS-5 mixture and its components were prepared by diluting the appropriate volume of each stock solution in culture medium to give the final ethanol concentration of 0.1% (v/v). Monolayers of HUVEC were maintained in culture dishes pre-coated with collagen type IV at 37°C, 5% CO_2 and 95% air in EGM supplemented with 2% fetal bovine serum (FBS), 10 ng/ml human epidermal growth factor, 1 μg/ml hydrocortisone, and 12 μg/ml bovine brain extract. Cells of third passage were used for experimentation. Cells were pretreated with NS-5 mixture or its components (set of 1-6 and 7-12) for 60 min and then set 7-12 were treated with LPS (100 ng/ml) for 30 min, followed by incubation of both sets with dihydroethidium dye (5 μM) specific for superoxide detection, and images were captured by epifluorescence microscope under constant exposure time and gain. The control cells received only 0.1% ethanol (v/v) 95% in culture medium. Data are mean fluorescence intensity of the cells (***n***=12-16) ± SD (standard deviation). Percentages of each treatment compared to control group (vehicle) of each treatment is above the column. Values in a column not sharing a common symbol are significantly different of various groups at set 1-6, ¶, § ***P*** < 0.02; and set 7-12, ¶, §, ‡ ***P*** < 0.01.

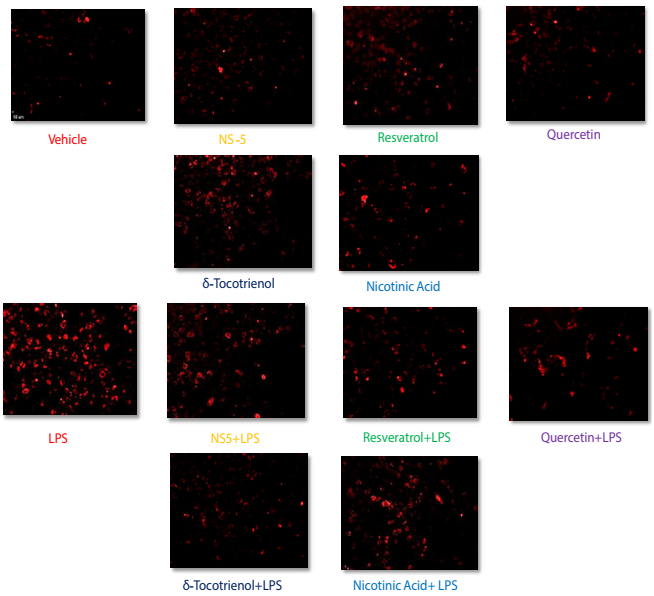

Figure 4: Dietary supplement of NS-5 mixture and its main components on dihdroethidium staining for superoxide production with and without LPS-stimulated HUVEC.

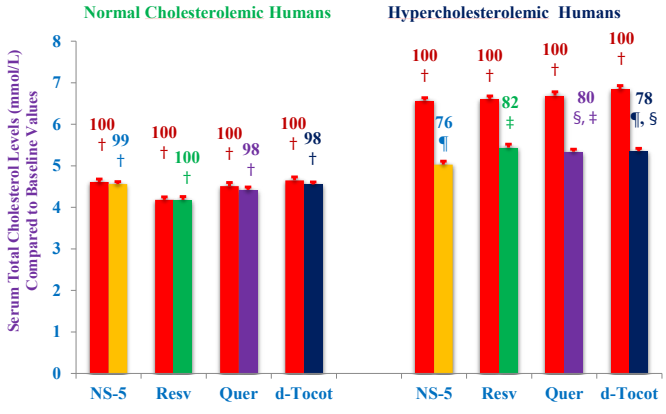

Figure 5: Serum total cholesterol levels were decreased in hypercholesterolemic humans who were administered NS-5 mixture or its individual component plus AHA Step-1 diet, but not in free-living normal cholesterolemic humans receiving NS-5 mixture or its components: Columns 1-4 represent subjects of group # 1 of normal cholesterolemic humans were administered one capsule of 250 mg/d of NS-5 mixture, or resveratrol, or quercetin, or δ-tocotrienol to normal cholesterolemic participants for four weeks. The columns 5-8 represent group # 2 of hypercholesterolemic participants were transferred to AHA Step-1 diet plus one capsule of 250 mg/d of NS-5 mixture or resveratrol, or quercetin or δ-tocotrienol for four weeks. Data are means ± SD (standard deviation). Percentages were compared to pre-dose vs post-dose of each treatment is above the column. Values in a column not sharing a common symbol are significantly different of various groups at ¶, § ***P*** < 0.01.

NS-5 (24%), resveratrol (18%), quercetin (20%), and δ-tocotrienol (22%), compared to pre-dose values (Figure 5).

There were also significant reductions in the serum levels of NO after consumption of NS-5 mixture (36, 42%; *P<0.*01) and its components, resveratrol (24, 28%; *P<0.*01), quercetin (31, 37%; *P<0.*01), δ-tocotrienol (32, 39%; *P<0.*01), respectively in normal cholesterolemic and hypercholesterolemic humans, compared to pre-dose values (Figure 6). Levels of serum CRP were also followed a similar pattern as observed with NO levels in these two groups. The NS-5 mixture consumption resulted in significant (*P<0.*01) reduction in CRP with NS-5 mixture (39, 50%), resveratrol (24, 40%), quercetin (35, 47%), and δ-tocotrienol (36, 38%), respectively compared to pre-dose values (Figure 7). The serum levels of total antioxidant status were increased after consumption of NS-5 mixture (29%; 33%, *P<0.*01), resveratrol (16, 22% *P<0.*05, *P<0.*01), quercetin (19 *P<0.*05, 27%, *P<0.*01), and

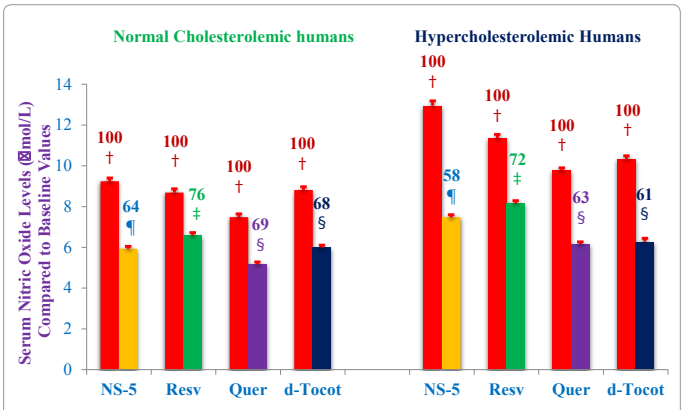

Figure 6: Serum nitric oxide (NO) levels were decreased in free-living normal cholesterolemic (NS-5 mixture or its individual component) and hypercholesterolemic subjects who were administered NS-5 mixture or its individual component plus AHA Step-1 diet: Columns 1-4 represent subjects of group # 1 of normal cholesterolemic humans were administered one capsule of 250 mg/d of NS-5 mixture, or resveratrol, or quercetin, or δ- tocotrienol to normal cholesterolemic participants for four weeks. The columns 5-8 represent group # 2 of hypercholesterolemic participants were transferred to AHA Step-1 diet plus one capsule of 250 mg/d of NS-5 mixture or resveratrol, or quercetin or δ-tocotrienol for four weeks. Data are means ± SD (standard deviation). Percentages were compared to pre-dose vs post-dose of each treatment is above the column. Values in a column not sharing a common symbol are significantly different of various groups at ¶, §, ‡ ***P*** < 0.01.

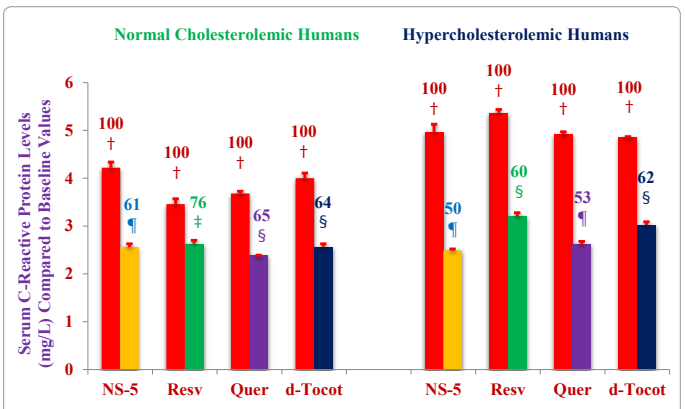

Figure 7: Serum C-reactive protein (CRP) levels were decreased in free-living normal cholesterolemic (NS-5 mixture or its individual component) and hypercholesterolemic humans who were administered NS-5 mixture or its individual component plus AHA Step-1 diet: Columns 1-4 represent subjects of group # 1 of normal cholesterolemic humans were administered one capsule of 250 mg/d of NS-5 mixture, or resveratrol, or quercetin, or δ-tocotrienol to normal cholesterolemic participants for four weeks. The columns 5-8 represent group # 2 of hypercholesterolemic participants were transferred to AHA Step-1 diet plus one capsule of 250 mg/d of NS-5 mixture or resveratrol, or quercetin or δ-tocotrienol for four weeks. Data are means ± SD (standard deviation). Percentages were compared to pre-dose vs post-dose of each treatment is above the column. Values in a column not sharing a common symbol are significantly different of various groups at ¶, § ***P*** < 0.01..

δ-tocotrienol (20% $P<0.05$, 30%, $P<0.01$), respectively compared to pre-dose values in both groups (Figure 8). All these results clearly indicated that the consumption of NS-5 mixture caused maximum reduction compared to its individual components predicted in our hypothesis.

Modulation of plasma cytokines/proteins, their gene expression, microRNAs by NS-5 mixture, and its components in normal cholesterolemic and hypercholesterolemic humans

As pointed out in the introduction, the present study was carried out to determine the effects of NS-5 mixture and its components on proteomic, genomic and microRNAs in humans, which was lacking in our earlier publications [1,2]. The effects of δ-tocotrienol on the functions of resistin, IL-1α, IL-2, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-γ, and miRNA-10b, R-16-1, R-18a, R-20a, R-29a, R-125a, R-133, R-223, R-372, R-214 have been reported in our recent publications [3,4]. The values of δ-tocotrienol of these cytokines and miRNAs were included in the present tables for comparison purposes, and their descriptions or functions were not included in this paper. Moreover, the effects of NS-5 mixture and its components on protein expression of cytokines, genes, and miRNAs were divided under different categories (cardiovascular, inflammation, cancer, diabetes and aging), and main functions were also included in for normal cholesterolemic (Tables 2,4,6), and for hypercholesterolemic humans (Tables 3,5,7). The values were based on percentages of pre-dose compared to post-dose values for each marker. The post-dose value was calculated based on their respective pre-dose value for each of estimation (regarded as 100%).

All cytokines (not described previously- IL-17α, IL-18, COX-2, GAPDH, IP-10, MIP-1α, NOS-2, VCAM-1, P53, FAS-1, VEGF, CCND-1, PAI-1) were down-regulated maximally with NS-5 mixture treatment group, compared to resveratrol, quercetin and δ-tocotrienol treatment groups, except IL-2 (resveratrol), IL-18, IL-10 (quercetin), TNF-α, VCAM-1, and IL-10 with δ-tocotrienol treatment group in normal cholesterolemic humans (Table 2). The Protein 53 and FAS-1 were up-regulated with all four treatments, which are associated with cancer (Table 2). In hypercholesterolemic humans, NS-5 mixture treatment group followed the same trend, as was observed with normal cholesterolemic humans, except GAPDH showed better down-regulation with resveratrol (35%), quercetin (29%), δ-tocotrienol (24%), IL-17α (quercetin), COX-2, P-53, FAS-1 (d-tocotrienol) compared to NS-5 treatment group (Table 3). Similarly, NS-5 mixture showed much more pronounced down-regulation or up-regulation in the gene expression of these cytokines compared to remaining other treatment groups (resveratrol, quercetin, δ-tocotrienol) both in normal cholesterolemic and hypercholesterolemic humans as shown in Tables 4 and 5, respectively.

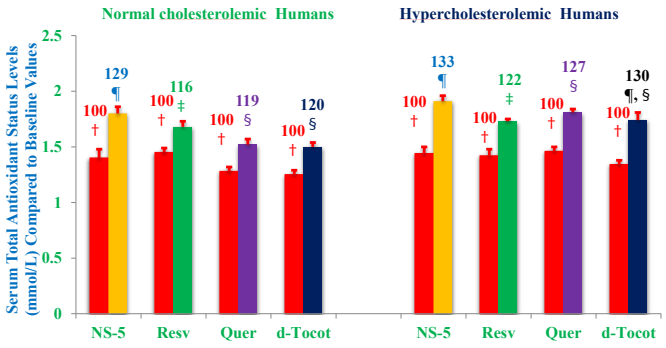

Figure 8: Serum total antioxidant status (TAS) levels were increased in free-living normal cholesterolemic (NS-5 mixture or its individual component) and hypercholesterolemic humans who were administered NS-5 mixture or its individual component plus AHA Step-1 diet: Columns 1-4 represent subjects of group # 1 of normal cholesterolemic subjects were administered one capsule of 250 mg/d of NS-5 mixture, or resveratrol, or quercetin, or δ-tocotrienol to normal cholesterolemic participants for four weeks. The columns 5-8 represent group # 2 of hypercholesterolemic participants were transferred to AHA Step-1 diet plus one capsule of 250 mg/d of NS-5 mixture or resveratrol, or quercetin or δ-tocotrienol for four weeks. Data are means ± SD (standard deviation). Percentages were compared to pre-dose vs post-dose of each treatment is above the column. Values in a column not sharing a common symbol are significantly different from various groups at ¶, § $P < 0.01$; ‡$P<0.05$.

The dysregulation of miRNAs play a crucial role in the development of cardiovascular disease cancer, diabetes and inflammation. Several studies have provided evidence showing that miRNAs participate in regulating cell cycle progression, proliferation, stem cell gene expression, and stress-induced responses. Cluster 1 - 23 miRNAs were also divided into cardiovascular, inflammation and aging groups in normal cholesterolemic humans (Table 6). miRNA 1-12 cluster was associated with cardiovascular disease, and significantly down-regulated with NS-5 mixture treatment (17% - 66%; mostly $P<0.001$) compared to resveratrol (8 - 42%; $P<0.01$), quercetin (7 - 59%; $P<0.01$ and 0.001) and δ-tocotrienol (13-60%; $P<0.001$), respectively based on pre-dose to post-dose treatments (Table 7). MicroRNAs associated with inflammation (miR-373, miR-93, miR-192, miR-216a and miR-503 ($P<0.001$) were up-regulated with the above mentioned treatments, and the best impact was again with NS-5 mixture group compared to resveratrol, quercetin and δ-tocotrienol treatments (Table 6). The maximum significant ($P<0.001$) down-regulation was observed in miRNA-146a associated with aging (79%), (56%), (67%), and (74%) with NS-5 mixture, resveratrol, quercetin and δ-tocotrienol treatments, respectively compared to their respective pre-dose treatments (Table 6).

On the other hand, a cluster of miRNAs 1-12 of cardiovascular disease (miR-7a, miR-10b, miR-15a, miR-16, miR-20a, miR-21, miR-29a, miR-92a, miR-26a, miR-133a, miR-200, miR-206) were significantly ($P<0.001$) up-regulated instead of being down-regulated as observed in normal cholesterolemic humans with NS-5 mixture, resveratrol, quercetin, and d-tocotrienol compared to their respective pre-dose percentage values in hypercholesterolemic humans (Table 7). The down-regulation of miRNAs associated with cardiovascular disease, inflammation and aging were similar to those found in normal cholesterolemic humans (Table 7). All these results clearly demonstrate that inhibition of superoxide production in HUVEC, biomarkers, or down-regulation of various cytokines, their gene expression, and miRNAs in normal cholesterolemic and hypercholesterolemic humans was maximal with NS-5 mixture, as compared to its individual components, resveratrol, quercetin and δ-tocotrienol, thus validating our original hypothesis.

Discussion

The dietary supplementation of NS-5 mixture and its components (resveratrol, quercetin, δ-tocotrienol, nicotinic acid) treatments caused a significant reduction in superoxide production (11% to 24%) in HUVEC. These reductions were much more pronounced when these compounds were tested in LPS-stimulated HUVEC (12% to 40%) compared to pre-dose values. The maximal inhibition in superoxide production was with NS-5 treatment group compared to its individual components, which proved our hypothesis that a combined mixture (NS-5) of various compounds will be more effective than its individual components. These findings were further supported by serum total cholesterol levels of NS-5 mixture treated group (24%) versus

			NS-5	Resveratrol	Quercetin	δTocotrienol	
#	Cytokines‡	Pre-dose	Post-dose	Post-dose	Post-dose	Post-dose¶	Functions
			%	%	%	%	
I	**Cardiovascular disease:**						
1	Resistin§	100	71 ± 1.4**	84 ± 1.5**	88 ± 1.8**	81 ± 1.6**	Regulatory role in insulin resistance, diabetes, atherosclerosis.
2	IL-2	100	67 ± 2.7**	51 ± 1.9**	65 ± 1.6**	75 ± 0.7**	For growth, proliferation, and differentiation of T cells to (Effector T) cells.
3	IL-6	100	75 ± 1.2**	77 ± 1.4**	83 ± 2.4**	83 ± 1.2**	Regulates the immune response, hematopoiesis and inflammation.
4	IL-8	100	68 ± 2.9**	69 ± 3.3**	77 ± 2.2*	75 ± 0.3**	Produced by macrophages and epithelial cells, potent angiogenic factor.
5	IL-12	100	74 ± 2.9**	82 ± 2.8*	84 ± 2.7*	75 ± 2.9**	It plays a key role in the activities of natural killer cells and T lymphocytes.
6	1L-17α	100	44 ± 3.3**	60 ± 2.4**	74 ± 3.1**	49 ± 3.4**	This gene is a pro-inflammatory cytokine produced by activated T cells.
7	IL-18	100	72 ± 1.4**	85 ± 1.7**	50 ± 3.9**	85 ± 1.8*	It acts as an angiogenic factor in many diseases.
8	COX-2	100	68 ± 3.2**	76 ± 1.5**	75 ± 3.6**	74 ± 2.9**	It is an enzyme, which is responsible for the production of prostanoids.
9	GAPDH	100	70 ± 1.1**	73 ± 2.3**	77 ± 2.8**	80 ± 2.5**	Key role in glycolysis and new host defense mechanism against HIV.
10	IP-10	100	58 ± 1.7**	83 ± 2.9*	82 ± 2.6*	57 ± 2.1**	It acts as potent inhibitor of angiogenesis *in vivo*.
II	**Inflammation:**						
11	TNF-α	100	63 ± 1.2**	71 ± 1.3*	68 ± 2.1*	42 ± 2.7*	Cytokines produced by macrophages/monocytes during inflammation.
12	INF-γ	100	67 ± 2.4**	75 ± 3.3**	73 ± 2.1**	67 ± 2.9**	Potent mediators of host defense and homoeostasis.
13	MIP-1α	100	62 ± 1.8**	70 ± 2.3**	73 ± 3.5**	68 ± 1.9**	A subfamily of chemokines that exhibit pro-inflammatory activities.
14	NOS-2	100	65 ± 1.8**	75 ± 1.5**	63 ± 3.9**	65 ± 2.4**	It is involved significantly in many vascular functions.
15	VCAM-1	100	61 ± 1.7**	68 ± 2.7**	63 ± 4.1**	50 ± 3.8**	It is involved in inflammatory-linkage.
16	MCP-1	100	78 ± 1.5**	87 ± 1.7**	80 ± 2.9*	75 ± 1.4**	It is a chemokine.
III	**Cancer**						
17	IGF-1	100	78 ± 1.1**	84 ± 2.2**	86 ± 2.7*	75 ± 3.1*	It is a potent mitogen.
18	P-53	100	197 ± 4.2**	135 ± 3.2**	156 ± 3.9*	141 ± 4.1*	It regulates the cell-cycle and acts as a tumor suppressor.
19	FAS-1	100	231 ± 6.7*	123 ± 3.4*	127 ± 2.1*	135 ± 3.2*	It forms death-inducing signaling complex (DISC) upon ligand binding
20	VEGF	100	66 ± 2.3*	71 ± 2.4*	79 ± 3.1*	73 ± 3.6*	Role in stabilizing the endothelial cell adhesion and signal transduction.
21	CCND-1	100	62 ± 1.8**	76 ± 2.3*	68 ± 3.5*	65 ± 1.5**	Controls progression of cells by activating cyclin-dependent kinase.
IV	**Diabetes**						
22	PAI-1	100	22 ± 2 .2**	46 ± 2 .3**	70 ± 1 .6**	63 ± 2 .8**	Associated with atherosclerosis and have increased levels in diabetics.
V	**Aging**						
23	IL-1α	100	57 ± 2 .1**	75 ± 2 .6**	76 ± 2 .9**	77 ± 1 .7**	Agonist mediating inflammatory and immuno-modulatory effects.
24	IL-10	100	72 ± 2.6**	73 ± 4.1**	66 ± 1.5**	62 ± 1.6**	Cytokine with pleiotropic effects in immuno-regulation and inflammation.

* - **Values in a row sharing a common asterisk are significantly different at *$P<0.01$; **$P<0.001$.
¶The effect of δ-tocotrienol on resistin, IL-2, IL-6, IL-8, IL-10, IL-12, TNF-α and IFN-γ has been reported recently (AAQ et al. BJMMR. 6(4): 351-366, JCEC. 2015; 6:4. 1000367.
‡The cytokines IL-12, IL-17α, IL-18 (inflammation); IL-2 (cancer); and resistin, IL-6, IL-17α (diabetes) also involved in these diseases.
§Resistin = Obesity-mediated insulin resistance and Type 2 diabetes Mellitus; IL-2: Interleukin-2; IL-6: Interleukin-6, 8, 12, 17α, 18; COX-2: Cyclooxygenase-2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TNF-α: Tumor Necrosis Factor-α; INF-γ: Interferon-γ; MIP-1α: Macrophage Inflammatory Protein-1α; NOS-2: Nitric Oxide Synthase-2; VCAM-1: Vascular Cell Adhesion Molecule-1; MCP-1: Monocyte Chemotactic Protein-1; IGF-1: Insulin-like Growth Factor-1; P-53: P-53 tumor suppressor protein; FAS-1: Fatty Acid Synthetase-1; VGEF: Vascular Endothelial Growth Factor; CCND-1: Cyclin D-1; PAI-1: Plasminogen Activator Inhibitor-1; IL-1α: Interleukin-1α; IL-10: Interleukin-10.
Table 2: Effects of NS- 5, resveratrol, quercetin, and δ-tocotrienol (Pre-dose vs Post-dose) on plasma cytokines/proteins of normal cholesterolemic humans.

resveratrol (18%), quercetin (20%), and δ-tocotrienol (22%) treated groups in hypercholesterolemic humans (Figure 4), and a similar trend in the reduction of serum levels of NO, CRP. An increase in TAS was observed in normal cholesterolemic and hypercholesterolemic humans (Figures 5-7).

These results suggest that NS-5 mixture and its components are potent and effective agents in the reduction of cellular superoxide production in HUVEC, and cardiovascular risk factors and inflammatory biomarkers in humans, which are modulated by redox sensitive transcription factor NF-κB [11,22]. NF-κB regulates several molecules involved in atherosclerosis, including ICAM-1 and VCAM-1 [23], and it was reported that these compounds reduced NF-κB activation, suggesting that these compounds may also act on adhesion molecule expression by blocking activation of NF-κB [11].

Atherosclerosis is associated with an impairment of endothelium-dependent relaxations, which represent the bioavailability of nitric oxide (NO) produced from endothelial NO synthase (eNOS). Among various mechanisms implicated in the impaired endothelium-dependent relaxations in atherosclerosis, superoxide generated from dysfunctional eNOS has attracted much attention. Experimental studies *in vitro* have revealed that NO from eNOS constitutes an antiatherogenic molecule. A deficiency in eNOS was demonstrated to accelerate atherosclerotic lesion formation in eNOS knockout mice [24]. In contrast, eNOS overexpression with hypercholesterolemia may promote atherogenesis via increased superoxide generation from dysfunctional eNOS as reported by Kawashima, and also observed in the present results [25].

Moreover, aging is a devastating physiological phenomenon that leads to deterioration in normal functioning of the body, resulting in multiple detrimental changes, ultimately decreasing the quality of life [26]. The anti-aging properties of resveratrol have conflicting reports. It was reported that a mixture of resveratrol combined with 5% quercetin and 5% rice bran phytate (longevinex; the ingredients were micronized to increase the bioavailability) fed rats produced desirable positive effects, and revealed superior cardiac performance, reduced infarct size, and induction of survival signals evidenced by increased Bcl2/Bax ratio and enhanced Akt phosphorylation. In contrast, LC3-II and

			NS-5	Resveratrol	Quercetin	δTocotrienol	
#	Cytokines‡	Pre-dose	Post-dose	Post-dose	Post-dose	Post-dose¶	Functions
			%	%	%	%	
I	**Cardiovascular disease:**						
1	Resistin	100	86 ± 1.6**	91 ± 1.7*	88 ± 2.1*	93 ± 2.7*	Regulatory role in insulin resistance, diabetes, atherosclerosis.
2	IL-2	100	89 ± 1.8*	94 ± 2.2*	89 ± 4.2*	92 ± 2.9*	For growth, proliferation, and differentiation of T cells.
3	IL-6	100	63 ± 1.8**	69 ± 2.4**	85 ± 1.3**	78 ± 1.3**	Cytokine regulates immune response, hematopoiesis and inflammation.
4	IL-8	100	75 ± 2.4**	80 ± 1.4**	79 ± 2.4**	75 ± 3.0**	Chemokine produced by macrophages, epithelial cells, and angiogenic factor.
5	IL-12	100	73 ± 2.4**	80 ± 1.2**	92 ± 1.3*	76 ± 0.9**	Plays a role in the activities of natural killer cells and T lymphocytes.
6	1L-17α	100	69 ± 2.1**	72 ± 2.1**	62 ± 0.8**	72 ± 1.5**	Pro-inflammatory cytokine produced by activated T cells.
7	IL-18	100	70 ± 3.4**	83 ± 1.1**	82 ± 2.3**	76±1.3**	It acts as an angiogenic factor in many diseases.
8	COX-2	100	73 ± 2.5**	82 ± 2.8**	81 ± 5.6**	70 ± 1.5**	It is an enzyme, which is responsible for the production of prostanoids.
9	GAPDH	100	85 ± 7.8*	65 ± 3.3**	71 ± 4.3**	74 ± 1.7**	Plays a role in glycolysis and new host defense mechanism against HIV.
10	IP-10	100	80 ± 1.1**	93 ± 0.9**	84 ± 0.7**	84 ± 2.7**	It acts as potent inhibitors of angiogenesis *in vivo*.
II	**Inflammation:**						
11	TNF-α	100	75 ± 1.7**	85 ± 2.4**	81 ± 1.8**	86 ± 2.6**	Cytokine produced by macrophages/monocytes during inflammation.
12	INF-γ	100	75 ± 1.7**	93 ± 1.9*	80 ± 3.1**	82 ± 2.5**	Potent mediators of host defense and homoeostasis.
13	MIP-1α	100	82 ± 2.1**	93 ± 0.7*	85 ± 2.2**	90 ± 2.3*	Chemokines that exhibit a variety of pro-inflammatory activities.
14	NOS-2	100	72 ± 2.8**	75 ± 1.7**	71 ± 1.2**	75 ± 1.9**	It is involved significantly in many vascular functions.
15	VCAM-1	100	51 ± 2.7**	64 ± 4.1**	60 ± 2.4**	60 ± 3.1**	It is involved in inflammatory-linkage.
16	MCP-1	100	71 ± 3.8**	82 ± 3.3*	73 ± 2.8**	79 ± 3.3*	It is a chemokine
III	**Cancer:**						
17	IGF-1	100	79 ± 2.7**	90 ± 1.5*	79 ± 1.2**	79 ± 1.1**	It is a potent mitogen.
18	P-53	100	135 ± 2.2**	126 ± 4.1**	120 ± 5.1**	150 ± 2.9**	It regulates the cell-cycle and acts as a tumor suppressor.
19	FAS-1	100	145 ± 4.0**	140 ± 4.5**	141 ± 1.9**	164 ± 4.9**	It forms death-inducing signaling complex (DISC) upon ligand binding
20	VEGF	100	79 ± 2.4**	79 ± 4.0**	82 ± 4.1**	78 ± 2.4**	Role in stabilizing the endothelial cell adhesion and signal transduction.
21	CCND-1	100	65 ± 2.7**	69 ± 1.6**	68 ± 3.8**	68 ± 1.3**	Controls progression of cells by activating cyclin-dependent kinase.
IV	**Diabetes:**						
22	PAI-1	100	82 ± 1.3**	86 ± 4.6**	59 ± 3.1**	84 ± 1.2*	Involved in atherosclerosis and diabetes.
V	**Aging:**						
23	IL-1α	100	60 ± 1.3**	68 ± 3.5**	82 ± 1.5**	75 ± 3.2**	It is an agonist mediating inflammatory and immuno-modulatory effects.
24	IL-10	100	34 ± 1.7**	69 ± 3.0**	52 ± 3.7**	65 ± 1.2**	Pleiotropic effects in immuno-regulation and inflammation.

* - **Values in a row sharing a common asterisk are significantly different at *$P<0.01$; **$P<0.001$.
¶The effect of δ-tocotrienol on resistin, IL-2, IL-6, IL-8, IL-10, IL-12, TNF-αand IFN-γ has been reported recently (AAQ et al. BJMMR. 6(4): 351-366, JCEC. 2015.
‡The cytokines IL-12, IL-17α, IL-18 (inflammation); IL-2 (cancer); and resistin, IL-6, IL-17α(diabetes) also involved in these diseases.
§Resistin: Obesity-mediated insulin resistance and Type 2 diabetes Mellitus; IL-2: Interleukin-2; IL-6: Interleukin-6, 8, 12, 17α, 18; COX-2: Cyclooxygenase-2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TNF-α: Tumor Necrosis Factor-α; INF-γ: Interferon-γ; MIP-1α: Macrophage Inflammatory Protein-1α; NOS-2: Nitric Oxide Synthase-2; VCAM-1: Vascular Cell Adhesion Molecule-1; MCP-1: Monocyte Chemotactic Protein-1; IGF-1: Insulin-like Growth Factor-1; P-53: P-53 tumor suppressor protein; FAS-1: Fatty Acid Synthetase-1; VGEF: Vascular Endothelial Growth Factor, CCND-1: Cyclin D-1; PAI-1: Plasminogen Activator Inhibitor-1; IL-1α: Interleukin-1α; IL-10: Interleukin-10.
Table 3: Effects of NS-5, resveratrol, quercetin, and δ-tocotrienol (Pre-dose vs Post-dose) on plasma cytokines/proteins in hypercholesterolemic humans.

Beclin were increased significantly after longevinex treatment [27]. This confirms the hypothesis that resveratrol in combination with other compounds is more effective rather than being administered alone.

Our earlier studies have revealed that δ-tocotrienol affects several different signaling pathways and other factors involved in inflammation (TNF-α, IL-1α, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, resistin, INF-γ, IGF-1, VEGF) in hypercholesterolemic subjects, which are important inflammatory biomarkers [3,4]. In our earlier studies, in regard to cytokines/proteins, the main focus was, their association with cardiovascular disease [3,4]. The present study demonstrates that NS-5 mixture and its components caused down-regulation or up-regulation of these and other several cytokines/proteins, including their gene expression in normal cholesterolemic and hypercholesterolemic humans.

It is well known that IL-17α plays important roles in inflammation and the immune response. It is involved in the pathogenesis of various diseases (allergies, autoimmune diseases, malignancy, and protective roles against infectious diseases) and promotes induction of cytotoxic T-lymphocytes responses against cancer [28]. It induces stromal cells to produce pro- inflammatory and hematopoietic cytokines, and also enhances the surface expression of ICAM-1/intracellular adhesion molecule-1. The increasing level of IL-17α is associated with cardiovascular disease via possible regulation of aortic VCAM-1 expression and T cell content [28], which was down-regulated significantly with treatment of NS-5 and its components. The treatments of NS-5, and its components have down-regulated interleukin-18 (IL-18), which is synthesized by various cell types as an inactive precursor molecule that becomes functional following cleavage by caspase-1, and may be a potential therapeutic target for angiogenesis- directed diseases.

			NS-5	Resveratrol	Quercetin	δ-Tocotrienol	
#	Genes¶	Pre-dose	Post-dose	Post-dose	Post-dose	Post-dose‡	Descriptions
			%	%	%	%	
I	Cardiovascular disease:						
1	Resistin	100	48 ± 3.22**	76 ± 1.12**	57 ± 1.35**	73 ± 2.81**	Pathogenesis of obesity-mediated insulin resistance and type 2 diabetes mellitus
2	IL-2	100	53 ± 2.07**	88 ± 2.48*	64 ± 2.59**	72 ± 2.88**	Interleukin-2
3	IL-6	100	56 ± 3.06**	71 ± 0.68**	85 ± 0.91**	68 ± 2.87*	Interleukin-6
4	IL-8	100	68 ± 1.22**	94 ± 2.90*	95 ± 0.76*	95 ± 0.21*	Interleukin-8
5	IL-12	100	85 ± 1.93**	78 ± 1.51**	70 ± 2.12**	81 ± 0.89**	Interleukin-12
6	1L-17α	100	66 ± 3.44**	83 ± 1.56**	83 ± 0.46**	85 ± 1.62**	Insulin-enhances nitric oxide, and NF-κB.
7	IL-18	100	45 ± 3.08**	59 ± 1.30**	85 ± 1.34**	79 ± 3.00**	Interleukin-18
8	COX-2	100	41 ± 2.78**	73 ± 1.44**	55 ± 1.36**	65 ± 1.62**	Cyclooxygenase-2
9	GAPDH	100	29 ± 2.59**	81 ± 1.36**	73 ± 0.88**	78 ± 2.96**	Glyceraldehyde-3-phosphate dehydrogenase
10	IP-10	100	21 ± 2.15**	88 ± 1.97*	74 ± 1.74**	72 ± 0.94**	Interferon-Inducible Protein-10
II	Inflammation:						
11	TNF-α	100	48 ± 2.30**	63 ± 1.31**	65 ± 2.44**	83 ± 2.08**	Tumor Necrosis Factor-α
12	INF-γ	100	59 ± 2.36**	67 ± 1.10**	71 ± 1.31**	75 ± 1.60**	Interferon-γ
13	MIP-1α	100	57 ± 3.94**	87 ± 1.13*	81 ± 3.20**	74 ± 2.24**	Macrophage Inflammatory Protein-1α
14	NOS-2	100	56 ± 0.41**	57 ± 1.94**	67 ± 1.72**	67 ± 2.11**	Nitric oxide synthase-2
15	VCAM-1	100	70 ± 2.01**	90 ± 3.76*	72 ± 2.93**	84 ± 0.70**	Vascular cell adhesion molecule
16	MCP-1	100	69 ± 4.99**	83 ± 2.83**	77 ± 2.17**	76 ± 2.02**	Monocyte Chemotactic Protein-1
III	Cancer:						
17	IGF-1	100	68 ± 3.56**	74 ± 2.33**	70 ± 2.99**	71 ± 2.93**	Insulin-like Growth Factor-1
18	P-53	100	217 ± 4.87**	113 ± 1.19*	120± 1.96**	239 ± 8.66**	P-53 tumor suppressor protein
19	FAS-1	100	157± 3.72**	119 ± 2.78**	151 ± 3.07**	129 ± 1.94**	Fatty acid synthetase-1
20	VEGF	100	71 ± 2.51**	73 ± 1.78**	83 ± 0.85**	90 ± 0.47*	Vascular Endothelial Growth Factor
21	CCND-1	100	40 ± 5.33**	71 ± 6.04**	54 ± 1.91**	67 ± 3.71**	Cyclin D-1
IV	Diabetes:						
22	PAI-1	100	26 ± 1.21**	81 ± 0.48**	71 ± 4.79**	71 ± 2.37**	Plasmenogen Activator Inhibitor-1
V	Aging:						
23	IL-1α	100	70 ± 1.67**	84 ± 1.52*	84 ± 2.09*	80 ± 0.76**	Interleukin-1α
24	IL-10	100	76 ± 1.44**	96 ± 1.58*	88 ± 4.12*	69 ± 1.42**	Interleukin-10

* - **Values in a row sharing a common asterisk are significantly different at *P<0.01; **P<0.001.
‡The effect of δ-tocotrienol on resistin, IL-2, IL-6, IL-8, IL-10, IL-12, TNF-α and IFN-γ has been reported recently (AAQ et al. BJMMR. 6(4): 351-366, and JCEC. 2015; 6:4. 1000367).
¶The mRNAs IL-12, IL-17α, IL-18 (inflammation); IL-2 (cancer); and resistin, IL-6, IL-17α (diabetes) also involved in these diseases.

Table 4: Effects of NS-5, resveratrol, quercetin, and δ-tocotrienol (Pre-dose vs Post-dose) on gene expression of messenger RNAs (mRNAs) of normal cholesterolemic humans.

The down-regulation by NS-5 mixture and its components on several cytokines/proteins were observed in COX-2, GAPDH, IP-10, MIP-1α, NOS-2, VCAM-1, MCP-1, VEGF, CCND-1, and PAI-1 and up-regulated in protein-53 and FAS-1 (Tables 2-7). Cyclooxygenase-2 is an enzyme that is responsible for the formation of prostanoids, and is a cardio-protective protein that can remove ischemia. GAPDH catalyzes the energy-yielding step in carbohydrate metabolism, and the production of ATP and pyruvate. IP-10 functions as an antimicrobial peptide in innate immunity, and MIP-1α, chemokines are the major factors produced by macrophages after stimulation with bacterial endotoxins. They stimulate human granulocytes, which can lead to acute neutrophilic inflammation, and also induce the synthesis and release of IL-1, IL-6 and TNF-α, from fibroblasts and macrophages.

Nitric oxide synthase-2 (NOS-2) synthesized the metastable free radical nitric oxide, which plays critical role as a mediator of vasodilation in blood vessels. The vasodilator action of nitric oxide plays a key role in renal control of extracellular fluid homeostasis and is essential for the regulation of blood flow and blood pressure [29]. VCAM-1 is expressed in individuals with minimum coronary disease and angina. The primary function of VCAM-1 is the mediation of leukocyte-endothelial cell adhesion and signal transduction. Monocyte Chemotactic Protein-1 (MCP-1) is one of the key chemokines that play a major role in selectively recruiting monocytes, neutrophils, and lymphocytes. It also regulates migration and infiltration of monocytes/macrophages. Migration of monocytes from the blood stream across the vascular endothelium is required for routine immunological surveillance of tissues, and in response to inflammation.

Serum VEGF level is high in an advanced disease of cancer patients. Angiogenesis is the growth of new blood vessels and is an important natural process occurring in the body. IL-6 and IL-8 have been shown to promote angiogenesis [30]. These results clearly demonstrate that NS-5 mixture, and its components possess anti-angiogenic activity by reducing expression of angiogenic promoters. Therefore, they may act as potential agents for the prevention of cancer progression through angiogenesis [31]. Cyclin-1 (CCND-1) is a family of proteins that control the progression of cells by activating cyclin-dependent kinase (CDK) enzyme. The oscillation of the cyclins, namely fluctuations in cyclins gene expression and destruction by the ubiquitin-mediated proteasome pathway, induce oscillation in CDK activity to drive the cell cycle. Plasminogen Activator Inhibitor-1 (PAI-1) is mainly

			NS-5	Resveratrol	Quercetin	δ-Tocotrienol	
#	**Genes**[¶]	Pre-dose	Post-dose	Post-dose	Post-dose	Post-dose[‡]	Descriptions
			%	%	%	%	
I	**Cardiovascular disease:**						
1	Resistin	100	39 ± 3.02**	43 ± 1.21**	52 ± 1.96**	72 ± 1.04**	Pathogenesis of obesity-mediated insulin resistance and type 2 diabetes mellitus
2	IL-2	100	28 ± 1.99**	73 ± 1.52*	79 ± 2.64**	71 ± 1.71**	Interleukin-2
3	IL-6	100	56 ± 2.20**	76 ± 2.02**	61 ± 0.89**	62 ± 1.36**	Interleukin-6
4	IL-8	100	65 ± 1.22**	69 ± 1.22**	77 ± 2.62**	67 ± 2.36**	Interleukin-8
5	IL-12	100	18 ± 0.62**	78 ± 1.03**	70 ± 2.06**	71 ± 3.40**	Interleukin-12
6	1L-17α	100	63 ± 2.17**	82 ± 1.86**	69 ± 0.89**	84 ± 0.54**	Insulin-enhances nitric oxide, and NF-κB.
7	IL-18	100	75 ± 1.24**	59 ± 1.30**	85 ± 1.34**	79 ± 3.00**	Interleukin-18
8	COX-2	100	69 ± 1.35**	69 ± 0.83**	61 ± 2.68**	70 ± 1.52**	Cyclooxygenase-2
9	GAPDH	100	82 ± 1.76**	88 ± 2.03*	88 ± 1.00*	71 ± 2.66**	Glyceraldehyde-3-phosphate dehydrogenase
10	IP-10	100	83 ± 1.61**	37 ± 1.85**	30 ± 8.50**	19 ± 2.64**	Interferon-Inducible Protein-10
II	**Inflammation:**						
11	TNF-α	100	52 ± 1.80**	57 ± 2.11**	27 ± 0.87**	87 ± 0.94*	Tumor Necrosis Factor-α
12	INF-γ	100	61 ± 2.57**	73 ± 2.48**	45 ± 3.0**	64 ± 1.90**	Interferon-γ
13	MIP-1α	100	62 ± 1.95**	73 ± 1.55**	67 ± 0.46**	64 ± 1.53**	Macrophage Inflammatory Protein-1α
14	NOS-2	100	61 ± 2.02**	62 ± 1.62**	76 ± 0.81**	64 ± 4.24**	Nitric oxide synthase-2
15	VCAM-1	100	83 ± 2.36**	83 ± 2.59**	72 ± 0.72**	84 ± 0.91**	Vascular cell adhesion molecule
16	MCP-1	100	35 ± 2.44**	60 ± 3.22**	36 ± 2.83**	39 ± 0.66**	Monocyte Chemotactic Protein-1
III	**Cancer:**						
17	IGF-1	100	64 ± 4.03**	74 ± 1.99**	65 ± 1.49**	61 ± 3.69**	Insulin-like Growth Factor-1
18	P-53	100	165 ± 2.43**	3.27**	126± 0.57**	197 ± 2.96**	P-53 tumor suppressor protein
19	FAS-1	100	155 ± 3.65**	148 ± 1.44**	129 ± 1.41**	132 ± 0.89**	Fatty acid synthetase-1
20	VEGF	100	65 ± 2.09**	73 ± 3.84**	71 ± 0.93**	78 ± 1.74*	Vascular Endothelial Growth Factor
21	CCND-1	100	27 ± 4.89**	48 ± 1.37**	71 ± 0.59**	63 ± 3.31**	Cyclin D-1
IV	**Diabetes:**						
22	PAI-1	100	33 ± 1.61**	76 ± 1.15**	57 ± 2.93**	72 ± 1.02**	Plasmenogen Activator Inhibitor-1
V	**Aging:**						
23	IL-1α	100	45 ± 3.15**	72 ± 1.84**	53 ± 1.99**	66 ± 2.76**	Interleukin-1α
24	IL-10	100	17 ± 1.91**	45 ± 1.79**	57 ± 2.66**	45 ± 0.66**	Interleukin-10

*Values in a row sharing a common asterisk are significantly different at *P*<0.01; *P*<0.001.
[‡]The effect of δ-tocotrienol on resistin, IL-2, IL-6, IL-8, IL-10, IL-12, TNF-α and IFNγ has been reported recently (AAQ et al. BJMMR. 6(4): 351-366, and JCEC. 2015. 6:4. 1000367).
[¶]The mRNAs IL-12, IL-17α, IL-18 (inflammation); IL-2 (cancer); and resistin, IL-6, IL-17α (diabetes) also involved in these diseases.

Table 5: Effects of NS-5, resveratrol, quercetin, and δ-tocotrienol (Pre-dose vs Post-dose) on gene expression of messengerRNAs (mRNAs) of hypercholesterolemic humans.

produced by endothelial cells, and also synthesized by adipose tissue, and an increased level of PAI-1 has been found with a number of atherosclerotic factors, and patients with insulin resistance syndrome and diabetes mellitus.

Protein53 (P53) is a tumor suppressor protein that works by regulating the cell cycle, and its activity stops the formation of tumors. FAS-1 protein forms the death-inducing signaling complex (DISC) upon ligand binding. It may act as a cell adhesion molecule. Vascular Endothelial Growth Factor (VEGF) plays an important role in regulating major angiogenic processes such as prolification, migration, differentiation and apoptosis [32].

The circulating miRNAs are novel biomarkers for diverse cardiovascular diseases, including acute myocardial infarction, heart failure, coronary artery disease, diabetes, stroke, hypertension, and acute pulmonary embolism [17]. The impacts of NS-5 mixture and its components on a cluster of 1-23 miRNAs has been shown in normal cholesterolemic humans and hypercholesterolemic humans (Table 7). The plasma high levels of Let-7a expression may be an indicator of impaired cell cycle function in old people with normal cholesterol levels, and this miRNA was significantly down-regulated with the treatment of NS-5, and its components. Most of the highly expressed miRNA that were lower in the blood of patients with coronary artery disease are known to be expressed in endothelial cells (miR-126 and members of the miR-17-92 cluster, namely miR-17, miR-19b, miR-20a, and miR-106a) and down-regulated in several cell types [33]. Plasma levels of miR-126, miR-29a, miR-92a were regulated with NS-5 mixture, and each of its components in hypercholesterolemic humans. In normal cholesterolemic humans, these miRNAs were down-regulated compared to their pre-dose values. MicroRNAs (miR-125a, miR-155, miR-216a) associated with inflammation that were up-regulated in normal cholesterolemic as well as patients with coronary artery disease were down-regulated with the treatments of NS-5 mixture, and its components.

It is reported that over 75 miRNAs have different type of expression. MicroRNA-20b and miRNA-21 were implicated in cardiac remodeling (anti-angiogenic), but now they are also linked with pancreatic cancer and osteogenic differentiation [34,35]. Moreover, miRNA-21 is also known as a good biomarker for inflammation. Plasma miRNA analyses

			NS-5	Resveratrol	Quercetin	δ-Tocotrienol
	‡MicroRNAs	Pre-dose	Post-dose	Post-dose	Post-dose	Post-dose
		%	%	%	%	%
I	**Cardiovascular disease:**					
a	**Down-regulation**					
1	miRNA-7a	100	43 ± 1.02**	74 ± 1.83**	78 ± 1.76**	61 ± 0.66**
2	miRNA-10b	100	34 ± 2.40**	87 ± 0.64*	91 ±1.13*	42 ± 4.17**
3	miRNA-15a	100	68 ± 2.07**	92 ± 1.89*	67 ± 1.49**	87 ± 0.24*
4	miRNA-16	100	61 ± 1.66**	87 ± 0.44*	74 ± 1.16**	71 ± 1.24**
5	miRNA-20a	100	61 ± 0.97**	83 ± 0.75*	93 ± 0.98*	71 ± 1.07**
6	miRNA-21	100	43 ± 2.14**	79 ± 0.62**	50 ± 1.50**	40 ± 2.06**
7	miRNA-29a	100	83 ± 0.97*	87 ± 2.32*	74 ± 1.09**	84 ± 0.41**
8	miRNA-92a	100	36 ± 2.25**	79 ± 0.88**	41 ± 1.68**	55 ± 1.91**
9	miRNA-126a	100	71 ± 0.79**	91 ± 1.45*	67 ± 6.08**	82 ± 0.52*
10	miRNA-133a	100	35 ± 1.52**	84 ± 1.52*	44 ± 2.44**	72 ± 0.76**
11	miRNA-200	100	64 ± 1.09**	70 ± 2.22**	79 ± 0.77**	84 ± 0.69*
12	miRNA-206	100	48 ± 0.98**	58 ± 1.98**	70 ± 1.41**	78 ± 0.91**
b	**Up-regulation**					
13	miRNA-499	100	127 ± 1.20**	122 ± 0.57**	144 ± 4.39**	132 ± 2.00**
II	**Inflammation:**					
c	**Up-regulation**					
14	miRNA-373	100	176 ± 1.96**	164 ± 4.20**	136 ± 1.79**	132 ± 1.30**
15	miRNA-93	100	341 ± 3.38**	331 ± 4.14**	129 ± 2.50**	310 ± 5.12**
16	miRNA-192	100	362 ± 1.96**	229 ± 7.91**	357 ± 5.26**	282 ± 1.98**
17	miRNA-216a	100	367 ± 5.89**	220 ± 5.44**	319 ± 6.81**	177 ± 5.06**
18	miRNA-503	100	259 ± 4.77**	240 ± 4.93**	121 ± 1.29**	130 ± 3.11**
d	**Down-regulation**					
19	miRNA-101a	100	55 ± 1.20**	63 ± 2.18**	84 ± 1.06*	66 ± 1.37**
20	miRNA-125a	100	64 ± 1.85**	68 ± 1.22**	87 ± 0.79*	86 ± 1.60*
21	miRNA-155	100	23 ± 2.53**	66 ± 4.81**	57 ± 0.63**	26 ± 2.05**
22	miRNA-223	100	62 ± 2.36**	40 ± 5.06**	46 ± 1.44**	82 ± 1.81*
III	**Aging:**					
e	**Down-regulation**					
23	miRNA-146a	100	21 ± 1.98**	44 ± 4.48**	33 ± 3.31**	26 ± 1.93**

* - **Values in a row sharing a common asterisk are significantly different at *$P<0.01$; **$P<0.001$.

‡The Effects of δ-tocotrienol on miRNAs-7a, miR-15a, miR-20a, miR-21, miR-29a, miR-92a, miR-200, miR-206 (in BJMMR. 2015; 6(4): 351-366), and miR-16-1, miR-125, miR-133a, miR-155, miR-223, miR-372, miR-10b, miR-18a, miR-214 (in JCEC. 2015; in press) have been reported. These miRNAs of δ-tocotrienol were included for comparison purpose only.

Table 6: The effects of various compounds on plasma circulating microRNAs (miRNA), the novel biomarkers for cardiovascular disease, cancer, diabetes, and aging process in normal cholesterolemic humans.

revealed reduction in the levels of R-15a, R-20b, R-21, and R-223 by the treatments of NS-5 and its components in normal cholesteromic humans only, as these miRNAs were associated with diabetes mellitus type 2 and also with cardiovascular disease [36]. It was reported that high glucose concentration reduced the level of miR-126a in endothelial cells. The reduction of miR-126a was confined to circulating vesicles in the plasma of type 2 diabetes patients [37]. These finding might explain the impaired peripheral angiogenic signaling in patients with diabetes mellitus. MicroR-126a also promotes blood vessel growth, one of the most predictive markers of diabetes.

As pointed out earlier miRNAs are novel post-transcriptional modulator of gene expression with potential roles as regulators of skeletal muscle mass and function possibly by contributing to reduced muscle cell renewal and regeneration in the aging human muscle. A characteristic of aging is loss of skeletal muscle known as sarcopenia [38]. Aging is the predominant risk factor for developing cardiovascular disease. Many miRNAs appear to be dysregulated during cellular senescence, aging and disease [38]. However, only few miRNAs have been linked to age-related changes in cellular and organ functions. More recent evidence has showed that miRNAs can contribute in the regulation of longevity, and miRNA-71 remarkably involved in longevity in a cell-non-autonomous manner. A member of the Let-7 family is down-regulated with advanced aging, which consists of the known function in the suppression of the steroid hormone receptor daf-12 [39].

Recently, a review has reported the relationship between various cytokines and miRNAs, where the regulation of miRNAs by pro- and anti-inflammatory cytokines and the regulatory cytokines were discussed, describing in detail how miRNAs mediate some of the known functions of these cytokines [19]. The regulatory correlation between plasma levels of several pro- and anti-inflammatory cytokines (TNF-α, IL-1α, IL-6, IL-10, IL-12, IFN-γ) and miRNAs (miR-29a, miR-92a, miR-125a, miR-155) modulated by treatments of NS-5 and its components was described in our earlier reports [3,4]. The first example of miRNA induction by IL-1 was demonstrated in a human monocytic leukemia cell line THO1, when miR-146a and miR-146b were induced in an NF-κB-dependent manner by repressing IL-1 signaling, IRAK1 and TRAF6, thereby creating a negative feed-back loop by switching off or

			NS-5	Resveratrol	Quercetin	δ-Tocotrienol
	‡MicroRNA	Pre-dose	Post-dose	Post-dose	Post-dose	Post-dose
		%	%	%	%	%
I	**Cardiovascular disease:**					
a	**Up-regulation**					
1	miRNA-7a	100	128 ± 1.41**	124 ± 2.55**	116 ± 1.24*	113 ± 1.74*
2	miRNA-10b	100	203 ± 1.03**	123 ± 2.00**	180 ±1.52**	153 ± 5.25**
3	miRNA-15a	100	147 ± 3.14**	131 ± 1.16**	127 ± 0.66**	120 ± 0.53**
4	miRNA-16	100	231 ± 2.83**	209 ± 1.90**	127 ± 1.04**	131 ± 1.26**
5	miRNA-20a	100	252 ± 2.4**2	209 ± 7.73**	146 ± 6.08**	176 ± 5.15**
6	miRNA-21	100	242 ± 0.94**	192 ± 5.20**	123 ± 1.02**	129 ± 3.33**
7	miRNA-29a	100	217 ± 1.49**	123 ± 1.63**	115 ± 5.33*	206 ± 2.48**
8	miRNA-92a	100	245 ± 1.09**	163 ± 2.64**	119 ± 0.88*	239 ± 4.07**
9	miRNA-126a	100	251 ± 1.78**	174 ± 4.65**	167 ± 2.84**	158 ± 2.10**
10	miRNA-133a	100	237 ± 2.52**	130 ± 5.23**	125 ± 3.69**	195 ± 3.40**
11	miRNA-200	100	168 ± 3.60**	125 ± 1.89**	136 ± 0.91**	147 ± 5.56**
12	miRNA-206	100	148 ± 2.78**	133 ± 1.25**	119 ± 1.44*	122 ± 2.77*
b	**Down-regulation**					
13	miRNA-499	100	42 ± 1.88**	58 ± 1.97**	71 ± 1.65**	54 ± 3.96**
II	**Inflammation:**					
c	**Up-regulation**					
14	miRNA-373	100	182 ± 4.61**	123 ± 2.05**	174 ± 2.41**	170 ± 4.39**
15	miRNA-93	100	391 ± 1.79**	150 ± 3.35**	122 ± 2.84**	332 ± 2.78**
16	miRNA-192	100	245 ± 4.76**	181 ± 1.32**	204 ± 3.61**	219 ± 3.45**
17	miRNA-216a	100	289 ± 6.85**	250 ± 3.98**	250 ± 4.95**	147 ± 2.47**
18	miRNA-503	100	345 ± 1.70**	271 ± 1.60**	163 ± 2.10**	127 ± 1.61**
d	**Down-regulation**					
19	miRNA-101a	100	71 ± 0.96**	82 ± 1.06*	42 ± 1.57**	84 ± 1.96*
20	miRNA-125a	100	36 ± 2.47**	64 ± 5.20**	53 ± 1.39**	67 ± 0.93**
21	miRNA-155	100	39 ± 2.92**	72 ± 2.53**	47 ± 3.37**	46 ± 2.16**
22	miRNA-223	100	22 ± 1.34**	74 ± 0.84**	67 ± 1.05**	29 ± 2.01**
III	**Aging:**					
e	**Down-regulation**					
23	miRNA-146a	100	33 ± 2.46**	71 ± 3.02**	53 ± 3.33**	36 ± 1.10**

* - **Values in a row sharing a common asterisk are significantly different at *$P<0.01$; **$P<0.001$.

‡The Effects of δ-tocotrienol on miRNAs-7a, miR-15a, miR-20a, miR-21, miR-29a, miR-92a, miR-200, miR-206 (in BJMMR. 2015; 6(4): 351-366), and miR-16-1, miR-125, miR-133a, miR-155, miR-223, miR-372, miR-10b, miR-18a, miR-214 (in JCEC. 2015; 4:6. 1000367). have been reported. These miRNAs of δ-tocotrienol were included for comparison purpose only.

Table 7: The effects of variouscom pounds on plasma circulating microRNAs (miRNA), the novel biomarkers for cardiovascular disease, cancer, diabetes, and aging process in hypercholesterolemic humans.

fine-tuning the pro-inflammatory response [40]. This was corroborated by later studies, which showed that over-expression of miR-146 could reduce the expression of IL-1-induced inflammatory cytokines in both epithelial and primary fibroblasts cells [41]. Induction of miR-155 has also been shown to modulate IL-1 signaling [42]. The enforced expression of miR-155 in rheumatoid arthritis synovial fibroblasts could block the production of IL-1-induced matrix metalloproteinase 3 [43]. From the studies, it appears that the IL-1-induction of miRNAs plays an important role in its negative regulation and highlights how manipulation of miRNAs could present new avenues for the therapy of IL-1α- associated diseases.

Similarly, TNF-α is a pro-inflammatory cytokine and is one of the principle mediators of the inflammatory disorders similar to those associated with IL-1α, such as rheumatoid arthritis, inflammatory bowel disease and psoriasis. Elevated levels of TNF-α are associated with many inflammatory disorders [3]. TNF-α can induce a subset of miRNAs and current evidence suggests that miRNAs play a role in mediating TNF-α messenger RNA stabilization. TNF-α can positively influence its own expression by inducing miR-155 and down-regulation of miR-125b treatments of NS-5, and its components as shown in the present study.

Recent evidence suggests a role for miRNAs in IL-6-mediated cell survival and IL-6 signaling modulation. Up-regulation of miR-21 and let-7 miRNA members was found in malignant myeloma, hepatocellular and cholineangiocyte cells due to over-expression of IL-6, which was down-regulated by the treatments of NS-5 and its components. IL-6 may also modulate its own signaling pathway via induction of the miR-17 - miR-92 cluster [4]. In several studies, it was demonstrated how the manipulation miRNA expression through over-expression or repression is sufficient to elicit a response independent of the original emphasizing an important function in miRNA biology [44,45].

Interleukin-10 is an anti-inflammatory cytokine that is crucial for dampening the inflammatory response after pathogen invasion as described earlier [3]. One particular mechanism of action is to down-regulate pro-inflammatory genes such as those encoding IL-1, TNF-α, and IL-6. The IL-10-mediated inhibition of miR-155 led to an increase in SHIP1 expression, supporting the role of IL-10 as an

anti-inflammatory mediator, as well as identifying a novel gene target for IL-10 [3,46]. Interferons (IFN) are cytokines that plays a key role in host defense against viral invasion and may have anti-viral and therapeutic potential against HIV infection [47]. All these highlight the hidden complexity of miRNAs induction and regulation of targets by cytokines, and suggest that similar mechanisms may exist for other cytokine signaling components [19].

Conclusions

The NS-5 mixture, and its components (resveratrol, quercetin, δ-tocotrienol, nicotinic acid) treatments caused significant reduction in superoxide production (12% to 19%) in HUVEC. These reductions were much more pronounced in LPS-stimulated HUVEC (26% to 40%), compared to pre-dose values. The maximum inhibition in superoxide production was with NS-5 treatment group compared to its individual components. These findings were further supported by serum total cholesterol levels of NS-5 treated group (24%) versus resveratrol, quercetin, and d-tocotrienol (18%-22%) treated groups only in hypercholesterolemic humans. A similar trend was observed in the reduction of serum levels of NO, and CRP while an increase in TAS was observed in normal cholesterolemic and hypercholesterolemic humans. There were significant reductions in the levels of pro-inflammatory cytokines and gene expressions of resistin, IL-2α, IL-6, IL-12, IL-18, TNF-α, and others, involved in the pathogenesis of atherosclerosis, diabetes, and ageing processes in both groups of subjects. The plasma circulating miRNAs associated with inflammation and cardiovascular disease (miR-92a, miR-126-a, miR-133a, miR-146-a, miR-155, miR-223, miR-101a, miR-499) of these treatments in normal cholesterolemic subjects were down-regulated compared to their respective pre-dose values. However, a cluster of 1-12 miRNAs (miR-7a, miR-10b, miR-15a, miR-16, miR-20a, miR-21, miR-29a, miR-126a, miR-133a, miR-200, miR-206) associated with cardiovascular disease were down-regulated in hypercholesterolemic humans, and up-regulated with NS-5 mixture and its components treatments. The miRNA-146a significantly increased during senescence, whereas the study compounds treatment significantly decreased these elevated levels of miRNA-146a. Several miRNAs have been implicated in the epigenetic regulation of key metabolic, inflammatory, cancer, and key anti-angiogenic pathways in type 2 diabetes, and those have been influenced by this study treatments. As far as safety and tolerability of NS-5 or other treatments are concerned, none of the participants reported any adverse events or reactions either during or after the course of the study using these naturally-occurring compounds. The present results indicated that NS-5 mixture is the most effective inhibitor/modulator of superoxide production and other several risk with cardiovascular and factors associated other diseases compared to its individual components (resveratrol, quercetin, and δ-tocotrienol), thus confirming our hypothesis, which is also supported by other investigators [27,48,49].

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Conflict of Interest

A.A. Qureshi, D.A. Khan, W. Mahjabeen, N. Silswal, and N. Qureshi have no conflict of interest to declare.

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