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

Asaf A Qureshi1,*, Dilshad A Khan2, Wajiha Mahjabeen3, Neerupma Silswal4 and Nilofer Qureshi1,3
1Department of Basic Medical Sciences, University of Missouri-Kansas City, 2411 Holmes Street, Kansas City, MO 64108, USA
2Department of Chemical Pathology & Endocrinology, Armed Forces Institute of Pathology, and National University of Medical Science, Rawalpindi, Pakistan, 64000
3Division of Pharmacology and Toxicology, School of Pharmacy, University of Missouri-Kansas City, 2464 Charlotte Street, Kansas City, MO 64108, USA

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 (11% to 24%; P<0.01) in HUVEC. These reductions were more pronounced with LPS-stimulated HUVEC (26% to 40%; P<0.01). 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.

**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-terostilbene, 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-glutamyltransferase (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
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.

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-xB 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. Lancaster, 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...
The participants (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 subgrouped by sex, and randomly divided into five groups of placebo, NS-5 mixture, resveratrol, quercetin, and δ-tocotrienol for treatments.

**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). 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.

**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% CO2, 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/gm 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 confluency, cells were pretreated with NS-5 mixture and its components. Upon reaching confluence, 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 subgrouped 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/m²) 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) glased 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 (Seimien, LA, California, USA) on Immulite 1000 (Immulate, 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 (Diameter 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 was carried for all the treatments. The superoxide productions were significantly inhibited by the previously reported method [21]. Serum TAS 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 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-UM00461 (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
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 cholesterolemics versus hypercholesterolemics 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 hypercholesterolemic 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

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

<table>
<thead>
<tr>
<th>#</th>
<th>Parameters</th>
<th>Normal cholesterolemic (Means ± SD)</th>
<th>Hypercholesterolemic (Means ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Age</td>
<td>61.51 ± 7.46</td>
<td>60.82 ± 6.06</td>
</tr>
<tr>
<td>2</td>
<td>Males/Females (n)</td>
<td>3/2</td>
<td>3/2</td>
</tr>
<tr>
<td>3</td>
<td>Height (meter)</td>
<td>1.71 ± 0.09</td>
<td>1.72 ± 0.08</td>
</tr>
<tr>
<td>4</td>
<td>Weight (kg)</td>
<td>72.97 ± 4.99</td>
<td>77.88 ± 8.96</td>
</tr>
<tr>
<td>5</td>
<td>BMI (kg/m²)</td>
<td>24.79 ± 2.05</td>
<td>26.43 ± 3.10</td>
</tr>
<tr>
<td>6</td>
<td>Systolic BP (mmHg)</td>
<td>136.43 ± 8.61</td>
<td>136.76 ± 7.67</td>
</tr>
<tr>
<td>7</td>
<td>Diastolic BP (mmHg)</td>
<td>84.71 ± 5.41</td>
<td>87 ± 6.96</td>
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<tr>
<td>8</td>
<td>Serum Creatinine (µmol/L)</td>
<td>90.77 ± 10.90</td>
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<tr>
<td>9</td>
<td>Serum ALT (U/L)</td>
<td>32.14 ± 9.72</td>
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<tr>
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<td>Serum Total Cholesterol (mmol/L)</td>
<td>6.57 ± 0.16</td>
<td>96.73 ± 12.47</td>
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<td>Serum Triglyceride (mmol/L)</td>
<td>1.71 ± 0.09</td>
<td>1.72 ± 0.08</td>
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<tr>
<td>12</td>
<td>Serum NO (µmol/L)</td>
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<td>4.91 ± 0.21</td>
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<td>13</td>
<td>Serum CRP (mg/L)</td>
<td>2.22 ± 0.09</td>
<td>4.96 ± 0.17</td>
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<td>14</td>
<td>Serum Triglyceride (mmol/L)</td>
<td>1.71 ± 0.09</td>
<td>1.72 ± 0.08</td>
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<td>15</td>
<td>Serum CRP (mg/L)</td>
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<td>4.96 ± 0.17</td>
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<tr>
<td>16</td>
<td>Serum TAS (mmol/L)</td>
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</table>

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 37°C, 5% CO₂ 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 sixth 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.

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% CO₂ 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 sodium ethidium 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.
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 4: Dietary supplement of NS-5 mixture and its main components on dihydroethidium staining for superoxide production with and without LPS-stimulated HUVEC.

Figure 5: Serum total cholesterol levels were decreased in free-living normal cholesterolemic 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 who were administered one capsule of 250 mg/d of NS-5 mixture, or resveratrol, or quercetin, or δ-tocotrienol for four weeks. The columns 5-8 represent group # 2 of hypercholesterolemic participants who were transferred to AHA Step-1 diet plus one capsule of 250 mg/d of NS-5 mixture 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 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 †, ¶, § $P<0.01$.

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 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 4: Dietary supplement of NS-5 mixture and its main components on dihydroethidium staining for superoxide production with and without LPS-stimulated HUVEC.
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 microRNAs were included in the present table 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 microRNAs were divided under different categories (cardiovascular, inflammation, cancer, diabetes and aging), and main functions were also included in 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 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, R-372, R-214) have been reported in our recent publications [3,4]. The functions of resistin, IL-1α, IL-2, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-γ, in our earlier publications [1,2]. The effects of δ-tocotrienol on the proteomic, genomic and microRNAs in humans, which was lacking in normal cholesterolemic and hypercholesterolemic 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 microRNAs in normal cholesterolemic and hypercholesterolemic humans as shown in Tables 4 and 5, respectively.

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, 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-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 δ-tocotrienol treatments, compared to their respective pre-dose percentage values in hypercholesterolemic humans (Table 7). The down-regulation of microRNAs 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 microRNAs 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...

Table 2: Effects of NS-5, resveratrol, quercetin, and δ-tocotrienol (Pre-dose vs Post-dose) on plasma cytokines/proteins of normal cholesterol-depleted rats. 

<table>
<thead>
<tr>
<th>Cytokines²</th>
<th>NS-5 Post-dose</th>
<th>Resveratrol Post-dose</th>
<th>Quercetin Post-dose</th>
<th>δTocotrienol Post-dose</th>
<th>Functions</th>
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<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
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<td>Cardiovascular disease:</td>
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</tr>
<tr>
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<td>IP-10</td>
<td>100</td>
<td>58 ± 1.7”</td>
<td>83 ± 2.9”</td>
<td>82 ± 2.6”</td>
</tr>
</tbody>
</table>

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 | 62 ± 1.7” | 75 ± 3.3” | 73 ± 2.1” | 67 ± 2.9” | Potent mediators of host defense and homeostasis. |
| 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 INF-γ has been reported recently (AAQ et al. BJMMR. 6(4): 351-366, JCEC. 2015; 6:4. 1000367. Resistin = Obesity-mediated insulin resistance and Type 2 diabetes Mellitus; Type 2 diabetes Mellitus; Interleukin-2; Interleukin-6; Interleukin-8; Interleukin-10; COX-2: Cyclooxygenase-2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TNF-α: Tumor Necrosis Factor-α Factor; 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. 

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 (longevity; 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, LC-3-II and
Resveratrol

It is an enzyme, which is responsible for the production of prostanoids.

Cytokines

Post-dose

IL-18 72 ± 1.5
It regulates the cell-cycle and acts as a tumor suppressor.

VEGF 135 ± 2.2
Plays a role in glycolysis and new host defense mechanism against

PAI-1 84 ± 1.2
100
90 ± 2.3
It is involved significantly in many vascular functions.

Plays a role in the activities of natural killer cells and T lymphocytes.

Post-dose

VEGF 135 ± 2.2
Plays a role in glycolysis and new host defense mechanism against

PAI-1 84 ± 1.2
100
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PAI-1 84 ± 1.2
100
90 ± 2.3
It is involved significantly in many vascular functions.

Plays a role in the activities of natural killer cells and T lymphocytes.
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<td>67 ± 1.72</td>
<td>67 ± 1.11</td>
<td>Nitric oxide synthase-2</td>
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<td>VCAM-1</td>
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<td>70 ± 2.01</td>
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<td>72 ± 2.93</td>
<td>84 ± 0.70</td>
<td>Vascular cell adhesion molecule</td>
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<td>MCP-1</td>
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<td>69 ± 4.49</td>
<td>83 ± 2.83</td>
<td>77 ± 2.17</td>
<td>76 ± 0.20</td>
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<tr>
<td>17</td>
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<td>68 ± 3.56</td>
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<td>71 ± 2.93</td>
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<td>P-S3</td>
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<td>217 ± 4.87 (\pm 0.01)</td>
<td>113 ± 1.19</td>
<td>120 ± 1.96</td>
<td>239 ± 6.86</td>
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<tr>
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<td>119 ± 2.78</td>
<td>151 ± 3.07</td>
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<td>71 ± 2.51</td>
<td>73 ± 1.78</td>
<td>83 ± 0.85</td>
<td>90 ± 0.47</td>
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<tr>
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<td>40 ± 5.33</td>
<td>71 ± 6.04</td>
<td>54 ± 1.91</td>
<td>67 ± 3.71</td>
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<td><strong>IV</strong></td>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>22</td>
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<td>26 ± 3.21</td>
<td>81 ± 0.48</td>
<td>71 ± 4.79</td>
<td>71 ± 2.37</td>
<td>Plasminogen Activator Inhibitor-1</td>
</tr>
<tr>
<td>23</td>
<td>IL-1α</td>
<td>100</td>
<td>70 ± 1.67</td>
<td>84 ± 1.52</td>
<td>84 ± 2.09</td>
<td>80 ± 0.76</td>
<td>Interleukin-1α</td>
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<tr>
<td>24</td>
<td>IL-10</td>
<td>100</td>
<td>76 ± 1.44</td>
<td>96 ± 1.56</td>
<td>88 ± 4.12</td>
<td>69 ± 1.42</td>
<td>Interleukin-10</td>
</tr>
</tbody>
</table>

\(\text{a}\) - Values in a row sharing a common asterisk are significantly different at \(P<0.01\); \(P<0.001\).

\(\text{b}\) - The effect of δ-tocotrienol on resistin, IL-2, IL-6, 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).

\(\text{c}\) - The mRNAs IL-12, IL-17α, IL-18 (inflammation); IL-2 (cancer); and resistin, IL-6, IL-17a (diabetes) also involved in these diseases.

\(\text{d}\) - 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 CDK activity to drive the cell cycle. Plasminogen Activator Inhibitor-1 (PAI-1) is mainly

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 prostanooids, and it 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 vasodilatation 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
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 proliferation, 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 each of its components in hypercholesterolemic humans. In normal cholesterolemic as well as patients with coronary artery disease are known to be expressed in endothelial cells (miR-126 and miR-216a) associated with inflammation that were up-regulated in patients with insulin resistance syndrome and diabetes mellitus.

Pathogenesis of obesity-mediated insulin resistance and type 2 diabetes mellitus.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Pre-dose</th>
<th>Post-dose</th>
<th>Descriptions</th>
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<tbody>
<tr>
<td>Resistin</td>
<td>100</td>
<td>39 ± 3.02</td>
<td>52 ± 1.96</td>
</tr>
<tr>
<td>IL-2</td>
<td>100</td>
<td>28 ± 1.99</td>
<td>73 ± 1.52</td>
</tr>
<tr>
<td>IL-6</td>
<td>100</td>
<td>56 ± 2.20</td>
<td>76 ± 2.02</td>
</tr>
<tr>
<td>IL-8</td>
<td>100</td>
<td>65 ± 1.22</td>
<td>69 ± 1.22</td>
</tr>
<tr>
<td>IL-12</td>
<td>100</td>
<td>18 ± 0.62</td>
<td>78 ± 1.03</td>
</tr>
<tr>
<td>IL-17α</td>
<td>100</td>
<td>63 ± 2.17</td>
<td>82 ± 1.86</td>
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<tr>
<td>INFγ</td>
<td>100</td>
<td>75 ± 1.24</td>
<td>59 ± 1.30</td>
</tr>
<tr>
<td>Resistin</td>
<td>100</td>
<td>62 ± 1.95</td>
<td>73 ± 1.55</td>
</tr>
<tr>
<td>NOS-2</td>
<td>100</td>
<td>61 ± 2.02</td>
<td>62 ± 1.62</td>
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<tr>
<td>MCP-1</td>
<td>100</td>
<td>83 ± 2.36</td>
<td>83 ± 2.59</td>
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<tr>
<td>IGF-1</td>
<td>100</td>
<td>64 ± 4.03</td>
<td>60 ± 1.90</td>
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<tr>
<td>P-53</td>
<td>100</td>
<td>165 ± 2.43</td>
<td>73 ± 3.84</td>
</tr>
<tr>
<td>FAS-1</td>
<td>100</td>
<td>155 ± 3.65</td>
<td>148 ± 1.44</td>
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<tr>
<td>VEGF</td>
<td>100</td>
<td>65 ± 2.09</td>
<td>73 ± 3.84</td>
</tr>
<tr>
<td>CCND-1</td>
<td>100</td>
<td>27 ± 4.89</td>
<td>48 ± 1.37</td>
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<td>33 ± 1.61</td>
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<tr>
<td>IL-1α</td>
<td>100</td>
<td>45 ± 3.15</td>
<td>72 ± 1.84</td>
</tr>
<tr>
<td>IL-10</td>
<td>100</td>
<td>17 ± 1.91</td>
<td>45 ± 1.79</td>
</tr>
</tbody>
</table>
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 cholesteromic humans.

reflected 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-a, IL-1α, IL-6, 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 TH01, when miR-146a and miR-146b were induced in an NF-kb-dependent manner by repressing IL-1 signaling, IRAK1 and TRAF6, thereby creating a negative feed-back loop by switching off or
TNF-α, and IL-6. The IL-10-mediated inhibition of miR-155 led to an increase in SHIP1 expression, sup

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 manipulate miRNAs and current evidence suggests that miRNAs play a role in the negative regulation of TNF-α. 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-152b 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 carcinoma and cholinergic cholangiocyte 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-17-25 and 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 play a key role in host defense against viral infection 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 NS-5 mixture 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 δ-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 cholesterol 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 cholesterol and 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. This indicates 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].

Acknowledgments

We thank Ms. AnneM. Trias for editing the manuscript, and the president of Mega Resvertrol (60 Newton Toast # 32, Danbury CT, USA) for supplying 250 mg capsules of trans for the present and other studies in our lab. We also thank American River Nutrition, Inc. for donating the DeltaGold softgels (125 mg per capsule, American River Nutrition, Inc. Hadley, MA USA). The study was supported by Advanced Medical Research (AMR), Madison, Wisconsin, USA. The study was carried out under an FDA-approved IND # 36906. This study was supported in part from grant numbers GM102631 and GM102631S1 (N.Q.).

Conflict of Interest

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

References