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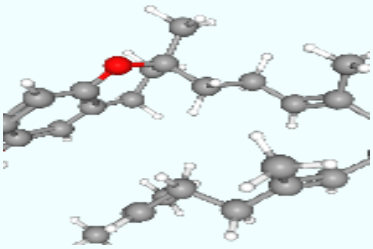
# A Novel Mixture of δ-Tocotrienol, Vitamin $D_3$, Resveratrol (NS-3) Significantly Decreases Diabetes Biomarkers Including Inflammatory in People with Type 2 Diabetes

Corresponding Author: Asaf A. Qureshi

## δ-TOCOTRIENOL

δ-Tocotrienol is a form of vitamin E that belongs to tocotrienols family. Tocotrienols and tocopherols constitute two main groups of vitamin E compounds.

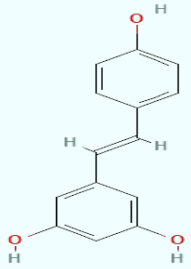


## VITAMIN $D_3$

Vitamin $D_3$ (cholecalciferol) is a crucial nutrient that plays various roles in the body, such as calcium absorption and bone health.

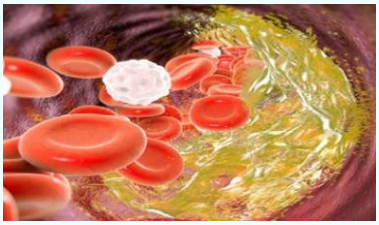

## RESVERATROL (Nutritional Supplement-NS-3)

Resveratrol is a natural compound found in certain plants, including the skin of red grapes, blueberries, raspberries, and peanuts. It belongs to a group of plant compounds called polyphenols and is known for its antioxidant properties.



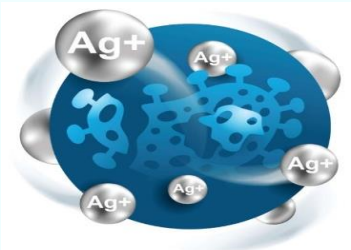
## ANTI-CHOLESTEROLEMIC AGENTS

Anti-cholesterolemic agents lower cholesterol in the body. Cholesterol is a fat, found in the blood, involves in many body functions. The elevated level of cholesterol (particularly, LDL-cholesterol) can increase the risk of cardiovascular disease.



## ANTI-INFLAMMATORY AGENTS

Anti-inflammatory agents reduce inflammation in the body. It is a natural response by immune system to injury or infection. Chronic inflammation can cause cardiovascular disease, diabetes, arthritis, and cancer.

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# *Re-Print: A Novel Mixture of δ-Tocotrienol, Vitamin $D_3$, Resveratrol (NS-3) Significantly Decreases Diabetes Biomarkers Including Inflammatory in People with Type 2 Diabetes

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**Running title:** ***Prevention of Type 2 Diabetes by a Mixture of Natural Products.***

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## Abstract:

**Aims:** Diabetes mellitus is a metabolic disorder identified by hyperglycemia due to insulin resistance. Impaired serum/plasma fasting glucose, HbA1c, hs-CRP are biomarkers, normally used to determine onset of diabetes. δ-Tocotrienol, vitamin $D_3$, and resveratrol (nutritional supplement-NS-3) are potent anticholesterolemic, anti-oxidative and anti-inflammatory agents. We hypothesized that a mixture of δ-tocotrienol, vitamin $D_3$, resveratrol (NS-3) will be more effective treatment for reducing diabetes biomarkers as compared to its individual components, in people with type 2 diabetes mellitus (T2DM).

**Methods:** To test our hypothesis, evaluation of NS-3 mixture and its individual components was carried out on diabetes inflammatory biomarkers using peripheral blood mononuclear cells (PBMC) obtained from healthy, normal and people with T2DM. A randomized placebo controlled double-blinded prospective trial of individual components (***n*** = 30/component), and NS-3 trial of people with T2DM (***n*** = 52/group), were given two capsules/d of cellulose/olive oil as placebo, individual components, or NS-3 mixture for 24-weeks.

**Results:** Significant down-regulation (15 - 74; ***P*** < 0.002) of gene expression was observed with individual components and NS-3 on diabetes biomarkers (IRS-1, SOD-2, GCKR, ICAM-1, VCAM-1, IL-6, IL-8) in PBMCs of T2DM, and in serum values of fasting glucose (11%), HbA1c (10%), hs-CRP (23%), fasting insulin (9%), HOMA-IR (20%), MDA (20%) of NS-3 treated people with T2DM after 24-weeks. Treatment with individual components showed significant decrease but were less effective than the mixture. RT-PCR analysis of blood RNAs obtained from NS-3 treated people with T2DM for 24-weeks resulted in significant (***P*** < 0.01) down-regulation of gene expression in diabetes biomarkers (IRS-1, SOD-2, GCKR, IGFPB-2) compared to pre-dose values.

**Conclusions:** Present results of *in vitro* and *in vivo* studies support our hypothesis that NS-3 mixture is more effective in lowering serum levels of several diabetes biomarkers including inflammatory gene expression markers compared to its individual components in people with T2DM.

**Keywords:** T2DM; PBMC; δ-tocotrienol; vitamin $D_3$; resveratrol; diabetes biomarkers; glucose; HbA1c; HOMA-IR; hs-CRP.

## Introduction

Diabetes mellitus is a complex, chronic metabolic disorder due to chronic inflammation, infection, and oxidative stress, are important factors for development of insulin resistance (IR) during the progression of type 2 diabetes mellitus (T2DM) [1]. It is a global health problem, and its prevalence is continuously increasing worldwide. The detection of T2DM often occurs late, although damage to organs or tissue starts early, even before the disease become clinical manifested, and chances of development of complications are more, and will also increase rate of morbidity, mortality, retinopathy with potential blindness, nephropathy that may lead to renal failure, and risk of foot, amputations. Therefore, a timely diagnosis can reduce the development of complications and augment the efficiency of treatment given to these individuals.

Hyperglycemia induced oxidative stress and inflammation, are associated with complications, such as diabetic nephropathy, retinopathy, and others [2]. The levels of glucose control and occurrence of diabetes associated complications occurred in different phases of disease, and strongly associated with enhanced oxidative stress [3]. Malondialdehyde (MDA) is the most assessed biomarker during diabetic complications, and chronic inflammation is thought to be an important factor for development of insulin resistance during the progression of T2DM [3]. Inflammation causes the release of different inflammatory markers such as hs-C-reactive protein (hs-CRP) and different cytokines such as interleukin (IL)-1α, IL-6, IL-8, ICAM-1, VCAM-1, and tumor necrosis factor-α (TNF-α). The increase in systemic markers of inflammation (hs-CRP, IL-6 and TNF-α) are associated with complications such as diabetic nephropathy and weakly associated with development of diabetic retinopathy [3,4].

Evaluation of other biomarkers and IL-1α in initiation and development of T2DM have been reported [5,6]. IL-1α plays its role in diabetes by enhancing β-cell failure, and it is a potential marker for onset and progression of T2DM *in vitro* studies [6]. Plasma monocytes chemoattractant protein-1(MCP-1), a chemokine is responsible for inducing insulin resistance and its levels are elevated in people with diabetes. Likewise, IL-8, an inducer of insulin resistance in diabetes has also been reported [7]. The vascular endothelial growth factor (VEGF) and IL-10 are also considered important factors in pathogenesis of diabetic nephropathy [8]. Excessive levels of these markers result in increased activation of nuclear factor kappa B (NF-κB). This causes the increase transcription of cytokine and chemokine genes responsible for development of insulin resistance, and impaired insulin action and ultimately results in production of hyperglycemia. Hyperglycemia induces altered protein, lipid, nucleic acid function, and gene expression causes cellular dysfunction thus leading to various complications associated with diabetes [3 - 9]. The risk factors like impaired fasting glucose, glucose tolerance and glycosylated hemoglobin (HbA1c) are used nowadays to diagnose the onset of diabetes mellitus, which are not sensitive enough and thus do not have the definite predictive values [10]. Beside these biomarkers, hs-CRP, adipokines and cytokines considered as potential novel biomarkers of metabolic diseases including type 2 diabetes mellitus [11].

The dietary supplements like δ-tocotrienol, vitamin $D_3$, and resveratrol have been established as potent anti-oxidative and anti-inflammatory agents, which are continuously re-evaluated for a long time in humans, animals, and in various tissues separately [12 - 25]. Data showing combined effects of these nutritional supplements in T2DM is still lacking, and due to these diverse results and limited number of studies, there is a need of large trials *in vivo* to determine impact of effects of nutritional supplements of naturally occurring compounds in people with T2DM. Thus, we have used several relatively inexpensive, commercially available naturally occurring, and FDA approved compounds with inhibitory properties of diabetes biomarkers including inflammation, such as levels of fasting glucose, HbA1c, hs-CRP, IRS-1, SOD-2, IGFPB-2, GCKR, PTPRN, IL-6, IL-8, ICAM-1, VCAM-1, and TNF-α.

The functions of IRS-1 are to lower blood glucose level and alleviates insulin resistance, SOD-2 is a free radical scavenging enzyme, PTPRN plays role in maintaining normal level of insulin, and GCKR for the accumulation of normal levels of insulin-containing vesicles in T2DM (based on Google search). Previously, we conducted several studies to evaluate the treatment response of combination of different nutritional supplements (d-tocotrienol, resveratrol, quercetin, pterostilbene, and nicotinic acid) in hypercholesterolemic subjects. All these studies showed significant post treatment changes in different inflammatory markers, cytokines, oxidative stress, lipid profile and different miRNAs in these individuals [26-28]. The oral hypoglycemic treatments, change of lifestyle (increasing physical activity), and dietary habits (consumption of fruits and vegetables) have been found most effective in preventing progression of diabetes and associated complications [29].

We have recently demonstrated that a mixture of δ-tocotrienol, resveratrol, quercetin, pterostilbene and nicotinic acid (NS-5 mixture) lowers serum total cholesterol levels and pro-inflammatory cytokines in hypercholesterolemic subjects more effectively than their individual components [26]. The hypothesis of present study is that a combination therapy would be more effective in inhibiting several diabetic biomarkers and that anti-inflammatory properties of naturally occurring compounds, δ-tocotrienol, vitamin $D_3$, resveratrol **(Figure 1)**, and their mixture (NS-3) would decrease the serum/plasma levels of fasting glucose, HbA1c, hs-CRP, MAD, other diabetic biomarkers, and levels of cytokines in people with T2DM more effectively than its individual components. To test our hypothesis, the first comparative evaluation of NS-3 mixture versus its individual components was tested *in vitro* using peripheral blood mononuclear cells (PBMCs) obtained from healthy normal, and those with T2DM. This was followed by clinical investigation of effects of a mixture of NS-3, and its individual components on oxidative stress, inflammation, different cytokines, and diabetic biochemical profile in people with T2DM to improve the quality of life of these individuals.

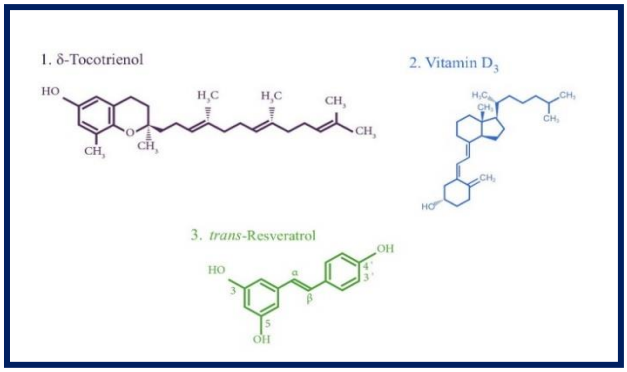


**Figure 1. Chemical structures of δ-tocotrienol, vitamin $D_3$, and resveratrol.**

## Materials and Methods

### Materials:

The 70% tocotrienol mixture (typical composition 90% δ-tocotrienol and 10% γ-tocotrienol) purified from annatto seeds was purchased from American River Nutrition, Inc. (Hadley, MA, USA); *trans*-Resveratrol from "Mega Resveratrol" (60 Newton Road # 32 Danbury CT, USA) and Vitamin $D_3$ purchased from Piping Rock, New York, USA. The peripheral blood mononuclear cells (PBMCs) from healthy normal subjects and T2DM subjects were purchased from Stem Cell Technologies, Vancouver, BC, Canada.

The 70 % δ-tocotrienol was purified to 98% as described earlier [30]. The capsulation of mixture of δ-tocotrienol (125 mg) + resveratrol (125 mg) + vitamin $D_3$ (125 µg [5000 IU]) = 250.125 mg/capsule; 90 capsules/bottle), and placebo capsule (125.125 mg cellulose + 125 mg olive oil, after removing micro-components of olive oil by washing with ethanol) = 250.125 mg/capsule. Capsules were prepared at Kabco Inc. New Jersey, USA. The bottles were blindly labeled as AMR-1 and AMR-2 by the capsulation company. RNeasy mini kits were obtained from QIAGEN Sciences (Germantown, MD, USA).

### Experimental:

#### Evaluation of a mixture of NS-3 and its components *in vitro* on various diabetes biomarkers and inflammatory cytokines in peripheral blood mononuclear cells (PBMCs) obtained from healthy normal and people with T2DM.

The stock solutions of each compound were prepared (1 mg/mL) in 95% ethanol and stored at -20 ºC. The working solutions (10 µM) of each compound and their mixture (NS-3) were prepared by diluting the appropriate volume of each stock solution in culture medium to give a final ethanol concentration 0.1% (v/v). The peripheral blood mononuclear cells (PBMCs) obtained from healthy normal or people with T2DM were added in each well (500,000 cells/well) in 96-well tissue culture plates and treated individually each compound as well as mixture of these compounds (triplicate of each compound and mixture), incubated at 37 ºC under 5% CO2 for 1h. After 1 h, total RNAs were isolated from cell lysates using Qiagen RNeasy Mini kit according to manufacturer's instructions for RT-PCR analyses of various biomarkers associated with diabetes. Quality of RNAs were assessed by spectrophotometric measurements. 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 using the ratio of 260/280 (2.02 - 2.08).

### Real-time PCR of purified treated RNAs

Real-time PCR (RT-PCR) was performed on total mRNAs isolated from untreated (control) and treated PBMCs by using One-Step qRT-PCR kit (Life Technologies, Foster City, California). All reactions were performed in triplicate using equal amount of mRNA per reaction in 96-well (500,000 cells/well) PCR plates. Gene expression from cell cultures was normalized ($2^{-\Delta\Delta C}$ analysis) to GAPDH.

#### Impact of a mixture NS-3 and its components on autophagy using peripheral blood mononuclear cells (PBMCs) obtained from healthy normal and people with T2DM.

The PBMC (200,000 cells/well in medium + 0.2% DMSO) were first differentiated with PMA (10 ng/mL) in media of 96-tissue culture white plate for 4 h at 37 ºC under 5% CO2 in an incubator, then washed with fresh media, followed by incubation with vehicle (medium + 2% DMSO, blank control), rapamycin, chloroquine, and mixture of rapamycin + chloroquine (as positive control) or δ-tocotrienol, vitamin $D_3$, resveratrol or mixture of these three compounds (NS-3) using a dose response of 0.0 µM, 10 µM, 20 µM, 40 µM, 80 µM of each compound or mixture for 4 h at 37 ºC, under CO2. The cells were washed with assay buffer followed by incubation with microscopy dual detection reagent (100 µL) for 30 min at 37 ºC in dark. The microscopy dual detection reagent was prepared according to the protocol of the manufacturer. After incubation, the PBMC were finally washed with assay buffer three times. The Fluorescence measurements were performed under Cytation-3-fluorometric reader using DAPI and FITC filer sets.

#### Impact of a mixture NS-3 and its components on diabetes biomarkers in healthy normal and people with T2DM.

### Study Design:

The present studies were double-blind, randomized, placebo-controlled trial (RCT). A non- probability convenience sampling technique was used. The study protocol was registered with WHO regional office in Asia (World Health Organization Sri Lanka Clinical Trials Registry, Sri Lanka Center; srilankactr@gmail.com), after ethical approval by the Institutional Review Board of Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan. The registry number was SLCTR/2018/019, dated 6.21.2018. The study was carried out according to the guidelines provided by the United States Food and Drug Administration (FDA, 2003) at (AFIP), Rawalpindi, and University of Health Sciences (UHS), Lahore. Pakistan. All participants of study # 1 and study # 2 have signed an informed consent form before start of the study. The purified total RNA samples were delivered at UMKC, School of Medicine after getting approval by the members "Compliance Officer (Christopher Winders)" and "Chemical Biological Safety Officer (Timothy Sturgis, RBP)" of Institution Board of UMKC School of Medicine, Kansas City, MO. USA.

### Study population:

People with T2DM (***n*** = 232) aged >30 years were selected over a period of 8 weeks for study # 1 and study # 2. The inclusion criteria for each study group were male and female participants diagnosed with T2DM, and exclusion criteria were acute illness, liver, renal, thyroid disorders or malignancy or history of taking anti-inflammatory drugs or vitamins (A, B, C, D, E, K) regularly or in the last 2-weeks. For the diagnosis of diabetes, their serum fasting glucose, random glucose, oral glucose tolerance (OGT) and HbA1c levels were measured. Individuals were labeled as diabetic if their fasting glucose was 126 mg/dL (7.0 mmol/L) or random blood glucose levels were 200 mg/dL (11.1 mmol/L), OGT $\geq$ 200 mg/dL (11.1 mmol/L) and HbA1c $\geq$ 6.5%.

All participants with T2DM in this project were screened at Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan. Their clinical history and general physical examination were carried out. The clinical history and general physical examination of each participant was recorded. A questionnaire was given to them. The questionnaire data was comprised of participant's height, weight, BMI, systolic and diastolic blood pressure at rest, history of smoking, physical activity, medicine intake. Drug history of participants was taken in detail (oral hypoglycemic drugs [Metformin, Glimepiride, Vildagliptin, Gliclazide, Glibenclamide], insulin, nitrates, oral aspirin, calcium antagonist, ACE inhibitors and diuretics). Height and weight were measured without shoes. Systolic and diastolic blood pressures were measured at rest. Body mass index (BMI, $kg/m^2$) was used as a measure of relative body weight. All relevant investigations have been carried out in the AFIP, Rawalpindi. The liver function tests, TSH and serum urea were analyzed to exclude liver, thyroid, and renal disorders respectively.

***In Study # 1***, baseline venous blood samples (12 h fast, 7:00 - 9:00 am) were drawn after 8-weeks (phase I). Then participants were randomly divided into four groups. The participants of Group A were provided capsules of AMR-1 (placebo group, ***n*** = 30 participants; 2 capsules/d of 250.125 mg/capsule of cellulose + olive oil (125.125 mg + 125.00 mg); group B (***n*** = 30) two capsules/d of δ-tocotrienol (250 mg/capsule); group C (***n*** = 30) two capsules/d of vitamin $D_3$ (5000 IU/capsule); and group D (***n*** = 30) capsules/d of resveratrol (250 mg/capsule) were given (one capsule after breakfast and second after dinner) for 24-weeks (phase II) as outlined in **Figure 2.**

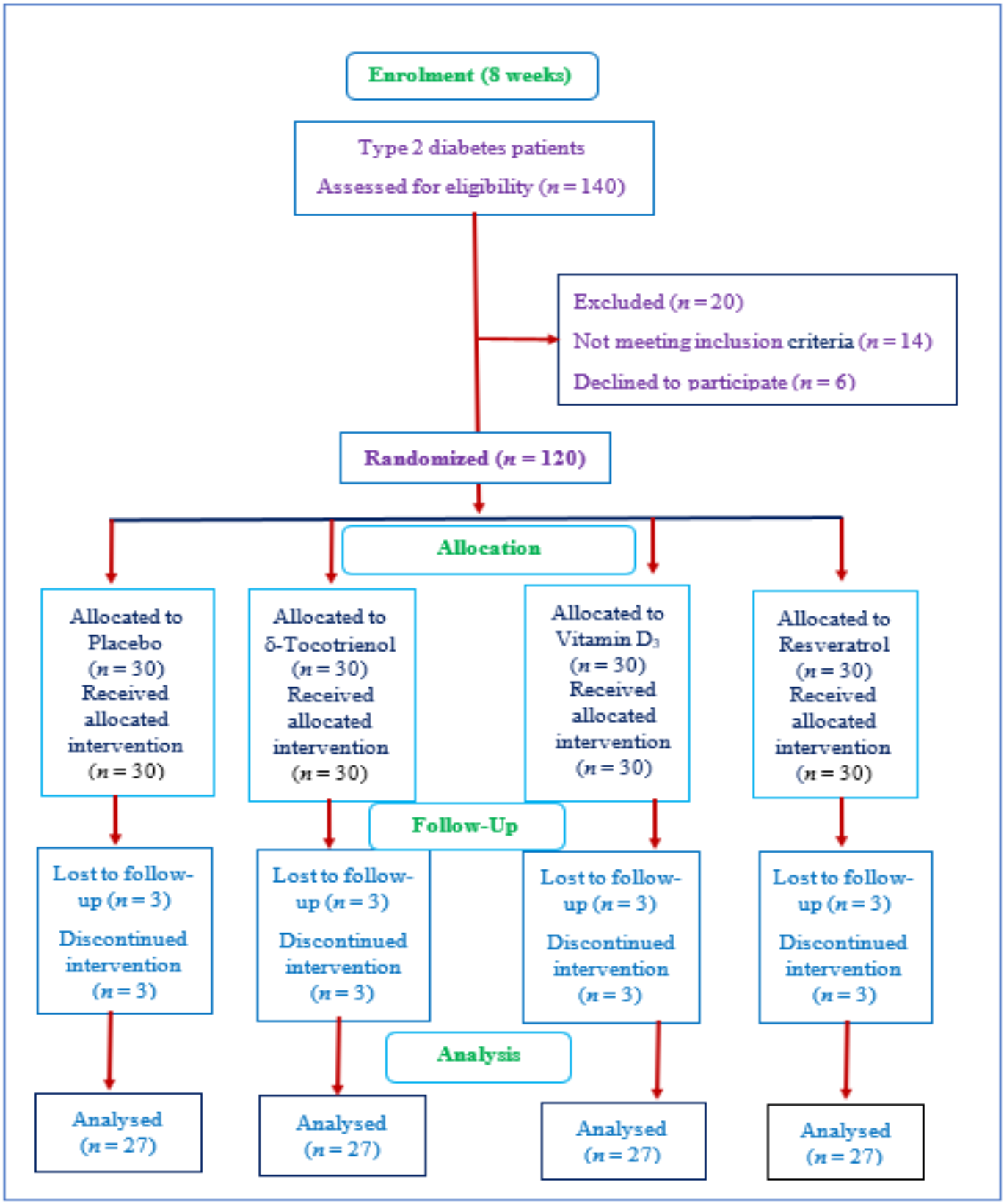


**Figure 2. Study # 1 protocol of effect of placebo vs. δ-tocotrienol, vitamin $D_3$, and resveratrol on diabetes biomarkers people with T2DM.**

***In study # 2*, ba**seline venous blood samples (12 h fast, 7:00 - 9:00 am) were also drawn after 8-weeks (phase I), then T2DM participants of placebo group (***n*** = 56) were given two capsules/d cellulose + olive oil (150.125 mg +150 mg/capsule) and NS-3 group (***n*** = 56) was given two capsules/d of a mixture of NS-3 (125 mg + mg + 5000 IU/capsule) for 24-weeks (phase II) as outlined in **Figure 3.** The capsules in each group were administered one capsule after breakfast and a second after dinner throughout the study. To ascertain full compliance of dietary recommendations and intake of nutritional supplements, participants were contacted by telephone. Two tubes (6 mL/tube) fasting venous blood sample were collected at the end of each phase, one sample into EDTA tubes for plasma and second set for serum. The blood tubes were centrifuged at 1200 x g for 10 minutes, followed by careful separation of plasma samples into three aliquots. One aliquot (2.0 mL) was immediately processed for total mRNAs purification. Plasma and serum (1.0 mL/tube) and purified total RNAs were stored at -80 ˚C for further analyses.

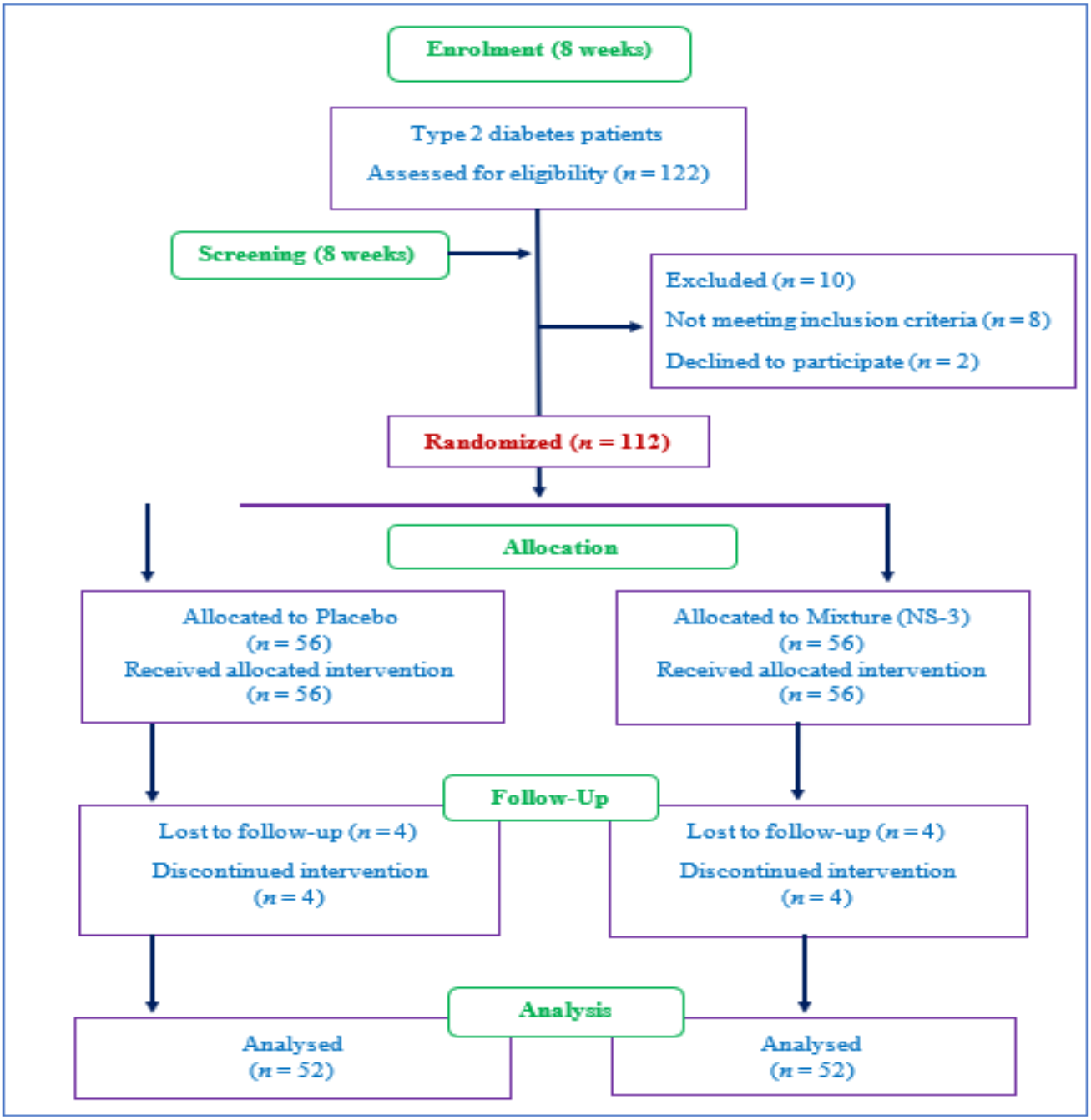


**Figure 3. Study # 2 protocol of effect of placebo vs. mixture of δ-tocotrienol, vitamin $D_3$, resveratrol (NS-3) on diabetes biomarkers people with T2DM.**

**Evaluation of a mixture of NS-3 on diabetes biomarkers and cytokines in RT-PCR of purified mRNAs of NS-3 treated people with T2DM.**

The total mRNAs were isolated from people with T2DM treated with a mixture NS-3 for 24-weeks by using One-Step qRT-PCR kit (Life Technologies, Foster City, California). The Real-time PCR assays of total mRNAs per reaction (200,000 RNAs/treatment) of pre-dose and post-dose were carried out as described above.

### Biochemical Analysis

Several biochemical assays were performed in all groups at the end of Phases I and II. The serum fasting glucose was determined by glucose oxidase method. Serum hs-CRP was analyzed by 2-site sequential chemiluminescent immune-metric assay kit (Seimen, LA) on Immulite 1000 (Immulite; Diagnostic Product Corporation). Serum hemoglobinA1c (HbA1c), bilirubin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, urea, uric acid, the estimation of these parameters was carried out in Department of Pathology, Rawalpindi, Pakistan. The serum/plasma levels of insulin, insulin resistance, oxidative stress, inflammatory biomarkers in people with T2DM were also determined.

### Sample Size Calculation in NS-3 Group

The sample size was carried out by G Power 3.1 software (Experimental Psychology institute, Dusseldorf, North Rhine-Westphalia, Germany) available online using 80% power and 95% confidence level. The levels of post-treatment of HbA1c, Hs-CRP and MDA in NS-3 treatment and placebo groups [Table 5] were analyzed based on previous studies [31,32].

### Statistical analyses:

The Statistical Package for Social Sciences (SPSS) software version 21.0 (IBM Corporation, New York, USA) was used for statistical analyses. Kolmogorov-VSmirnov test was applied on data to assess distribution for all the variables, mean, SD, median, and interquartile range (IQR) calculate for descriptive statistics. Serum cytokine levels, oxidative markers and glycosylated HbA1c among the people with T2DM in both groups were compared using Mann Whitney U test. Pearson correlation was also applied between HbA1c, MDA, hs-CRP, cytokines (Interleukin-6, IL-8, ICAM-1, VCAM-1, TNF-α, and IFN-γ). Analysis of covariance (ANOVA) was used to compare means of pre-treatment versus post-treatment. Data reported as mean ± SD (Standard Deviation) in Tables. The statistical significance level was set at 5% ($P < 0.05$).

## Results

**Evaluation of a mixture of NS-3 and its individual components on biomarkers of type 2 diabetes *in vitro* using PBMCs from normal and people with T2DM.**

The results of RT-PCR tested in PBMCs of people with T2DM indicated significant ($P > 0.05$ - 0.02) down-regulation in gene expression of important diabetes biomarkers (IRA-1, SOD-2, IGFBP-2, PTPRN, GCKR, resistin) with δ-tocotrienol (20% - 74%), vitamin $D_3$ (37% - 73%), resveratrol (58% - 74%), and NS-3 mixture (15% - 56%) as compared to their respective controls **(Table 1)**. Similarly, significant down-regulation was also observed with these compounds and NS-3 mixture in ICAM- 1, VCAM-1, IL-6, IL-8 (23% - 70%), respectively compared to their respective controls **(Table 1)**. These results clearly supported our original hypothesis that the NS-3 mixture effectively down-regulates gene expression of T2DM biomarkers compared to its individual components, except for δ-tocotrienol showed significant up-regulation in IFN-α (and with 80 μM [data is not shown]), and IL-8 (108%). The summary of these results was presented as percentage down- or up-regulation in **Figures 4A and 4B.** The above reported biomarkers were also tested in PBMCs obtained from healthy normal subjects under same conditions showed non-significant changes in these biomarkers compared to their respective controls (data not shown).

**Table 1: RT-PCR analysis of RNAs obtained from PBMCs of people with T2DM after treatment with a mixture of NS-3 and its components *in vitro* on diabetes biomarkers including inflammatory.**

| Biomarkers | Control | δ-Tocotrienol | Vitamin $D_3$ | Resveratrol | Mixture (NS-3) |
|---|---|---|---|---|---|
| 1. Insulin Receptor Substrate-1 (IRS-1) | 0.99 ± 0.04* | 0.33 ± 0.06 | 0.84 ± 0.07* | 0.74 ± 0.06 | 0.31 ± 0.06 |
| 2. Superoxide Dismutase (SOD-2) | | 0.75 ± 0.06 | 0.80 ± 0.08* | 0.89 ± 0.07* | 0.56 ± 0.08 |
| 3. Insulin Like FactorBinding Protein-2 (IGFBP-2) | | 0.20 ± 0.06 | 0.73 ± 0.07 | 0.70 ± 0.08 | 0.39 ± 0.06 |
| 4. Protein Tyrosine Phosphtase Receptor Type (NPTPRN) | | 0.81 ± 0.08* | 0.96 ± 0.07* | 0.60 ± 0.07 | 0.30 ± 0.11 |
| 5.Glucokines Regulator (GCKR) | 1.00 ± 0.04* | 0.45 ± 0.09 | 0.37 ± 0.10 | 0.58 ± 0.09 | 0.29 ± 0.06 |
| 6. Resistin | | 0.50 ± 0.06 | 0.57 ± 0.08 | 0.69 ± 0.08 | 0.50 ± 0.13 |
| 7. Intercellular Adhesion Molecule-1 (ICAM-1 | | 0.45 ± 0.09 | 0.67 ± 0.08 | 0.89 ± 0.08* | 0.58 ± 0.07 |
| 8. Vascular Adhesion Molecule-1 (VCAM-1 | | 0.23 ± 0.06 | 0.49 ± 0.10 | 0.54 ± 0.09 | 0.15 ± 0.06 |
| 9. Interleukin-6 (IL-6) | 1.00 ± 0.04* | 0.69 ± 0.09 | 0.85 ± 0.13* | 0.81 ± 0.19* | 0.26 ± 0.08 |
| 10. Interleukin-8 (IL-8) | | 1.08 ± 0.12* | 0.82 ± 0.12* | 0.84 ± 0.09* | 0.61 ± 0.08 |
| 11. Tumor Necrosis Factor-α (TNF-α) | | 0.98 ± 0.11* | 0.70 ± 0.10 | 0.98 ± 0.09* | 0.66 ± 0.10 |
| 12. Interferon-γ (IFN-γ) | | 1.38 ± 0.15 | 0.65 ± 0.08 | 0.75 ± 0.15* | 0.51 ± 0.12 |

ANOVA test is applied for analyses; *Values in a row sharing a common asterisk are non significant; all other are significant as compared with control.

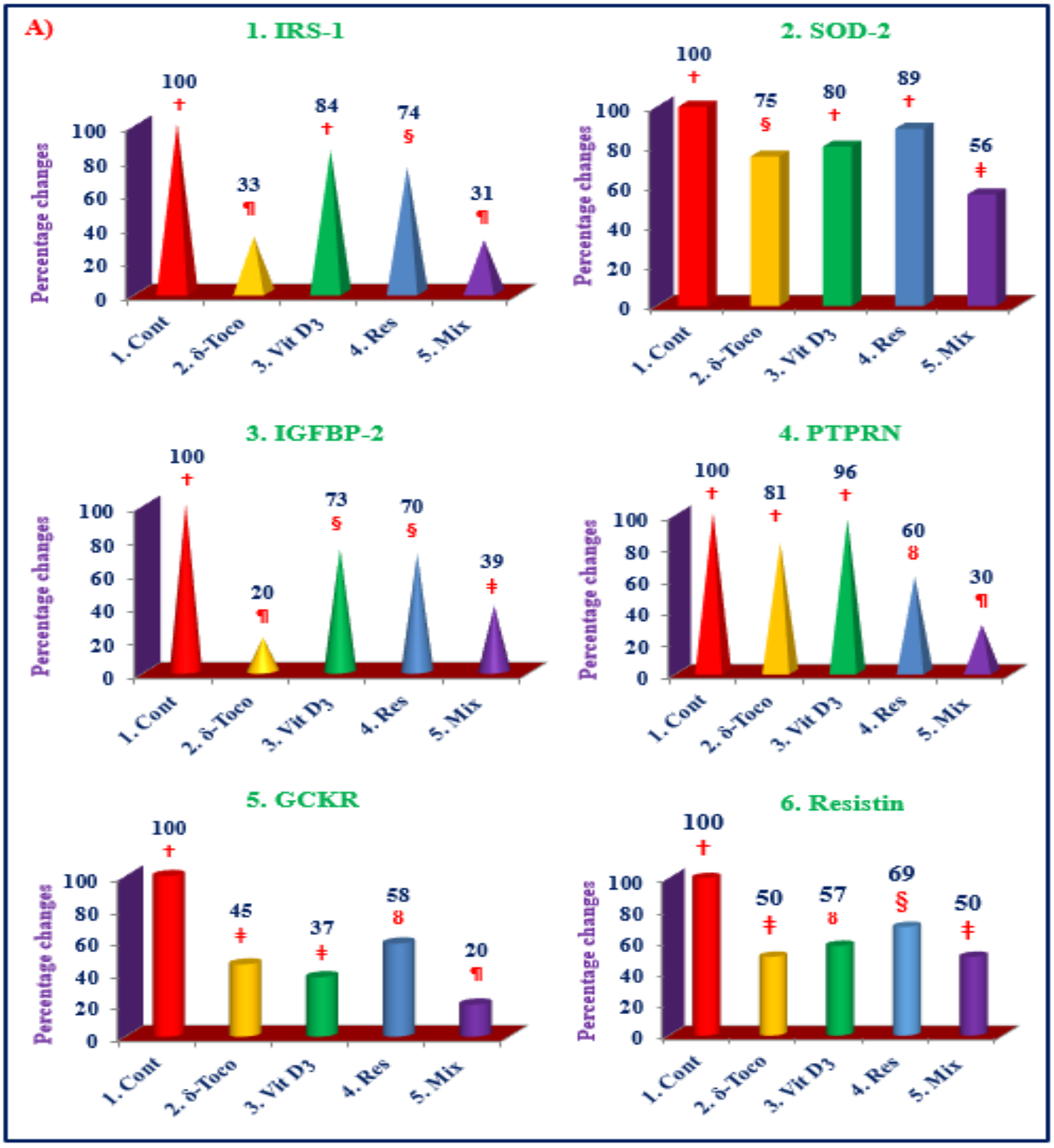


**Figure 4A. Effect of a mixture of NS-3 and its components *in vitro* on diabetes biomarkers and cytokines in PBMCs obtained from people with T2DM.**

The assay procedure of estimating effect of NS-3 mixture on diabetes biomarkers and cytokines in peripheral blood mononuclear cells (PBMCs) of people with T2DM has been described in detail in method section. Data are means ± SD. Values in a column sharing a common symbol is significantly different at compared to †control, §$P < 0.05$, ϑ$P < 0.02$, ≠$P < 0.01$, ¶$P < 0.001$.

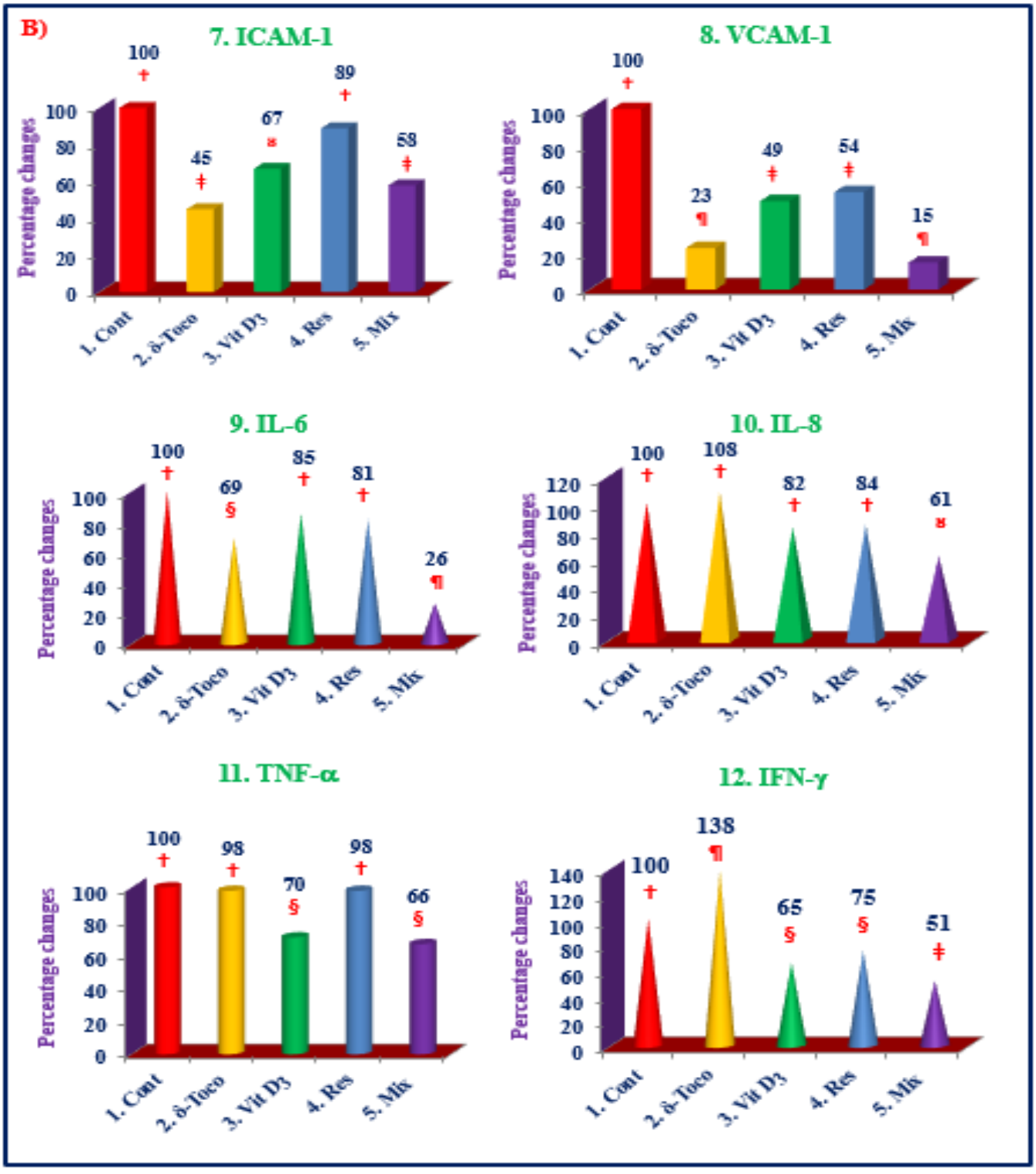


**Figure 4B. Effect of a mixture of NS-3 and its components *in vitro* on diabetes biomarkers and cytokines in PBMCs obtained from people with T2DM.**

The assay procedure of estimating effect of NS-3 mixture on diabetes biomarkers and cytokines in peripheral blood mononuclear cells (PBMCs) of people with T2DM has been described in detail in method section. Data are means ± SD. Values in a column sharing a common symbol is significantly different at compared to †control, §$P < 0.05$, ϑ$P < 0.02$, ≠$P < 0.01$, ¶$P < 0.001$.

**Toxicity of a mixture of NS-3 and its components on PBMCs obtained from people with T2DM by MTT or autophagy.**

The toxicity of a mixture of NS-3 and its components were treated PBMC from people with T2DM with different concentrations (10 µM - 80 µM) of these compounds for 4 h. The MTT and LDH cell death assays indicated that cells were more than 95% viable with these concentrations. Moreover, the effect of these compounds, and mixture (NS-3) was also examined on autophagy assays using PBMCs obtained from people with T2DM. None of the concentrations of these compounds or their mixture (NS-3) used induced autophagy in these cells after 4 h incubation **(Figure 5,** reported only for 20 µM**)**. However, rapamycin + chloroquine treatment (positive control) resulted in increased green fluorescence, as evidence of autophagy vacuole accumulation **(Figure 5)**. These results clearly demonstrated that these compounds can safely be used for human studies. Both findings prompted us to investigate the comparative effect of a mixture of NS-3 as well as its individual components on various diabetes biomarkers, and later gene expression of miRNAs and mRNAs associated with diabetes *in vivo* in people with T2DM.

**Evaluation of δ-tocotrienol, vitamin $D_3$, and resveratrol in people with T2DM.**

***In the first study,*** people with T2DM (***n*** = 120) were carried out of four groups: of placebo, δ-tocotrienol, vitamin $D_3$ and resveratrol (***n*** = 30/group) fed individual capsules for 24-weeks as outlined in **(Figure 2)**. Three subjects in each group were not able to complete the treatment. The physical characteristics of all the participants of pre-dose values are reported in **Table 2.** There were no changes in the body weight, height, body mass index, waist circumference, systolic blood pressure, diastolic blood pressure at the end of post-dose treatment **(Table 2)**.

**Table 2: Impact of placebo, δ-tocotrienol, vitmin $D_3$, and resveratrol on demographic charateristics of people with T2DM (*n* = 108).**

| # | Biomarkers | [a]Placebo | [a]δ-Tocotrienol | [a]Vitamin $D_3$ | [a]Resveratrol |
|---|---|---|---|---|---|
| | | (*n* = 27) | (*n* = 27) | (*n* = 27) | (*n* = 27) |
| 1 | Sex: | | | | |
| | Male | 15 | 15 | 15 | 15 |
| | Female | 12 | 12 | 12 | 12 |
| 2 | Age (years) | 53.67 ± 11.71 | 58.33 ± 9.92 | 58.41 ± 10.35 | 51.67 ± 8.02 |
| 3 | Weight (kg) | 89.59 ± 13.59 | 89.30.33 ± 12.21 | 86.37 ± 7.52 | 85.96 ± 13.13 |
| 4 | Height ($m^2$) | 3.34 ± 0.32 | 3.21 ± 0.38 | 2.89 ± 0.30 | 2.96 ± 0.28 |
| 5 | Body mass index ($kg/m^2$) | 27.62 ± 3.62 | 27.93 ± 3.62 | 30.06 ± 3.28 | 29.13 ± 4.55 |
| 6 | Waist Circumference (cm) | 39.70 ± 4.05 | 39.15 ± 5.14 | 39.07 ± 4.63 | 37.19 ± 2.20 |
| 7 | Duration of diabetes (years) | 9.07 ± 5.18 | 9.04 ± 5.21 | 9.48 ± 2.93 | 7.15 ± 2.73 |
| 8 | Systolic blood pressure (mmHg) | 137.04 ± 7.50 | 137.22 ± 9.54 | 142.22 ± 12.89 | 139.81 ± 6.12 |
| 9 | Diastolic blood pressure (mmHg) | 89.20 ± 7.50 | 90.00 ± 6.93 | 91.67 ± 8.20 | 96.30 ± 5.47 |
| 10 | Fasting blood sugar (mmol/L) | 7.65 ± 1.83 | 7.62 ± 1.84 | 7.55 ± 1.22 | 7.59 ± 1.31 |
| 11 | Hemoglobin A1c (%) | 8.46 ± 2.46 | 8.42 ± 1.24 | 8.86 ± 0.77 | 8.58 ± 1.30 |
| 12 | hs-C reactive protein (mg/L) | 3.48 ± 1.68 | 3.59 ± 1.64 | 3.37 ± 0.72 | 3.69 ± 0.73 |
| 13 | Total cholesterol (mmol/L) | 5.42 ± 0.54 | 5.42 ± 0.66 | 5.44 ± 0.60 | 5.67 ± 71 |
| 14 | Triglycerides (mmol/L) | 2.27 ± 1.56 | 2.22 ± 1.44 | 2.20 ± 0.56 | 2.19 ± 0.57 |

[a]Two capsules of cellulose/olive oil (250.125 mg/capsule; placebo) or two capsules of δ-tocotrienol or resveratrol (250 mg/capsule) or vitamin $D_3$ (5000.00 IU/capsule) were administered to people with T2DM for 24-weeks.

There were also insignificant changes in placebo group in serum/plasma values in various biomarkers of post-dose as compared to pre-dose values after the treatment (**Table 3A**).

However, there were significant (***P*** < 0.001 - 0.05) decreases in serum/plasma values of fasting glucose (7%), HbA1c (8%), hs-CRP (12%), fasting insulin (9%), HOMA-IR (14%), MDA (11%), TNF-α (14%), IL-6 (9%), total cholesterol (9%), LDL-Chol (4%), triglycerides (8%) of post-dose values as compared their respective pre-dose values at the end of δ-tocotrienol treatment (**Table 3A**). Similar significant (***P*** < 0.001 - 0.032) percentages decreases in all these parameters were also observed in serum/plasma levels of post-dose versus pre-dose values at the end of treatments with vitamin $D_3$, fasting glucose (5%), HbA1c (7%), hs-CRP (6%), fasting insulin (8%), HOMA-IR (11%), TNF-α (12%), IL-6 (14%), and with resveratrol, fasting glucose (5%), HbA1c (8%), hs-CRP (11%), fasting insulin (6%), HOMA-IR (7%), TNF-α (13%), IL-6 (15%) as compared to their respective pre-dose values (**Table 3B**).

**Table 3A: Impact of placebo vs δ-tocotrienol on various biomarkers of diabetes in people with T2DM (*n* = 27/group).**

| Biomarkers | [a]Placebo (*n* = 27) | Placebo | *P*-value | [a]δ-tocotrienol (*n* = 27) | δ-Tocotrienol | *P*-values |
|---|---|---|---|---|---|---|
| | Pre-dose | Post-dose | | Pre-dose | Post-dose | |
| 1. Fasting blood sugar (mmol/L) | 7.62 ± 1.84 (100)[b] | 7.57 ± 1.66 (99) | 0.303 | 7.35 ± 1.98 (100)[b] | 6.85 ± 2.00 (93) | 0.001 |
| 2. Hemoglobin A1c (%) | 8.42 ± 1.24 (100) | 8.42 ± 1.23 (100) | 0.619 | 8.44 ± 1.09 (100) | 7.79 ± 1.70 (92) | 0.001 |
| 3. hs-C reactive protein (mg/L) | 3.59 ± 1.64 (100) | 3.47 ± 1.81 (100) | 0.751 | 3.53 ± 2.05 (100) | 3.10 ± 1.93 (88) | 0.019 |
| 4. Fasting Insulin (IU/L) | 15.79 ± 4.83 (100) | 15.79 ± 4.64 (100) | 0.208 | 15.46 ± 5.40 (100) | 14.10 ± 5.18 (91) | 0.000 |
| 5. HOMA-IR | 5.51 ± 2.62 (100) | 5.44 ± 2.40 (99) | 0.791 | 5.23 ± 2.60 (100) | 4.51 ± 2.53 (86) | 0.001 |
| 6. Malondialdehyde (μmol/L) | 3.57 ± 0.61 (100) | 3.58 ± 0.59 (100) | 0.731 | 3.63 ± 0.72 (100) | 3.22 ± 0.68 (89) | 0.001 |
| 7. Tumor Necrosis Factor-a (pg/L) | 8.22 ± 2.64 (100) | 8.04 ± 2.48 (98) | 0.032 | 9.61 ± 4.11 (100) | 8.25 ± 0.25 (86) | 0.001 |
| 8. Interleukin-6 (pg/L) | 14.33 ± 3.89 (100) | 14.21 ± 3.22 (99) | 0.570 | 15.22 ± 3.64 (100) | 13.86 ± 3.46 (91) | 0.001 |
| 9. Total cholesterol (mmol/L) | 5.42 ± 0.68 (100) | 5.25 ± 0.74 (99) | 0.910 | 5.73 ± 0.77 (100) | 5.20 ± 0.74 (91) | 0.000 |
| 10. HDL-C (mmol/L) | 0.89 ± 0.34 (100) | 0.91 ± 0.35 (102) | 0.355 | 0.81 ± 0.14 (100) | 0.82 ± 0.12 (101) | 0.315 |
| 11. LDL-C (mmol/L) | 3.50 ± 0.76 (100) | 3.43 ± 0.94 (98) | 0.767 | 3.22 ± 0.67 (100) | 3.10 ± 0.54 (96) | 0.001 |
| 12. Triglycerides (mmol/L) | 2.25 ± 1.44 (100) | 2.21 ± 0.75 (98) | 0.387 | 2.61 ± 1.55 (100) | 2.40 ± 1.47 (92) | 0.752 |

[a]Two capsules of cellulose/olive oil (250.125 mg/capsule; placebo) or two capsules of δ-tocotrienol (250.125 mg/capsule) were administered to people with T2DM for 24-weeks.

[b]Percentage of control values are in parentheses.

**Table 3B: Impact of vitamin $D_3$ & resveratrol on various diabetes biomarkers in people with T2DM (*n* = 27/group).**

| Biomarkers | [a]Vitamin $D_3$ (*n* = 27) | Vitamin $D_3$ | *P*-value | [a]Resveratrol (*n* = 27) | Resveratrol | *P*-value |
|---|---|---|---|---|---|---|
| | Pre-dose | Post-dose | | Pre-dose | Post-dose | |
| 1. Fasting blood sugar (mmol/L) | 7.55 ± 1.22 (100) | 7.16 ± 1.11 (95) | 0.039 | 7.39 ± 1.31 (100)[b] | 6.98 ± 1.58 (94) | 0.026 |
| 2. Hemoglobin A1c (%) | 8.81 ± 0.77 (100) | 8.19 ± 0.90 (93) | 0.001 | 8.58 ± 1.30 (100) | 7.86 ± 1.36 (92) | 0.001 |
| 3. hs-C reactive protein (mg/L) | 3.37 ± 0.72 (100) | 3.09 ± 0.72 (94) | 0.023 | 3.69 ± 0.73 (100) | 3.28 ± 0.70 (89) | 0.001 |
| 4. Fasting Insulin (IU/L) | 15.84 ± 2.32 (100) | 14.97 ± 2.27 (92) | 0.001 | 15.59 ± 2.91 (100) | 14.65 ± 2.88 (94) | 0.001 |
| 5. HOMA-IR | 5.32 ± 1.05 (100) | 4.74 ± 0.91 (89) | 0.001 | 5.37 ± 1.38 (100) | 4.98 ± 1.33 (93) | 0.001 |
| 6. Malondialdehyde (μmol/L) | 3.59 ± 0.78 (100) | 3.48 ± 0.62 (97) | 0.089 | 3.82 ± 0.94 (100) | 3.49 ± 0.94 (91) | 0.001 |
| 7. Tumor Necrosis Factor-α (pg/L) | 9.71 ± 1.33 (100) | 8.55 ± 1.23 (88) | 0.001 | 10.37 ± 2.06 (100) | 9.02 ± 2.49 (87) | 0.001 |
| 8. Interleukin-6 (pg/L) | 14.92 ± 2.23 (100) | 12.90 ± 2.53 (86) | 0.001 | 16.19 ± 2.22 (100) | 13.69 ± 3.83 (85) | 0.001 |
| 9. Total cholesterol (mmol/L) | 5.44 ± 0.60 (100) | 5.36 ± 0.73 (99) | 0.309 | 5.69 ± 0.71 (100) | 5.50 ± 0.81 (97) | 0.654 |
| 10. HDL-C (mmol/L) | 0.85 ± 0.20 (100) | 0.88 ± 0.20 (104) | 0.506 | 0.83 ± 0.20 (100) | 0.83 ± 0.19 (100) | 0.912 |
| 11. LDL-C (mmol/L) | 3.59 ± 0.69 (100) | 3.52 ± 0.94 (98) | 0.878 | 3.84 ± 0.80 (100) | 3.69 ± 0.80 (96) | 0.451 |
| 12. Triglycerides (mmol/L) | 2.20 ± 0.56 (100) | 2.14 ± 0.58 (97) | 0.361 | 2.19 ± 0.57 (100) | 2.14 ± 0.63 (98) | 0.713 |

[a]Two capsules of vitamin $D_3$ (5000/capsule) or two capsules of resveratrol (250.125 mg/capsules were administered to people with T2DM for 24 weeks.

[b]Percentage of control values are in parentheses.

***In the second study***, people with T2DM (***n*** = 112) were selected for placebo group (***n*** = 56) and for a mixture NS-3 (δ-tocotrienol + vitamin $D_3$ + resveratrol, (***n*** = 56) group. They were fed placebo or NS-3 capsules for 24-weeks. Four subjects in each group did not complete the study towards the end. The physical characteristics of all the participants (***n*** = 52) of pre-dose values are reported in **Table 4.** There were no changes in the body weight, height, body mass index, waist circumference, systolic blood pressure, diastolic blood pressure at the end of post-dose treatment **(Table 4)**. There was also no change in all the parameters values of serum/plasma of post-dose compared to pre-dose levels of the placebo after 24-week treatment **(Table 4)**. However, there were significant (***P*** < 0.001 - 0.04) decreases in serum/plasma values of fasting glucose (11%), HbA1c (10%), hs-CRP (23%), fasting insulin (9%), HOMA-IR (20%), MDA (20%), microalbuminuria (5%), creatinine (8%), total cholesterol (8%), LDL-cholesterol (10%), triglycerides (8%) TNF-α (25%), and IL-6 (25%) of post-dose values compared their respective pre-dose values at the end of NS-3 mixture treatment **(Table 5)**. The serum levels of HDL-cholesterol were not changed in both sets of study (**Tables 4**). These results clearly indicate that treatment by a mixture of NS-3 to people with T2DM resulted significant reductions in the levels of fasting glucose, hs-CRP, HOMA-IR, MDA, TNF-α and IL-6 (**Table 5**).

**Table 4: Impact of a mixture of NS-3 on demographic characteristics of people with T2DM ( *n* = 104)**

| Various biomarkers | [a]Placebo (*n* = 52) | [b]Mixture (*n* = 52) | *p* -value |
|---|---|---|---|
| Sex | | | |
| Male | 26 (50) | 26 (50) | 0.082 |
| Female | 26 (50) | 26 (50) | 0.081 |
| Age (Years) | 50.48 ± 12.50[c] | 49.73 ± 12.08 | 0.951 |
| Weight (kg) | 86.77 ± 12.56 | 82.63 ± 11.71 | 0.207 |
| Height ($m^2$) | 3.25 ± 0.37 | 3.10 ± 0.35 | 0.125 |
| Body mass index ($kg/m^2$) | 26.78 ± 3.26 | 26.82 ± 4.05 | 0.999 |
| Waist Circumference (inches) | 38.67 ± 4.2 | 36.98 ± 4.41 | 0.116 |
| Systolic BP (mmHg) | 137.60 ± 8.55 | 137.94 ± 14.38 | 0.987 |
| Diastolic BP (mmHg) | 88.27 ± 6.63 | 88.92 ± 7.49 | 0.886 |
| Duration of Diabetes (years) | 8.42 ± 4.72 | 8.14 ± 5.13 | 0.953 |
| Fasting blood glucose (mmol/L) | 7.65 ± 1.66 | 7.39 ± 1.71 | 0.703 |
| HbA1c (%) | 8.53 ± 1.16 | 8.20 ± 1.30 | 0.301 |
| hs-CRP (mg/L) | 3.46 ± 1.51 | 3.65 ± 1.31 | 0.802 |
| Fasting Insulin (mIU/L) | 15.96 ± 4.37 | 15.90 ± 5.78 | 0.950 |
| HOMA-IR | 5.58 ± 2.44 | 5.44 ± 2.85 | 0.792 |
| Malondialdehyde (MDA; µmol/L) | 3.75 ± 0.65 | 3.81 ± 0.52 | 0.720 |
| Microalbuminuria (mg/mmol) | 11.32 ± 0.96 | 12.56 ± 1.95 | 0.821 |
| Creatinine (µmol/L) | 89.79 ± 12.30 | 89.75 ± 18.10 | 0.990 |
| Total Cholesterol (mmol/L) | 5.37 ± 0.71 | 5.36 ± 0.72 | 0.997 |
| HDL-C (mmol/L) | 0.92 ± 0.29 | 0.92 ± 0.34 | 0.987 |
| LDL-C (mmol/L) | 3.49 ± 0.83 | 3.36 ± 0.79 | 0.734 |
| Triglycerides (mmol/L) | 2.13 ± 1.27 | 2.36 ± 1.00 | 0.562 |
| TNF-α (pg/mL) | 8.98 ± 4.37 | 9.65 ± 5.60 | 0.500 |
| IL-6 (pg/mL) | 14.97 ± 7.82 | 14.86 ± 8.01 | 0.944 |

[a]Two capsules of cellulose/olive oil (250.125 mg/capsule) were administered to people with T2DM for 24-weeks.

[b]Two capsules of NS-3 mixture (250.125 mg/capsule) were administered to people with T2DMs for 24-weeks.

[c]Mean ± SD (Standard Deviation).

**Table 5: Impact of a Mixture of NS-3 on various biomarkers of diabetes in serum/plasma of people with T2DM ( *n* = 104).**

| # | Various biomarkers | [a]Placebo (*n* = 52) Pre-dose | Placebo (*n* = 52) Post-dose | [c]*P* -values | [b]Mixture (*n* = 52) Pre-dose | Mixture (*n* = 52) Post-dose | [c]*P* -values |
|---|---|---|---|---|---|---|---|
| 1 | Fasting glucose (mmol/L) | 7.65 ± 1.66 | 7.59 ± 1.49 (99)[d] | 0.098 | 7.39 ± 1.71 | 6.56 ± 1.66 (89)[d] | 0.000 |
| 2 | Fasting HbA1c (%) | 8.53 ± 1.16 | 8.50 ± 1.17 (98) | 0.764 | 8.20 ± 1.30 | 7.40 ± 0.93 (90) | 0.000 |
| 3 | hs-CRP (mg/L) | 3.46 ± 1.51 | 3.42 ± 1.68 (99) | 0.690 | 3.65 ±1.31 | 2.82 ± 1.07 (77) | 0.000 |
| 4 | Fasting Insulin (mIU/L) | 15.96 ± 4.37 | 15.94 ± 4.26 (100) | 0.601 | 15.90 ± 5.78 | 14.42 ± 5.56 (91) | 0.000 |
| 5 | HOMA-IR | 5.58 ± 2.44 | 5.50 ± 2.25 (99) | 0.060 | 5.44 ± 2.85 | 4.35 ± 2.25 (80) | 0.000 |
| 6 | Malondialdehyde (MDA; µmol/L) | 3.75 ± 0.65 | 3.76 ± 0.63 (100) | 0.960 | 3.81 ± 0.65 | 3.04 ± 0.47 (80) | 0.030 |
| 7 | Microalbuminuria (mg/mmol) | 11.32 ± 0.96 | 11.03 ± 9.45 (97) | 0.345 | 12.56 ± 1.19 | 11.90 ± 1.21(95) | 0.015 |
| 8 | Creatinine (µmol/L) | 89.79 ± 12.30 | 88.77 ± 12.69 (99) | 0.109 | 89.75 ± 18.10 | 82.40 ± 16.97 (92) | 0.000 |
| 9 | Total cholesterol (mmol/L) | 5.37 ± 0.71 | 5.34 ± 0.99 (99) | 0.844 | 5.36 ± 0.72 | 4.95 ± 0.72 (92) | 0.000 |
| 11 | HDL-C (mmol/L) | 0.92 ± 0.29 | 0.92 ± 0.30 (100.00) | 0.255 | 0.92 ± 0.34 | 0.94 ± 0.28 (102) | 0.498 |
| 12 | LDL-C (mmol/L) | 3.49 ± 0.83 | 3.49 ± 1.21 (100.00) | 0.956 | 3.36 ± 0.79 | 3.02 ± 0.78 (90) | 0.000 |
| 13 | Triglycerides (mmol/L) | 2.12 ± 1.27 | 2.03 ± 0.89 (96) | 0.621 | 2.36 ± 0.00 | 2.18 ± 0.98 (92) | 0.038 |
| 14 | TNF-α (pg/mL) | 8.98 ± 4.37 | 8.77 ± 4.12 (98) | 0.154 | 9.65 ± 5.60 | 7.28 ± 4.41 (75) | 0.000 |
| 15 | IL-6 (pg/mL) | 14.97 ±7.82 | 14.82 ± 7.22 (99) | 0.591 | 14.86 ± 8.01 | 11.13 ± 6.96 (75) | 0.003 |

[a]Two capsules of cellulose/olive oil (250.125 mg/capsule; placebo) were administered to people with T2DM for 24-weeks.

[b]Two capsules of a mixture of NS-3 (250.125 mg/capsule) were administered to people with T2DM for 24-weeks.

[c]The calculation of post treatment variables is based on an analysis of covariance (ANCOVA), adjusted for one covariates: baseline (pre-treatment) variables.

[d]Percentage of control values are in parentheses.

The results of comparison of between NS-3 and placebo groups analysis revealed that supplementation of NS-3 significantly reduced [mean difference at 95% confidence level of fasting glucose -1.076-(-0.534), HbA1c -1.240-(-0.714), hs-CRP-1.105-(-0.379), fasting insulin -2.218-(-0.707), HOMA-IR -1.422-(-0.667) and MAD -0.341-(-0.451) were significantly ($P < 0.05$) decreased as compared to placebo group (**Table 6).**

**Table 6: Comparison of between-groups of serum/plasma biovhemical levels in placebo and mixture groups in people with T2DM ($n$ = 104).**

| # | Various biomarkers | [a]Placebo ($n$ = 52) Pre-dose | [b]Mixture ($n$ = 52) Pre-dose | [c]Mean difference (95% CI) | $P$-values | Placebo ($n$ = 52) Post-dose | Mixture ($n$ = 52) Post-dose | Mean difference (95% CI) | $P$-values |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Fasting glucose (mmol/L) | 7.65 ± 1.66 | 7.39 ± 1.71 | -0.92 - 0.39 | 0.432 | 7.59 ± 1.49 (99)[d] | 6.56 ± 1.66 (89)[d] | -1.076 - (-0.534) | 0.000 |
| 2 | Fasting HbA1c (%) | 8.53 ± 1.16 | 8.20 ± 1.30 | -0.96 - 0.14 | 0.168 | 8.50 ± 1.17 (98) | 7.40 ± 0.93 (90) | -1.240-(-0.714) | 0.000 |
| 3 | hs-CRP (mg/L) | 3.46 ± 1.51 | 3.65 ±1.31 | -0.36 -0.74 | 0.503 | 3.42 ± 1.68 (99) | 2.82 ± 1.07 (77) | -1.105-(-0.379) | 0.000 |
| 4 | Fasting Insulin (mIU/L) | 15.96 ± 4.37 | 15.90 ± 5.78 | -2.06-1.93 | 0.950 | 15.94 ± 4.26 (100) | 14.42 ± 5.56 (91) | -2.218-(-0.707) | 0.000 |
| 5 | HOMA-IR | 5.58 ± 2.44 | 5.44 ± 2.85 | -1.17-0.89 | 0.792 | 5.50 ± 2.25 (99) | 4.35 ± 2.25 (80) | -1.422-(-0.667) | 0.000 |
| 6 | Malondialdehyde (MDA; µmol/L) | 3.75 ± 0.65 | 3.81 ± 0.65 | -0.38-0.66 | 0.515 | 3.76 ± 0.63 (100) | 3.04 ± 0.47 (80) | -0.341-(0-0.451) | 0.000 |
| 7 | Microalbuminuria (mg/mmol) | 11.32 ± 0.96 | 12.56 ± 1.19 | -2.97-5.45 | 0.561 | 11.03 ± 9.45 (97) | 11.90 ± 1.21(95) | -1.142-(-462) | 0.403 |
| 8 | Creatinine (µmol/L) | 89.79 ± 12.30 | 89.75 ± 18.10 | -6.06-5.98 | 0.990 | 88.77 ± 12.69 (99) | 82.40 ± 16.97 (92) | -1.021-(-4.42) | 0.000 |
| 9 | Total cholesterol (mmol/L) | 5.37 ± 0.71 | 5.36 ± 0.72 | -0.29-0.27 | 0.939 | 5.34 ± 0.99 (99) | 4.95 ± 0.72 (92) | -0.654-(-0.125) | 0.004 |
| 11 | HDL-C (mmol/L) | 0.92 ± 0.29 | 0.92 ± 0.34 | -0.11-0.13 | 0.895 | 0.92 ± 0.30 (100) | 0.94 ± 0.28 (102) | -0.035-(-0.045) | 0.796 |
| 12 | LDL-C (mmol/L) | 3.49 ± 0.83 | 3.36 ± 0.79 | -.044-0.19 | 0.432 | 3.49 ± 1.21 (100) | 3.02 ± 0.78 (90) | -0.72-(-0.050) | 0.023 |
| 13 | Triglycerides (mmol/L) | 2.12 ± 1.27 | 2.36 ± 0.00 | -0.21-0.68 | 0.301 | 2.03 ± 0.89 (96) | 2.18 ± 0.98 (92) | -0.24-(-0.351) | 0.790 |
| 14 | TNF-α (pg/mL) | 8.98 ± 4.37 | 9.65 ± 5.60 | -1.29-2.62 | 0.500 | 8.77 ± 4.12 (98) | 7.28 ± 4.41 (75) | -2.77-(-1.201) | 0.000 |
| 15 | IL-6 (pg/mL) | 14.97 ±7.82 | 14.86 ± 8.01 | -3.19-2.97 | 0.944 | 14.82 ± 7.22 (99) | 11.13 ± 6.96 (75) | -5.69-(-1.570) | 0.001 |

[a]Two capsules of cellulose/olive oil (250.125 mg/capsule; placebo) were administered to people with T2DM for 24-weeks.

[b]Two capsules of a mixture of NS-3 (250.125 mg/capsule) were administered to people with T2DM for 24-weeks.

[c]The calculation of post treatment variables is based on an analysis of covariance (ANCOVA), adjusted for one covariates: baseline (pre-treatment) variables.

[d]Percentage of control values are in parentheses.

In summary, the results of the impact of NS-3 mixture and its components on some important biomarkers associated with diabetes are shown in **Table 7**. The mixture (NS-3) displayed maximum reduction in serum/plasma values of fasting glucose, HbA1c, hs-CRP, HOMA-IR, MDA as compared to individual components (**Table 7**).

**Table 7: Summary of impact of placebo supplement or a mixture of (NS-3) or its components after treatment for 24-weeks on various biomarkers of diabetes in serum of people with T2DM.**

| | Biomarkers | Fasting Glucose Pre-dose | Fasting Glucose Post-dose | Fasting HbA1c Pre-dose | Fasting HbA1c Post-dose | hs-CRP Pre-dose | hs-CRP Post-dose | HOMA-IR Pre-dose | HOMA-IR Post-dose | MDA Pre-dose | MDA Post-dose |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Values in----------> | mmol/L | mmol/L | % | % | mg/L | mg/L | | | µmol/L | µmol/L |
| 1 | Control[a] (placebo) | 7.62 (100)[b] | 7.57 (99) | 8.42 (100) | 8.42 (100) | 3.59 (100) | 3.47 (100) | 5.51 (100) | 5.44 (99) | 3.57 (100) | 3.58 (100) |
| 2 | δ-tocotrienol[a] | 7.35 (100) | 6.85 (93) | 8.44 (100) | 7.79 (92) | 3.53 (100) | 3.10 (88) | 5.23 (100) | 4.51 (86) | 3.63 (100) | 3.22 (89) |
| 3 | Vitamin $D_3$[a] | 7.55 (100) | 7.16 (95) | 8.81 (100) | 8.19 (93) | 3.37 (100) | 3.09 (92) | 5.32 (100) | 4.74 (89) | 3.59 (100) | 3.48 (97) |
| 4 | Resveratrol[a] | 7.39 (100) | 6.98 (94) | 8.58 (100) | 7.86 (92) | 3.69 (100) | 3.28 (89) | 5.37 (100) | 4.98 (93) | 3.82 (100) | 3.49 (91) |
| 5 | Mixture (2 + 3 + 4)[a] | 7.39 (100) | **6.56 (89)** | 8.20 (100) | **7.40 (90)** | 3.65 (100) | **2.82 (77)** | 5.44 (100) | **4.35 (80)** | 3.81 (100) | **3.04 (80)** |

[a]Two capsules of placebo (cellulose/olive oil; 250.125 mg/capsule) or two capsules of δ-tocotrienol (250.125 mg/capsule), or vitamin $D_3$ (5000 IU = 0.125/capsule) or resveratrol (250.125 mg/capsule) were administered to people with T2DM for 24-weeks.

[b]Percentage of control values are in perentheses.

None of the subjects reported any side- effects with the NS-3 mixture during six months of trial period, and these results supported our original hypothesis that a mixture of natural products (δ-tocotrienol, vitamin $D_3$, resveratrol) is more effective in lowering serum/plasma levels of diabetes biomarkers and pro-inflammatory cytokines than their individual compounds (**Tables 4, 5**).

**Impact of a mixture of NS-3 on diabetes associated biomarkers and cytokines in real-time PCR of purified treated RNAs obtained from people with T2DM.**

In order to confirm *in vitro* results of RT-PCR effects of NS-3 and its individual components on diabetes biomarkers using RNAs purified from PBMC isolated from T2DM subjects, the RT-PCR of pre-dose RNAs

versus post-dose RNAs after NS-3 administered to persons with T2DM for 24- weeks was also carried out for several T2DM biomarkers, which indicated significant down- regulated gene expression of IRS-1 (79%), SOD-2 (90%), GCKR (87%), IGFPB-2 (88%), IL-4 (82%), IL-6 (76%) and iNOS (78%) as compared to pre-dose values (**Figure 6**). These *in vivo* results of down-regulated gene expression in diabetes biomarkers were more significant than in vitro results as reported in **Figures 4A and 4B**. These results prompted us to carry out mRNA-sequencing and miRNA-sequencing of these RNAs. This will be the subject of the next publication.

## Discussion

The hypothesis of our study is that synergistic effect of a mixture (NS-3) of natural compounds on biological functions will be more effective than its individual components in lowering the diabetes biomarkers. The results of a mixture of NS-3 when tested *in vitro* in PBMCs of people with T2DM indicated more effective down-regulation on gene expression of several diabetes biomarkers and cytokines as compared to its individual components (**Table 1**, **Figures 4A and 4B**), except δ- tocotrienol treatment showed up-regulation in interferon-γ (138%, 10 µM; 287%, 80 µM) only in PBMC of diabetes and not in normal healthy PBMC. It is well known IFN-γ is a cytokine associated with both innate and adaptive immunity against viral infection, and functions as a primary activator of macrophages and stimulate natural killer cells and neutrophils, thus δ-tocotrienol might be a very good candidate to induce immunity in humans.

Next, the toxicity of NS-3 mixture and its components was carried out by MTT test with concentration (10 µM - 80 µM) in diabetic PBMC showed the cells were > 95 % viable with these concentrations, and under same conditions as of MTT test, the NS-3 mixture and its components did not induce autophagy in these PBMC (**Figure 5**).

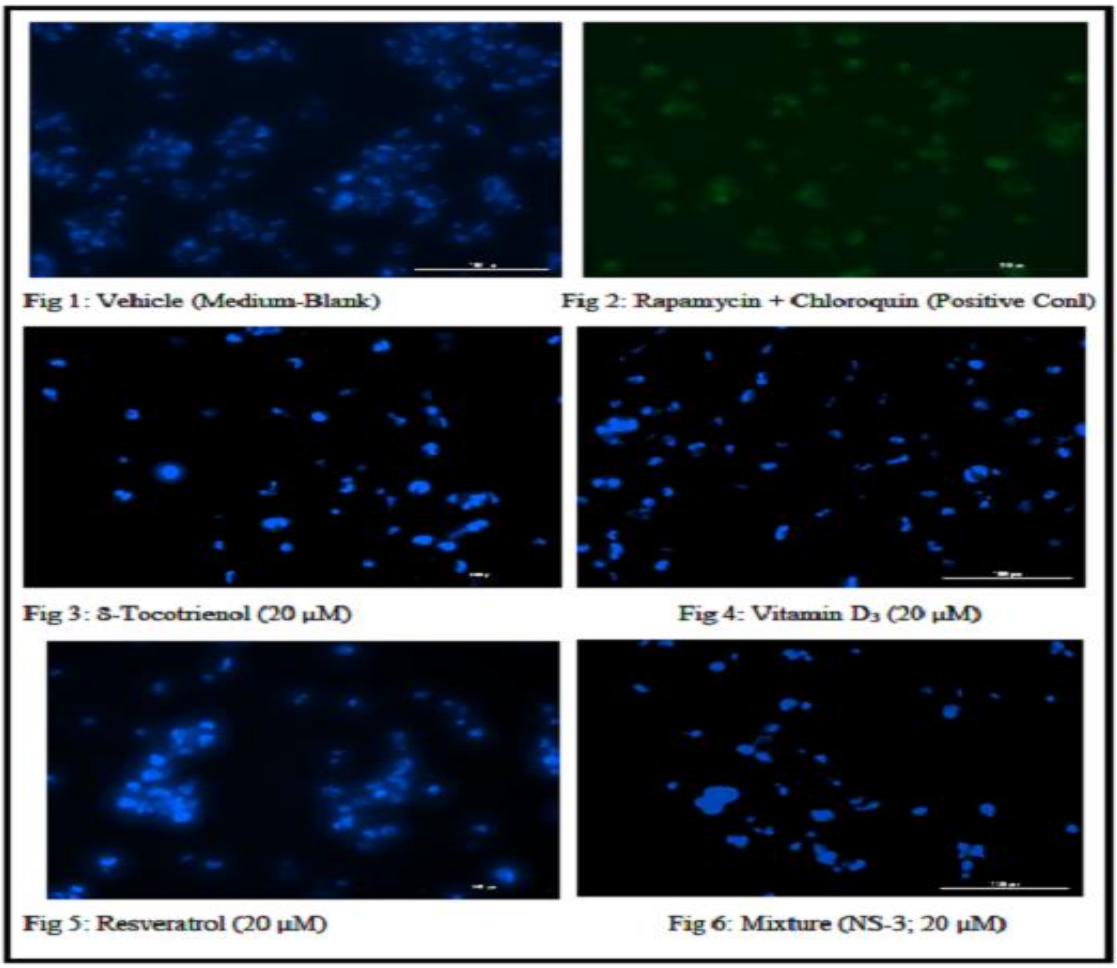


**Figure 5. Effect of a mixture of NS-3 and its component on autophagy assay in PBMCs of people with T2DM.**

The PBMC (200,000 cells/well in medium + 0.2% DMSO) were first differentiated with PMA (10 ng/mL) in media of 96-tissue culture white plate for 4 h at 37 ºC under 5% CO2 in an incubator, then washed with fresh media, followed by incubation with vehicle (medium + 2% DMSO, blank 26 control), a mixture of rapamycin + chloroquine (as positive control), δ-tocotrienol, vitamin $D_3$, resveratrol, or NS-3 using a dose of 20 µM of each compound or mixture for 4 h at 37 ºC under CO2. The cells were washed with assay buffer followed by incubation with microscopy dual detection reagent (100 µL) for 30 min at 37 ºC in dark. After incubation the PBMC were finally washed with assay buffer three times. The Fluorescence measurements were performed under Cytation-3-fluorometric reader using DAPI and FITC filer sets.

The results clearly demonstrate that NS-3 mixture safely can be used for human studies. A mixture of NS-3 showed much more significant decreases in the levels of serum/plasma of fasting glucose, HbA1c, hs-CRP, fasting insulin, HOMA-IR, MDA as compared to its individual components (**Table 5**). There were no side-effects reported by any of the subjects. These *in vitro* (PBMC) and *in vivo* results supported our hypothesis that a mixture NS-3 of δ-tocotrienol, vitamin $D_3$, resveratrol is more effective in lowering the diabetes biomarkers and pro-inflammatory cytokines compared to its individual components. The RT-PCR estimation of NS-3 treated *in vivo* of pure RNAs of pre-dose vs post-dose on several key biomarkers (IRS-1, SOD-2, IGFBP-2, GCKR), and IL-4, IL-6, iNOS of diabetes showed significant down-regulation in gene expression (>70%) in these biomarkers and cytokines compared to pre-dose values (**Figure 6**).

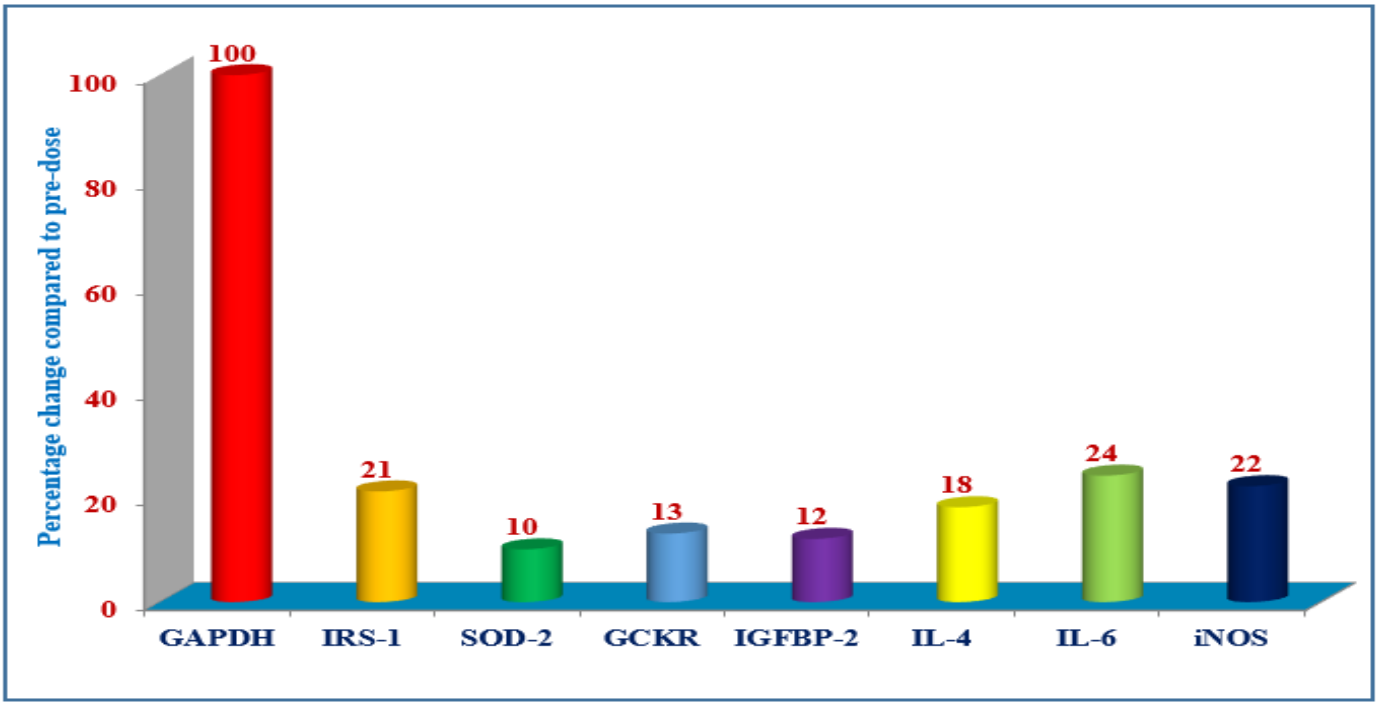


**Figure 6. Effect of a mixture of NS-3 on diabetes biomarkers and cytokines by RT-PCR of total RNAs obtained from NS-3 administered people with T2DM after 24-weeks.**

Real-time PCR (RT-PCR) was performed on total RNAs isolated from people with T2DM treated with a mixture of NS-3 for 24-weeks. All reactions were performed in triplicate using equal amount of mRNA per reaction (200,000 RNAs/treatment) of pre-dose as well as post-dose. Real-time PCR assays were completed by a Step-one plus RT-PCR system as described in **Figures 4A and 4B.** Data are means ± SD. Values in a column sharing a common asterisk are significantly different at ***P*** < 0.001 compared to control.

The number of pre-diabetes and people with T2DM will ultimately increase to 482 million people by the year 2040. Therefore, more effective methods and new biomarkers will be required to diagnose pre-diabetes and diabetes people and their complications. The current parameters, hs-CRP, HbA1c, and HOMA-IR as well as some new biomarkers will be required to diagnose pre-diabetes and diabetes and its complications. Recently, a comprehensive review of novel biomarkers for prediabetes, diabetes, and associated complications has been reported [33]. They have discussed the functions of nineteen novel general (such as HbA1c, GA, OGT, adiponectin, LP(a), THBS1, GPLD1, miRNAs) and seven inflammatory (hs-CRP, IL-6, WBCs, fibrinogen, PAI-1, IL-18, IL- IRA) biomarkers in detail [33]. The present results clearly indicate that six biomarkers (IRS-1, SOD-2, IGFBP-2, PTPRN, GCKR, resistin), and cytokines (ICAM-1, VCAM-1, IL-6, IL-8, TNF-α, IFN-γ) may be used in future for diagnosing early onset of people of pre-diabetes and with T2DM.

The current-well-designed, long-term population studies that show that current-well-designed, long-term population studies that show association between plasma glucose levels, HbA1c and hs-CRP, but have some limitations, such as moderate sensibility and specificity are

inaccurate in certain clinical conditions. Therefore, these novel biomarkers (gene expression of IRS-1, SOD-2, GCKR, IGFBP-2, resistin) might be useful for diagnosing and identifying early onset of people with T2DM. IRS-1 regulates body growth and peripheral insulin action. Phosphorylation in IRS-1, increases body weight, lowers blood glucose level, and alleviates insulin resistance (IR) in T2DM. SOD-2 is a free radical scavenging enzyme that forms a major component in guarding against oxygen radical species produced during cellular metabolism. It functions as a homodimer that binds copper and zinc. IGFBP-2 is a pleiotropic polypeptide that functions as an autocrine and/or paracrine

growth factor. PTPRN plays a role in vesicle-mediated secretory processes and is required for the accumulation of normal levels of insulin-containing vesicles and preventing their degradation. It is also required for normal accumulation of the neurotransmitter norepinephrine, dopamine, and serotine in the brain. Glucokinase regulator (GCKR) is associated with elevated T2DM risk, and acts as an allosteric switch for glucokinase in blood glucose control by the liver and resistin is involved in insulin resistance (IR) in T2DM (based on Google Search). In the future, the present suggested biomarkers may predict individual risk of diabetes complications after long-term population studies, and RT-PCR technique will be used routinely for diagnostic purposes in clinical laboratories soon. Recently, the function of miRNA as regulatory marker of glucose and lipid metabolism has been reported in studies of different tissues and animals [34, 35]. This will be the subject of our next publication.

## Conclusions:

The present study confirms the important roles played by IRS-1, SOD-2, IGFBP-2, PTPRN, GCKR as novel biomarkers, and cytokines (ICAM-1, VCAM-1, IL-6, IL-8, IFN-γ) in diagnosing T2DM. The *in vitro* (PBMC) and *in vivo* (clinical study) results supported our hypothesis that a mixture NS-3 of δ-tocotrienol, vitamin $D_3$, resveratrol will be more effective in lowering the diabetes biomarkers and pro-inflammatory cytokines as compared to its individual components. Although, present mixture of NS-3 effectively lowers the elevated levels of all the tested diabetes and inflammatory biomarkers, there are some limitations of present study. The elevated plasma level of HbA1c decreased only 10% in T2DM and not below its normal value of < 7%. There are many commercial products of synthetic compounds (Jardiance, Farxiga, Trulicity, Ozempic) available in the market, which are very effective in lowering the elevated plasma level of HbA1c in people with T2DM, but most of them have severe side-effects (nausea, diarrhea, vomiting, abdominal pain, discomfort, frequent bowel movements, indigestion, decreased appetite, and fatigue). Therefore, there is still a need to find out a mixture of natural products, which should be able to lower elevated plasma level of HbA1c below normal value of < 7.0%. This might be achieved by carrying out a dose-response (400 mg/d, 800 mg/d, 1200 mg/d) study by changing composition of present mixture or by adding some other potent natural compounds in the present mixture and then carrying out a study of (NS-4) in people with T2DM for 24-weeks. The other limitation is that in our first pilot preliminary study, few T2DM participants (***n*** = 30 for each component) were enrolled for individual components compared to (NS-3) mixture (***n*** = 56). However, Dr. Wajiha Mahjabeen (Ph.D. student) of Dr. Dilshad A Khan has completed the clinical trial of "Effects of δ-tocotrienol, vitamin $D_3$, resveratrol (***n*** = 55 for each compound) feeding on levels of glycemic control, oxidative stress, inflammatory biomarkers, and miRNA expression in type 2 diabetes mellitus for her Ph.D. thesis, and obtained results for δ-tocotrienol-fasting glucose 93%, HbA1c 94%, hs-CRP 90%, HOMA-IR 87%, MDA 91%). The decrease in T2DM biomarkers with NS-3 mixture is significantly better **(Table 7)** than these values (personal communication). In summary, the present study using a mixture of natural products (NS-3) has demonstrated a product which can effectively decrease several current well-established diabetes biomarkers, including some new ones in people with T2DM, without any side-effects.

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**Competing interest:** All authors declare that they have no competing interests.

## Author's contributions

AAQ and DAK conceived, and NQ, NS, and BMD planned the study. DAK and WM carried out human study and prepared total mRNAs. WM collected the data and carried out most of the estimation of all the parameters/biomarkers. DAK and AAQ carried out analysis of the data. NS carried out RT-PCR analyses of PBMC and autophagy assays. AAQ wrote the manuscript. NQ and BMD edited and proof-read the manuscript. All authors have read and approved the final manuscript.

## Availability of data and materials

All data generated or analyzed during this study are included in this article and is available in USB.

## Consent of publication

All contributing authors agree to the publication of this article.

## Abbreviations:

**T2DM** = type 2 diabetes mellitus,

**PBMC** = peripheral blood mononuclear cells,

**IRS-1** = insulin receptor substrate-1,

**SOD-2** = superoxide dismutase-2,

**IGFBP-2** = insulin like factor binding protein-2,

**PTPRN** = protein tyrosine phosphatase receptor type N,

**GCKR** = glucokinase regulators,

**ICAM-1** = intercellular adhesion molecule-1,

**VCAM-1** = vascular cell adhesion molecule-1,

**IL-6** = interleukin-6,

**IL-8** = interleukin-8,

**TNF-α** = tumor necrosis factor-α,

**IFN-γ** = interferon-γ,

**NF-κB** = nuclear factor kappa B.

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