Research Article **Open Access**

BCHD
Bioactive Compounds in Health and Disease

Assessment of squalene effect on antioxidant enzymes and free radicals in patients with type 2 diabetes mellitus

Danik Martirosyan[1*], Mohammad Reza Ashoori[2], Anna Serani[3, 1], Kevin Zhang [1,4], and Hossein Mirmiranpour[5]

[1]Functional Food Center (FFC), Functional Food Institute, Dallas, TX, USA; [2]Department of Laboratory Sciences, School of Allied Medical Sciences, Zanjan University of Medical Sciences, Zanjan, Iran; [3]Georgia Institute of Technology, Atlanta, Georgia, USA; [4]Georgetown University, Washington, D.C., USA; [5]Endocrinology and Metabolism Research Center (EMRC), Valiasr Hospital, School of Medicine, Tehran University of Medical Science, Tehran, Iran

***Corresponding Author:** Danik Martirosyan, PhD, Functional Food Center, Functional Food Institute, Dallas, TX, 75254,USA

Submission Date: September 12th, 2022; **Acceptance Date**: November 8th, 2022; **Publication Date**: November 11, 2022

Please cite this article as: Martirosyan D., Ashoori M.R., Serani A., Zhang K., Mirmiranpour H. Assessment of squalene effect on antioxidant enzymes and free radicals in patients with type 2 diabetes mellitus. Bioactive Compounds in Health and Disease 2022; 5(11):236-250. DOI: https://www.doi.org/10.31989/bchd.v5i11.1005

ABSTRACT

Background: Diabetes mellitus as a metabolic disease can have serious consequences. Due to their chemical properties, bioactive compounds can play a role in diabetes management. Squalene is a natural oil and bioactive compound. The anti-inflammatory and antioxidant effects of squalene have been discussed in recent studies. Squalene plays a role in controlling diabetes by maintaining the oxidant/antioxidant balance.

Objective: The main purpose of this study was to evaluate the antioxidant effect of different doses of squalene, on different days, on the levels of some oxidative indices and the activity of antioxidant enzymes in groups of people with type 2 diabetes and compare them with each other and healthy people.

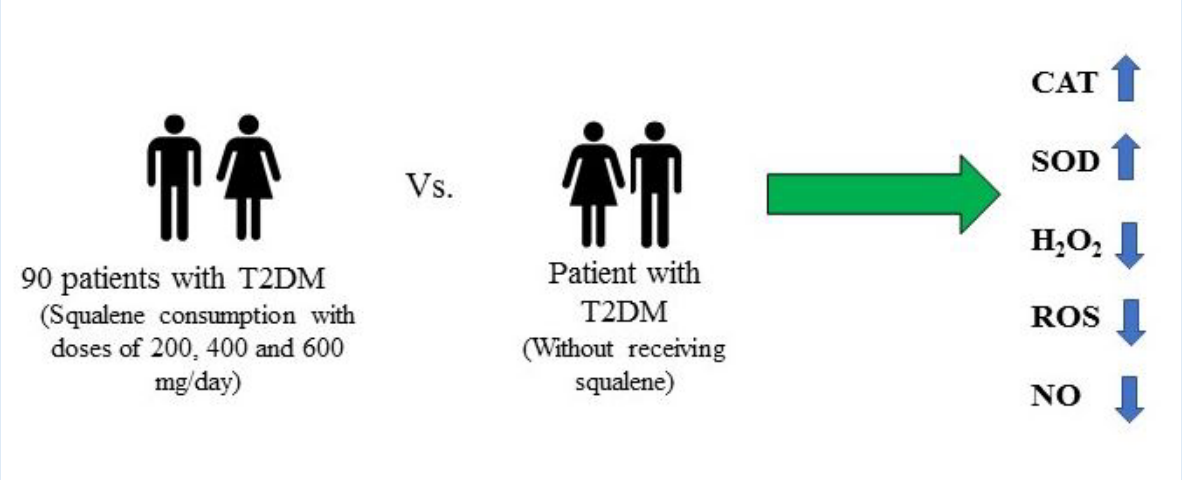
Methods: 150 individuals were recruited in this study. These individuals were separated into five groups. Group one contained 30 individuals, representing the healthy control group. Groups 2, 3, 4 and 5 included subjects with type 2 diabetes. Each of the subjects in groups 3, 4 and 5 received squalene in doses of 200, 400 and 600 mg as an oral capsule (liquid filled oral), respectively for 84 days. Subjects in Group 2 did not receive squalene. Catalase, superoxide dismutase, glutathione peroxidase (as antioxidant indicators) activities and the levels of hydrogen peroxide, nitric oxide and reactive oxygen species (as oxidant indicators) were assayed.

Results: In 84 days, a statistically significant difference (P value < 0.05) was observed in all the diabetic groups compared to the healthy group. In the comparison between groups receiving squalene with each other, there was a significant

increase (P value < 0.05) in catalase and superoxide dismutase activity, depending on squalene dose and time. There was not a statistically significant (P value > 0.05) increase in glutathione peroxidase activity. Statistically significant changes in oxidative indices were not dose-dependent or time-dependent.

Conclusion: Based on the findings of this study, a dose of 600 mg of squalene in 84 days is effective in increasing catalase and superoxide dismutase activity and reducing hydrogen peroxide levels. Squalene can play an important role in controlling and reducing the consequences of diabetes caused by changes in the oxidant/antioxidant balance.

Keywords: squalene, type 2 diabetes, antioxidant enzymes, bioactive compounds, free radicals



INTRODUCTION: Squalene is a natural, bioactive compound commonly found in shark oil, olive oil, and some vegetable oils [1-4]. This 30-carbon triterpenoid, or isoprenoid, is reported to have antioxidant, anti-inflammatory, and anti-atherosclerotic properties [5]. Through varying mechanisms, squalene may increase anti-inflammatory enzymes, reduce pro-inflammatory enzymes, alter gene expression, and act directly as a free radical scavenger [1, 6]. Additionally, squalene is involved in lipid metabolism as a precursor to cholesterol and functions as a feedback inhibitor to regulate cholesterol metabolism [7]. The wide distribution of squalene in nature and its potentially massive clinical upside makes squalene a growing area of research. It was found that squalene functions effectively to reduce levels of proteinuria. In a clinical study on 150 diabetic patients, it was found that squalene reduced the level of proteinuria. Additionally, the amount of proteinuria reduced was increased with higher doses of squalene. Therefore, the results from this study indicate the effectiveness of squalene as a bioactive compound supporting the reduction of proteinuria [8].

Currently, literature surrounding squalene's anti-inflammatory properties remain limited to primarily in vitro and in vivo studies, often with diseases associated with inflammation and oxidative stress [7]. In vitro, squalene has been shown to decrease reactive oxygen species production and improve cellular glutathione homeostasis, protecting bone marrow progenitor cells [9]. In an isoproterenol-myocardial infarction (MI) mouse

model study, squalene appears to equilibrate abnormally low antioxidant levels [10]. Specifically, isoproterenol-induced MI mice showed significantly lower activities of glutathione-dependent antioxidant enzymes and antiperoxidative enzymes (CAT and SOD). Upon treatment with squalene, all alterations were mitigated, maintaining antioxidant enzyme rates, such as glutathione peroxidase (GPx), near normal without observable adverse effects [10]. In another MI-induced rat model, squalene supplementation was found to elevate the endogenous antioxidants of vitamin C and vitamin E compared to MI controls [11]. Squalene's effectiveness as an anti-inflammatory supplement may prove to be potent in other oxidative stress-related diseases, such as diabetes.

Diabetes is one of the top ten causes of death globally, with a rapidly growing burden in developed countries [12, 13]. Diabetes is a major inflammatory disease characterized by hyperglycemia. However, its etiology spans beyond blood glucose to oxidative stress, hyperlipidemia and other factors [14]. Indeed, imbalances between reactive oxygen species (ROS), other free radicals and antioxidants lead to "oxidative stress," a precursor to diabetes [15, 16]. Free radicals are molecules that possess an unpaired electron that result in the molecule's highly reactive nature. The human body produces free radicals, however, in excess, it can become harmful to the body's overall health. While oxygen is needed in aerobic respiration, excess oxidative stress damages key macromolecules (e.g. DNA and proteins) which may contribute to diseases, including cancer and heart disease [17]. In diabetic patients, hyperglycemic-induced oxidative stress is believed to cause local and systemic inflammation [12, 18]. Additionally, hyperglycemia is known to lead to the inactivation of superoxide dismutase (SOD), catalase [18], and other antioxidant enzymes, promoting further oxidative stress [19]. Increasing the expression of antioxidant enzymes may be critical in managing oxidative stress, improving diabetes treatment outcomes [20, 21]. Therefore, oxidative stress can be reduced by increasing these enzymes in number and overall activity.

To the researchers' knowledge, no study has investigated the effect of squalene on SOD, CAT, and GR antioxidant levels in type II diabetic patients; However, a number of squalene studies demonstrated alternative benefits to address diabetes. In obese/diabetic *KK-A^y* mice fed soybean oil, squalene significantly enhanced the expressions of enzymes involved in fatty acid metabolism and increased liver DHA levels six-fold, compared to controls. DHA, a key PUFA in cell membranes and brain function, is known to reduce the risk of inflammatory diseases [22]. In addition to increasing fat metabolism, squalene has been observed to manage fasting blood glucose levels in type 2 diabetes-induced rats [23].

In this 84-day study, diabetic patients were administered varying doses of squalene to assess its effects on antioxidant enzymes and free radicals. The antioxidant enzymes investigated include SOD, CAT, and GPx; the free radicals researched include hydrogen peroxide (H_2O_2), nitric oxide (NO) and other reactive oxygen species (ROS).

METHODS:

Materials: Squalene (S3626, in liquid form and extracted from shark liver, with a purity of more than 98%) was purchased from Sigma-Aldrich Co (USA). The human glucose assay kit was purchased from MyBioSource Inc (USA). The activity assay kits of antioxidant enzymes, including catalase and glutathione peroxidase (GPX), were procured from Biocore Diagnostik Company (Germany). The activity assay kit of antioxidant enzyme superoxide dismutase (SOD) was purchased from BioVision Company (USA). Serum levels of free radicals, including hydrogen peroxide (H_2O_2) and nitric oxide [6] were measured by the kits procured from ZellBio GmbH

Company (Germany). The human reactive oxygen species (ROS) assay kit was purchased from MyBioSource Inc Company (USA).

Methods:

Participants: In this randomized clinical trial study, among the participants in this study 150 volunteers selected based on the following grouping:

Group 1: 30 healthy people (as control)

Group 2: 30 patients with T2DM, without consumption of squalene

Group 3: 30 patients with T2DM, treated with 200 mg/day squalene

Group 4: 30 patients with T2DM treated with 400 mg/day squalene

Group 5: 30 patients with T2DM treated with 600 mg/day squalene

Patients in groups 3, 4, and 5 consumed squalene (as an oral capsule (liquid filled oral)) once a day, during lunch for 84 days. Patients with T2DM were selected among the people referred to Vali-Asr medical laboratory in Tehran, Iran. Concurring to the World Health Organization (WHO) criteria, individuals with fasting plasma glucose ≥ 126 mg/dL or glycated hemoglobin (HbA1c) ≥ 6.5% were considered as diagnosed T2DM. Patients with T1DM and other diseases, a history of surgery, as well as young patients with T2DM were excluded from the study. All participants, especially subjects with T2DM, were informed about how to conduct the study and the type of substances they consume. Informed consent was obtained of all the participants in this study.

General Features and Sampling: After grouping, blood samples were taken from all participants. Sampling was performed in five time periods on days 1, 14, 28, 56, and 84. Anthropometric data including age, sex, weight, height, body mass index (BMI), systolic blood pressure, and diastolic blood pressure of all study participants were recorded. Blood samples were taken from all participants after 12 hours of overnight fasting. After collecting blood samples, they were centrifuged (250 g for 10 min); Following centrifugation the serum was separated from the centrifuged samples. Then, isolated serum samples were used to assess the biochemical parameters.

Biochemical Measurement: In each of the time periods above, biochemical parameters were measured in all five groups. Two laboratory methods, according to the instructions of the relevant kits, were used to measure these parameters: Enzyme–linked immunosorbent assay (ELISA) for glucose and ROS and colorimetric method for H_2O_2, NO and antioxidants enzymes.

Statistical Analysis: Statistical analysis was done by SPSS (version 23, IBM, USA) software for Windows. All results were expressed as mean ± standard deviation. The Kolmogorov-Smirnov test was used to analyze the normal distribution of data. P-values < 0.05 were considered significant. An independent-sample T-test was used to compare the mean of general characteristics of the participants. Statistical significance was analyzed by a one-way ANOVA to compare the mean of the obtained data. After the one-way ANOVA test, Tukey post hoc was used.

RESULTS: Significant findings were found in each parameter throughout the duration of 84 days. Table 1 shows the activity of antioxidant enzymes and the levels of oxidants in the healthy control group, the diabetic group, and the diabetic groups treated with different doses on different days. As the data in the table shows, a significant correlation was observed in the comparison of antioxidant and oxidant parameters between the control

group and the untreated diabetic group with squalene. This significant difference in the mentioned parameters was also observed between the groups treated with squalene and the healthy control group (P value < 0.05).

The activity and levels of the mentioned parameters among the groups treated with squalene and within the groups were also investigated (Table 1). The details of the comparison of the obtained results are shown in Table 2.

Table 1: Comparison between the levels of biochemical parameters of the control group with other groups

Parameter Group	CAT (U/ml)	SOD (U/ml)	GPx (U)	H_2O_2 (µM)	ROS (U/L)	NO (µM)
Healthy control	2.11 ± 0.14	6.91 ± 0.86	105.78 ± 8.53	200.1 ± 13.8	2209.16 ± 8.6	50.43 ± 3.49
Diabetic control (No squalene)	1.31 ± 0.14	2.92 ± 0.86	62.78 ± 8.53	302.9 ± 12.64	3208.7 ± 8.85	80.06 ± 3.42
P value	< 0.05					
Diabetic + 200 mg/day squ (14th day)	1.34 ± 0.14	2.95 ± 0.8	63.44 ± 8.46	292.83 ± 12.62	3107.26 ± 9.51	79.03 ± 3.34
Diabetic + 400 mg/day squ (14th day)	1.35 ± 014	2.96 ± 0.84	64.66 ± 8.53	290.76 ± 12.71	3086.46 ± 9.19	78.06 ± 3.45
Diabetic + 600 mg/day squ (14th day)	1.37 ± 0.14	2.99 ± 0.81	65.29 ± 8.53	289.73 ± 12.59	3068.03 ± 9.33	77.03 ± 3.43
Diabetic + 200 mg/day squ (28th day)	1.33 ± 0.12	2.96 ± 0.86	64.88 ± 8.5	289.86 ± 12.63	3075.66 ± 15.44	78.03 ± 3.37
Diabetic + 400 mg/day squ (28th day)	1.35 ± 0.12	3.04 ± 0.86	66.52 ± 8.47	288.76 ± 12.64	3046.53 ± 10.69	77.06 ± 3.43
Diabetic + 600 mg/day squ (28th day)	1.37 ± 0.13	3.08 ± 0.85	68.56 ± 7.72	286.73 ± 12.74	3027.26 ± 8.85	76.03 ± 3.37
Diabetic + 200 mg/day squ (56th day)	1.38 ± 0.14	2.97 ± 0.84	66.53 ± 8.54	286.76 ± 12.61	3025.16 ± 9.06	75.03 ± 3.43
Diabetic + 400 mg/day squ (56th day)	1.41 ± 0.14	3.04 ± 0.87	66.8 ± 8.5	285.73 ± 12.49	3016.63 ± 8.93	74.1 ± 3.37
Diabetic + 600 mg/day squ (56th day)	1.42 ± 0.14	3.1 ± 0.86	67.09 ± 8.5	284.66 ± 12.7	3008.03 ± 9.83	73.16 ± 3.4
Diabetic + 200 mg/day squ (84th day)	1.4 ± 0.14	2.99 ± 0.86	66.54 ± 8.53	285.76 ± 12.71	3000.56 ± 30.19	74.03 ± 3.47
Diabetic + 400 mg/day squ (84th day)	1.41 ± 0.14	3.92 ± 0.86	66.86 ± 8.53	284.73 ± 12.72	2990.9 ± 27.35	73.16 ± 3.43
Diabetic + 600 mg/day squ (84th day)	1.43 ± 0.14	3.99 ± 0.91	67.49 ± 8.53	282.86 ± 12.67	2986.13 ± 10.71	72.03 ± 3.47

Data are given as mean ± SD. CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; NO, nitric oxide

Table 2: Multiple comparisons between the levels of biochemical parameters between the groups in different days

Parameter Group	CAT (U/ml)	SOD (U/ml)	GPx (U)	H_2O_2 (µM)	ROS (U/L)	NO (µM)
Diabetic14day vs. Diabetic200squ14day	0.88	0.99	0.99	0.01	0.00	0.64
Diabetic14day vs. Diabetic400squ14day	0.69	0.99	0.82	0.002	0.00	0.11
Diabetic14day vs. Diabetic600squ14day	0.38	0.98	0.66	0.001	0.00	0.004
Diabetic200squ14day vs. Diabetic400squ14day	0.98	1.00	0.94	0.92	0.00	0.69
Diabetic200squ14day vs. Diabetic600squ14day	0.81	0.99	0.83	0.77	0.00	0.11
Diabetic400squ14day vs. Diabetic600squ14day	0.95	0.99	0.99	0.98	0.00	0.64
Diabetic28day vs. Diabetic200squ28day	0.94	0.99	0.76	0.00	0.00	0.10
Diabetic28day vs. Diabetic400squ28day	0.73	0.95	0.30	0.00	0.00	0.00
Diabetic28day vs. Diabetic600squ28day	0.29	0.88	0.04	0.000	0.00	0.00
Diabetic200squ28day vs. Diabetic400squ28day	0.96	0.98	0.87	0.98	0.00	0.69
Diabetic200squ28day vs. Diabetic600squ28day	0.61	0.95	0.32	0.77	0.00	0.11
Diabetic400squ28day vs. Diabetic600squ28day	0.87	0.99	0.77	0.92	0.00	0.64
Diabetic56day vs. Diabetic200squ56day	0.23	0.99	0.32	0.00	0.00	0.00
Diabetic56day vs. Diabetic400squ56day	0.03	0.94	0.26	0.00	0.00	0.00
Diabetic56day vs. Diabetic600squ56day	0.02	0.84	0.21	0.00	0.00	0.00
Diabetic200squ56day vs. Diabetic400squ56day	0.85	0.98	0.99	0.98	0.00	0.71
Diabetic200squ56day vs. Diabetic600squ56day	0.78	0.93	0.99	0.91	0.00	0.15
Diabetic400squ56day vs. Diabetic600squ56day	0.99	0.99	0.99	0.98	0.00	0.71
Diabetic 84 day vs.	0.07	0.98	0.32	0.00	0.00	0.00

Parameter Group	CAT (U/ml)	SOD (U/ml)	GPx (U)	H_2O_2 (µM)	ROS (U/L)	NO (µM)
Diabetic 200 squ84day						
Diabetic 84 day vs. Diabetic 400 squ84day	0.04	0.00	0.25	0.00	0.00	0.00
Diabetic 84 day vs. Diabetic 600 squ84day	0.00	0.00	0.14	0.00	0.00	0.00
Diabetic200squ84day vs. Diabetic400squ84day	0.99	0.00	0.99	0.98	0.30	0.76
Diabetic200squ84day vs. Diabetic600squ84day	0.84	0.00	0.97	0.81	0.05	0.11
Diabetic400squ84day vs. Diabetic600squ84day	0.93	0.99	0.99	0.94	0.82	0.58
P value						

CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; NO, nitric oxide. P value < 0.05 indicates significance.

Table 2 shows the comparison of the results obtained from the mentioned parameters in a significant and non-significant form (P value). Table 2 shows the results based on the dose and days of treatment. For example, the activity of the antioxidant enzyme CAT increased significantly (P value < 0.05) in the dose of 400 and 600 squalene in 56 days, compared to the group not treated with squalene. This shows that CAT activity is time-dependent with squalene treatment. Catalase activity in 84 days in groups treated with doses of 400 and 600 mg of squalene increased significantly (P value < 0.05) compared to the group untreated with squalene. SOD activity significantly increased after a certain treatment time of squalene. A significant increase (P value < 0.05) was observed on day 84 and in doses of 200, 400 and 600 mg of squalene. A significant increase in GPx activity was observed only in the treatment with 600 mg of squalene for 28 days (P value < 0.05).

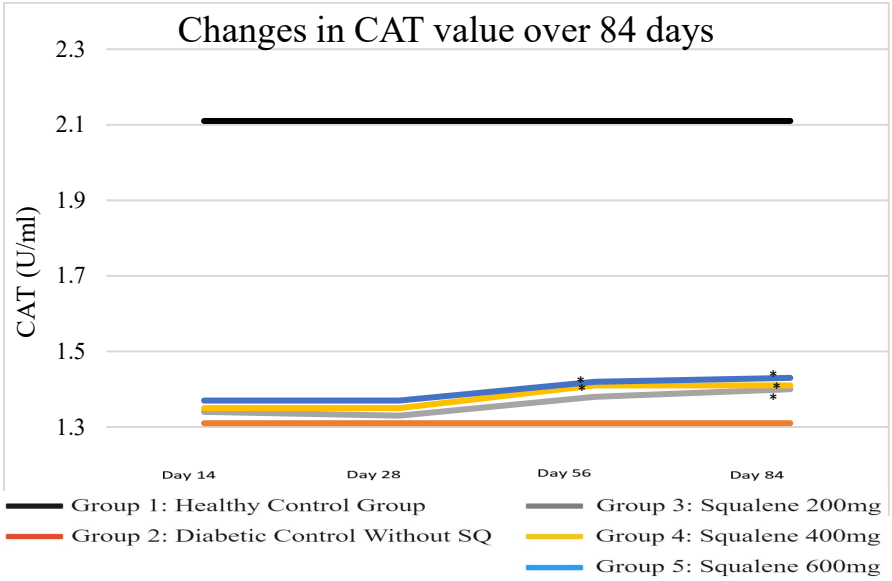


Figure 1: This figure depicts the changes in CAT level of all five groups studied over the study period of 84 days. *Indicates statistical significance (p < 0.05); **Indicates high statistical significance (p < 0.01)

As shown in figure 1, CAT levels remained slightly stationary throughout the first 28 days of the study period. All three experimental groups, which consumed variable levels of squalene, showed no significant change in CAT level throughout these first 28 days. However, from days 28 to 56, there was an observable spike in CAT levels for the three experimental groups which consumed squalene. Group 5, which consumed 600 mg of squalene daily (highest dose), showed the highest CAT on day 56 compared to group 4 (400 mg of squalene daily) and group 3 (200 mg of squalene daily). On day 56, groups 5 and 4 showed significant changes in CAT level. Past this date, CAT levels continued to show slight increases in level as recorded on day 84. On day 84, groups 4 and 5 showed statistically significant changes similar to day 56.

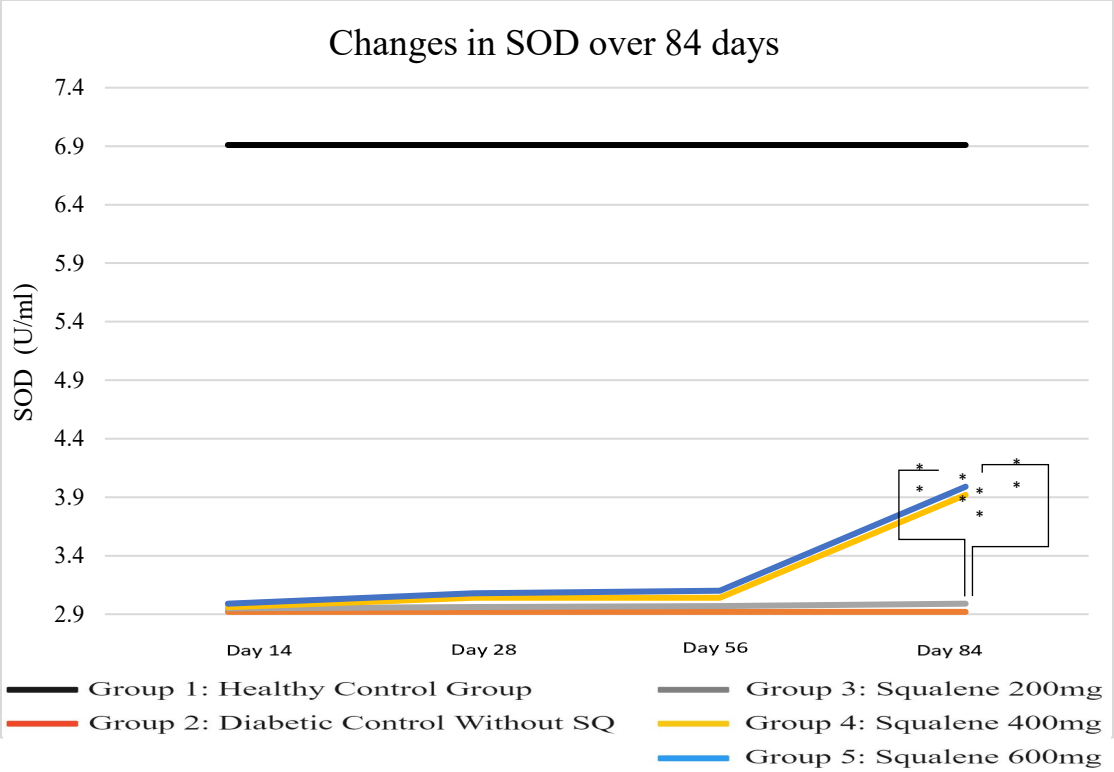


Figure 2: This figure depicts the changes in SOD level of all five groups studied over the study period of 84 days. *Indicates statistical significance ($p < 0.05$); **Indicates high statistical significance ($p < 0.01$); Connecting bars indicate significance between experimental diabetic groups. Further explanation included in caption.

Figure 2 illustrates the changes in SOD level of all five groups studied over the duration of the 84-day study period. As shown in this figure, all three experimental groups which consumed squalene (groups 3, 4, and 5) showed only a slight increase in SOD level for the first 56 days of the experiment. There had not been any statistically significant changes in SOD for any of these experimental groups throughout the first 56 days.

However, after day 56 there had been a large spike in SOD level for group 4 (diabetic group which consumed 400 mg of squalene daily) and group 5 (diabetic group which consumed 600 mg of squalene daily). On day 84, statistically significant findings were reported for both of these experimental groups, compared to both the diabetic control group and group 3 (diabetic group which consumed 200 mg of squalene daily).

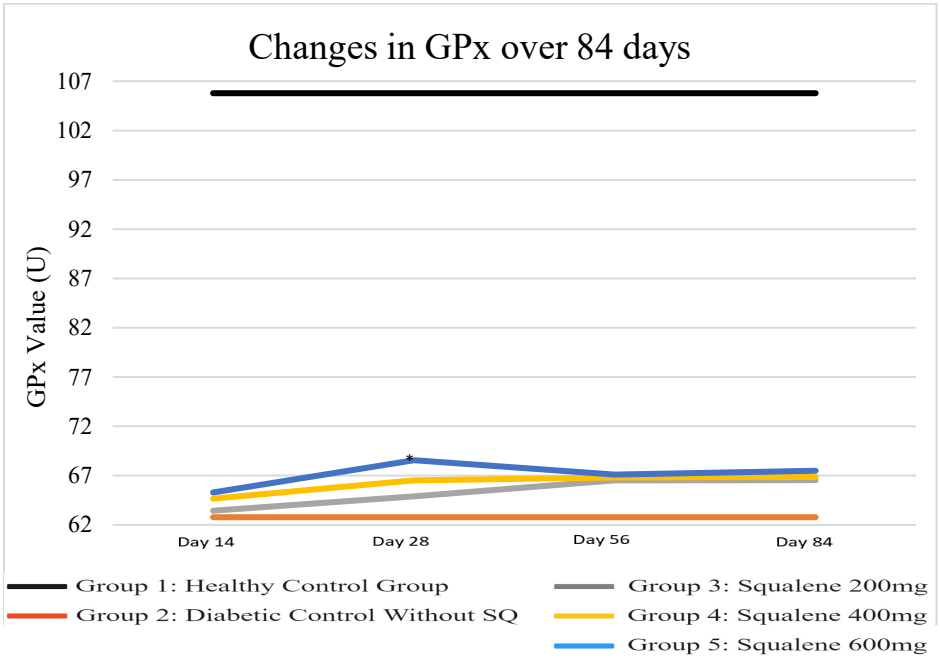


Figure 3: This figure depicts the changes in GPx level of all five groups studied over the study period of 84 days. *Indicates statistical significance ($p < 0.05$); ** Indicates high statistical significance ($p < 0.01$)

Figure 3 illustrates the changes in GPx level of all five groups studied over the duration of the 84-day study period. As shown in this graph, GPx level showed an increase in all three experimental groups (groups 3, 4, and 5) from days 14 to 28. However, of these groups, only group 5 (diabetic group which consumed 600 mg of squalene daily) had shown statistically significant changes in GPx level on day 28. From days 28 to 56, GPx levels for group 5 showed a decrease. During this same time period, groups 3 and 4 showed a slight increase. From days 56 to 84, there had been a slight increase in GPx level for all three experimental groups.

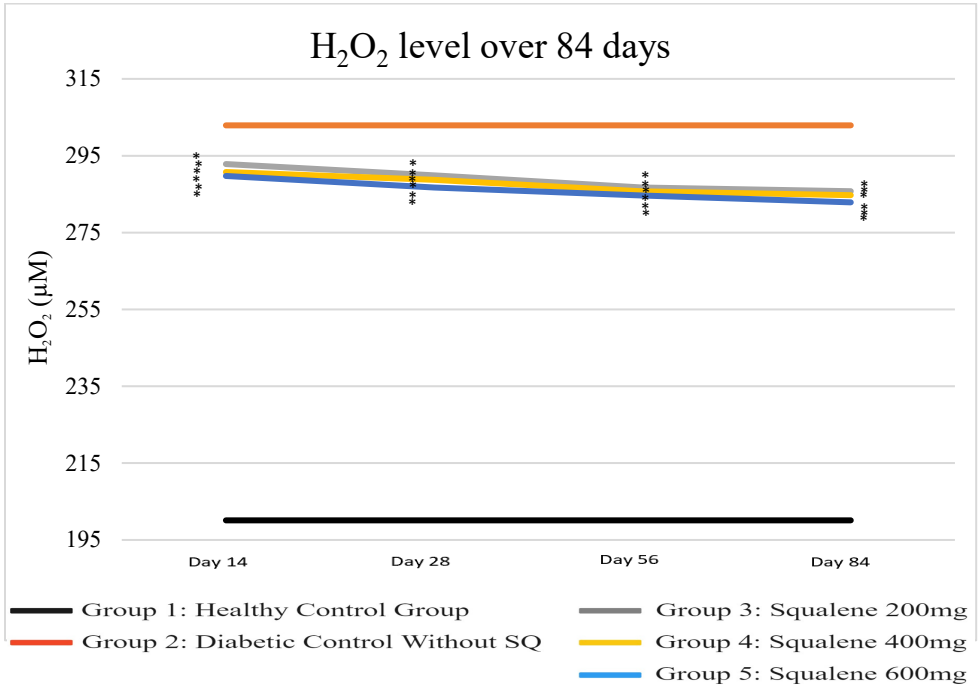


Figure 4: This figure depicts the changes in H_2O_2 level of all five groups studied over the study period of 84 days. *Indicates statistical significance ($p < 0.05$); ** Indicates high statistical significance ($p < 0.01$)

Figure 4 illustrates the changes in H_2O_2 level of all five groups studied over the duration of the 84-day study period. As shown in this figure, statistically significant changes in H_2O_2 levels were found for all three experimental groups (groups 3, 4, and 5) on days 14, 28, 56, and 84. As depicted in the graph, H_2O2 levels showed a decrease in H_2O_2 levels throughout all 84 days with consumption of squalene.

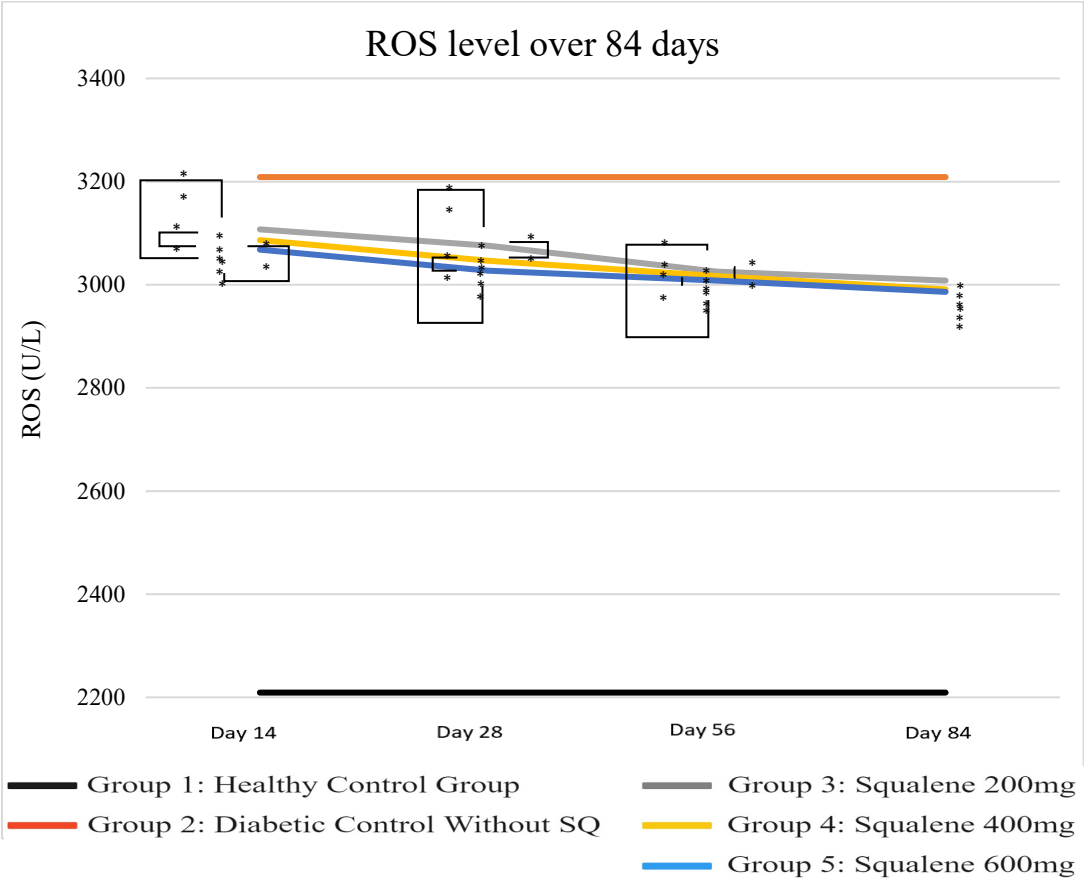


Figure 5: This figure depicts the changes in ROS level of all five groups studied over the study period of 84 days. *Indicates statistical significance ($p < 0.05$); **Indicates high statistical significance ($p < 0.01$); Connecting bars indicate significance between experimental diabetic groups. Further explanation included in caption.

Figure 5 illustrates the changes in ROS level of all five groups studied over the period of 84 days. As illustrated above, statistically significant changes in ROS level were found for all three experimental groups (groups 3, 4, and 5) on days 14, 28, and 56 as compared to the diabetic control group. Additionally, statistical significance was also found in group 3 (diabetic group which consumed 200 mg of squalene daily), in comparison to group 5 (diabetic group which consumed 600 mg of squalene daily) on days 14, 28, 56, and 84. This was also found between group 4 (diabetic group which consumed 400 mg of squalene daily) and group 3 (diabetic group which consumed 200 mg of squalene daily),as well as between group 4 and group 5. On day 84, statistical significance was found for all experimental groups (groups 3, 4, and 5) compared to the diabetic control group.

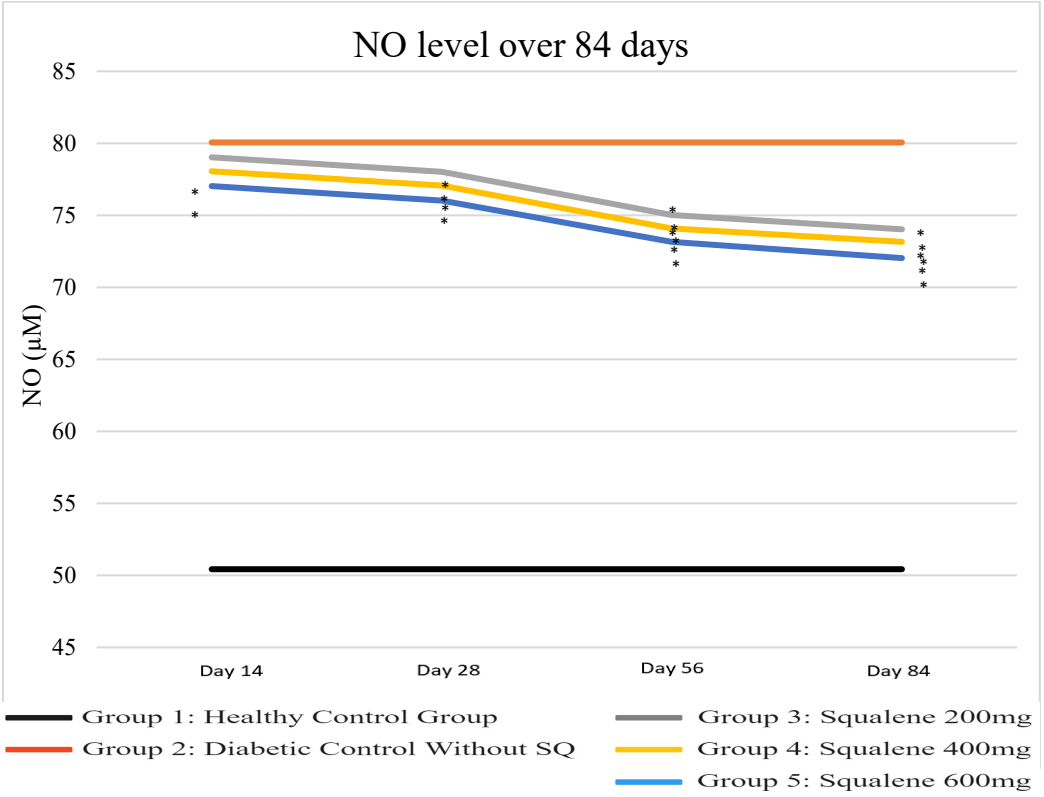


Figure 6: This figure depicts the changes in NO level of all five groups studied over the study period of 84 days. *Indicates statistical significance ($p < 0.05$); **Indicates high statistical significance ($p < 0.01$)

Figure 6 illustrates the changes in NO level over the duration of the study. As shown, on day 14, statistical significance was only noted for changes in NO level of group 5 (diabetic group which consumed 600 mg of squalene daily). On day 28, however, statistical significance was found for both groups 4 (diabetic group which consumed 400 mg of squalene daily) as well as group 5. On from days 28 to 56, we see a slight drop in NO levels of all the experimental groups (groups 3, 4, and 5). Statistical significance was found for all of these groups on day 56 as compared to the diabetic control. Similarly, statistical significance as compared to the diabetic control was also found for all of these groups on day 84, the final data collection day.

DISCUSSION: Our research focused on the effect of squalene on free radicals and antioxidant enzymes. In order to determine its effect, our primary targeted parameters included changes in superoxide dismutase (SOD), human reactive oxygen species (ROS), catalase, glutathione peroxidase (GPx), nitric oxide, and hydrogen peroxide (H_2O_2). SOD is a biological enzyme that is crucial to organisms due to its ability to inactive superoxide. In one study, mice with mitochondrial SOD (MnSOD) deficiency were studied. These knockout mice were found to have been impacted with anemia, degeneration of neurons, perinatal death and myocardial injury [24]. SOD was also included in our study in order to identify squalene's potential effect on SOD. NO has been a particular area of interest due to its highly reactive nature when NO interacts with O_2^- or O_2. From this interaction, reactive nitrogen oxide species are formed in the body [25]. GPx also proves to play an important role in defending against oxidative stress. Indeed, GPx1 null mutant mice exhibited increased sensitivity to paraquat, an oxidative stressor [26]. H_2O_2 was also studied due to its destructive effect on the cell [27]. ROS was included as a parameter due to its ability to damage cells. Finally,

catalase was included as a parameter due to its ability to decrease H_2O_2 levels.

SOD exhibited a unique trend throughout the duration of 84 days, as seen in Figure 2. As shown, SOD exhibited little growth in all three experimental groups studied throughout days 14 to 56. However, after day 56, SOD levels in both experimental groups 4 (diabetic group which consumed 400 mg of squalene daily) and 5 (diabetic group which consumed 600 mg of squalene daily) had peaked. Group 3 (diabetic group which consumed 200 mg of squalene daily), however, did not show the same trend of immediate rise as the other two groups. We predict that this may be due to squalene's biochemical mechanisms, with attention to dosage and duration. This is because SOD only peaked in the diabetic groups that consumed a minimum of 400 mg of squalene daily. Future research may focus on squalene's biochemical effect on SOD to identify the reason for this effect. This will also give more insight into identifying the minimum squalene amount required to produce such effects, as well as the minimum duration of time needed to be prescribed. In order to understand more about the dynamics of this peak from days 56 to 84, an extension of time should be added to the overall study duration. This will allow for understanding if SOD levels continue to increase at the same rate from days 56 to 84. If it does not, this will allow for a better understanding of identifying a maximum prescription time for squalene to help raise SOD levels. As such, two areas of primary focus with this parameter are overall prescription time as well as dosage amounts.

ROS exhibited unique statistical significance compared to the other antioxidant enzymes and free radicals recorded in this study. From days 14 to 56, all the experimental groups (groups 3, 4, and 5) had observed statistical significance as compared to the diabetic control. Additionally, statistical significance was also found between groups 3 and 4, groups 4 and 5, and groups 3 and 5 within days 14 to 56. On day 84, groups 3, 4, and 5 only exhibited statistical significance, in comparison to the diabetic control group (This is shown in figure 5). These results indicate that squalene affects ROS levels, as statistical significance was found in each experimental group that consumed squalene throughout all days recorded in the experiment.

GPx provided interesting results between days 14 to 28 compared to days 28 to 84. On days 14 to 28, all three experimental groups (groups 3, 4, and 5) had an increase in GPx level, therefore getting closer to the value for the healthy group. However, from days 28 to 56, only groups 3 and 4 had an increase. Group 5 had a decrease in GPx level. From days 56 to 84, the GPx value for groups 3, 4 and 5 showed an increase. However, this overall end value on day 84 for group 5 was not as high as on day 28. The value difference in GPx for group 4 was also very close to day 84, as it was on day 28. These results indicate that if squalene is prescribed between 400 and 600 mg, by day 28, there should be the most observable changes. After the 28^{th} day, there are slight changes, but they are not as noticeable. This is not the case for group 2. This is because group 2 had a large peak occur between days 28 and 56. Therefore, the most noticeable changes would occur around this duration of time. Prescription time plays an important role in observing the range of days that will provide optimal effects. One of the consequences of uncontrolled diabetes is cardiovascular diseases. Uncontrolled diabetes is accompanied by hyperlipidemia. Ibrahim and his colleague Mohamed stated in a review article that squalene can be a complementary factor for cardiovascular health. They stated that the antioxidant activity of squalene is related to cardiovascular health [28]. In a study conducted by Gabás-Rivera et al., squalene was administrated to mice at a dose of 1gr/kg for eleven weeks. They reported that dietary squalene contributes to the reduction of ROS in apolipoproteins [29]. The effect of squalene on ROS level

in the present study agreed with the study of Gabás-Rivera et al. A study was conducted by Ravi Kumar et al. on Male obese/diabetic model, *KK-A* ^{y} mice [30]. They reported that treated diabetic mice with 2% squalene had an increase in the activity of liver enzymes CAT and GPx, compared to the control group. In our study, the serum level of the mentioned enzymes was investigated and significant changes in the activity of the enzymes were observed in some groups.

The parameters CAT, NO, and H_2O_2 all showed trends of values getting closer to the healthy range without major fluctuations on certain dates or with certain dosages. With the given statistical significance, this is indicative that squalene effectively impacts CAT, NO, and H_2O_2 levels to reach a healthy range.

CONCLUSION: As per this experiment, squalene has shown promising results in bringing the studied parameters in diabetic patients to closer levels to that of the healthy control group. This brings forth the possible application of squalene as a method of aiding treatment for individuals with irregular oxidative enzyme levels. Future research should focus on defining a precise measure for dosage levels, as specific cases arose where certain parameters displayed peaks or drops in levels specific to only particular experimental squalene groups. Another key area of importance for future studies is to extend the timeline of the experiment. Duration played an important role in this study as certain parameters showed changes in value near the end of the study. For example, SOD showed an incredible peak in data. In order to understand if this pattern of increasing will continue, a longer duration of time must be implemented. This will set a defined prescription time. One final area for future studies to focus on is with the biochemical background of squalene as it reacts with the defined parameters. In future studies, squalene may potentially be used as a bioactive compound to aid in bringing these oxidative enzymes closer to a healthier level.

List of abbreviations: MI: myocardial infarction, GPx: glutathione peroxidase, ROS: reactive oxygen species, SOD: superoxide dismutase, BMI: body mass index, H_2O_2: hydrogen peroxide, NO: nitric oxide, HbA1c: glycated hemoglobin, T2DM: type two diabetes mellitus

Authors' contributions: Conceptualization, H.M. and D.M.; Methodology, H.M; Validation and formal analysis, M.A; Supervision, D.M. and H.M; Writing the manuscript and drawing the graphs and tables of the results, A.S, DM and K.Z.

Competing interests: The authors declare that they have no competing interests.

Acknowledgements: The authors of this article acknowledge the staff of the Vali-Asr medical laboratory.

REFERENCES

1. Kim, S.-K. and F. Karadeniz: Biological importance and applications of squalene and squalane. Adv Food Nutr Res 2012, 65:223-233. DOI: https://doi.org/10.1016/B978-0-12-416003-3.00014-7
2. Martirosyan, D., J. Hutcheson, D. Sajitharan, S. Williams, and C. Mohan: The effect of amaranth oil on proteinuria in lupus prone mice. FFS 2021, 1(10):39-49. DOI: https://doi.org/10.31989/ffs.v1i10.848
3. Martirosyan, D., M.R. Ashoori, A.S. Mikaeili, S. Pezeshki, A. Serani, M. Lee, and H. Mirmiranpour: Inflammatory factors and immunoglobulins alterations in subjects with type 2 diabetes mellitus treated with squalene. FFS 2022, 2(8):181-197. DOI: https://doi.org/10.31989/ffs.v2i8.979
4. Ronco, A.L. and E. De Stéfani: Squalene: a multi-task link in the crossroads of cancer and aging. FFHD 2013, 3(12): 462-476.DOI: https://doi.org/10.31989/ffhd.v3i12.30
5. Lou-Bonafonte, J.M., R. Martínez-Beamonte, T. Sanclemente, J.C. Surra, L.V. Herrera-Marcos, J. Sanchez-Marco, C. Arnal, and J. Osada: Current insights into the biological action of

squalene. Mol Nutr Food Res 2018, 62(15):1800136. DOI: https://doi.org/10.1002/mnfr.201800136

6. Cárdeno, A., M. Aparicio-Soto, S. Montserrat-de la Paz, B. Bermúdez, F.J. Muriana, and C. Alarcón-de-la-Lastra: Squalene targets pro-and anti-inflammatory mediators and pathways to modulate over-activation of neutrophils, monocytes and macrophages. J Funct Foods 2015, 14:779-790. DOI: https://doi.org/10.1016/j.jff.2015.03.009
7. Ibrahim, N.I., S. Fairus, M.S. Zulfarina, and I. Naina Mohamed: The efficacy of squalene in cardiovascular disease risk-a systematic review. Nutrients 2020, 12(2):414. DOI: https://doi.org/10.3390/nu12020414
8. Mirmiranpour, H., M.R. Ashoori, A.S. Mikaeili, S. Pezeshki, A. Serani, A. Boez, and D. Martirosyan: The effect of squalene on proteinuria in patients with type 2 diabetes mellitus. BCHD 2022, 5(6):117-135. DOI: https://doi.org/10.31989/bchd.v5i6.945
9. Das, B., R. Antoon, R. Tsuchida, S. Lotfi, O. Morozova, W. Farhat, D. Malkin, G. Koren, H. Yeger, and S. Baruchel: Squalene selectively protects mouse bone marrow progenitors against cisplatin and carboplatin-induced cytotoxicity in vivo without protecting tumor growth. Neoplasia 2008, 10(10):1105-IN4. DOI: https://doi.org/10.1593/neo.08466
10. Farvin, K.S., R. Anandan, S.H.S. Kumar, K. Shiny, T. Sankar, and T. Thankappan: Effect of squalene on tissue defense system in isoproterenol-induced myocardial infarction in rats. Pharmacol Res 2004, 50(3):231-236. DOI: https://doi.org/10.1016/j.phrs.2004.03.004
11. Farvin, K., A. Surendraraj, and R. Anandan: Protective effect of squalene on endogenous antioxidant vitamins in experimentally induced myocardial infarction in rats. Asian J Biochem 2009, 4(4):133-139. DOI: https://doi.org/10.3923/AJB.2009.133.139
12. Oguntibeju, O.O.: Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links. Int J Physiol Pathophysiol Pharmacol 2019, 11(3):45. DOI: https://pubmed.ncbi.nlm.nih.gov/31333808/
13. Khan, M.A.B., M.J. Hashim, J.K. King, R.D. Govender, H. Mustafa, and J. Al Kaabi: Epidemiology of type 2 diabetes–global burden of disease and forecasted trends. J Epidemiol Glob Health 2020, 10(1):107. DOI: https: doi.org/10.2991/jegh.k.191028.001
14. Ashoori, M.R., S.Z. Bathaie, and H. Heidarzadeh: Long-term, high-dose aspirin therapy increases the specific activity of complex III of mitochondrial respiratory chain in the kidney of diabetic rats. Physiol Pharmacol 2015, 19(3):158-166. DOI: https://ppj.phypha.ir/article-1-1099-en.html
15. Asmat, U., K. Abad, and K. Ismail: Diabetes mellitus and oxidative stress—A concise review. Saudi Pharm J 2016, 24(5): 547-553.DOI: https://doi.org/10.1016/j.jsps.2015.03.013
16. Martirosyan, D., H. Ghomi, M.R. Ashoori, A. Rezaeinezhad, A.S. Mikaeili, F. Jahanbakhshi, and H. Mirmiranpour: Study of the effect of gallic acid and cold plasma on the levels of inflammatory factors and antioxidants in the serum sample of subjects with type 2 diabetes mellitus. BCHD 2021, 4(8):167-179. DOI: https://doi.org/10.31989/BCHD.V4I8.824
17. Halliwell, B.: Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? The lancet 1994, 344(8924):721-724. DOI: https://www.doi.org/ 10.1016/s0140-6736(94)92211-x.
18. Martirosyan, D., H. Mirmiranpour, and M.R. Ashoori: Synergistic effect of laser irradiation and cinnamic acid as a functional food on oxidative stress in type 2 diabetes mellitus. BCHD 2020, 3(9):154-165. DOI: https: doi.org/10.31989/bchd.v3i9.746
19. Martirosyan, D., M.R. Ashoori, and H. Mirmiranpour: The effect of low level-laser irradiation on antioxidant enzymes and mineral levels in serum of patients with type 2 diabetes mellitus. BCHD 2020, 3(5):82-89. DOI: https: doi.org/10.31989/bchd.v3i5.705
20. Al-Aubaidy, H.A. and H.F. Jelinek: Oxidative DNA damage and obesity in type 2 diabetes mellitus. Eur J Endocrinol 2011, 164(6):899-904. DOI https://doi.org/10.1530/EJE-11-0053
21. Al-Aubaidy, H.A. and H.F. Jelinek: Oxidative DNA damage: antioxidant response in postprandial hyperglycaemia in type 2 diabetes mellitus. BJDVD 2011, 11(2):87-91. DOI: https://doi.org/10.1177/1474651411405259
22. Ravi Kumar, S., I. Yamauchi, B. Narayan, A. Katsuki, M. Hosokawa, and K. Miyashita: Squalene modulates fatty acid metabolism: Enhanced EPA/DHA in obese/diabetic mice (KK-Ay) model. Eur J Lipid Sci Technol 2016, 118(12):1935-1941. DOI: https://doi.org/10.1002/ejlt.201600006
23. Widyawati, T., S. Syarifah, and I. Sumantri. Squalene decreased fasting blood glucose level of type ii diabetic rats. in IOP Conference Series: EES. 2021. IOP Publishing. DOI: https://doi.org/10.1088/1755-1315/912/1/012088

24. Lebovitz, R.M., H. Zhang, H. Vogel, J. Cartwright Jr, L. Dionne, N. Lu, S. Huang, and M.M. Matzuk: Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. Proc Natl Acad Sci 1996, 93(18):9782-9787. DOI: https://doi.org/10.1073/pnas.93.18.9782
25. Akaike, T., M. Suga, and H. Maeda: Free radicals in viral pathogenesis: molecular mechanisms involving superoxide and NO. Proc Soc Exp Biol Med 1998, 217(1):64-73. DOI: https://doi.org/10.3181/00379727-217-44206
26. de Haan, J.B., C. Bladier, P. Griffiths, M. Kelner, R.D. O'Shea, N.S. Cheung, R.T. Bronson, M.J. Silvestro, S. Wild, and S.S. Zheng: Mice with a homozygous null mutation for the most abundant glutathione peroxidase, Gpx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide. J Biol Chem 1998, 273(35):22528-22536. DOI: https://doi.org/10.1074/jbc.273.35.22528
27. Chae, H.Z., S.W. Kang, and S.G. Rhee: Isoforms of mammalian peroxiredoxin that reduce peroxides in presence of thioredoxin. Methods in enzymology. 1999, Elsevier. p. 219-226. DOI: https://doi.org/10.1016/S0076-6879(99)00128-7
28. Ibrahim, N.I. and I. Naina Mohamed: Interdependence of Anti-Inflammatory and Antioxidant Properties of Squalene–Implication for Cardiovascular Health. Life 2021, 11(2):103. DOI: https://doi.org/10.3390/life11020103
29. Gabás-Rivera, C., C. Barranquero, R. Martínez-Beamonte, M.A. Navarro, J.C. Surra, and J. Osada: Dietary squalene increases high density lipoprotein-cholesterol and paraoxonase 1 and decreases oxidative stress in mice. PloS one 2014, 9(8): e104224. DOI: https://doi.org/10.1371/journal.pone.0104224
30. Ravi Kumar, S., B. Narayan, Y. Sawada, M. Hosokawa, and K. Miyashita: Combined effect of astaxanthin and squalene on oxidative stress in vivo. Mol Cell Biochem 2016, 417(1):57-65. DOI:https://doi.org/10.1007/s11010-016-2713-2