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# Improving the anti-diabetic and anti-hyperlipidemic activities of extra virgin olive oil by the incorporation of diallyl sulfide

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**Abstract**

Development of novel functional foods is trending as one of the hot topics in food science and food/beverage industries. In the present study, the anti-diabetic, anti-hyperlipidemic and histo-protective effects of the extra virgin olive oil (EVOO) enriched with the organosulfur diallyl sulfide (DAS) (DAS-rich EVOO) were evaluated in alloxan-induced diabetic mice. The ingestion of EVOO (500µL daily for two weeks) attenuated alloxan-induced elevated glucose, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase, lactate dehydrogenase (LDH), urea and creatinine. It also normalized the levels of triglycerides (TG), total cholesterols (TC), low-density lipoprotein-cholesterol (LDL-c) and their consequent atherogenic index of plasma (AIP) in diabetic animals. Additionally, EVOO prevented lipid peroxidation (MDA) and reduced the level of hydrogen peroxide (H2O2) in diabetic animals. Concomitantly, it enhanced the activity of the antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD), reducing thereby tissue oxidative stress injury. The overall histologic (pancreas, liver, and kidney) alterations were also improved after EVOO ingestion. The manifest anti-diabetic, lipid-lowering and histo-protective properties of EVOO were markedly potentiated with DAS-rich EVOO suggesting possible synergistic interactions between DAS and EVOO lipophilic bioactive ingredients. Overall, EVOO and DAS-rich EVOO show promise as functional foods and/or adjuvants for the treatment of diabetes and its complications.

**Keywords:** Antioxidant activity, Diabetes, Diallyl sulfide, Extra virgin olive oil, Lipid profile

## 1. Introduction

Olive oil is the highest grade of edible oils which is prized for its unique organoleptic properties and health benefits. Extra virgin olive oil (EVOO) is the key functional food of the well-known Mediterranean diet commonly associated with lower incidences of cardiovascular diseases, cancers, and immune disorders [1]. Among the wide range of nutraceuticals present in EVOO, fatty acids (*i.e* oleic acid, linoleic acid, linolenic acid and palmitoleic acid), vitamin E (α-, β-, and γ-tocopherols), phytosterols (*i.e.*β-sitosterol, $\Delta^5$-avenasterol, stigmasterol, campesterol, campestanol, ergosterol, cycloartanol, and cycloartenol), pigments (i.e. β-carotene, lutein, violaxanthin, neoxanthin, and chlorophyll derivatives) and phenolic compounds (*i.e.* hydroxytyrosol, tyrosol, oleuropein, oleuropein aglycone, oleocanthal, ligstroside, oleacein, pinoresinol, acetoxypinoresinol, apigenin, luteoline, sinapic acid, ferulic acid, vanillic acid, *p*-coumaric acid, protocatechuic acid, and hydroxyl-isochroman) have received a great deal of attention because of their interesting biological activities [2, 3]. Previous pharmacological studies pointed to the antioxidant, antimicrobial [4], anti-obesity [5], histoprotective

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[2], anti-gout [6] and anti-tumor [7] activities, among others.

Some of the EVOO-derived bioactive compounds are effective in alleviating diabetes and its complications [8]. The advantages of oleuropein [9], tyrosol [10], hydroxytyrosol [11] and oleic acid [12] in preventing diabetes and its associated complications namely oxidative stress and hyperlipidemia have been reported.

Owing to the wide array of bioactive ingredients and potential biological activities, the investigation of EVOO is a boundless field, notably focusing on the isolation and purification process, analytical and authentication techniques, conception of new EVOO-based products and their biological properties [13]. In the latter case, the available data showed that enrichment of EVOO could represent an attractive option not only for ensuring an optimal intake of EVOO polyphenols but also for the development of new functional foods with better oxidative stability [14], good sensory attributes and improved biological activities [15]. In terms of bioactivity, a previous pharmacokinetic study showed that enrichment of EVOO with its own phenolic compounds significantly increased their bioavailability and their concentrations in human plasma [16].

Later, a randomized controlled trial showed that the consumption of EVOO enriched with its own phenolic compounds improved endothelial function through the increased ischemic reactive hyperemia and reduced oxidized LDL in hypertensive patients [17]. These effects were correlated with the up-regulation of proteins related to cholesterol homeostasis, protection against oxidation, blood [18] and enhanced superoxide dismutase-mediated DNA protection against oxidation [19]. Other publications indicated that the intake of EVOO enriched with its own phenols increased the serum HDL cholesterol and enhanced macrophage cholesterol efflux *in vivo* [20]. More recently, we have demonstrated that the incorporation of diallyl sulfide (DAS) into EVOO boosted its antioxidant, anti-inflammatory and hepatoprotective effects in mice [13]. The DAS is one of the chief organosulfur compounds of garlic oil which was credited with a long list of health beneficial effects including antioxidant, antimicrobial, anticancer and anti-inflammatory effects [21]. The hepatoprotective, chemoprotective and antitumoral effects of DAS and its metabolites diallyl sulfoxide (DASO) and diallyl sulfone ($DASO_2$) have also been reported [22].

Prompted by these antecedents and considering the lipophilic property of DAS which could greatly improve its bioavailability, the present study was planned to investigate the anti-hyperglycemic and antihyperlipidemic potential of DAS-rich EVOO in alloxan-induced type 2 diabetic mice model. The DAS-rich EVOO was made by the incorporation of DAS at 1% into EVOO. Such low and non-toxic dose of DAS was well tolerated and did not induce significant changes in standard toxicological parameters of mice [13]. Practically, in addition to the enrichment of the wide assortment of EVOO-based functional foods, the DAS-rich EVOO may provide a prospective therapeutic solution for the prevention and management of diabetes, expanding thereby the application range, and empowering the economic profitability of EVOO-based products.

## 2. Materials and Methods

### 2.1. Reagents and materials

Acetic acid, alloxan monohydrate, ammonium molybdate, bovine serum albumin (BSA), diallyl sulfide (DAS), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), sodium phosphate, trichloroacetic acid (TCA) and Tris-HCl were purchased from Sigma-Aldrich (Steinheim, Germany). Solvents of analytical grade were purchased from Carlo Erba Reactif-CDS (Val de Reuil, France).

### 2.2. Preparation of the DAS enriched EVOO

The DAS-rich EVOO was prepared by a direct addition of DAS (1%, v/v) to the mono-varietal “Chemlali” EVOO as previously described [13].

### 2.2. Experimental animals and diets

Male Suisse albino mice (weight 30 ± 5g) were purchased from the Society of Pharmaceutical Industries, Ben Arous, Tunisia) and housed under standard housing conditions (22 ± 2° C; relative humidity 50% and 12 h light/dark cycle). They were fed commercial pellet diet made up of 12% moisture, 23% crude protein, 3% fat, 2.5% soybean oil, 12% soybean meal, 3% corn gluten meal, 10% fish meal, 10% wheat middling, 1.5% molasses, 7% crude fiber, 0.5% salt, minerals and vitamins (El Badr Utique-TN, Bizerte, Tunisia) and water ad libitum. All protocols followed in the study were approved by the "Comité d’éthique de la Recherche en Sciences de la Vie et de la Santé" (CER-SVS) at the Institut Supérieur de Biotechnologie de Monastir (ISBM), University of Monastir, (Ethical approval: CER-SVS/ISBM 016/2021).

### 2.3. Induction of diabetes

For the induction of diabetes, a single intraperitoneal (i.p) injection of alloxan monohydrate (160 mg/kg body weight) was used. Animals with fasting blood glucose levels higher than 13 mM /L at 10th after the injection of alloxan were considered diabetic [23].

### 2.4. Experimental design

Experimental animals were randomly divided into 6 groups (6 mice each) designated as follows: Normal control receiving basal diet; Diabetic control receiving basal diet; Normal control receiving 500 µL of EVOO; Normal control receiving 500 µL of DAS-rich EVOO; Diabetic mice receiving 500 µL of EVOO; and Diabetic mice receiving 500 µL of DAS-rich EVOO.

After 10 days of induction of diabetes, fresh oil samples were administered orally by gavage once per day for 15 days. At the end of the experiment, mice were anesthetized using anesthetic ether and sacrificed by cervical dislocation. The blood samples were collected into heparin-coated tubes and centrifuged at 3000 × g for 10 min at 4 °C. The serum was collected for biochemical estimations.

### 2.5. Measurement of serum biochemical parameters

The commercial standard enzymatic kit (Biomaghreb, Ariana, Tunisia) detected serum levels of glucose, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase, lactate dehydrogenase (LDH), albumin, urea, uric acid, creatinine, triglycerides (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C). The atherogenic index of plasma (AIP) as a predictor of cardiovascular risk was calculated as the logarithmic transformation of TG to HDL-C ratio and was categorized as: Low (AIP<0.11); Intermediate (0.11<AIP<0.21) and

High (AIP>0.21) [24].

### 2.6. Estimation of oxidative stress markers

Malondialdehydes (MDA), $H_2O_2$, sulfhydryl group (-SH) were determined in liver and kidney tissues supernatants obtained after homogenization of both organ tissues in 50 mM phosphate buffer saline ($KH_2PO_4$/ $K_2HPO_4$; pH 7.4) followed by centrifugation at 10000 × g for 10 min (4° C). The MDA content was measured according to the method of Draper and Hadley [25]. Briefly, a 1 mL aliquot of the supernatant was mixed with 1 mL of a saturated solution of TBA reagent (0.5 N HCl, 120 mM TBA buffered in 26 mM Tris) and heated in boiling water for 30 min. After cooling, the absorbance of the mixture was measured at 532 nm and the MDA content was determined using the extinction coefficient of the complex TBA-MDA $1.56 \times 10^5$ $M^{-1}cm^{-1}$.

The $H_2O_2$ content was determined following the method of Dingeon et al. [26] by measuring the absorbance at 505 nm of the quinoneimine chromophore which represents the end product of the reaction between hydrogen peroxide with *p*-hydroxybenzoic acid and 4-aminoantipyrine in the presence of peroxidase.

For the (-SH) group, the method of Ellman [27] was used and the results were expressed as nmol of thiol group per mg protein. Briefly, 100 µL aliquot of supernatant was mixed with 100 µL of 10% SDS and 800 µL of 10 mM phosphate buffer (pH 8) and the absorbance was read at 412 nm (A0). Thereafter, 100 µL of DTNB were added and the reaction mixture was incubated at 37°C for 1 h and the absorbance (A1) was determined. The thiol group (-SH) was determined from the A1 to A0 deduction using the extinction coefficient of $13.6 \times 10^3$ $mol^{-1}.cm^{-1}$.

### 2.7. Estimation of the activities of antioxidant enzymes

Catalase (CAT; E.C: 1.11.1.6.) activity was determined by surveying the decomposition of $H_2O_2$ at 240 nm for 3 min and was expressed as nmol $H_2O_2$ decomposed per mg of protein per minute [28]. The reaction mixture contained 33 mM $H_2O_2$ in 50 mM phosphate buffer (pH 7.0) and the activity of CAT was calculated using the extinction coefficient of $H_2O_2$ (40 $mM^{-1}$. $cm^{-1}$).

Glutathione peroxidase (GPx; E.C: 1.11.1.9) activity was measured at 412 nm using the method of Flohé and Günzler [29]. Briefly, a 0.2 mL aliquot of supernatant was mixed with 0.2 mL of phosphate buffer (0.1 M; pH 7.4), 0.2 mL of 4 mM GSH and 0.4 mL of 5 mM $H_2O_2$. After incubation at 37°C for 1 min, the reaction was stopped by addition of 0.5 mL of 5% TCA (w/v) and the absorbance of the mixture was determined at 412 nm after addition of 0.5 mL DTNB (10 mM). The GPx activity was expressed as nmol of GSH consumed per min per mg protein.

The ability of superoxide dismutase (SOD, E.C: 1.15.1.1) to inhibit the radical-mediated chain-propagating auto-oxidation of epinephrine in alkaline pH medium was determined spectrophotometrically at 480 nm [30]. The reaction mixture (2 mL) consisted 0.2 mL supernatant of 10 µL bovine catalase (0.4 U/mL), 20 µL epinephrine (5 mg/mL) and 62.5 sodium carbonate/bicarbonate buffer pH 10.2. Changes in absorbance were recorded at 480 nm and the activity was reported in units of SOD per mg protein. One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of auto-oxidation of epinephrine by 50%.

Protein concentration was determined by using the method of Bradford with bovine serum albumin (BSA) as a standard [31].

### 2.8. Histopathological study

Fresh tissues from liver, pancreas and kidney were immediately excised from sacrificed animals and fixed in 10% neutral buffered formalin solution. Thereafter, fixed tissues were dehydrated in ethanol, embedded in paraffin, sectioned to 4-5µm thickness, and then stained with hematoxylin and eosin (HE) for observation of pathological changes using an Olympus light microscope (Tokyo, Japan).

### 2.9. Statistical analysis

Data were expressed as mean ± standard deviation of at least three determinations. Group means were compared using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. All analyses were performed using the Statistical Package for the Social Sciences (SPSS v 18.0) software (IBM, Chicago, IL, USA). Results at $p<0.05$ were considered statistically significant.

## 3. Results

### 3.1. Serum biochemical markers

Data from Table 1 showed that intraperitoneal injection of alloxan monohydrate to control mice increased the blood glucose, ALT, AST, alkaline phosphatase, LDH, urea and creatinine by 131, 103, 111.43, 81.2, 39.4, 67.7 and 37.4%, respectively in diabetic control over the non-diabetic control mice. In contrast, it induces 34.2 and 42.3% reduction in albumin and uric acid, respectively. All serum parameters were significantly ($p<0.05$) normalized after the administration of DAS-rich EVOO and to a lesser extend EVOO. These results suggest that EVOO and DAS-rich EVOO have anti-diabetic effects.

### 3.2. Plasma lipid profiles

In diabetic mice, there was 83 and 29% increase in plasma triglyceride and plasma cholesterol, respectively over the non-diabetic control (Table 2). The increment of plasma cholesterol was allied to 61% increase in LDL-C *versus* 27% decrease in HDL-C. These changes will put diabetic animals at high cardiovascular risk as revealed by the 2.5-, 2.24-, and 3.27-fold increase in TC/HDL-C ratio, LDL-C/HDL-C ratio, and the AIP, respectively. These alterations were improved by the ingestion of EVOO and DAS-rich EVOO being significantly ($p<0.05$) the most efficient in reducing the risk of cardiovascular disease in non-diabetic control and diabetic mice. This indicates that DAS incorporation into EVOO improved its lipid-lowering property.

### 3.2. Oxidative status

As illustrated in Fig. 1. diabetic animals experienced severe oxidative bursts as revealed by the 36.77 and 62% increase of MDA content in liver (7.81±0.58 nmol/min/mg protein) and kidney (8.44±0.73 nmol/min/mg protein), respectively over the non-diabetic (5.71±0.62 and 5.21±0.54 nmol/min/mg protein in liver and kidney, respectively) control (Fig. 1A). Similar trend was observed for $H_2O_2$ which was significantly ($p<0.05$) increased in liver (1.94-fold) and kidney (2-fold) of diabetic mice (Fig.

**Table 1.** Effect of EVOO and DAS-enriched EVOO on blood glucose level and liver (ALT, AST, AKP and LDH), and kidney (urea, uric acid, albumin, and creatinine) biochemical parameters in control and experimental groups of mice.

| Treatment | Blood glucose (mM) | ALT (UI/L) | AST (UI/L) | AKP (UI/L) | LDH (UI/L) | Urea (mM/L) | Uric acid (mM/L) | Creatinine (µM/L) | Albumin (g/dL) |
|---|---|---|---|---|---|---|---|---|---|
| Non diabetic Control | 5.97$^{d}$±0.51 | 33$^{c}$ ± 2.14 | 35$^{c}$±3.08 | 101$^{c}$±8.86 | 876$^{d}$±48 | 6.74$^{c}$±0.28 | 0.26$^{a}$±0.02 | 123$^{c}$±2.88 | 4.51$^{a}$±0.46 |
| Diabetic control | 13.81$^{a}$±2.3 | 67$^{a}$±3.24 | 74$^{a}$±5.22 | 183$^{a}$±10.63 | 1221$^{a}$±81 | 11.3$^{a}$±0.88 | 0.15$^{b}$±0.01 | 169$^{a}$±9.46 | 2.97$^{b}$±0.24 |
| Non diabetic+EVOO | 6.02$^{d}$±0.46 | 27$^{d}$±1.83 | 36$^{c}$±1.42 | 109$^{c}$±8.54 | 907$^{d}$±34 | 7.53$^{b}$±0.69 | 0.28$^{a}$±0.01 | 121$^{c}$±4.98 | 4.54$^{a}$±0.46 |
| Non diabetic+DAS-rich EVOO | 5.63$^{d}$±0.63 | 27$^{d}$±1.64 | 33$^{c}$±2.08 | 104$^{c}$±9.33 | 886$^{d}$±18 | 7.69$^{b}$±0.53 | 0.28$^{a}$±0.01 | 116$^{d}$±2.24 | 4.62$^{a}$±0.32 |
| Diabetic + EVOO | 7.54$^{b}$±0.77 | 41$^{b}$±3.18 | 51$^{b}$±3.28 | 123$^{b}$±5.82 | 983$^{b}$±27 | 8.13$^{b}$±0.62 | 0.26$^{a}$±0.01 | 132$^{b}$±4.92 | 4.16$^{a}$±0.38 |
| Diabetic + DAS-rich EVOO | 6.79$^{c}$ ± 0.27 | 37$^{b}$± 2.32 | 46$^{b}$±3.52 | 118$^{b}$±6.44 | 946$^{c}$±12 | 7.79$^{b}$±0.89 | 0.28$^{a}$±0.01 | 126$^{c}$±3.72 | 4.53$^{a}$±0.29 |

*AKP: alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDH: Lactate dehydrogenase; EVOO: Extra virgin olive oil; DAS: Diallyl sulfide. ** Values are mean ± SD of triplicate (n = 3); Means followed by different superscript letters within column are significantly different at $p<0.05$.

**Table 2.** Changes in lipid profile in control and experimental groups of mice.

| Treatment | TC (mM/L) | TG (mM/L) | HDL-c (mM/L) | LDL-c (mM/L) | TC/HDL-c | LDL-c/ HDL-c | AIP |
|---|---|---|---|---|---|---|---|
| Non diabetic control | 1.84$^{d}$±0.12 | 0.87$^{c}$±0.06 | 0.57$^{a}$±0.02 | 0.54$^{d}$±0.06 | 3.5$^{d}$±0.22 | 0.95$^{c}$±0.06 | 0.18$^{d}$±0.02 |
| Diabetic control | 2.38$^{a}$±0.15 | 1.59$^{a}$±0.18 | 0.41$^{b}$±0.03 | 0.88$^{a}$±0.04 | 8.81$^{a}$±0.64 | 2.13$^{a}$±0.12 | 0.59$^{a}$±0.04 |
| Non diabetic + EVOO | 1.82$^{d}$±0.08 | 0.77$^{c}$±0.08 | 0.54$^{a}$±0.05 | 0.59$^{c}$±0.02 | 3.45$^{d}$±0.42 | 1.15$^{d}$±0.04 | 0.17$^{e}$±0.01 |
| Non diabetic + DAS-rich EVOO | 1.68$^{d}$±0.11 | 0.69$^{d}$±0.04 | 0.57$^{a}$±0.02 | 0.54$^{d}$±0.02 | 2.9$^{e}$±0.33 | 1$^{e}$±0.02 | 0.08$^{f}$±0.01 |
| Diabetic + EVOO | 2.12$^{b}$±0.09 | 1.06$^{b}$±0.09 | 0.47$^{b}$±0.04 | 0.72$^{b}$±0.08 | 5.22$^{b}$±0.45 | 1.55$^{b}$±0.04 | 0.35$^{b}$±0.02 |
| Diabetic + DAS-rich EVOO | 2.02$^{c}$±0.18 | 0.95$^{b}$±0.11 | 0.49$^{b}$±0.05 | 0.70$^{b}$±0.04 | 4.47$^{c}$±0.39 | 1.42$^{c}$±0.08 | 0.29$^{c}$±0.02 |

*TC: Total cholesterol; TG: Triglycerides, HDL-c: Digh density lipoprotein chomesterol; LDL-c: Low densiry lipoprotein density; AIP: Atherogenic index of plasma (AIP =[Log TG/HDL-c]). **Values are mean ± SD of triplicate ( n = 3); Means followed by different superscript letters within column are significantly different at $p<0.05$.

1B). In parallel experiments, sulfhydryl groups (-SH) in protein cysteine residues were markedly declined (≈35%) in liver (0.82±0.04mM) and kidneys (0.56±0.03 mM) of diabetic mice in comparison of non-diabetic (1.26±0.05 and 0.88 in liver and kidney, respectively) mice (Fig. 1C).

In connection with oxidative stress, the activity of indigenous antioxidant enzymes was determined. As shown in Fig.2, the activity of the enzymes CAT, GPx and SOD were significantly ($p<0.05$) decreased in diabetic mice. In diabetic mice, the activity of CAT was decreased by 28 and 40% in liver and kidneys, respectively. For GPx, the magnitude of decline was found to be 45 and 48% in liver and kidney, respectively. Likewise, the activity of SOD dropped by 41 and 39% in liver and kidney, respectively. The decline of the activity of the antioxidant enzymes in both liver and kidneys of diabetic mice has exacerbated oxidative bursts in these animals. Regarding the renal GPx and SOD, the incorporation of DAS significantly improved their activity in DAS-rich EVOO treated group (7.9±0.34 nmol GSH/min/mg protein and 5.29±0.32 U/mg protein for GPx and SOD respectively) as compared with the EVOO treatment in diabetic group (6.7±0.82 nmol GSH/min/mg protein and 4.64±0.47 U/mg protein for GPx and SOD respectively). Such trends was however, not evident in hepatic tissues (8.3-8.7 nmol GSH/min/mg protein). In general, ingestion of EVOO and DAS-rich EVOO significantly ($p<0.05$) attenuated the oxidation-induced alterations of the oxidative status. At this point, it seems that the anti-diabetic activity of EVOO and DAS-rich EVOO was mediated through alleviating oxidative stress.

### 3.3. Histopathology

Examination of HE-stained sections of pancreas of control, EVOO-, and DAS-rich EVOO-treated animals showed normal appearance of the pale and well-shaped islet of Langerhans (Fig. 3 A-C). In the alloxan-diabetic group, the islet of Langerhans was irregularly outlined and showed degenerative changes with a decrease in the density of β-cells (Fig.3 D). Some cells are foamy with vacuolated cytoplasm.

Other cells showed nuclear shrinkage and pyknosis. Treatment with EVOO and DAS-rich EVOO significantly reduced these histologic alterations with better recovery being recorded for DAS-rich EVOO (Fig. 3 E, F). Histological examination of the liver samples from the untreated control, EVOO-, and DAS-rich EVOO-treated animals showed normal hepatic architecture and polyhedral hepatocytes with regular nucleus and cytoplasm (Fig. 3 G-I). In diabetic animals, noticeable pathological changes including distension of hepatocytes, extended vacuolization, and sinusoid widening were observed in liver (Fig. 3 J). EVOO and DAS-rich EVOO alleviated liver alterations in diabetic mice with the effect being more pronounced with DAS-rich EVOO (Fig. 3 K, L). Microscopic observation

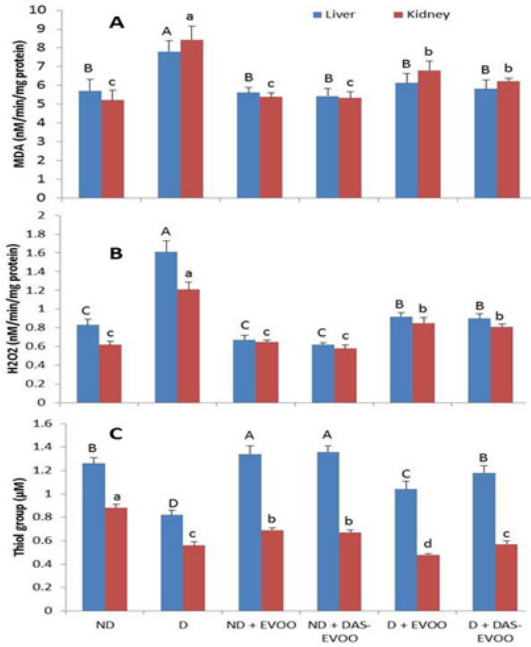


**Fig. 1.** Changes in oxidative stress markers A) MDA, B) $H_2O_2$ and C) thiol group (-SH) in different experimental groups of mice. ND: Non-diabetic control; D: diabetic control; ND + EVOO: Non-diabetic control receiving 500µL of EVOO daily; ND + DAS-EVOO: Non-diabetic control receiving 500µL of DAS-rich EVOO daily; D+EVOO: Diabetic mice receiving 500µL of EVOO daily; D+DAS-EVOO: Diabetic control receiving 500µL of DAS-rich EVOO daily. Data are expressed as mean ± SD of triplicate ($n$ = 3), Error bars marked with different capital (liver) and lowercase (kidneys) letters denote significant differences at $p<0.05$

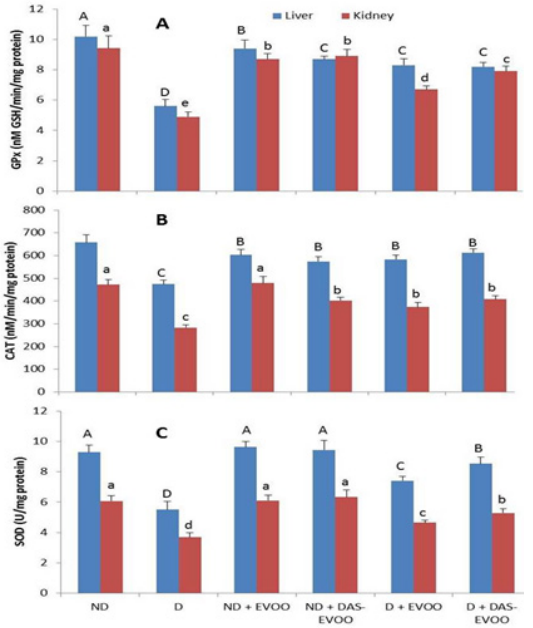


**Fig. 2.** Changes in the activity of antioxidant enzymes A) GPx, B) CAT and C) SOD in liver and kidneys of different experimental groups of mice. ND: Non-diabetic control; D: diabetic control; ND + EVOO: Non-diabetic control receiving 500µL of EVOO daily; ND + DAS-EVOO: Non-diabetic control receiving 500µL of DAS-rich EVOO daily; D+EVOO: Diabetic mice receiving 500µL of EVOO daily; D+DAS-EVOO: Diabetic control receiving 500µL of DAS-rich EVOO daily; GSH: reduced glutathione. Data are expressed as mean ± SD of triplicate (n = 3), Error bars marked with different capital (liver) and lowercase (kidneys) letters denote significant differences at $p<0.05$.

of kidney section of the untreated control, EVOO-treated and DAS-rich EVOO-treated control showed normal appearance of glomeruli which was regularly delimited by the Bowman's capsule. Proximal convoluted and distal convoluted tubules were well-defined (Fig. 3 M-O). However, the diabetic mice showed glomerular atrophy with consequent congestion, widening of Bowman's space; thickening of membrane basement and extended vacuolization of mesangial cells (Fig. 3 P). In EVOO and DAS-rich EVOO-treated diabetic mice, these alterations were not evident, and the renal architecture was nearly similar to the untreated control (Fig. 3 Q, R). From the histologic examination, it can be concluded that EVOO and DAS-rich EVOO have pancreatic, hepato-, and reno-protective effects in alloxan-induced diabetic animals. The histo-protective effect of EVOO was markedly improved by the incorporation of DAS.

## 4. Discussion

Diabetes mellitus (DM) and its associated complications hyperglycemia and hyperlipidemia is one of the most metabolic disorders worldwide. In diabetic patients, the deregulation of the metabolism of carbohydrates, lipids, and proteins as well as the increase in free radical production *versus* a drop of antioxidant defense is associated with increased risk of vascular, retina, nervous, and renal diseases [32]. For the management of DM, insulin and oral hypoglycemic agents including sulfonylureas (glipizide, glyburide, gliclazide, glimepiride), meglitinides (repaglinide and nateglinide), biguanides (metformin), thiazolidinediones (rosiglitazone, pioglitazone), and α-glucosidase inhibitors (acarbose, miglitol), among others are commonly used [33]. Unfortunately, these agents can cause many adverse effects including hepatotoxicity, nausea, diabetic macular edema, congestive heart failure, abdominal discomfort, mild anorexia, diarrhea, and flatulence [33]. The-

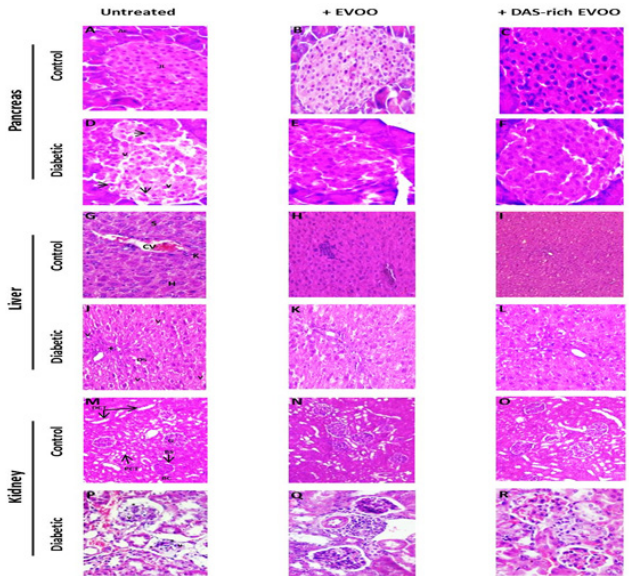


**Fig. 3.** Histopathological observation of pancreatic, hepatic and renal tissues from different experimental groups of mice. Ac: acini; IL: islet of Langerhans; CV: central vein; H: hepatocyte; K: Kupffer cell; S: sinusoid; DS: dilated sinusoid; G: glomerulus; BC: Bowman's capsule; BS: Bowman's space; DCT: distal convoluted tubules; PCT: Proximal convoluted tubules; V: vacuolization; Arrowheads in pancreas indicate β-cells; asterisk (*) in kidney indicates the membrane basement thickening.

refore, the use of bio-based drugs holds promise for the management of DM. Because of its manifest antioxidant, anti-inflammatory and hepatoprotective effects [13], we hypothesized that DAS-rich EVOO may provide a new potential candidate for the management of DM and its associated complications. To test this hypothesis, alloxan-induced diabetes is adopted as a well-established experimental model for the diabetes study. Consequently, the injection of a single diabetogenic dose of alloxan caused a significant increase of blood glucose and a substantial elevation in the activity of serum markers enzymes ALT, AST, alkaline phosphatase and LDH indicating liver injury. Likewise, renal dysfunction reflected in the increment of the level urea and creatinine was also noticed. The toxic effects of alloxan were further confirmed by histopathological observations showing damages in pancreas (reduction of β-cells number, and extended vacuolization in the islets of Langerhans, nuclear shrinkage and pycnosis of some pancreatic cells), in liver (hepatocytes distension and sinusoid broadening) and in kidney (congested and atrophied glomerules, enlarged Bowman's space and vacuolated mesangial cells).

Additional analyses showed that these pathophysiological manifestations were intimately linked with enhanced oxidative stress and insufficient antioxidant response. Indeed, a remarkable increase in MDA (a product of lipid peroxidation) and $H_2O_2$ (indicator of the intensity of oxidative stress and extended production of free radicals) was observed in hepatic and renal tissues of diabetic mice. Concomitantly, the increase in oxidative markers was accompanied by a general decrease of CAT, GPx and SOD indicating their incapacity to counteract the alloxan-induced generation of free radicals and $H_2O_2$. Accumulation of $H_2O_2$ and excessive generation of free radicals will lead to enhanced degradation of macromolecules including lipids and proteins as reflected in the elevated MDA content and the drop of (-SH) group causing serious histological alterations in pancreatic, hepatic, and renal tissues in diabetic animals. Alloxan-induced oxidation of (–SH) group has been described as the main mechanism behind the decrease of hexokinase enzyme (a key enzyme responsible for the insulin-dependent catalysis of glucose phosphorylation in pancreatic β-cells and liver) leading ultimately to β-cells destruction and loss of glucose homeostasis [34].

The ingestion of EVOO normalized all serum biochemical markers and alleviated alloxan-induced histopathologic alterations in pancreas, liver and kidney indicating its hypoglycemic and histoprotective effects. The hypoglycemic effect of EVOO might be mediated through the recovery or regeneration of pancreatic β-cells and/or their stimulation to produce insulin. The reduction of insulin resistance which leads to improved insulin-mediated suppression of the hepatic production of glucose, and/or enhancing insulin-mediated entry of glucose into muscle, adipocytes and liver tissues was suggested too [35]. A conformational modification of the insulin receptors was reported as the main mechanism behind the antidiabetic effect of oleic acid-rich EVOO [12].

The histoprotective effects of EVOO might be, in turn, associated with its antioxidant activity conferring thus a good protection of hepatocyte and renal tissues against free radicals produced by alloxan. The presence of putative antioxidants acting lonely or in combination could, at least in part, explain the antidiabetic and histoprotective effects of EVOO. Among the active components identified in EVOO, some of them namely phenolic compounds and vitamin E have received particular attention because of their well-recognized antioxidant and antidiabetic activity [8]. Basic research has found that tyrosol can effectively ameliorate streptozotocin-induced diabetes in rats by increasing insulin secretion from remnant β-cells, reducing hyperglycemia-induced production of free radicals, decreasing lipid peroxidation, and improving the activity of antioxidant enzymes [10]. The tyrosol-induced glucose homeostasis was mediated through an increased activity of the key metabolic enzyme's phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and glucose-6-phosphatase *versus* a reduction of the activity of hexokinase and glucose-6-phosphate dehydrogenase [36]. Similar observations have been described for cinnamic acid [37] and ferulic acid [38].

The antioxidant-based antidiabetic and histoprotective effects of hydroxytyrosol, oleuropein [39], apigenin [40], luteolin [41], pinoresinol [42], vannilic acid [43] and tocopherols [44] has also been confirmed using both *in vitro* and *in vivo* approaches. Common mechanisms of these compounds include the improvement of β-cell functionality, inhibition of lipid peroxidation, stimulation of the activity of antioxidant enzymes (CAT and SOD), reducing insulin resistance, increasing the glucose uptake by peripheral tissues, reducing inflammatory cytokine (tumor necrosis factor-α TNF-α and interleukins IL-1β/IL-6), increasing liver function and insulin signaling pathways, inhibition of hepatic gluconeogenesis, inhibition of α-amylase and α-glucosidase, and reducing protein glycation [45].

The ameliorative effects of EVOO were potentiated by incorporation of DAS presumably through its ability to enhance the antioxidant response and prevent oxidative stress-mediated histopathological damage. The chemoprotective effect of DAS and its metabolites diallyl sulfoxide (DASO) and diallyl sulfone ($DASO_2$) has already been reported [22]. The protective effect of these metabolites was mediated through a competitive inhibition of the cytochrome P450-dependant drug metabolizing enzyme CYP2E1 and its associated enzymes namely anilin-4-hydroxylase and *p*-nitrophenol hydroxylase which are involved in the elimination of xenobiotics like acetaminophen, $CCl_4$, alloxan, and streptozotocin [22].

Although the mechanism by which DAS-rich EVOO exerts its antidiabetic activity is unknown, most hypothesis point to i) increment of the activity of endogenous antioxidant enzymes CAT, SOD and GPx, ii) declination of lipid peroxidation, iii) protection of pancreatic, liver, and renal tissues from alloxan-induced free radical production, and vi) inhibition of CYP2E1-dependant drug metabolizing enzymes. Evidence supporting the antioxidant and histoprotective effects of DAS is given by Kalayarasan et al. [46] using gentamicin-induced nephrotoxicity in rats as experimental model. In that study, it has been found that DAS improved the antioxidant function through the activation of antioxidants enzymes CAT, SOD, GPx, and glutathione reductase (GR). It also inhibited lipid peroxidation and the expression of pro-inflammatory cytokines (*i.e.* iNO, NF-κB and TNF-α) conferring thus a better protection of renal tissues against gentamicin-induced nephrotoxicity [39]. In support of these findings, we have recently reported that the incorporation of DAS into EVOO greatly enhanced its antioxidant activity *in vitro* and effi-

ciently prevented the $CCl_4$-induced liver injuries in mice and reduced the production of inflammatory cytokines [13]. We have also postulated that the increased antioxidant and its subsequent anti-inflammatory effects of DAS-rich EVOO resulted from the synergistic action between DAS and the native EVOO bioactive components namely phenolic antioxidants, tocopherols, and pigments.

Dyslipidemia, which features elevated serum TG, TC and LDL-C *versus* a reduced HDL-C is the most important complication associated with alloxan-induced diabetes. These modifications of plasma lipid profile in diabetic animals are generally linked with coronary atherosclerosis, thus increasing risk of cardiovascular diseases [47]. In the present study, the rise of TG, TC, and LDL-c cholesterol levels indicates derangement of lipid metabolism. The decreased HDL-c level owing to its reduced production by intestine and liver, and/or its glycation by elevated glucose concentrations might exacerbate dyslipidemia and ultimately led to and increased incidence of cardiac dysfunction in diabetic mice [48]. To test this assumption, the atherogenic index of plasma (AIP), a reliable predictor of atherosclerosis and coronary heart disease, was calculated. The result indicates that AIP was 3.19-fold higher in alloxan-diabetic animals pointing that they are at high risk of atherosclerotic coronary heart disease. Alterations of the lipid profiles were successfully restored by ingestion of EVOO and DAS-rich EVOO being the most effective.

Despite that the lipid-lowering property of EVOO [46], phenol-enriched EVOO [17], garlic oil and its derived organosulfur compounds (diallyl disulphide (DADS) and diallyl trisulfide (DATS)) has been repeatedly described [49, 50], no data is available on the effect of DAS-rich EVOO on the lipid profile of diabetic animals. Considering the unique publication pointing to the incapacity of DAS to lower the lipid accumulation in 3T3-L1 adipocytes [51], it is plausible to hypothesize that the manifest amelioration of lipid profile in response to DAS-rich EVOO resulted from the synergistic effects of DAS with other EVOO components having a lowering lipid property. The hypolipidemic effect of some components present in EVOO has been reported in literature. In a previous study, Pal and colleagues [52] reported that the α-tocopherol significantly decreased the cholesterol content in human HepG2 hepatoma cells either by a competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR), a rate-limiting enzyme for cholesterol synthesis and the induction of LDL receptors. Using the same cellular model, Guo et al. [53] reported that apigenin dose-dependently inhibited adipogenesis following the activation of 5'-adenosine monophosphate-activated protein kinase (AMPK) thus ameliorating the lipid profile.

In other studies, the lipid-lowering activity of hydroxytyrosol, oleuropein and oleuropein aglycone [54] has been shown to be mediated through the inhibition of lipid peroxidation *in vivo*. Using the model of gentamycin-induced liver injury in rats, Babaeenezhad and colleagues [55] reported that cinnamic acid (50 mg/kg/day) significantly improved the lipid profile by amelioration of the level of TG, TC and VLDL in addition to its stimulatory effect on the activity of antioxidant enzymes CAT and GPx *versus* its inhibitory effects on lipid peroxidation and nitric acid production. In that study, the inhibition of pancreatic lipase, the up-regulation of lecithin-cholesterol acyltransferase (a key enzyme responsible for the esterification of cholesterol and the production of HDL), the increase of the activity of plasma lipoprotein lipase (LPL), and the activation of lipolytic genes have been proposed as underlying mechanisms of the lipid-lowering activity of cinnamic acid [56]. A concomitant up-regulation of hepatic carnitine palmitoyltransferase 1a (CPT1a) and peroxisome proliferator-activated receptor alpha (PPARα) has been described as the main mechanism underlying the modulatory effect of lipid metabolism of ferulic acid [57]. Recently, Sun et al. [58] have successfully deciphered the mechanism of the hypolipidemic effect of luteolin pointing to its capacity to enhance the activity of antioxidant enzymes (CAT, SOD and GSH-Px) and enzymes associated with lipid catabolism including FAβO (a key enzyme that promote the oxidation of fatty acids) and cholesterol 7α-hydroxylase (CYP7A1, a rate-limiting enzyme that converts cholesterol in non-hepatic tissues to bile acids) in addition to its inhibitory effects on HMG-CoAR, hepatic lipase (HL, enzyme responsible for the clearance of TG from VLDL pools) and diacylglycerol acyltransferase (DGAT, enzyme responsible for the synthesis of triacylglycerol through the catalytic binding of diacylglycerol and acyl fatty acids).

Even though our study does not provide a realistic appraisal of the mechanism of action of the hypolipidemic activity of DAS-rich EVOO, the hypothesis of a synergistic action between DAS and other bioactive components in EVOO makes sense. In support of this conclusion, we have previously reported that DAS synergistically improves the anti-inflammatory effect of EVOO in $CCl_4$-induced liver injuries in mice [13]. More recently, Asdaq et al. [50] showed that DADS-rich garlic oil was more effective than DADS alone in ameliorating dyslipidemia in rats fed high-fat diet. The recognized inhibitory effect of DAS on the activity of fatty acid synthase (FAS) [59] could represent an additional way to empower the activity of putative hypolipidemic components of EVOO. Consistent with these findings, a synergistic antioxidant action between DAS and lipophilic antioxidants like α-tocopherol and β-carotene has been reported [60].

Owing to its lipophilic aspect, antioxidant and preventing lipid peroxidation activity [12], DAS might potentially contribute to the decrease of membrane fluidity which hampers the diffusion and propagation of free radicals thus a good protection of cellular structures from oxidative injuries. The crucial role of the interaction between lipophilic antioxidants and lipid bilayers in stabilizing biological membranes and their components from oxidative damage has been extensively studied and reviewed [61]. In this context, it has been reported that the modification of biophysical properties of the lipid bilayer (decreased membrane fluidity) is one of the main mechanisms underlying the antimicrobial, antiplatelet and anti-proliferative effects of DAS [62-65].

Consequently, it is plausible to propose that DAS can effectively stabilize the lipid bilayer and abrogate lipid peroxidation and the propagation of free radicals which will cooperatively improve the antioxidant-mediated antidiabetic and anti-hyperlipidemic activities of the bioactive components of EVOO. The membrane-interacting properties of EVOO active ingredients (especially α-, γ-tocopherol, tyrosol, hydroxytyrosol, oleuropein, oleuropein aglycone, ligustrazine, cinnamic acids, ferulic acid, etc.) and their presumed synergistic effects with DAS deserves further atomic-level studies.

## 5. Conclusions

From the results of this study, it can be concluded that the incorporation of DAS into EVOO enhanced the natural antidiabetic and antihyperlipidemic effects of EVOO, and significantly recovered the adverse metabolic alterations in alloxan-induced diabetic mice. The synergistic effects between DAS and EVOO active compounds leading to the amelioration of serum biochemical parameters, histologic features and oxidative status in diabetic animals have been proposed as underlying mechanisms behind the potentiation of the antidiabetic, anti-hyperlipidemic and histoprotective effects of EVOO by the incorporation of DAS. These results suggest that both DAS and EVOO could be combined to potentiate their beneficial effects and effectively used in alleviating diabetes and its associated comorbidities.

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## Interest conflict

The authors declare no competing interests.

## Consent for publications

The author read and proved the final manuscript for publication.

## Availability of data and material

All data generated during this study are included in this published article.

## Authors' Contribution

All authors had equal role in study design, work, statistical analysis and manuscript writing.

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