

## **Antidiabetic Effect of Oleuropein from *Olea europaea* Leaf against Alloxan Induced Type 1 Diabetic in Rats**

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### **ABSTRACT**

*This study have been designed to study the effect of extracted pure oleuropein from Oleaeuropaealeaf against alloxan induced type 1 diabetic rats. Diabetic male rat was induced by injecting single subcutaneous injection of 100 mg/kg b.w of alloxan. Respectively, pure oleuropein compound (5, 10, 15 and 20 mg/kg.b.w) was orally administered once per a day during a period of 40 days of experiment. Then, the serum blood was collected for the determination of glucose level, haematological analysis, enzymatic and non-enzymatic antioxidant. Further, pancreatic tissue was evaluated for histological examination. Oleuropein showed a significant role in attenuating the blood glucose levels and elevation of in-vivo antioxidant after treating diabetic rats with 5 and 10 mg/kg. b.w. The haematological test did not show any significant differences. The histological sections of diabetic rats treated with 5mg/kg/ b.w of oleuropein showed regularity in size appearance of pancreatic islet with normal distribution of islet cells. Oleuropein as a natural active compound have antioxidant activity to attenuate the effect of alloxan against diabetic disease. Therefore, it can be recommended to use oleuropein as an additive food to cure type 1 diabetic.*

**Keywords:** Oleuropein, Type 1 Diabetic, Alloxan, Antioxidant, Blood glucose and HBA1C

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## INTRODUCTION

Diabetes mellitus type 1 (T1D), known as insulin-dependent diabetes or juvenile diabetes, is a metabolic disorder that results from the autoimmune destruction of  $\beta$ -cells, the insulin-producer in the pancreas, (1). The subsequent lack of insulin leads to increase blood and urine glucose. In the present study allolxan, 2,4,5,6-Tetraoxypyrimidine; 2,4,5,6-pyrimidinetetrone, have been used as diabetogenic substance. It is the most prominent diabetic chemical inducer in animal diabetic research (2). The mechanism of diabetic allolxan have been reported by Lenzen 2008 (3). Briefly, allolxan is affected on pancreatic beta cell via the glucose transporter GLUT2 that causes  $\beta$ -cell toxicity and death through reactive oxygen state, ROS. It will then a state of insulin-dependent diabetes mellitus (T1D) will appear.

Over many years, scientists interested to find out the effect of natural product particularly phenolic compound for their brilliant biological activity as

antioxidant (4) and anti-inflammatory (5) agents against variety of diseases including diabetes. Olive tree *Olea europaea* is documented as a high antioxidant plant and is considered to be a valuable component in healthy diet all over the world (6) this appreciated healthy diet could be through the composition of phenolic content (7). The main active secondary metabolite in olive leaf extract is oleuropein in Fig. 1 (8). Studies have reported various pharmacological benefits of oleuropein. It is used as anti-inflammatory agents (9), antioxidant (10), Anti-proliferative and apoptotic on human breast cancer MCF-7 cells (11) and anti-colorectal cancer (12).

In the past decade, various phenolic compounds have been used as immunomodulatory agents to suppress autoimmunity against T1D. So far, there is no effective therapeutic research of oleuropein against T1D. The present study aimed to assess oleuropein as ROS suppresser and  $\beta$  cell protector in allolxan-induced diabetic rats.

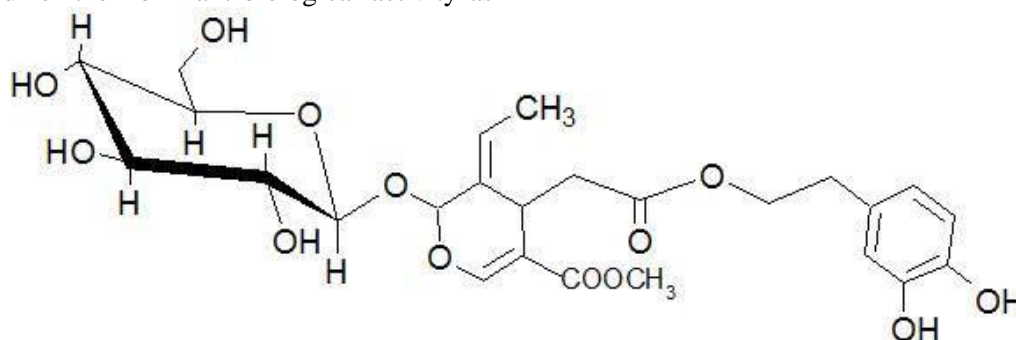


Figure 1: Chemical structure of oleuropein

## MATERIALS AND METHODS

### Experimental Animal

Adult male albino rats *Rattus norvegicus* were obtained from animal house of Biology Department, College of Education, University of Salahaddin. In this study, 48 healthy rats 200-250 g weight were used. Animals were received the humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals". The rats were kept at  $25 \pm 2^\circ\text{C}$  and 50–60% humidity, with 12 h light/dark cycle.

### Diabetic Induction

Allolxan monohydrate (BDH Chemical Ltd. England) was used to induce diabetes as described by Antathan (13). Briefly, animals were injected with single subcutaneous injection of freshly prepared 100 mg/kg body weight of allolxan in 0.1M citrate

buffer pH = 4.5. The control animals received citrate buffer only (Nimenibo-Vadia, 2003). Allolxan treated animals were allowed to drink 5% of D-Glucose (Merck KGG, a Darmstadt Germany) overnight to prevent the potentially fatal hypoglycaemia occurring as a result of massive insulin release following allolxan injection (Wohaeib and Godin, 1987). Diabetes mellitus was confirmed by testing blood glucose using indicator sticks (Accu-check Roche Diagnostics GmbH, Mannheim, Germany). After 3 days, the diabetes animals were detected based on loss of body weight, polyuria, glycosuria, polydipsia, polyphagia and blood glucose levels (14). Rats with blood Glucose level of  $\geq 300$  mg/dl were considered as diabetic and have been used in this study (15).

### Experimental Design

In this experiment, the total of 48 rats ( $n = 8$ ) were used and they were fed with standard diet and allowed to drink water *ad libitum*. The animals were grouped randomly into six groups as followings:

Group I-Normal control: rats of this group received no induction and treatment.

Group II-Diabetic control: diabetic rats of this group received no treatment

Group III- treated diabetic group: diabetic rats of this group received 5 mg/kg of body weight of pure oleuropein compound.

Group IV- treated diabetic group: diabetic rats of this group received 10 mg/kg of body weight of pure oleuropein compound.

Group V- treated diabetic group: diabetic rats of this group received 15 mg/kg of body weight of pure oleuropein compound.

Group VI- treated diabetic group: diabetic rats of this group received 20 mg/kg of body weight of pure oleuropein compound.

Following 40 days of treatment, prior the fasting the amount of blood glucose was recorded in all experimental groups. Next, all rats were fasted overnight and then the rats were all sacrificed by overdose of ketamine (100 mg/mL) and xylazine (100 mg/mL) in a ratio of 4:1 (v/v) intramuscularly. Blood samples were collected by cardiac puncture for serum biochemical and physiological analysis, the pancreas was taken and preserved in 10% buffered formalin for histopathological examination.

### Plant preparation

Oleuropein extracted from olive leaves (OL) using steam blanching process at optimum conditions. Briefly, the fresh OL were treated under optimum conditions (10 min steam blanching of 20–21 mm olive leaves fraction). Then, the resultant OL from steam blanched were dried and the amount of 10 g of dried steam blanched were grounded in 80 ml 70% (v/v) ethanol/water at 40 °C for 30 min. Afterward, the extract were stirred at 5000 rpm for 10 min and the pellets were re processed under the same conditions. The extract was then eluted via 0.45-lm syringe filters and chromatographed using reversed-phase HPLC DAD HITACHI equipment coupled to an autosampler L-2200, pump L-2130, column oven L-2300 and diode array detector L-2455 controlled by Agilent EZChrom Elite software. The column was a Pinnacle II RP C 18, 3  $\mu$ m, 150  $\times$  4.6 mm (Restek), protected by a Kromasil 100 – 5 C 18 guard cartridge starter kit for 3.0 / 4.6

mm id. Column oven was set at 40 °C. Eluent (A) and (B) were 0.02 M sodium acetate adjusted at pH 3.2 with acetic acid and pure acetonitrile, respectively. The flow rate was 1 ml/min. The elution gradient profile was as follows: started (A) 90%; 2 min, 85%; 9 min, 75%; 12 min, 65%; 15 min, 55%; 18 min, 40%; 20 min, 90%. The elution was monitored at 280 nm for oleuropein.

### Determination of blood glucose and glycosylated haemoglobin (HbA1c) level

Blood samples from all experimental rats were analysed for glucose level using glucometer (Accu-Chek, Roch diagnostic GmbH, Mannheim, Germany) (16). Further, Glycosylated haemoglobin was determined using HbA1c determination kit (Nycocard® HbA1c - Indea) (17). Briefly, the kit contains test devices with a porous membrane filter and test tubes prefilled with reagent and a washing solution. The reagent contains agents that lyse erythrocytes and precipitate haemoglobin specifically, as well as a blue boronic acid conjugate that binds cis-diols of glycosylated haemoglobin. When blood is added to the reagent, the erythrocytes immediately lyse and all haemoglobin will precipitate. The boronic acid conjugate binds cis-diol configuration of glycosylated haemoglobin. Then, aliquot of the reaction mixture is added to the test device and all the precipitated haemoglobin conjugate-bound and unbound remains on top of the filter. The precipitate was recorded by measuring the blue (glycosylated haemoglobin) and the red (total haemoglobin) color intensity by using Nycocard® READER II. The ratio between them is proportional to the percentage of HbA1c in the sample.

### Haematological analysis

Complete blood counts of all blood samples were carried out at Erbil Medical laboratory. The blood parameters were included total white blood cell, red blood cell, haemoglobin, platelet and packed cell volume. The coulter counter measurement has been used (SysmX K- 1000), TOA Medical Electronics Co., LTD. KOBEL. JAPAN (18).

### Determination of reduced glutathione (GSH)

Following the methods described by Al-Zamely *et al*, 2001, Ellman's reagent has been used for the determination of serum GSH. Briefly, equal volume of serum sample (150  $\mu$ L) was mixed with 4% sulfosalicylic acid and centrifuged at 2000 rpm for 5 min. Then 4.5 ml of Ellman's reagent (0.1189 g

of bis 2-nitrobenzoic acid. 5-5 dithio (DTNB) in 100 ml of buffer at pH 7.4 was added to 150  $\mu$ L of supernatant which has been obtained from centrifugation. The mixture allowed for 5 min at room temperature and then the absorbance was measured at 412 nm spectrophotometrically. The GSH concentration was calculated based on the following calculation:

GSH concentration( $\mu$ mol/L) =

$$\frac{A_{\text{test}} - A_{\text{blank}}}{L \times E_0} \times 10^6$$

E<sub>0</sub>: Extinction coefficient= 13600 M<sup>-1</sup> cm<sup>-1</sup> L = light bath 1cm

#### Determination of plasma vitamin E ( $\alpha$ -tocopherol)

Plasma vitamin E was measured depending on colorimetric method using Emmeric Engel reaction as described by(19). This method was applied based on the reduction by tocopherols of ferric to ferrous ions, which then form a red complex with  $\alpha, \alpha'$  - dipyridyl. Tocopherols and carotenes are then extracted into xylene and the extinction read to 460nm to measure the carotenes. A correction is made for these after adding ferric chloride and reading at 520nm. The concentration of vitamin E was estimated using the following equation:

Tocopherol (mg/L) =

$$\frac{A_{\text{test at 520 nm}} - A_{\text{test at 460 nm}} \times 0.29}{A_{\text{standard at 520 nm}}}$$

$\times$ conc. of standard (10 mg/L)

#### Histological examination of Pancreas

At the end of 40 days of experiment, all animals were dissected then thepancreatic tissues were washed and fixed in10% formalin fixative solution. Subsequently, the tissue were processed (Dehydrated, Cleaned and then Infiltrated) automatically using Automated Tissue Processing Leica (TP1020). Then, the tissues were embedded

in paraffin wax using Leica HISTOEMBEDDER. The embedded tissues were sectioned with microtome to produce 5  $\mu$ m paraffin wax tissue sections. The sections were stained with Haematoxylin & Eosin followed by mounting with DPX mounting media. Next, the mounted sections were evaluated for microscopic examination using light microscope (AmScoop microscope eyepiece camera. China) (20).

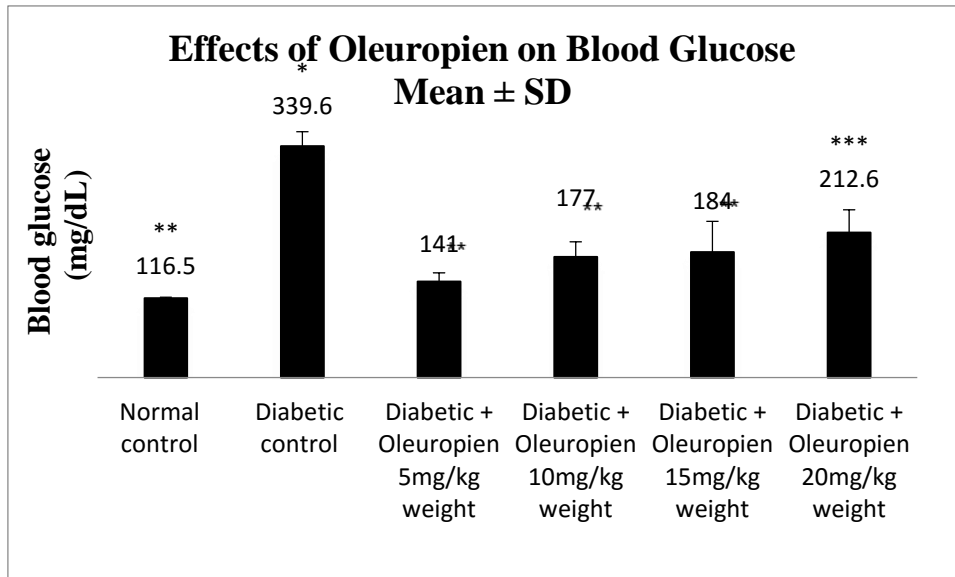
#### Statistical analysis

All the data were analyzed by Statistical Package Social Science (SPSS) version 20.0. Duncan post Hoc test was used to show the mean differences between all samples at  $p < 0.05$ . Microsoft Excel 2010 was used to illustrate the graphs.

## RESULTS

#### Estimation of serum glucose and glycosylated haemoglobin (HbA1c) level

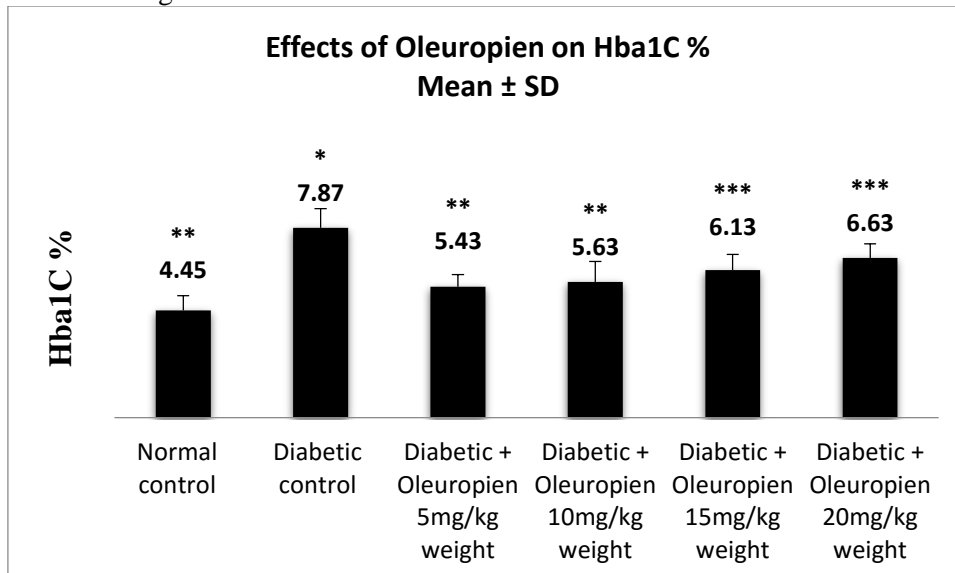
The results of this study illustrated the effects of oleuropein on serum glucose level in experimental animals treated for 40 days. As shown in Figure 1, the mean  $\pm$  SD of serum glucose levels of normal control rats is about 116.5 $\pm$ 1.603mg/dL while the mean  $\pm$  SD of serum glucose levels of diabetic controlrats is about 339.6  $\pm$  21.345 mg/dL. Rats treated with oleuropein significantly  $p < 0.05$  showed decreasing in the serum glucose levels. After treating diabetic rats with 5 mg/kg of oleuropein, the serum glucose levels was decreased about 141 $\pm$  12.751 mg/dLas in Figure 2. The significantly differences  $p < 0.05$  has been also illustrated after administration 10 mg/kg and 15 mg/kg of oleuropein (177  $\pm$  22.33 mg/dLand 184  $\pm$  45.118 mg/dL) respectively as compared with diabetic control rats as in Figure 2. Although, the mean  $\pm$  SD (212.6  $\pm$  33.553 mg/dL) of rats treated with 20 mg/kg of oleuropein did not show significantly differences  $p < 0.05$  with the serum glucose levels of diabetic control rats. It's shown from Figure 2 to be more close to the serum glucose levels of diabetic control rats.



**Figure 2:** Effects of oleuropein on serum glucose levels in experimental animals for 40 days. Data are presented as mean ± SD (n = 8). P <0.05 are represented as statistically significant different. \* p<0.05 significant differences as compared with normal control, \*\*p<0.05 significant differences as compared with diabetic control and \*\*\*p<0.05 significant differences as compared with normal control and diabetic control.

Further confirmation has been detected regarding the glucose level by using HbA1c determination kit. The percentage of serum Hba1C of all experimental animals has been illustrated in Figure 3. The mean ± SD of serum Hba1c % of normal control rats is about 4.45 ± 0.61% while the mean ± SD of serum Hba1c % of diabetic control rats is about 7.87 ± 0.8 %.

Rats treated with oleuropein significantly p<0.05 showed decreasing in the serum Hba1c%. Same as in the previous test, the diabetic rats treated with 5 mg/kg and 10 mg/kg of oleuropein are caused decreasing in the serum Hba1c% about 5.43 ± 0.51% and 5.63 ± 0.85% respectively in comparison with diabetic control rats. While diabetic rats treated with 15 mg/kg and 20 mg/kg oleuropein were not show any significantly differences p<0.05 with the serum Hba1c% of diabetic control rats.



**Figure3:** Effects of oleuropein on serum Hba1C% in experimental animals for 40 days. Data are presented as mean ± SD (n = 8). P<0.05 are represented as statistical significant different. \* P<0.05 significant differences as compared with normal control, \*\*p<0.05 significant differences as compared with diabetic control and \*\*\*p<0.05 significant differences as compared with normal control and diabetic control.

### Determination of haematological analysis

The plasma haematological data as in Table 1, including RBCs, Hb, PCV and PLTs, of all experimental rats did not show any statistical

differences  $p < 0.05$  between all the groups in the present work. That means neither alloxan nor oleuropein did not affect in this experiment.

**Table 1:** Effects of oleuropein on hematological analysis including (RBCs, Hb, PCV and PLTs) in plasma of all experimental rats. Data are presented as mean  $\pm$  SD (n = 8). P < 0.05 are represented as significant.

Animal groups	RBCs ( $\times 10^6/\mu\text{L}$ )	Hb (g/dl)	PCV %	PLTs ( $\times 10^3/\mu\text{L}$ )
Normal control	6.75 $\pm$ 0.15	12.82 $\pm$ 0.23	40.35 $\pm$ 0.90	456.13 $\pm$ 22.55
Diabetic control	6.69 $\pm$ 0.12	11.83 $\pm$ 0.25	36.42 $\pm$ 1.23	471.28 $\pm$ 23.57
Diabetic + Oleuropein 5mg/kg BW	7.40 $\pm$ 0.03	14.34 $\pm$ 0.21	45.16 $\pm$ 0.43	469.71 $\pm$ 22.09
Diabetic + Oleuropein 10mg/kg BW	7.44 $\pm$ 0.14	14.02 $\pm$ 0.18	44.25 $\pm$ 0.67	434.00 $\pm$ 13.19
Diabetic + Oleuropein 15mg/kg BW	7.19 $\pm$ 0.04	12.84 $\pm$ 0.23	40.51 $\pm$ 0.75	469.14 $\pm$ 26.56
Diabetic + Oleuropein 20mg/kg BW	7.18 $\pm$ 0.05	12.14 $\pm$ 0.26	40.02 $\pm$ 0.64	460.12 $\pm$ 27.92

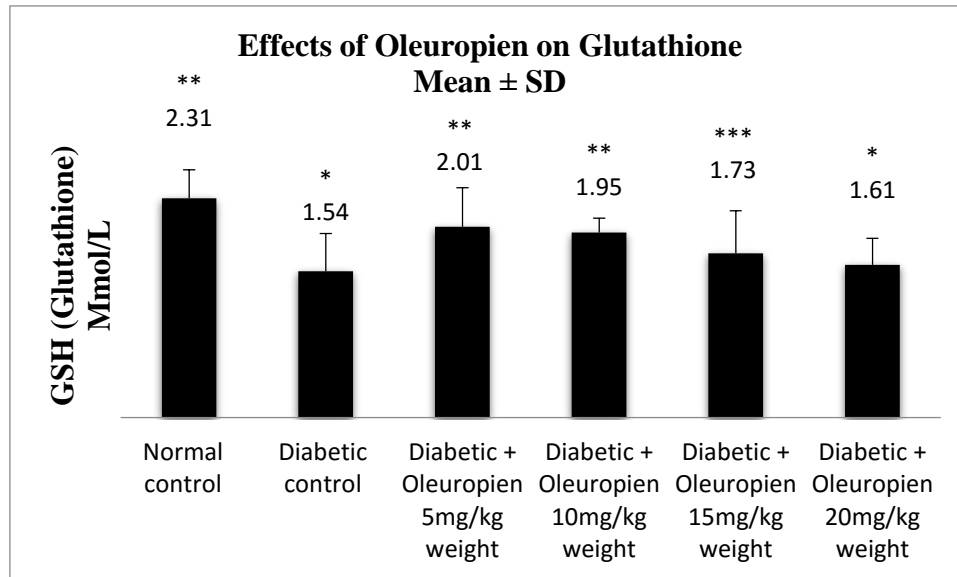
RBCs: red blood cells, Hb: hemoglobin, PCV: Packed cell volume, PLTs: Platelets Count

### Determination of reduced glutathione (GSH)

Reduced glutathione have been charted in Figure 4. The data presented that mean  $\pm$  SD of serum GSH in normal control is about 2.31  $\pm$  0.3 mmol/L while mean  $\pm$  SD of serum GSH in diabetic control rat is about 1.54  $\pm$  0.4. Rats treated with 5 and 10 mg/kg of oleuropein significantly  $p < 0.05$  showed elevation of serum GSH about 2.01  $\pm$  0.41 and 1.95  $\pm$  0.15 respectively in comparison with serum GSH in diabetic control. Rats treated with 15 mg/kg of oleuropein did not show any significant differences  $p < 0.05$  with normal control and diabetic

control rat. While, rats treated with 20 mg/kg of oleuropein did not show any statistical significant  $p < 0.05$  elevation in serum GSH.

Both normal rats and oleuropein-treated diabetic with 5 and 10 mg/kg showed a constant augmentation in the serum GSH levels and serum glucose levels. Further, the negative correlation coefficient ( $R^2 = -0.83$ ) have been observed between serum GSH and serum glucose. Meaning that increasing in the serum glucose is lowering the antioxidant ability and vice versa.

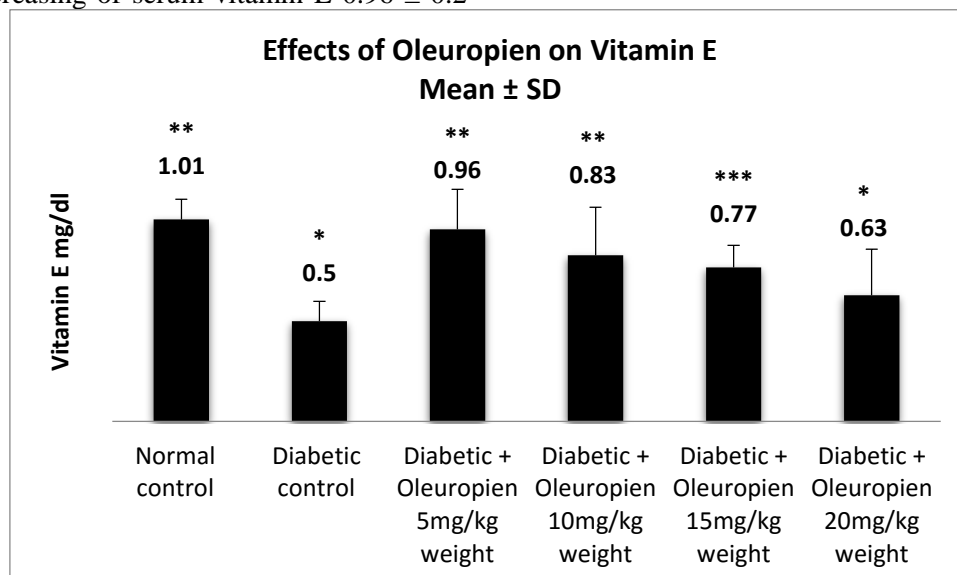


**Figure 4:** Effects of oleuropein on serum GSH (Glutathione) Mmol/L in experimental animals for 40 days. Data are presented as mean  $\pm$  SD (n = 8).  $p < 0.05$  are represented as statistical significant different. \*  $p < 0.05$  significant differences as compared with normal control, \*\* $p < 0.05$  significant differences as compared with diabetic control and \*\*\* $p < 0.05$  significant differences as compared with normal control and diabetic control.

#### Determination of serum vitamin E ( $\alpha$ -tocopherol)

Serum vitamin E mg/dl has been illustrated in Figure 5. It has shown that mean  $\pm$  SD of normal control rats is about  $1.01 \pm 0.1$  mg/dl while the mean  $\pm$  SD of untreated diabetic rats (diabetic control rats) is about  $0.5 \pm 0.1$  mg/dl. In comparison with diabetic control rats, diabetic rats treated with 5 and 10 mg/kg of oleuropein significantly  $p < 0.05$  showed increasing of serum vitamin E  $0.96 \pm 0.2$

and  $0.83 \pm 0.24$  mg/dl respectively. Although, diabetic rats treated with 15 mg/kg of oleuropein did not show significantly differences  $p < 0.05$  with normal control and diabetic control rats. The diabetic rats treated with 20 mg/kg of oleuropein did not show any elevation of serum vitamin E  $0.63 \pm 0.23$  mg/dl and the data is not significantly differences  $p < 0.05$  with diabetic control rats.



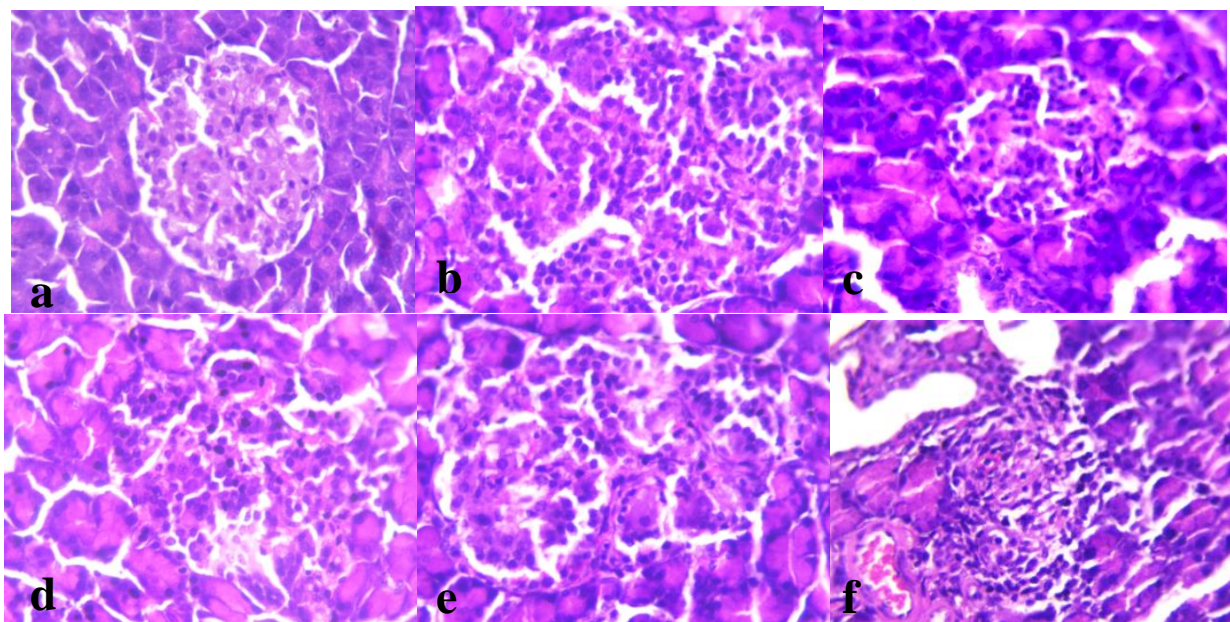
**Figure5:** Effects of oleuropein on serum vitamin E mg/dl in experimental animals for 40 days. Data are presented as mean  $\pm$  SD (n = 8).  $P < 0.05$  are represented as statistical significant different. \*  $p < 0.05$  significant differences as compared with normal control, \*\* $p < 0.05$  significant differences as compared with diabetic control and \*\*\* $p < 0.05$  significant differences as compared with normal control and diabetic control.



### Histological Examination of Pancreas

The pancreas tissue from normal group rats, after receiving standard diet with distilled water, were showed normal pattern of pancreatic islet with normal distribution of islet cells as shown in Figure 6a. Pancreas with diabetic control, after injecting 100 mg/kg bw showed expansion and dilatation of pancreatic islet with irregular distribution of islet cells as in Figure 6b. Diabetic rats treated with 5mg/kg bw of oleuropein showed regularity appearance of islet with normal distribution of islet cells Figure 6c. Treating diabetic rats with 10 mg/kg bw of oleuropein showed mild expansion of

pancreatic islet with less haemorrhaged associated with accumulation of zymogene granule in acini Figure 6d. Expansion of pancreatic islet with prominent hyperplastic islet cells have been seen after treating diabetic rats with 15 mg/kg bw of oleuropein in Figure 6e. Diabetic rats treated with 20 mg/kg bw of oleuropein showed irregular pattern and prominent hyperplastic of islet cells associated with lymphatic infiltration and appearance of vacuoles (H&E 400X).



**Figure 6:** Photomicrograph of normal architecture, regular in shape and size of pancreatic islet cells (a); alloxan diabetic rats with 100 mg/kg bw (b); treated diabetic rats with 5 mg/kg bw of oleuropein (c); 10 mg/kg bw of oleuropein treated diabetic rat (d); diabetic rats treated with 15 mg/kg bw of oleuropein (e) and diabetic rats treated with 20 mg/kg bw of oleuropein (f) (H&E 400X).

### DISCUSSION

Type one diabetic is one of the diseases that correlated with oxidative stress positively. Variety disorders have been reported based on the formation of free radicals in T1D such as nephropathy, retinopathy, nephropathy and vascular disorders (13). Further, hyperglycemia manifested to be appeared with the elevation of free radicals in the body (20). In another hand, dietary and supplementary antioxidant are able to decrease the complications accompanied with oxidative stress in T1D (21).

In the present work, alloxan have been used to induce diabetic rats and oleuropein to eliminate the

complications associated with diabetic disorder. Obviously, elevation of glucose levels have been observed in diabetic control rats in comparison with normal control rats in this experiment. This is in agreement with the previous study reported by Khaled Hamden 2009 (22) and (3). The mechanism behind this elevation explained to be through the inhibition of insulin secretion and damage to  $\beta$  cells (23). While after treating diabetic rats with oleuropein particularly 5 mg/kg and 10 mg/kg, the decreasing in the serum glucose levels have been determined.

Further, the negative correlation has been also detected between serum glucose levels with that in serum enzymatic and non-enzymatic antioxidant.



Previously, oleuropein have been reported as a good antioxidant *in vitro* through chelating of Cu and Fe metallic ions which then catalyse free radical formation(24)(25)and *in vivo* through the inhibition of enzymatic oxidation such as lipoxygenases(10). In agreement with the present experiment, a significant increasing in the serum GSH has shown after treating diabetic rats with 5mg/kg and 10mg/kg of oleuropein. Previous experiment has been observed that GSH capable to prevent the diabetic when it has injected together with alloxan in rats (3). That means GSH is able to decrease the generation of free radicals, formed from alloxan, in the body and then positively protecting the  $\beta$  cell from any destruction(3).

Moreover, non-enzymatic antioxidant elevation also has been observed in the present study after treating diabetic rats with 5mg/kg and 10mg/kg of oleuropein. Vitamin E or  $\alpha$ -tocopherol as an essential antioxidant has been proved to be depressed when glucose level is increased in the body(26). The same results been detected in this study, it has been shown a negative correlation between serum glucose level and serum vitamin E levels of diabetic rats treated with oleuropein. Meaning that oleuropein is able to decrease glucose level and increasing the antioxidant capability. However, oleuropein have also been assessed for haematological analysis there is no any significant changes been detected in this study as presented in Table 1.

Further confirmation has been shown through pancreatic micrographs regarding the protection role of oleuropein as being presented in Figure 6. The damage and irregular distribution of  $\beta$  cells has been observed in diabetic control. While normal appearance and regeneration of  $\beta$  cells been observed in diabetic rats treated with 5mg/kg and 10 mg/kg bw of oleuropein. This protection could be related to the elimination of pancreatic toxicity through the inhibition of free radical activities. As in the previous paragraph, it has been explained that oleuropein is able to scavenge oxidative stress of alloxan diabetic induction. Study revealed that regeneration of  $\beta$  cells and reactivation of insulin secretion after treating diabetic rats with phenolic compound could be due to the possibility that some islet  $\beta$  cells are able to be reactivated by phenolic compound to labour its insulin secretion(27).

## 5. CONCLUSIONS

Eventually, this study proved that oleuropein is a significant hypoglycemic and antioxidant active

compound. This significant role is could be through the antioxidant activity of oleuropein by attenuating free radical effect of alloxan. As well as, the reactivating of  $\beta$  cell and raising the enzymatic and non-enzymatic antioxidant. So that, further study is needed to confirm that oleuropein is a good natural active compound for T1D therapy upon human.

## ACKNOWLEDGMENTS

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## CONFLICT OF INTEREST

No conflict in this work.

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