International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2018.963.972

Research Article
Biological Activity of Olive Leaf Extract and Regulation of Tissue Transglutaminase Expression in Diabetic Wound Healing

[1]Gadah Al-basher and [2]Fatimah Al-otibi

[1]Department of Zoology, College of Science, King Saud University, 11451 Riyadh, Saudi Arabia
[2]Department of Microbiology, College of Science, King Saud University, 11451 Riyadh, Saudi Arabia

Abstract

Background and Objective: Impaired wound healing was seriously associated with diabetes. More complications such as microbial infections, delay in fibrogenesis is process and collagen deposition were shown to be linked with hyperglycemia. In this study, an aqueous extract of olive leaf was analyzed for antioxidant activity, wound healing property and then its effect on the activity and expression of tTG was tested *in vivo* by applying on the wounds of rats with streptozotocin (STZ)-induced diabetes. **Methodology:** Fifty healthy male wistar rats were treated intravenously with STZ (55 mg kg^{-1} body weight) to induce diabetes. To evaluate wound healing activity of olive leaf extracts, animals with diabetic wounds were treated topically twice daily with ointments of olive extracts at doses of 2 and 5% (w/w). Wound closer, epithelialization, hydroxyproline (HPX), tissue transglutaminase (tTG) and total antioxidant capacity (TAC) were estimated as parameters of wound healing capacity of olive extracts in diabetic treated and non-treated rats. Also, *in vitro* phytochemical screening analyses were performed to estimate active constituents present in OLE. **Results:** In diabetic group, wound healing time was found to be (18.8±0.61) and (16.8±1.3) in OLE wound treated rats at a doses of 2 and 5% compared to non-treated (27.9±0.96) and standard drug (21.5±0.6) respectively. Wound contraction, scar formation and epithelialization processes positively correlated with the increase in the levels of HPX, tTG as markers of collagen deposition and TAC activity and negatively with diabetes (HbA1c)in treated wound tissues. Due to antioxidant and anti-diabetic activity of OLE constituents, particularly oleuropein, collagen deposition and accelerated epithelialization processes were estimated in diabetic wound healing. **Conclusion:** Finally, the data showed that OLE as ointment in doses of 2 and 5% efficiently accelerates wound healing process via promoting antioxidant capacity, expression of both HPX and tTG, which are essential for collagen deposition and re-epithelialization process. These findings suggested that methanolic extract olive leaf enriched with of oleuropein could be a suitable therapeutic agent for diabetic wound healing.

Key words: Diabetes, olive leaf extract, oleuropein, diabetic wounds, streptozotocin (STZ)

Received: January 25, 2018 Accepted: June 28, 2018 Published: September 15, 2018

Citation: Gadah Al-basher and Fatimah Al-otibi, 2018. Biological activity of olive leaf extract and regulation of tissue transglutaminase expression in diabetic wound healing. Int. J. Pharmacol., 14: 963-972.

Corresponding Author: Gadah Al-basher, Department of Zoology, College of Science, King Saud University, 11451 Riyadh, Saudi Arabia
Tel: +966503488325

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus is a global health crisis, persistently affecting the humanity[1]. Significant rise in number of populations who affected from diabetes mellitus (DM) was reported with increasing of its prevalence and complications with high mortality and morbidity rates during short times[2,3].

On metabolic level, people with hyperglycemia were exposed to long-term damage, dysfunction and even failure of many tissues and organs such as eyes, kidneys, nerves, heart and blood vessels[4].

Failure of wound healing therapeutic strategies was one of the most serious complications reported in patients with diabetes. Whereas, more wound healing problems appear such as microbial infection and gangrenes which leading even to amputations of diabetic foot were significantly associated with hyperglycemia[5,6]. The increase in blood sugar results in microvascular complications, neuropathy, microangiopathy and prevents cell reproduction, collagen generation, hydroxyproline production and decrease in wound tensile strength which subsequently leading to a delay in wound healing[7,8].

In addition, during tissue injury cells synthesize and release many molecules at the wound site, transglutaminase activity was shown to increase during wound healing in rats[9-11]. In addition, a significant increase in both activity and expressions of Ttg was observed by day 3 post-wounding process in endothelial cells, macrophages and skeletal muscle cells of rats[12]. Thus, tTG as rapid stabilizing matrix component released following tissue damage could be a reliable biomarker for diabetic and non-diabetic wound healing process[13,14].

For the management of diabetes and its related complications such as wound healing, herbal treatments are quite acceptable therapeutic strategies due to its anti-diabetic and limited adverse effects in comparison with the conventional anti-diabetic drugs[15-18]. Also, herbal plants were shown to enhance fibrogenesis, wound closer, by increasing of wound tensile strength, tissue remodeling and production of hydroxyproline[19-21].

Olive is one of the previously recommended herbal plants as a source used in wound treatment and diabetes[22-24]. This may be related to antioxidant, antimicrobial, blood pressure-lowering, hypocholesterolemic, anti-diabetic and anti-inflammatory effects of its phenolic components, particularly oleuropein which constitutes the main phenolic component in olive leaf followed by hydroxytyrosol, oleuropein aglycone and tyrosol[21-34].

It is hypothesized that olive leaf extracts enriched with oleuropein as active constituents will be possible to regulate the activity and the expression of tissue transglutaminase (tTG), hydroxyproline and may aid an easily accessible wound care product for the treatment of diabetic wounds. Thus, in this study, an aqueous extract of olive leaf was analyzed for antioxidant activity, wound healing property and then, its effect on the activity and expression of tTG was tested *in vivo* by applying on the wounds of rats with streptozotocin (STZ)-induced diabetes.

MATERIALS AND METHODS

This study was conducted within four months from August, 2016 to November, 2016 at research labs, Zoology Department, college of science, King Saud University, Riyadh, Saudi Arabia. Animals were obtained from the authorized experimental animal care center, college of science, King Saud University, Riyadh, Saudi Arabia. The experiment and the procedures were approved according to Ethics Committee of the Experimental Animal Care Society at King Saud University (Permit Number: PT 1204).

Materials: Olive leaves were purchased from the local spice shop (Othaim Markets) in Riyadh, Saudi Arabia. STZ and all reagents used for the determination of oxidative indices were purchased from Sigma chemicals (St Louis, Mo, USA). Other reagents of analytical grade were purchased from normal commercial sources.

Extract preparation: A total of 100 gram of olive leaves was ground with a mortar and pestle under liquid nitrogen and then 10 mL of 80% methanol (80% MeOH) solvent were added as previously reported[35]. The mixture was allowed to stand in the dark for 24 h. The extract was centrifuged at 5000 g for 10 min, at room temperature and the supernatants were then filtered using a filter paper and further squeezed to discharge the remaining solutes. Then, the extract was concentrated by a rotary evaporator, dried and mixed with pure Vaseline (100 g) to make a Vaseline-based 2% (5/100 g Vaseline) and 0.5% (10/100 g Vaseline) ointment[36-37]. All extraction procedures run under dim light to avoid effect of light on olive active constituents. For comparing the wound healing potential of olive leaf extract, povidone iodine ointment (5%; w/w) used as a standard wound healing drug[38].

Phytochemical screening of olive leaf extract: Various phytochemical screening tests were estimated by using standard procedures for the identification of the phyto constituents present in methanolic extract of olive leaves (OLE)[38].

Assessment of both total polyphenolic and flavonoid contents: UV-spectrophotometric analysis using diluted Folin-Ciocalteu (0.5/3 mL DW) and aluminum chloride ($AlCl_3$; 0.5 mL/2% ETOH ethanol) as reagents were recommended to determine both total polyphenols (TPC) and flavonoid contents (TFC) respectively in olive leaf methanolic extract[39,40]. The absorbance of the reaction mixtures produced was measured at 650 nm for polyphenols and 420 nm for flavonoids respectively. All measurements were carried out in triplicate; standard calibrated curves of gallic acid and quercetin were used to estimate poly phenolic and flavonoids compounds in olive leaf samples. According to the linear equation calculated from calibration curve of the standard gallic acid (determination coefficient; 2 = 0.9971) and quercetin (determination coefficient 2 = 0.9964) graphs, total poly phenolic contents were expressed as gallic acid Equivalents/100 mg and flavonoid content as quercetin mg of quercetin equivalents (QE)/g of dry olive leaf extract (OLE). Oleuropein as the main active constituent was estimated in olive leaves extract by using HPLC method. Oleuropein content was estimated in 25% (w/w) of the OLE product and was kept in the dark at -20°C until studied[21,41].

Animals and study design: Fifty healthy male wistar rats, weighing between 150 and 200 g were included in this study. They housed in polyethylene cages in groups of 10 rats per cage and kept at a constant temperature (22±2°C), humidity (55%) and 12 h light-dark conditions. The animals were provided with diet and free access to drinking water. Animals with no history of surgery, infection and other medical interventions, randomly assigned to five groups of 10 each.

Acute toxicity test: Toxicity studies were conducted as per internationally accepted protocol drawn under OECD guidelines in Wistar albino rats at a dose level of extracts up to 2000 mg kg^{-1} b.wt. In this study, the toxic effect of the methanolic extracts of olive leaves studied at a dose levels up of 2 and 5% mg kg^{-1} b.wt., in 10 rats. During the period of the experiment, animals examined for any signs of intoxication, lethargy, behavioral modification and morbidity[42,43].

Induction of diabetes: Following overnight fast diabetes induced in each rat as previously reported[44]. Rats injected intravenously with a diluted streptozotocin (STZ) (55 mg kg^{-1} body weight) in 0.05 M citrate buffer. Three days following injection, rats presented HbA1c ($\geq$6.5) and blood glucose level more than 250 mg dL^{-1} (13.9 mmol L^{-1}) were considered as diabetic.

Induction of wound experimentally: Fifty rats were anaesthetized with a combination of ketamine hydrochloride (50 mg kg^{-1}) and xylazine hydrochloride (10 mg kg^{-1}) of body weight as previously reported[45]. After controlling anesthetic depth with chin and skeletal muscle tone, their dorsum shaved and was disinfected with povidone-iodine. Then, a full thickness excision wounds with a circular area of 314 mm^2 were created along the marking on shaved dorsal region according to previously described method[46-47].

Post wounding treatments: Post wounding, the rats were randomly classified into five groups of 10 animals and subjected to treatment with both olive leaf extract and standard wound healing agent as follows; group I (diabetic untreated), group II (Vaseline treated), group III (5% betadine-povidone iodine cream), groups IV and V which were treated with 2 and 5% (w/w) ointments of olive leaf extract, respectively. All the treatments were applied topically twice daily from the day of the operation until the complete healing. Following wound formation, open wounds were measured regularly for contraction and epithelialization processes by caliper every 3 days. At the end of the study periods, tissue samples were collected and stored until reused for biochemical and wound healing analysis.

Wound area assessment: The rate of wound contraction rate and complete epithelialization time were estimated as main parameters of wound-healing rate in treated and non-treated rats. Wound closer was measured on days 0, 3rd, 7th, 10th, 15th and 21st by caliper for all groups. The percentage of wound contraction was calculated according to by the following Eq.[48]:

$$\text{Contraction (xth day \%)} = 100\text{-}\frac{\text{Total wound area on xth day}}{\text{Total wound area on day 0}}\times 100$$

Epithelialization period was calculated as the number of days required for falling off the dead tissue remnants of the wound without any residual raw wound[49,50].

Assessment of total antioxidant capacity (TAC) in tissue wounds: Spectrophotometer analysis with the aid of colorimetric assay kit (BioVision Incorporated, CA, USA) was used to estimate the concentrations of total antioxidant capacity (TAC) in tissues from excised wounds. The results were determined at 570 nm and calculated as a function of Trolox concentration as follows:

$$\frac{Sa}{Sv} = \frac{\text{nmol}}{\mu\text{L or mM trolox equivalent}}$$

Where:

Sa = Sample amount (in nmol) which has been read from the standard curve

Sv = Undiluted sample volume added to the wells

Assessment of hydroxyproline (HPX) and transglutaminases (tTG) in tissue wounds: Hydroxyproline content was estimated in tissues from excised wounds of all rats. Following draying to constant weight in a hot air oven at 60°C, tissues were hydrolyzed in 6 N HCl for 4 h at 130°C. The hydrolysate produced was subjected to Chloramine-T oxidation at pH 7.0 for 20 min and then after 5 min, 0.4 M perchloric acid was added to block the reaction. In each sample, the developed color due to reaction with Ehrlich reagent at 60°C was analyzed at 557 nm in ultraviolet (Systronics-2203) spectrophotometer. A standard curve of the pure L-hydroxyproline was used to calculate the hydroxyproline content in the tissue samples[51]. Transglutaminases (tTG) content was determined in excised tissues according to colorimetric assay kit (Catalog # K571-100, biovision Co, Milpitas Blvd., Milpitas, CA 95035, USA). A total of 150-300 µL cold homogenization buffer containing protease inhibitors cocktail were used to homogenize tissues on ice. The tissue homogenate centrifuged at 16,000 X g for 20 min at 4°C and the clarified supernatant was transferred to a fresh pre-chilled tube and keep on ice until reused. By adding stop solution, a colored hydroxamate product was estimated colorimetrically at 525 nm as measure of tTG activity in tissues of excised wounds. Activity of tTG was calculated from standard curve generated for the resulted hydroxamate product as shown from the following Eq:

$$\text{Sample's transglutaminase activity} = \frac{Bx1.5/T}{mg} = \frac{nmol/T}{mg}$$

Where:

B = Hydroxamate amount from Standard Curve (nmol)

1.5 = Nmoles of hydroxamate product generated in 150 µL reaction volume

T = Incubation time in min

Mg = Amount of protein/reaction in mg

Statistical analysis: The data were statistically analyzed using statistical software, GraphPad, InStat 3. The results are expressed as Mean±standard deviation. One-way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons were used to study the significance of studied variables. The data were considered significant at $p<0.05$ levels.

RESULTS

Phytochemical screening of olive leaf extract (OLE): The methanolic OLE showed 12.8% w/w of yield. Based on phytochemical screening analysis, flavonoids, glycosides, triterpenoids, carbohydrates, steroids, saponins, polyphenols and proteins were estimated in OLE (Table 1). Considerable amounts of phenolic contents {252.6±13.5 mg of gallic acid equivalents (GAE)/g} and flavonoid {529±25.6 mg of quercetin equivalents (QE)/g} contents were estimated in methnolic OLE extract. Oleuropein as a major phenolic active constituent comprises 25% w/w (112.5±2.8) of the total OLE (Table 1).

Effect of OLE on wound contraction, scar formation and epithelialization period: A preliminary treatment effect was observed during treatment with OLE extract from day 3 up to day 21 as shown in Fig. 1. The enhancement reported on day 21 of OLE extract at doses of 2 and 5% favor the potential curative effect of tested olive extract (Table 2). In diabetic rats tested with extract at doses of 2 and 5%, showed a maximum significant effect by increasing in the rate of wound closer, scar formation without any raw wound residues compared to non-treated diabetic control ($p<0.001$), ointment base treated ($p<0.001$) and standard groups ($p<0.01$) that proportionally

Table 1: Phytoconstituents screening, percentage yield and quantitative phytochemical contents of olive leaf extract (OLE mg/100 mg)

Item	OLE mg/100 mg
yield (%)	12.80
Phytochemical screening (+/-)	
Alkaloids	-
Flavonoids	+
Tannins	-
Glycosides	+
Triterpenoids	+
Carbohydrates	+
Steroids	+
Saponins	+
Polyphenols	+
Proteins	+
Phytochemical constituents (Mean±SD)	
Total polyphenolic content 1	252.6±13.5
Total flavonoid content 2	529.0±25.6
Oleuropein % (µmol g^{-1})	25% (112.5±2.8)

(+/-) presence or absence of phytoconstituents, phytochemical constituents represent as Mean±SD (n = 5). [1]Expressed as mg of gallic acid equivalents (GAE)/g of the dry extract. [2]Expressed as mg of quercetin equivalents (QE)/g of the dry extract

Fig. 1: Photographs represent the percentage of wound closer rates on different post excision days (3rd-21st)
Group I: Diabetic untreated rats, group II; diabetic rats treated with vaseline, Group III: Standard control rats treated with betadine 5% w/w povidone iodine cream, Group IV: Diabetic rats treated with vaseline plus 2% OLE and Group V: Diabetic rats treated with vaseline plus 5% OLE

Table 2: Effect of olive leaf extract (OLE) on % wound contraction and epithelialization period of wounds in excision wound model

	Wound contraction (%)						Epithelialization period
Group	3rd day	7th day	10th day	15th day	21st day	Scar area (mm^2)	(days)
I	125.2±1.2	122.4±0.8 (22%)	85.1±1.3 (41%)	55.3±0.9 (62.5%)	38.9±1.5(71%)	92.2±2.6	27.9±0.96
II	154.1±0.67[a]	148.0±2.1[a] (22.6%)	115.0±1.8[a] (45.2%)	57.3±1.3 (69.1%)[c]	32.6±1.2 (74%)[c]	96.8±3.2[c]	26.3±0.35[b]
III	158.9±0.45	142.2±1.2(38.9%)[a]	98.1±1.5(59%)[b]	78.2±0.6 (73.5%)[c]	29.1±0.6 (87%)[c]	99.1±1.3[c]	21.5±0.6[b]
IV	165.3±3.1	112.0±1.1 (40.9%)[a]	76.1±0.75 (72%)[b]	48.3±0.6 (84.2%)[c]	26.3±0.3 (90%)[c]	99.6±2.8[c]	18.8±0.61[c]
V	172.2±1.2	98.0±1.7(56.7%)	59.4±2.1 (81.5%)[b]	29.3±1.2 (91.2%)[c]	20.7±0.9 (97.8%)[c]	99.8±2.9[c]	16.8±1.3[c]

[a]p<0.05, [b]p<0.01, [c]p<0.001. All values are represented as Mean±SD, n = 10 animals in each group. Data were analyzed by one-way ANOVA, followed by Tukey-Kramer Multiple Comparisons Test. Group I: Un treated diabetic rats, Group II: Diabetic rats treated with vaseline, Group III: Standard diabetic rats treated with betadine 5% w/w povidone iodine cream, Group IV: Diabetic rats treated with vaseline plus 2% olive leaf extract and Group V: Diabetic rats treated with vaseline plus 5% olive leaf extract

confer healing process. In addition, OLE extract showed a relation with wound epithelialization. At doses of 2 and 5%, there was a contribution role in accelerating epithelialization rate with complete epithelialization process in short period of time ($p<0.01$) as compared with control and the ointment base treated group (Table 2, Fig. 2).

Effect of OLE on HPX, tTG and TAC content in tissue wounds: Tissue samples from diabetic wound treated with 2 and 5% doses of OLE and betadine 5% w/w povidone iodine cream as standard drug showed significant increase ($p<0.01$) in the levels of HPX and tTG as biomarkers related to the formation of collagen fibrils around the wound area. In addition, TAC was shown to be elevated in wound tissues following OLE treatment in doses of 2 and 5% compared to control diabetic non-treated rats and standard control treated diabetic wounds. Both HPX and tTG significantly increased in OLE treated group animals in regular dependent manner in comparison with to control and ointment base treated group (Table 3). Regarding to collagen stability or wound strength, diabetic wounds treated with standard 5% povidone iodine OLE extract 2 and 5% showed higher collagen stability or wound strength compared to those of ointment base treated and non-treated diabetic control respectively (Table 2). Correlation analysis showed that in excised wound healing, wound closer, scar formation and epithelialization of wound tissues correlated positively ($p = 0.001$) with HPX, tTG, TAC and negatively ($p = 0.001$) with diabetes (HbA1c) (Table 4).

Table 3: Effect of olive leaf extract (OLE) on dry granulation tissue, protein, hydroxyproline and tissue transglutaminase (tTG) in excision wound model

			Fibrogenesis markers		
Group	Dry weight of tissues (mg)	Protein (mg/g dry tissue)	Hydroxyproline (µg/100 mg tissues)	tTG (nmol/mg)	TAC (nmol)
I	265.6±11.5	46.2±2.3	145.1±11.4	51.1±2.3	8.9±3.1
II	281.5±13.7[c]	49.2±4.1[c]	148.2±2.1[c]	51.4±2.4[c]	9.5±2.8[c]
III	436.6±18.6[a]	52.9±2.6[a]	198.6± 14.2[c]	78.6±4.7[c]	16.4±3.8[c]
IV	518.2±10.4[b]	69.5± 2.8[b]	215.0±19.1[c]	96.1±8.1[c]	18.9± 5.2[c]
V	396.3±9.4	78.8±3.7[c]	258.6±7.3[c]	118.1±6.3[c]	26.9±6.1[c]

Values are Mean±SEM of 10 rats in each group. [a]p<0.05, [b]p<0.01 and [c]p<0.001 compared to respective diabetic and standard groups. Statistical analysis was done by one-way analysis of variance followed by Tukey-Kramer Multiple Comparisons Test. Group I: Un treated diabetic rats, Group II: Diabetic rats treated with vaseline, Group III: Standard diabetic rats treated with betadine 5% w/w povidone iodine cream, Group IV: Diabetic rats treated with vaseline plus 2% olive leaf extract and Group V: Diabetic rats treated with vaseline plus 5% olive leaf extract

Table 4: Correlation between the rates of wound closer, scar formation, epithelialization period, hydroxyproline and tissue transglutaminase (tTG) in diabetic and non-diabetic rats treated with olive leaf extract (OLE)

Item	Wound closer (% contraction)	Scar formation (mm^2)	Epithelialization period (days)
Hydroxyproline (µg g^{-1} of tissues)	0.215[b]	0.425[b]	0.135[b]
tTG (nmol/T/mg)	0.68[b]	0.85[b]	0.89[b]
TAC (nmol)	0.412[b]	0.360[b]	0.235[b]
Diabetes (HbA1c)	-0.36[b]	-0.41[b]	-0.51[b]

Data presented as coefficient (R), [a]Significance at <0.01, [b]Significance at <0.001

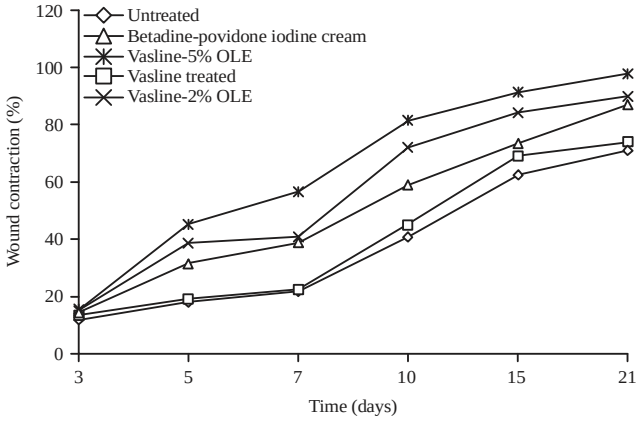

Fig. 2: Rate of wound closure on each day in treated and non treated diabetic rats following olive leaf extract (OLE; 2, 5%) compared to standards and vaseline treated rats

DISCUSSION

This study showed that ointments of olive leaf extracts at doses of 2 and 5% significantly improve wound healing process in rats with diabetes. Wound closer, epithelialization process and expression of HPX and tTG as biomarkers of collagen deposition were significantly increased in rats treated with OLE at doses of 2 and 5%, respectively. These results may be related to antioxidant, anti-diabetic and fibrogentic expression activity of flavonoids and other polyphenols constituents of OLE. Previous research reports showed that the activity of herbal plants as non-drug remedy depend mainly up on the biological activities of flavonoids and other polyphenols constituents that contribute in modulation of many biological process especially *in vivo* oxidative balances, inflammatory as well as damage of cells and tissues[52-53].

In this study, phytochemical screening of methanolic OLE showed the presence of flavonoids, glycosides, triterpenoids, carbohydrates, steroids, saponins, polyphenols and proteins were estimated in OLE. These metabolites originally used by plants for protection against herbivores. In addition, it may provide with some pharmacological activity when tested on animals. Supporting to currently obtained results, the health promoting compounds extracted from olive (*Oleaeuropaea L.*) leaves were applied previously as potential anti-cancer[54], anti-inflammatory[55], anti-microbal[56], antioxidants[57-58], anti-diabetic[27] or wound healing agents for centuries to prevent and treat many diseases[59]. In addition, Oleuropein as a main active product constitutes up to 25% of OLE total product followed by hydroxytyrosol, oleuropein aglycone and tyrosol as measured by HPLC analysis[42,43]. These products were previously reported to occur in olive leaf[60,61] and were responsible for many biological activities of OLE[29-35].

In this current study, in incisional diabetic wounds, topical application of OLE extract at doses of 2 and 5% showed significant improvement in wound healing parameters particularly; wound closure, scar formation and epithelialization process. The data showed that OLE ointment produces wound closure, scar formation without residual and complete epithelialization of wounded tissues in relatively short times compared to standard control povidone iodine and Vaseline treated diabetic groups. Due to previously mentioned biological activities of olive plant[24-30], topical

application of OLE treats microvascular complications produced because of hyperglycemia. The release of blood glucose leads to many complications such as neuropathy, microangiopathy, prevents hydroxyproline production and subsequently a reduction in collagen reproduction by cells which finally leads to a decrease in wound tensile strength and healing process[7,8], these all complications were restored upon application of OLE in wound healing. Olive leaves was shown to have many beneficial effects on human health[35,62-65] and that OLE has been reported to possess wound-healing properties in diabetic patients[34].

Impaired wound healing was shown to be most serious problem linked with diabetes mellitus and microbial infection which finally leads even to amputations of diabetic foot[5,6]. In many studies, Olive in different plant forms was shown to be as an important source for many active constituents used in treating diabetes and wound healing[24-30].

It is of significance that treating of diabetic wounds with OLE accelerates the healing process in short periods. In this study, diabetic wounds treated with OLE at doses of 2 and 5% applied topically recovered faster starting from the 3rd day on wards in comparison with diabetic wounds treated with vaseline and povidone iodine as standard control. In previous studies, speed up of wound healing process starting from 12th day[39] onwards when 5% embelin was topically applied and also in excised diabetic wounds , wound contraction and healing starts in 4th and 7th days onwards[41], when a plant based formula was used. Similarly, it was reported that diabetic wounds could be treated faster when aloe vera leaves extracted was applied to diabetic wounds compared to diabetic controls[42]. Consistent to these currently obtained results, data from other study showed that diabetic wound healing starts at 3rd day on wards when OLE was applied as addressing material containing 24 g of oleuropein[62]. In addition, it concluded that treating of diabetic and non-diabetic wounds by OLE has been determined to be more effective in comparison with classic wound healing treatments.

To explore more mechanisms of OLE in wound healing process, hydroxyproline (HPX) and tissue transglutaminase (tTG) as markers of collagen deposition and total antioxidant capacity (TAC) as measure of OLE antioxidant property were estimated in tissues from excised diabetic wounds following treating with OLE. In this study, there was significant increase in the levels of HPX, tTG and TAC was reported in diabetic wounds treated with OLE in doses of 2 and 5% respectively in comparison with diabetic wounds treated with Vaseline based and standard controls. The potential antidiabetic activity of OLE especially oleuropein significantly increases the production of hydroxyproline as well as formation of collagen fibrils which finally enhance wound closure, tensile strength and healing process in a shorter times compared to non-treated diabetic wounds[7,8]. In addition, tTG appears to play a significant role in several physiological processes such as wound healing. It was proposed as a means of stabilizing collagen extracellular matrix (ECM) scaffolds for tissue engineering applications in vascular diseases via increment in spreading, contractile response as indicated by elevated F-actin polymerization and myosin light chain phosphorylation. Also, it was suggested that tTG increases matrix mechanical stiffness, which apparently alters the contractile and proliferative response during wound healing and increase tensile strength and healing process[66]. In addition, a significant increase in both activity and expressions of tTGwas observed by day 3 post-wounding process in endothelial cells, macrophages and skeletal muscle cells in all stages of dermal wound healing of rats[12].

In this study, consistent to other studies, administration of Olive oil was shown to improve cutaneous wound healing of pressure ulcers in mice through the acceleration of the ROS and NO synthesis which significantly reduces oxidative damage via free radical scavenging activity of olive phenolic compounds and consequently leading to a reduction in inflammation status and promotes both dermal reconstruction and wound closure of treated wounds[67]. In addition, *in vivo* and *in vitro* experimental studies showed that uses of olive oil for wound treatments significantly improves wound contraction, re-epithelialization, hydroxyproline levels and blood vessel density and over all cutaneous wound healing in chronically stressed mice[68-69].

It was concluded also that *in vivo* administration of olive oil provides antioxidant activity against free radical of lipid peroxidation, which produces oxidative cell damage and increase fibroblast migration and collagen deposition around wounds[68].

The data of this study was confirmed with others who reported that, Oleuropein was shown to increase deposition of collagen fiber, reduced cell infiltration in the wound site and provides more advanced re-epithelialization ($p<0.05$) in the experimental group as compared to the control group on days 3 and 7 post incision[70].

Finally, in diabetic wounds treated with OLE at doses of 2 and 5%, wound closure, scar formation and epithelialization of wound tissues correlated positively ($p = 0.001$) with increased levels of HPX, tTG, TAC and negatively ($p = 0.001$) with diabetes (HbA1c).

Although oleuropein was reported as skin wound healing agent in previous rat models[31,70], this study confirmed that

besides antioxidant capacity of olive leaf extract enriched with oleuropein, the data showed that OLE has fibro genetic expression activity of both HPX and tTG which could be a suitable therapeutic agent for diabetic wound healing. The significance of this study is that treating of diabetic and non-diabetic wounds by OLE has been determined to be more effective in comparison with classic wound healing treatments and expression of tTG during healing process which signifies the importance of OLE in promoting healing process via fibrogenesis and apoptotic process.

CONCLUSION

The data showed that OLE as ointment in doses of 2 and 5% efficiently accelerates wound healing process via promoting antioxidant capacity, expression of both HPX and tTG which essentials for collagen deposition and re-epithelialization process. These findings suggest for the first that methanolic extract of oleuropein-rich olive leaf could be a suitable option as a therapeutic agent for diabetic wound healing. In addition, this study signifies the importance of OLE in promoting healing process via fibrogenesis and apoptotic process.

SIGNIFICANT STATEMENT

Although oleuropein was reported as skin wound healing agent in previous rat models, this study confirmed that besides antioxidant capacity of olive leaf extract enriched with oleuropein, our data showed that OLE has fibro genetic expression activity of both HPX and tTG which could be a suitable therapeutic agent for diabetic wound healing. In addition, it concluded that treating of diabetic and non-diabetic wounds by OLE has been determined to be more effective in comparison with classic wound healing treatments.

ACKNOWLEDGMENT

This research project was supported by a grant from the research center of the female scientific and medical colleges, Deanship of Scientific Research (DSR-KSU-36), King Saud University.

REFERENCES

1. Colagiuri, R., 2010. Diabetes: A pandemic, a development issue or both? Expert Rev. Cardiovasc. Ther., 8: 305-309.
2. Kamtchouing, P., S.M. Kahpui, D.P.D. Dzeufiet, L. Tedong, E.A. Asongalem and T. Dimo, 2006. Anti-diabetic activity of methanol/methylene chloride stem bark extracts of *Terminalia superba* and *Canarium schweinfurthii* on streptozotocin-induced diabetic rats. J. Ethnopharmacol., 104: 306-309.
3. Wild, S., G. Roglic, A. Green, R. Sicree and H. King, 2004. Global prevalence of diabetes: Estimates for the year 2000 and projections for 2030. Diabetes Care, 27: 1047-1053.
4. American Diabetes Association, 2010. Diagnosis and classification of diabetes mellitus. Diabetes Care, 33: S62-S69.
5. Falanga, V., 2005. Wound healing and its impairment in the diabetic foot. Lancet, 366: 1736-1743.
6. Sugimoto, T., L. Huang, T. Minematsu, Y. Yamamoto and M. Asada *et al*., 2013. Impaired aquaporin 3 expression in reepithelialization of cutaneous wound healing in the diabetic rat. Biol. Res. Nurs., 15: 347-355.
7. Altay, P. and G. Basal, 2010. Wound dressings. Electron. J. Text. Technol., 4: 109-121.
8. Blakytny, R. and E. Jude, 2006. The molecular biology of chronic wounds and delayed healing in diabetes. Diabetic Med., 23: 594-608.
9. Davis, G.E., K.J. Bayless, M.J. Davis and G.A. Meininger, 2000. Regulation of tissue injury responses by the exposure of matricryptic sites within extracellular matrix molecules. Am. J. Pathol., 156: 1489-1498.
10. Bowness, J.M., A.H. Tarr and T. Wong, 1988. Increased transglutaminase activity during skin wound healing in rats. Biochim. Biophys. Acta (BBA)-Gen. Subj., 967: 234-240.
11. Upchurch, H.F., E. Conway, M.K. Patterson and M.D. Maxwell, 1991. Localization of cellular transglutaminase on the extracellular matrix after wounding: Characteristics of the matrix bound enzyme. J. Cell. Physiol., 149: 375-382.
12. Haroon, Z.A., J.M. Hettasch, T.S. Lai, M.W. Dewhirst and C.S. Greenberg, 1999. Tissue transglutaminase is expressed, active and directly involved in rat dermal wound healing and angiogenesis. FASEB J., 13: 1787-1795.
13. Gross, S.R., Z. Balklava and M. Griffin, 2003. Importance of tissue transglutaminase in repair of extracellular matrices and cell death of dermal fibroblasts after exposure to a solarium ultraviolet A source. J. Investig. Dermatol., 121: 412-423.
14. Nicholas, B., P. Smethurst, E. Verderio, R. Jones and M. Griffin, 2003. Cross-linking of cellular proteins by tissue transglutaminase during necrotic cell death: A mechanism for maintaining tissue integrity. Biochem. J., 371: 413-422.
15. Kanner, J., S. Gorelik, S. Roman and R. Kohen, 2012. Protection by polyphenols of postprandial human plasma and low-density lipoprotein modification: The stomach as a bioreactor. J. Agric. Food Chem., 60: 8790-8796.
16. Modak, M., P. Dixit, J. Londhe, S. Ghaskadbi and T.P.A. Devasagayam, 2007. Indian herbs and herbal drugs used for the treatment of diabetes. J. Clin. Biochem. Nutr., 40: 163-173.

17. Ali, H., P.J. Houghton and A. Soumyanath, 2006. α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. J. Ethnopharmacol., 107: 449-455.
18. Marles, R. and N.R. Farnsworth, 1994. Plants as Sources of Antidiabetic Agents. In: Economic and Medicinal Plant Research, Wagner, H., H. Hikino and N.R. Farnsworth (Eds.). Academic Press Ltd., United Kingdom, pp: 149-187.
19. Bailey, C.J. and C. Day, 1989. Traditional plant medicines as treatments for diabetes. Diabetes Care, 12: 553-564.
20. Hui, H., G. Tang and V.L.W. Go, 2009. Hypoglycemic herbs and their action mechanisms. Chin. Med., Vol. 4, No. 1. 10.1186/1749-8546-4-11.
21. El, S.N. and S. Karakaya, 2009. Olive tree (*Olea europaea*) leaves: Potential beneficial effects on human health. Nutr. Rev., 67: 632-638.
22. Bagheri, S., H. Ahmadvand, A. Khosrowbeygi, F. Ghazanfari, N. Jafari, H. Nazem and R.H. Hosseini, 2013. Antioxidant properties and inhibitory effects of *Satureja khozestanica* essential oil on LDL oxidation induced-$CuSO_4$ *in vitro*. Asian Pac. J. Trop. Biomed., 3: 22-27.
23. Lin, Y. and Z. Sun, 2010. Current views on type 2 diabetes. J. Endocrinol., 204: 1-11.
24. Wiwanitkit, V., 2011. Thai ethnopharmacological herbs for diabetes treatment: Data collection and informatics tracing for therapeutic property. Diabetes Metab. Syndrome: Clin. Res. Rev., 5: 103-104.
25. Alzweiri, M., A. Al Sarhan, K. Mansi, M. Hudaib and T. Aburjai, 2011. Ethnopharmacological survey of medicinal herbs in Jordan, the Northern Badia region. J. Ethnopharmacol., 137: 27-35.
26. Cakilcioglu, U., S. Khatun, I. Turkoglu and S. Hayta, 2011. Ethnopharmacological survey of medicinal plants in Maden (Elazig-Turkey). J. Ethnopharmacol., 137: 469-486.
27. Boudjelal, A., C. Henchiri, M. Sari, D. Sarri, N. Hendel, A. Benkhaled and G. Ruberto, 2013. Herbalists and wild medicinal plants in M'Sila (North Algeria): An Ethnopharmacology survey. J. Ethnopharmacol., 148: 395-402.
28. Sargın, S.A., E. Akcicek and S. Selvi, 2013. An ethnobotanical study of medicinal plants used by the local people of Alasehir (Manisa) in Turkey. J. Ethnopharmacol., 150: 860-874.
29. Polat, R. and F. Satil, 2012. An ethnobotanical survey of medicinal plants in Edremit Gulf (Balikesir-Turkey). J. Ethnopharmacol., 139: 626-641.
30. Saric-Kundalic, B., C. Dobes, V. Klatte-Asselmeyer and J. Saukel, 2010. Ethnobotanical study on medicinal use of wild and cultivated plants in middle, South and West Bosnia and Herzegovina. J. Ethnopharmacol., 131: 33-55.
31. Koca, U., I. Suntar, E.K. Akkol, D. Yilmazer and M. Alper, 2011. Wound repair potential of *Olea europaea* L. leaf extracts revealed by *in vivo* experimental models and comparative evaluation of the extracts' antioxidant activity. J. Med. Food, 14: 140-146.
32. Visioli, F., A. Poli and C. Gall, 2002. Antioxidant and other biological activities of phenols from olives and olive oil. Med. Res. Rev., 22: 65-75.
33. Perrinjaquet-Moccetti, T., A. Busjahn, C. Schmidlin, A. Schmidt, B. Bradl and C. Aydogan, 2008. Food supplementation with an olive (*Olea europaea* L.) leaf extract reduces blood pressure in borderline hypertensive monozygotic twins. Phytother. Res., 22: 1239-1242.
34. Hamdi, H.K. and R. Castellon, 2005. Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor. Biochem. Biophys. Res. Commun., 334: 769-778.
35. Abaza, L., N.B. Youssef, H. Manai, M.F. Haddada, K. Methenni and M. Zarrouk, 2011. Chetoui olive leaf extracts: Influence of the solvent type on phenolics and antioxidant activities. Grasas Aceites, 62: 96-104.
36. Neyestani, T., 2007. The inhibitory effects of black and green teas (*Camellia sinensis*) on growth of pathogenic *Escherichia coli in vitro*. Iran. J. Nutr. Sci. Food Technol., 1: 33-38.
37. Hsu, S., 2005. Green tea and the skin. J. Am. Acad. Dermatol., 52: 1049-1059.
38. Bhaskar, A. and V. Nithya, 2012. Evaluation of the wound-healing activity of *Hibiscus rosa sinensis* L. (Malvaceae) in Wistar albino rats. Indian J. Pharmacol., 44: 694-698.
39. Khandelwal, K.R., 2005. Practical Pharmacognosy Techniques and Experiments. 22nd Edn., Nirali Prakashan, Pune, India.
40. Sadasivam, S. and A. Manickam, 1996. Biochemical Methods. 2nd Edn., New Age International (P) Ltd. Publishers, New Delhi, India.
41. Ordonez, A.A.L., J.D. Gomez, M.A. Vattuone and M.I. Lsla, 2006. Antioxidant activities of *Sechium edule* (Jacq.) Swartz extracts. Food Chem., 97: 452-458.
42. Tsarbopoulos, A., E. Gikas, N. Papadopoulos, N. Aligiannis and A. Kafatos, 2003. Simultaneous determination of oleuropein and its metabolites in plasma by high-performance liquid chromatography. J. Chromatogr. B, 785: 157-164.
43. Ghosh, M.N., 1984. Fundamentals of Experimental Pharmacology. 2nd Edn., Scientific Book Agency, Calcutta, India, pp: 178-210.
44. Gonzalez, M., A. Zarzuelo, M.J. Gamez, M.P. Utrilla, J. Jimenez and I. Osuna, 1992. Hypoglycemic activity of olive leaf. Planta Medica, 58: 513-515.
45. Davidson, E.P., L.J. Coppey, B. Dake and M.A. Yorek, 2011. Treatment of streptozotocin-induced diabetic rats with alogliptin: Effect on vascular and neural complications. Exp. Diabetes Res., Vol. 2011. 10.1155/2011/810469.

46. Udegbunam, S.O., R.I. Udegbunam, C.C. Muogbo, M.U. Anyanwu and C.O. Nwaehujor, 2014. Wound healing and antibacterial properties of methanolic extract of *Pupalia lappacea* Juss in rats. BMC Complementary Alternat. Med., Vol. 14. 10.1186/1472-6882-14-157.
47. Morton, J.J. and M.H. Malone, 1972. Evaluation of vulneray activity by an open wound procedure in rats. Arch. Int. Pharmacodyn. Ther., 196: 117-126.
48. Mukherjee, P.K., R. Verpoorte and B. Suresh, 2000. Evaluation of *in-vivo* wound healing activity of *Hypericum patulum* (Family: Hypericaceae) leaf extract on different wound model in rats. J. Ethnopharmacol., 70: 315-321.
49. Boateng, J.S., K.H. Matthews, H.N.E. Stevens and G.M. Eccleston, 2008. Wound healing dressings and drug delivery systems: A review. J. Pharm. Sci., 97: 2892-2923.
50. Manjunatha, B.K., S.M. Vidya, K.V. Rashmi, K.L. Mankani, H.J. Shilpa and S.D.J. Singh, 2005. Evaluation of wound-healing potency of *Vernonia arborea* Hk. Indian J. Pharmacol., 37: 223-226.
51. Woessner, Jr. J.F., 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch. Biochem. Biophys., 93: 440-447.
52. Tan, S.P., S.E. Parks, C.E. Stathopoulos and P.D. Roach, 2014. Extraction of flavonoids from bitter melon. Food Nutr. Sci., 5: 458-465.
53. Thaipong, K., U. Boonprakob, K. Crosby, L. Cisneros-Zevallos and D.H. Byrne, 2006. Comparison of ABTS, DPPH, FRAP and ORAC assays for estimating antioxidant activity from guava fruit extracts. J. Food Compos. Anal., 19: 669-675.
54. Goulas, V., V. Exarchou, A.N. Troganis, E. Psomiadou, T. Fotsis, E. Briasoulis and I.P. Gerothanassis, 2009. Phytochemicals in olive leaf extracts and their antiproliferative activity against cancer and endothelial cells. Mol. Nutr. Food Res., 53: 600-608.
55. Fabiani, R., A. de Bartolomeo, P. Rosignoli, M. Servili, G.F. Montedoro and G. Morozzi, 2002. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. Eur. J. Cancer Prev., 11: 351-358.
56. Beauchamp, G.K., R.S.J. Keast, D. Morel, J. Lin and J. Pika *et al*., 2005. Phytochemistry: Ibuprofen-like activity in extra-virgin olive oil. Nature, 437: 45-46.
57. Bisignano, G., A. Tomaino, R.L. Cascio, G. Crisafi, N. Uccella and A. Saija, 1999. On the *in-vitro* antimicrobial activity of oleuropein and hydroxytyrosol. J. Pharm. Phrmacol., 51: 971-974.
58. Vuong, Q.V., S. Hirun, P.D. Roach, M.C. Bowyer, P.A. Phillips and C.J. Scarlett, 2013. Effect of extraction conditions on total phenolic compounds and antioxidant activities of *Carica* papaya leaf aqueous extracts. J. Herbal Med., 3: 104-111.
59. Elamin, M.H., M.H. Daghestani, S.A. Omer, M.A. Elobeid and P. Virk *et al*., 2013. Olive oil oleuropein has anti-breast cancer properties with higher efficiency on ER-negative cells. Food Chem. Toxicol., 53: 310-316.
60. Goldsmith, C.D., C.E. Stathopoulos, J.B. Golding and P.D. Roach, 2014. Fate of the phenolic compounds during olive oil production with the traditional press method. Int. Food Res. J., 21: 101-109.
61. Samancioglu, S., A. Esen, G. Ercan, N.H. Mansoub, S. Vatansever and I. Ince, 2016. A new dressing material in diabetic wounds: Wound healing activity of oleuropein-rich olive leaf extract in diabetic rats. Gazioantep Med. J., 22: 14-21.
62. Benavente-Garcia, O., J. Castillo, J. Lorente, A. Ortuno and J.A. Del Rio, 2000. Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. Food Chem., 68: 457-462.
63. Meirinhos, J., B.M. Silva, P. ValentAo, R.M. Seabra and J.A. Pereira *et al*., 2005. Analysis and quantification of flavonoidic compounds from Portuguese olive (*Olea europaea* L.) leaf cultivars. Nat. Prod. Res., 19: 189-195.
64. Wahrburg, U., M. Kratz and P. Cullen, 2002. Mediterranean diet, olive oil and health. Eur. J. Lipid Sci. Technol., 104: 698-705.
65. Bouallagui, Z., J. Han, H. Isoda and S. Sayadi, 2011. Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells. Food Chem. Toxicol., 49: 179-184.
66. Susalit, E., N. Agus, I. Effendi, R.R. Tjandrawinata, D. Nofiarny, T. Perrinjaquet-Moccetti and M. Verbruggen, 2011. Olive (*Olea europaea*) leaf extract effective in patients with stage-1 hypertension: Comparison with Captopril. Phytomedicine, 18: 251-258.
67. Spurlin, T.A., K. Bhadriraju, K.H. Chung, A. Tona and A.L. Plant, 2009. The treatment of collagen fibrils by tissue transglutaminase to promote vascular smooth muscle cell contractile signaling. Biomaterials, 30: 5486-5496.
68. Donato-Trancoso, A., A. Monte-Alto-Costa and B. Romana-Souza, 2016. Olive oil-induced reduction of oxidative damage and inflammation promotes wound healing of pressure ulcers in mice. J. Dermatol. Sci., 83: 60-69.
69. Rosa, A.D.S., L.G. Bandeira, A. Monte Alto Costa and B. Romana Souza, 2014. Supplementation with olive oil, but not fish oil, improves cutaneous wound healing in stressed mice. Wound Repair Regener., 22: 537-547.
70. Mehraein, F., M. Sarbishegi and A. Aslani, 2014. Evaluation of effect of oleuropein on skin wound healing in aged male BALB/c mice. Cell J., 16: 25-30.