Journal of Traditional and Complementary Medicine 11 (2021) 427–434

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Original Article

Egyptian Olea europaea leaves bioactive extract: Antibacterial and wound healing activity in normal and diabetic rats

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ARTICLE INFO

Article history:
Received 13 March 2020
Received in revised form
29 December 2020
Accepted 20 February 2021
Available online 3 March 2021

Keywords:
Olea europaea
Antimicrobial activity
MRSA
Oleuropein
Wound healing

ABSTRACT

Background and aim: *In vitro* activity evaluation of Egyptian *Olea europaea* leaves extracts, and *in vivo* healing activity assessment of the newly prepared ointment of *Olea europaea* leaves extracts mingled with Shea butter.

Experimental procedure: Different extraction methods and solvents were used to extract Egyptian *Olea europaea* bioactive agent(s). Antibacterial, scavenging activity and *in-vivo* evaluation of wound repair potentiality of *Olea europaea* extract were examined in normal and diabetic experimental rat models with induced circular excisions.

Results and conclusion: Olive leaves extract of Tanta was selected as the most active agent against Methicillin-resistant *S. aureus* (MRSA), with MIC value 15.6 μg/ml. Moreover, checkerboard dilution technique approved that the interaction between Tanta LEM crude extract and Ciprofloxacin was synergistic. Scavenging activity of the extract against DPPH free radicals was 87.55% at concentration of 50 μg/ml. *In vivo* normal and diabetic experimental rats treated with Shea butter: Tanta LEM extract (1:3 w/v) showed the maximum wound contraction and healing activity.

1. Introduction

The olive tree (*Olea europaea* L.) is an important tree found in the Mediterranean countries. Olive extract is marketed as a natural medicine with wide-ranging health benefits.[1] Affirmation took place on the antioxidant activity of the extract and its corresponding health benefits such as cardioprotective and chemopreventive effects.[2] Also the leaves are important for their secondary metabolites such as the secoiridoid compounds oleacein and oleuropein, the former responsible for hypotensive activity and the latter for hypoglycemic activity.[3] Several reports have shown that olive leaf extract has the capacity to lower blood pressure in animals,[4] relieve arrhythmia and prevent intestinal muscle spasms.[5] Olive leaves and its disease-preventing effects have been attributed to its fatty acid profile, as well as the presence of a number of bioactive components such as tocopherols, phospholipids, and phenolic compounds.[6] Olive extract contains biophenols with varied therapeutic properties. The phenolics content of olive depends on cultivar, climate, irrigation regimes, degree of ripeness of the fruit, and elaboration process.[7]

Antimicrobial properties of phenolic compounds were mainly related to olive leaves.[3] Antibiotics are important biochemicals that are widely used as current medicine for a long time.[8] The misuse of antibiotics leads to increasing the bacterial resistance strains. Increasing the bacterial drug resistant strains had caused an increasing interest in herbal medicine and antimicrobial activity of plant extracts.[9] Abeed et al.[10] described the antimicrobial activity of olive leaf extracts against methicillin-resistant *Staphylococcus aureus*. Olive leaves extract is used to make an ointment, which is applied onto cuts and wounds for rapid healing. In Bulgaria, Italy, and Portugal, leaves were applied to heal burns.[11] Studies have also focused on the composition of olive leaf extracts because of the availability and low cost of the raw material.[12]

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

https://doi.org/10.1016/j.jtcme.2021.02.008

List of abbreviations

Tanta LEM (Tanta Leaves sample extracted with Ethanol using Maceration method)
Methicillin-resistant *S. aureus* (MRSA)
Transmission electron microscopic (TEM)
Column chromatography (CC)

Therefore, the aim of the present study was to examine the antimicrobial activity of *Olea europaea* extract against a wide range of pathogenic bacteria. Also show the wound healing activity assessment in normal and diabetic rats using the combination of shea butter with *Olea europaea* leaves extract.

2. Materials and methods

2.1. In-vitro study

2.1.1. Sample collection

Olea europaea leaves and stem were collected from four different geographic areas in Egypt, namely Tanta (30° 47′ 18.49″ N, 31° 00′ 6.91″ E), Alexandria (31° 12′ 20.7108″ N, 29° 55′ 28.2936″ E), Marsa Matrouh (31° 19′ 60.00″ N, 27° 12′ 60.00″ E) and Siwa Oasis (29°12′11.41″ N, 25°31′10.36″ E) during September 2017. Samples (leaves and stem) were cleaned and rinsed with sterile water and air dried. All samples were identified by Botany Department, Faculty of Science, Alexandria University.

2.1.2. Extraction of bioactive material

Leaves and stem of *Olea europaea* were extracted using ethanol, acetone, and ethanol/acetone (1:1) individually using maceration, sonification and soxhlet extraction technique[13] (data shown in supplementary file).

2.1.3. Microorganisms

ATCC strains were kindly provided from the U.S Naval Medical Research Unit (NMRU) in Cairo namely: *Staphylococcus aureus* 25923, *Staphylococcus epidermidis* 12228, *Pseudomonas aeruginosa* 27853, *Klebsiella pneumoniae* 13883, *Proteus mirabilis* 35659, *and Escherichia coli* 25922. Multi-drug resistant (MDR) strains namely *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis* (MRSE), *Pseudomonas aeruginosa, E. coli, Klebsiella pneumoniae, Acinetobacter baumannii and Enterococcus faecalis* were kindly provided and identified from El-Shatby paediatric hospital and each kept in brain heart infusion glycerol broth at −4 °C till testing. Each MDR strain was enumerated (1, 2, 3 etc.) to differentiate between the isolated strains from different clinical samples.

2.1.4. Evaluation of resistance prevalence of some MDR bacteria

Antibiotics of different groups were used namely: Amikacin (AK, 30 μg), Ampicillin (AMP, 10 μg), Ampicillin/Sulbactam (SAM, 10/10 μg), Amoxacillin/Clavulanate (AMC, 20/10 μg), Aztreonam (ATM, 30 μg), Cefazolin (KF, 30 μg), Cefoxitin (FOX, 30 μg), Cefotaxime (CTX, 30 μg), Ceftazidime (CAZ, 30 μg), Cholramphenicol (C, 30 μg), Ciprofloxacin (CIP, 5 μg), Colistin (CT, 10 μg), Doxycycline (DO, 30 μg), Meropenem (MEM, 10 μg) and Pipracillin (PRL, 100 μg). The antibiotic discs activity was assessed according to CLSI guidelines.[14]

2.1.5. Antibacterial effect of Olea europaea extracts

Antibacterial activity was carried out using the disc-diffusion method; discs were saturated with 25 μl of each *Olea europaea* extract then placed on the surface of the inoculated Müeller-Hinton agar plates.[14] Further antibacterial activity evaluation was done by assessing the minimal inhibitory concentration (MIC) [15], minimum bactericidal concentration (MBC) values[16] and the bacterial time-kill curve.[17]

2.1.6. Transmission electron microscopic (TEM) examination of the Olea europaea treated cells

Olea europaea extract treated MRSA cells were subjected to TEM examination (JEM-100 CX Joel).[18]

2.1.7. GC-MS (gas chromatography- mass spectroscopy) analysis for Olea europaea crude extract

GC-MS analysis was evaluated for the chemical analysis and identification of the *Olea europaea* extract components according to Ahmad et al.[19]

2.1.8. Evaluation of the active fraction(s) of the most promising Olea europaea extract

The ethanolic extract of the most promising *Olea europaea* sample was fractionated by silica gel column chromatography (700-230 mesh) using CH_2Cl_2–MeOH (8:2) and then the total fractions were collected. The fractions were checked by TLC (DC Kieselgel 60 F254 25 Alufolien 20 × 20 cm, Merck) using $CHCl_3$-EtOAc (7:3) followed by incubation for 24 h in iodine vapour.[20] Moreover, the evaluation of the chemical structure of the active fraction(s) analysis was done using GC-MS.

2.1.9. Checkerboard dilution technique to combine Olea europaea extract and commonly known antibiotics

The broth microdilution checkerboard technique was employed to study the synergistic effect between the most promising *Olea europaea* extract and commonly known antibiotic according to Al-Ani et al.[21] Fractional inhibitory concentration index (FICI) was computed with the following equation:

$$FICI = FIC\ of\ agent\ A + FIC\ of\ agent\ B$$

where;

$$\text{FIC of agent A} = \frac{\text{MIC of antimicrobial agent A in combination}}{\text{MIC of antimicrobial agent A alone}}$$

$$\text{FIC of agent B} = \frac{\text{MIC of antimicrobial agent B in combination}}{\text{MIC of antimicrobial agent B alone}}$$

FICI was considered as a synergistic when it was ≤0.5, as additive when it was >0.5–1, indifferent when it was ≥1–4.0 and antagonistic when it was >4.

2.1.10. Test for antioxidant activity

Various concentrations of *Olea europaea* extract (0.3 ml) were mixed with 2.7 ml of methanolic solution containing DPPH radicals (6×10^{-5} mol/L). The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm. The DPPH scavenging effect was calculated as a percentage of DPPH discolouration using the equation:

$$\text{Scavenging percentage} = \frac{\text{ADPPH} - \text{AS}}{\text{ADPPH}} \times 100$$

where AS is the absorbance of the solution when the sample extract has been added at a particular level and ADPPH is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (EC50) was calculated from the graph of scavenging effect percentage against extract concentration.[7]

2.2. In-vivo study

2.2.1. Animals

The present experiment was performed using Female rats (*Rattus norvegicus albinus*) (average body weight 180–200 g) which was in an approved animal care centre in accordance with the Animal Care and Use Committee (ACUC) at the Faculty of Science, Alexandria University and was in accordance with the International Principles for Laboratory and Care of the European Community Directive of 1986; AU/04/19/04/13/03/02. The animals were left for 3 days at room conditions for acclimatization. The rats were maintained on a standard pellet diet and water throughout all the experiment.

2.2.2. Diabetes induction

Diabetes was induced by intraperitoneal injection of Alloxan monohydrate to overnight fasted animals at a dose of 100 mg/kg body weight. Diabetes was confirmed by recording blood glucose values.[22]

2.2.3. Circular excision wound model

Animals were anesthetized with Ketamine and Xylazine then the back hair of the rats were shaved and cleaned with 70% alcohol. A circular wound was created on the dorsal interscapular region of each animal by excising the skin carefully with a 5-mm biopsy punch; circular wounded areas were left open until the end of the experiment.[23]

2.2.4. Animal groups

Both diabetic and non-diabetic rats were divided into eight groups according to their treatment as follows: Group 1: negative control rats; Group 2: positive control rats; Group 3: rats treated with shea butter; Group 4: rats treated with ciprofloxacin; Group 5: rats treated with the most promising *Olea europaea* extract (Tanta LEM); Group 6: rats treated with ointment 1 (1:1 shea butter: Tanta LEM/CIP); Group 7: rats treated with ointment 2 (1:2 shea butter: Tanta LEM/CIP); Group 8: rats treated with ointment 3 (1:3 shea butter: Tanta LEM/CIP). Seven rats groups of diabetic and non-diabetic rats (Group 2:8) were infected with MRSA (20 μL of 7.27×10^9 CFU/ml) by intradermal injection.[24]

2.2.5. Wound healing activity

For the first time a combination of shea butter with *Olea europaea* leaves extract was used as an ointment to test the wound healing activity of *Olea europaea* extract in normal and diabetic rats. Each ointment (0.5 g) was applied on the wounded area once a day until the wounds were completely healed. The progressive changes in wound area were monitored, and wound area was measured every day.

2.2.6. Bacterial load assessment

At time intervals, rats were sacrificed, the area around the wound lightly swabbed with 70% ethanol, and excised. The tissue was weighed then homogenized in tryptic soy broth (1 ml). The homogenized tissue was then serially diluted in polyphosphate buffer before plating onto mannitol salt agar plates. The plates were incubated for 24 h at 37 °C, then a confirmatory test (coagulase test) of staphylococci were done before viable MRSA cells (CFU/g tissue) were counted and bacterial reduction in the skin wound post-treatment was determined for each group.[24] The number of CFU/g of tissue was calculated as follows:

$$\mathbf{CFU}\Big/\mathbf{g} = \mathbf{plate\ count}\left(\frac{1}{\mathbf{dilution}}\right) \times \frac{10}{\mathbf{wieght\ of\ tissue}}$$

2.2.7. Histological study

At the end of the experiment, a sample from the healed skin tissue of each group of normal and diabetic rats was taken and subjected to histological examination.[24] The tissues were stained by haematoxylin and eosin and were examined by light microscopy.

2.3. Statistical analyses

Results were the mean of three trials and expressed as means ± standard deviation. The means of the treatments were considered significant when $0.05 > p > 0.01$.

3. Result

3.1. In-vitro study

3.1.1. Evaluation of resistance prevalence of MDR strains

Results revealed that all the tested MDR strains were resistant to ciprofloxacin (100%) followed by cefazolin (90%) and Amikacin (80%) respectively. However, the highest susceptibility percentage was noticed with Meropenem (30%) followed by cefotaxime and aztreonam (40%). All the other tested antibiotics showed inferior results.

3.1.2. Antibacterial effect of Olea europaea extracts

Bioactive agents in leaves and stem of *Olea europaea* were extracted using three solvents with three extraction methods one at a time. The most susceptible ATCC bacterium was *S. aureus* with an average inhibition zone diameter of 8.5 mm. MRSA was the most susceptible MDR bacteria with an average inhibition zone diameter of 10 mm. Tanta leaves ethanolic extract using maceration method (Tanta LEM extract) was the most potent extract (Supplementary data). Tanta LEM, Alexandria LEM, Marsa Matrouh LEM and Siwa Osasis LEM extracts exhibited a bactericidal activity with average MBC ranging from 31.2 μg/ml to 1000 μg/ml against MDR bacterial strains (Table 1). MIC index showed that Tanta LEM extract was proved to be a bactericidal agent against different MDR bacteria, while the most susceptible organism was MRSA. The bacterial time-kill curve was evaluated and the results revealed that while exposing MRSA cells to 15.6 μg/ml MIC of Tanta LEM extract, the bacterial growth was sharply decreased after 6 h and the bacterial growth was completely eradicated after 8 h incubation (Supplementary data).

3.1.3. TEM of Tanta LEM treated MRSA cells

TEM analysis was evaluated to examine Tanta LEM extract treated MRSA cells. Results revealed that MRSA control cells had typical features of the cell wall and cell membrane. While Tanta LEM treated MRSA cells were completely destroyed accompanied by cellular debris and release of intracellular components were observed (Fig. 1).

3.1.4. GC-MS analysis of Tanta LEM

Tanta LEM extract was prepared and analysed using GC-MS as shown in Fig. 2 and Table 2 Dasycarpidan-1-methanol, acetate (ester), Linoleic acid ethyl ester, Vitamin D, Ethyl Oleate, Tributyl acetylcitrate, 2-Methoxy-4-vinylphenol, Benzoic acid 4-formyl-methyl ester, 3-(2,6,6-Trimethyl-cyclohex-1-enyl)-propionic acid

Table 1
Minimum inhibitory concentrations of all tested *Olea europaea* leaf samples[a] against MDR bacteria.

Microorganisms	MIC values (μg/mL)											
	Tanta LEM			Alexandria LEM			Marsa Matrouh LEM			Siwa Oasis LEM		
	MIC	MBC	MIC Index	MIC	MBC	MIC Index	MIC	MBC	MIC Index	MIC	MBC	MIC Index
K. pneumoniae	125	375	3	125	500	4	62.5	250	4	125	375	3
MRSA 1[b]	15.6	31.2	2	125	375	3	250	1000	4	15.6	31.2	2
A. baumannii	15.6	62.4	4	250	750	3	125	500	4	125	250	2
E. coli 1[b]	62.5	250	4	125	625	5	250	1000	4	15.6	31.2	2
E.faecalis	62.5	250	4	15.6	62.4	4	125	1000	8	250	750	3
E. coli 2[b]	15.6	31.2	2	125	625	5	250	750	3	62.5	125	2
P. aeruginosa 1[b]	125	375	3	250	750	3	125	375	3	62.5	125	2
MRSA 2[b]	15.6	31.2	2	52.5	210	4	62.5	375	6	15.6	62.4	4
MRSA 3[b]	15.6	31.2	2	125	750	6	125	250	2	62.5	125	2
P. aeruginosa 2[b]	15.6	62.4	4	250	750	3	125	500	4	125	500	4

[a] Ethanol extraction using maceration technique.
[b] The code used 1, 2, and 3 was applied to enumerate different clinical isolates however the full data was demonstrated in supplementary file.

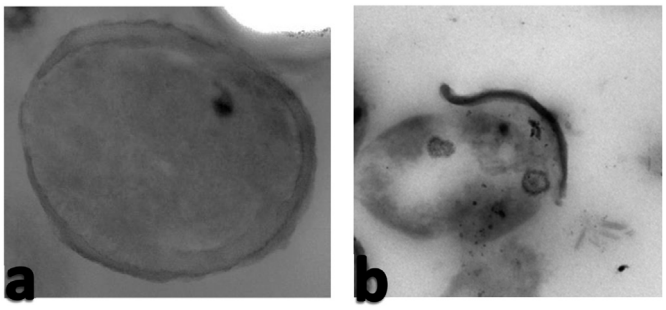

Fig. 1. TEM for MRSA cells; untreated (a) and treated (b) with Tanta LEM extract.

methyl ester, Benzenepropanoic acid 3,4-dihydroxy-methyl ester, Hexadecanoic acid ethyl ester, Phenyl 2,3,4,6-tetra-O-acetyl-β-d-glucopyranoside, Cellobioseoctaacetate, dl-α-Tocopherol, Ergosta-5,22-dien-3-ol acetate (3β,22E) and 1-Monolinoleoylglycerol trimethylsilyl ether had been identified on referring with the corresponding acquisition time. Table 2 showed that Dasycarpidan-1-methanol, acetate and Linoleic acid ethyl ester had the highest percentage (19.1 and 11.3% respectively), followed by Vitamin D (10.2%).

3.1.5. Evaluation of the active fraction(s) of Tanta LEM

Column chromatography (CC) was used for Tanta LEM extract separation. Each fraction obtained from CC was further separated using TLC for the major components detection. The eluted bands of TLC were tested for its antibacterial activity using disc-diffusion method and MIC. Results revealed that the eluent number four[4] with R_f value 0.90 showed an inhibitory effect against MRSA growth with inhibition zone diameter and MIC of 10 mm and 31.2 μg/ml, respectively. While eluents 1, 2, 3, 5 and 6 with R_f values of 1.45, 1.25, 1.11, 0.86 and 0.5, respectively showed no inhibition zone against the tested bacterium. The most active fraction (eluent 4) of Tanta LEM was further investigated using GC-MS analysis which proved that it was composed of Linoleic acid ethyl ester and Tributyl acetylcitrat. Unfortunately the entire separated eluents showed less activity than the parent crude extract, and this may be due to the combine action of different compounds originally present in the plant extract, hence further experiments were done with the parent crude extract.

3.1.6. Checkerboard dilution technique

The interaction of Tanta LEM crude extract and commonly known antibiotics which were commonly used as a treatment for wound infections were evaluated against MRSA using the checkerboard dilution technique one at a time. Data in Table 3 revealed

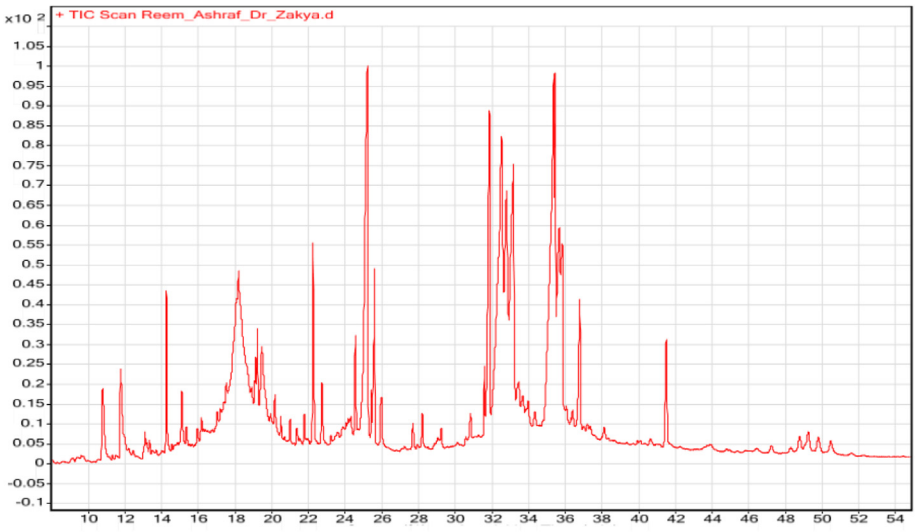

Fig. 2. GC-MS chromatogram of the crude Tanta LEM extract.

Table 2
Retention time (RT) and probable compounds according to MS library.

Group	R.T. (min)	Suggested compound	M.W.	M.F.	Content %
Ester	11.78	Benzoic acid, 4-formyl-, methyl ester	164	$C_9H_8O_3$	6.30
	18.18	Dasycarpidan-1-methanol, acetate (ester)	540	$C_{25}H_{32}O_{13}$	19.10
	19.13	3-(2,6,6-Trimethyl-cyclohex-1-enyl)-propionic acid, methyl ester	210	$C_{13}H_{22}O_2$	4.60
	19.45	Benzenepropanoic acid, 3,4-dihydroxy-, methyl ester	196	$C_{10}H_{12}O_4$	5.80
	22.24	Hexadecanoic acid, ethyl ester	284	$C_{18}H_{36}O_2$	5.50
	25.23	Linoleic acid ethyl ester	308	$C_{20}H_{36}O_2$	11.30
	25.53	Ethyl Oleate	310	$C_{20}H_{38}O_2$	8.10
	27.28	Tributyl acetylcitrate	402	$C_{20}H_{34}O_8$	4.30
	31.87	Phenyl 2,3,4,6-tetra-O-acetyl-β-d-glucopyranoside	424	$C20H24O_{10}$	6.50
	35.36	Cellobioseoctaacetate	678	$C_{28}H_{38}O_{19}$	4.00
	49.24	Ergosta-5,22-dien-3-ol, acetate, (3β,22E)	440	$C_{30}H_{48}O_2$	2.50
Aldehyde	15.94	9,10-Secochola-5,7,10(19)-trien-24-al, 3-hydroxy-, (3β,5Z,7E) (Vitamin D)	356	$C_{24}H_{36}O_2$	10.20
Alcohol	41.49	dl-α-Tocopherol	430	$C_{29}H_{50}O_2$	3.60
	10.79	2-Methoxy-4-vinylphenol	150	$C_9H_{10}O_2$	4.80
Aromatic Hydrocarbon	49.78	1-Monolinoleoylglycerol trimethylsilyl ether	498	$C_{27}H_{54}O_4Si_2$	3.40

M.W.: Molecular Weight, M.F.: Molecular Formula.

that the combined action of Tanta LEM crude extract and Ciprofloxacin was synergistic with FICI 0.40. While the combination of Tanta LEM and vancomycin, amikacin, imipenem one at a time was indifferent with FICI 3.35, 1.40, 1.05, 2.00 and 1.02 respectively.

3.1.7. Antioxidant activity of Tanta LEM

Scavenging activity of Tanta LEM extract against DPPH radicals increased in a concentration-dependent way. The maximum scavenging activity of the extract under test was 87.55% at IC50 equalled 50 μg/ml, while Ascorbic acid (standard) scavenging activity reaches IC50 equalled 62.8 μg/ml.

3.2. In-vivo study

3.2.1. Gross symptoms

During the period of this investigation all rat groups were closely inspected daily in order to detect any apparent behavioural changes or clinical signs of toxicity. It was of considerable interest that animals from both control and experimental groups did not exhibit any case of mortality.

3.2.2. Morphological study

As mentioned before wounds were excised (1 cm in diameter) include full thickness skin (i.e. epidermis, dermis, and the hypodermis). Morphologically, it was noticed that bleeding following the wounding was minimal in all rat groups, no clear cut clotting were observed by naked eye on wounds of experimental rats of all groups at the first few minutes post-injury. Therefore, wounds of all experimental animals have bloody appearance soon after excision.

3.2.3. Wound healing activity

Wound contraction in non-diabetic and diabetic rats showed gradual reduction of wound area as shown in ointments treated groups (ointment 1,2 and 3) while there is a slightly reduction in wound area as shown in control groups. Wound contraction in non-diabetic rats is faster than wound contraction in diabetic rats. Non-diabetic rats at day 9 post-injury treated with ointment 1, 2 and 3 revealed 70, 90 and 100% contraction in wound size respectively, while diabetic rats at day 9 revealed 30, 50 and 70% contraction in wound size respectively. Moreover, diabetic rats at day 13 post-injury revealed 70, 80 and 100% contraction in wound size respectively. However all normal and diabetic control groups only showed a slight improvement in wound size. In conclusion, non-diabetic and diabetic group 8 (treated with 1:3 shea butter: Tanta LEM/CIP) rats were the most significant among all treatment regimens (Fig. 3).

3.2.4. Bacterial load assessment

At the beginning of the experiment rats received an intradermal injection containing 7.27×10^9 CFU/ml MRSA. Furthermore, at specific time interval, the viable MRSA cells were counted (CFU/g tissue) and bacterial reduction in the skin wound post-treatment was determined for each group. As shown in Table 4, all treatments significantly reduced the bacterial counts compared with the control groups. Among non-diabetic and diabetic rat groups, the group treated with ointment 3 had the highest reduction in $\log_{10}$ CFU/g (0.1 in non-diabetic and 0.3 in diabetic rats). In contrast, all control groups had a slightly reduction in $\log_{10}$ CFU/g in both non-diabetic and diabetic rat groups.

3.2.5. Histological study of wounded rat skin of each group

Non-diabetic wounded rat skin in vertical sections was found to be consisting of the principle layers namely: epidermis and dermis. Wounded non-diabetic and diabetic rats (Fig. 4a) showed some epithelial appendages such as the sebaceous glands with wide ducts (oil-producing gland) that developed at the neck of the hair

Table 3
Synergy test of Tanta LEM crude extract in combination with commonly known antibiotics.

Antibacterial agent	Single drug		Combined drug		FIC	FICI	Interpretation
	MIC (μg/ml)	IZ (mm)	MIC (μg/ml)	IZ (mm)			
Tanta LEM crude extract	15.60	15.00	3.90		0.25		
Vancomycin	125.00	12.00	143.75	12.00	1.15	1.40	Indifferent
Ciprofloxacin	250.00	20.00	37.50	37.00	0.15	0.40	Synergy
Amikacin	250.00	10.00	200.00	10.00	0.80	1.05	Indifferent
Imipenem	62.00	16.00	108.50	16.00	1.75	2.00	Indifferent

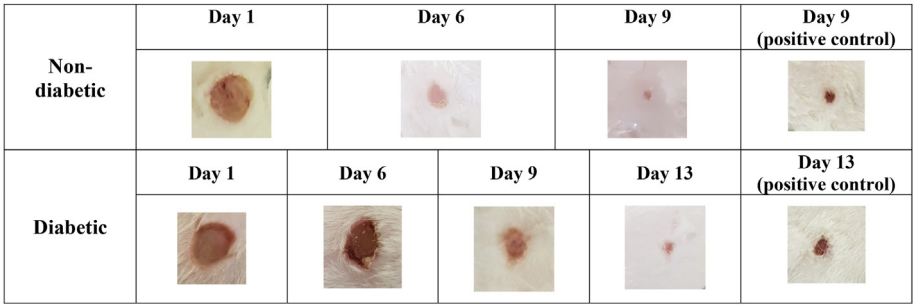

Fig. 3. Morphological appearance of wound healing during time intervals in diabetic and non-diabetic rats*. * Group 8 (treated with 1:3 shea butter: Tanta LEM/CIP (w/v)).

Table 4
Bacterial cell load in non-diabetic and diabetic animal tissues after treatment at time intervals.

Group	MRSA viable log_{10} CFU/g tissue			
Non-diabetic rat group	Day 3	Day 6	Day 9	Day 13
1: Negative control	9.1	8.3	7.2	3.5
2: Positive control	9.9	9.5	9.1	8.4
3: Treated with shea butter	9.2	7.1	6.2	5.0
4: Treated with Ciprofloxacin	8.5	6.1	5.5	1.8
5: Treated with Tanta LEM	8.5	5.7	4.3	1.5
6: Treated with ointment 1[a]	7.3	5.5	3.1	0.9
7: Treated with ointment 2[b]	6.8	3.9	1.9	0.5
8: Treated with ointment 3[c]	5.9	2.9	0.1	0.0
Diabetic rat group				
1: Negative control	9.7	9.1	8.5	8.1
2: Positive control	9.8	9.8	9.7	9.5
3: Treated with shea butter	9.2	8.5	7.9	7.5
4: Treated with Ciprofloxacin	9.1	7.8	6.8	6.1
5: Treated with Tanta LEM	8.9	8.1	7.6	7.2
6: Treated with ointment 1[a]	7.9	6.3	5.4	4.8
7: Treated with ointment 2[b]	7.8	5.2	3.2	2.3
8: Treated with ointment 3[c]	6.1	3.4	1.4	0.3

[a] (1:1 w/v shea butter: Tanta LEM/Ciprofloxacin).
[b] (1:2 w/v shea butter: Tanta LEM/Ciprofloxacin).
[c] (1:3 w/v shea butter: Tanta LEM/Ciprofloxacin).

follicle opening. After healing, non-diabetic wounded rat skin showed an irregular distribution of collagen fibres in the dermis layer, however, wounded diabetic rat skin showed thinning in the epidermis layer compared to normal wounded rat skin (Fig. 4b). As the process of wound healing, the infected wound of the diabetic rats (Fig. 4c) appeared housing numerous polymorphonuclear leucocyte indicating inflammation. On the other hand, examination of the non-diabetic wounded rats infected with MRSA showed new blood vessel formation within the wound area indicating angiogenesis (Fig. 4d), which was not showed in the diabetic wounded rats infected with MRSA. While treatment with ointment 3 revealed almost complete healing. Formation of the demarcation line appeared in healthy wounded rats treated with ointment 3 which separate the necrotic tissue from vital tissue was detected (Fig. 4e). Moreover, infected wounds treated with ointment 3 showed micro-vessel and intense number of new blood vessel within the newly formed dermis. However, diabetic wounded rat treated with ointment 3 revealed that the dermal features were consistently different when compared to the healthy one (presence of moderate inflammatory cells, which appeared in the newly formed dermis and high density of microvessel) (Fig. 4f). It was revealed that the treatment with ointment 3 enhanced the neo-angiogenesis process.

4. Discussion

Olea europaea samples were collected from four geographically different areas in Egypt. The most promising extract was Tanta leaves ethanolic extract using maceration method (Tanta LEM extract). Kuley et al.[25] reported that.

Bacillus subtilis was the least susceptible organism, whereas *E. coli, K. pneumoniae* and *S. aureus* were the most susceptible for olive leaf extract. Similar observations were detected with Tanta LEM extract. Olive leaf extract may lower the risk of bacterial infections mainly due to the protective action provided by its phenolic compounds. In fact, the mode of action of phenolics has been shown to be concentration dependent.[26] Tanta LEM extract (15.6 μg/ml) eradicate the bacterial growth completely at 8 h while TEM study revealed that the degradation of the ultrastructural features of cell wall indicated that the mechanism of action of Tanta LEM extract was cell wall synthesis inhibition, where the extract attacks the peptidoglycan layer and deforms it leading to cell lysis. Al-Habib et al.[27] reported that *Olea europaea* extract may lead to cell wall or cell membrane disruption to Gram-positive bacteria, which was more susceptible compared to Gram-negative bacteria. The antioxidant activity of Tanta LEM extract was 87.55% at concentration of 50 μg/ml which could be explained by Osman & Tantawy[28] who mentioned that olive leaves were considered as the highest scavenging activity radical of different parts of olive trees. The wound healing activity of *Olea europaea* can be explained by Rosa et al.,[29] where olive oil administration proved to increase cell migration, TGF- β protein expression, and collagen deposition. Olive oil may stimulate collagen formation by fibroblasts through increased synthesis of TGF- β, leading to improved wound contraction. In addition, treatment with oleic acid reduces the local inflammatory response, leading to faster wound contraction and reepithelialization. Another important factor in the formation of granulation tissue is angiogenesis, the high amount of blood vessels were observed in stressed + olive oil group which may have induced the reduction of observed in HIF- 1α levels. The presence of phenolic compounds in plant extracts are known to have antibacterial, antioxidant and several biological activity which maybe the reason behind the promising activity of *Olea europaea* extract. Ouni et al.[30] declared that the genotypic potential, environmental, agronomic, and processing systems can all significantly affect the phytochemical composition and content of *Olea europaea*. The presence of biologically important phytochemicals contributes to the medicinal value of *Olea europaea* leaves extract.[31] GC-MS analysis of Tanta LEM extract revealed that the main chemical class identified was Esters followed by Alcohols then Aldehydes.[32] Malheiroa et al.[33] reported that esters were the main chemical class identified when *Olea europaea* cultivars was analysed during different sampling dates. While alcohols and aldehydes were present in low concentrations. Moreover, Mahabaleshwara et al.[34]

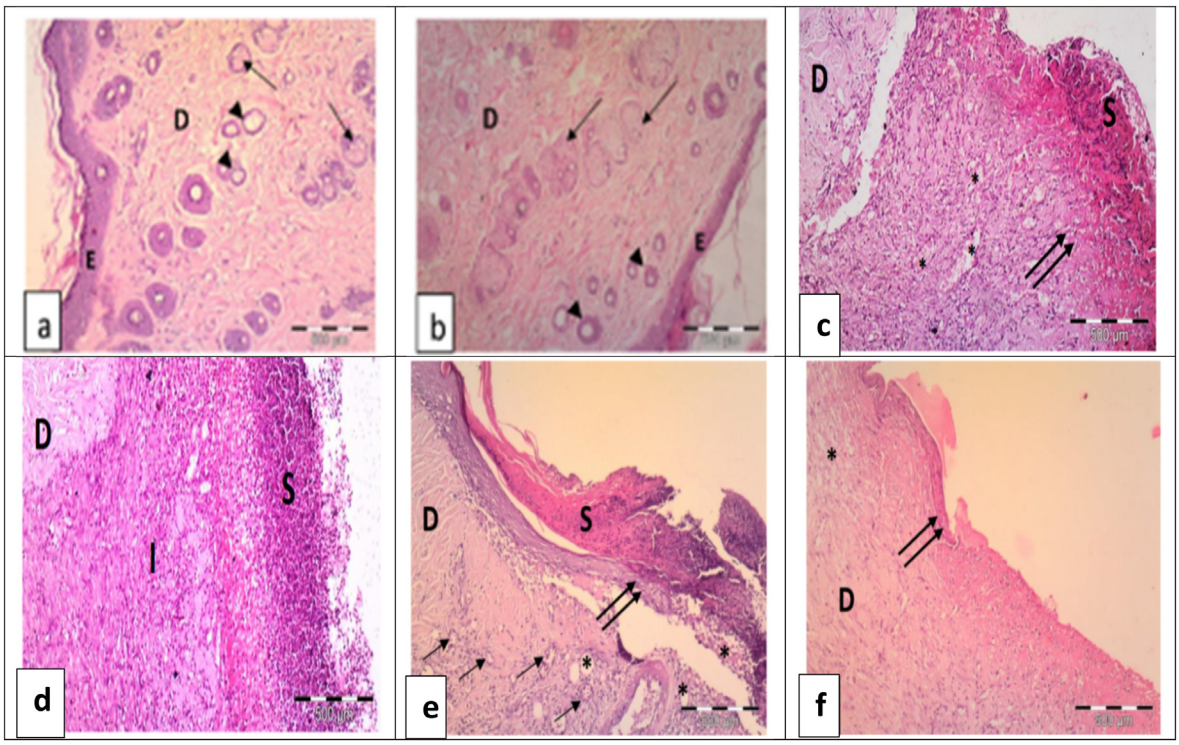

Fig. 4. Light micrograph (X 100) of V.S. in wounded skin of a) Non-diabetic wounded rat skin of group 1 (negative control), showing dermis (D) with irregular distributed collagen fibers, hair follicle (arrowheads) and sebaceous gland (arrows). b) Diabetic wounded rat skin of group 1 (negative control), showing thinning of epidermis (E), dermis (D) with irregular distributed collagen fibers, hair follicle (arrowheads) and sebaceous gland (arrows). c) Non-diabetic wounded rat skin of group 2 (positive control), showing new blood vessel formation within the wound area indicating angiogenesis. (D). d) Diabetic wounded rat skin of group 2 (positive control), showing dermis (D) with irregular distributed collagen fibers. Double arrows demonstrate the epithelial migration of keratinocytes beneath the scab (S), inflammatory cells (I) in newly formed dermis. Note: intense number of new blood vessel observed (*). e) Non-diabetic rats after treatment with ointment 3, showing, epithelial migration of keratinocytes (double arrows) beneath the scab (S), microvessel (*), intense number of new blood vessel observed (arrows) and dermis (D). f) diabetic rats after treatment with ointment 3, showing, epidermal moving (double arrows), moderate inflammatory cells in newly formed dermis (D) and high density of microvessel (*), epidermal moving (double arrows), moderate inflammatory cells in newly formed dermis (D) and high density of microvessel (*).

revealed that the GC-MS of olive leaf methanol extract has contained different identifiable compounds such as: Hexadecanoic acid, methyl ester; Isopropyl Palmitate; Ethyl Oleate; Dasycarpidan1-methanol,acetate (ester); Hexadecanoic acid,2-methylpropyl ester; Isopropyl linoleate; Benzoic acid, 2-[2-methoxyethoxy)-5-(2,2- dimethylpropanoylamino]. Hussein et al.[35] showed that linoleic acid was known to have potential antibacterial and antifungal activity while Tributyl acetylcitrat has anticancer and antimicrobial activity. It is well known that the management of wounds involves dressing, pain killers, and the administration of antibiotic, anti-inflammatory, and antibacterial agents. The use of plants in healing wounds could be attributed to their antioxidant as well as antimicrobial effects. In diabetic patients, high blood glucose levels lead to poor blood circulation, making it hard for blood – needed for skin repair – to reach the wounded areas, therefore increasing the risk of microbial infections which could take up to several months for healing the wound, in contrast to non-diabetic patients. Olive leaf extract polyphenols had antioxidant activity which in turn affect and facilitate the wound healing process, as well as the antibacterial activity of olive leaves which was tested by injecting the bacteria in the wound area of diabetic rats.[36] Based on the present and earlier investigations, it was inferred that to enhance the antibiotic effect, its combination with other antibiotics on one hand and plant extracts on the other should be used against a particular pathogen. In many instances the combinations of drugs as well as drug and plant extracts exercise high degree of inhibition by either causing microbicidal or microbistatic activity.[37] Wound healing is an important but complicated process, containing a complex series of reactions and interactions of inflammatory mediators and cell growth interactions which governed by sequential overlapping phases, including hemostasis, inflammation phase, proliferation phase, and remodeling phase.[38] In the present study, Wound contraction in non-diabetic rats was faster than wound contraction in diabetic rats. Non-diabetic rats at day 9 post-injury treated with ointment 3 revealed 100% contraction in wound diameter, while diabetic rats at day 9 revealed 70% contraction in wound diameter. Eber et al.[39] showed that the antimicrobial effect of olive leaves also has a beneficial effect on wound healing after 20 days. The phenolic compounds found in olive leaves were shown to inhibit the growth of various human pathogenic strains.[40] Therefore olive extracts aid the healing process by providing an optimal healing environment by their effect against bacteria, which resulted in decreased rates of infection. This is the first work to emphasis the effect of the *Olea europaea* leaves extract in combination with shea butter to treat wounded non-diabetic and diabetic rats, and this combination has not been reported in any previous studies. In addition, shea butter enhanced the healing process by vitamins and phytonutrients absorption into the subcutaneous layer of the skin. Moreover this combination can be industry useful as its eco-friendly, organic and natural product for wound healing.

The present study concluded that Tanta LEM extract had showed antibacterial activity against MRSA, and it was noticed that after the exposure of MRSA cells to Tanta LEM extract for 8 h the cells were completely eradicated and became as a ghost cells. Besides its antibacterial activity, the extract proved to have a potent scavenging activity. Tanta LEM crude extract analysis showed that

oleuropein and linoleic acid ethyl ester were the most abundant, while the active fraction of Tanta LEM proved to contain linoleic acid ethyl ester and tributyl acetylcitrat. Moreover, the combination between both Tanta LEM crude extract and active fraction with Ciprofloxacin was found to be synergistic. Finally the combination of Shea butter with Tanta LEM (ointment 3) was used for the first time and it was noticed to have the highest wound healing activity in normal and diabetic rats wound infection.

Declaration of competing interest

The contribution represents original work, has not been previously published or simultaneously submitted for publication, the manuscript has been read and approved by all authors. The authors report no financial or personal conflicts of interest.

Acknowledgment

Sincere gratitude should be expressed to Botany Department, Faculty of Science, Alexandria University for identifying all *Olea europaea* samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2021.02.008.

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