Supporting Information

Pyrus communis L. (Pear) and *Malus domestica* Borkh. (apple) leaves lipoidal extracts

as sources for beta-sitosterol rich formulae and their wound healing evaluation

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Abstract

P. communis L. (cv. Le-Conte) (pears) and *M. domestica* Borkh. (cv. Anna) (apples) are economic fruit crops cultivated in Egypt. Their leaves were assessed for their beta-sitosterol content and found to have 9.4 mg/ g dried leaves wt and 5 mg/g dried leaves, respectively. So we used the lipoidal leaves extracts in formulation of eight beta-sitosterol rich emulgels from which the most stable formulae were tested for their antimicrobial activity and finally, the formulae which exerted antimicrobial activity were biologically evaluated for wound healing against well known wound healing ointment Mebo® which composed mainly of 0.25% beta-sitosterol in base of sesame oil and beeswax and wound contraction was statistically different in both formulae F3 and F8 from both control and Mebo® groups which indicated better wound healing activity of these formulae ensured by further histopathological study of the healed wounds.

Experimental:

-Plant material

Pyrus communis L. (cv Le-Conte) *Malus domestica* Borkh. (cv. Anna) leaves were provided by Horticulture Research Institute, Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Giza, Egypt in December 2014. Voucher samples were kept at Pharmacognosy Department Herbarium, Faculty of Pharmacy, Cairo University, Cairo, Egypt under no. 12.03.2017.01 and 12.03.2017.2.

-Experimental animals

Adult male Sprague Dawley rats (180-200 g) were obtained from the breeding colony maintained at the animal house of the National Organization for Drug Control and Research (NODCAR, Cairo, Egypt). Rats were held at controlled conditions of 25 ± 2 °C temperature and 40-60 % relative humidity with 12 hours alternating dark and light cycles. Animals had free access to water ad libitum and diet. Two weeks before the experiment, rats were habituated. NODCAR standard guidelines were followed in the experimental animals conducting and this is meeting the requirements of the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996).The study protocol was approved by The Institutional Research Ethics Committee (NODCR-REC) at the National Organization for Drug Control and Research (NODCAR/I/35/20).

-Material for microbiological studies

Mueller-Hinton agar; it was recommended by National Committee for Clinical Laboratory Standards (NCCLS) for good batch-to-batch reproducibility. Filter paper discs with a diameter of 8.0 mm; Schleicher and Schuell Co., Spain, Slipping calipers available in NCCLS.

Drugs were obtained from RCMB (The Regional Center for Mycology and Biotechnology).

Gentamycin; antibacterial agent, Ketoconazole; antifungal agent.

Microorganisms were obtained from RCMB

Staphylococcus aureus (RCMB 010010) and *Staphylococcus epidermidis* RCMB 009 (2); Gram (+ ve) bacteria, *Escherichia coli* (RCMB010052) ATCC 25955; *Salmonella typhimurium* RCMB 006 (1) ATCC 14028; Gram (-ve) bacteria, *Aspergillus flavus* (RCMB 002002) and *Candida albicans* RCMB 005003 (1) ATCC 10231; as fungi.

-Chemicals for pharmaceutical formulae

Oily phase: Sesame oil, Imtenan Co., Egypt, Gelling agent: Carbopol 954. Sigma Aldrich, surfactants: Tween 60, Acros Organics Co., USA, Tween 80 Adwia Co., Egypt, Brij 35, Fischer Scientific Co., USA and Brij L23, Alfa Aesar Co., Germany. Preservatives: Methyl paraben and Propyl paraben. Al Nasr Co., Egypt. Triethanolamine for PH adjustement.

-Authentic and chemicals for Beta-sitosterol assay

USP beta-sitosterol standard (92.8%) from Phytochemistry lab, national Organization for drug Control and Research (NODCAR).
n-Hexane for extraction of the plant material was of analytical grade, Tetrahydrofuran, Ethanol and Acetonitrile are all of HPLC grade

-Instruments

For GC/MS analysis of leaves unsabonifiable fraction (USM)

Shimadzu GCMS-QP2010 (Tokyo, Japan) equipped with Rtx-5MS fused bonded column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Restek, USA). Pharmacognosy department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.

For beta-sitosterol assay

HPLC apparatus Agilent 1200, Germany, with column BDS Kromasil C18 (4.6 x 100 mm, 3 µm particle size), using UV detector at 210 nm and 40°C column temp and 20 µl injection volume.

For determination of formulae viscosity

Viscometer: Brookfield DVIII ultra programmable rheometer, USA, spindle 40.

Methods:

1- Preparation of the plant material

A- Extraction of the plant material

For *P. communis* L. and *M. domestica* Borkh. leaves

Air dried leaves of *P. communis* L. **(1 kg)** and air dried leaves of *M. domestica* Borkh. **(1.13 kg)** were separately cold macerated with 80% ethanol till complete exhaustion. The ethanolic extracts were separately evaporated under reduced pressure, at a temperature not exceeding 50 ºC to yield **(193g)** of *P. communis* L. leaves semisolid dark green extract and **(200g)** of *M. domestica* Borkh. leaves dark green semisolid extract.

b- Fractionation:

A part of *P. communis* L. leaves extract obtained **(145 g)** and **(110 g)** of *M. domestica* Borkh ethanolic extract were suspended separately in (500 ml) distilled water and successively extracted with *n*-hexane (8 x 500 mL) to yield **(19.2 g)** and **(6 g)** of pear and apple *n*-hexane extract, respectively.

2-Saponification and GC/MS analysis of leaves *n*-hexane extracts

Fractionation of the total lipoidal matter by the saponification process (Elsaid and Amer, 1965)

About 1g of each of the prepared *n*-hexane extracts for leaves was refluxed with 10% alcoholic potassium hydroxide for saponification for 4 hours then cooled and filtered. The saponified solution (filterate) was dried by evaporation. The residue was mixed with 20 ml distilled water and then extracted with sequent portions of diethyl ether (4 x 50 ml).

Collection and washing of the ethereal extracts were performed with distilled water several times till being free from alkalinity and then dehydrated by filtering over anhydrous sodium sulfate and finally, dried by evaporation, yielding yellow residue (0.515 g), (0.476 g) of *P. communis* L. and *M. domestica* (USM) respectively which later were subjected to GC/MS analysis.

3-HPLC analysis of beta-sitosterol

A solution of (1 mg/mL) was prepared from the *n*-hexane extracts in 40% of tetrahydrofuran and a solvent mixture (Acetonitrile: ethanol: water) (45:40:15) and analyzed by HPLC. The

method used for HPLC assay of beta-sitosterol was reported (Vorobyova et al. 2014**).** Beta-sitosterol reference standard (USP) stock solution of (1 mg/mL) was prepared in 40% tetrahydrofuran and the solvent mixture. Stock solution aliquots were diluted with the solvent mixture, and a calibration curve was created over the range of (1- 0.2 mg/mL) **fig (1)**.

The HPLC system was operated adopting the conditions described by **(Vorobyova et al. 2014),** the retention time (RT) of beta-sitosterol with these operating conditions was 4.33 min.

From the linear regression analysis, the leaves' and fruits' beta-sitosterol contents of the *n*-hexane extracts were expressed as mg/mL of the solution used.

Was calculated from the following equations:

A = 5510 C (equation 1)

Where, A = peak area,

C = corresponding concentration (mg/ml)

Calculation:

Beta-sitosterol content (SC) of dried leaves and fresh fruits material can be calculated from the following equation:

SC = C.V. m (equation 2)

Where,

SC = total content of beta-sitosterol compound in g of plant material.

C = concentration of beta-sitosterol established from the calibration curve of value (0.4 mg/mL) and (0.46 mg/mL) for *P. communis* L., *M. domestica* Borkh. dried leaves *n*-hexane fractions and, respectively.

V = dilution factor of the injected solution (1 mg/mL).

m = wt of *n*-hexane extract (mg/g) plant material which is (23.4 mg) and (10.5 mg) for dried *P. communis* L. and *M. domestica* Borkh leaves.

4-Preparation of *n*- hexane extracts emulgels:

Eight emulgels were prepared using, Carbopol 954 (1%) as gelling agent. The polymer was dispersed into a beaker containing the calculated amount of purified water and (0.2%) and (0.02%) of methyl paraben and propyl paraben, respectively as preservatives and stirred using a magnetic stirrer until no lumps were observed. Another beaker containing sesame oil (20%) as the oily phase, in addition tween 60 (1%) with brij L23 (1%), for the third combination we use tween 80 (1%) with brij 35 (1%) and the fourth combination includes tween 80 (1%) with brij L23 (1%) as emulsifying agents with addition of *n*-hexane fractions of pear and apple separately equivalent to 0.25% beta-sitosterol content according to the previous beta-sitosterol estimation, so we use pear leaves *n*-hexane fraction with (0.63%) and apple leaves *n*-hexane fraction with (0.54%) . Sesame oil with the added emulsifying agents and *n*-hexane fraction was then added to carbopol 954 beaker with continuous stirring to obtain the required emulgel .For emulgels containing carbopol 954, the polymer was sparse in the purified water and refrigerated overnight. In formulae containing carbopol 954 suitable amount of triethanolamine was added for neutralization of the free carboxylic acid groups of carbopol 954 to pH 6.5 - 7 ± 0.2 (Lucero et al. 1994).

5-Evaluation of the prepared emulgels:

The physical properties of the prepared gels were examined including visual inspection, spreadability, pH determination and rheological properties moreover, accelerated stability testing, biological evaluation including, antimicrobial activity and finally, wound healing activity.

A-Physical properties:

- Visual inspection:

The prepared formulations were physically examined for their characteristics namely; color, clarity, homogeneity and phase separation.

- Test for spreadability:

The emulgels spreadability was measured by spreading of 0.5 g of the gel on 2 cm diameter circle on a glass plate which is remarked and then a second glass plate was employed. Weight of half kilogram was permitted to rest on the upper glass plate for 5 min. The diameter of the circle was determined after spreading of the gel (Dantas et al. 2016).

- pH determination:

The prepared emulgels pH was measured for 10 % water solutions. The results are the average of three readings.

- Rheological properties assessment:

A rotational Brookfield viscometer of cone and plate structure was used for evaluation of the rheological properties (Arora et al. 2014) the following method was used:

The tested formula (about 0.5 g) was introduced to the plate and left till the temperature of the cone reached ($25^{o}C \pm 1^{o}C$). Measures were taken over a wide range of 0.1 to 1 rpm.

b- Accelerated stability testing:

The eight emulgel formulae were tested for their stability by centrifugation of 10 g of each formulation at 3000 rpm for 15 min (Simovic et al. 1998) and by the freeze-thaw cycle in which each formulation kept at -4 $^{\circ}C$ for 8 hours and then at 40 $^{\circ}C$ for 16 hours for 3 days (Shahin et al. 2011). The formulations which passed from the accelerated stability tests were then subjected to the biological evaluation.

6-Biological evaluation of the prepared formulations

a - Evaluation of antimicrobial activity

The agar disc diffusion method was used in the antimicrobial tests (Hindler et al. 1994).

Prepared emulgel formulations of pear and apple *n*-hexane fractions which passed from the accelerated stability tests were individually tested against *Aspergillus flavus*, *Candida albicans* as fungi and against *Staphylococcus aureus, staphylococcus epidermidis* (Gram + ve bacteria) and against *Escherichia coli*, *Salmonella typhimurium* (Gram – ve bacteria). Ketoconazole and gentamycin were used as standards for fungi and gram + ve and gram - ve bacteria, respectively, as positive controls. Antimicrobial activity was explained as inhibition diameter zones in millimeters (mm).

b- Evaluation of wound healing process

This activity was evaluated using excision wound method (Mukherjee and Suresh 2000, Esimone et al. 2006). Rats' anesthesia was carried out by chloral hydrate (300 mg/ kg body weight) via intra peritoneal injection. The dorsal surface of rat was shaved, cleaned with 70% ethanol. Excision wounds (approximately 22 mm diameter) were made by cutting out a

predetermined dorsal area of skin from the shaved area using pointed scissors and toothed forceps. The entire wound was left open**.**

Adult female rats were randomly divided into 4 groups. Each group consisted of 6 rats. The two chosen formulae were applied topically after the skin excision and continued daily for successive 14 days.

Topical application of one ml of the tested formulated gel to each animal once daily. All groups of animals were treated similarly. The animals' treatment was achieved according to the next scheme:

Group 1: Rats having wounds were kept untreated, served as control group.

Group 2: Rats having wounds treated topically with Mebo® ointment applied after excision of the skin, served as reference.

Group 3: Rats having wounds treated topically with the chosen emulgel formula of apple hexane extract (5.4 mg/ml) applied after excision of the skin.

Group 4: Rats having wounds treated topically with the chosen emulgel formula of pear hexane extract (6.3 mg/ml) applied after excision of the skin.

Wound constriction rate was measured as percentage of wound size reduction at every other day from each rat wound till wound closure. Transparency paper and a marker were used for monitoring of the gradual decrease in the wound size periodically then graphical assessment of the wound area was carried out to monitor wound closure percentage, which indicates the new epithelial tissue formation for wound coverage. Wound constriction was expressed as the original wound size reduction percentage (Mukherjee and Suresh 2000, Esimone et al. 2006). Calculation of the wound healing rate was carried out as the following:

The Percentage (%) wound contraction = (wound area on day 0 - wound area on day n) / wound area on day 0 × 100

7- Method for histopathological study

Rats' skin autopsy samples were taken from the different groups and then fixed in 10% formal saline for twenty four hours. Washing with tap water was done then serial dilutions of alcohol (methyl, ethyl and absolute ethyl alcohol) were used for dehydration. After specimens' clearance in xylene they were embedded in paraffin at 56 °C for twenty four hours in hot air oven. Paraffin bees wax tissue blocks preparation for sectioning at 4 microns thickness was carried out by sledge microtome. Collection of the obtained tissue sections was done on glass

slides which then deparaffinized and finally, stained by hematoxylin & eosin stain for light electric microscope routine examination (Banchrof et al. 1996).

The histopathological study was done on five groups:

Group (1): The negative control of normal non-injured rats' skin.

Group (2): The positive control of injured non-treated rats' skin.

Group (3): Rats' skin that treated by commercial product Mebo®.

Group (4): Rats' skin that treated by formula 3.

Group (5): Rats' skin that treated by formula 8.

The study was carried out as described in the methods. The results of the microscopical examination of the skin of the five groups were presented in **figures (3S-7S).**

Lists of figures

Fig (1S): Standard calibration curve of Beta-sitosterol

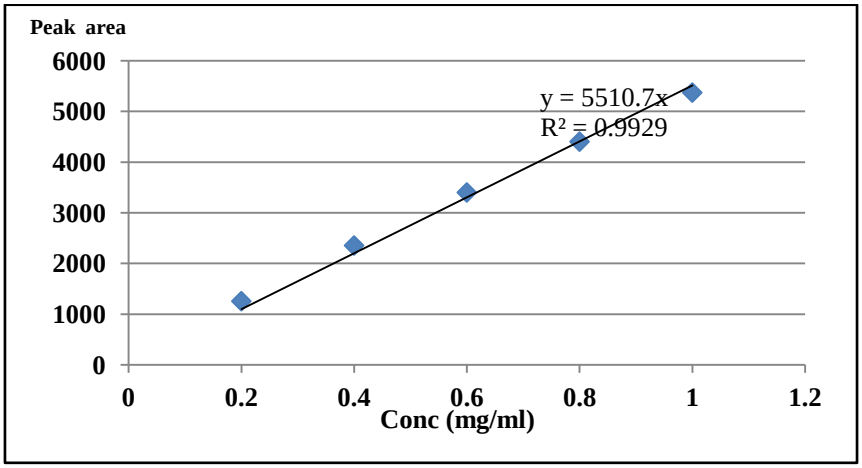


Fig (2S): Emulgel formulae

Fig (3S): Photographs of macroscopic appearance of wound excised from rats that were untreated (control), treated with mebo® and treated with F3 and F8 emulgels

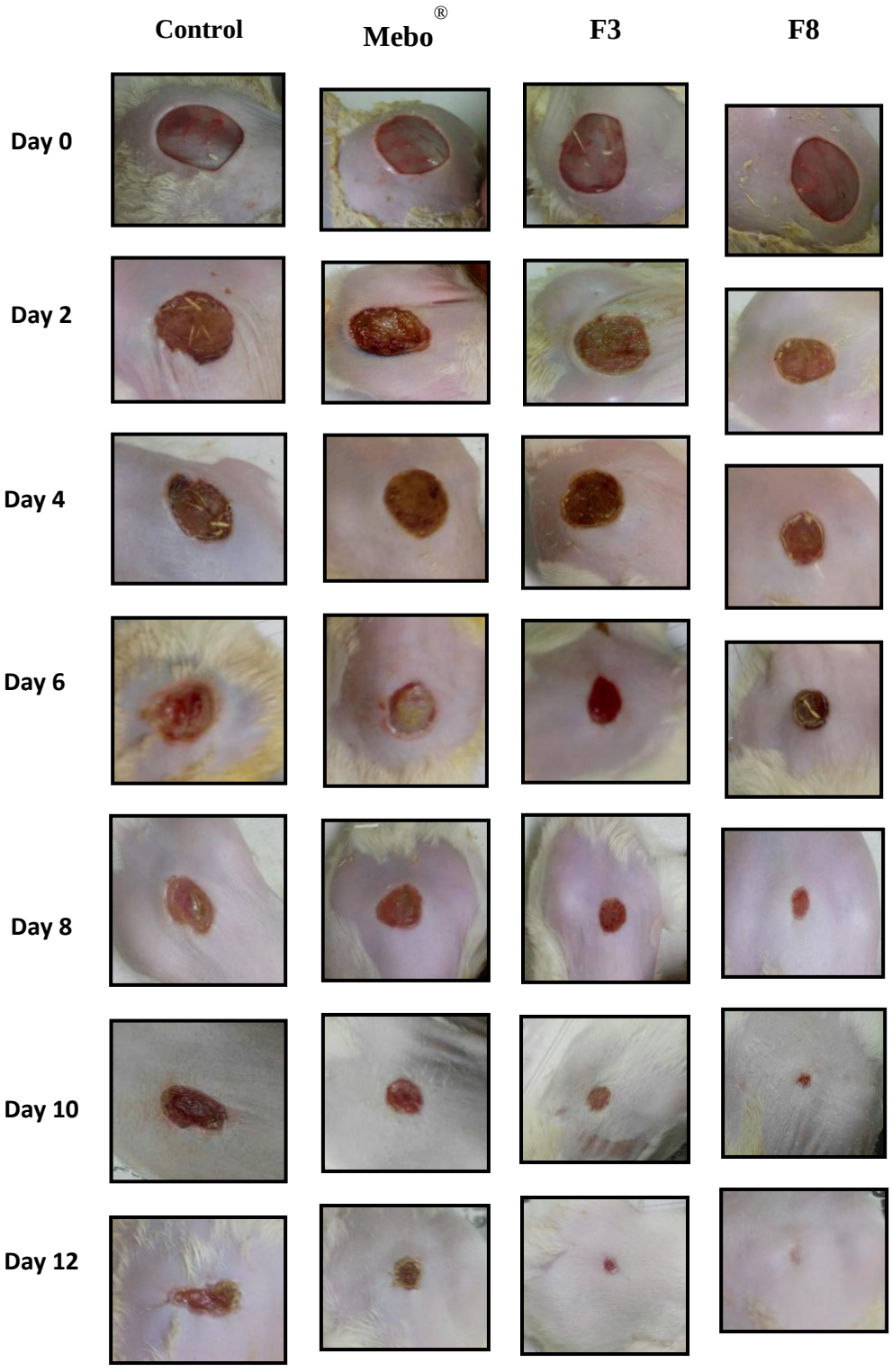


Fig (4S): Illustrated diagram of wound contraction in rats

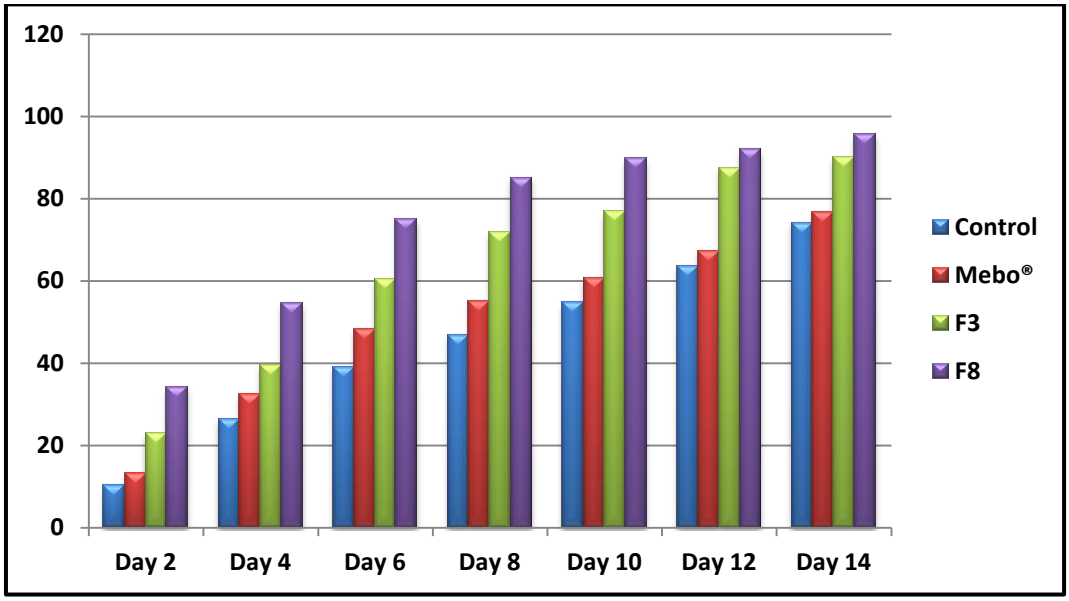
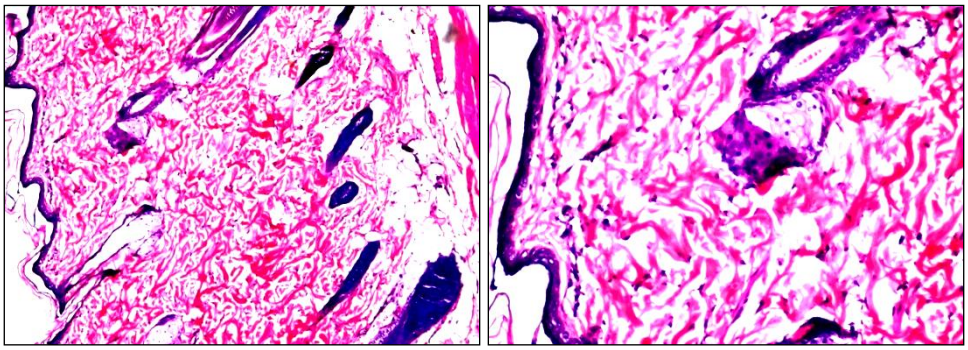
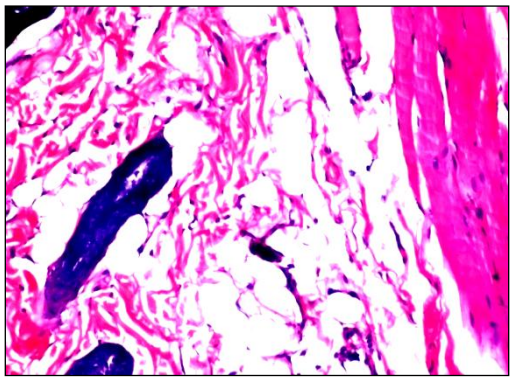


Fig (5S): Histology of skin of rats in group (1) H & E (hematoxylin & eosin stain)

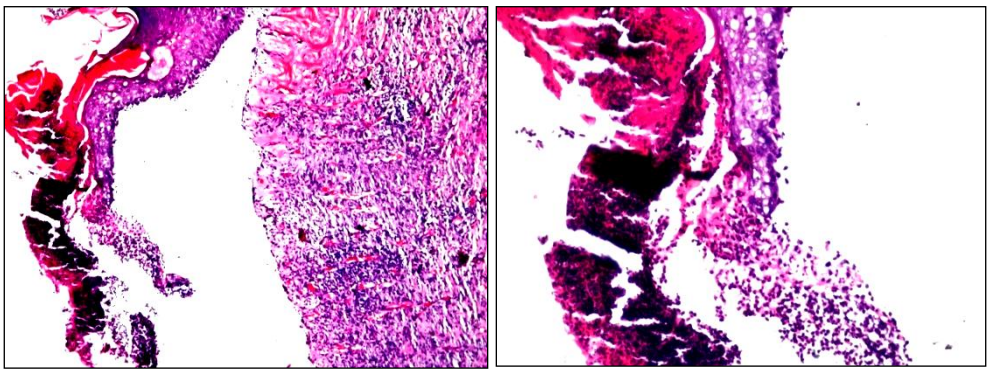


(A) H & E, X= 16 **(B)** H & E, X= 40

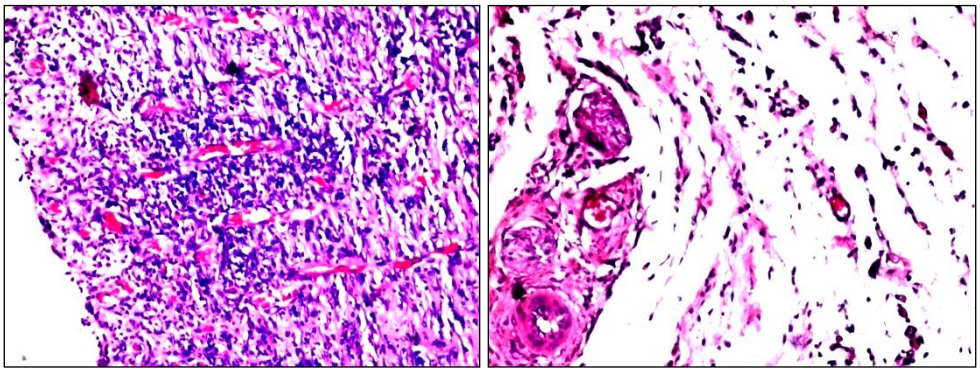


(C) H & E, X= 40

Fig (6S): Histology of skin of rats in group (2)

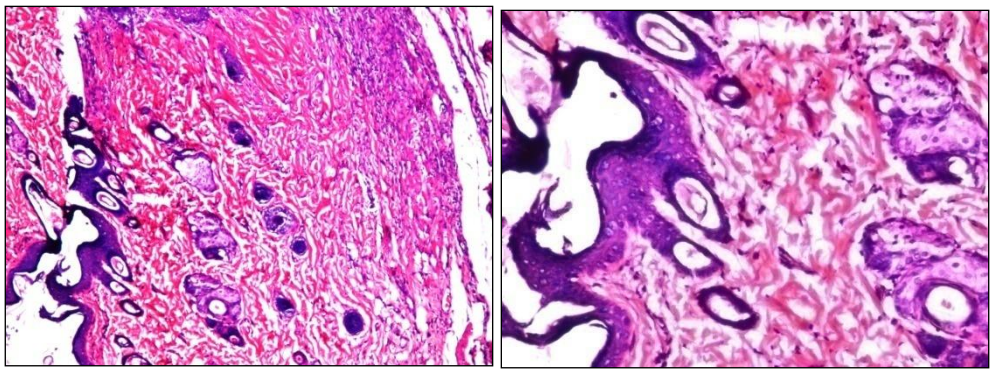


(A) H & E, X= 16 **(B)** H & E, X= 40

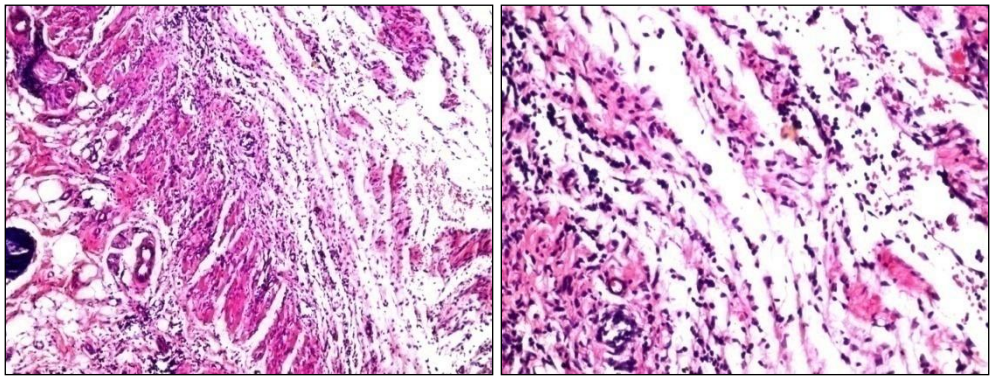


(C) H & E, X= 40 **(D)** H & E, X= 40

Fig (7S): Histology of skin of rats in group (3)

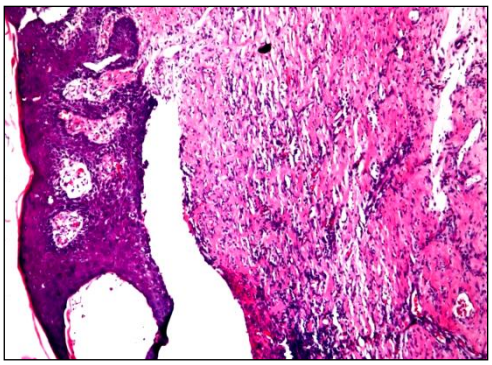


(A) H & E, X= 16 **(B)** H & E, X= 40

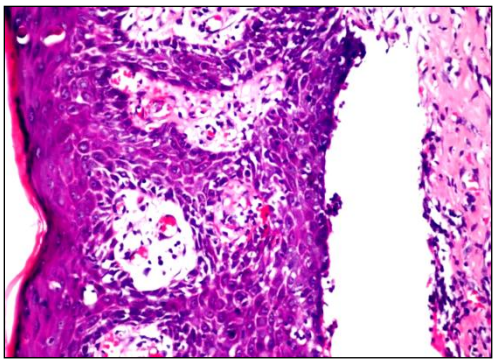


(C) H & E, X= 16 **(D)** H & E, X= 40

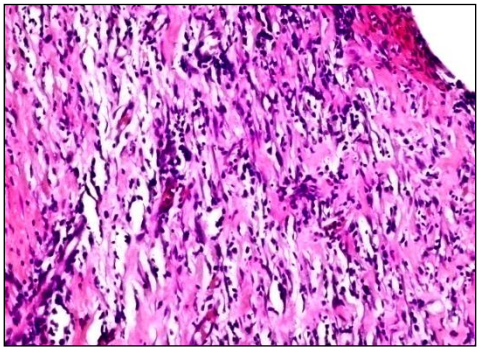
Fig (8S): Histology of skin of rats in group (4)



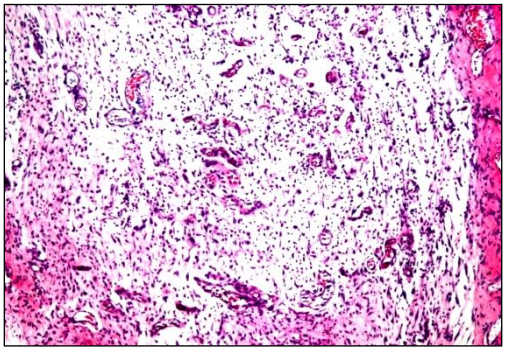
(A) H & E, X= 16



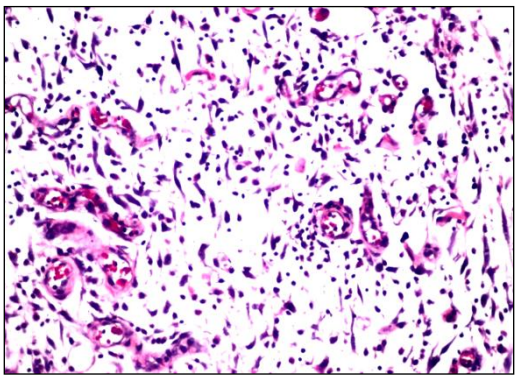
(B) H & E, X= 40



(C) H & E, X= 40

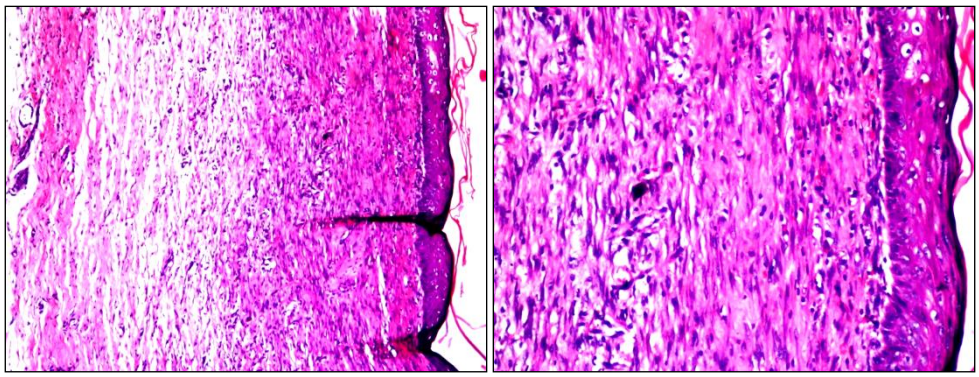


(D) H & E, X= 40

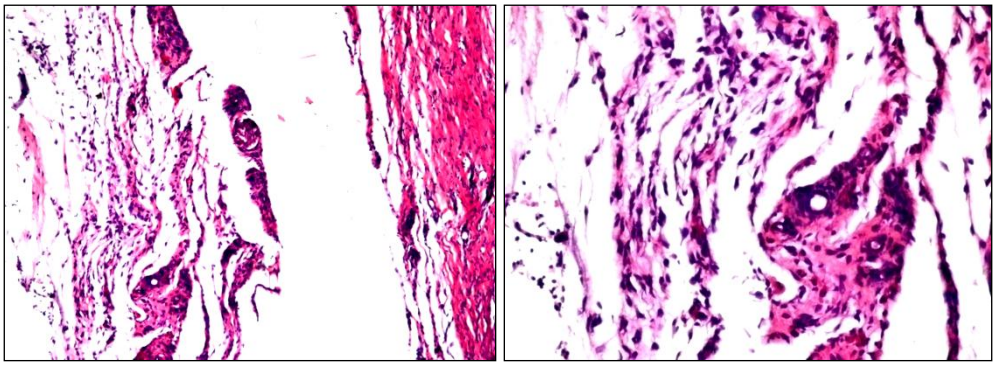


(E) H & E, X= 40

Fig (9S): Histology of skin of rats in group (5)



(A) H & E, X= 16 **(B)** H & E, X= 40



(C) H & E, X= 16 **(D)** H & E, X= 40

List of tables

Table (1S): GC/MS analysis of USM of leaves and fruits of *P. communis* L. *and M. domestica* Borkh.

No.	Compound	*P. communis* leaves	*M. domestica* leaves	RRT*
1	1,3-dimethyl cyclohexane	-	4.5	0.06
2	1,4-dimethyl cyclohexane	-	5.1	0.061
3	Octane	-	9.7	0.07
4	Phytol	4.7	25.7	0.66
5	5-methyl tricosane	-	9.8	0.74
8	Squalene	11	3.9	0.87
9	Nonacosane	0.7	-	0.88
10	Betulin	3.9	-	0.88
11	Stigma-3, 5-diene	4	-	0.905
13	Hentriacontane	0.21	-	0.927
16	Alpha-tocopherol	3	-	0.94
19	Beta-Sitosterol	64	26.7	1
20	Lanosterol	1.8	-	1.01
21	Cycloartenol	0.62	-	1.025
22	Lupeol	5	-	1.03
% of Identified compounds		99%	85.4%	

RRT*: relative retention time to Beta-sitosterol =1 with RT= 57.1 min.

Table (2S): Results of estimation of the Beta-sitosterol content in pear and apple dried leaves and their *n*-hexane extracts

Plant extract	Peak area	wt *n*- hexane ext. mg/g of plant material	SC in mg/g plant dry wt
pear leaves	2141	23.4	9.4
apple leaves	2528	10.5	5

SC: Beta-sitosterol concentration

Table (3S): Composition of *n*-hexane fractions emulgels formulated with emulsifying agents.

Formula No.	C_{954} (%)	Tween 60 (%)	Brij 35 (%)	Tween 80 (%)	Brij L23 (%)	PH (%)	AH (%)	TEA
1	1	-	1	1	-	-	0.54	q.s
2	1	-	-	1	1	-	0.54	q.s
3	1	1	1	-	-	-	0.54	q.s
4	1	1	-	-	1	-	0.54	q.s
5	1	-	1	1		0.63	-	q.s

6	1	-	-	1	1	0.63	-	q.s
7	1	1	1	-	-	0.63	-	q.s
8	1	1	-	-	1	0.63	-	q.s

C_{954}: Carbopol 954

Brij L23: polyethylene (23) lauryl ether

Brij 35: Polyoxyethylene (23) lauryl ether

N.B: Water was added to make 100 g of each emulgel formula.

PH: pear leaves hexane extract.

AH: apple leaves hexane extract.

TEA: Triethanolamine.

Table (4S): Physical evaluation of the prepared emulgels

Formula code	Appearance	pH	Spreadability	η at Minimum Shear Rate (cp)	η at Maximum Shear Rate (cp)
F1	Yellowish green homogenous emulgel	6.98	5.2	65662	13155
F2	Yellowish green homogenous emulgel	7	7.4	27486	14760
F3	Yellowish green homogenous emulgel	6.44	6.4	86328	31611
F4	Yellowish green homogenous emulgel	6.04	6.46	59906	22871
F5	Yellowish green homogenous emulgel	6.35	5.23	77412	22812
F6	Yellowish green homogenous emulgel	6.05	4.63	140000	24460
F7	Yellowish green homogenous emulgel	6.8	6.84	81356	18693
F8	Yellowish green homogenous emulgel	6.05	5.8	247000	42792

Table (5S): Antimicrobial activity of the prepared formulae

Tested microorganisms	Formulations					Control
	F2	**F3**	**F4**	**F7**	**F8**	
Aspergillus flavus	NA	8	NA	NA	NA	Ketoconazole 16
Candida albicans	NA	NA	NA	NA	NA	20
Staphylococcus aureus	NA	NA	NA	8	9	Gentamycin 24
Staphylococcus epidermidis	NA	NA	NA	NA	NA	28
Salmonella typhimurium	NA	NA	NA	NA	NA	17
Escherichia coli	NA	NA	NA	NA	NA	30

NA: no activity, Ketoconazole (100 µg/ml), Gentamycin (4 µg/ml).

Concentration of the samples tested was 10 mg/ml concentration.

Table (6S): Wound contraction in rats treated with Mebo® and the prepared emulgels

% of Wound Contraction	Control	Mebo®	F3	F8
Day 2	10.31± 1.836	13.02± 3.096	22.83± 1.481[abc]	34.22± 1.485[ab]
Day 4	26.47± 2.535	32.45± 4.330[a]	39.56± 1.082[abc]	54.46± 2.246[ab]
Day 6	38.96± 1.660	48.04± 2.810	60.27± 0.9281[ab]	75.00± 2.064[ab]
Day 8	46.84± 3.383	55.22± 1.072	71.74± 2.392[abc]	85.11± 1.575[ab]
Day 10	54.55± 3.192	60.56± 3.111	76.88± 2.754[ab]	89.70± 0.4622[ab]
Day 12	63.52± 3.415	67.27± 1.532	87.44± 0.4714[ab]	91.96± 1.365[ab]
Day 14	74.08± 1.814	76.56± 1.355	90.00± 0.577 [ab]	95.55± 0.774 [ab]

Each value indicates the mean SEM of (6) observations. [abc] $P < 0.05$ compared to control, MEBO®, and formulation 8 groups, respectively.

One way ANOVA was used for statistical analysis followed by Tukey-Kramer Multiple Comparison Test.

[a] Significant from control group at $P < 0.05$.

[b] Significant from Mebo® group, [C] significant from F8 group at $P < 0.05$.

Table (7S): The histopathological alterations severity in the skin of different experimental groups

Skin layer	Group no. / Histopath. alterations	1	2	3	4	5
Epidermis	Focal ulceration and necrosis	-	+++	-	-	-
	Acanthosis	-	-	++	+++	+
Dermis	Newly formed capillaries	-	++	-	-	-
	Inflammatory cell infiltration	-	+++	-	++	-
	Fibrosis	-	-	-	-	++
	Granulation tissue formation	-	-	-	+++	-
Subcutaneous tissue	Oedema	-	++	+	++	+
	Inflammatory cell infiltration	-	++	++	++	+

(+++): Sever (75-100%), (++): Moderate (50-75%), (+): Mild (25-50%), (-): Nil (0-25%).