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Biomaterials Based on Essential Fatty Acids and Carbohydrates for Chronic Wounds

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ABSTRACT

Chronic wounds such as decubitus ulcer remain challenging due to their integrated and overlapping phases. The matrix metalloproteinases (MMPs) enzymes, whose main function is to degrade all kinds of extracellular matrix (ECM) proteins, aid cellular migration and extracellular remodeling. MMPs, in the wound bed, allow the lysis of the dead tissues, by which the macrophages task becomes easier to digest the dead cells. MMPs activities should be monitored and inhibited as the healing process proceeds. If MMPs are not inhibited in time, they will break down tissue to attack the ECM itself creating chronic wounds. In the current work, conjugated linoleic acid (CLA) and ricinoleic acid (RA) are extracted from commercial oils as MMPs inhibitors. A pharmaceutical carrier is formulated containing chitosan fine particles, impregnated silver nanoparticles into microcrystalline cellulose, CLA and RA. Carrier and the active ingredients were prepared and characterized by spectral and morphological analysis. The final formulation was examined for antimicrobial, cytotoxicity, and in-vivo wound healing activity. Results showed a strong inhibitory activity against the tested pathogenic microorganisms for the silver contacting samples. The rates of wound closures during wound healing in diabetic male-rats of formulas containing ricinoleic acid was faster than that containing conjugated linoleic acid.

INTRODUCTION

Chronic wounds such as diabetic foot ulcer and decubitus ulcer are common problems in people suffering from type 2 diabetes. These can cause pain, nerve damage, leading to eventual foot or leg amputation. In the pathological view, chronic wounds are those in which the body cannot heal the wound to limit the area of air exposure and microbial invasion in normal time. Generally speaking, a chronic wound is the wound that has remained unhealed for more than six weeks. In chronic wounds, there is many factors responsible for impairing the ability of the body to heal the wound and maintain the skin integrity such as; impaired venous drainage; metabolic abnormalities; and genetic disorder (Anders *et al.*, 2010; Bansal, *et al.*, 2005). Wound healing is a very complicated process of a well-organized sequence of biological events. Much has been published (Benbow, 2007; Vowden and Vowden, 2002) on wound healing and show that the wound healing process, either in the acute (normal) or chronic wounds, must go through basic phases. Hemostasis, inflammatory phase, the proliferative phase and the maturation phase are the main four phases of the wound healing (Lazarus *et al.*, 1994). The second stage, the inflammation phase which is the most important phase is associated with edema, warmth, and pain. The inflammatory response causes the blood vessels to leak blood plasma into the surrounding tissue in the injured part. The inflammation phase is characterized by influx of white blood cells (leukocytes and macrophages) which are needed to destroy bacteria, cleanse the wound of debris and stimulate the biochemical actions that are necessary for repair. Macrophages (specialized white blood cells) are considered to be

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local biochemical control centers for healing, whereas their role is mainly focused on digesting cellular debris and stimulate any other immune cells to respond to the pathogens (any agent that can cause disease). The overall function of inflammation is to neutralize and destroy any toxic agents at the site of injury and to restore the tissue homeostasis (Gurtner *et al.*, 2008b).

In the normal wound healing, the inflammatory cells work to clean out the wound bed of dead cells. The matrix metalloproteinases (MMPs) enzymes belong to the proteases family whose main function is to degrade all kinds of extracellular matrix (ECM) proteins, aid cellular migration and extracellular remodeling. It is believed that MMPs presence in the wound bed to allow lysis of dead tissue, by which the macrophages task becomes easier to digest the dead cells. MMPs should be inhibited and decrease their activities as the healing process proceeds. If MMPs are not monitored and inhibited in time, they will break down tissue and attack the ECM and that is the main reason for chronic wounds, such as ulcers (Von Reedern *et al*., 1998). It has been investigated that the addition of acid-based inhibitors (malonic, boronic, etc.) will decrease the MMPs activity in the wound site. It is indicated that by slightly increasing the acidity of the wound environment, MMP levels may be reduced, consequently decreasing the inflammatory level (Boyle, 2008; Rushton, 2007).

Recently, essential fatty acids (EFA), whose basic structures are a carboxylic acid group with a long saturated or unsaturated linear chain, have been reported to stimulate the wound healing process. EFA such as linoleic and ricinoleic acids have a broad spectrum of activity for healing which are necessary for the maintenance of epidermal integrity in the skin. Declair *et al*. (Declair, 1997) studied the topical application of EFAs to improve the hydration and elasticity of the skin, help prevent skin breakdown in patients with poor nutritional status, and may repair damaged skin.

The current work aims to produce wound healing carrier, made of microcrystalline cellulose (MCC) loaded by MMPs inhibitors and antimicrobial agents, to reduce the chronic wound risks. Linoleic and ricinoleic acids will be used to decrease the MMPs activity and the chitosan fine particles to inhibit the bacterial invasion.

MATERIALS AND METHODS

Materials

Castor oil and sunflower oil was obtained from a local grocery (Crystal, Egypt) were used. Silver nitrate, ethyl alcohol, acetone, urea, sodium hydroxide, potassium hydroxide, magnesium sulfate, ethyl acetate (EtOAC) were purchased from sigma and used without further purification. Cotton linter; trade name Medical cotton: Egyptian company of weaving and spinning in Mahalla El Kobra was used.

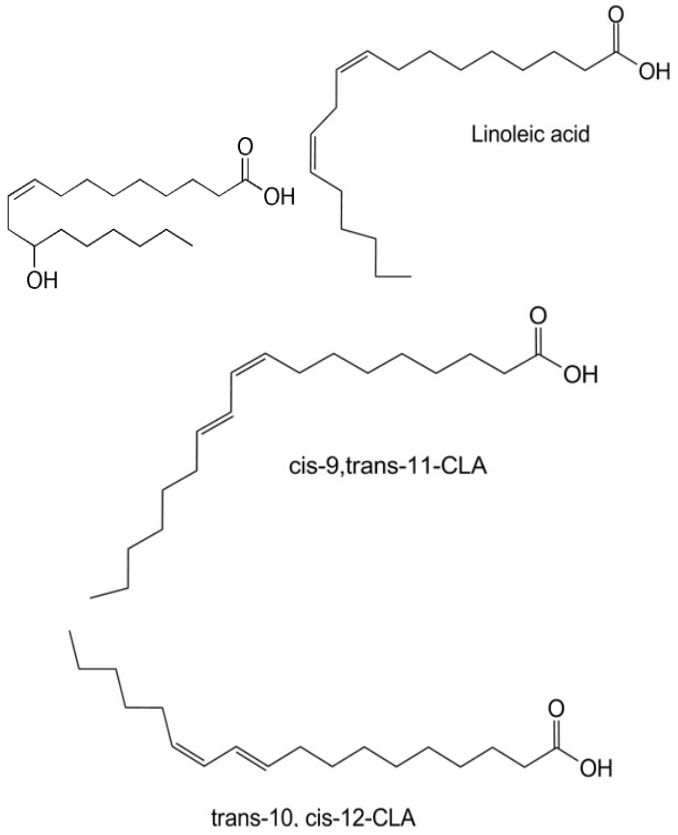
Methods

Preparation of ricinoleic acid from castor oil

Pure ricinoleic acid (RA) can be obtained by using alkali treatment of commercial castor oil (cold processed) in the presence of alcohol and followed by an acidification step to liberate free RA. Preparation of RA was carried out using the method described elsewhere (Vaisman *et al*., 2007).

Preparation of conjugated linoleic acid (CLA)

Conjugated linoleic acid was synthesized by alkali isomerization of extracted and purified linoleic acid (LA). Extraction and purification were carried out according to the method described by Nada *et al*. (2011). CLA was chemically synthesized from the purified LA as described by Yeong *et al*. (Kim *et al.*, 2005). Chemical structures of the prepared free fatty acids are shown in Scheme (1).



Scheme. 1: Chemical structure of ricinoleic acid, Linoleic acid and its conjugated isomers (cis-9, trans-11 CLA–trans–10, cis–12–CLA)

Preparation of Microcrystalline cellulose (MCC)

Microcrystalline cellulose (MCC) was prepared by using the acid hydrolysis procedure of cotton linter. Typically, 10 gm of cellulose sources (cotton linter; trade name Medical cotton: Egyptian company of weaving and spinning in Mahalla El Kobra; and/or cellulose powder, purchased from sigma), which were used with no additional purification, was placed in 1-L three-necked round-bottomed flask containing a Tefelon-coated magnetic stirring bar, a cooling condenser, and 300 mL of 2.5 M solution of (smoking) hydrochloric acid at 105 ^{o}C for 1 hour. The reaction was stopped by neutralization using sodium bicarbonate solution and cooling in ice-bath for 5 min. The white suspension was filtered, and the filtrate was collected, air-dried and stored at 4^{o}C for further use (Battista and Smith, 1962)

Impregnation Ag-NPs into MCC

MCC was prepared according to the method of Abou-Zeid *et al*. (2015) In typical procedure, 10 g MCC powder was suspended in 1000 mL of 0.005 M silver nitrate and sonicated for 10 minutes. D-glucose (2 g/l) was added to each mixture and stirred for 6 hours. After 6 hours the mixture was allowed to settle down and the excess reaction mixture was decanted off. The AgNPs impregnated cellulose materials was washed with deionized water and dried in a vacuum oven at 30 °C overnight.

Preparation of chitosan fine particles

In our studies, chitosan different-sized particles were prepared by mixing chitosan solution with gelatin (type A). In typical procedure, 0.5% chitosan was prepared by using 2% of acetic acid solution, then, chitosan solution was mixed with gelatin solution 1%. The mixture was poured dropwise into dispersion medium (commercial oil). The dispersion was stirred with magnetic stirrer in range 600 rpm at room temperature over night. At the end, the microspheres were collected by filtration and washed by hexane several times. The microspheres were air-dried and kept at 4 ^{o}C for further uses.

Preparation of the curing formulations

Cream and ointment are pharmaceutical carriers of a medication for topical use (on the skin) that contains a water base or oil base system respectively. In general, creams and ointments comprise different proportions of oil to water. Ointments have about 80 % of oil and are known as oil in water (o/w) emulsion, while creams have about 50% or less oil and are known as water in oil (w/o) emulsion. In emulsions the more oil, the stickier and greasier the final product. Therefore, creams may work better on larger areas of the skin because of their "spreadability" factor, compared to ointments. Such emulsions are unstable and will be broken down into two separate phases by time. Therefore, thickening agent is necessary to stabilize such formulations. Thickeners can be added to either the oil phase or the water phase.

Thickeners like coconut oil, bees wax and glycerol are added to the oil phases while thickener like polypropylene glycol is added to the water phases (Adeyeye *et al*., 2002).

Antibacterial and antifungal activity

Four common pathogenic microorganisms strains from the Chemistry of Natural and Microbial Products Dept., Pharmaceutical Industries Div., National Research Centre, Cairo, Egypt were employed. They include *Bacillus subtitls* (*B. subtilis*) as Gram-positive (G +ve) bacteria: and *Escherichia coli* (*E. coli*) as Gram-negative (G-ve) bacteria;yeasts such as *Candida albicans* and fungi (*Asperagillus niger*).

The antimicrobial activities of the cream and ointment samples which are loaded by the active ingredients (conjugated linoleic acid, ricinoleic acid, chitosan/gelatin beads, and silver nanoparticle impregnated microcrystalline cellulose) were evaluated using agar disc diffusion assay. Briefly, a 24 hours old culture of bacteria and 48 hours old culture of fungi was mixed with sterile physiological saline (0.9%) and the turbidity was adjusted to the standard inoculum of Mac-Farland scale 0.5 (10^6 colony forming units (CFU) per ml). Petri dishes containing 25 mL of Mueller Hinton assay medium (Lab M., Bury, Lancashire, UK) and Sabouraud-dextrose agar (Lab M., Bury, Lancashire, UK) was used for antibacterial and antifungal activity respectively. The inoculums were spread on the surface of the solidified media. Subsequently, pure oil, cream and ointment samples were warmed up to 40^{o}C and an amount of 40 µL/disc of each solution was added to filter paper discs (6 mm in diameter) to be placed on the inoculated plates. Standard antibiotics (Streptomycin (S-10 µg), Tetracyciline (TE- 30 µg), Neomycin (N-30µg) and Nystatin (NY-100 µg)) were used as positive control for bacteria and fungi respectively.

Diameter of inhibition zone was recorded in millimeters after incubating bacterial strains at 37°C (24 h) and fungal strains at 25°C (48 h). Plates were examined for evidence of antimicrobial activities, represented by a zone of inhibition of microorganism's growth around the paper disk and diameters of clear zones were expressed in millimeters (mm) (Rashad *et al*., 2005).

In our studies, the both cream and ointment were prepared by mixing (using regular hand mixer) the oil phase and the water ingredients separately and heated to 70 ^{o}C. The aqueous phase and the oil phase ingredients are listed in Table 1.

Table 1: Curing formulations.

	Cream samples contain						**Ointment samples contain**						**Pure fatty acids contain**		
	Group –a						**Group -b**						**Group - c**		
Code	**a1**	**a2**	**a3**	**a4**	**a5**	**a6**	**b1**	**b2**	**b3**	**b4**	**b5**	**b6**	**c1**	**c2**	**c3**
LA(g)	10	10	10	10	10	10	18	18	18	18	18	18	26	26	26
H_2O(g)	13	13	13	13	13	13	6	6	6	6	6	6	0	0	0
CLA(g)	1.5	3	0	1.5	3	0	1.5	3	0	1.5	3	0	1.5	3	0
RA(g)	1.5	0	3	1.5	0	3	1.5	0	3	1.5	0	3	1.5	0	3
BW (g)	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	0	0	0
Chit-b (mg)	200	200	200	200	200	200	200	200	200	200	200	200	0	0	0
Ag-(mg)	0	0	0	200	200	200	0	0	0	200	200	200	0	0	0
pH	-	-	-	-	-	-	-	-	-	-	-	-	4.8	4.2	3.8

LA, linoleic acid; H_2O, water; CLA, conjugated linoleic acid; RA, ricinoleic acid; BW; Bees wax, Chito-B, chitosan/gelatin beads; Ag, microcrystalline cellulose impregnated with silver nanoparticles.

Cytotoxicity assessment

The cytotoxicity test was conducted as described by Mosmann (Mosmann, 1983) using the hTERT-BJ1 human immortalized skin fibroblasts cell line. The choice of fibroblast cell line was based on their role on producing the protein associated in extracellular matrix (ECM) synthesis and their crucial role in wound healing process. hTERT-BJ1 cell line was kindly provided by Prof. Stig Linder, Karolinska Institute, Sweden. Cells were maintained in DMEM:F12 Medium/10% FBS and were incubated at 37°C in 5% CO_2 and 95% humidity. Cells were sub-cultured using trypsin versene 0.15% . The culture samples (ricinoleic acid, conjugated linoleic acid and cream/ointment) were warmed up to 40^{o}C and an increasing amounts of each sample (10, 20 and 30 µL) was added to a filter paper discs (6 mm in diameter) to be placed on the seeded cells. 100 ppm adriamycin (induces cell death) was used as positive control and unloaded filter disc was used as negative control. Prepared discs were sterilized under ultraviolet (UV) light for 20 min each side in a laminar flow chamber. BJ1cells were seeded into wells, in 96-well plate, at a density of 30000 cells per well. After incubation for 24 hrs, prepared and sterilized discs, in triplicates, were placed into wells and left for 24hrs (Cheng *et al.*, 2014).

The culture samples were taken off and medium was aspirated and replaced by 50 uL of MTT solution. Then, the solution was incubated for 4 hrs at 37^{o}C. The solution was aspirated and 200 µL of DMSO (Dimethyl sulfoxide) containing 125 µL of glycine buffer (pH= 10) was added to dissolve the formazan crystals. The solution was shaked for 10 min to obtain a clear DMSO solution. The absorbance of the DMSO solutions at 595 nm was measured using a UV-Vis spectrophotometer (Varian Cary 3E UV-Visible Spectroscopy). Cell viability was calculated according to the following equation (El-menshawi *et al*., 2013)

$$[av(x)/av(NC)]x100$$

Where: Av: average, X: absorbance of sample, NC: absorbance of negative control. Absorbance was measured at 595 nm with reference 690 nm.

In-vivo test

The preclinical studies of the produced formulations either for creams or ointments are crucial in which samples will be examined on animal models and in controlled laboratory environment to simulate the clinical environment. Experiments perform on animal models have to provide justifiable comparisons to the human beings. However, the question of what animal model best mimics the human chronic wounds has been a subject of debate by investigators, peer review panels, and editors of journals. The similarities in wound development and wound healing of chronic wounds make diabetic wounds of male rats a relevant model for simulating the chronic wounds (Salcido *et al*., 2007).

Experimental Animals

Male rats weighing 100– 120 g were used for *in vivo* studies and obtained from the animal house in the national research center, Cairo, Egypt. The rats were housed in polypropylene cages in standard environmental conditions and were fed commercial rat feed and water ad libitum. All rats were handled in accordance with the NIH guidelines for the care and use of laboratory animals and with the recommendations of the institutional ethical committee.

Induction of diabetes

Diabetes was induced in rats by a single intraperitoneal injection of streptozotocin (70mg/kg body weight) in 0.1 M citrate buffer, pH 4.0. Fasting blood glucose levels were checked (with glucose oxidase reagent strips) 3 days after streptozotocin injection, and animals with glucose levels greater than 200mg/dl were used for the study (Chithra *et al*., 1998).

Wound creation

Wounds were created on the 3th day after induction of diabetes. Excision wounds were used for the study of wound contraction. All wounds were of full-thickness type extending up to the adipose tissue. Rats were anaesthetized by open mask method with anaesthetic ether and their backs were shaved with electric clippers. Excision wounds of size (about) 1 cm^2 were made by cutting 1 x 1 cm piece of the skin from the shaven area in each side (Chithra *et al.*, 1998).

Grouping of animals

After wound creation, experimental animals were divided into four groups as follows: Group a- (wounds treated by cream curing formulations), Group b- (wounds treated by ointment curing formulations), Group c- (wounds treated by pure fatty acids), Group d- (wounds treated with standard cream (Dermazin) as positive). Each rat has two identical excision wounds on the shaven back to be used for the treated and untreated trails.

Histopathological test

Animals were sacrificed on the tenth day after wound creation and the entire wound on each animal was cut out. Specimens of all animals were dissected immediately after death and fixed in 10% neutral-buffered formal saline for 72 hours at least. All the specimens were washed in tap water for half an hour and then dehydrated in ascending grades of alcohol (70% - 80% - 90% and finally absolute alcohol), cleared in xylene, impregnated in soft paraffin wax at 55 °C and embedded in hard paraffin. Serial sections of 6 µm thick were cut and stained with Haematoxylin and eosin (Carleton *et al*., 1980) for histopathological investigation. Images were captured and processed using Adobe Photoshop version 8.0.

Statistical analysis

Results were expressed as a mean value with its standard deviation (mean ± S.D.) of each sample that is repeated three times (n=3). Statistical analysis was performed with student's t-test and differences were considered as significant at p-values under 0.05.

RESULTS

Rheological properties of the cream and ointment emulsions

Emulsion viscosity is an important performance characteristic of cream and ointment emulsions. In low viscosity emulsions are likely to run off the treating surfaces, whereas too viscous emulsions may not distribute well over the surface. Viscosity is also related to other important properties like settlement - low viscosity emulsions are subjected to settle (separate) during storage (James, 1997). Creams and ointments were prepared by using extracted linoleic acid as oil phase and bees wax as thickening agent with distilled water. Bees wax in different concentrations was used to produce different viscosities of both cream and ointment emulsions in order to provide stabile emulsions. Table 2 shows the dynamic viscosity cream and ointment emulsions as it increases with increasing the bees wax concentrations. C1 and T1 were used for further use.

Table 2: Formulations of cream and oil emulsions and their viscosity.

	Cream			Ointment		
Code	**C1**	**C2**	**C3**	**T1**	**T2**	**T3**
LA(g)	50	50	50	80	80	80
H_2O(g)	50	50	50	20	20	20
Bees wax (g)	5.6	2.8	1.4	5.6	2.8	1.4
Viscosity (Pa.s)	322	151	82	126	52	6.4

* Viscosity was measure at 1 shear rate(1/s), 25°C.

Antimicrobial activity

The antimicrobial activities of the produced cream/ointment and its constituents compared to standard antibiotics against different microorganisms are shown in Table 3. It is clear from the results that sample (1) which contains silver nanoparticles has a good antimicrobial activity against gram positive bacteria (*Bacillus Subtilis*) and a weak inhibition zone with *Candida albicans*. The former phenomenon may attribute to the complicated structure of the outer cell wall of *Bacillus subtilis*, *Candida albicans*, and *Aspergillus niger* which is considered as a potential barrier against foreign molecules.

While sample (5) that represents the final formula containing the active ingredients (conjugated linoleic acid, ricinoleic acid, chitosan/gelatin beads, and silver nanoparticle impregnated microcrystalline cellulose) shows better antimicrobial activities against all microorganism used. However, samples (2, 3 and 4) which represent the conjugated linoleic acid, ricinoleic acid and the carrier emulsion respectively did not show any antimicrobial activities against any microorganisms used in the test. As seen from the results, final formula shows more effective inhibition zones in all microorganisms. In general, silver and chitosan containing samples showed a moderate inhibitory activity compared to the standard antibiotics. This due to the quick diffusion the used antibiotics to the agar plate than the studied samples (Kumbar *et al*., 2014).

Table 3: Antimicrobial activity (zone of inhibition in mm) of produced cream/ointment and its constituents compared to standard antibiotics against different pathogenic microorganisms.

	Microorganisms and inhibition zones diameters (mm)			
Sample name	***B. subtilis***	***E. coli***	***C. albicans***	***A. niger***
1	10	---	8	---
2	---	---	---	---
3	---	---	---	---
4	---	---	---	---
5	12	10	12	12
Standard antibiotics				
S (10µg)	14	12	--	--
TE (30µg)	18	23.5	--	--
N(30 µg)	--	--	16	15
NS(100 µg)	--	--	--	15

Impregnated microcrystalline cellulose with silver nanoparticle (1), conjugated linoleic acid (2), ricinoleic acid (3), carrier emulsion, moderate viscosity of the cream (C1), (4), and the final formula, containing the active ingredients (conjugated linoleic acid, ricinoleic acid, chitosan/gelatin beads, and silver nanoparticle impregnated microcrystalline cellulose), (5), Streptomycin (S), Tetracyciline (TE), Neomycin (N), and Nystatin (NY).

Cytotoxicity test

Biocompatibility of the produced cream and its constituents was demonstrated by fibroblast cell viability and material toxicity is defined as greater than 30% cell death (A. A. Nada *et al.*, 2014). Results shown in Figure (1) reveal that silver nanoparticles- impregnated microcrystalline cellulose (Ag) has adverse effect on cell viability especially with higher amounts (20 µL and 30 µL). Data also investigated the influence of the conjugated linoleic acid (CLA) on cell viability in increasing amounts (10-30 µL). Results demonstrated that CLA at lower amount (10 µL) enhanced the cell viability. However at higher concentrations, CLA showed an adverse effect on cell viability decreased from 76% to 33% cell survival of CLA 20 and 30 µL respectively.

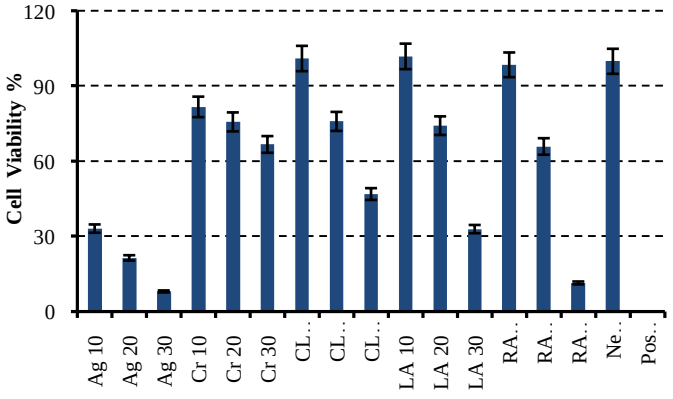


Fig. 1: hTERT-BJ1 Cell viability in percentage with selected samples (Ag, CLA; conjugated linoleic acid, LA; linoleic acid, RA; ricinoleic acid, negative control; cellulose filter paper, positive control; 100 ppm adriamycin). Biocompatibility of the produced cream and its constituents was demonstrated by fibroblast cell viability. Silver nanoparticles- impregnated microcrystalline cellulose (Ag) has adverse effect on cell viability while LA, RA and CLA at lower amount (10 µL) enhanced the cell viability.

Likewise, LA and RA at 10 µL showed nontoxic nature while higher amount (30 µL) exhibited cell survival only 33% and 11.5% respectively.

In-vivo test

The curing formulations were prepared as described in Table 1. Each rat received about 1 g of the curing formulation, once a day, on the wound surface. Readings were taken at time points 0-2-6- and 10 days. The biocompatibility was evaluated in male rats with fresh wound in area size of 1 cm^2 Fig (2a). The wound areas of treated and untreated wounds are recorded in Table 4 in which readings were taken in days 1, 2, 6 and 10. In Figure 3, the wound contraction in percentage indicates that pure fatty acids formulations are superior to either the cream or ointment samples. For the cream formulations, samples with silver nanoparticles showed better results for the all combinations (cream and ointment) while samples with ricinoleic acid (RA) induced the wound contraction higher than those with conjugated linoleic acid (CLA) and higher than those with RA and CLA mixture. The same trend was noticed in ointment and oil formulations. To extend, pure RA showed wound contraction more than 90 % after ten days while pure RA and CLA mixture showed 80 %. Recent study results provide evidence that RA shows a pro- or anti-inflammatory action following its topical application, and it represents a useful alternative to irritant substances that relieve pain. Accordingly, RA induced the healing process by eliminating the inflammation phase (Gaginella and Philips, 1975; Vieira *et al.*, 2000a; Vieira *et al.*, 2000b; Vieira *et al.*, 2001).

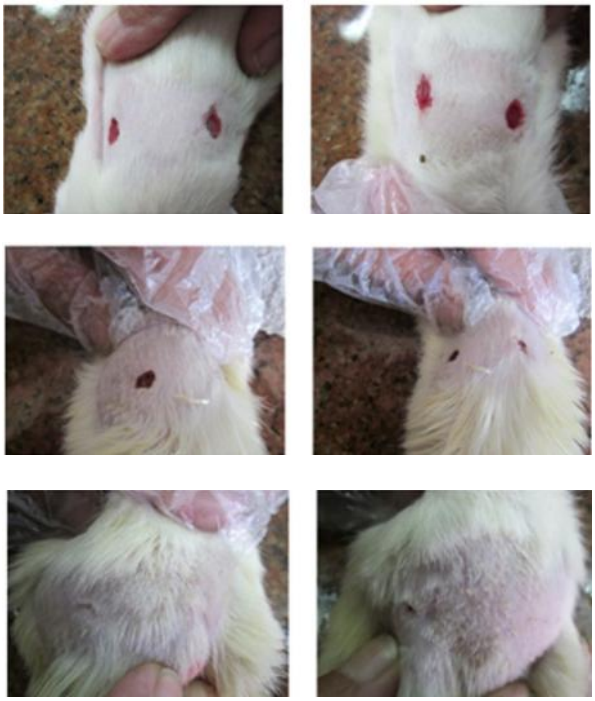


Fig. 1: a) Selected samples of fresh wounds of male rats; b) after two days; c) after ten days. Excision wounds of size (about) 1 cm^2 were made by cutting 1 x 1 cm piece of the skin from the shaven area in each side. Wounds were created on the 3th day after induction of diabetes.

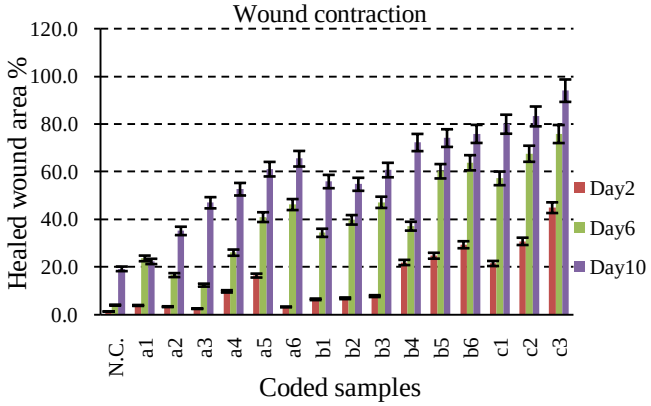


Fig. 3: Percentage of wound closure rate. Wound contraction in percentage indicates that pure fatty acids formulations are superior to either the cream or ointment samples. For the cream formulations, samples with silver nanoparticles showed better results for the all combinations (cream and ointment) while samples with ricinoleic acid (RA) induced the wound contraction higher than those with conjugated linoleic acid (CLA) and higher than those with RA and CLA mixture.

Table 4: Wound area (cm2) of the treated rats by different formulations.

	Wound area (cm2) zero time	Wound area (cm2) 2 days	Wound area (cm2) 6 days	Wound area (cm2) 10 days
N.C.	1.57±0.001	1.52±0.003	1.46±0.002	1.18±0.003
a1	1.57±0.003	1.51±0.002	1.20±0.001	1.22±0.003
a2	1.51±0.001	1.46±0.003	1.26±0.001	0.98±0.001
a3	1.62±0.001	1.58±0.001	1.42±0.002	0.86±0.002
a4	1.54±0.002	1.39±0.002	1.14±0.001	0.73±0.001
a5	1.59±0.001	1.33±0.001	0.94±0.001	0.62±0.001
a6	1.56±0.003	1.51±0.002	1.20±0.001	1.20±0.001
b1	1.72±0.002	1.61±0.003	1.13±0.002	0.76±0.001
b2	1.61±0.001	1.50±0.004	0.97±0.001	0.73±0.001
b3	1.55±0.003	1.43±0.003	0.82±0.003	0.61±0.001
b4	1.51±0.001	1.18±0.001	0.95±0.001	0.42±0.001
b5	1.58±0.004	1.19±0.001	0.63±0.001	0.41±0.001
b6	1.57±0.003	1.51±0.002	1.20±0.001	1.20±0.001
c1	1.54±0.001	1.21±0.003	0.66±0.001	0.31±0.001
c2	1.66±0.002	1.15±0.002	0.54±0.001	0.28±0.001
c3	1.65±0.004	0.91±0.001	0.40±0.001	0.10±0.001

N.C.; negative control, samples codes are defined in Table 1.

Histopathological test

For the histopathological analysis, Figure (4) shows a photomicrograph of sections of skin: **(A)** from a rat of group negative control (NC) of (a1) shows hyperplasia of epidermal layer's cells (arrow) at wound site with focal hyperplasia of granular layer's cells (arrowhead). Most of the epidermal cells at site of hyperplasia are ballooned with dark nuclei. **(B)** from a rat of group (a1) shows normalization of the pattern of epidermal layer including granular cell layer, although ballooned cells with dark nuclei are still observed (arrow). **(C)** from a rat of group (NC-a4) shows marked reduction of epidermal layer thickness at the bottom of the wound area (arrowhead), while hyperplasia of this layer's cells is seen on both sides of wound (arrow). **(D)** from a rat of group (a4) shows marked reduction of epidermal layer thickness, normalization of granular cell layer. Hair follicles show hyperplasia of its cells (arrow). **(E)** from a rat of group(NC-b1) shows marked focal increase in epidermal layer thickness due to focal aggregation of cellular infiltration and granulation tissue (arrow). Most of keratinocytes show vacuolar degeneration and

karrhyolysis (arrowhead). **(F)** from a rat of group (b1) shows noticeable reduction of epidermal cell layer thickness even more than normal on both sides of wound (arrow).

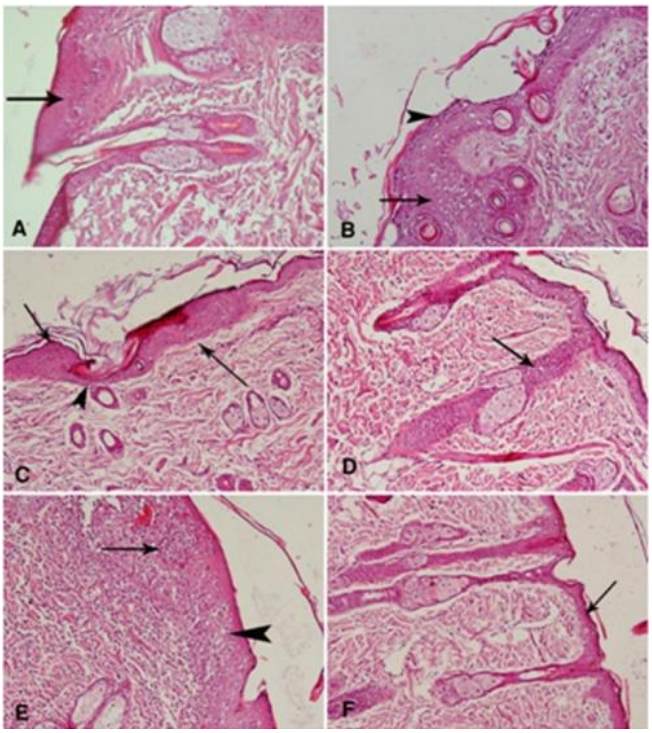


Fig. 4: A photomicrograph of sections of skin: (A) from a rat of group (NC-a1); (B) from a rat of group (a1); (C) from a rat of group (NC-a4); (D) from a rat of group (a4); (E) from a rat of group(NC-b1); (F) from a rat of group (b1). Figure shows hyperplasia of epidermal layer's cells (arrow) at wound site with focal hyperplasia of granular layer's cells (arrowhead).

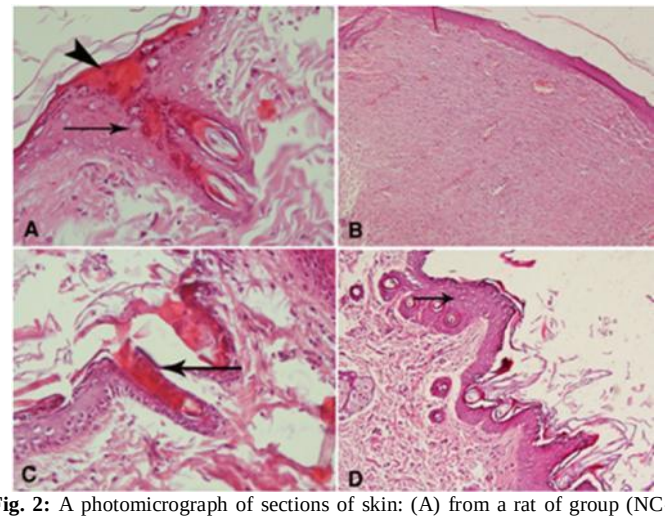


Fig. 2: A photomicrograph of sections of skin: (A) from a rat of group (NC-b4); (B) from a rat of group (b4); (C) from a rat of group (NC-c1); (D) from a rat of group (c1). Figure shows noticeable increase in keratin filling the gap of wound (arrowhead) and granular cell layer thickness (arrow).

Figure (5) shows a photomicrograph of sections of skin: **(A)** from a rat of group (NC-b4) shows noticeable increase in keratin filling the gap of wound (arrowhead) and granular cell layer thickness (arrow). **(B)** from a rat of group (b4) shows marked increase of fibrous connective tissue at the expense of other elements of dermal tissue over a wide area. Dilated blood capillaries and normal thickness of epidermal layer are seen. **(C)** from a rat of group (NC-c1) shows marked increase of keratin in an attempt to fill the wound gap (arrow) with deformity of the dermal tissue below. **(D)** from a rat of group (c1) shows focal hyperplasia of epidermal layer's cells (arrow).

Figure (6) shows a photomicrograph of sections of skin tissue: **(A)** from a rat of Group (NC-c2) shows detachment of epidermal layer with condensation of connective tissue fibers (arrowhead). Regenerated epithelial cells are seen at site of wound (arrow). **(B)** from a rat of group (c2) shows hyperplasia of epidermal cells at wound side (arrow) that extends to line the wound gap with no increase in keratin. The dermal layer below is deformed. **(C)** from a rat of group (NC-c3) shows hyperplasia of epidermal layer's cells (arrow) with abnormality of shape and arrangement of keratinocytes. **(D)** from a rat of group (c3) shows normalization of epidermal layer that extends to line the wound gap and dermal layer with some condensation of fibrous elements below the bottom of the wound.

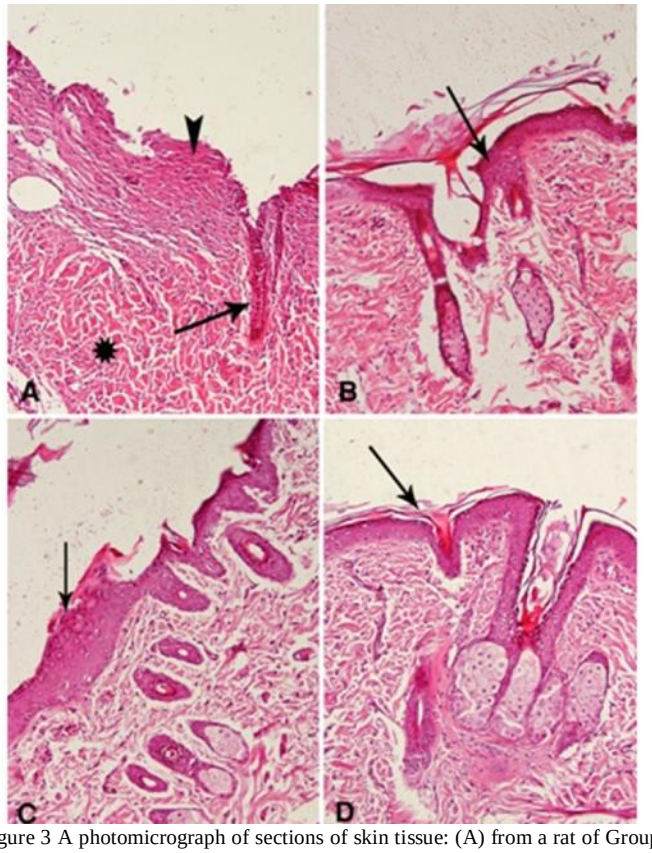


Figure 3 A photomicrograph of sections of skin tissue: (A) from a rat of Group (NC-c2); (B) from a rat of group (c2); (C) from a rat of group (NC-c3); (D) from a rat of group (c3). Figure shows detachment of epidermal layer with condensation of connective tissue fibers (arrowhead). Regenerated epithelial cells are seen at site of wound (arrow).

DISCUSSION

New pharmaceutical carriers were produced based on oil/water emulsions and using bees wax as thinking agent as described in Table (1). Moderate viscosity of the cream (C1) and

ointment (T1) samples were selected for further studies (Table 2). Antimicrobial assessment for the prepared samples were carried out against selected microorganisms strains (Table 3) and showed a strong activity for samples containing silver nanoparticles and chitosan microspheres. These activities may attribute to the presence of two antimicrobial agents namely silver nanoparticles and the chitosan beads in which their charge characters possessed a positive charge. These positive charges of chitosan and Ag-NPs increase the eruption of the microorganism's cell wall, leakage of glucose and lactate dehydrogenase from cells, and subsequently possesses antimicrobial activities (Tsai and Su, 1999). hTERT-BJ1 human immortalized skin fibroblasts cell line indicates the cytotoxicity profile of the produced samples. Adverse effect on cell viability was shown with higher amounts of silver nanoparticles. Therefore, results emphasize the hypothesis that higher concentrations of Ag-NPs reduce fibroblast fission and their viability(Peng *et al.*, 2012). Accordingly, the cream sample which contains different concentration of Ag-NPs has the same trend in the cell viability. However, cream sample shows more than 60 % of cell survival for all different concentrations (Figure 1). Pure LA, CLA and RA showed nontoxic nature for the fibroblast cell with lower concentrations while cell viability decreased with increasing their concentrations. This cytotoxicity effect of higher concentrations of the free fatty acids may attributed to their surfactant characteristics and correlate with their capability to inhibit water transfer to cells and isolate cells. Add to that, RA showed significant cell death more than other for its laxative actions (fluid secretion) (Gaginella et al., 1977). The visual appearance, of the topical application of the full thickness wounds, suggested that all formulations were very benign to the hosts. Lacking of redness with daily administration indicates that formulations did not induce extensive acute inflammatory responses which strongly correlated with diabetic wounds (Fig 2 b, c).The healing promotion seemed to be a pH dependent wherein the highest wound healing contraction came with sample C3 which has pH 3.8 which is lower than those of samples C2 (4.2) and C1 (4.8) (Boyle, 2008). It has been reported that adjusting pH of the wound bed is very important for wound healing process. It is believed that lower pH induces the healing process in terms of shorten the inflammation phase time and accelerating the achievement of the reactive oxygen species (ROS) which has very important role in the wound healing process (Gottrup and Sci, 2004; Gurtner et al., 2008a; Hopf and Rollins, 2007).

CONCLUSION

The pharmaceutical carrier was formulated based on water in oil cream and ointment containing chitosan fine particles, impregnated silver nanoparticles microcrystalline cellulose, conjugated linoleic acid, and ricinoleic acid. Cream, ointment, and the active ingredients were prepared and characterized by spectral and morphological analysis. The final formulation was examined for antimicrobial test, cytotoxicity test, and in-vivo test. Results showed high activity against the microbial strains for the silver contacting samples. The rates of wound closures during wound healing in diabetic male-rats of formulas containing ricinoleic acid was faster than that containing conjugated linoleic acid.

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