

<http://www.pjbs.org>

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Oral Dose of Citrus Peel Extracts Promotes Wound Repair in Diabetic Rats

¹M. Ahmad, ¹M.N. Ansari, ²A. Alam and ¹T.H. Khan

¹Department of Pharmacology, College of Pharmacy, Salman Bin Abdulaziz University,
Kingdom of Saudi Arabia

²Department of Pharmacognosy, College of Pharmacy, Salman Bin Abdulaziz University,
Kingdom of Saudi Arabia

Abstracts: Diabetic patients wound healing is slower than the healthy individuals. Three citrus peel extracts; Lemon (*Citrus limon*), Grapes fruits (*Citrus paradise*) and Orange (*Citrus sinensis*) promote wound healing in experimental animals. This study investigated the effect of oral treatment with citrus peel extracts on wound repair of the skin of diabetic rats. The extracts were estimated for vitamin C and total carotenoid contents prior to animal study. Diabetes mellitus was induced in rats by intraperitoneal injection of a single dose of streptozotocin (STZ, 75 mg kg⁻¹ b.wt.). One week after diabetes induction, full thickness excision wounds were made in hyperglycemic rats and were divided groups, each containing 6 rats. The different test group animals were treated with different citrus peel extract orally at the dose of 400 mg kg⁻¹ body weight daily for 12 days. The blood glucose, body weight and rate of wound closure of each rat were measured every 3rd day during the experimental period. At the end of experiment, granular tissues of wounds were removed and estimated for hydroxylproline and total protein content. The results showed significant reduction in blood glucose and time to wound closure. Tissue growth and collagen synthesis were significantly higher as determined by total protein and hydroxyl proline content. From our experimental data, we propose that oral administration of citrus peel extracts has a therapeutic potential in the treatment of chronic wounds in diabetes.

Key words: Antidiabetic, streptozotocin, wound healing, citrus peel, vitamin

INTRODUCTION

Different citrus plant species (family Rutaceae) has recently been explored for their antibacterial, antifungal, anti-inflammatory, antiseptic, tonic, anticarcinogenic, antiatherogenic, antifungal, antiviral, antidiabetic, wound healing, antioxidant and anticoagulant activities. They exhibited a varying degree of pharmacological potential (Kawaii *et al.*, 2000; Mahgoub, 2002; Burt, 2004; Ortuno *et al.*, 2006; Chatha *et al.*, 2011; Dhanavade *et al.*, 2011). Out of the citrus peel extracts in different solvents such as ethanol, methanol and acetone, the ethanolic extract shows marked pharmacological activities (Keles *et al.*, 2001). The peel of *Citrus* fruits is a rich source of flavonoid glycosides, coumarins, β and γ -sitosterol, glycosides and volatile oils (Sultana *et al.*, 2007). *Citrus* peels contain health beneficiary flavanone glycosides (hesperidin, neohesperidin, naringin; diosmin, eriocitrin and tangeretin), flavones glycosides, hydroxylated polymethoxy flavones, methylated flavonoids flavonols, proanthocyanidins and dihydrochalcones (Rouseff *et al.*, 1987; Hollman and Arts, 2000; Ross *et al.*, 2000;

Nogata *et al.*, 2006). Peels are generally waste products (approximately half of the fruit weight) of citrus fruits juice processing industries in spite of the fact that it contains more vitamins and other active constituents than the juice itself.

Vitamin C present in citrus fruits helps in enhancing the immunity of the body. Daily consumption of citrus fruits will help the body to keep away the germs, cold, cough and flu. Presence of vitamin C in sweet orange, lemon and grapefruits has already been estimated (Ali *et al.*, 2010). It plays an important role in the wound healing process. Vitamin C is a cofactor in the hydroxylation of proline and lysine for procollagen formation. It also provides tensile strength to newly built collagen; otherwise, new tissue could not stretch without tearing. Vitamin C deficiency will markedly delay the wound healing by impairing the collagen formation and will also impair the previously healed wounds dehiscence (Sonel *et al.*, 1997; Shukla *et al.*, 1997). The β -carotene, most abundant and most efficient provitamin A in our foods, is yellow orange and red pigments that are widely distributed in plants. The biological properties of β -Carotene or vitamin A include anti-oxidant activity,

increased fibroblast proliferation, modulation of cellular differentiation and proliferation, increased collagen and hyaluronate synthesis and decreased MMP mediated extracellular matrix degradation (Burgess, 2008).

Diabetes mellitus is life threatening disease and has become the third largest killer of humans after cancer and cardiovascular diseases, even with the use of several synthetic recent drugs for effective treatment options (Li *et al.*, 2004). It constitutes one of the most important public health problems due to its high prevalence and enormous social and economic consequences (Silva *et al.*, 2007). Diabetics is a multisystem disorder that also effects the wound healing process and wound healing is significantly much slower in diabetics than in healthy individuals (Falanga, 2004). Delayed cutaneous wound healing is a chronic complication in diabetes and is caused primarily by hyperglycemia, diminished expression of cytokines, oxidative stress, vascular insufficiency and microbial infections (Lerman *et al.*, 2003; Sivan-Loukianova *et al.*, 2003; Tandara and Mustoe, 2004; Hirsch *et al.*, 2008; Abu-Al-Basal, 2010). Crude flavonoids, eriocitrin and hesperidin suppresses the oxidative stress in the diabetic rats, thus improves insulin sensitivity (Miyake *et al.*, 1998). Citrus flavonoids possess health promoting phytochemicals such as flavanones, polymethoxyflavones and C-glycosylflavones which support antidiabetics and wound healing properties (Miyake *et al.*, 2003; Ahmad *et al.*, 2006). One of the recent study revealed that vitamin C significantly reduced the blood glucose level in diabetes (Owu *et al.*, 2012). The beneficial effect of antioxidants and antioxidant enzymes in scavenging free radicals generated during oxidative stress and wounds has been reported (Fitzmaurice *et al.*, 2011). These antioxidant systems prevent the generation and actions of reactive oxygen species and provide a potential mechanism for wound healing and ameliorating diabetic complications. The factors that can cause delay of wound healing include oxygenation, infection, stress, diabetes, obesity, medications, alcoholism, smoking, nutrition, age and sex hormones alteration in normal biological processes in the human body (Guo and Dipietro, 2010).

The present study was undertaken to determine whether treatment with citrus peel extracts would restore in excision wound changes from rats with diabetes mellitus.

MATERIALS AND METHODS

Chemicals: Streptozotocin (STZ) was purchased from Fluka (Germany) and EDTA, [5, 5-dithiobis-(2-nitrobenzoic acid)], Beta-carotene, L-ascorbic acid; 2, 4-Dinitrophenyl

hydrazine, Thiourea, Drabkin's solution, Haemoglobin, Bovin Serum Albumin, L-4-Hydroxyproline, Chloramine-T, 4-(Dimethylamino) benzaldehyde were purchased from Sigma (Germany). All other chemicals and reagents used in this study were of analytical grade.

Collection of material: The fruits of *Citrus limon*, *Citrus paradise* and *Citrus sinensis* were collected from the local retail market of Riyadh, Saudi Arabia in April 2012. The peels were washed thoroughly 2-3 times with running tap water. The skin of the fruit was scored into quarters with sharp knife and then peeled off with fingers, keeping the pith attached. The removed peels of citrus fruits were shade-dried for about one month. The powder of the peels was obtained after grinding and sieving and was packed in air tight bottles.

Preparation of extracts: The ethanolic extract of each of the different citrus fruits peels was prepared by soaking 100 g of the dried powder of either *Citrus limon*, *Citrus paradise* and *Citrus sinensis* in 900 ml ethanol, with occasionally shaking, for 24 h then the solvent was filtered. The process was repeated for three times. The extracts were concentrated using rotary evaporator and finally dried in lyophilizer under vacuum (Abdelbaky *et al.*, 2009).

Estimation of total carotenoids and vitamin C

Estimation of total carotenoids: Total carotenoids were determined by the method of Jensen². One gram sample was extracted with 100 mL of 80% methanol solution and centrifuged at 4000 rpm for 30 min. The supernatant was concentrated to dryness. The residue was dissolved in 15 mL of diethyl ether and after addition of 15 mL of 10% methanolic KOH the mixture was washed with 5% ice-cold saline water to remove alkali. The free ether extract was dried over anhydrous sodium sulphate for 2 h. The ether extracts were filtered and its absorbance was measured at 450 nm by using ether as blank (Jensen, 1978).

Estimation of vitamin C: The 0.01 g dried extract sample was homogenized with acetic acid solution and transferred into a 100 mL volumetric flask and was shaken gently until a homogenous dispersion was obtained. Then it was diluted up to the mark by acetic acid solution. Then the solution was filtered using Whatmann filter paper-1 and the filtrated sample solution a few drops of bromine water were added until the solution became colored. A fresh 100 µg mL⁻¹ stock solution of ascorbic acid was prepared and diluted to get varying concentrations of 1, 2, 3, 4, 5, 6 and 7 µg mL⁻¹ standard solutions. Then a few drops of thiourea solution and 2, 4-Dinitrophenyl

hydrazine solution were added in both standard and sample. The absorbance was measured with UV-Vis spectrophotometer (Shimadzu UV 2450) at 280 nm. A standard curve of absorbance against concentration was then plotted. The total vitamin C was expressed in mg/100 g (Mohammed *et al.*, 2009).

Experimental animals: Male Wistar rats weighing between 200-250 g were obtained from the experimental animal care center, College of Pharmacy, Salman Bin Abdulaziz University, Al-Kharj, Saudi Arabia. The protocol for the study was approved by the Ethical Committee of the College of Pharmacy, Salman Bin Abdulaziz University, Al-Kharj, Kingdom of Saudi Arabia. The animals were housed under standard laboratory conditions at room temperature of 22±2°C, humidity (55%) and were exposed to 12 h light/dark cycle. Animals were provided with Purina chow diet and water *ad libitum*. The animals were divided into five groups, namely, normal control (Group 1), the diabetic control (Group 2), diabetic rats treated with Citrus limon extract (Group 3), diabetic rats treated with Citrus paradise extract (Group 4) and diabetic rats treated with Citrus sinensis extract (Group 5). Each group consisted of 6 rats.

Induction of diabetes mellitus: Diabetes mellitus was induced in all rats except group 1, by a single intraperitoneal injection of STZ (75 mg mL⁻¹ b.wt.) dissolved in citrate buffer (pH 4.5). Group 1 control rats were given normal saline only. Body weight and basal blood glucose levels were measured just prior to STZ injection using electronic animal balance (Shimadzu BL 3200HL) and an automated glucose analyzer (glucometer Acu-checkmini plus, Roche, Germany), respectively. A drop of blood samples were collected from the tail tip. Fasting blood glucose levels were measured (with glucose oxidase reagent strips) 3 days after streptozotocin injection and animals with glucose levels greater than 200 mg dL⁻¹ were used for the study. The diabetic animals were used for the experiment after one weeks of diabetes mellitus induction.

Wound creation: The excision wound model was used to monitor wound healing in diabetics rats, accounting by biochemical parameters and the rate of wound contraction. The dorsal skin of each rat was shaved with an electric clipper. After 24 hours, all animals were anaesthetized by diethyl ether and the shaved area was sterilized with 70% alcoholic solution. Excision wounds were made by cutting out a predetermined dorsal area (approximately 2.5 cm diameter) of skin from the shaved area using toothed forceps and pointed scissors.

Wound treatment: Citrus fruit peel extracts were administered orally, using a gastric tube; each rat in Groups 3, 4 and 5 was administered *Citrus limon*, *Citrus paradise* and *Citrus sinensis* respectively at a dose of 400 mg kg⁻¹, daily for 12 days. Rats in the control groups (Groups 1 and 2) received 2 mL kg⁻¹ of normal saline. Progressive changes in the wound area and body weight were monitored periodically at every 3rd day.

Percentage of wound reduction: To determine the rate of wound contraction, excision wounds were measured by using vernier calliper (Fig. 1) and the change in wound size was calculated as the percentage of wound area that had healed. The reduction in the wound size was calculated according to the following formula:

$$\text{Wound reduction (\%)} = \frac{W_0 - W_t}{W_0} \times 100$$

where, W₀ is wound diameter on day zero; W_t is wound diameter on day t. The time taken for 50% of wound closure (WC₅₀) was calculated by a plot of percentage wound closure against days.

Collection of granular tissues: In the excision wound model, granulation tissue formed on the wound was excised on the 12th postoperative day and its weight recorded. The tissue was dried in an oven at 70°C and the dry weight was again noted.

Estimation of total protein contents: Estimation of protein was done by method described by Lowry *et al.* (1951). Different dilutions (0.05 to 1 mg mL⁻¹) of Bovin Serum Albumin (BSA) were prepared by mixing stock BSA solution (1 mg mL⁻¹) for standard curve and absorbance was measured at 660 nm.

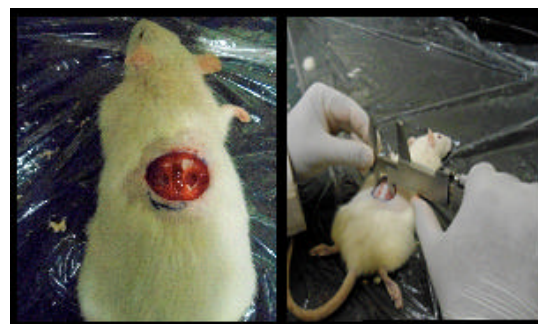


Fig. 1: Photographical representation of wound model and measurement of area of wound contraction using vernier calliper

Estimation of hydroxyproline: The dried granulation tissue (≈ 100 mg) was hydrolyzed in 6 M HCl for 16 h at 120°C then washed three times with distilled water. The samples were reconstituted in 2.0 mL of acetate-citrate buffer. Five hundred micro liter Chloramine-T (0.05 M) was added to 1 mL of each sample and then the samples were incubated for 20 min at room temperature, followed by the addition of 0.5 mL 15% perchloric acid and 15% 4-dimethyl aminobenzaldehyde in 1-propanol. The absorbance read at 550 nm after incubation at 60°C for 20 min. Hydroxyproline concentrations were calculated from the linear standard curve and presented as $\mu\text{g g}^{-1}$ dry tissue weight (Lee *et al.*, 2012).

Collection of blood: Blood samples were collected every 3rd day interval from the tail by giving a small incision at the tail tip.

Estimation of blood glucose: One drop of fresh blood was drawn onto the test strip channel. After beeping sound of meter, the test began automatically and result appeared in 9 sec (Obelis and Sae, 2009). The blood glucose level of all the 30 rats used for anti-diabetic study was determined before induction of diabetes and periodically at every 3rd day interval.

Body weight measurement: The body weight of rats was determined using the weighing balance (Metilado, Switzerland). The rats were weighed initially before induction of diabetes and periodically at every 3rd day interval of 12 day treatment.

Statistical analysis: The results are expressed as Mean \pm SEM). The results were analyzed using GraphPad Prism software version 5 (GraphPad Software, San Diego, California, USA) by one-way and two way variance (ANOVA) with Bonferroni's multiple comparison tests.

RESULTS

Percentage yield, total carotenoids and vitamin-C content: The percolated extract of the citrus peel using 90% alcoholic solvent was yielded. The percentage yield of the extract obtained for *Citrus limon*, *Citrus paradise*

and *Citrus sinensis* showed 13.6%, 15.8% and 12.5% respectively in terms of dry powder. The total carotenoids were found maximum in *Citrus sinensis* 162.7 \pm 0.04 mg/100 g and minimum in *Citrus limon* 144.3 \pm 0.02 mg/100 g. The Vitamin C was found approximately similar quantity in *Citrus limon* (52.67 \pm 1.30, mg/100 g) and *Citrus paradise* (55.67 \pm 2.16, mg/100 g) peel extract (Table 1).

Excised wound healing

Percentage of wound reduction: The observed excised wound healing activity was significantly increased in *Citrus sinensis* peel extract when compared with those who received the saline in both non diabetics and diabetics control treatments. All the peel extract produced a significant reduction in wound area. The WC₅₀ values showed that diabetic control took more time while peel extract treatments took lesser time for healing (Table 2). The Model and effect of citrus peel extracts on diabetic's animals before and after 12 days experiments are shown in Fig. 1 and 2.

Total protein and hydroxyproline contents: In the excised granulation tissue of wound, a significant increase in the total protein and hydroxyproline were observed when compared with the both control and diabetic's controls (Table 3). Total protein, hydroxyproline and granulation tissue weight, increased significantly ($p < 0.001$), following treatment with the *Citrus limon*, *Citrus paradise* and *Citrus sinensis* extract respectively when compared with the diabetic control group.

Blood glucose: During the STZ-induced hyperglycemic rats excised wound healing experiment, all citrus peel extract have shown significant ($p < 0.001$) decrease in the blood glucose level when compared to the control and diabetics control rats (Table 4).

Table 1: Average total carotenoids and vitamin C content in grapefruits, orange and lemon peel alcoholic extracts

Citrus Plant	Total carotenoids (mg/100 g)	Vitamin C (mg/100 g)
<i>Citrus limon</i>	144.3 \pm 0.02	52.67 \pm 1.30
<i>Citrus paradise</i>	153.7 \pm 0.03	55.67 \pm 2.16
<i>Citrus sinensis</i>	162.7 \pm 0.04	66.50 \pm 1.15

Data expressed as Mean \pm SEM of triplicate analyses

Table 2: Effect of different citrus peel extract on percentage wound contraction on normal and hyperglycemic rats during the period of wound healing

Treatment	3rd day	6th day	9th day	12th day	WC ₅₀
Normal control	3.58 \pm 0.29	8.95 \pm 0.35	17.18 \pm 0.44	28.99 \pm 0.58	28.31
Diabetic control	4.65 \pm 0.10	4.79 \pm 0.36	10.50 \pm 0.23	13.28 \pm 0.74	56.03
<i>C. limon</i>	4.33 \pm 0.01	13.38 \pm 0.24 ^{ab}	26.89 \pm 1.21 ^{ab}	49.47 \pm 2.45 ^{ab}	17.48
<i>C. paradise</i>	3.85 \pm 0.33	12.82 \pm 0.12 ^{ab}	28.88 \pm 0.26 ^{ab}	55.18 \pm 0.98 ^{ab}	15.97
<i>C. sinensis</i>	4.19 \pm 0.22	13.65 \pm 0.41 ^{ab}	30.27 \pm 1.17 ^{ab}	58.27 \pm 1.22 ^{ab}	15.27

Data expressed as Mean \pm SEM (n = 5); ^a $p < 0.001$ compared with normal control group and ^b $p < 0.001$ compared with diabetic control group, WC₅₀: Each value is the time taken for 50 percent wound closure

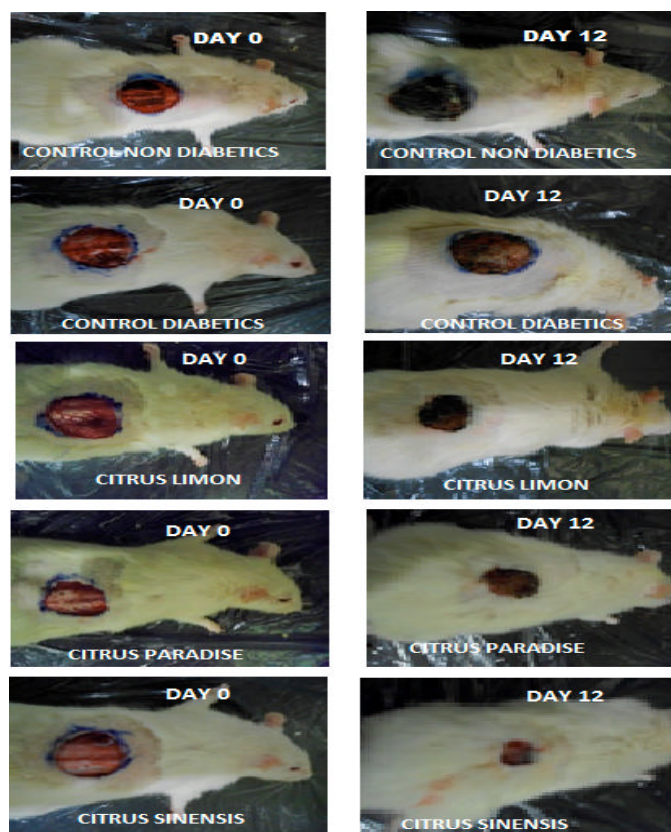


Fig. 2: Photographical representation of contraction rate on day 0 and on day 12 of non diabetics, diabetics control and experimental (*citrus* peel extract oral treatment) wounds

Table 3: Effect of the citrus peel extracts on total protein and hydroxyproline in excised wound model

Group	Total protein (mg g ⁻¹)	Hydroxyproline (mg g ⁻¹)
Normal control	68.56±0.35	11.57±0.17
Diabetic control	35.56±0.35	06.56±0.35
<i>Citrus limon</i>	79.24±0.68 ^{ab}	21.93±0.43 ^{ab}
<i>Citrus paradise</i>	82.56±0.96 ^{ab}	24.93±0.29 ^{ab}
<i>Citrus sinensis</i>	85.65±0.96 ^{ab}	25.96±0.29 ^{ab}

Data expressed as Mean±SEM (n = 5); ^ap<0.001 compared with normal control group and ^bp<0.001 compared with diabetic control group

Table 4: Effect of different citrus peel extract on blood sugar level of normal and hyperglycemic rats during the period of wound healing

Treatment	1st day	3rd day	6th day	9th day	12th day
Normal control	76.80±1.6	74.60±4.02	79.20±2.35	78.60±1.66	78.40±1.96
Diabetic control	147.2±1.59	145.60±0.92	142.00±1.22	135.80±2.22	131.60±2.30
<i>Citrus limon</i>	140.0±3.51	135.00±3.44	128.00±4.12	115.00±1.91	105.00±1.98 ^{ab}
<i>Citrus paradise</i>	164.0±3.90	150.00±3.90	143.00±4.20	134.00±5.00	125.00±4.20 ^{ab}
<i>Citrus sinensis</i>	158.0±3.00 ^a	149.00±3.00	136.00±2.40	124.00±1.20	117.00±0.97 ^{ab}

Data expressed as Mean±SEM (n = 5); ^ap<0.001 compared with normal control group and ^bp<0.001 compared with diabetic control group

Table 5: Effect of different citrus peel extract on body weight of normal and hyperglycemic rats during the period of wound healing

Treatment	1st day	3rd day	6th day	9th day	12th day
Normal Control	237.6±6.990	237.6±6.07	240.4±6.56	244.4±6.50	249.2±6.733
Diabetic Control	235.4±7.110	232.0±6.35	224.4±6.38	214.8±6.53	205.0±6.500
<i>Citrus limon</i>	274.0±9.390	273.6±9.62	277.0±9.55	279.8±9.84	286.4±11.30
<i>Citrus paradise</i>	255.6±7.800	257.2±8.42	260.4±8.49	265.0±8.29	267.0±8.470
<i>Citrus sinensis</i>	249.6±12.06	252.4±11.91	252.4±9.20	255.0±7.79	259.2±7.430

Data expressed as Mean±SEM (n = 5)

Body weight: The body weights of normal rats (group 1) were significantly less than the STZ control group receiving saline (group 2). The improvement of body weight was shown in wounded rats after treatment with the citrus peel extract when compared with diabetic control group (Table 5).

DISCUSSION

The diabetes is a well known factor for altering the normal wound healing programmed phase in normal biological process in the human body by altering the body weight and persistent of glucose in blood (Guo and Dipietro, 2010). The previous findings show that diabetic patients need to pay special attention to their critical illness, wound, or ulcer (Meyer, 1996). Impaired wound healing is a common complication of diabetes mellitus (LoGerfo and Coffman, 1984). When wound occurs, it is exposed to external environmental and is prone to attack by microbes which invade the wound and generate the free radicals (Ghosh *et al.*, 2012). Common wound contaminants include *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Pseudomonas aeruginosa* and *Clostridium perfringens*, *Clostridium tetani* and *Coliform bacilli*. It has been reported that alcoholic extract of citrus peel possess antimicrobial potential which may help in the speeding up the process of wound healing (Negi and Jayaprakasha, 2001; Kumar *et al.*, 2011; Shivhare *et al.*, 2010). The delay of wound repair in diabetic rats is associated with reduced collagen (hydroxyproline) and other protein synthesis in granular tissue due to depression of fibroblast. The antioxidants activated the fibroblasts that are necessary for local collagen and other protein synthesis (Buemi *et al.*, 2004). Facilitating oxygen diffusion, increasing lymphatic drainage, diminishing oxygen free radical overproduction and increasing the collagen synthesis were found, together, to improve healing (Inan *et al.*, 2006). The vitamin A and C deficiencies result in impaired healing and have been linked to decreased collagen synthesis and fibroblast proliferation, decreased angiogenesis, increased capillary fragility, an impaired immune response and increased susceptibility to wound infection (Arnold and Barbul, 2006; Campos *et al.*, 2008). The β -carotene and vitamin-C are estimated in our study is in agreement of previous reported in Citrus peel extracts (Sun *et al.*, 2011). The previous research also reported the peel extracts contains flavones and flavonols. The β -Carotene is converted to vitamin A in intestinal mucosal cells only in the presence of bile acids and stored in liver (Olson, 1961). It has been documented that β -Carotene

and vitamin A antioxidants possess the therapeutic potential like anti-inflammatory, anti-fungal, antibacterial, antidiabetics and wound healing (Akkol *et al.*, 2009; Nayak *et al.*, 2009). Moreover, flavonoids and their derivatives are known to decrease lipid peroxidation by improving vascularity and by preventing or slowing down the progress of cell necrosis (Raygude *et al.*, 2012). Vitamin C does work better when accompanied by flavonoid molecules, as their presence in cells spares vitamin C and provides greater antioxidant activity (McAnulty *et al.*, 2011). These vitamins and flavonoids rich citrus peel extracts have been shown to be powerful antioxidants and anti-inflammatories and health-protective properties, especially when it comes to diabetes and wound (Montanari *et al.*, 1998).

The main finding of our study is that oral administration of citrus peel extract to a diabetic rat with full-thickness excision skin wound increases the rate of wound healing. In our study, we observed that defective wound repair in diabetic rats is associated with reduced collagen (hydroxyproline) and other protein. Our finding showed that citrus peel alcoholic extract promoted wound repair in diabetic rats and these findings are in agreement with earlier reports on the beneficial effects of citrus peel on wound healing of ischemic skin wounds (Sandhya *et al.*, 2011) diabetic (Naim *et al.*, 2012) and diabetic wounds. Our data suggest that granular tissue deposited on healed wound accounted by increased hydroxyproline and total protein content may be due to increase the delivery of oxygen and other nutrients after oral treatment of citrus peel extract. Our data also suggested that altered normal biological process in the human body due to diabetes can be treated with the oral administration of citrus peel extracts (Aruoma *et al.*, 2012).

CONCLUSION

The ethanolic extract of all citrus fruit peel significantly attributed to healing potential in diabetic condition. The main mechanism behind the significant healing potential is antioxidants due to Vitamins and flavonoids. Further, more animal studies are needed and clinical studies are required before clinical implementation.

ACKNOWLEDGMENTS

The authors would like to acknowledge Mohammad Ayman Salkini, Lecturer, College of Pharmacy, Salman Bin Abdulaziz University, Al-Kharj, KSA, for support and help to carry out the study.

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