CHAPTER V
WOUND HEALING EFFECT OF NATURAL PRODUCTS LOADED WITH ZEIN BASED NANOFIBER MATS IN STREPTOZOTOCIN-DIABETIC RATS

1. Introduction

Wound healing retardation is one of the most commonly found complications of diabetes mellitus. It is a complex process involves multifactors including inflammation, migration of different cell types, fibroplasia, collagen deposition, and wound contraction. It has been reported that the healing process of diabetic wound is slow and characterized by delayed cellular infiltration and granulation tissue formation, decreased collagen organization, diminished blood flow, increased blood viscosity and reduced angiogenesis (Babaei et al., 2013).

Accumulative lines of evidence have demonstrated that oxidative stress plays the crucial role on the impairment of wound healing process in diabetes mellitus (Mohammad et al., 2008). Substances possess antioxidant activity can improve wound healing both in experimental animal and diabetic patients (Singh et al., 2014; Lan et al., 2013). In addition, it has been demonstrated that electrospun nanofiber can enhance the benefit of wound healing agent (Kim and Yoo, 2013; Fu et al., 2014). Based on the benefit of substances possessing antioxidant activity and electrospun polymeric nanofiber mentioned earlier, we hypothesized that the natural products (quercetin or tomato) loaded nanofiber facilitate wound healing in animal model of diabetic wound. To determine the effect and possible underlying mechanism of the natural products (quercetin or tomato)-loaded nanofiber mats on diabetic wound, this study was carried out.

2. Materials and Methods

2.1 Diabetic Induction

The solution of streptozotocin (STZ) was freshly prepared in 0.1 M citrate buffer, pH 4.5 and administered to the fasted rats at dose of 50 mg/kg BW (Kumar et al., 2006). Forty-eight hours later, blood samples were collected and

glucose levels were determined to confirm the development of diabetes. STZ injection should rapidly induced the characteristic signs of diabetes, such as increased intake of water and food, frequent urination and increased blood glucose concentration. Forty-eight hours after STZ injection, blood samples were collected and glucose levels were determined to confirm the development of diabetes. Only the animals which showed blood glucose levels >250 mg/dl were used in the experiment. This study has been reviewed and approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of National Research Council of Thailand. The record number is AEKKU 11/2552 and the reference number is 0514.1.12.2/8.

2.2 Diabetic wound model

The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). An incision was made at the dorsal surface of anterior-dorsal at T1 level, 1x1cm a full thickness skin flap, completely transdermal, was removed (Dash et al, 2001).

2.3 Experimental protocol

This study was divided in to 2 separated parts. The first part was focused on the determination of wound healing effect of quercetin loaded nanofiber mat in diabetic rats whereas the second part was focused on the determination of wound healing effect of tomato extract loaded nanofiber mat in diabetic rats.

2.3.1 Determination of wound healing effect of quercetin loaded nanofiber mat in diabetic rats

In this part, rats were induced diabetic condition with single shot injection of streptozotocin. Then, diabetic rats were divided in to various groups as described following;

Group 1	Diabetic rat which received no treatment
Group 2	Diabetic rats which received shame operate
Group 3	Diabetic rats with wound excision which received vehicle or zein based nanofiber treatment

Group 4-6 Diabetic rats with wound excision which received zein based nanofiber mats loaded with quercetin at concentrations of 5%, 10% and 15%

The animals had been treated with the assigned intervention for 15 days. They were assessed the wound healing index via wound sticking, adsorption of exudates and elimination of pus, percent of wound area ratio, percent of wound contraction assessment and the granulation tissue was isolated and determined the collagen synthesis level of hydroxy-proline every 3 days throughout the study period. at the end of the study period, they were determined, the lesion skin were isolated and determined the collagen area, collagen thickness and collagen synthesis, oxidative stress status including the activities of superoxide dismuatase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and the level of malondialdehyde (MDA) in the lesion skin.

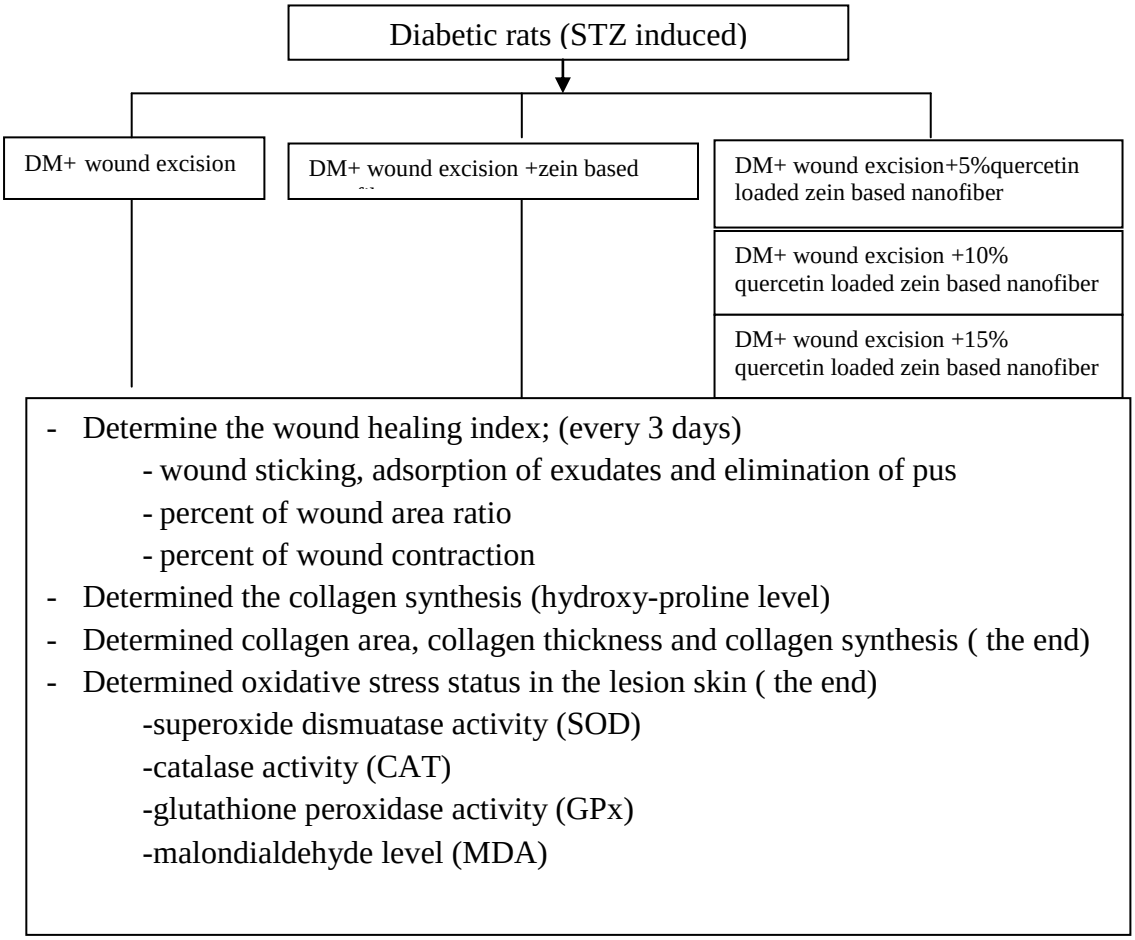


Figure 5-1 Schematic diagram showing experimental protocol for the determination of the effect of zein based nanofiber mats loaded with various concentrations of quercetin in diabetic wound

2.3.2 Determination of wound healing effect of tomato extract loaded nanofiber mat in diabetic rats

Diabetic rats were divided in to various groups as mentioned earlier in 2.3.1 except that rats in group 4-6 were treated with zein based nanofiber mats loaded with tomato extract at concentrations of 5%,10% and 15%. The animals were also assessed all parameters mentioned earlier at the same time schedule.

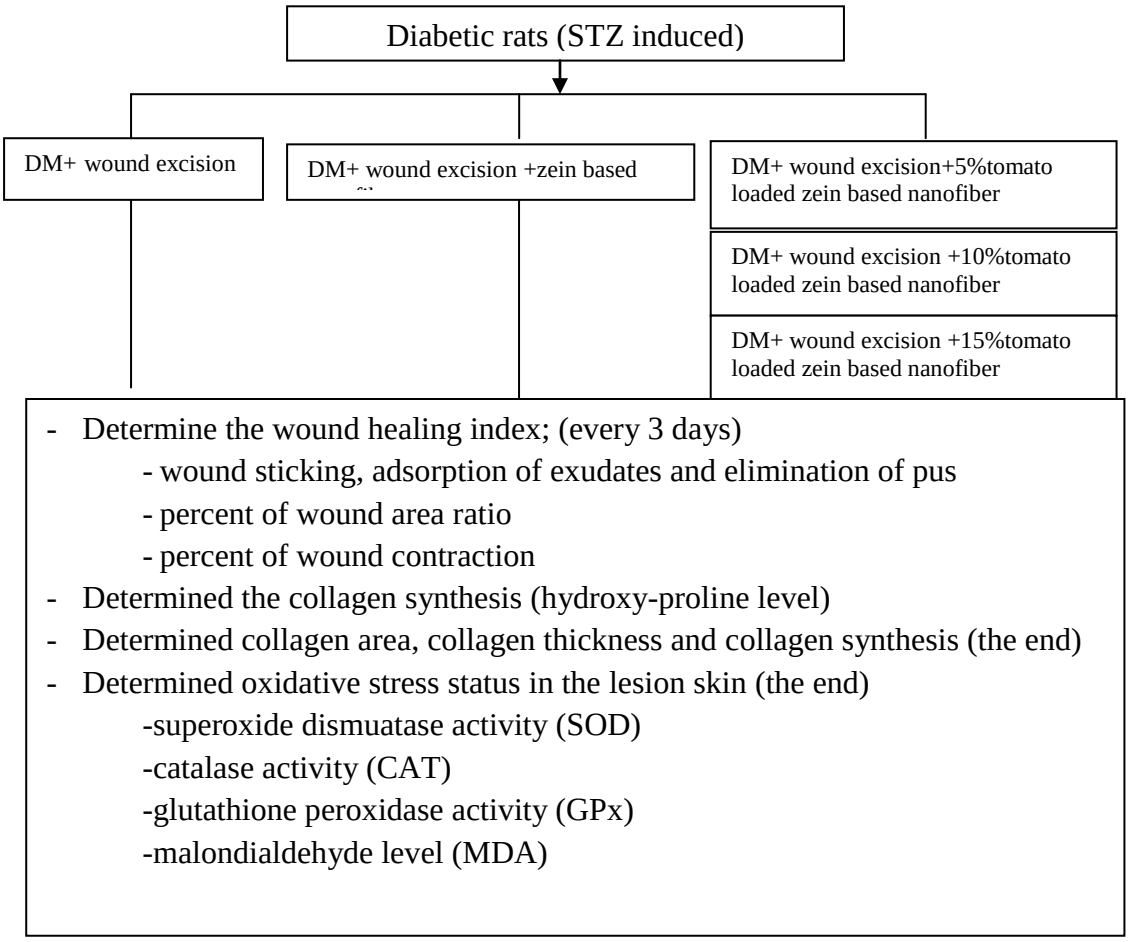


Figure 5-2 Schematic diagram showing experimental protocol for the determination of the effect of zein based nanofiber mats loaded with various concentrations of tomato extract in diabetic wound

2.4 Determination of Wound Healing Index

2.4.1 Wound adsorption score determination

The wound was evaluated for wound sticking, adsorption of exudates and elimination of pus every 3 days since the start of intervention to the end of study and graded as wound adsorption score according to the following criteria (Aoyagi et al., 2007).

4 = for very good

3 = good

2 = neutral

1 = negative responses

2.4.2 Determination of wound healing rate

2.4.2.1 Percent of wound area ratio

Ratio of the wound surface area to the initial area, wound area ratio, was calculated as the healing index according to the following equation: (Fukawa et al., 1982 and Yamashita et al., 1989)

$$\text{Ratio of the wound surface area (\%)} = 100 \times \frac{\text{Wound length x Wound width}}{\text{Initial wound length x Initial wound width}}$$

2.4.2.2 Percent of wound contraction

Percent of wound contraction was also evaluated and calculated by using the following equation

$$\text{Wound contraction (\%)} = 100 \times \frac{\text{Wound area day 0- Wound area day N}}{\text{Wound area day N}}$$

2.5 Determination of the Possible Underlying Mechanisms on Wound Healing

Based on the previous information that oxidative stress and collagen play the crucial role on wound healing process, thus, the current study also focused on the alteration of both parameters as described following.

2.5.1 Determination of histopathological change with Masson's trichrome method

In this study, Masson's trichrome staining method, a three-colour staining protocol, was used to determine collagen deposition area and thickness. The trichrome is applied by immersion of the fixated sample into Weigert's iron hematoxylin, and then three different solutions. According to this process, cell compositions were distinguishable by different colors. The collagen fibers were stained blue, the nuclei was stained black and the background was stained red. Then, the area of collagen deposition and collagen thickness were determined and analyzed using Image Pro Plus version 3.0 software. (Michelle's protocol, 2005)

2.5.2 Determination of hydroxyproline level

Wound granulation was harvested and determined the hydroxyproline level by colorimetric method which modified method of Edwards and Obrien (Edwards and Obrien, 1980). In brief, the wound granulation was hydrolyzed in HCl (10 mg tissue/l ml 6 mol/l HCl) and heated at 12O°C in a pressure vessel (seal tube) for 24 h. Then the hydrolysate was let dried in the vacuum desicator overnight and the freshly prepared buffer (citric acid 4.43%, glacial acetic 1.07%, sodium acetate10.67%,sodium hydroxide .03% and propranolol 26.67%) was added to the dried hydrolysate in order to dilute samples to the assay sensitivity range. Then the standard hydroxyproline (500 µg hydroxyproline per ml buffer) was added and allowed to react with Chloramine-T reagent at room temperature for 20 minutes. After this process, aldehyde-perchloric acid reagent was added to sample and placed in hot water bath at 60°C for 15 minutes. The sample was allow to be cooled and the absorbance at 550 nm was recorded via spectrophotometer.

2.5.3 Homogenate preparation

At the end of experiment, homogenate of the skin at the lesion area was prepared in 1 ml of 0.1 M phosphate buffer, pH 7.4. The obtained homogenate was adjusted to 10 % w/v and centrifuged at 10,000 g, 4°C for 1 hour. The supernatant was harvested and processed for the estimation of biochemical parameters.

2.5.4 Determination of malondialdehyde (MDA)

In this study, thiobarbituric acid reacting substances (TBARS) assay was used for determining malondialdehye (MDA) level. In brief, the mixture containing 100 µl of sample, 100 µl of 8.1% (w/v) sodium dodecyl sulphate, 750 µl 20% (v/v) acetic acid (pH 3.5), and 750 µl of 0.8% thiobarbituric acid (TBA) was prepared. The solution was heated in a water bath at 95°C for one hour and cooled immediately under running tap water. Then, 500 µl of chilled water and 2500 µl of butanol and pyridine [15:1 v/v] were added into each tube and mixed thoroughly with vortex. After mixing, the solution was centrifuged at 800 x g for 20 minutes. The upper layer was harvested and the absorbance at 532 nm was recorded. 1,3,3-tetra ethoxy propane (TEP) was used as the reference (Ohkawa et al., 1979). The level of MDA was expressed as U/mg.protein.

2.5.5 Determination of superoxide dismutase (SOD) assay

SOD activity assessment was performed via nitrobluetetrazolium (NBT) reduction assay. According to this method, the xanthine - xanthine oxidase system was used as a superoxide generator. In brief, 20 µl of sample was mixed with 200 µl of reaction mixture consisting of 57 mM phosphate buffer solution (KH2PO4), 0.1 mM EDTA, 10 mM cytochrome C solution and 50 µM of xanthine solution and 20 µl of xanthine oxidase solution (0.90mU/ml) at 25ºC. The absorbance at 415 nm was determined. A system devoid of enzyme was served as the control and three parallel experiments were conducted (Sun et al., 1988). SOD activity was expressed as U/mg.protein.

2.5.6 Determination of catalase (CAT) assay

CAT activity was determined based on the ability of the enzyme to break down H_2O_2. In brief, 10 µl of sample was added to the reaction mixture which contained 50 µl of 30 mM hydrogen peroxide (in 50 mM phosphate buffer, pH 7.0), 25 µl of H2SO4 and 150 µl of KMnO4. The solution was mixed thoroughly and the absorbance at 490 nm was measured. A control which was used in this study was a system devoid of the substrate (hydrogen peroxide). The difference in absorbance per unit time was expressed as the activity. An amount of enzyme required to decompose 1.0 M of hydrogen peroxide per minute at pH 7.0 and 25º is regarded as one unit (Goth, 1991).The value of CAT activity was expressed as U/mg.protein.

2.5.7 Determination of glutathione peroxidase (GPx) assay

This assay was performed based on the glutathione recycling method by using 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) and glutathione reductase. According to this method, 2-nitro-5-thiobenzoic acid and GSSG were generated from the reaction between DTNB and GSH. In brief, 20 µl of sample was added to the reaction mixture consisting of 10 µl of dithiothreitol (DTT) in 6.67 mM potassium phosphate buffer (pH 7), 100 µl of sodium azide in 6.67 mM potassium phosphate buffer (pH 7), 10 µl of glutathione solution and 100 µl of hydrogen peroxide and mixed thoroughly. After the mixing process, the mixture was incubated at room temperature for 5-10 minutes. Then, 10 µl of DTNB (5,5-dithiobis-2-nitrobenzoic acid) was added and the optical density at 412 nm was recorded at25 ºC over a period of 5 min. Activity was presented as nmoles/min/mg lens protein (Rotruck *et al.*, 1973). GPx activity was expressed as U/mg.protein.

2.6 Statistical Analysis

All data were expressed as mean ± SEM. Comparisons between groups were performed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using SPSS statistical software. P-value<0.05 was considered significant.

3. Results

3.1 Effect of quercetin loaded with zein based nanofiber mats on healing process of wound in diabetic rats

According to Table 5-1, it was found that diabetic rats which received zein based nanofiber mats failed to show the significant change of wound adsorption score throughout the study period when compared to diabetic rats which received NSS. Diabetic rats which received 5%quercetin loaded zein based nanofiber mat also failed to show the significant improvement of this parameter throughout a 15 day-study period when compared to diabetic rats which received zein based nanofiber mat. Diabetic rtas which receive zein based nanofiber mat loaded with quercetin at concentration of 10% and 15% significantly enhanced wound adsorption score at 3 days of treatment (P-value<.05 all; compared to diabetic rats which received zein based nanofiber) and no significant changes were observed at the other time window. It was found that both diabetic rats which received zein based nanofiber mat and diabetic rats which received zein based nanofiber mat loaded with quercetin at all concentrations used in this study showed the full adsorption score since 12 days of treatment whereas the full adsorption score of diabetic rats which received NSS was observed at 15 days of treatment.

The effect of quercetin-loaded nanofiber mats on wound healing index was also explored and results were shown in Table 5-2. When compared to diabetic rats which received NSS, no significant change of wound healing index in diabetic rats which obtained zein based nanofiber mat throughout a 15 day-study period. No significant improvement of wound healing index was observed in diabetic rats which received 5%quercetin loaded zein based nanofiber mat when compared to diabetic rats which obtained zein based nanofiber mat throughout a study period. Interestingly, both diabetic rats which received 10%quercetin loaded zein based nanofiber mat and

diabetic rats which received 15%quercetin loaded zein based nanofiber mat significantly enhanced wound healing index at 6, 9, 12 and 15 days of treatment (P-value<.01 and.05; .05 and.01; .01 and .05 and .05 all; compared to diabetic rats which received zein based nanofiber mat).

Table 5-3 showed the effect of quercetin loaded zein based nanofiber mat on percent of wound contraction. Diabetic rats which received zein based nanofiber mat received zein based nanofiber mat failed to show the significant change of percent of wound contraction when compared to diabetic rats which received NSS. At 6 and 9 days of treatment, diabetic rats which received zein based nanofiber mat loaded with all concentrations of quercetin showed the significant improvement of percent of wound contraction (P-value<.05 all; .001, .01 and .01; compared to diabetic rats which received zein based nanofiber mat). When the treatment was prolonged to 12 days, it was found that the significant increase in percent of wound contraction was observed in diabetic rats which received zein based nanofiber mat loaded with 10% and 15% quercetin (P-value<.01 all; compared to diabetic rats which received zein based nanofiber mat). In addition, diabetic rats which received zein based nanofiber mat loaded with all concentrations of quercetin used in this study showed full healing within 15 days of treatment whereas both diabetic rats which received NSS and diabetic rats which received zein based nanofiber mat failed to show the full healing at a15 day-study period.

3.2 Possible underlying mechanism of healing enhancing effect of quercetin loaded with zein based nanofiber mats in diabetic rats

Based on the previous findings that collagen and oxidative stress play the crucial role on the wound healing process (Forbes and Cooper, 2013), the effect of quercetin loaded zein based nanofiber mat on collagen and oxidative stress markers were explored and the results were shown in Table 5-4 –Table 5-5 and Figure 5-3. It was found that no changes in collagen thickness and hydroxyproline content of the diabetic rats which received zein based nanofiber mat. Diabetic rats which received 5%quercetin loaded zein based nanofiber mat failed to produce the significant increase in collagen thickness whereas both diabetic rats which received zein based nanofiber mat loaded with 10% and 15% quercetin significantly enhanced collagen thickness (P-value<.05 all; compared to diabetic rats which received zein based

nanofiber mat). The content of collagen evaluated by using the content of hydroxyproline, a main amino acid in collagen type I, as index was also performed. Table 5-5 revealed that diabetic rats which received zein based nanofiber mat significantly enhanced the hydroxyproline level in granulation tissue at 3 days of treatment (P-value<.05; compared to diabetic rats which received NSS) and no significant changes of this parameter were observed at the other time windows of treatment. Diabetic rats which received quercetin loaded zein based nanofiber mats at the concentration range used in this study also significantly enhanced the level of hydroxyproline in granulation tissue at 3 days of treatment (P-value<.01, .01 and .001 respectively; compared to diabetic rats which received zein based nanofiber mat) and no significant changes of this parameter were observed in any quercetin treatment groups at the other time windows of treatments. In addition, diabetic rats which received 10%quercetin loaded zein based nanofber showed full healing process within 12 days so no data of hydroxyproline level on day 12 of treatment of this group was presented. The content of hydroxyproline, MDA level and the activities of SOD, CAT and GPx in the skin of wound raea were also determined at the end of study and data were shown in Table 5-6. Diabetic rats which received zein based nanofiber mat failed to produce the significant changes of all parameters mentioned earlier when compared to diabetic rats which received NSS. Diabetic rats which received 10%quercetin loaded zein based naofiber showed the significant increase in hydroxyproline level, SOD and GPx activities but decreased MDA level (P-value<.05, .05, .001 and .001 respectively; compared to diabetic rats which received zein based nanofiber mat). When compared to diabetic rats which received zein based nanofiber mat, both diabetic rats which received quercetin at concentratins of 5% and 15%significantly decreased MDA level in the skin at the wound area (P-value<.001 all; compared to diabetic rats which receive zein based nanofiber mat) but no significant changes of the scavenger enzyme activities and hydroxyproline level were observed.

Table 5-1 The effect of quercetin-loaded nnaofiber mats on the healing process of wound in diabetic rats evaluated by using wound adsorption score as index

	Wound adsorption score					
Group	**1-day**	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**
DM+wound excision+0.9%NSS	2.00±0.00	1.25±0.25	1.75±0.47	2.75±0.25	3.75±0.25	4.00±0.00
DM+wound excision+zein based nanofiber	2.00±0.00	1.40±0.24	2.20±0.20	3.40±0.24	4.00±0.00[a]	4.00±0.00
DM+wound excision+5%quercetin loaded zein based nanofiber	2.00±0.00	1.40±0.24	2.00±0.00	3.40±0.24	4.00±0.00[a]	4.00±0.00
DM+wound excision+10%quercetin loaded zein based nanofiber	2.00±0.00	2.00±0.32[a#]	2.80±0.20[a]	3.80±0.20[a]	4.00±0.00[a]	4.00±0.00
DM+wound excision+15%quercetin loaded zein based nanofiber	2.00±0.00	2.00±0.00[a#]	2.40±0.24	3.80±0.20[a]	4.00±0.00[a]	4.00±0.00

Rats were transdermally administered quercetin-loaded nanofiber at a period of 15days .At the end of experiment,they were determined wound adsorption score**.**
Data were presented as mean ± S.E.M. (n=6 per group).
[a] P-value < .05 all; compared with DM+ wound excision group and
[#] P-value < .05 all; compared with DM+ wound excision +zein based nanofiber group.

Table 5-2 The effect of quercetin-loaded nnaofiber mats on the healing process of wound in diabetic rats evaluated by using wound healing index as indicator

Group	Wound healing index					
	1-day	3-day	6-day	9-day	12-day	15-day
DM+wound excision+0.9%NSS	0.00±0.00	8.90±1.53	10.11±5.48	24.25±5.95	57.63±3.68	70.67±4.12
DM+wound excision+zein based nanofiber	0.00±0.00	10.08±3.28	15.74±2.94	25.81±2.66	61.46±1.58	80.53±1.49
DM+wound excision+5%quercetin loaded zein based nanofiber	0.00±0.00	18.50±1.73	31.86±2.19^{a}	43.30±1.90	70.64±1.58	92.33±0.55^{aa}
DM+wound excision+10%quercetin loaded zein based nanofiber	0.00±0.00	19.02±1.75	46.09±2.54$^{aa\#\#}$	55.81±1.22$^{a\#}$	83.04±1.06$^{aa\#\#}$	98.94±0.12$^{aaa\#}$
DM+wound excision+15%quercetin loaded zein based nanofiber	0.00±0.00	13.36±1.35	43.3±2.11$^{aa\#}$	55.75±2.90$^{aa\#\#}$	80.00±1.55$^{aa\#}$	97.24±0.22$^{aaa\#}$

Rats were transdermally administered quercetin-loaded nanofiber at a period of 15days. At the end of experiment,they were determined wound healing index. Data were presented as mean ± S.E.M. (n=6 per group).

$^{a, aa, aaa}$P-value < .05, .01 and .001 respectively; compared with DM+ wound excision group and

$^{\#, \#\#}$P-value < .05 and .01 resspectively; compared with DM+ wound excision +zein based nanofiber group.

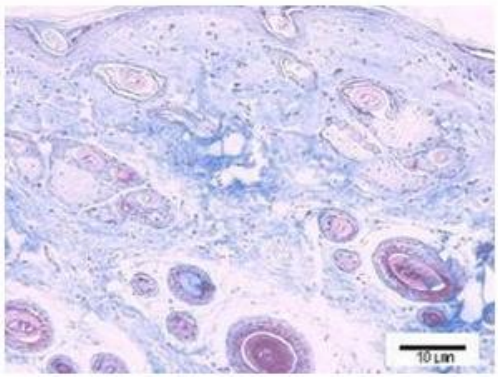
Table 5-3 The ffect of quercetin loaded zein based nanofiber mats on percent of wound contraction of diabetic rats

Group	Percent of wound contraction					
	1-day	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**
DM+wound excision+0.9%NSS	0.00±0.00	10.15±1.84	17.27±8.32	18.40±8.61	166.57±18.74	357.43±46.70
DM+wound excision+zein based nanofiber	0.00±0.00	12.16±4.35	19.61±4.41	21.76±4.64	134.04±15.95	467.07±36.24
DM+wound excision+5%quercetin loaded zein based nanofiber	0.00±0.00	24.56±2.93	51.79±5.29 a#	82.75±6.41 aa###	294.34±34.73	Full healing
DM+wound excision+10%quercetin loaded zein based nanofiber	0.00±0.00	24.25±2.94	91.09±9.98 aa#	128.24±5.88 aa##	525.48±48.23 aa##	Full healing
DM+wound excision+15%quercetin loaded zein based nanofiber	0.00±0.00	16.26±1.81	83.48±6.52 aa#	148.15±11.71 aaa##	464.82±29.29 aa##	Full healing

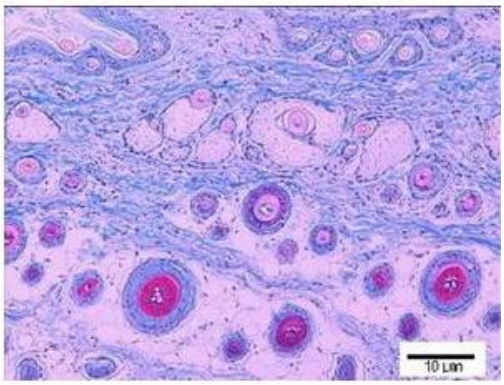
Rats were transdermally administered quercetin-loaded nanofiber at a period of 15days. At the end of experiment,they were determined percent of wound contraction. Data were presented as mean ± S.E.M. (n=6 per group).

[a, aa, aaa]P-value < .05, ,01 and .001 respectively; compared with DM+ wound excision group and

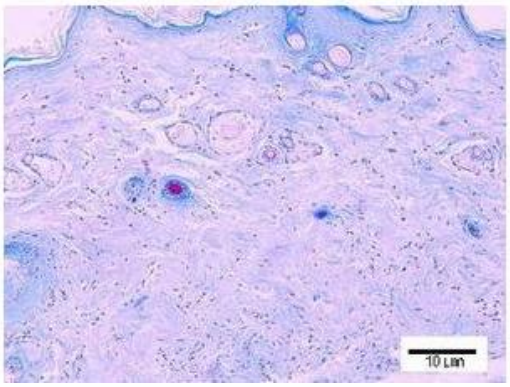
[#, ##, ###]P-value < .05 , .01 and .001 resspectively; compared with DM+ wound excision +zein based nanofiber group.



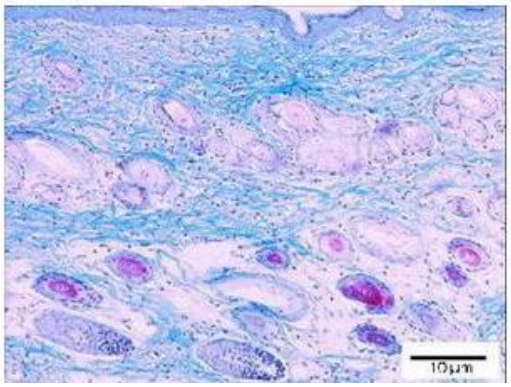
DM+wound excision



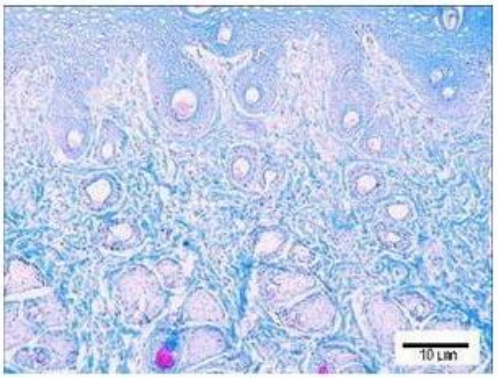
DM+wound excision zein based



DM+wound excision+5%quercetin loaded zein based nanofiber



DM+wound excision+10%quercetin loaded zein based nanofiber



DM+wound excision+15%quercetin loaded zein based nanofiber

Figure 5-3 Photographs of skin at the wound area of diabetic rats which received zein based nanofiber mats loaded with various concentrations of quercetin which stained with Masson's trichrome at 40X magnification

Table 5-4 Effect of zein based nanofiber mats loaded with quercetin at concentrations of 5%,10% and 15% on collagen thickness of diabetic rats

	Collagen thickness (µm)
DM+wound excision	44.31±1.32
DM+wound excision+zein based nanofiber	46.06±1.78
DM+wound excision+5%quercetin loaded zein based nanofiber	51.01±1.54[aa]
DM+wound excision+10%quercetin loaded zein based nanofiber	51.20±1.18[aa#]
DM+wound excision+15%quercetin loaded zein based nanofiber	50.83±0.79[aa#]

Rats were transdermally administered quercetin-loaded nanofiber at a period of 15days. At the end of experiment,they were determined collagen thickness in tissue stained with Masson's trichrome. Data were presented as mean ± S.E.M. (n=6 per group).

[aa]P-value < .01 all; compared with DM+ wound excision group and

[#]P-value < .05 all; compared with DM+ wound excision +zein based nanofiber group.

Table 5-5 Effect of quercetin loaded zein based nanofiber mat on content of hydroxyproline in granulation tissue in diabetic rats

Group	Hydroxyproline level (µg/10mg tissue)			
	Day 3	**Day 6**	**Day 9**	**Day 12**
DM+wound excision+0.9%NSS	20.63±0.29	18.92±2.75	15.28±3.11	20.33±0.83
DM+wound excision+zein based nanofiber	18.79±0.13[a]	23.38±2.04	21.92±0.88	19.67±0.17
DM+wound excision+5%quercetin loaded zein based nanofiber	25.54±0.21[aa##]	20.63±3.21	26.56±2.19[a]	19.71±2.70
DM+wound excision+10%quercetin loaded zein based nanofiber	26.46±0.29[aa##]	19.25±0.58	18.75±1.62	-
DM+wound excision+15%quercetin loaded zein based nanofiber	36.29±0.13[aaa###]	19.54±2.13	20.46±0.13	20.33±0.00

Data were presented as mean ± S.E.M. (n=6 per group).

[a, aa, aaa] P-value < .05, .01and .001respectively; compared with DM+ wound excision group and

[#, ###] P-value < .05 and .001respectively; compared with DM+ wound excision +zein based nanofiber

Table 5-6 Effect of qyercetin extract loaded zein based nanofiber mat on hydroxyproline content and oxidative stress markers including MDA level and the activities of SOD, CAT and GPx in the wound area of diabetic rats

Group	Hydroxyproline levels (µg/10mg tissue)	MDA level (nmol/min.g.protein)	SOD activity (u/mg. protein)	CAT activity (u/mg. protein)	GPx.activity (u/mg. protein)
DM+wound excision	17.08±0.99	1.30±0.50	0.009±0.00	0.007±0.00	0.08±0.01
DM+wound excision+zein based nanofiber	17.82±1.12	0.76±0.05	0.015±0.00	0.007±0.00	0.11±0.00
DM+wound excision+5%quercetin loaded zein based nanofiber	17.95±1.48	0.28±0.05 aa###	0.014±0.00	0.008±0.00	0.12±0.01 aaa
DM+wound excision+10%quercetin loaded zein based nanofiber	22.96±2.19 a#	0.32±0.06 a###	0.022±0.00 #	0.006±0.00	0.19±0.03 aaa###
DM+wound excision+15%quercetin loaded zein based nanofiber	18.34±2.44	0.08±0.04 aa###	0.012±0.00	0.007±0.00	0.11±0.01 aa

Rats were transdermally administered quercetin loaded zein based nanofiber mat at a period of 15 days. Then, wound areas of diabetic rats which received various treatments were deermined hydroxyproline content, MDA level and the activities of SOD, CAT and GPx.. Data were presented as mean ± S.E.M. (n=6 per group).

a, aa,aaa P-value <.05,.01 and .001respectively; compared with DM+ wound excision group and

#, ##, ###P-value <.05, .01and .001respectively; compared with DM+ wound excision +zein based nanofiber.

3.3 Effect of Tomato Extract Loaded with Zein Based Nanofiber Mats on Wound Healing Process of Diabetic Rats

Table 5-7 showed that zein based nanofiber mat failed to produce the significant changes of wound adsorption score in diabetic rats when compared to diabetic rats which received NSS. The significant improvement of wound adsorption scores at 3 days of treatment were observed in diabetic groups which received zein based nanofiber loaded with tomato extract at all concentrations used in this study (P-value<.05 all; compared to diabetic rats which received zein based nanofiber mat). Interestingly, diabetic rats which received 5% and 15%tomato extract loaded zein based nanofiber mats also showed the significant improvement of wound adsorption scores at 6 and 9 days of treatment (P-value<.01 all and .05 all; compared to diabetic rats which received zein based nanofiber mat).Diabetic rats in both groups showed the full adsorption score since 9 day-study period whereas diabetic rats which received 10%tomato extract loaded zein based nanofiber achieved the full adsorption score at 12 day-study period as similar as that of observed in diabetic rats which received either NSS or zein based nanofiber mat which served as delivery system.

The effect of zein based nanofiber mats loaded with various concentrations of tomato extract ranging from 5%, 10% and 15% on wound healing index was also explored and results were shown in Table 5-8. Diabetic rats which received zein based nanofiber showed the significant increase in wound healing index at 12 and 15 days of treatment (P-value<.05 and .001 respectively; compared to diabetic rats which received NSS). 15% tomato extract loaded zein based nanofiber mat significantly increased wound healing index of diabetic rats at 9 and 12 days of treatment (P-value<.01 and .05 respectively; compared to diabetic rats which received zein based nanofiber mat). In addition, it produced the full percentage of wound healing index since 12 days of treatment. No significant changes were observed in the other treatments groups at all times window of treatments used in this study.

The percent of wound contraction of diabetic rats which received tomato extract loaded zein based nanofiber mat was also evaluated and results were shown in Table 5-9. It was demonstrated that diabetic rats which received zein based nanofiber mat failed to show the significant change compared to diabetic rats which received NSS throughout the 15 day-study period. Only diabetic rats which obtained

15%tomato extract loaded zein based nanofiber mat significantly enhanced percent of wound contraction at 9 days of treatment (P-value<.05; compared to diabetic rats which received zein based nanofibermat) whereas the other treatment groups failed to show the significant changes of this parameter when compared to diabetic rats which received zein based nanofiber mat. The data also showed that diabetic rats which received 15%tomato extract loaded zein based nanofiber mat enhanced wound healing process until the wound was fully healed and the wound contraction could not be measured since 12 days of treatment while this phenomenon was observed at 15 days of treatment in other treatments groups

3.4 Possible Underlying Mechanism of Healing Enhancing Effect of Tomato Extract Loaded with Zein Based Nanofiber Mats in Diabetic Rats

Figure 5-4 and Table 5-10 showed that diabetic rats which received zein based nanofiber mat and diabetic rats which received zein based naofiber mat loaded with various concentrations of tomato extract showed trends of the increased collagen thickness but no significant was observed. However, the biochemical assay of hydroxyproline content, the main amino acid in collagen type I which plays the crucial role on wound healing showed the different effect. It was found that diabetic rats which received zein based nanofiber mat showed the significant increase in hydroxyproline content in granulation tissue at 9 and 12 days of treatment (P-value<.01 and .05 respectively; compared to diabetic rats which received NSS). Both diabetic rats which received 5%tomatoextract loaded zein based nanofiber and diabetic rats which received 15%tomato extract showed the elevation of hydroxyproline contents in granulation tissue at 3 and 6 days of treatment (P-value<.05 all and .001 all respectively; compared to diabetic rats which received zein based nanofiber mat) as shown in Table 5-11.

The effect of tomato extract loaded nanofiber mat on oxidative stress markers and hydroxyproline content of skin at the wound area were also determined. Data were shown in Table 5-12. Diabetic rats which received zein based naofiber mat failed to produce the significant change of hydroxyproline content in the skin at the wound area. No significant changes of MDA level and the activities of SOD, CAT and GPx in the skin at the wound area. Diabetic rats which received 5%tomato extract loaded zein based nanofiber mat also failed to produce the significant changes of all parameters mentioned earlier when compared to diabetic rats which received zein

based nanofiber mat. It was found that diabetic rats which received 10% tomato extract loaded zein based nanofiber mat significantly enhanced SOD while diabetic rats which received 15% tomato extract loaded zein based nanofiber mat showed the significant elevation of CAT and GPx (P-value<.01, .05 and .05 respectively; compared to diabetic rats which received zein based nanofiber mat). Unfortunately, no significant change of MDA level in the skin at the wound area was observed.The other treatment groups failed to show the significant changes of this parameter when compared to diabetic rats which received zein based nanofiber mat. The data also showed that diabetic rats which received 15%tomato extract loaded zein based nanofiber mat enhanced wound healing process until the wound was fully healed and the wound contraction could not be measured since 12 days of treatment while this phenomenon was observed at 15 days of treatment in other treatment groups

Table 5-7 Effect of tomato loaded nanofiber mats on wound adsorption score of diabetic rats

Group	Wound adsorption score					
	1-day	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**
DM+wound excision	2.00±0.00	1.25±0.13	1.75±0.424	3.25±0.13	4.00±0.00	4.00±0.00
DM+wound excision+zein based nanofiber	2.00±0.00	1.40±0.11	2.20±0.09	3.40±0.11	4.00±0.00	4.00±0.00
DM+wound excision+5%tomato loaded zein based nanofiber	2.00±0.00	2.00±0.00[a#]	4.00±0.00[aa##]	4.00±0.00[a#]	4.00±0.00	4.00±0.00
DM+wound excision+10%tomato loaded zein based nanofiber	2.00±0.00	2.00±0.00[a#]	2.00±0.00	3.60±0.11	4.00±0.00	4.00±0.00
DM+wound excision+15%tomato loaded zein based nanofiber	2.00±0.00	2.00±0.00[a#]	4.00±0.00[aa##]	4.00±0.00[a#]	4.00±0.00	4.00±0.00

Rats were transdermally administered tomato-loaded nanofiber at a period of 15days. At the end of experiment, they were determined wound adsorbtion score. Data were present as mean+ SEM (n=6/group).

[a] P-value < 0.05 all; compared with DM+ wound excision group and

[#] P-value < 0.05 all; compared with DM+ wound excision +zein based nanofiber group.

Table 5-8 The effect of tomato-loaded nanofiber mats on wound healing process of diabetic rats evaluated by using wound healing index as indicator

Group	Wound healing index					
	1-day	**3-day**	**6-day**	**9-day**	**12-day**	**15-day**
DM+wound excision	0.00±0.00	21.15±5.37	27.06±8.28	40.06±7.92	56.80±3.31	75.97±5.00
DM+wound excision+zein based nanofiber	0.00±0.00	23.49±3.02	38.21±3.94	52.89±4.08	80.83±4.34[a]	95.07±1.52[aaa]
DM+wound excision+5%tomato loaded zein based nanofiber	0.00±0.00	23.95±5.38	42.30±1.76	55.59±1.62	85.39±2.08[aa]	99.37±0.27[aaa]
DM+wound excision+10%tomato loaded zein based nanofiber	0.00±0.00	29.11±3.25	46.10±5.15	58.23±4.29	89.22±2.91[aa]	98.89±0.48[aaa]
DM+wound excision+15%tomato loaded zein based nanofiber	0.00±0.00	26.39±0.64	44.67±1.33	86.85±1.51[aaa##]	100±0.00[aaa#]	100±0.00[aaa]

Rats were transdermally administered tomato-loaded nanofiber at a period of 15days .At the end of experimen, they were determined wound healing index. Data were presented as mean ± S.E.M. (n=6 per group)

[a,aa,aaa]P-value < .05, .01 and .001 respectivly; compared with DM+ wound excision group and

[#, ##] P-value < .05 and .01respectivly; compared with DM+ wound excision +zein based nanofiber group.

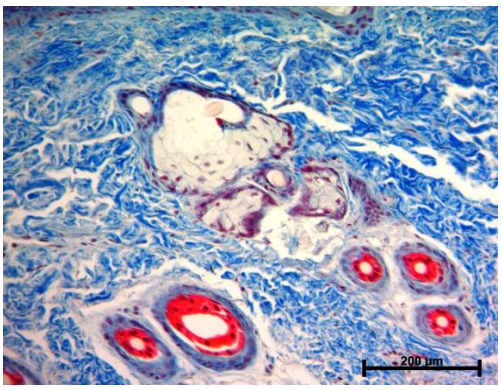
Table 5-9 The effect of tomato loaded zein based nanofiber mats on percent of wound contraction of diabetic rats

Group	Percent of wound contraction					
	Day 1	Day 3	Day 6	Day 9	Day 12	Day 15
DM+wound excision	0.00±0.00	20.32±8.06	51.68±21.42	90.93±30.78	141.18±21.15	487.64±87.33
DM+wound excision+zein based nanofiber	0.00±0.00	24.91±5.20	69.73±10.53	134.35±39.36	486.25±52.29	Full healing
DM+wound excision+5%tomato loaded zein based nanofiber	0.00±0.00	32.82±9.40	81.8±8.08	140.52±9.53	682.96±70.34	Full healing
DM+wound excision+10%tomato loaded zein based nanofiber	0.00±0.00	43.01±7.10	113.84±25.45	207.38±25.26	873.02±176.55[a]	Full healing
DM+wound excision+15%tomato loaded zein based nanofiber	0.00±0.00	36.16±1.19	98.9±1.38	302.59±5.78[aa#]	Full healing	Full healing

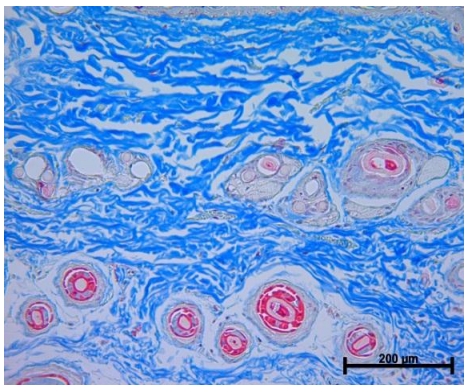
Rats were transdermally administered tomato-loaded nanofiber mats at a period of 15days .At the end of experiment, they were determined percent of wound contraction(%).Data were present as mean±SEM (n=6/group).

[a, aa] P-value < .05, .01 respectively; compared with DM+ wound excision group and

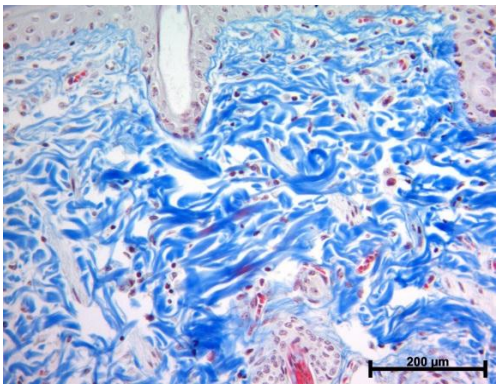
[#]P-value <.05; compared with DM+ wound excision +zein based nanofiber group.



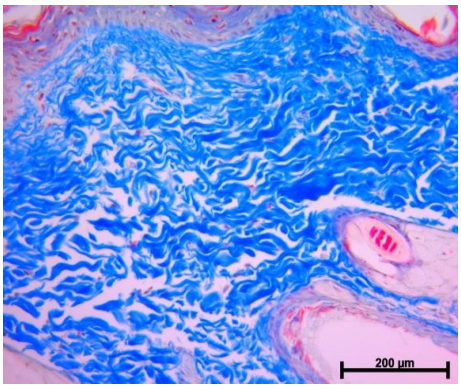
DM+wound excision



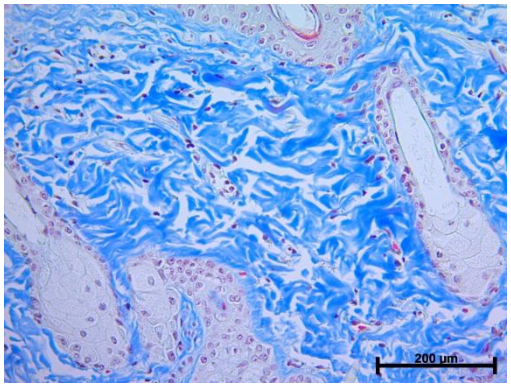
DM+wound excision+zein based nanofiber



DM+wound excision+5%tomato loaded zein based nanofiber



DM+wound excision+10%tomato loaded zein based nanofiber



DM+wound excision+15%tomato loaded zein based nanofiber

Figure 5-5 Photographs of skin at the wound area of diabetic rats which received zein based nanofiber mats loaded with various concentrations of tomato extract which stained with Masson's trichrome at 40X magnification

Table 5-10 Effect of zein based nanofiber mats loaded with tomato at concentrations of 5%, 10% and 15% on collagen thickness of diabetic rats

	Collagen thickness (µm)
DM+wound excision	466.47±15.03
DM+wound excision+zein based nanofiber	476.62±23.12
DM+wound excision+5%tomato loaded zein based nanofiber	507.10±26.81
DM+wound excision+10%tomato loaded zein based nanofiber	532.65±19.94[a]
DM+wound excision+15%tomato loaded zein based nanofiber	512.25±14.67

Data were present as mean±SEM (n=6/group).

[a] P-value < .05 all; compared with DM+wound excision group.

Table 5-11 Effect of tomato loaded zein based nanofiber mat on content of hydroxyl proline in glanulation tissue in diabetic rats

Group	Hydroxyproline level (µg/10mg tissue)			
	Day 3	Day 6	Day 9	Day 12
DM+wound excision	87.17±18.61	122.33±8.43	379.00±6.64	334.78±11.56
DM+wound excision+zein based nanofiber	47.72±4.39	96.75±4.80	458.83±8.17^{aa}	398.50±31.11^{a}
DM+wound excision+5%tomato loaded zein based nanofiber	145.08±33.78$^{\#}$	437.20±10.79$^{aaa\#\#\#}$	437.20±10.79^{a}	425.77±2.36^{aa}
DM+wound excision+10%tomato loaded zein based nanofiber	133.30±28.68	289.94±13.43$^{aaa\#\#\#}$	379.13±11.26	404.19±6.91^{a}
DM+wound excision+15%tomato loaded zein based nanofiber	140.57±29.66$^{\#}$	404.19±6.91$^{aaa\#\#\#}$	463.35±22.62^{aa}	411.85±6.82^{aa}

Data were presented as mean ± S.E.M. (n=6 per group).

$^{a, aa, aaa}$ P-value < .05, .01and .001respectively; compared with DM+ wound excision group and

$^{\#, \#\#\#}$ P-value < .05 and .001respectively; compared with DM+ wound excision +zein based nanofiber

Table 5-12 Effect of tomato extract loaded zein based nanofiber mat on hydroxyl proline content and oxidative stress markers including MDA level and the activities of SOD, CAT and GPx in the wound area of diabetic rats

Group	Hydroxyproline levels (µg/10mg tissue)	MDA level (nmol/min.g.protein)	SOD activity (u/mg. protein)	CAT activity (u/mg. protein)	GPx.activity (u/mg. protein)
DM+wound excision	17.08±0.10	0.020±0.007	2.454±0.455	6.520±10.37	5.1950±1.54
DM+wound excision+zein based nanofiber	17.82±0.13	0.003±0.001	2.034±0.314	8.350±2.024	5.0690±1.05
DM+wound excision+5%tomato loaded zein based nanofiber	18.71±0.18	0.002±0.001	2.753±0.316	7.730±1.243	0.3550±1.13
DM+wound excision+10%tomato loaded zein based nanofiber	24.86±0.14 aa##	0.002±0.002	9.811±0.502 aa##	4.060±0.161	12.333±1.21
DM+wound excision+15%tomato loaded zein based nanofiber	22.19±0.13 a#	0.002±0.001	3.604±0.125	15.52±0.122 a#	63.138±2.56 a#

Rats were transdermally administered tomato loaded zein based nanofiber mat at a period of 15 days. Then, wound areas of diabetic rats which received various treatments were deermined hydroxyproline content, MDA level and the activities of SOD, CAT and GPx.. Data were presented as mean ± S.E.M. (n=6 per group).

[a, aa] P-value <.05and .01respectively; compared with DM+ wound excision group and

[#, ##] P-value <.05and .01respectively; compared with DM+ wound excision +zein based nanofiber.

4. Wound Healing Activity of Quercetin-Loaded Nanofiber in Diabetic Condition

Wound healing process comprises of integrated cellular and biochemical events leading to reestablishment of structural and functional integrity with regain of strength of injured tissue. The aim of wound treatment is to either shorten the time required for healing or to minimize the undesired consequences (Myers et al, 1980). Under normal circumstance, there are four distinct inter-related phases including haemostasis, inflammation, proliferation and remodeling (Stadelmann et al., 1998). The impairment in any phases will give rise to the delay wound healing or chronic wound

Diabetic wounds are chronic wounds that are difficult and frustrating to manage (Singer and Clark, 1999). To date, the precise mechanisms of poor wound healing in diabetic wound is not completely understood. Accumulating lines of evidence from studies involving human and animal models reveal abnormalities in all phases of healing process. It is believed that wound healing impairment in diabetic condition occurs as a result of impaired blood flow and oxygen release from increased blood sugar, decreased collagen and fibronectin synthesis from protein malnutrition, impaired local immune and cell defenses and decreased growth factor (Perez Gutierrez et al., 2006; Teoh et al., 2009).

Numerous natural products have been reported to possess wound healing activity. Recent finding has demonstrated that a flavonoid derivative "Quercetin 3-O-glucoside" appears to be the potential compound to enhance wound healing (Süntar et al., 2010). However, no scientific evidence concerning the efficacy of quercetin loaded nanofiber is available. The data obtained from the current study clearly revealed that 10% and 15%quercetin loaded nanofiber could enhance wound adsorption score, wound healing index and percent of wound contraction whereas 5%quercetin loaded zein based nanofiber mat enhanced only the percent of wound contraction. Therefore, this study clearly demonstrated the wound enhancing effect of zein based nanofiber mat loaded with quercetin at all concentrations used in this study.

It has been reported that collagen, an important connective tissue protein, provides strength and integrity to the dermis (Raghow, 1994) and important for

wound healing process (Purna and Babu, 2000). The decreased collagen content also plays the important role in all phases of wound healing process (Karmer et al., 2010). In addition, previous study demonstrated that flavonoids could suppress lipid peroxidation and gave rise to the increased viability of collagen fibrils by activating the DNA synthesis and preventing the cell damage (Getie et al., 2002; Shetty et al., 2008). This was also in agreement with our findings. Since the current data showed that quercetin loaded zein based nanofiber mat enhanced the hydroxyproline content in granulation tissue and in the skin of wound area together with the increased collagen thickness, it was possible that quercetin might suppress lipid peroxidation which in turn increased collagen synthesis and collagen deposition resulting in the increased collagen thickness and the enhanced wound healing process. Since flavonoids also contribute the important roles on other processes of wound healing process such as antibacterial effect (Xing et al., 2012), the contribution of antibacterial effect can't be excluded.

Although diabetic rats which received zein based nanofiber mat loaded with quercetin at all concentrations used in this study decreased lipid peroxidation, the significant increases in SOD and GPx, the important scavenging enzymes, were observed only in diabetic rats which received 10%quercetin loaded zein based nanofiber mat. Therefore other factors such as the decreased oxidative stress formation and the increased non-enzymatic acavenging system might also play the role. However, this required further exploration.

In this study, no dose dependent manner of quercetin-loaded nanofiber was observed. The possible explanation might be due to the complex phenomena of both the wound healing process and the changes of oxidative stress. Since both phenomena mentioned earlier are under the influences of many factors, no simple linear relationships between the mentioned processes and the concentration of quercetin.

In conclusion, the present data have revealed the potential of 10% quercetin- loaded nanofiber patch as wound healing enhancer in diabetic condition. Therefore, quercetin, the commonly found flavonoid in vegetables and fruits, doesn't only provide the health benefit as food but it can be developed as wound care product which still has high growth rate in the market. Therefore, this study doesn't provide only health benefit of quercetin but also ahows the potential to create more value of quercetin with nanotechnology. However, further researches are required to provide better understanding about the

detail of the underlying mechanism and kinetic changes of the quercetin loaded zein based nanofiber mat .

5. Wound Healing Activity of Tomato Extract-Loaded Nanofiber in Diabetic Condition

Wound healing impairment is a common complication of diabetes mellitus. The pathophysiologic relationship between diabetes and impaired healing is complex. Numerous factors including delayed vascularization, reduction in blood flow (Abiko and Selimovic, 2010), decreased or impaired growth factor production (Galkowska et al., 2006),decreased innate immunity, decreases collagen synthesis and deposition (Perez Gutierrez et al., 2006; Teoh et al., 2009) and elevation of oxidative stresses (Luo et al., 2004).

The current data showed that tomato-loaded nanofiber showed the potential to enhance wound healing process in diabetic condition. Although oxidative stress is regarded as one of the important factors to regulate wound healing, no change of MDA level, a valid indicator of oxidative stress was observed in this study. Therefore, the wound enhancing effect of tomato extract loaded zein based naofiber observed in this study might be less likely to involve the improved oxidative stress status. However, this point is still cannot be cut off because the determination of oxidative stress markers was performed at the end of study which was in the remodeling phase and this change might have been accomplished.

In this study, we have found that the collagen type I was increased in rats subjected to all concentrations of tomato extract treated groups. However, only rats subjected to 15%tomato extract-loaded nanofiber showed the significant increased collagen thickness both in granulation tissue and in the skin of wound area together with the markedly increased of wound contraction. Based on the role of collagen mentioned above, it was suggested that the wound healing enhancing activity induced by tomato extract-loaded nanofiber might be partly associated with the enhanced collagen.

Although angiogenesis and the enhanced blood flow also play the important role on wound healing process, this study did not focus on these changes. However, it has been reported that lycopene, a main pigment in tomato extract, can up-regulate the

level of endothelial nitric oxide synthase (eNOS) (Gao et al., 2012) which in turn increase the blood flow. Therefore, the role of the improved angiogenesis and blood flow should be considered and required further exploration.

The results obtained from this study failed to show dose dependent manner. The possible explanation might be due to several reason as described following; 1) the masking effect of non-active ingredient 2) lack of simple linear relationship between parameters indicating wound healing and the concentration of tomato extract.

In conclusion, tomato-loaded nanofiber shows the potential to enhance wound healing process in diabetic complication. Therefore, it is warrant for further study to determine the precise underlying mechanism. However, the toxicity evaluation should also be concerned.

6. Conclusion

Numerous natural products show health benefits to mitigate the commonly found disorders which are still considered as challenge health-problems. These products can be increased their values as health product. In addition, electrospun nanofiber is served as the potential delivery tool of natural product in the form of both pure substance and crude extract via transdermal route. With this method, the therapeutic dose was very much decreased. In addition, toxicity risk is also decreased. However, safety evaluation following repetitive exposure is essential.