CHAPTER III
THE DEVELOPMENT AND THE ASSESSMENT CHRONIC TOXICITY OF THE NATURAL PRODUCTS LOADED NANOFIBER

1. Introduction

Currently, the development and application of polymer based-nanofiber in medicine has gained much attention due to a number of advantages including improved therapeutic index, possibility for localized delivery and reduced toxicity of drugs. Based on the unique features of electrospun fibers such as their ability to incorporate a wide range of drugs, their high surface area leading to efficient drug release, their interconnecting porous structure with high permeability and the ease of fabricating the delivery vehicle in the required architecture or form, the application of nanofiber as the delivery system is continually increasing (Cui et al., 2010). However, the effective delivery appears to depend on various factors including good mechanical properties, biocompatibility, effective drug release (Carracciolo et al., 2013) together with safety use without toxicity.

Natural product has been long term used for treating numerous ailments since pre-historical time. Currently, numerous natural products have been used for treating and preventing many diabetic complications such as peripheral neuropathy (Khongrum et al., 2012; Galuppo et al., 2014) and diabetic wound (Dorai, 2012; Sharma et al., 2013). Although it has been proven that many natural products have a strong therapeutic value, the poor solubility and bioavailability have severely limited their application. During the last decade, nanofiber loaded natural product has been used for the treatment and prevention of numerous disorders such as wound healing (Merrell et al., 2009) and memory decline (Pangpookiew et al., 2012). Since the nanofibers loaded with natural product possessing antioxidant can attenuate and prevent the oxidative related disorders such as the reduction of memory capacitance and the retardation of wound healing, the possibility to attenuate the oxidative related challenges such as diabetic neuropathy and

diabetic wound with the nanofiber loaded natural products possessing antioxidant effect has been raised.

It has been found that abundant of vegetables and fruits containing quercetin, the most abundant flavonol-type flavonoids in fruits and vegetables (Hertog&Hollman, 1996) are commonly found in Northeast of Thailand. Quercetin has been reported to exhibit numerous health benefits such as anticarcinogenic (Lamson&Brignall, 2000), anti-inflammatory (Rogerio et al., 2007) and antioxidant effects (Takahama, 1988). Therefore, the possibility of nanofiber loaded with quercetin to mitigate diabetic complications has been considered. In addition to quercetin, tomato (*Solanum lycopersicum* Linn.) plants in the family Solanaceae is regarded that as one economic plant in the Northeast of Thailand. It also possesses antioxidant effect (Engelhard et al, 2006). Based on its antioxidant effect, it has the potential to be developed as anti-diabetic complication. Although both quercetin and tomato have the potential to mitigate diabetic complication, no scientific data are available. In order to increase the potential benefit of both quercetin and tomato, this study was set up to develop the nanofiber loaded with quercetin and nanofiber loaded with tomato and to determine the potential benefit to mitigate diabetic complications in vitro. However, it is very much important that prior to the human and pet applications, the novel materials and novel products should be assessed the toxicity. Thus, the subchronic toxicity of the selected natural product loaded nanofiber mat was also investigated to assure the safety for application.

To date, the application of nanotechnology into medicine has gained much attention. It has been believed that nanotechnology will bring significant advances in the diagnosis and treatment of disease. In recent years, the growth and research in application of nanotechnology in medicine especially in drug delivery system in order to improve the preventive and therapeutic strategies. In order to improve these strategies, an amount of drugs or active substances should be delivered and retained in a sufficient amount for a period of time. In addition, it is also expected to avoid from the toxicity of the free drug to non-target organs. To achieve these goals, numerous nanotechnology has been applied to enhance the effectiveness of delivery system including the polymeric structure application. Electrospinning, the most popular and preferred technique for fabrication of nanofibers which is simplicity, cost-effectiveness, flexibility, high potential to scale up, and high ability to spin a broad

range of polymers have been applied to drug delivery technique due to the high surface area of nanofibers as well as three-dimensional open porous structure help to reduce the constraint towards drug diffusion, resulting in a more efficient drug-release system (Luo et al, 2012). However, the effective delivery system is not focused only on the effective to deliver an active substance to the target but also on safety consumption.

During the last decade zein, a gluatamine rich protein has been prepared as polymer nanocomposite in order to increase the delivery effectiveness. Zein based polymer has been used as an effective delivery tool to deliver the active substance such as quecetin to the target organ not only in the peripheral system but also in the central nervous system (Pangpookiew et al, 2012). During facing numerous processes, the substances can change their properties and produce toxicity either by themselves or by the interaction of various substances. Since no data concerning the zein based polymer loaded with quercetin are available until now, the determination of chronic toxicity of zein based polymer loaded with quercetin is very much essential in order to assure about the safety for application of zein based polymer loaded with quercetin especially in the application for chronic pathological states. Thus, the current study was set up to determine the subchronic toxicity of zein based polymer loaded with quercetin.

2. Materials and Method

2.1 Preparation of the tomato extract

Tomato cultivar VF134-1.2 was used as the raw material in the present study. The whole cleaned tomatoes were thoroughly blended, boiled at 70 ° C for 10 minutes and the supernatant was separated from the residues. Then, the residues were further extracted with 50% hydro-alcoholic solvent by maceration method at a ratio of 30 ml: 1 g. After the maceration at room temperature for 24 hr and filtered through Whatman filter paper number 1. The solutions were kept for the determination of antioxidant activity and aldose suppression effect.

2.2 Preparation of quercetin loaded zein polymer

Firstly, zein was dissolved in DMF (N, N-Dimethylformamide, C3H7NO) at a ratio of 1:2 weight/volume (g/ml). Then, the solution was stirred at a period of 60

minutes until the content was completely mixed. Quercetin was added to the solution and stirred until all contents thoroughly mixed to produce 5%, 10% and 15%. The obtained solution was loaded into a 10 ml plastic syringe equipped with a needle spinner made of stainless steel at diameter of 0.7 mm. The needle was connected to a high voltage supply (DEL Electronics Corp., USA (0-100 kV). The solution was fed at a rate of 0.2 ml/h using a syringe pump (TERUMO Terufusion Syringe pump TE-331, Japan). A piece of flat aluminum foil was placed 12 cm below the tip of the needle, and used for nanofibers collection. The voltage for electrospinning was 13kV. All electrospinning processes were performed at room temperature and below 40% relative humidity.

2.3 Determination of the 1, 1-Dipheny-2-picrylhydrazyl (DPPH) radical

The ability to scavenge stable free radicals of the nanofiber loaded with various concentratin of quercetin and tomato extract were assessed using DPPH assay. The working solutions of quercetin was prepared in absolute ethanol whereas that of tomato was prepared in 50% ethanol and the working solution of vitamin C was prepared in distill water. Quercetin and tomato-loaded eletrospun zein based nanofibers (2x2 cm^2) were prepared in 70%ethanol. Various concentrations of ascorbic acid similar to that of the plant extract were prepared and used as standard. The solution containing 1 ml of 0.1 nm DPPH in methanol solution and 450 µl of 50 nm Tris-HCl buffer (pH 7.4). After 30 min of incubation at room temperature, the reduction in the number of DPPH free radicals was measured and reading the absorbance at 517 nm. It this assay results were expressed as the ration percentage of the absorbance decrease of DPPH radical solution following equation: [Absorbance of control-Absorbance of test sample]/Absorbance of control x100.

2.4 Ferric reducing / antioxidant power (FRAP) assay

The FRAP method was used for the determination of total antioxidant capacity base on the reduction of Fe^{3+}.TPTZ complex to blue Fe^{2+}.TPTZ complex by electron donating substance under acidic condition. In this part, the working solution of quercetin, tomato and vitamin C were prepared as mentioned in 2.3. The FRAP reagent (900µl) containing TPTZ, $FeCl_3$ and acetate buffer, was mixed with 90 µl of distilled water and 30 µl of the working solutions of quercetin or tomato extract or vitamin C or the nanofiber mat loaded with either quercetin or tomato extract (cut into

2x2 square centimeter). The absorbance values at 593 nm were recorded for 10 min at 37 C°. Data were expressed as μmol/L.

2.5 Determination of aldose reductase activity

Aldose reductase activity was evaluated using spectrophotometric method. An assay mixture containing 0.7 mL of phosphate buffer (0.067 mol), 0.1 mL of NADPH (25 × 10-5 mol), 0.1 mL of DL-glyceraldehyde (substrate, 5 × 10-4 mol) and 0.1 mL of nerve supernatant were prepared. Absorbance was recorded against a reference cuvette containing all other components except the substrate, DL-glyceraldehyde. The final pH of the reaction mixture was adjusted to pH = 6.2. The determination was performed after adding the substrate or DL-glyceraldehyde by measuring the decrease in NADPH absorbance at 390 nm over a 4- minute period (Patel and Mishra, 2009). The enzyme activity was expressed as nmol/min/mg.

2.6 Determination of morphology of the nanofiber mats loaded either with quercetin or with tomato

The morphology of nanofiber mats loaded either with quercetin or with tomato extract were determined using scanning electron microscope (SEM). The average diameter of nanofiber was determined by analyzing the SEM images with and image analyzing software. (Image –Pro Plus, Media Cybernatics Inc)

2.7 Determination of quercetin content

The content of quercetin was determined by using immersion method. In brief, quercetin-loaded nanofiber sample at 2x2 cm^2 was dissolved in DMF at room temperature. The amount of quercetin in the sample solution was determined using Folin-Ciocalteu method.

2.8 Determination of quercetin release

The cumulative release of quercetin from quercetin-loaded eletrospun zein based nanofibers was assessed using immersion method. The nanofiber loaded with quercetin at 1x1 cm^2 was immersed in 20 ml of B/T/M releasing medium at 37 ^{0}C. At a specified immersion period between 0 and 72 h, 0.3 ml of the test medium was withdrawn and an equal amount of the fresh medium was refilled. The amount of quercetin in the sample solution was determined using Folin-Ciocalteu method.

2.9 Determination chronic toxicity of quercetin-loaded nanofiber

The current data from in vitro and in vivo study concerning the antioxidant activity and the suppression effect of aldose reductase of the nanofiber loaded with quercetin revealed that quercetin-loaded nanofiber showed higher potential to combat the diabetic complications. Therefore, the determination of chronic toxicity of quercetin-loaded nanofiber mats was performed. Healthy young adult male and female Wistar rats, weighing 200-250 g, were purchased from National Laboratory Animal Center, Salaya, Nakorn Pathom, Thailand. The animals were acclimatized to laboratory conditions for 7 days prior to the experiments. The rats were maintained at a room temperature of 22–24°C, with a 12 h light/dark cycle. All procedures in this study were carried out according to the guideline and approval of the Ethical Committee on Animals Experiments of Khon Kaen University (AEKKU 11/2552).

The animals of both sexes were randomly divided in to 3 separated groups as following; 1) control 2) zein based nanofiber treated rats 3) 15% quercetin-loaded zein based nanofiber treated rats. In this study, the control rats received no treatment whereas the rats which treated with all of types of nanofiber mats (1x1 cm^2) were placed either with zein based nanofiber mat or with 15%quercetin-loaded nanofiber mats at the dorsal back of the animals. The nanofiber mats were changed every day throughout a 6 month-study period. At the end of study, they were sacrificed, venous blood was collected for the determination of hematological and biochemical changes at Srinagarind hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Vital organs were excised, weighed and determined gross pathology. In addition, they were preserved in a fixation medium of 10% solution of buffered formalin for histological study.

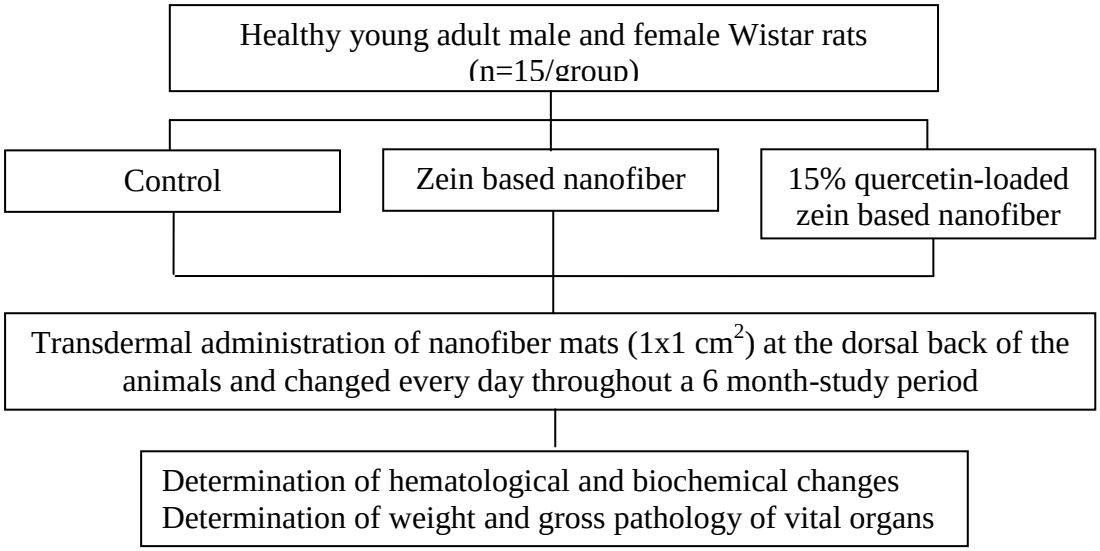


Figure 3-1 Schematic diagram shows the experimental protocol of the determination of chronic toxicity of quercetin-loaded nanofiber mats

2.9 Histopathological study

All vital organs isolated from each individual were fixed in 10% buffered formalin, routinely processed and embedded in molten paraffin wax. The sections were cut at 5 µm and stained with haematoxylin and eosin (H&E). The slides were examined under a light microscope and the magnified images of the tissues structure were captured for further study.

2.10 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 Biological activity related with diabetic complications of the nanofiber loaded either with quercetin or with tomato extract

Based on the previous findings, it was found that the complications of diabetes mellitus such as peripheral neuropathy and diabetic wound are associated with oxidative stress and the function of polyol pathway, the antioxidant activity and

the suppression effect of aldose reductase were determined. Table 3-1 showed the antioxidant activity determined via DPPH and FRAP assays and the suppression activity effect of aldose reductase of vitamin C, quercetin, tomato extract and the nanofiber mats loaded either with various concentration of quercetin or with various concentration of tomato extract. According to the ability to scavenge the stable free radicals via DPPH, quercetin showed the lowest EC_{50} (0.086 mg/ml). The EC50 of quercetin is lower than the well-known antioxidant, vitamin C. The zein based nanofiber mat loaded with various concentrations of quercetin showed higher EC_{50} (0.105, 1.320 and 3.130 mg/ml) than quercetin. However, 5%quercetin-loaded zein based nanofiber mat still showed lower EC50 (0.105 mg/ml) than that of vitamin C (0.905 mg/ml). Zein based nanofiber mats loaded with all concentrations of tomato extract also showed higher EC50 (22.124, 23.269 and 9.989 mg/ml) than tomato extract and vitamin C. It was found that 10%quercetin-loaded zein based nanofiber mat showed the lowest EC_{50} via FRAP assay (0.008 mg/ml). Both 5% and10% quercetin extract loaded zein based nanofiber mats still showed EC_{50} (0.025 and 0.008 mg/ml) lower than that of quercetin (0.168 mg/ml). Zein based nanofiber mats loaded with 5% and 10%tomato extract showed lower EC_{50} (1.364 and 1.504 mg/ml) than that of tomato extract (1.573 mg/ml) whereas zein based nanofiber mats loaded with 15%tomato extract showed ED_{50} (1.706 mg/ml) higher than that of tomato extract.

Data concerning the suppression effect of aldose reductase of this study showed that the zein based nanofiber mat loaded with 5%quercetin showed the lowest ED50 (0.0003 mg/ml). It was found that5% and 15%loaded zein based nanofiber mat showed EC50 lower than that of quercetin but 10%loaded nanofiber showed higher ED50 than that of quercetin. It was found that the zein based nanofiber mats loaded with all concentrations of tomato showed lower ED50 (0.569, 0.129 and .536 mg/ml) than that of tomato extract (11.821 mg/ml).

3.2 Morphology of zein based loaded nanofiber mat

Figure 3-2(A) showed the morphology of zein based nanofiber mat and zein based nanofiber mats loaded with quercetn at concentrations of 5, 10 and 15%. It was shown that the zein based nanofiber mats loaded with all concentrations of quercetin showed the bigger diameters than zein-based nanofiber mats (P-value<.001 all; compared to zein based nanofiber mat). However, no significant difference in

diameters among various groups of quercein-loaded zein based nanofiber was observed as shown in figure 3-2(B).

Figure 3-3(A) showed the morphology of zein based nanofiber mat and zein based nanofiber mats loaded with tomato at concentrations of 5, 10 and 15%. It was shown that the zein based nanofiber mats loaded with all concentrations of tomato showed the bigger diameters than zein-based nanofiber mats (P-value<.05 and .001 respectively; compared to zein based nanofiber mat). In addition, 15%tomato-loaded zein based nanofiber mat showed the bigger diameters than 5% and 10%tomato-loaded zein based nanofiber mat (P-value<.001 all; compared to 15%tomato-loaded zein based nanofiber mat) as shown in figure 3-3(B).

Table 3-1 The antioxidant activity determined via DPPH and FRAP assays and the suppression activity effect of aldose reductase of vitamin C, quercetin, tomato extract and the nanofiber mats loaded either with various concentration of quercetin or with various concentration of tomato extract

Material	Aldose reductase (EC50: mg/ml)	DPPH (EC50: mg/ml)	FRAP (EC50: mg/ml)
Vitamin C	0.008	0.905	0.097
Quercetin	0.027	0.086	0.168
Tomato extract	11.821	5.379	1.573
5% Quercetin loaded zein based nanofiber mats	0.0003	0.105	0.025
10% Quercetin loaded zein based nanofiber mats	0.028	1.320	0.008
15% Quercetin loaded zein based nanofiber mats	0.001	3.130	1.673
5% Tomato loaded zein based nanofiber mats	0.569	22.124	1.364
10% Tomato loaded zein based nanofiber mats	0.129	23.269	1.504
15% Tomato loaded zein based nanofiber mats	0.536	9.989	1.706

(A)

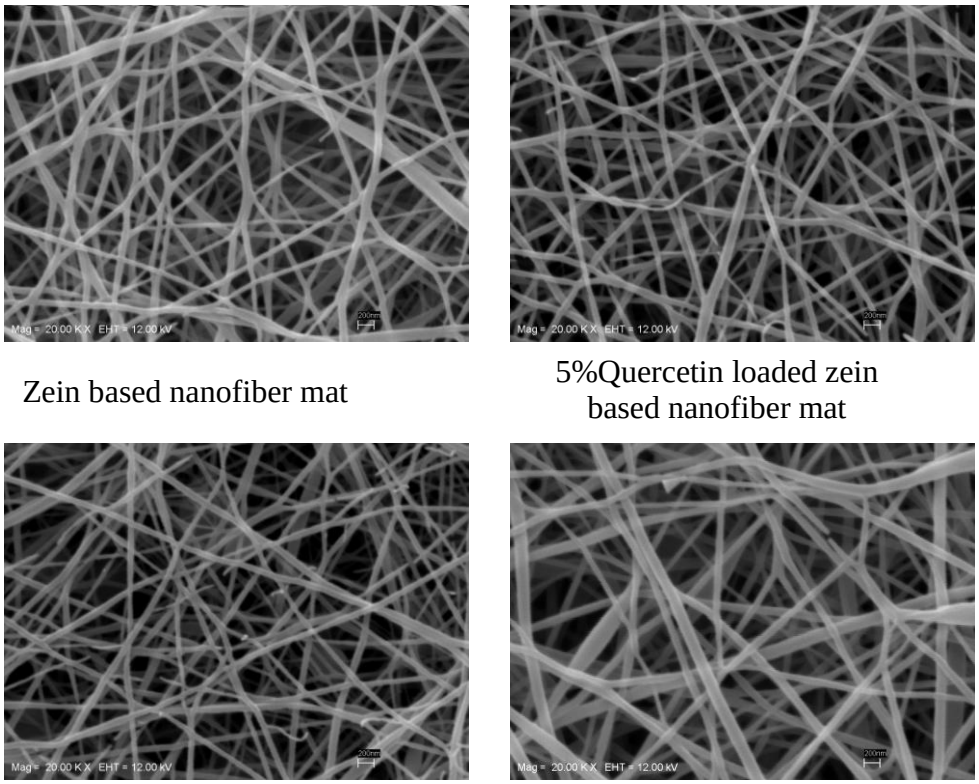


Figure 3-2 Morphology of zein based nanofiber and zein based nanofiber loaded with 5%, 10% and 15%quercetin . A) Photographs of zein based nanofiber and zein based nanofiber loaded with 5%,10% and 15%quercetin from scanning electron microscope (SEM). B) Bar graph illustrating the sizes of zein based nanofiber mat and zein based nanofiber mat loaded with 5%,10% and 15%quercetin evaluated via SEM. Data were expressed s mean±SEM (n=8/group) $^{###}$P-value<.001; compared to zein based nanofibr mat

(B)

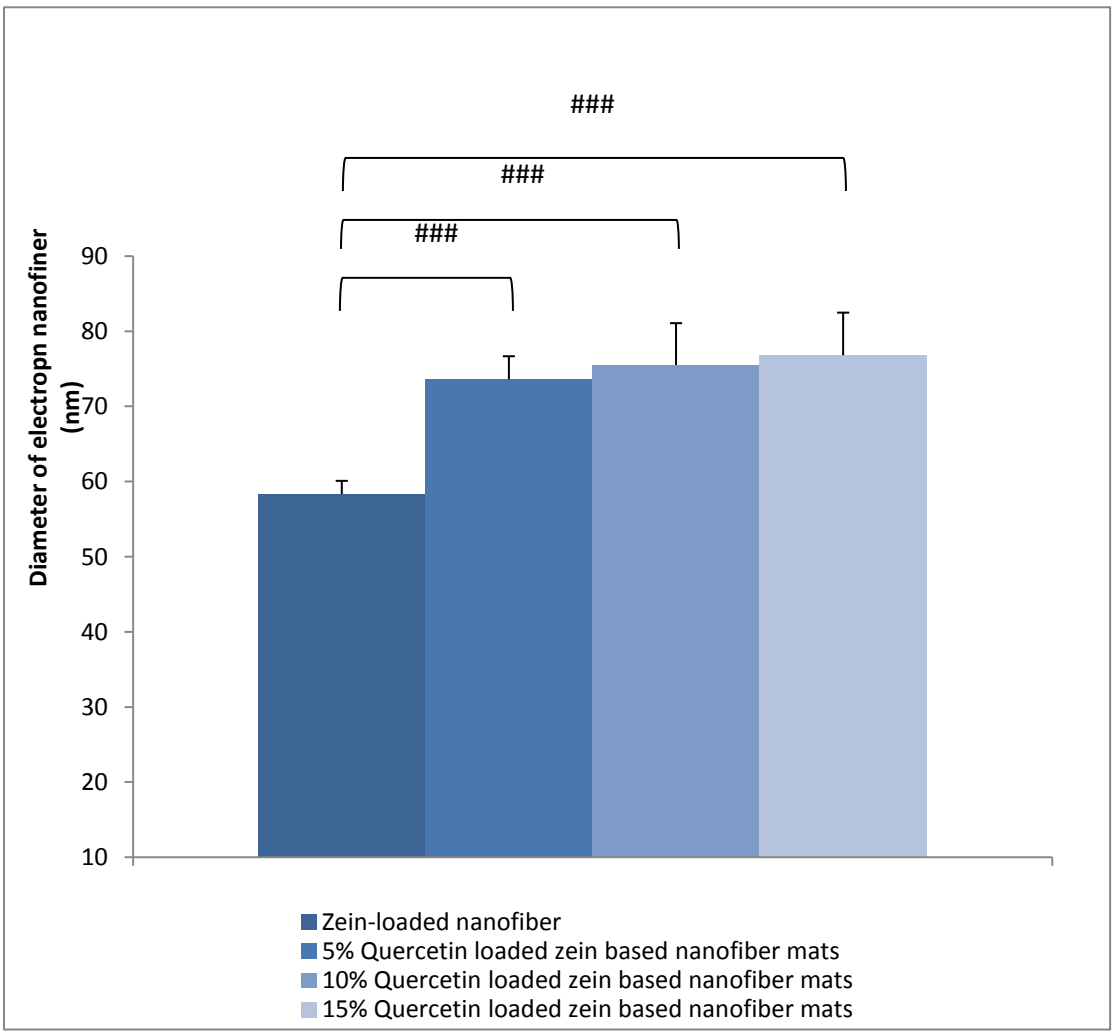
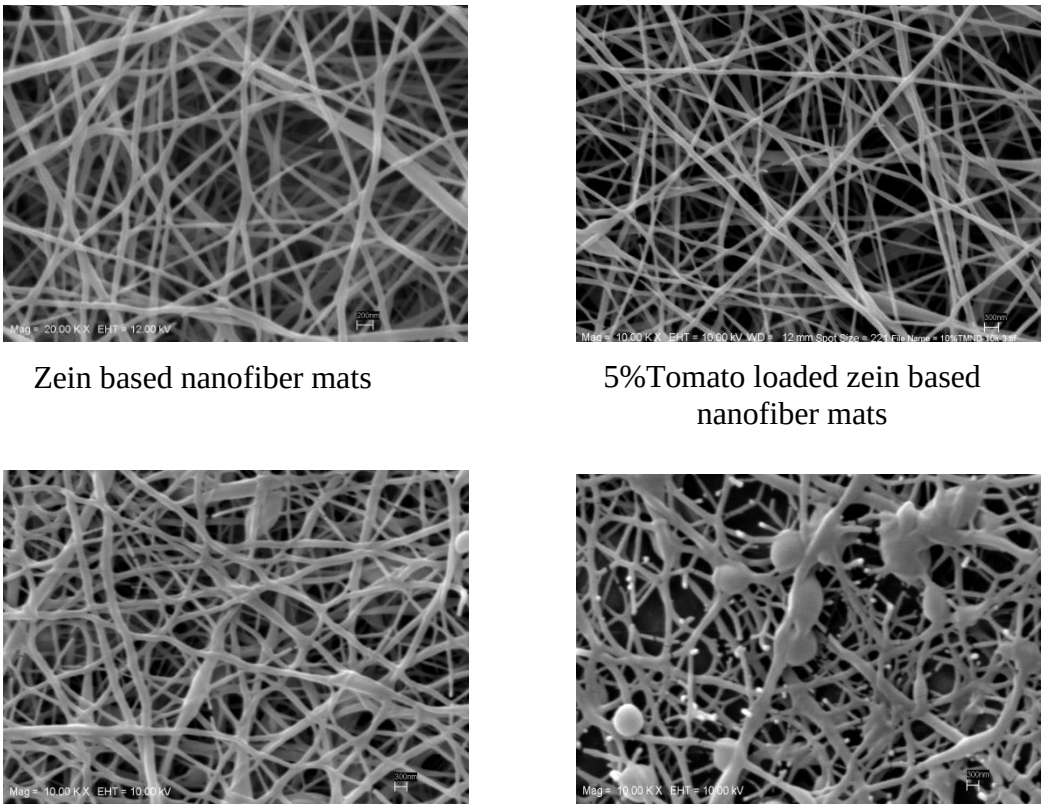


Figure 3-2 Morphology of zein based nanofiber and zein based nanofiber loaded with 5%, 10% and 15%quercetin. A) Photographs of zein based nanofiber and zein based nanofiber loaded with 5%,10% and 15%quercetin from scanning electron microscope (SEM). B) Bar graph illustrating the sizes of zein based nanofiber mat and zein based nanofiber mat loaded with 5%,10% and 15%quercetin evaluated via SEM. Data were expressed s mean±SEM (n=8/group) $^{###}$P-value<.001; compared to zein based nanofibr mat (Cont.)

(A)



Zein based nanofiber mats

5%Tomato loaded zein based nanofiber mats

10%Tomato loaded zein based nanofiber mats

15%Tomato loaded zein based nanofiber mats

Figure 3-3 Morphology of zein based nanofiber and zein based nanofiber loaded with 5%, 10% and 15%tomato extract. A) Photographs of zein based nanofiber and zein based nanofiber loaded with 5%,10% and 15% tomato extract from scanning electron microscope (SEM). B) Bar graph illustrating the sizes of zein based nanofiber mat and zein based nanofiber mat loaded with 5%,10% and 15% tomato extract evaluated via SEM. Data were expressed s mean±SEM (n=8/group) $^{\#,\#\#\#}$P-value<.05 and .001 respectvely; compared to zein based nanofibr mats ^{aaa}P-value< .001; compared to 15%tomato loaded zein based nanofibr mats

(B)

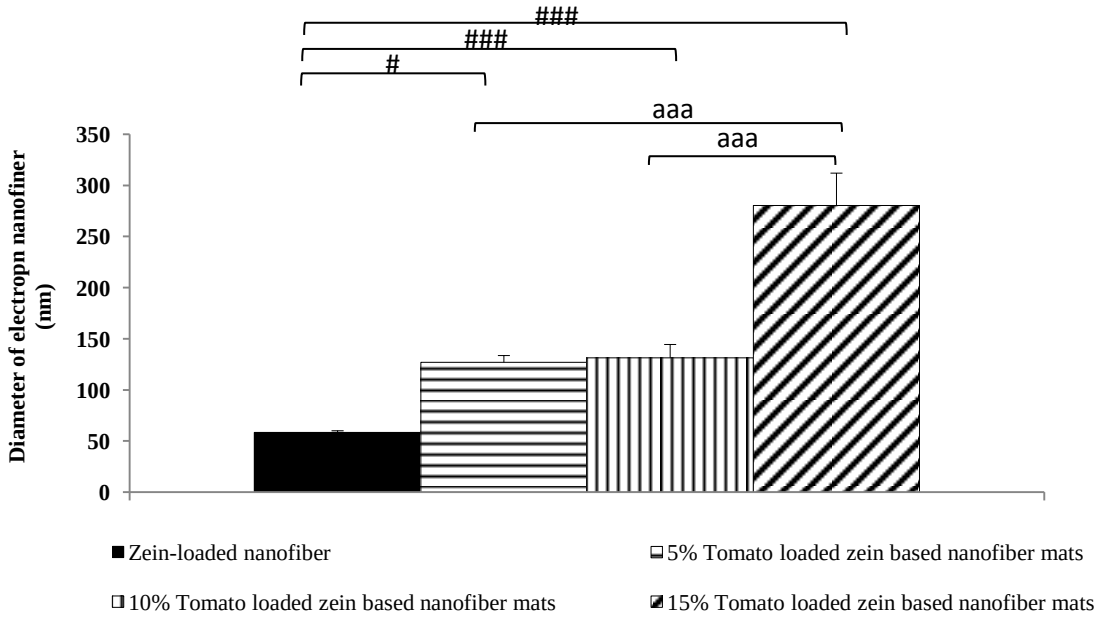


Figure 3-3 Morphology of zein based nanofiber and zein based nanofiber loaded with 5%, 10% and 15%tomato extract. A) Photographs of zein based nanofiber and zein based nanofiber loaded with 5%,10% and 15% tomato extract from scanning electron microscope (SEM). B) Bar graph illustrating the sizes of zein based nanofiber mat and zein based nanofiber mat loaded with 5%,10% and 15% tomato extract evaluated via SEM. Data were expressed s mean±SEM (n=8/group) $^{\#,\#\#\#}$P-value<.05 and .001 respectvely; compared to zein based nanofibr mats ^{aaa}P-value< .001; compared to 15%tomato loaded zein based nanofibr mats (Cont.)

3.3 Quercetin loading and release

Since the efficiency of quercetin-loaded zein based nanofiber depended on percentage of entrapment of quercetin. Figure 3-4, it was found that zein based polymer was successfully loaded with quercetin at various concentrations. Loading efficiency was varied depending on the concentration of loaded quercetin. Percentage of loading of 5%, 10% and 15% quercetin-loaded nanofiber were 64.126, 63.471 and 72.846 respectively.

In order to assure that the loaded quercetin could be released, the evaluation of the release characteristic of quercetin from quercetin-loaded zein base nanofiber mats was also performed. The result show that quercetin which loaded in 5 and 10% quercetin-loaded zein base nanofiber mats showed the gradual release and reached the plateau within the first 24hr while the quercetin which had been loaded in 15% quercetin-loaded zein base nanofiber mats showed the continually released throughout the 72 hr-period as shown in figure 3-5.

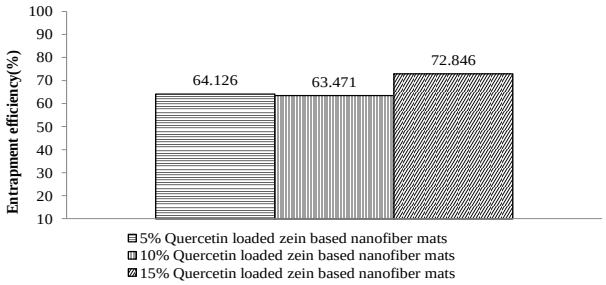


Figure 3-4 Entrapment efficiency of quercetin loaded zein based nanofiber

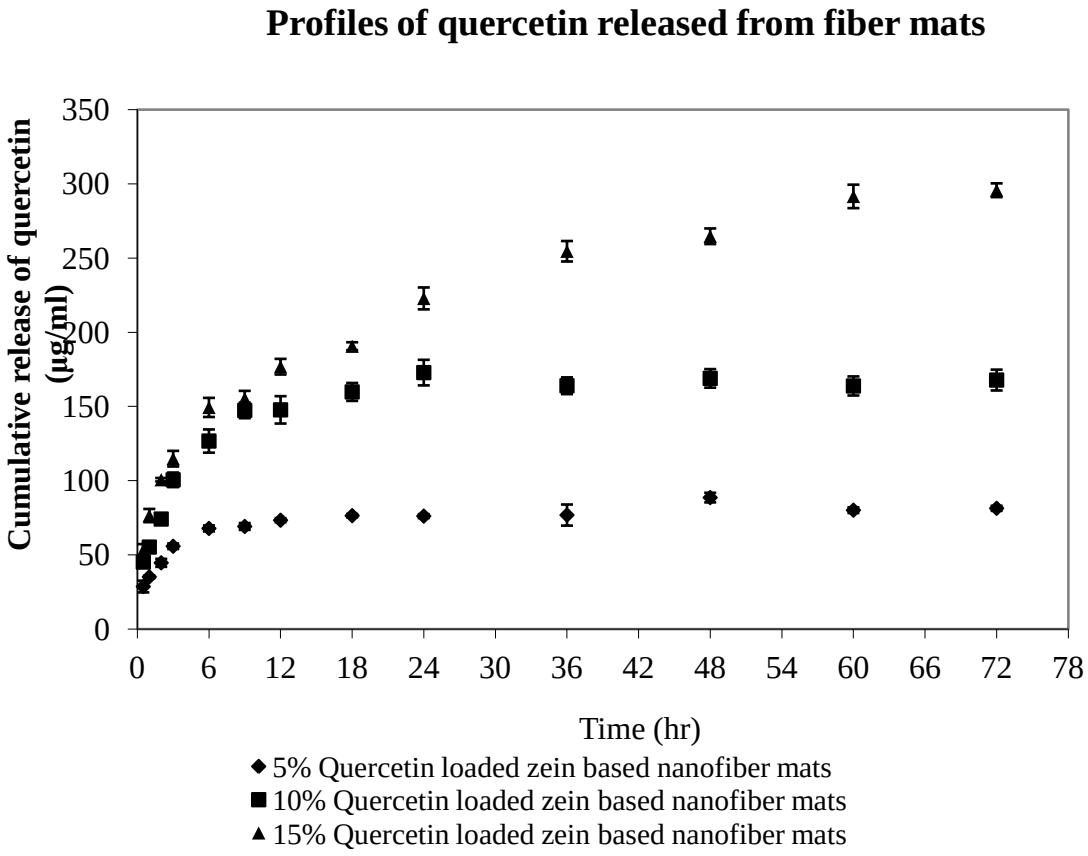


Figure 3-5 Cumulative release of quercetin from quercetin-loaded zein based nanofiber mats

3.4 Chronic toxicity of quercetin-loaded zein based nanofiber mats

The results obtained from the present study demonstrated that the 15% quercetin-loaded zein based nanofiber mats and zein based nanofiber mats did not produce any mortality throughout the study period of 6 months. The present data also showed no toxicity signs such as tremors, convulsions, salivation, fur, eyes, diarrhea, lethargy, sleep and coma, changes in physical appearance, injury, pain, and signs of illness as shown in table 3-2 to 3-5. In addition, no changes of histomorphology of liver, kidney and spleen were observed as shown in figure 3-6. The relative organ weights of various organs of both female and male rats were shown in table 3-6 and table 3-7. It was found that no significant difference of organ weights among group

was observed. The significant changes of organ histomorphologies of male and female rats also didn't showed the significant difference as shown in figure 3-6.

The hematological study showed no significant changes in hematological parameters among groups. No significant changes of liver function and kidney functions were observed as shown in table 3-8 and table3-9. No significant changes of blood hematological values were observed as shown in table 3-10 and table 3-11.

Table 3-2 The effect 6-month administration of zein based nanofiber mat via transdermal route on toxicity signs of male rats (1)

No.	Response	Unmarked		Head		Body		Tail		Head&Tail		Haed&Body	
		Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	Alertness	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2	Grooming	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
3	Hyperactivity	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
4	Tremors	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
5	Convulsion	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
6	Salivation	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	Fur	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	Eye	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9	Diarrhea	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
10	Lethalgy	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
11	Sleep and coma	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
12	Injury	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
13	Pain response	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
14	Signs of illness	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent

Table 3-3 The effect 6-month administration of zein based nanofiber mat via transdermal route on toxicity signs of female rats (2)

No.	Response	Unmarked		Head		Body		Tail		Head&Tail		Haed&Body	
		Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	Alertness	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2	Grooming	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
3	Hyperactivity	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
4	Tremors	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
5	Convulsion	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
6	Salivation	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	Fur	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	Eye	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9	Diarrhea	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
10	Lethalgy	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
11	Sleep and coma	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
12	Injury	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
13	Pain response	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
14	Signs of illness	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent

Table 3-4 The effect6-month administration of 15%quercetin loaded zein based nanofiber mat via transdermal route on toxicity signs of male rats

No.	Response	Unmarked		Head		Body		Tail		Head&Tail		Haed&Body	
		Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	Alertness	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2	Grooming	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
3	Hyperactivity	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
4	Tremors	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
5	Convulsion	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
6	Salivation	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	Fur	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	Eye	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9	Diarrhea	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
10	Lethalgy	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
11	Sleep and coma	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
12	Injury	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
13	Pain response	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
14	Signs of illness	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent

Table 3-5 The effect6-month administration of 15%quercetin loaded zein based nanofiber mat via transdermal route on toxicity signs of female rats

No.	Response	Unmarked		Head		Body		Tail		Head&Tail		Haed&Body	
		Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	Alertness	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2	Grooming	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
3	Hyperactivity	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
4	Tremors	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
5	Convulsion	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
6	Salivation	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	Fur	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	Eye	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9	Diarrhea	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
10	Lethalgy	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
11	Sleep and coma	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
12	Injury	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
13	Pain response	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
14	Signs of illness	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent

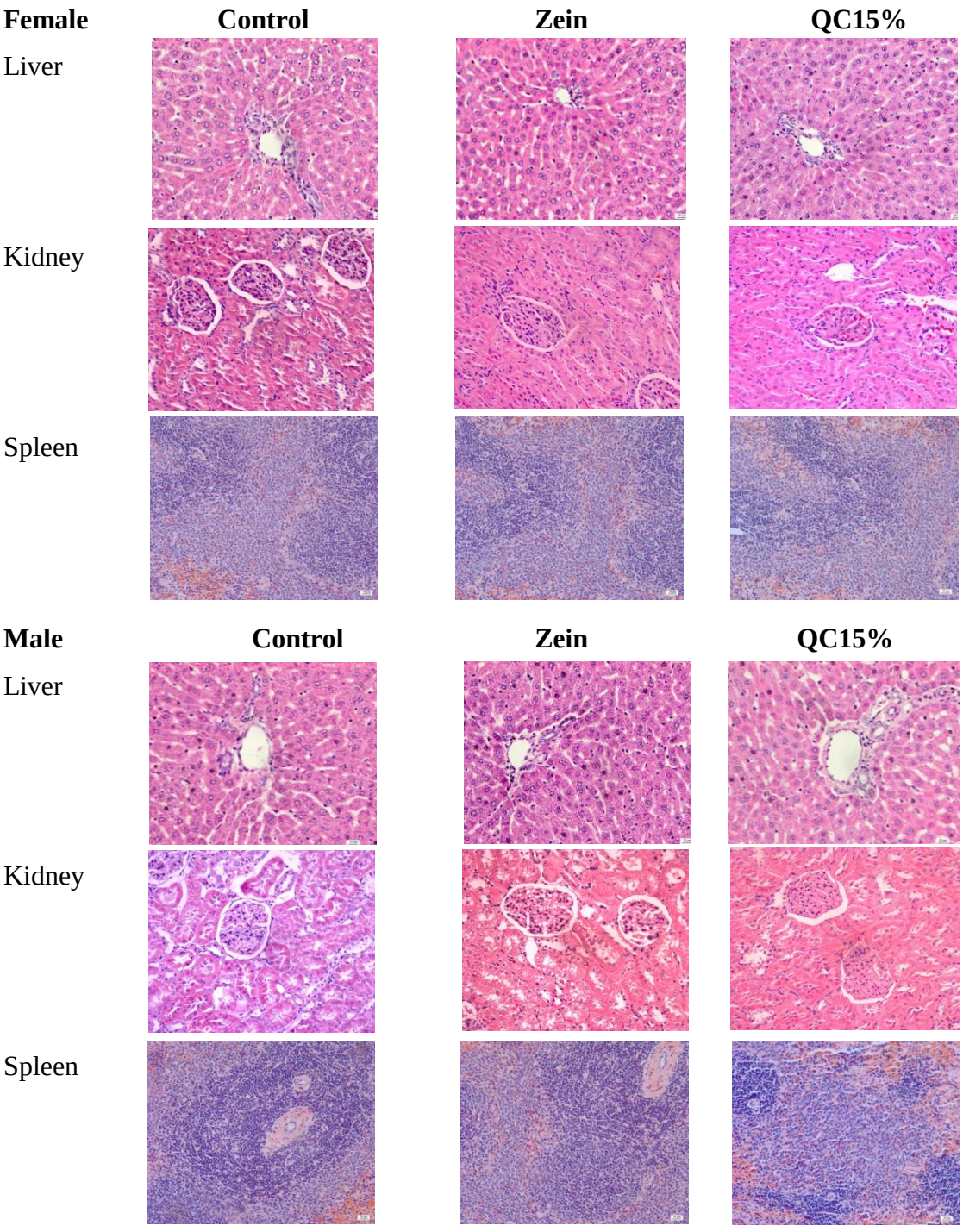


Figure 3-6 Histopathological changes of vital organs such as liver, kidney and spleen of male and female rats which subjected to a 6-month administration of either zein based nanofiber mat or 15%quercetin loaded zein based nanofiber mat in comparison with control rats. (N=15/group) (40X)

Table 3-6 The relative organ weight of female rats which received 15%quercetin loaded zein based nanofiber mat via transdermal route for 6 months. (N=15/group) Data were shown as mean±SEM

Female relative organ weight (g/kg, n=15)			
	Control	**Zein**	**QC15%**
Body weight	256 ± 2.34	260.47 ± 5.33	259.31 ± 6.75
Brain	7.86 ± 0.18	7.65 ± 0.17	7.26 ± 0.19
Lung	6.80 ± 0.64	10.48 ± 1.25	12.22 ± 2.03
Heart	3.73 ± 0.20	4.00 ± 0.15	3.49 ± 0.43
Liver	31.05 ± 1.87	29.49 ± 1.13	29.22 ± 1.05
Rt. Kidney	3.52 ± 0.08	3.48 ± 0.12	3.23 ± 0.11
Lt. Kidney	3.61 ± 0.16	3.49 ± 0.09	3.28 ± 0.10
Spleen	2.67 ± 0.08	2.90 ± 0.08	2.88 ± 0.11
Pancreas	5.91 ± 0.36	5.80 ± 0.46	5.15 ± 0.29
Rt. Ovary	0.53 ± 0.04	0.50 ± 0.03	0.50 ± 0.04
Lt. Ovary	0.46 ± 0.03	0.54 ± 0.04	0.50 ± 0.04
Rt. Salivary gl.	0.43 ± 0.05	0.51 ± 0.05	0.44 ± 0.02
Lt. Salivary gl.	0.39 ± 0.05	0.52 ± 0.05	0.47 ± 0.02
Thymus	1.663 ± 0.16	1.79 ± 0.13	1.98 ± 0.10

Table 3-7 The relative organ weight of male rats which received 15%quercetin loaded zein based nanofiber mat via transdermal route for 6 months. (N=15/group) Data were shown as mean±SEM

Male relative organ weight (g/kg, n=15)			
	Control	**Zein**	**QC15%**
Body weight	407.06 ± 6.06	410.33 ± 6.78	429.00 ± 11.51
Brain	4.92 ± 0.09	5.04 ± 0.22	4.69 ± 0.14
Lung	5.30 ± 0.15	8.98 ± 2.27	11.01 ± 3.32
Heart	3.23 ± 0.14	3.53 ± 0.08	3.52 ± 0.13
Liver	24.56 ± 1.03	26.81 ± 0.60	25.90 ± 0.35
Rt. Kidney	2.79 ± 0.08	3.06 ± 0.11	2.95 ± 0.09
Lt. Kidney	2.88 ± 0.11	3.23 ± 0.13	3.05 ± 0.13
Spleen	2.63 ± 0.19	2.20 ± 0.12	2.02 ± 0.12
Pancreas	4.72 ± 0.26	4.91 ± 0.61	3.82 ± 0.29
Rt. Testis	4.93 ± 0.11	4.92 ± 0.10	4.67 ± 0.08
Lt. Testis	5.08 ± 0.16	5.00 ± 0.18	4.78 ± 0.08
Rt. Salivary gl.	0.43 ± 0.03	0.39 ± 0.04	0.38 ± 0.06
Lt. Salivary gl.	0.46 ± 0.04	0.37 ± 0.04	0.35 ± 0.03
Thymus	1.26 ± 0.04	1.97 ± 0.37	1.57 ± 0.16

Table 3-8 Biochemical changes of biomarkers indicated liver and kidney functions of female rats (N=15/group) Data were expressed as mean±SEM

Female blood biochemical values (n=15)			
	Control	**Zein**	**QC15%**
BUN(mg/dl)	23.59 ± 0.56	26.60 ± 1.23	25.29 ± 0.83
ALT(U/L)	40.80 ± 5.02	39.73 ± 5.68	35.07 ± 1.96
AST(U/L)	127.33 ± 7.14	95.36 ± 11.10	111.21 ± 10.32
ALP(U/L)	25.00 ± 1.00	28.00 ± 1.95	32.00 ± 2.26
Tot Bili(mg/dl)	0.30 ± 0.08	0.31 ± 0.02	0.31 ± 0.01

Table 3-9 Biochemical changes of biomarkers indicated liver and kidney functions of male rats (N=15/group) Data were expressed as mean±SEM

Male blood biochemical values (n=15)			
	Control	**Zein**	**QC15%**
BUN(mg/dl)	24.64 ± 1.99	22.67 ± 0.44	23.40 ± 0.52
ALT(U/L)	43.47 ± 2.44	42.56 ± 2.47	41.90 ± 4.52
AST(U/L)	148.50 ± 3.15	129.57 ± 8.20	135.29 ± 7.55
ALP(U/L)	38.55 ± 2.16	40.75 ± 3.35	43.33 ± 6.12
Tot Bili(mg/dl)	0.10 ± 0.00	0.12 ± 0.01	0.12 ± 0.01

Table 3-10 Blood hematological values changes of female rats (N=15/group). Data were expressed as mean±SEM

Female blood hematological values (n=15)			
	Control	**Zein**	**QC15%**
WBC(x1000/uL)	3.15 ± 0.29	3.55 ± 0.17	4.11 ± 0.34
NEUT(%)	24.71 ± 1.05	29.23 ± 1.87	26.34 ± 0.80
LYMPH(%)	70.88 ± 2.70	64.53 ± 1.71	66.34 ± 0.98
MONO (%)	4.43 ± 1.56	4.72 ± 0.53	4.54 ± 0.45
EO (%)	2.37 ± 0.90	1.67 ± 0.37	1.49 ± 0.23
BASO (%)	0.13 ± 0.10	1.21 ± 0.53	1.14 ± 0.36
RBC(x1e6/uL)	7.52 ± 0.10	7.26 ± 0.11	7.38 ± 0.10
HGB(g/dL)	14.64 ± 0.19	14.19 ± 0.17	14.25 ± 0.12
HCT (%)	44.40 ± 0.99	42.68 ± 0.57	42.75 ± 0.54
MCV (fL)	58.70 ± 0.49	58.04 ± 0.46	58.28 ± 0.70
MCH (pg)	19.10 ± 0.22	19.55 ± 0.15	19.87 ± 0.35
MCHC (g/dL)	29.60 ± 1.96	33.47 ± 0.19	33.52 ± 0.15

Table 3-11 Blood hematological values changes of female rats (N=10/group). Data were expressed as mean±SEM

Male blood hematological values (n=10)			
	Control	**Zein**	**QC15%**
WBC(x1000/uL)	4.39 ± 0.39	3.93 ± 0.47	4.44 ± 0.49
NEUT(%)	22.96 ± 2.17	26.37 ± 1.15	27.43 ± 1.34
LYMPH(%)	68.91 ± 2.91	66.43 ± 1.43	66.57 ± 1.41
MONO (%)	8.20 ± 1.81	3.84 ± 0.30	4.30 ± 0.47
EO (%)	3.19 ± 0.76	1.17 ± 0.18	1.25 ± 0.11
BASO (%)	0.34 ± 0.03	0.43 ± 0.05	0.43 ± 0.04
RBC(x1e6/uL)	7.92 ± 0.30	7.88 ± 0.38	8.07 ± 0.14
HGB(g/dL)	16.14 ± 0.24	15.44 ± 0.31	15.30 ± 0.35
HCT (%)	48.30 ± 1.62	45.32 ± 1.18	44.88 ± 0.96
MCV (fL)	58.46 ± 1.32	55.23 ± 0.42	54.44 ± 0.96
MCH (pg)	19.91 ± 0.62	18.23 ± 0.17	18.49 ± 0.24
MCHC (g/dL)	33.16 ± 1.33	32.89 ± 0.41	33.46 ± 0.22

4. Discussion and Conclusion

The current results demonstrated that quercetin and tomato loaded nanofiber showed the alteration of EC50 of antioxidant and aldose reductase suppression effects. The possible explanation might be associated with chemical interaction between the zein based polymer and quercetin and between zein based polymer and tomato extract. In addition the alteration of properties of quercetin and tomato extract might be changed during the electrospining process. The photograph of SEM obtained from this study also demonstrated that all concentrations of quercetin and tomato extract were successfully incorporated into the nanofibers. No loaded substances were observed in the space between fibers. The efficiency of loading of quercetin was also investigated. It was found that the efficiency was varied between 63-73% show the efficiency of loading via this condition was not too low. It is also in the range that observed in the other studies which loaded other substance such as Retin-A-loaded

nanofiber (Taepaiboon et al., 2007). It was also shown that the loaded quercetin could be released from the zein based nanofiber mat. The releases of quercetin from zein based nanofiber loaded with various concentrations of quercetin were more than 50% within 6 hours, reached the peaked within 24 hours and maintained their values until 72 hours. Therefore, these results suggested that within 72 hours the substances still be released. However, the peak of release was observed within 24 hours. Although this study clearly demonstrated that the development of quercetin loaded zein based nanofiber mat was successful, it is very essential to assure about the safety. Therefore, the chronic toxicity of 15%quercetin loaded nanofiber via transdermal route was evaluated. The current data showed that no adverse effect level (NOAEL) via transdermal route was 15%quercetin and the continuous application for 6 months showed no toxicity. Based on these data, quercetin loaded zein based nanofber was safe up to 15%quercetin loaded zein based nanofber and it is safe for long term used up to 6 month. In addition, quercetin loaded zein based nanofiber also showed high potential to combat diabetic complications which related to oxidative stress and polyol pathway. Thus quercetin loaded zein based nanofiber might be the potential novel candidate for treating the challenge diabetic complications such as diabetic neuropathy and diabetic wound. However, in vivo study concerning this aspects are stll necessary.