CHAPTER III
THE DEVELOPMENT AND THE ASSESSMENT ACUTE TOXICITY OF THE NOVEL HERBAL RECIPE FROM THE SELECTED PLANTS

1. Introduction

Vegetable has been long term consumed for health promotion. They contain high content of phenolic compounds which can counteract the reactive oxygen stress (ROS). The antioxidant activities of phenolics are related to the number of different mechanisms, such as free-radical scavenging, hydrogen donation, singlet oxygen quenching, metal ion chelation, and acting as a substrate for radicals such as superoxide and hydroxyl. However, the current life styles of people have changed. The consumption of vegetable was decreased and the foods which are ready to be used have gained attention. In addition, the heath concern is also increased. Therefore, there is a growing of interest concerning the application a vegetable-based functional food targets at the protection against chronic diseases such as diabetes and its complications.

According to the traditional folklore, vegetables can also be used as medicine. They have been long term used either as single herb or as polyherbal recipe. However, in traditional folklore especially in Oriental Medicine such as Ayurveda and Traditional Chinese Medicine (TCM), polyherbal formulations are more commonly used than single herb application based on the belief that polyherbalism can approach multi-targets and provide better benefit due to the synergistic effect of the ingredients (Nadeem *et al.*, 1996).

Vegetables possessing medicinal properties such as purple waxy corn (*Zea mays* L.), ginger (*Zingiber officinale* Roscoe), mulberry (*Morus alba* L.), red onion (*Allium cepa* L.) and papaya (*Carica papaya* L.) are widely consumed in the Northeast Thailand and some plants also show the potential to suppress aldose reductase activity. Moreover, these plants are widely available in the Northeast regions of Thailand with cheap cost. Therefore, the development of novel health product from these plants has gained much attention in order to provide the alternative

choice to strengthen the health status via dietary therapy which is cheap and easy to approach. In addition, the agricultural products can be increased their values in the market. Since no scientific data concerning the potential against diabetic conditions and the recipe of the polyherbal targeting at diabetic complications such as diabetic neuropathy and diabetic cataract have available, this study aimed to determine the protective potential against diabetic complication of the selected plants the novel herbal recipe. Moreover, acute toxicity is also evaluated.

2. Materials and Methods

2.1 Screening the potential of the selected plants to protect against diabetic complications

2.1.1 Plant materials and preparation

The selected plants including *Zea mays* Linn. var. *ceratina* Kulesh, *Zingiber officinale* Roscoe, *Morus alba* Linn, *Allium cepa* Linn and *Carica papaya* Linn were collected from Khon Kaen province during September-November 2012. The seeds of *Zea mays* Linn (purple color, open pollinated cultivar) were obtained from Faculty of Agriculture KKU. The old leaves and fruit of ripe *Morus alba* Linn were collected from Queen Sirikit Sericulture Center Udon Thani. The rhizome of aged *Zingiber officinale* Roscoe, bulb of *Allium cepa* Linn and fruit of ripe *Carica papaya* Linn were obtained from Lom Sak-Phetchabun, Sisaket and Khon Kaen province, respectively. They were cleaned, cut into small pieces and dried at 60 ^{0}C for 24 hrs. They were ground to fine powder by using a homogenizer. The powder was stored at 25 ^{0}C in the dark bottle until used.

2.1.2 In *vitro* screening of antioxidative activity of the plants extract

The dried powders of the mentioned plants were extracted with 50% hydro-alcoholic solvent by maceration method. The samples were macerated at room temperature for 24 hrs and filtered through Whatman filter paper number 1. The solutions were used to assess the total phenolic compounds, flavanoid and an antioxidative activity.

1) Determination of total phenolic compounds

A total polyphenol compound was measured using Folin-Ciocalteu colorimetric method (Quettier-Deleu *et al.*, 2000). 20 µl of each plant

extracts was mixed with 0.2 ml of Folin-Ciocalteu reagent and 2 ml of distill water, and incubated at room temperature for 5 min. Then, 1 ml of 20% sodium carbonate was added and incubated at room temperature for 2 hr. The total polyphenolic compounds was determined by measuring the absorbance 765 nm with a GENESYS™ 20 spectrophotometer. Gallic acid was used as a standard and the total phenolics were expressed as gallic acid equivalents (mg/L GAE/mg extract). All determinations were performed in triplicate.

2) Determination of flavanoids

The aluminum chloride method was used for the determination of the total flavonoid content. 100 µl of each plant extracts was mixed with 100 µl of 2% AlCl3. Absorbance at 415 nm was recorded after 15 min of incubation. Quercetin was used as a standard and the total flavanoids were expressed as milligram quercetin equivalent (mg/L quercetin/mg extract).

3) Determination of anthocyanins

Total anthocyanins content was estimated by a pH-differential method (Giusti and Wrolstad, 2005). This method was performed based on the concept that monomeric anthocyanins undergo a reversible structural transformation as a function of pH (colored oxonium form at pH 1.0 and colorless hemiketal form at pH 4.5) and the difference in absorbance at the "vis-max of the pigment is proportional to the concentration of pigment. In brief, two types of buffers were prepared. The first type of buffer was prepared buffer is prepared by dissolving 1.86g of KCl in 980 mL distilled water and adjust pH to pH 1 by the drop wise addition of HCl, then the volume is completed to 1L with distilled water. The second type of buffer was prepared by dissolving 54.43g of sodium acetate in 970 mL distilled water and adjusted pH to 4.5 through the addition of HCl drop by drop, and then the volume is completed to 1L using distilled water. Then, 3 ml of extract was diluted in 5 ml of two different buffers; 0.025 M potassium chloride pH = 1.0 and 0.4 M sodium acetate pH = 4.5, respectively. After 30 minutes of incubation at room temperature, absorption (A) was measured at wavelength of 510 and 700 nm respectively. The absorbance of the diluted sample (A) was calculated as follows:

$$A = (A510 – A700)\ pH\ 1.0 – (A510 – A700)\ pH\ 4.5$$

The monomeric anthocyanin pigment concentration in the original sample using the following formula:

Monomeric anthocyanin pigment (mg/L) = (A x MW x DF x 1000)/(εx1)

MW is the molecular weight, DF is the dilution factor, and ε is the molar absorptivity,

MW = 449.2 and ε= 26,900 l $mol^{-1}cm^{-1}$

The content of total anthocyanins was expressed in mg of cyanidin-3-glucoside equivalents/mg extract.

4) Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical

The capacity to scavenge the stable free radical DPPH was determined by using DPPH assay (Tai *et al.*, 2011). The working solutions of each plant extract ranging from 10, 25, 50, 100, 250, 500 and 1000 µg/mL were prepared in methanol. Various concentrations of ascorbic acid similar to that of the plant extract were prepared and used as standard. The solution containing 1 mM DPPH solution in methanol was incubated at room temperature for 30 min in a dark condition. The absorbance at 517 nm was recorded. Equal amount of methanol and DPPH solution were mixed and processed as similar as that of the plant extract and the obtained solution was served as control. The radical scavenging activity was calculated using the following formula:

% DPPH scavenging effect = [(A control - A extract)/A control] x 100

5) Determination of ferric reducing antioxidant power (FRAP) assay

The assay was based on the reducing power of a compound (antioxidant) (Benzie and Strain, 1996). A potential antioxidant will reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); the latter forms a blue complex (Fe^{2+} /TPTZ), which will increase the absorption at 593 nm. Briefly, the FRAP reagent was prepared by mixing 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H2O$ and 16 mL $C_2H_4O_2$) at pH 3.6, 10 mM TPTZ (2,4,6-tripyridyls-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution in distilled water. Then acetate buffer (25 mL) and TPTZ (2.5 mL) were mixed together with $FeCl_3 \cdot 6H_2O$ (2.5 mL). The FRAP reagent (1450 µl)

and sample solutions (50 µl) were added to each well and mixed thoroughly and measured at 0 minute after vortexing. Then, samples were incubated at 37° for 10 minutes. Ascorbic acid standards were processed in the same way. All determinations were performed in triplicates.

6) Determination of aldose reductase activity

Aldose reductase activity was evaluated using spectro-photometric 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 lens 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).

2.2 Development of the herbal recipe containing the combination extract from selected plants (PWCG)

Dried grains of purple waxy corn or *Zea mays* L. (purple color; KKU open pollinated cultivar) and rhizomes of ginger or *Zingiber officinale* Roscoe were selected for the development of the novel herbal recipe used in this study. They were harvested during September 2012 and authenticated by Associate Kamol Lertrat, and Dr. Bhalang Suriharn, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand. The voucher specimens (voucher specimen 2012001 and 2012002) were kept at the Integrative Complementary Alternative Medicine Research and Development Center, Khon Kaen University. The dried grains of purple waxy corn and rhizomes of ginger were extracted with 50% hydro-alcoholic solvent by maceration method at a ratio of 2:5 and 1:5 (weight:volume), respectively. The samples were macerated at room temperature for 3 days. Both of the yielded extracts were concentrated by lyophilization and kept at 4°C for further study. The percentage yields of purple waxy corn and ginger extracts were 5.72 and 25.26 respectively. The combination extract was prepared by mixing the purple waxy corn and ginger extracts at a ratio of 1:4 (This ratio provided the highest anti-cataract potential).

2.3 Determination of the chromatogram of the novel herbal recipe containing the combination extract from selected plants (PWCG)

The analysis of sample was carried out via gradient high performance liquid chromatography (HPLC) system consisting of 515 HPLC pump and 2998 Photodiode array detector of Waters company, USA. Chromatographic separation was performed using Purospher®STAR,C-18 encapped (5µm), LiChroCART®250-4.6 and HPLC-Cartridge, Sorbet Lot No. HX255346 (Merk,Germany). Two mobile phases consisting of methanol and 2.5% acetic acid in deionized (DI) water were used to induce gradient elution. The injection volume was 20 µL and the flow rate was 1.0 mL/min. During HPLC analysis the solvent gradient was programmed as shown in Table 1 and data analysis was performed using Empower™ 3

Table 3-1 Gradient program of HPLC analysis of the novel herbal product containing the combination extract of purple waxy corn and ginger (PWCG)

Column:	Purospher®STAR,C-18 encapped (5µm), LiChroCART®250-4.6, HPLC-Cartridge, Sorbet Lot No. HX255346 (Purchased from Merck)
Mobile Phase:	A. MeOH B. 2.5% Acetic acid
Gradient:	0 min, 10% A 17 min, 70%A 18-20 min, 100%A 20.5-25 min10%A
Flow Rate:	1mL/min
Injection Volume:	20 µL
Detection:	UV absorbance at 270 nm
Standard:	[6]-Gingerol ≥98% (HPLC), Sigma-Aldrich Gallic acid monohydrate≥99% (HPLC) , Sigma-Aldrich Cyanidin chloride ≥95% (HPLC) , Sigma-Aldrich Quercetin ≥95% (HPLC), Sigma-Aldrich
Software:	Empower™ 3

2.4 Determination acute toxicity

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 maintained and treated in accordance with the guideline and approval of the Ethical Committee on Animals Experiments of Khon Kaen University (AEKKU 98/2555). Lighting was controlled to supply 12 h of light and 12 h of dark for each 24-h period. They were maintained at room temperature approximately 23 °C with constant humidity and they were allowed to acclimatize to laboratory conditions for a week before starting the experiment. They were given water ad libitum throughout the study period.

Acute oral toxicity test was carried out according to the Organization for Economic Co-operation and Development (OECD) guidelines (Organization for Economic Co-operation and Development (OECD), 2012). The experimental group was treated with a single administration of PWCG which was dissolved in distilled water at dose of 5000 mg/kg BW once daily whereas control group was treated with distilled water which was used as vehicle. After the administration, food was withheld for further 1-2 h. Rats were observed for 24-h, with a special attention given at the first 4-h and daily thereafter, for a total of 14 days. The rats were weighed and assessed for mortality. The toxicity signs including tremors, convulsions, salivation, fur, eyes, diarrhea, lethargy, sleep and coma, changes in physical appearance, injury, pain, and signs of illness were assessed once daily during the period. The time of death, if any, was recorded. At the end of the experiment, all rats were sacrificed. The organs were excised, weighed, and examined macroscopically. Principal vital organs were preserved in a fixation medium of 10% solution of buffered formalin for histopathological study. Blood was collected and the determinations of hematological and clinical chemistry changes were performed at Srinagarind hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

All vital organs isolated from each individual were fixed in 10% buffered formalin, routinely processed and embedded in OCT. OCT sections (5 μm) were cut on glass slides 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.

2.5 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 The antioxidative activity of the selected plants extract

The antioxidant activity of the selected plants extract including the grains of *Zea mays* Linn. var. *ceratina* Kulesh, rhizome of aged *Zingiber officinale* Roscoe, old leaves and fruit of ripe *Morus alba* Linn, bulb of *Allium cepa* Linn and fruit of ripe *Carica papaya* Linn were determined by total phenolic compounds, DPPH, FRAP activity and aldose reductase activity in normal lens and nerves as summarized in table 3-2. The highest content of total phenolic compounds and flavonoids were observed in the extract of aged rhizome of *Z.officinale* (19.03±0.16 mg/L GAE/mg extract) and the ripen fruit of *M.alba* (1.493±0.003 mg/L quercetin/mg extract) respectively whereas the lowest content of total phenolic compounds and flavonoids were observed in the extract of *M.alba* leaves (1.92±0.16 mg/L GAE/mg extract) and *Z.mays* seed (0.211±0.004 mg/L quercetin/mg extract) respectively. The plants which exhibited the highest antioxidant activity via DPPH and FRAP assays were the extract of the aged rhizome of *Z.officinale* (EC_{50} 1.03 mg/ml) and the extract of ripen fruit of *M.alba* (EC_{50} 6.11 mg/ml) whereas the extract which showed the lowest antioxidant effect via both DPPH and FRAP assays was *M.alba* leaves extract (EC_{50} 11.43 and 166.93 mg/ml respectively). However, the extracts which showed the highest aldose reductase suppression activity in lens and nerve were the extract from *A.cepa* (EC_{50} 1.42 mg/ml) and *C.papaya* (EC_{50} 1.40 mg/ml). Based on the potential benefit concerning the phenolics and flavonoids contents, the antioxidant and aldose suppression activities mentioned earlier together with the available registered patent related to the selected plants, the extracts obtained from the seed of *Z.mays* and the aged rhizome of *Z.officinale* were selected for the development of the novel herbal recipe. Various ratios of *Z.mays* and *Z.oficinale* were formed and determined the total phenolics and flavonoids contents together with the antioxidant and aldose reductase

activities as mentioned earlier. The current data showed that the combination extract of *Z.mays* and *Z.officinale* at a ratio of 1:4 showed the highest potential benefit as shown in table 3-3

Table 3-2 The antioxidative activity of the selected plants extract

Selected plants	Total phenolic compounds (mg/L GAE/mg extract)	Flavanoids (mg/L quercetin/mg extract)	DPPH (EC50: mg/ml)	FRAP (EC50: mg/ml)	Aldose reductase (EC50: mg/ml)	
					Lens	Nerves
Grains of *Zea mays* L. (purple color)	6.88±0.12	0.211±0.004	2.13	72.90	2.22	4.72
Aged rhizome of *Zingiber officinale Roscoe*	19.03±0.16	0.583±0.008	1.03	16.87	2.57	1.99
Old leaves of *Morus alba* L.	1.92±0.16	0.289±0.005	17.43	166.93	7.80	15.56
Ripen fruit of *Morus alba* L.	15.85±0.12	1.493±0.003	2.02	6.11	2.14	2.57
Bulb of *Allium cepa* L.	3.79±0.01	0.492±0.007	8.98	38.63	1.42	14.23
Ripen fruit of *Carica papaya* L.	4.64±0.07	0.607±0.006	10.35	20.86	3.50	1.40

Table 3-3 The antioxidative activity of the novel herbal recipe (purple waxy corn: ginger) ratio

The novel herbal recipe (PWC:G ratio)	Total phenolic compounds (mg/L GAE/mg extract)	Anthocyanin (mg of cyanidin-3-glucoside equivalents/mg extract)	DPPH (EC50: mg/ml)	FRAP (EC50: mg/ml)	Aldose reductase (EC50: mg/ml)	
					Lens	Nerves
0:1	45.22 ± 0.90	542.71 ± 0.08	1.06	1.45	0.38	0.72
1:0	21.22 ± 0.08	2371.24 ± 0.08	4.84	8.18	2.55	1.32
1:1	35.52 ± 0.00	1093.78 ± 0.01	3.73	4.52	0.89	1.04
1:2	35.12 ± 0.49	734.75 ± 0.02	3.50	4.31	0.68	0.81
1:3	38.12 ± 0.49	517.67 ± 0.05	1.79	3.55	0.27	1.98
1:4	44.82 ± 2.37	392.42 ± 0.03	2.58	3.32	0.20	2.14
1:5	42.12 ± 0.16	434.17 ± 0.02	3.55	2.78	0.98	0.68
2:1	30.52 ± 0.49	1269.12 ± 0.07	4.90	5.14	0.73	0.31
2:3	36.12 ± 0.00	801.55 ± 0.22	4.94	4.01	0.73	0.36
2:5	38.82 ± 0.73	317.28 ± 0.03	4.36	3.46	0.45	0.72
3:1	27.22 ± 0.57	1235.72 ± 0.12	5.43	4.26	0.47	1.31
3:2	30.52 ± 0.65	960.19 ± 0.09	5.49	11.24	0.31	1.45
3:4	38.42 ± 2.86	425.82 ± 0.13	4.87	3.72	0.96	0.84
3:5	36.82 ± 0.41	759.8 ± 0.08	5.05	3.33	0.42	0.87

Table 3-3 The antioxidative activity of the novel herbal recipe (purple waxy corn: ginger) ratio (Cont.)

The novel herbal recipe (PWC:G ratio)	Total phenolic compounds (mg/L GAE/mg extract)	Anthocyanin (mg of cyanidin-3-glucoside equivalents/mg extract)	DPPH (EC50: mg/ml)	FRAP (EC50: mg/ml)	Aldose reductase (EC50: mg/ml)	
					Lens	Nerves
4:1	33.32 ± 2.61	1644.84 ± 0.09	6.27	5.73	0.98	0.58
4:3	28.62 ± 0.08	834.94 ± 0.03	7.90	5.37	0.95	0.40
4:5	36.72 ± 1.96	701.35 ± 0.00	4.77	3.95	0.86	0.56
5:1	26.12 ± 0.24	1686.59 ± 0.10	5.26	6.13	1.95	0.18
5:2	29.82 ± 2.37	993.58 ± 0.06	5.28	5.53	0.23	0.23
5:3	26.12 ± 0.16	1043.68 ± 0.03	4.64	4.80	4.02	0.60
5:4	30.62 ± 0.57	1244.07 ± 0.03	4.56	3.50	0.83	2.18

3.2 The biological activities of novel herbal recipe (PWCG)

The fingerprint chromatogram of the PWCG at ratio 1:4 (purple waxy corn: ginger) was shown in figure 3-1. The PWCG contained Quercetin, Gingerol, Anthocyanin and Gallic acid at concentrations of 14.421 mg Quercetin equivalent (QE)/100 mg PWCG extract, 10.701mg Gingerol/100 mg PWCG extract, 0.089 mg Cynanidin-3- glucoside/100 mg PWCG and 0.022 mg Gallic acid/100 mg PWCG, respectively. In addition, the contents of total phenolic compounds and anthocyanins in the combination extract were 44.82 ± 2.37 mg/L GAE/mg extract and 392.42 ± 0.03 mg/L cyanidin-3-glucoside equivalents/ mg extract, respectively. The EC50 via DPPH and FRAP assay were 2.58 and 3.32 mg/mL, respectively. In addition, The EC50 of the aldose reductase inhibition in normal nerve and lens were 2.14 and 0.20 mg/mL, respectively as shown in table 3-1.

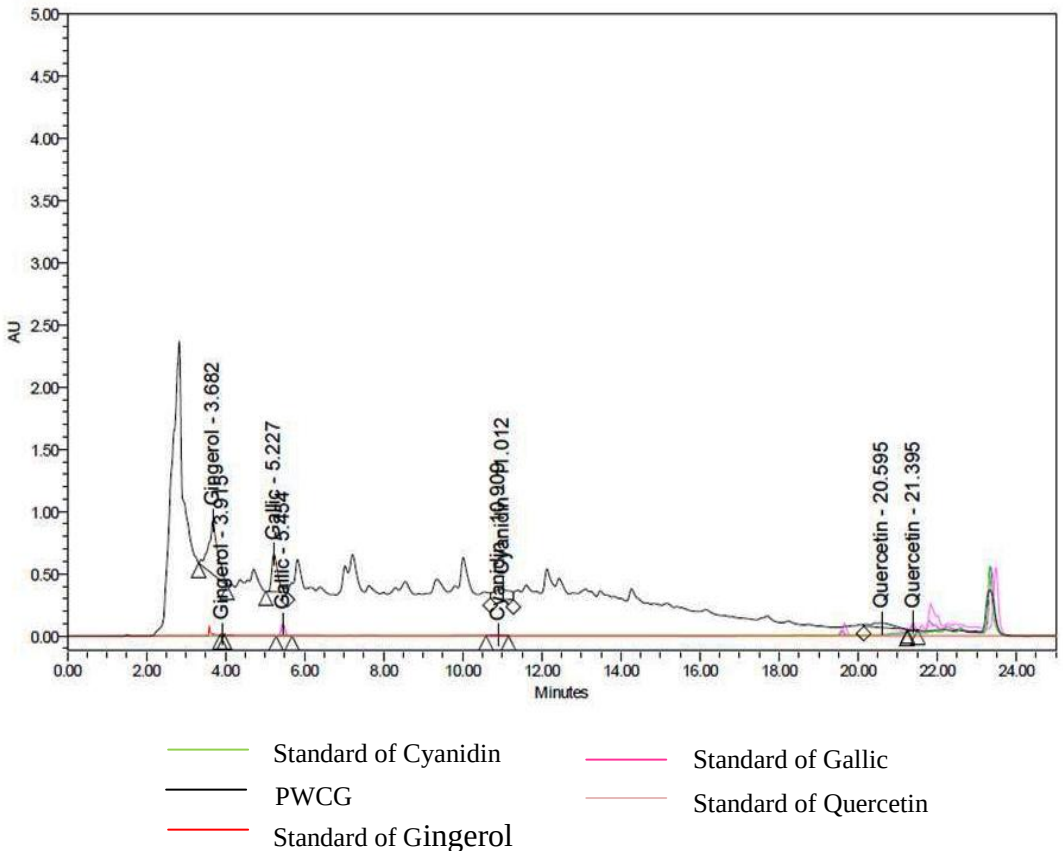

Figure 3-1 Fingerprint chromatogram of the combination extract of purple waxy corn and ginger or PWCG

3.3 Acute toxicity

The results obtained from the present study demonstrated that the PWCG did not produce any mortality throughout the study period of 14 days. Our data also showed no signs of changes including tremors, convulsions, salivation, fur, eyes, diarrhea, lethargy, sleep and coma, changes in physical appearance, injury, pain, and signs of illness and no changes of histomophology of heart, liver, kidney, lung, spleen and pancreas as shown in table 3-4 and figure 3-2, respectively. The relative organ weights of various organs were shown in table 3-5. It was found that the kidney weight of female was slightly increased while the relative weight of stomach in male was slightly decreased (p-value<0.01 and 0.05 respectively; compared to control group) However, no significant changes of organ histomorphologies of male and female rats were observed as shown in figure 3-2. In addition, it was found that PWCG at dose of 5 $g.kg^{-1}$ BW significantly decreased white blood cell count in female rats and decreased creatinine level in male rats (p-value<0.01 and 0.05 respectively; compared to control group). In addition, the elevated of high density lipoprotein (HDLC) was also observed in both male and female rats (p-value<0.01 and 0.05 respectively; compared to control group) as shown in table 3-6. However, all changes were still in normal range (Inala *et al.*, 2002). Therefore, the oral LD50 should be more than 5 $g.kg^{-1}$ BW.

Table 3-4 Effect of PWCG on acute oral toxicity test in rats

No.	Response	Unmarked		Head		Body		Tail		Head & Tail		Head & 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 Relative organ weights, food and water consumptions of male and female Wistar rats treated with PWCG at dose of 5 $g.kg^{-1}$ BW for 14 days

Organs (g)		Male		Female	
		Control	PWCG (5 $g.kg^{-1}$ BW)	Control	PWCG (5 $g.kg^{-1}$ BW)
Brain		2.08±0.00	1.94±0.02	1.84±0.01	1.93±0.02
Liver		9.97±0.61	10.66±0.55	7.98±0.17	8.03±0.23
Lung		2.92±0.08	2.54±0.25	1.93±0.58	2.93±0.51
Heart		1.09±0.12	1.22±0.07	0.78±0.11	0.98±0.03
Kidney	**Rt.**	1.14±0.14	1.30±0.09	0.79±0.05	0.98±0.05
	Lt.	1.09±0.10	1.20±0.05	0.73±0.02	1.06±0.03**
Spleen		0.87±0.07	0.85±0.05	0.67±0.01	0.73±0.03
Pancreas		1.57±0.16	1.53±0.19	1.23±0.22	1.41±0.22
Testis /Ovary	**Rt.**	1.77±0.01	1.72±0.07	0.11±0.02	0.13±0.01
	Lt.	1.70±0.02	1.72±0.07	0.11±0.08	0.12±0.02
Thymus		0.64±0.01	0.57±0.06	0.33±0.00	0.48±0.05
Salivary gland	**Rt.**	0.19±0.16	0.12±0.03	0.09±0.01	0.14±0.04
	Lt.	0.16±0.03	0.15±0.02	0.07±0.03	0.11±0.03
Adrenal gland	**Rt.**	0.07±0.04	0.06±0.01	0.05±0.00	0.07±0.01
	Lt.	0.07±0.02	0.06±0.02	0.04±0.00	0.08±0.01
Stomach		2.41±0.22	1.89±0.10*	1.88±0.21	1.69±0.09
Urinary bladder		0.24±0.09	0.21±0.03	0.12±0.02	0.09±0.01
Intestinal		7.42±0.68	9.24±0.69	7.77±0.28	7.39±0.25
Food intake rate (g)		131.79±4.64	113.99±3.49	77.14±1.70	79.23±2.77
Water intake rate (mL)		258.75±8.69	205.52±7.02	186.25±5.34	143.19±7.47

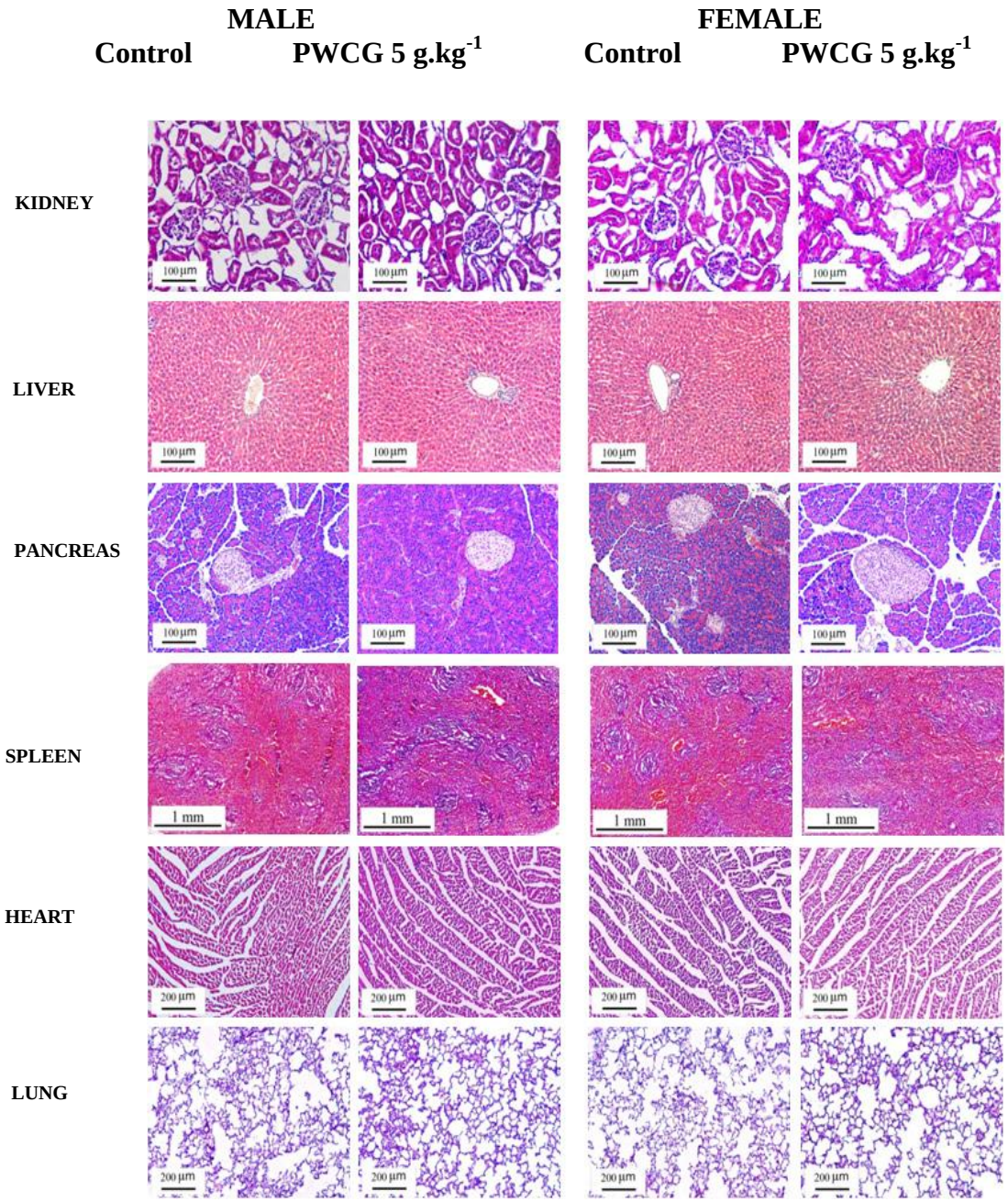

Figure 3-2 Histopathological changes of male and female Wistar rats treated with PWCG at dose of 5 $g.kg^{-1}$ BW for 14 days

Table 3-6 Hematological and blood biochemical parameters of male and female Wistar rats treated with PWCG at dose of 5 $g.kg^{-1}$ BW for 14 days

Hematological parameters	**Male**		**Female**	
	Control	**PWCG (5 $g.kg^{-1}$ BW)**	**Control**	**PWCG (5 $g.kg^{-1}$ BW)**
Red blood cell (10^6/uL)	7.24±0.21	6.37±0.79	7.19±0.32	7.60±0.15
Haemoglobin (g/dL)	14.23±0.48	13.56±0.56	13.83±0.61	14.73±0.24
Hematocrit (%)	42.28±1.53	37.08±4.46	39.95±1.89	42.98±0.58
White blood cells (10^3/Ul)	4.20±0.55	3.02±0.41	4.35±0.68	2.03±0.23**
Platelet count (10^3/uL)	762.00±66.10	894.00±67.30	803.00±6.36	800.50±8.50
Mean platelet volume (fL)	5.25±0.06	5.30±0.15	5.38±0.09	5.63±0.10
Neutrophils (%)	10.25±4.02	11.36±4.58	19.25±3.81	13.15±3.63
Lymphocytes (%)	84.55±5.69	86.94±4.96	78.65±4.18	77.03±6.25
Monocytes (%)	2.63±2.13	0.36±0.17	0.48±0.32	1.13±0.43
Eosinophil (%)	0.18±0.14	0.56±0.14	0.53±0.22	0.50±0.21
Basophil (%)	2.40±1.40	0.78±0.47	1.10±0.63	1.45±0.88
Mean orpuscular volume (fL)	58.40±0.69	58.34±0.52	55.45±0.61	56.55±0.47
Mean corpuscular Hemoglobin (pg)	19.68±0.25	22.82±3.12	19.25±0.27	19.35±0.23
Mean corpuscular Hemoglobin concentration (g/dL)	33.65±0.10	39.04±5.09	34.63±0.20	34.23±0.17
Red blood cell distribution width (%)	13.40±0.19	13.00±0.34	13.58±0.31	13.85±0.30
Blood biochemical parameters				
Albumin (g/dL)	4.38±0.14	4.06±0.19	4.34±0.13	3.87±0.06
ALAT (IU/L)	10.79±0.70	11.34±1.01	9.66±0.50	10.74±0.27
ASAT (IU/L)	9.56±0.52	10.18±1.19	8.67±0.44	8.77±0.33
Bilirubin (mg/dL)	0.27±0.12	0.41±0.05	0.21±0.01	0.33±0.00
CK-MB (U/L)	0.37±0.01	0.40±0.05	0.35±0.02	0.35±0.01
Creatinine (mg/dL)	0.71±0.03	0.58±0.04*	0.60±0.04	0.52±0.02
HDLC (mg/dL)	44.53±3.39	53.09±5.66**	60.68±1.46	65.57±2.17*
LDH (U/l)	290.16±8.04	292.03±18.00	252.68±17.47	289.31±7.03
LDLC (mg/dL)	55.69±7.20	67.22±18.85	44.56±14.42	43.33±0.33
Urea (mg/dL)	11.00±1.32	17.81±1.17	10.58±1.16	8.66±1.11

4. Discussion

It was found that the antioxidant activity of the extracts of the selected plants which evaluated via DPPH and FRAP assays failed to show the tight association with the contents of total phenolics and flavonoids. Therefore, we did suggest that the antioxidant activity observed in this study can be attributed to the presence of various bio-actives components such as tannins, polyphenols, alkaloids, flavonoids and steroids found in these extracts. The combination extract of *Z.mays* and *Z.officinale* (PWCG) significantly showed better aldose reductase suppression activity and antioxidant effect than *Z.mays* or *Z.officinale* alone. Since PWCG contained high concentrations of quercetin, gingerol and anthocyanins which were shown to exhibit antioxidant and aldose reductase activities (Benzie and Strain, 1996; Einbonda *et al.*, 2004; Kim *et al.*, 2013; Masuda *et al.*, 2004; Varma *et al.*, 1975) the antioxidant and aldose reductase activity observed in this study might be associated with these substances.

Since the evaluation of toxic properties of a substance is crucial when considering for public health protection, we also investigated the acute toxicity of PWCG. Acute toxicity test can provide the information the range of doses that could be used. On the basis of the previous study, it has been demonstrated that the oral route administration is the most convenient and commonly used one when studying acute toxicity. The absorption might be slow, but this method costs less and is painless to the animals. It was found that during the 14 days of period acute toxicity evaluation, rats which are orally treated with PWCG at single dose of 5000 mg/kg showed no signs of distress and toxicity symptoms. In addition, no death was observed. All rats also gained weight and displayed no significant changes in behavior. Food and water consumption also showed no changes. In this study, it was found that female rats which received PWCG at dose of 5000 $mg.kg^{-1}$ BW showed the increased weight of kidney. However, no significant change of creatinine (Cr) and urea. The histomorphology also failed to show the significant changes. Therefore, the PWCG is safe for consumption up to the dose of 5000 $mg.kg^{-1}$ BW. Since the increased in kidney weight was observed at 14 days after the single administration of PWCG, the enhanced kidney weight observed in this study might not be associated with PWCG. However, this required the confirmation via subchronic and chronic toxicity.

5. Conclusion

This study has demonstrated that the novel herbal recipe (PWCG) shows the potential to protect against diabetic complications. It is also safe for consumption up to 5 $g.kg^{-1}$BW. However, the subchronic and chronic toxicity are required to confirm the safety for the repetitive exposure to PWCG.