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Protective effect of Djulis (*Chenopodium formosanum*) and its bioactive compounds against carbon tetrachloride-induced liver injury, *in vivo*

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ABSTRACT

The protective effect of water extracts of Djulis (*Chenopodium formosanum*) (WECF) and its bioactive compounds against carbon tetrachloride-induced liver injury in rats was investigated. Rutin, kaempferol, betanin and another nine compounds were present in WECF using HPLC-DAD and HPLC-MS/MS analyses. Oral administration of WECF to rats at 2.5 mg/kg bw for 28 consecutive days before a single dose of CCl_4 demonstrated significantly lowered aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, and attenuated histopathological changes in CCl_4-treated rats. WECF inhibited lipid peroxidation, restored glutathione (GSH), enhanced superoxide dismutase (SOD), and reduced DNA damage in CCl_4-treated rats. Rutin, kaempferol and betanin at 1.0 μg/kg bw restored GSH and reduced DNA damage in CCl_4-treated rats. In addition, betanin increased SOD activity. Overall, WECF protects rat liver from CCl_4-treated liver injury due mainly to attenuating oxidative stress. The presence of bioactive compounds in WECF may partly be responsible for the hepatoprotection of WECF.

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Abbreviations: WECF, water extracts of Djulis; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; CCl_4, carbon tetrachloride; BUN, blood urea nitrogen; CRE, creatinine; CYP2E1, cytochrome P450 2E1; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reaction substances; H_2O_2, hydrogen peroxide
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1. Introduction

The World Health Organization (WHO) survey indicates that among men, liver cancer is one of the five most common sites of cancer diagnosed in 2012 (WHO Media Centre, 2015). Liver is the largest organ in the body and plays an important role in metabolising ingesta, synthesising vitals and detoxifying noxious substances (Cho et al., 2013; Park, Choi, Eom, & Choi, 2013). Liver injury is caused by a variety of deleterious facts such as oxidative stress, tobacco smoking, alcohol drinking, Hepatitis B or C viruses, and aflatoxins. For example, intoxication with a high dose of carbon tetrachloride (CCl_4) causes cellular necrosis, oxidative stress and inflammation, which leads to acute liver injury (Cho et al., 2013); alcohol metabolism-associated oxidative stress contributes to the pathogenesis of alcoholic-liver disease (Tamura et al., 2013). It is well known that oxidative stress is associated with liver disease such as hepatotoxicity, which can ultimately lead to liver cancer (Hanlon, Webber, & Barnes, 2007). Therefore, use of natural treatments for liver disease from natural sources is considered to be effective against hepatotoxicity due to the presence of various biological compounds such as phytochemicals, which can reduce oxidative stress (El Arem et al., 2014). Moreover, several studies and epidemiological evidence show that increased dietary intake of natural bioactive compounds (e.g., chemopreventive agents, phytochemicals, and antioxidants) is associated with lower incidence of human diseases (Ness & Powles, 1997). Hence, many researchers strive to search for natural materials to attenuate the development of liver disease by enhancing antioxidation and improving redox stages via a dietary modification rather than medicinal treatments. In recent years, there has been a substantial increase in studies on the effects of natural plant-derived compounds in liver disease prevention and treatment. In particular, a search of phytochemicals which may attenuate oxidative stress can be an effective and complementary therapeutic strategy for preventing and treating liver disease (Wang, Lee, Chen, Yu, & Duh, 2012). Therefore, investigation of the protective effect of bionatural sources against liver disease induced by oxidative stress has recently received much attention.

Djulis (*Chenopodium formosanum*), which is called "Hang Li" due to its bright red colour, is traditionally used as a native cereal and as one of the ingredients of a local wine brewed by aboriginal people in Taiwan. With respect to the biological effect of Djulis, betanin, isobetanin, amaranthine and isoamaranthine, which demonstrate antioxidant activity in an acellular model, are purified from Djulis (Tsai, Sheu, Wu, & Sun, 2010). A previous work by the authors demonstrated that water extracts of Djulis (WECF) consisting of bioactive compounds, including rutin, kaempferol, betanin and another 20 compounds, could prevent oxidative stress by enhancing the antioxidant indices and reducing apoptosis, *in vitro*, according to HPLC-DAD and HPLC-MS/MS analyses (Chyau, Chu, Chen, & Duh, 2015). The effectiveness of Djulis in protection against cell injury in a cellular model system has been established. However, it remains unclear whether Djulis exhibits a hepatoprotective effect against oxidative stress, *in vivo*. Therefore, this *in vivo* study aims to determine the hepatoprotective effect of Djulis and its bioactive compounds, rutin, kaempferol and betanin, against liver injury induced by oxidative stress in an animal model, and the mechanism of hepatoprotection is also elucidated in this study.

2. Materials and methods

2.1. Sample preparation

The Djulis (*Chenopodium formosanum*), purchased from Kullku Farm, Pingtung, Taiwan, was ground to a fine powder. The powder (100 g) was extracted with boiling water (1000 ml) and stirred for 40 min. The extract was filtered and the residue was re-extracted under the same conditions. The combined filtrate was freeze-dried. The dehydrated powder was suspended in water and this water extract of Djulis was abbreviated to be WECF (Chyau et al., 2015).

2.2. HPLC/ESI-MS-MS analysis of Djulis

The HPLC/electrospray ionisation (ESI) mass spectrometric analysis of water extracts of Djulis was according to previous method (Peng et al., 2011) with some modification. Djulis sample analysis was conducted on a Luna C18(2) column (2.00 mm × 150 mm, 3.0 μm, Phenomenex, Inc., Torrance, CA) using an HPLC system consisted of a Finnigan Surveyor module separation system and a photodiode-array (PDA) detector (Thermo Electron Co., MA, USA). The elution solvent system was performed by gradient elution using two solvents: Solvent A (water containing 0.1% formic acid) and Solvent B (acetonitrile containing 0.1% formic acid). The flow rate during the elution process was set at 0.2 ml/min. A linear gradient elution was carried out with 20–30% B in 15 min, 30–95% B in 65 min and finally 95% B isocratic elution for 10 min. The absorption spectra of eluted compounds were scanned within 210–600 nm using the in-line PDA detector monitored at 280, 360 and 530 nm, respectively. The compounds having been eluted and separated were further identified with a Finnigan LCQ Advantage MAX ion trap mass spectrometer. The system was operated in electrospray ionisation (ESI) with both positive and negative ionisation modes. Samples of 20 μl of extracts were directly injected into the column using a Rheodyne (model 7725i) injection valve. The typical operating parameters were as follows: spray needle voltage, 4 kV; ion transfer capillary temperature, 280 °C; nitrogen sheath gas, 45 and auxiliary gas, 5 (arbitrary units). Mass spectra were acquired in an *m/z* range of 150–1000, with five microscans and a maximum ion injection time of 200 ms. For MS/MS analysis, helium collision gas was introduced in accordance with the manufacturer's recommendations. The MS/MS fragment spectra were produced using normalised collision energies with an increment of 40% and also with wideband activation "off".

2.3. Animal treatment

Male Wistar rats (5-week-old) were obtained from BioLASCO, Taiwan Co., Ltd. in this study. Animals were cared and used after the experimental protocols approved by institutional animal ethics committee (Chia-Nan University, Tainan, Taiwan, ROC). The animals were all fed a commercial rodent chow diet

in entire experiment. The rats were randomly divided into ten groups (6 rats/group) that were control, CCl_4 treatment, silymarin, rutin, betanin, kaempferol with CCl_4 treatment, and high dose, medium dose, low dose of WECF with CCl_4 treatment, respectively. The last group was fed high dose (2.5 mg/kg bw) of WECF without CCl_4 treatment. All animals were maintained in a controlled environment at 21 ± 2 °C, 50 ± 5% relative humidity and a cycle of 12 h dark/light and provided with food and water *ad libitum*. However, they were acclimatised for 1 week prior to use. To study the protective effect against CCl_4-induced acute hepatic damage, rutin (1.0 μg/kg bw), betanin (1.0 μg/kg bw), kaempferol (1.0 μg/kg bw), and high dose (2.5 mg/kg bw), medium dose (1.0 mg/kg bw) and low dose (0.5 mg/kg bw) of WECF were gavaged for 28 consecutive days. Furthermore, the positive control was oral-administered silymarin (100 mg/kg bw), the negative control was treatment with CCl_4 by intraperitoneal (IP) alone. However, the control group was treatment with olive oil (without CCl_4), and the normal saline instead the sample administration. On day 28, the rats were IP injected with CCl_4 that was dissolved in olive oil (CCl_4/olive oil, 1/1: v/v, 2 ml/kg bw) before 18 h of the end of experiment and fasted overnight. At the end of treatment, rats were asphyxiated by carbon dioxide and then blood was collected with a heparinised syringe. Blood was centrifuged at 1000 ×*g* for 10 min and serum obtained was frozen at –20 °C until analysis. Immediately after killing, the liver was weighed and two portions were removed: one from the left lateral lobe and the other from the largest lobe. The samples were weighed separately, quickly frozen with liquid nitrogen, and stored at –80 °C until used (Chen et al., 2013). In addition, the liver and kidney were dried and their weights were measured. Relative organ weights (%) were expressed as weight of organ (g) per final body weight (g) × 100.

2.4. Serum biochemical assays

Kits for alanine transaminase (ALT) and aspartate transaminase (AST) were obtained from Denka Seiken Co., LTD. (Tokyo, Japan). Blood urea nitrogen (BUN) and creatinine (CRE) levels in serum were determined by commercial kits from urea liquid and creatinine liquid, respectively (Sentinel Diagnostics, Milan, IT). Protein levels were determined by the Bradford Protein Assay Kit (Bio-Rad Laboratories).

2.5. Assay for antioxidative status in liver

The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were, respectively, determined using commercial kits from Randox Laboratories Ltd., Crumlin, Antrim, UK. The activity of catalase (CAT) and the content of reduced glutathione (GSH) in liver were, respectively, determined by commercial kits from Cayman Chemical Company, Ann Arbor, MI, USA. These assays were completed according to methodology recommended by the manufacturer and detected by a biochemical autoanalyser (Toshiba, TBA-200FR, Holliston, MA, USA). The SOD activity of the sample was reported as 50% inhibition of nitro-blue tetrazolium (NBT) reduction/min/g protein. GPx and CAT activity was calculated using the absorbance expressed as μmol of GSH consumed/min/g protein and μmol of hydrogen peroxide (H_2O_2) consumed/min/g protein, respectively. The concentration of GSH was calculated using the absorbance expressed as μg/g protein.

2.6. Measurement of lipid peroxidation products

Liver tissues were homogenised in cold Tris–HCl (pH 7.4) (1:10, w/v) of 20 mmol/l. The homogenate was centrifuged for 30 min at 2500 ×*g* and 4 °C. The homogenate was stored at –80 °C for the following experiments. Measurement of lipid peroxidation products was carried out by the method of Buege and Aust (1978). In brief, 1 ml of the homogenate was mixed with 1 ml of 7.5% (w/v) cold trichloroacetic acid (TCA) to precipitate proteins and then centrifuged at 190 ×*g*. The supernatant was reacted with 1 ml of 0.8% (w/v) thiobarbituric acid (TBA) in boiling water for 45 min. Lipid peroxidation products were estimated by measuring the concentration of thiobarbituric acid reaction substances (TBARS) in fluorescence at 530 nm ex/552 nm em.

2.7. Assay for detoxification enzyme in liver

Cytochrome P450 2E1 (CYP2E1) concentrations were measured by ELISA kit E90988Ra (Uscn, Life Science Inc., USA) according to the manufacturer's protocol. Each set of experiments was carried out in a single lot of ELISA reagents. The concentration of CYP2E1 was calculated using the absorbance expressed as *n*g/g protein.

2.8. Comet assay

Comet assay was determined and modified by the methods of Szeto et al. and Braz et al. (Braz & Favero Salvadori, 2007; Szeto, Chu, & Benzie, 2006). Each step was carried out under indirect light. Slides were coded and analysed without knowledge of the identity of the sample. Volumes of 10 μl of freshly lymphocytes were added to 0.5% low melting point agarose, respectively, at 37 °C. The mixtures were layered onto slides precoated with 1.5% normal agarose, covered with a coverslip, and left for 5 min at 4 °C to solidify the agarose. Afterwards, the coverslips were carefully removed and the slides immersed in a lysis solution for 15 min. Then, the slides were immersed in a freshly prepared alkaline buffer in a horizontal electrophoresis tank. After a 20 min DNA unwinding period, electrophoresis was conducted at 30 V and 300 mA for 20 min. Following 10 min neutralisation with 0.4 M Tris (hydroxymethyl) aminomethane (Tris, pH 7.5), the slides were fixed in methanol, and stored at 4 °C. Before analysis, the slides were stained with 10 μl propidium iodide (2.5 μg/ml) and scored using a fluorescent microscope (Nikon, Tokyo, Japan) at 200× magnification. Images from 100 "nucleoids" (10–20 from each of two replicate slides per sample) were analysed using the Comet Assay II free software (Perceptive Instruments, Haverhill, Suffolk, UK). Tail moment and tail intensity were used to estimate DNA damage. As tail intensity (% DNA tail) gave similar results, only tail moment values were presented.

2.9. Histopathology

Liver tissues, trimmed into 2 mm thickness, were fixed with buffered formaldehyde for 24 h. The fixed tissues were embedded in paraffin, sectioned and rehydrated. The histological

examination by the above conventional method was evaluated the index of CCl_4-induced necrosis by assessing the morphological changes in the liver sections stained with haematoxylin and eosin (H&E). Sections were studied under light microscope (DIALUX 20 EB, Wetzlar, Germany) at 40 and 100× magnifications. The results were approved by pathologist without saying of its treatment nature.

2.10. *Statistical analysis*

The results are expressed as mean ± standard deviation (SD), and ANOVA was conducted by using the SPSS software (version 12.0; SPSS, Chicago, IL, USA). When a significant F ratio was obtained ($p < 0.05$) a post hoc analysis was conducted between groups by using a multiple comparison procedure with an LSD test. Statistical significance was accepted at a level of $p < 0.05$.

3. Results

According to a previous work by the authors, bioactive compounds are present in water extracts of WECF. However, when extracting bioactive compounds from plants and natural resources, the contents of bioactive compounds may vary each time due to climate changes, harvest and storage conditions. It is therefore necessary to analyse the chemical constituents of WECF in the present work before experiments were conducted.

3.1. *Bioactive compounds*

Qualitative and quantitative analyses obtained by HPLC-MS/MS and HPLC-DAD, respectively, are summarised in Table 1 and shown in Fig. 1. The unknown pigment and polyphenol concentrations in WECF were calculated according to the established calibration curves by using the external method of HPLC analysis as shown in Equations (1) and (2), respectively.

$$y = 17.019\,x + 3208.2 \quad (1)$$

$$y = 68.866\,x + 6036.1 \quad (2)$$

where x is concentration (mg/g) and y is peak area.

In which, commercial standards of betanin and kaempferol 3-glucoside (Sigma) were used for pigment (compounds 1–3) and polyphenol (compounds 4–17) quantification, respectively. The results show that 12 compounds in WECF were identified and quantified. The phenolic compounds, rutin and kaempferol glycosides, were detected. The compound that had a $[M - H]^+$ at *m/z* 611 and $[M - H]^-$ at 609, and λ_{max} 235, 349 nm was identified as rutin. Seven derivatives of kaempferol were detected:

kaempferol-3-*O*-[6‴-p-coumaroyl-glucosyl-β-(1→4)-rhamnoside] (8), kaempferol-3-*O*-[xylopyranosyl (1,3)-rhamnopyranosyl (1,6)]-galactoside (9), kaempferol-3-*O*-[2-*O*-xylopyranosyl-6-*O*-rhamnopyranosyl]-galactoside (11), kaempferol-3-*O* robinoside (12), kaempferol-3,7-di-*O*-rhamnoside (13), Kaempferol-3-*O*-rutinoside (14) and kaempferol-3,4,-dixyloside (15). Besides these phenolic compounds, amaranthine (1), betanin (2) and isobetanin (3) which are responsible for the red colour in Djulis were also identified in WECF. Among the 12 compounds identified, compound (11), compound (14), compound (8), compound (15), compound (10) and compound (2) are the six most abundant compounds in WECF. Many studies reported that silymarin has hepatoprotective properties that

Table 1 – Compound name, retention time and area percentage of methanol soluble compounds in water extracts of Djulis.

Peak No.	Compound	t_R (min)	λ_{max} (nm)	$[M + H]^+$	$[M - H]^-$	Amount (mg/g)[d]
1	Amaranthine[b]	16.41	538, 274	727	–	0.06
2	Betanin[a]	19.89	534, 233	551	–	0.43
3	Isobetanin[b]	22.65	534, 235	551	–	0.11
4	Unknown	25.62	275, 234	713	711	1.33
5	Unknown	31.72	350, 265			1.15
6	Camellianoside[b]	33.25	268, 350	743	741	0.29
7	Unknown	33.63	258, 355	–	–	0.55
8	Kaempferol 3-*O*-[6‴-*p*-coumaroyl-glucosyl-β-(1→4)-rhamnoside] [b]	33.91	267, 349	741	739	2.56
9	Kaempferol-3-*O*-[xylopyranosyl(1,3)-rhamnopyranosyl(1,6)]-galactoside[b]	34.42	266, 351	727	725	0.33
10	Rutin[a]	35.06	235, 349	611	609	1.41
11	Kaempferol-3-*O*-[2-*O*-xylopyranosyl-6-*O*-rhamnopyranosyl]-glucoside[b]	35.34	267, 351	727	725	4.41
12	Kaempferol-3-*O*-robinoside[b]	36.36	265, 346	595	593	1.01
13	Kaempferol-3,7-di-*O*-rhamnoside[c]	36.71	268, 350	579	577	1.11
14	Kaempferol-3-*O*-rutinoside[a]	37.56	236, 350	595	593	5.22
15	Kaempferol-3,4′-dixyloside[c]	39.04	266, 325	551	549	1.75
16	Unknown	43.08	266, 324	–	–	0.26
17	Unknown	55.34	278, 233	–	–	3.22

[a] The identification was confirmed further by authentic compound.
[b] Compounds were tentatively identified according to mass spectra and the matched data from literatures.
[c] Compounds were limitedly identified from mass spectra and UV-visible absorbance spectra.
[d] Contents of compounds 1–3 and compounds 4–17 were expressed as mg of betanin and kaempferol 3-glucoside equivalent in one gram dried weight of extract, respectively.

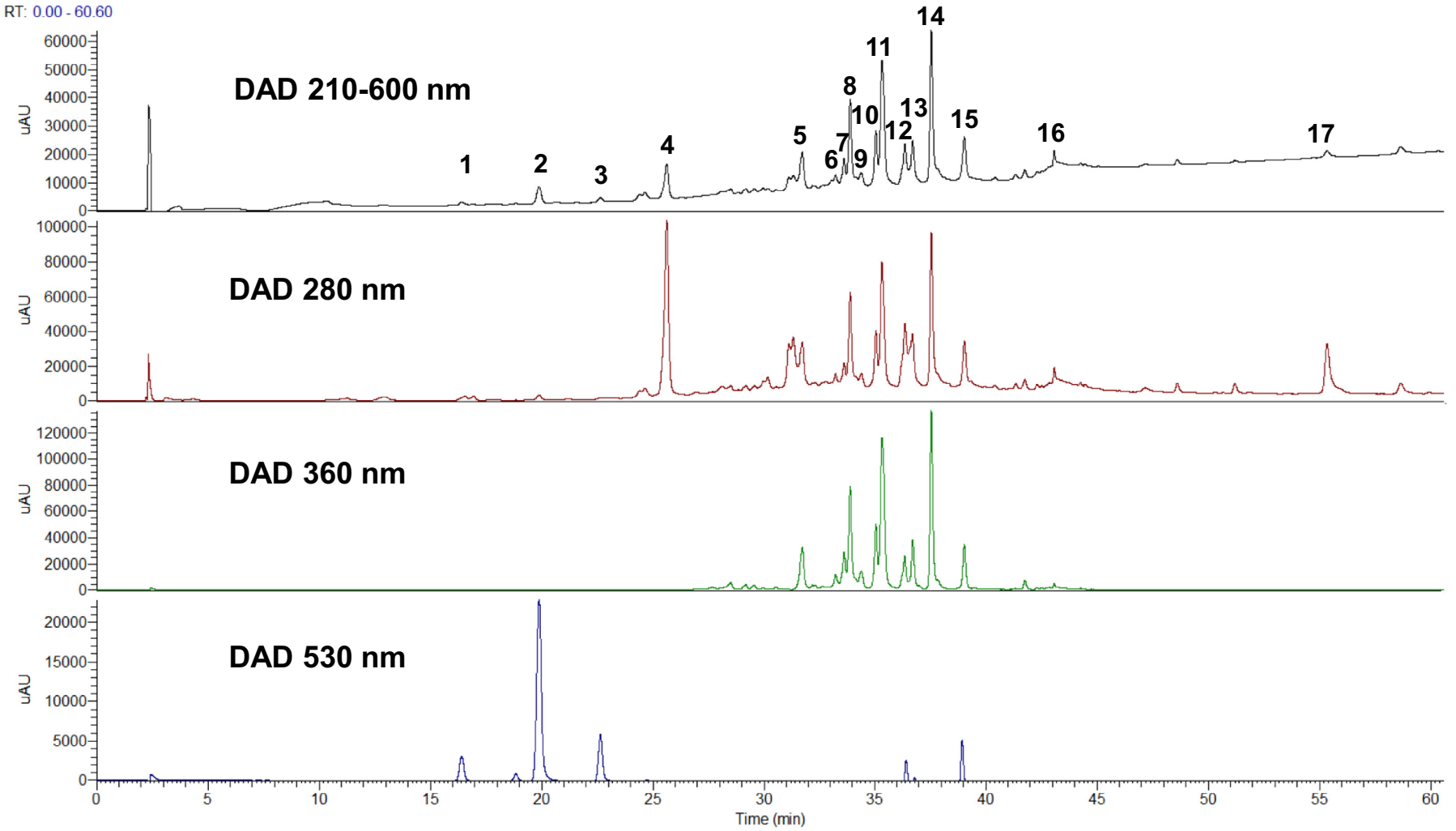

Fig. 1 – HPLC-photodiode array detection chromatograms at full scan of 210–600 nm, 280 nm, 360 nm and 530 nm, respectively, from water extracts of Djulis (*Chenopodium formosanum*) (WECF). Peak numbers refer to Table 1.

Table 2 – Effects of water extracts of Djulis (*Chenopodium formosanum*) (WECF) and its bioactive compounds on serum biochemical values in rats treated with CCl_4.

Groups	AST (U/L)	ALT (U/L)	BUN (mg/dL)	CRE (mg/dL)
Control	116 ± 10	35 ± 1.5	15 ± 2	0.64 ± 0.06
50% CCl_4	1409 ± 821[a]	391 ± 199[a]	18 ± 3	0.62 ± 0.06
Silymarin (100 mg/kg bw) + CCl_4	622 ± 681[b]	203 ± 254	20 ± 3	0.67 ± 0.05
Rutin (1.0 μg/kg bw) + CCl_4	868 ± 654	257 ± 193	21 ± 3	0.65 ± 0.04
Betanin (1.0 μg/kg bw) + CCl_4	948 ± 619	217 ± 155	21 ± 4	0.67 ± 0.07
Kaempferol (1.0 μg/kg bw) + CCl_4	1205 ± 633	496 ± 3001	19 ± 3	0.64 ± 0.04
WECF (0.5 mg/kg bw) + CCl_4	1320 ± 1001	522 ± 448	21 ± 2	0.67 ± 0.04
WECF (1.0 mg/kg bw) + CCl_4	747 ± 383	214 ± 130	20 ± 3	0.63 ± 0.04
WECF (2.5 mg/kg bw) + CCl_4	315 ± 161[b]	94 ± 43[b]	21 ± 4	0.64 ± 0.06
WECF (2.5 mg/kg bw)	119 ± 39	36 ± 10	16 ± 0	0.62 ± 0.02

Values are means ± SD for six rats per group. Results were all statistically analysed with LSD test.
[a] Significant difference from the control group ($p < 0.05$).
[b] Significant difference from the CCl_4 group ($p < 0.05$).
AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; CRE, creatinine.

protect liver cell against toxin (Lee & Pan, 2013). In addition, flavonoid glycosides can be metabolised into aglycones by the colon microflora (Calderón-Montaño, Burgos-Morón, Pérez-Guerrero, & López-Lázaro, 2011). Therefore, kaempferol, rutin and betanin, a representative of red pigments, and silymarin were selected as reference compounds in the following *in vivo* experiments.

3.2. *Effects on body, liver and kidney weight changes*

The gain in body weight of rats was recorded daily during the experimental period. No significant differences between the 10 groups were found ($p > 0.05$), indicating that the gavage was well tolerated (Chen et al., 2013) (data not shown). In addition, the relative liver and kidney weights of rats in each group on the day of sacrifice were determined. There were no significant differences ($p > 0.05$) in the relative liver and kidney weights of the rats among rats fed with or without WECF (0.5–2.5 mg/kg bw) or supplementation of reference compounds for 28 consecutive days, compared with the control (data not shown).

3.3. *Effects of WECF on serum biochemical markers*

The protective effects of WECF and its bioactive compounds, rutin, kaempferol and betanin on serum biochemical markers in CCl_4-intoxicated rats are shown in Table 2. Both serum AST and ALT levels were significantly increased in CCl_4-treated rats when compared with the control group. However, pretreatment with WECF at 2.5 mg/kg bw and silymarin (100 mg/kg bw) resulted in significant attenuation in the elevation of AST. For ALT, WECF at 2.5 mg/kg bw reduced significantly the elevation of ALT levels ($p < 0.05$). Rutin, kaempferol and betanin at 1.0 μg/kg bw had a tendency to reduce the elevation of AST and ALT levels, though not significantly, when compared with the group treated with CCl_4 alone. In addition, no significant difference was observed between the rats pretreated with WECF at 2.5 mg/kg bw without CCl_4 treatment and the control group, indicating that WECF at a dose of 2.5 mg/kg bw showed no harmful effect on liver and kidney (Table 2). In addition, the results imply that the administration of WECF at 2.5 mg/kg bw attenuated the effect of CCl_4, revealing that WECF reduces the hepatotoxicity of rats induced by CCl_4. Additionally, no significant differences ($p > 0.05$) were found in BUN and CRE levels in each group, suggesting that kidney of the rats pretreated with WECF and its bioactive compounds, rutin, kaempferol and betanin, was not affected by the presence or absence of CCl_4.

3.4. *Effects of WECF on liver histopathological changes*

To further investigate the effect of WECF on liver damage in CCl_4-intoxicated rats, histopathological changes were determined using haematoxylin and eosin staining. As shown in Fig. 2, the livers of the control group showed normal architectures (Fig. 2a). However, CCl_4 exposure caused extensive hepatic necrosis and leukocyte infiltration (Fig. 2b), which were a remarkable recovery of the hepatocytes from necrosis in the rats treated with different concentrations of WECF (Fig. 2g–i). Necrotic cells and vacuolisation were absent in the group pretreated with 2.5 mg/kg bw of WECF without CCl_4 treatment (Fig. 2j) and were reduced in the group pretreated with rutin, betanin, and kaempferol at 1.0 μg/kg bw (Fig. 2c–e). Moreover, as shown in Fig. 2i and j, both liver plate and structure of hepatocytes were almost intact, and the boundary between hepatocytes was clear, indicating that WECF has a protective ability against hepatic damage induced by CCl_4.

3.5. *Effect of WECF on lipid peroxidation*

To assess the effect of WECF and its bioactive compounds, rutin, kaempferol and betanin, on liver lipid peroxidation, thiobarbituric acid reactive substance (TBARS) analysis was conducted. As shown in Fig. 3, TBARS value was increased in CCl_4-intoxicated rats compared with the control group. Administration of WECF at a dose of 0.5–2.5 mg/kg bw or rutin, kaempferol, betanin at 1.0 μg/kg bw to CCl_4-intoxicated rats resulted in a significant decrease in TBARS value compared with rats treated with CCl_4 alone. This observation indicated that pretreatment with WECF and its bioactive compounds, rutin, kaempferol and betanin, could prevent lipid peroxidation in CCl_4-intoxicated rats.

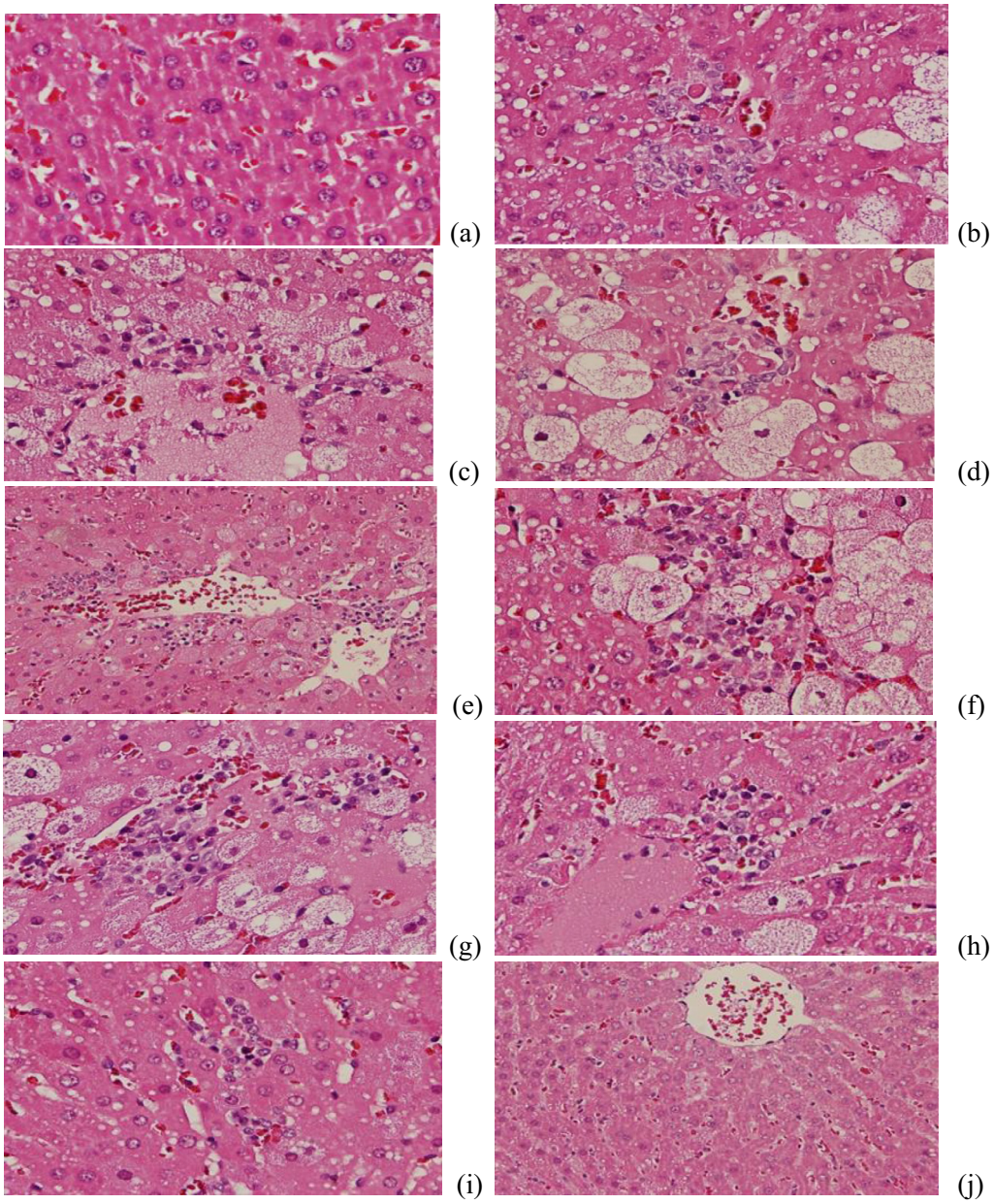

Fig. 2 – Effects of water extracts of Djulis (*Chenopodium formosanum*) (WECF) and its bioactive compounds on the liver histological damage after CCl_4 treatment in rats. (a) Control group; (b) 50% CCl_4; (c) Rutin (1.0 μg/kg bw) + CCl_4; (d) Betanin (1.0 μg/kg bw) + CCl_4; (e) Kaempferol (1.0 μg/kg bw) + CCl_4; (f) Silymarin (100 mg/kg bw) + CCl_4; (g) WECF (0.5 mg/kg bw) + CCl_4; (h) WECF (1.0 mg/kg bw) + CCl_4; (i) WECF (2.5 mg/kg bw) + CCl_4; (j) WECF (2.5 mg/kg bw).

3.6. *Effects of WECF on glutathione (GSH) and antioxidant enzyme activity*

Effects of WECF and its bioactive compounds, rutin, kaempferol and betanin, on GSH, SOD, GPx and CAT are shown in Table 3. After CCl_4 treatment, the levels of GSH, SOD, GPx and CAT were decreased by 0.89-, 0.67-, 0.66- and 0.99-fold compared with the control group, respectively. Apparently, the CCl_4-intoxicated group demonstrated a reduced GSH level in the rat liver, whereas groups with WECF at 0.5–2.5 mg/kg bw had significantly increased GSH levels. In addition, rutin, kaempferol and betanin at 1.0 μg/kg bw demonstrated significant increase in GSH levels. This observation indicates that WECF and its bioactive compounds, rutin, kaempferol and betanin, recovered depleted GSH levels and provided significant protection against GSH reduction in CCl_4-intoxicated rat liver. The SOD activity shows a significant increase in WECF at 0.5–2.5 mg/kg bw treated-groups, whereas the rats treated with CCl_4 demonstrated a significant decrease in SOD activity compared with the control group. The group administrated with betanin at dose of 1.0 μg/kg bw for 28 consecutive days showed significant increase in CCl_4-induced reduction in SOD activity. However, the group administrated with rutin and kaempferol at 1.0 μg/kg bw showed a restoration of SOD activity in CCl_4-induced rat liver injury, though there was no statistically significant difference ($p > 0.05$) when compared with the rats

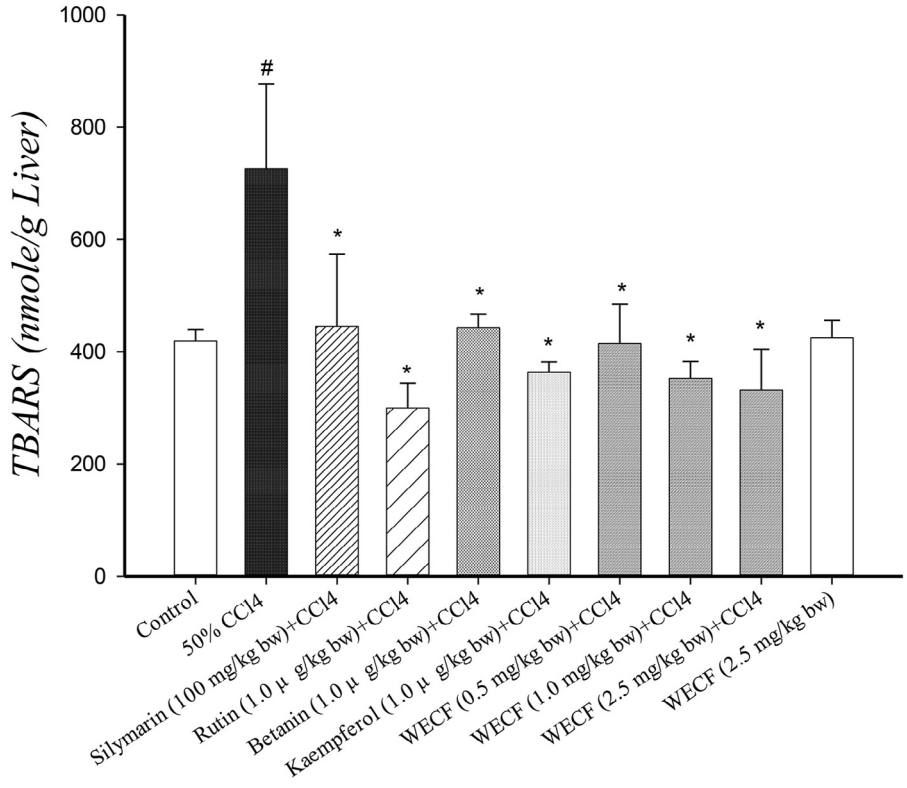

Fig. 3 – Effects of water extracts of Djulis (*Chenopodium formosanum*) (WECF) and its bioactive compounds on thiobarbituric acid reaction substance (TBARS) formation in the liver microsomes of rats treated with CCl_4. Values are means ± SD for six rats per group. #Significant difference from the control group ($p < 0.05$). *Significant difference from the CCl_4 group ($p < 0.05$).

treated with CCl_4 alone. Additionally, no effect of treatment with WECF or its bioactive compounds on CAT and GPx activity was observed.

3.7. *Effects of WECF on CYP2E1 activity*

Fig. 4 shows the effect of WECF and its bioactive compounds, rutin, kaempferol and betanin, on CYP2E1 activity in CCl_4-induced rat liver. There was no significant difference in CYP2E1 activity between rats treated with CCl_4 alone and the control group ($p > 0.05$). In rats pretreated with rutin, kaempferol, and betanin at 1.0 μg/kg bw, the CYP2E1 activity was similar to that observed in CCl_4-treated rats. Obviously, no effect of these bioactive compounds on CYP2E1 activity was observed. However, CYP2E1 activity was significantly increased by WECF at 1.0 and 2.5 mg/kg bw, compared with that of rats treated with CCl_4 alone. Moreover, pretreatment of WECF at 2.5 mg/kg bw in rats without treatment of CCl_4 significantly enhanced CYP2E1 activity, suggesting that WECF at 2.5 mg/kg bw could induce CYP2E1 activity in rat liver microsomes.

Table 3 – Effects of water extracts of Djulis (*Chenopodium formosanum*) (WECF) and its bioactive compounds on glutathione (GSH) and antioxidant enzyme activities in rats treated with CCl_4.

Groups	GSH (μg/g liver)	SOD (U/g liver)	GPx (U/g liver)	CAT (ng/g liver)
Control	1558 ± 14	3480 ± 1355	157 ± 66	458 ± 30
50% CCl_4	1384 ± 259	2335 ± 461	104 ± 13	455 ± 42
Silymarin (100 mg/kg bw) + CCl_4	1996 ± 160[a]	3415 ± 516	133 ± 12	468 ± 55
Rutin (1.0 μg/kg bw) + CCl_4	2289 ± 337[a]	2595 ± 636	148 ± 45	552 ± 66
Betanin (1.0 μg/kg bw) + CCl_4	2081 ± 422[a]	4573 ± 886[a]	140 ± 24	533 ± 58
Kaempferol (1.0 μg/kg bw) + CCl_4	1967 ± 226[a]	2848 ± 647	108 ± 37	554 ± 98
WECF (0.5 mg/kg bw) + CCl_4	1999 ± 236[a]	4534 ± 1823[a]	112 ± 18	493 ± 58
WECF (1.0 mg/kg bw) + CCl_4	1948 ± 292[a]	4446 ± 1033[a]	156 ± 20	483 ± 62
WECF (2.5 mg/kg bw) + CCl_4	2428 ± 430[a]	4647 ± 496[a]	145 ± 40	456 ± 59
WECF (2.5 mg/kg bw)	1969 ± 49	2548 ± 311	149 ± 38	390 ± 76

Values are means ± SD for six rats per group. Results were all statistically analysed with LSD test.
[a] Significant difference from the CCl_4 group ($p < 0.05$).
GSH, glutathione; SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase.

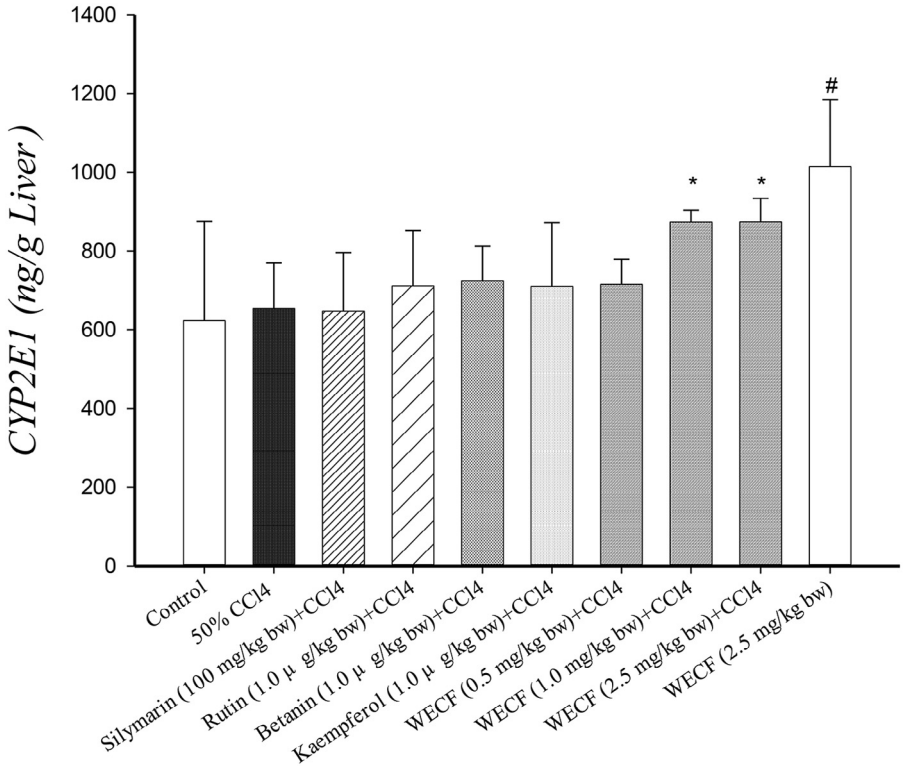

Fig. 4 – Effects of water extracts of Djulis (*Chenopodium formosanum*) (WECF) and its bioactive compounds on CYP2E1 activity in the liver microsomes of rats treated with CCl_4. Values are means ± SD for six rats per group. #Significant difference from the control group ($p < 0.05$). *Significant difference from the CCl_4 group ($p < 0.05$).

3.8. *Effect of WECF on CCl_4-induced DNA damage*

To evaluate the genotoxicity of WECF and its bioactive compounds, rutin, kaempferol and betanin, in rats, blood lymphocytes from rats were purified and employed to study the damaging effect of WECF and its bioactive compounds on DNA, as analysed by the Comet assay. The data from the photomicrograph for comet (Fig. 5A) and genotoxicity (Fig. 5B) of blood lymphocyte indicated that CCl_4-treated rats had significantly increased DNA strand breakage in comparison with the control group. Conversely, treatment with WECF at 0.5–2.5 mg/kg bw and rutin, kaempferol and betanin at 1.0 μg/kg bw for 28 consecutive days significantly decreased ($p < 0.05$) DNA fragmentation induced by CCl_4 as compared with the CCl_4-treated groups, indicating that WECF and its bioactive compounds, rutin, kaempferol and betanin, show a protective effect against DNA damage induced by CCl_4, as assessed by the Comet assay. There was no significant difference change in DNA fragmentation in each concentration of WECF and its bioactive compounds compared with the group treated with CCl_4 alone. No significant difference in DNA fragmentation between the groups pretreated with WECF at 2.5 μg/kg bw in the absence of CCl_4 and the control group indicated that WECF showed no genotoxicity towards rats.

4. Discussion

Numerous studies have shown that many plant phytochemicals confer inhibitory effects against oxidative damage (Hollman & Katan, 1999). The health benefits of phytochemicals depend on the amount consumed and on their bioavailability (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). According to extensive *in vitro* and *in vivo* experiments and epidemiological evidence, many plants and fruits such as noni juice (Lin, Chang, Yang, Tzang, & Chen, 2013), date (El Arem et al., 2014), Pu-erh tea (Duh, Wang, Liou, & Lin, 2010), and sweet orange peel (Chen et al., 2013) demonstrate a protective effect against hepatotoxicity. A previous study by the authors demonstrated that WECF provided significant protection against cytotoxicity indices by oxidative stress *in vitro* model (Chyau et al., 2015). This study further explored whether WECF can prevent an *in vivo* protective effect against hepatotoxicity.

Fig. 1 shows the HPLC-MS total ions and the HPLC-DAD chromatograms of WECF. Table 1 reports all the identified compounds with the retention time (t_R), UV_{max} and pseudomolecular ions and the fragmented ions of MS/MS analysis for the 12 identified compounds of WECF. A previous study by the authors identified 16 compounds in WECF, revealing that the number of identified compounds may vary each time. These identified compounds consist mostly of free flavonoid (rutin) and flavonoid glycosides such as kaempferol glycosidic-linked compounds. Additionally, betacyanin was also characterised in WECF, mainly betanin and isobetanin. The phenolic composition of WECF obtained by HPLC-DAD and HPLC/MS/MS analyses was consistent with previous work by the authors (Chyau et al., 2015). Besides these identified compounds, the presence of five unreported compounds in WECF (Table 1) could be related to the lack of scientific studies on them, and to the

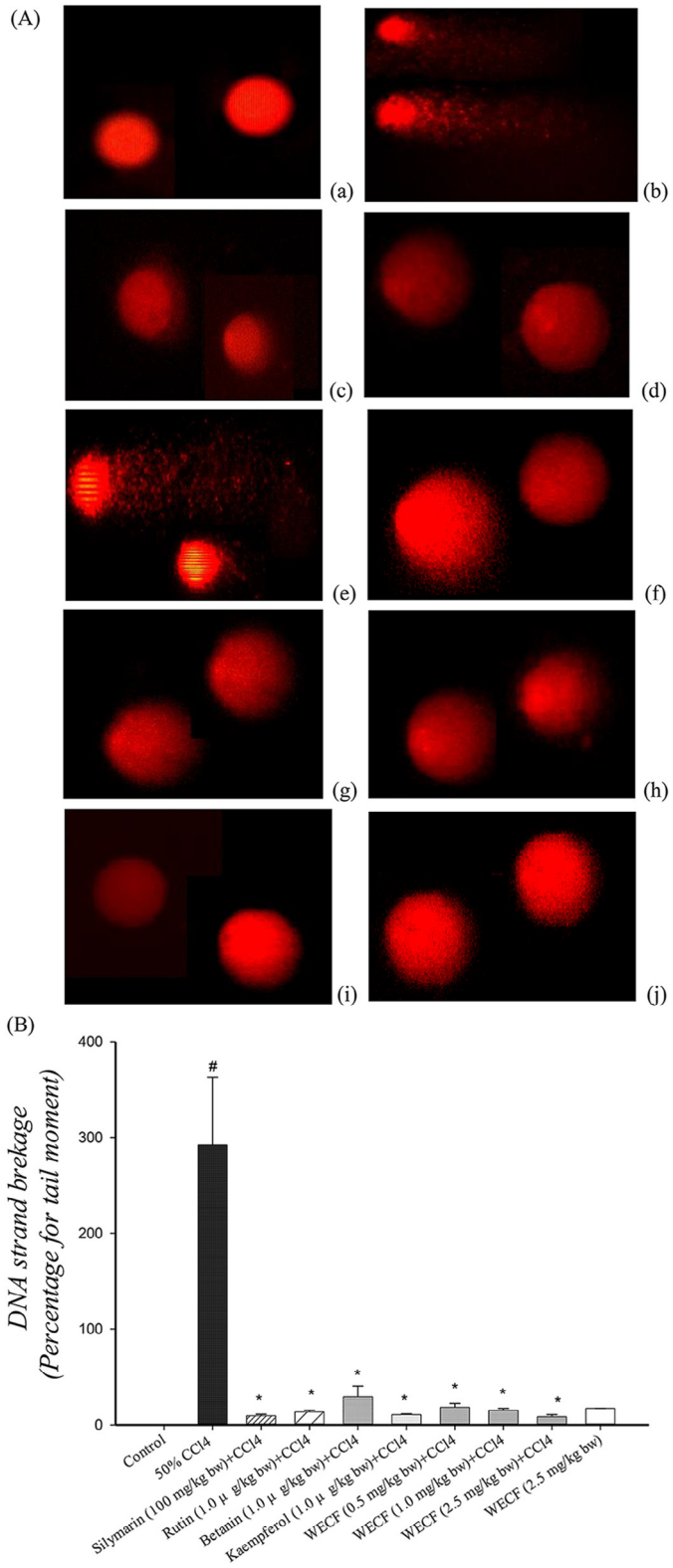

Fig. 5 – Effects of water extracts of Djulis (*Chenopodium formosanum*) (WECF) and its bioactive compounds on DNA strand breakage. Photomicrographs of comets (A) in lymphocyte stained with propidium iodide in different groups: (a) Control group; (b) 50% CCl_4; (c) Rutin (1.0 μg/kg bw) + CCl_4; (d) Betanin (1.0 μg/kg bw) + CCl_4; (e) Kaempferol (1.0 μg/kg bw) + CCl_4; (f) Silymarin (100 mg/kg bw) + CCl_4; (g) WECF (0.5 mg/kg bw) + CCl_4; (h) WECF (1.0 mg/kg bw) + CCl_4; (i) WECF (2.5 mg/kg bw) + CCl_4; (j) WECF (2.5 mg/kg bw) and genotoxicity of WECF on DNA strand breakage (B) in blood lymphocytes from rats treated with CCl_4. Tail moment = percent of DNA in the tail × tail length (Tm). Data were presented as mean ± SEM. Results were all statistically analysed with LSD test. #Significant difference from the control group ($p < 0.05$). *Significant difference from the CCl_4 group ($p < 0.05$).

extraction procedures performed by previous analytical works (Spinola, Pinto, & Castilho, 2015).

When the liver is damaged, the cell membrane integrity is lost, and causes the release of some hepatospecific enzymes such as AST and ALT into the plasma (Dong, Xu, Yin, Qi, & Peng, 2015). As shown in Table 2, CCl_4 intoxication increased significantly serum AST and ALT activities in treated groups, indicating that CCl_4 caused liver severe damage. In other words, the range of increase in enzyme activity in serum can reflect the severity of hepatic damage (El Arem et al., 2014). However, co-administration of WECF at 2.5 mg/kg bw plus CCl_4 significantly ameliorated liver damages resulting in less markers released from liver tissues into blood. Apparently, WECF administration improved physiological integrity of hepatocytes of rats induced by CCl_4, thereby normalising the values of these enzymes in serum. This result implies that the WECF has a hepatoprotective effect against liver damage of rats induced by CCl_4. In addition, significant difference in histological changes in livers could be observed between rats treated with the CCl_4 group alone and those pretreated with bioactive compounds of WECF, rutin, kaempferol and betanin. Obviously, CCl_4-induced liver injury was significantly ameliorated in rats pretreated with WECF and its bioactive compounds. These results are in accordance with the histopathological changes attenuated by pretreatment with WECF or its bioactive compounds, rutin, kaempferol and betanin.

CCl_4 is a well-known hepatotoxicant that causes cellular necrosis, oxidative stress and inflammation (Chen et al., 2013; Cho et al., 2013). It has been documented that a single exposure to CCl_4 would result in accumulation of CCl_4 in hepatic parenchyma cells. CCl_4 is then metabolised to become $\cdot CCl_3$ radicals by cytochrome P-450 enzymes, leading to the production of ROS, thereby initiating lipid peroxidation (Chen et al., 2013). Therefore, CCl_4 is widely employed to induce liver injury in experimental animals to determine the sufficiency of potential hepatoprotective agents (Pinto, Rodríguez-Galdón, Cestero, & Macías, 2013). Moreover, lipid peroxidation is a biomarker for estimation of the oxidative status in the body (El Arem et al., 2014). Therefore, development of antioxidant action and inhibition of free radical generation are two main determinants for protection against liver damage induced by CCl_4. This study demonstrated that CCl_4 treatment induced significant increase in TBARS level. This increase may result from several radicals or oxidative stress in the rats, which induces an increase in superoxide radicals, leading to an increase in lipid peroxidation (El Arem et al., 2014). According to the data shown in Fig. 3, the pretreatment with WECF or its bioactive compounds, rutin, kaempferol and betanin, effectively protected the rats against lipid peroxidation by reducing TBARS formation. It was speculated that the mechanism behind the inhibitory effects of WECF and its bioactive compounds, rutin, kaempferol and betanin, against lipid peroxidation may involve radical-scavenging effect that consequently leads to hepatoprotection.

Glutathione is crucial for antioxidant defence due to its characteristics of reductant and nucleophile. In general, the intracellular concentration of GSH in most mammalian cells is relatively high (in the millimolar range). However, glutathione deficiency is associated with oxidative stress and, therefore, may play a key role in aging and the pathogenesis of many diseases (Kovacs-Nolan et al., 2014; Wang et al., 2012). According to Table 3, the GSH concentration in CCl_4-intoxicated rat liver was decreased. However, at selected concentration, WECF and its bioactive compounds, rutin, kaempferol and betanin, restored the GSH levels, respectively, indicating that treatment with WECF or its bioactive compounds, rutin, kaempferol and betanin, is effective in reducing oxidative stress which further results in GSH depletion by CCl_4. The possible reason is that GSH allows the detoxification of free radicals and ROS, consequently, its concentration decrease (Kovacs-Nolan et al., 2014). Therefore, supplemental ingested GSH can benefit the treatment of these liver diseases and increase liver GSH concentration for detoxification (Kovacs-Nolan et al., 2014). Thus, it was speculated that increased GSH levels in the presence of WECF or its bioactive compounds, rutin, kaempferol and betanin, in the face of an oxidative damage enhanced the detoxification of free radical and ROS, thereby resulting in a hepatoprotective effect in CCl_4-induced rat liver.

The main antioxidant-defended mechanisms in mammals involve both non-enzyme antioxidant, such as GSH, and antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). These antioxidant enzymes provide the first line of defence against superoxide and hydrogen peroxides (Chen, Chu, Chyau, Chu, & Duh, 2012; Lin et al., 2013). Many studies reported that antioxidant enzymes were abnormally decreased in rats induced by oxidative stressors, favouring the accumulation of ROS such as hydrogen peroxide and superoxide radicals (Marazza, LeBlanc, de Giori, & Garro, 2013). Results of the current study indicated decreased SOD activity in rat liver in response to CCl_4 treatment, while WECF pretreatment increased the SOD activity when compared with rats treated with CCl_4 alone. It was worth noting that the chief determinant of such effect was the kind of bioactive compounds involved since the administration of rutin and kaempferol at 1.0 μg/kg bw in rats did not significantly affect SOD activity in liver, whereas betanin at 1.0 μg/kg bw significantly affected SOD activity (Table 3). SOD activity was increased in rats pretreated with WECF but there were no significant differences in liver CAT and GPx activities among groups.

Polyphenolic compounds such as quercetin have been demonstrated to increase expressions of antioxidant enzymes in the liver. These genes are regulated by the transcription factor Nrf2, which responds to oxidative stress by binding to the antioxidant response element (ARE) as a promoter of genes that code for antioxidant enzymes (Kobori et al., 2015). In other words, antioxidant enzymes are induced by Nrf2 activation and other signal transduction pathways (Cho et al., 2013). Although the mechanism of Nrf2 signalling by WECF and its bioactive compounds, rutin, kaempferol and betanin, was not investigated in this study, many reports noted that liver injury induced by oxidative stressors is attenuated by augmentation of antioxidant enzyme activities via Nrf2 activation (Cho et al., 2013). Therefore, the mechanism of Nrf2 activation and other signal transduction pathways by WECF merits further exploration in future studies.

In this *in vivo* study, WECF and its bioactive compounds, rutin, kaempferol and betanin, inhibited lipid peroxidation, significantly increased SOD activity, and restored both GSH concentration and normal oxidative status. In accordance with the known fact, the amelioration of liver injury in CCl_4-

intoxicated rats may correlate with the overall improvement of antioxidant defence mechanisms influenced by the treatments with WECF and its bioactive compounds, rutin, kaempferol and betanin.

Cytochrome P450 2E1 (CYP2E1), a member of the cytochrome P450 mixed-function oxidase system, has been indicated as a marker of oxidative stress and inflammation associated with liver injury since expression of CYP2E1 parallels increased ROS production, which further activates many xenobiotic to hepatotoxic metabolites (El-Batch, Hassan, & Mahmoud, 2011; Lai et al., 2014). The present study showed that pretreatment of rats with WECF at 1.0 or 2.5 mg/kg bw caused significant induction of CYP2E1 activity compared with rats treated with CCl_4 alone. However, CYP2E1 activity was not affected in rats pretreated with rutin, kaempferol and betanin at 1.0 μg/kg bw. It was worth noting that rats pretreated with WECF alone, at a dose of 2.5 mg/kg bw, showed a significant increase in CYP2E1 activity, indicating that WECF is a potential inducer of CYP2E1 in rat primary hepatocyte. As mentioned above CCl_4 is catalysed by CYP2E1 to produce the unstable free radicals of trichloro-methyl radical ($\cdot CCl_3$), peroxyl trichloromethyl ($\cdot OOCCl_3$), and ROS (Dong et al., 2015). Hence, CYP2E1 has been reported as an effective generator of ROS. Therefore, any agent with an inhibitory effect on CYP2E1 activity may be able to alleviate oxidative stress effects (El-Batch et al., 2011). Although a significant increase in CYP2E1 activity induced by WECF indicates a pro-oxidant effect, WECF restored the hepatic GSH level, enhanced the SOD activity, and reduced lipid peroxidation in CCl_4-induced rats, which explains the antioxidant property. Therefore, it was speculated that pretreatment with WECF induces significant antioxidant activity, which overwhelms the pro-oxidant activity caused by treatment with CCl_4 or by activation of CYP2E1 induced by WECF, resulting in the protection against hepatotoxicity in CCl_4-induced rats. In the present study, induction of CYP2E1 activity was found to be associated with WECF but not with rutin, kaempferol or betanin, suggesting that other bioactive compounds in WECF with the exception of rutin, kaempferol and betanin may render effects that can contribute to induce CYP2E1 activity. According to the results, CCl_4 not only initiated lipid peroxidation and altered redox status of liver but also induced DNA damage. The data from the photomicrograph for comet (Fig. 5A) and genotoxicity (Fig. 5B) of blood lymphocyte show that CCl_4 injection can result in oxidative damage to DNA. However, administration of the WECF or its bioactive compounds, rutin, kaempferol, and betanin, increased the resistance of DNA to CCl_4-induced DNA strand breaks and improved the DNA repair after CCl_4 challenge in blood lymphocytes, which implied that WECF and its bioactive compounds, rutin, kaempferol and betanin, could protect against DNA damage induced by CCl_4.

In this study, amaranthine, camellianoside, betanin, isobetanin and eight polyphenols, which included seven kaempferol glycosides and flavonol quercetin, rutin, were identified from Djulis. A previous study by the authors demonstrated that rutin, kaempferol and betanin contribute to protect against *t*-BHP-induced oxidative stress, *in vitro* (Chyau et al., 2015). The results show that rutin, kaempferol and betanin at a dose of 1.0 μg/kg bw significantly restored GSH levels, attenuated DNA damage and inhibited TBARS formation in CCl_4-induced rats, in addition to the restoration of SOD activity by betanin at 1.0 μg/kg bw. Therefore, it is thought that restoration of GSH levels and enhancement of SOD activity, causing the decrease in the intracellular TBARS and ROS, are two main prospective activities against liver injury afforded by the antioxidant activity of rutin, kaempferol and betanin. However, the results of this *in vivo* study did not support the claimed hepatoprotective effect of rutin, kaempferol and betanin at a dose of 1.0 μg/kg bw in reducing the elevation of AST and ALT for CCl_4-induced rats. It was speculated that a compensatory hepatic response had occurred and thereby leading to lessening CCl_4-induced liver injury, which probably explains why rutin, kaempferol and betanin at a dose of 1.0 μg/kg bw did not display significant positive effect on AST and ALT activities (Chiu et al., 2015). On the other hand, other bioactive compounds, along with rutin, kaempferol and betanin, are identified (Fig. 1 and Table 1). In addition, the HPLC analysis shows that there are other unidentified peaks in WECF, which may render effect and contribute to the hepatoprotective effect by a direct or a synergistic action. Hence, hepatoprotection in CCl_4-induced rats by WECF might be attributed to a direct or the synergistic antioxidant potential of combinations of bioactive compounds present in WECF.

Conclusively, the data presented here support the view that WECF can protect liver from CCl_4-induced hepatotoxicity and genotoxicity *in vivo* due mainly to enhancing its antioxidant defence system involving nonenzymatic and enzyme antioxidants, improving redox status of liver in CCl_4-induced rats and thereby ameliorating oxidative stress responses, thus diminishing liver damage in CCl_4-induced rats. In addition, it is most likely that the hepatoprotective effect of WECF is attributed to the bioactive compounds present in WECF. Taken together, results of this *in vivo* study demonstrate that Djulis can protect against liver injury induced by oxidative stressors, suggesting that consumption of Djulis may protect liver tissue from oxidative injury.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2016.08.025.

REFERENCES

Braz, M. G., & Favero Salvadori, D. M. (2007). Influence of endogenous and synthetic female sex hormones on human blood cells *in vitro* studied with comet assay. *Toxicology In Vitro: An International Journal Published in Association with BIBRA, 21*, 972–976.

Buege, J. A., & Aust, S. D. (1978). Microsomal lipid peroxidation. *Methods in Enzymology*, *52*, 302–310.
Calderón-Montaño, J. M., Burgos-Morón, E., Pérez-Guerrero, C., & López-Lázaro, M. (2011). A review on the dietary flavonoid kaempferol. *Mini Reviews in Medicinal Chemistry*, *11*, 298–344.
Chen, S. Y., Chyau, C. C., Chu, C. C., Chen, Y. H., Chen, T. H., & Duh, P. D. (2013). Hepatoprotection using sweet orange peel and its bioactive compound, hesperidin, for CCl_4-induced liver injury *in vivo*. *Journal of Functional Foods*, 5, 1591–1600.
Chen, Z. T., Chu, H. L., Chyau, C. C., Chu, C. C., & Duh, P. D. (2012). Protective effects of sweet orange (*Citrus sinensis*) peel and their bioactive compounds on oxidative stress. *Food Chemistry*, *135*, 2119–2127.
Chiu, W. C., Huang, Y. L., Chen, Y. L., Peng, H. C., Liao, W. H., Chuang, H. L., Chen, J. R., & Yang, S. C. (2015). Synbiotics reduce ethanol-induced hepatic steatosis and inflammation by improving intestinal permeability and microbiota in rats. *Food & Function*, *6*, 1692–1700.
Cho, B. O., Ryu, H. W., So, Y., Jin, C. H., Baek, J. Y., Park, K. H., Byun, E. H., & Jeong, I. Y. (2013). Hepatoprotective effect of 2,3-dehydrosilybin on carbon tetrachloride-induced liver injury in rats. *Food Chemistry*, *138*, 107–115.
Chyau, C. C., Chu, C. C., Chen, S. Y., & Duh, P. D. (2015). Djulis (*Chenopodium formosaneum*) and its bioactive compounds protect against oxidative stress in human HepG2 cells. *Journal of Functional Foods*, *18*, 159–170.
Dong, D., Xu, L., Yin, L., Qi, Y., & Peng, J. (2015). Naringin prevents carbon tetrachloride-induced acute liver injury in mice. *Journal of Functional Foods*, *12*, 179–191.
Duh, P. D., Wang, B. S., Liou, S. J., & Lin, C. J. (2010). Cytoprotective effects of Pu-erh tea on hepatotoxicity *in vitro* and *in vivo* induced by tert-butyl-hydroperoxide. *Food Chemistry*, *119*, 580–585.
El Arem, A., Ghrairi, F., Lahouar, L., Thouri, A., Saafi, E. B., Ayed, A., Zekri, M., Ferjani, H., Haouas, Z., Zakhama, A., & Achour, L. (2014). Hepatoprotective activity of date fruit extracts against dichloroacetic acid-induced liver damage in rats. *Journal of Functional Foods*, *9*, 119–130.
El-Batch, M., Hassan, A. M., & Mahmoud, H. A. (2011). Taurine is more effective than melatonin on cytochrome P450 2E1 and some oxidative stress markers in streptozotocin-induced diabetic rats. *Journal of Agricultural and Food Chemistry*, *59*, 4995–5000.
Hanlon, P. R., Webber, D. M., & Barnes, D. M. (2007). Aqueous Extract from Spanish black radish (*Raphanus sativus* L. Var. niger) induces detoxification enzymes in the HepG2 human hepatoma cell line. *Journal of Agricultural and Food Chemistry*, *55*, 6439–6446.
Hollman, P. C., & Katan, M. B. (1999). Dietary flavonoids: Intake, health effects and bioavailability. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *37*, 937–942.
Kobori, M., Takahashi, Y., Akimoto, Y., Sakurai, M., Matsunaga, I., Nishimuro, H., Ippoushi, K., Oike, H., & Ohnishi-Kameyama, M. (2015). Chronic high intake of quercetin reduces oxidative stress and induces expression of the antioxidant enzymes in the liver and visceral adipose tissues in mice. *Journal of Functional Foods*, *15*, 551–560.
Kovacs-Nolan, J., Rupa, P., Matsui, T., Tanaka, M., Konishi, T., Sauchi, Y., Sato, K., Ono, S., & Mine, Y. (2014). *In vitro* and *ex vivo* uptake of glutathione (GSH) across the intestinal epithelium and fate of oral GSH after *in vivo* supplementation. *Journal of Agricultural and Food Chemistry*, *62*, 9499–9506.
Lai, Y. S., Chen, W. C., Ho, C. T., Lu, K. H., Lin, S. H., Tseng, H. C., Lin, S. Y., & Sheen, L. Y. (2014). Garlic essential oil protects against obesity-triggered nonalcoholic fatty liver disease through modulation of lipid metabolism and oxidative stress. *Journal of Agricultural and Food Chemistry*, *62*, 5897–5906.
Lee, B. H., & Pan, T. M. (2013). Dimerumic acid, a novel antioxidant identified from Monascus-fermented products exerts chemoprotective effects: Mini review. *Journal of Functional Foods*, 5, 2–9.
Lin, Y. L., Chang, Y. Y., Yang, D. J., Tzang, B. S., & Chen, Y. C. (2013). Beneficial effects of noni (*Morinda citrifolia* L.) juice on livers of high-fat dietary hamsters. *Food Chemistry*, *140*, 31–38.
Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: Food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79*, 727–747.
Marazza, J. A., LeBlanc, J. G., de Giori, G. S., & Garro, M. S. (2013). Soymilk fermented with *Lactobacillus rhamnosus* CRL981 ameliorates hyperglycemia, lipid profiles and increases antioxidant enzyme activities in diabetic mice. *Journal of Functional Foods*, 5, 1848–1853.
Ness, A. R., & Powles, J. W. (1997). Fruit and vegetables, and cardiovascular disease: A review. *International Journal of Epidemiology*, *26*, 1–13.
Park, H. Y., Choi, H. D., Eom, H., & Choi, I. (2013). Enzymatic modification enhances the protective activity of citrus flavonoids against alcohol-induced liver disease. *Food Chemistry*, *139*, 231–240.
Peng, C. H., Liu, L. K., Chuang, C. M., Chyau, C. C., Huang, C. N., & Wang, C. J. (2011). Mulberry water extracts possess an anti-obesity effect and ability to inhibit hepatic lipogenesis and promote lipolysis. *Journal of Agricultural and Food Chemistry*, *59*, 2663–2671.
Pinto, C., Rodríguez-Galdón, B., Cestero, J. J., & Macías, P. (2013). Hepatoprotective effects of lycopene against carbon tetrachloride-induced acute liver injury in rats. *Journal of Functional Foods*, 5, 1601–1610.
Spinola, V., Pinto, J., & Castilho, P. C. (2015). Identification and quantification of phenolic compounds of selected fruits from *Madeira Island* by HPLC-DAD-ESI-MS(n) and screening for their antioxidant activity. *Food Chemistry*, *173*, 14–30.
Szeto, Y. T., Chu, W. K., & Benzie, I. F. F. (2006). Antioxidants in fruits and vegetables: A study of cellular availability and direct effects on human DNA. *Bioscience, Biotechnology, and Biochemistry*, *70*, 2551–2555.
Tamura, A., Sasaki, M., Yamashita, H., Matsui-Yuasa, I., Saku, T., Hikima, T., Tabuchi, M., Munakata, H., & Kojima-Yuasa, A. (2013). Yerba-mate (*Ilex paraguariensis*) extract prevents ethanol-induced liver injury in rats. *Journal of Functional Foods*, 5, 1714–1723.
Tsai, P. J., Sheu, C. H., Wu, P. H., & Sun, Y. F. (2010). Thermal and pH stability of betacyanin pigment of Djulis (*Chenopodium formosanum*) in Taiwan and their relation to antioxidant activity. *Journal of Agricultural and Food Chemistry*, *58*, 1020–1025.
Wang, B. S., Lee, C. P., Chen, Z. T., Yu, H. M., & Duh, P. D. (2012). Comparison of the hepatoprotective activity between cultured *Cordyceps militaris* and natural *Cordyceps sinensis*. *Journal of Functional Foods*, 4, 489–495.
WHO Media Centre. *Cancer*. World Health Organization. <http://www.who.int/mediacentre/factsheets/fs297/en/:> updated February 2015.