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Antioxidant and hepatoprotective effects of *Ajuga nipponensis* extract by ultrasonic-assisted extraction

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ABSTRACT

Objective: To investigate suitable condition for extraction of the active components from *Ajuga nipponensis (A. nipponensis)*.

Methods: Orthogonal experimental design was used to determine the optimal extraction parameters for ecdysterones and flavonoids. Finally, the hepatoprotective abilities of *A. nipponensis* extracts were evaluated by CCl_4-induced animal models.

Results: Maximum yields of flavonoids (7.87 ± 0.10) mg/g and ecdysterones (0.73 ± 0.02) mg/g could be obtained when the extraction time was 50 min, the extraction temperature was 60 °C, and the ratio of sample to 70% (v/v) ethanol was 1:20 (w/w). The antioxidant property of *A. nipponensis* was correlated to the concentration of its extracts. At 5 mg/mL, *A. nipponensis* extract scavenged 84.8% of DPPH radical and had absorbance values of 2.43 ± 0.04 reducing power. Upon CCl_4-induced liver injury, glutamic oxaloacetic transaminase and glutamic pyruvic transaminase decreased significantly after the mice were treated with *A. nipponensis*. Histological researches also explained that *A. nipponensis* reduced the extent of liver lesions induced by CCl_4.

Conclusions: *A. nipponensis* exhibited potent antioxidant activity in chemical experimental models and hepatoprotective effect against CCl_4-induced liver damage.

1. Introduction

The liver is a vital organ in human which regulates metabolism, clears poisonous substances from blood and conversion of excess glucose to glycogen. Chronic liver failure affects many people globally with viral infection being the main cause. Nonetheless, alcohol and drugs abuse resulting in liver failure are also of high prevalence. Carbon tetrachloride (CCl_4) and alcohol poisoning are commonly used to cause liver failure in animal models. The toxicity of CCl_4 depends on its reduction to form trichloromethyl radicals via liver mitochondria, following by the production of more toxic trichloromethylperoxyl radical in the presence of oxygen [1]. On the other hand, ethanol feeding readily increases oxygen availability in liver lobules, resulting in hepatic hypoxia and greater O_2^- and H_2O_2 production. Besides, long-term alcohol consumption can up-regulate cytochrome P450 2E1 in hepatocytes and nicotonamide adenine dinucleotide phosphate oxidase in Kupffer cells, leading to alcoholic liver diseases [2].

It is believed that plants are the best sources of natural antioxidants, and these bioactive phytochemicals are valued as playing primary roles in relieving oxidation process by quenching free radicals, chelating metal ions, and scavenging oxygen in the biological systems [3], thus prevents free radical mediated oxidation of proteins, lipids and DNA which is implicated in the pathogenesis of many diseases. Flavonoids have multiple reduction capacities to facilitate the donation of electrons from hydroxyl moieties to oxidizing radical species [4]. On the other hand, the contribution of flavonoids to hepatoprotective properties has been clarified by several

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researches [1] that *Ajuga nipponensis (A. nipponensis)* is a widespread folk edible medicine in Asia for treating traumatic injury and inflammation [5,6]. Among numerous active compound contained in *A. nipponensis* and ecdysterones are unique for their great variety [7]. Although it lacks active hydroxyl groups which are critical characteristics of antioxidants such as tea polyphenols [8], the antioxidant properties of ecdysterones have been indicated by recent studies [4]. Further research indicates that ecdysteroids could also inhibit the peroxidation of rat liver microsomes induced by hydroxyl radicals, as monitored by the formation of thiobarbituric acid reactive substances, and prevent radical-induced decrease of membrane fluidity [9]. Apart from ecdysterones, flavonoids are another potential bio-constitutes of *A. nipponesis* [10].

Extraction is a critical method for isolating the effective component in plants. There are several parameters concerned in the extraction of antioxidative components from plant tissues. This includes the types of solvent, the ratio of solvent-to-sample, the extraction temperature and the extraction time [11]. Antioxidants extracted by traditional techniques may undergo degradation during extraction, but the ultrasonic-assisted extraction (UAE) will enhance production yield as well as avoid thermal damages. UAE possesses greater solvent penetration into plant tissues due to the formation of cavitational bubble collapse, thus improving its solvent extraction ability [12].

Our Previous studies also show the UAE was the better way to isolate of flavonoids and ecdysteroids from *Ajuga* plants, and show the potential forhypoglycaemic health food [6]. This study was aimed to develop an optimum extraction method of *A. nipponensis* and evaluate its antioxidant and hepatoprotective effects. We used orthogonal experimental design to determine the optimal extraction parameters for ecdysterones and flavonoids of *A. nipponensis*. Several factors including extraction time, extraction temperature and the ratio of sample-to-solvent were taken into consideration. Finally, the hepatoprotective abilities of *A. nipponensis* extracts were evaluated by CCl_4-induced animal models.

2. Materials and methods

2.1. Sample preparation of plant materials

A. nipponensis was provided by Taiwan Agricultural Research Institute, Council of Agriculture, Executive Yuan (Taiwan) with assistance from Instructor Te-Hsun Lin (Department of Medicinal Botany and Healthcare, Da-Yeh University, Taichung, Taiwan). Prior to extraction, the raw *A. nipponensis* was freeze-dried, then crushed by the homogenizer, and sieved over a 60-mesh screen.

In UAE, 3.0 g of *A. nipponensis* powders were mixed with 100 mL of 70: 30 ratio of ethanol, and the mixture was sonicated in the ultrasonic extraction tank for 50 min at 50 °C with a frequency of 28 kHz. The extracts were suction filtered to discard the residue and the filtrates were then filtered through a 0.45 μm filter.

2.2. Orthogonal array experimental design

An orthogonal experiment [$L_9(3)^4$] test design was used to optimize the extraction conditions of *A. nipponensis* by UAE. In the present study, extraction was accomplished with 70% ethanol according to the pre-test results. The effect of extraction time, the extraction temperature and the ratio of sample to extraction solvent on extraction yield was investigated. Each factor had three optimization levels. Levels for extraction time factor 1, 2, 3 representative of 45, 50 and 55 min, extraction temperature factor 1, 2, 3 representative of 40, 50 and 60 °C, and ratio of *A. nipponensis* to70% ethanol factor 1, 2, 3 representative of 1: 10, 1: 20 and 1: 30.

2.3. Determination of flavonoid contents

Flavonoid contents were determined using the aluminum chloride colorimetric method. The procedure was performed based on the protocol from the previous research method [13]. In brief, 0.5 mL of *A. nipponensis* extracts prepared at various concentrations (0.01, 0.05, 0.1, 0.5 and 1 mg/mL) were reacted with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride hexahydrate, 0.1 mL of 1 M potassium acetate (CH_3COOK), and 2.8 mL of deionized water for 40 min at room temperature. Finally, the absorbance of the mixture was measured at 415 nm. Quercetin was used as the standard.

2.4. Determination of ecdysterone contents

The ecdysterone contents were analyzed with an RP-18 column by a Hitachi L-7400 HPLC system (Tokyo, Japan). The flow rate was 1.0 mL/min, and the composition ratio of the mobile phase was methanol/water at 40: 60 (v/v). Ecdysterone was detected at 248 nm and 20-hydroxyecdysone was used as the standard [(0.01–0.1) mg/mL].

2.5. DPPH scavenging assay

The antioxidant abilities of *A. nipponensis* extracts were determined by their capacities to neutralize radicals of DPPH [di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium]. Each 100 μL of 1 mM DPPH solution was placed into test tubes, and 100 μL varying *A. nipponensis* extracts concentrations in methanol each 20 μL of tested sample was dissolved in 80 μL of methanol were added. Reaction was completed at room temperature for 30 min, and the absorbance was measured at 517 nm. All experiments were performed in triplicate. Butylated hydroxyanisole (BHA) was used as a positive control. The capability to scavenge the DPPH radical was calculated by the following equation (%): = [(Ac – As)/Ac], where Ac is the absorbance of the control reaction and as is the absorbance in the present of samples or BHA. Therefore, lower values represent higher antioxidant ability of each tested sample.

2.6. Reducing power assay

An aliquot of sample (5 mL) at different concentrations was mixed with 2.5 mL of 0.2 M phosphate buffer and 2.5 mL of 1% potassium ferricyanide. The mixture was then reacted at 50 °C for 20 min. After cooling, 2.5 mL of 10% trichloroacetic acid was added. After that, 5 mL of the mentioned reaction liquid was mixed with 5 mL of distilled water and 1 mL of 0.1% iron (III) chloride. Finally, the absorbance was detected at 700 nm. All experiments were performed in triplicate. BHA was used as a positive control.

2.7. Experimental animals and hepatotoxic induction by CCl_4

An acute CCl_4-induced toxicity model was performed according to the previous research method [14]. Thirty ICR male mice weighting (20 ± 0.5) g were obtained from National Laboratory Animal Center, Taipei, Taiwan, and housed in a controlled environment at (23 ± 1) °C as well as a relative humidity of 40%–60% with a 12 h/12 h light/dark photoperiod. After adaption for a week, the mice were divided into four groups (n = 6 per group) and fed through gastrostomy tube. Normal control and hepatotoxic control groups were fed with saline (1 mL/100 g BW); positive control group was given silymarin (100 mg/kg BW); and treatment group was fed with 250 mg/kg and 500 mg/kg BW of *Ajuga nipponenesis* extract respectively. Silymarin is a well-known liver protective agent [15]. On the eighth day, the normal control group was injected with soybean oil (0.3 mL/100 g BW) and the other groups were inject with 2% CCL_4/soybean oil (0.3 mL/100 g BW) in the intraperitoneal area 6 h prior to blood collection. Blood was collected through tail bleeding. The activities of serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were immediately determined after centrifugation of collected blood at 3 000×g for 10 min. The Liver tissues were collected from the same lobes and then fixed in a 10% buffered formaldehyde solution and embedded in paraffin. They were further cut into 2 μm sections, stained with hematoxylin and eosin, and then examined under light microscopy.

Liver tissues were collected from the same lobes and trimmed to 2 mm thickness. Then these tissues were fixed in 10% buffered formaldehyde solution and embedded in paraffin and further cut into 2 μm sections, stained with hematoxylin and eosin (H&E), and then examined under light microscopy.

2.8. Statistical analysis

Results were expressed as mean ± SD. Differences among the groups were subjected to a one-way ANOVA (analysis of variance) followed by Duncan's multiple range, Tukey's HSD or Fisher's tests. Statistical significance was accepted when a P-value was less than 0.01 or 0.05.

3. Results

3.1. Optimization of extracting flavonoids and ecdysterones from A. nipponensis by UAE

Optimization of the experimental condition is a critical step in an extraction process. UAE parameters were optimized to obtain the best extraction of flavonoids and ecdysterones from *A. nipponensis*. Thus, the extraction time, extraction temperature and the ratio of sample to solvent were considered as important factors during the UAE process of *A. nipponensis*. As shown in Table 1 given certain parameters, the results varied; and Table 2 showed that the parameters influence (R) on the amount of flavonoid contents in *A. nipponensis* extraction in the order of C > A > B, and parameters influence (R) on ecdysterone contents in the order of C > B > A. The ratio of sample to solvent was found to be the most important determinant of the contents of antioxidant compounds. In summary, the maximum yield of flavonoids (7.87 ± 0.10) mg/g and ecdysterones (0.73 ± 0.02) mg/g from *A. nipponensis* were obtained when the extraction time was 50 min, the extraction temperature was 60 °C, and the ratio of sample to 70% ethanol was 1: 20, as indicated in Table 3.

Table 1
Definition and level of factors in orthogonal array experimental design.

Levels	Factors		
	A	B	C
1	45	40	1:10
2	50	50	1:20
3	55	60	1:30

A: Extraction time (min); B: Extraction temperature (°C); C: Extraction proportions of sample/solvent.

3.2. Effects of A. nipponensis extracts on DPPH scavenging and reducing power assays

After determining the most efficient extraction process of *A. nipponensis* extracts by UAE, we also detected the antioxidant abilities of *A. nipponensis* extracts processed by defined extraction conditions because the antioxidant bioactivities of plants might mainly contribute to their biological functions including hepatoprotective properties. The DPPH scavenging effects increased with increased *A. nipponensis* extraction concentration (Figure 1A). Results indicated that when the concentration of group 6 was 5 mg/mL, it exhibited the best antioxidant activities with a DPPH scavenging ability approximately 84.86% ± 1.76%, similar to BHA 90.08% ± 2.46%. Flavonoids have a diphenylpropane structure, in which B or A ring of the structure contains catechol, C-3 includes either hydroxy or gallic acid (galloyl group), C-2 and C-3 are linked by a double bond and the C-4 is in a ketoform. These provided flavonoid its free radical scavenging capacity [16].

As indicated in Figure 1B the higher concentration of BHA and *A. nipponensis* extracts the greater antioxidant property. Results indicated that when the concentration was 5 mg/mL, the absorbance values of BHA and *A. nipponensis* extracts reducing

Table 2
Results of orthogonal array design experiments by range analysis.

		A	B	C
Flavonoid	k1	5.80	6.08	5.21
	k2	6.92	6.14	6.97
	k3	6.20	6.70	6.75
	R	1.11	0.62	1.76
Ecdysterone	k1	0.62	0.57	0.53
	k2	0.61	0.59	0.66
	k3	0.61	0.67	0.65
	R	0.01	0.10	0.13

A: Extraction time (min); B: Extraction temperature (°C); C: Extraction proportions of sample/solvent. k1, k2, and k3 were average results corresponding to their respective factor group 1, 2, and 3. R was extreme difference of the maximum and minimum k1, k2, and k3 for each factor reflecting the contribution of the factor to the extraction result.

Table 3
Orthogonal array of the experiments on flavonoid and ecdysterone contents of *A. nipponensis* extracts by UAE.

Experiment no.	Factors			Results	
	A	B	C	Flavonoid content (mg/g)	Ecdysterone content (mg/g)
1	1	1	1	4.37 ± 0.14	0.51 ± 0.05
2	1	2	2	5.92 ± 0.18	0.65 ± 0.07
3	1	3	3	7.12 ± 0.21	0.69 ± 0.03
4	2	1	3	6.75 ± 0.11	0.61 ± 0.01
5	2	2	1	6.13 ± 0.16	0.49 ± 0.06
6	2	3	2	7.87 ± 0.10	0.73 ± 0.02
7	3	1	2	7.12 ± 0.13	0.60 ± 0.09
8	3	2	3	6.37 ± 0.11	0.64 ± 0.08
9	3	3	1	5.12 ± 0.17	0.59 ± 0.04

A: Extraction time (min); B: Extraction temperature (°C); C: Extraction proportions of sample/solvent. Each value presents mean ± SD (n = 3).

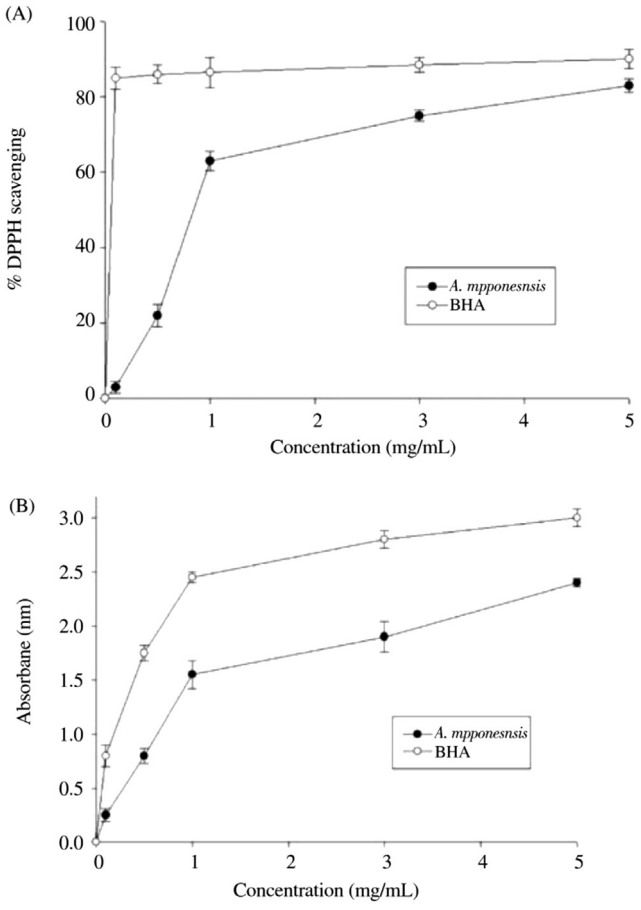

Figure 1. *A. nipponensis* treatment effects on DPPH scavenging and reducing power. (A) The DPPH scavenging effects of *A. nipponensis* at different concentrations compared with BHA; (B) Reducing power effect of *A. nipponensis* at different concentrations compared with BHA. *A. nipponensis* was extracted by 70% ethanol (sample: extraction solvent (v/v) = 1: 20) at 60 °C for 50 min. BHA was used as the positive control.

power are (3.00 ± 0.09) mg/mL and (2.43 ± 0.04) mg/mL respectively.

3.3. Hepatoprotective effects of A. nipponensis extracts by UAE in CCl_4-induced acute liver injury

Figures 2 and 3 represented the results of serum GPT and GOT levels in experimental animals. Consistent with previous evidences, the GPT and GOT levels increased after CCl_4 treatments (Figures 2 and 3). However, the data showed that in CCl_4-induced model, *A. nipponensis* extracts treatments (500 mg/kg body weight, *p.o.*) significantly reduced the serum GPT and GOT levels compared to the hepatotoxic control groups ($P < 0.01$ or 0.05), implying its ability to restore the liver injury.

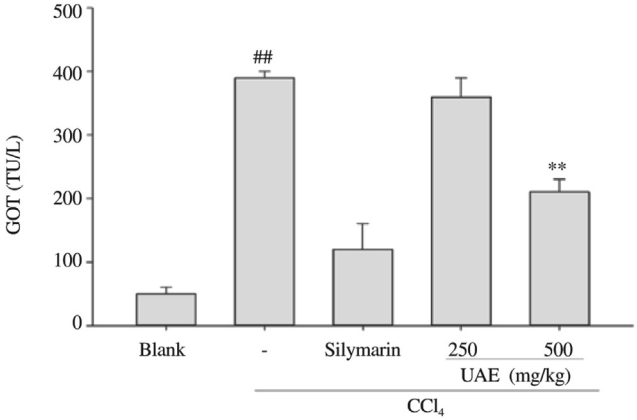

Figure 2. GOT values of CCl_4-induced mice after administration of *A. nipponensis* extracts. Each value presents mean ± SD (n = 6). $^{**}P < 0.01$. Statically significant was calculated relative to the hepatotoxic control group. $^{\#\#}P < 0.01$. Statically significant was calculated relative to the normal group.

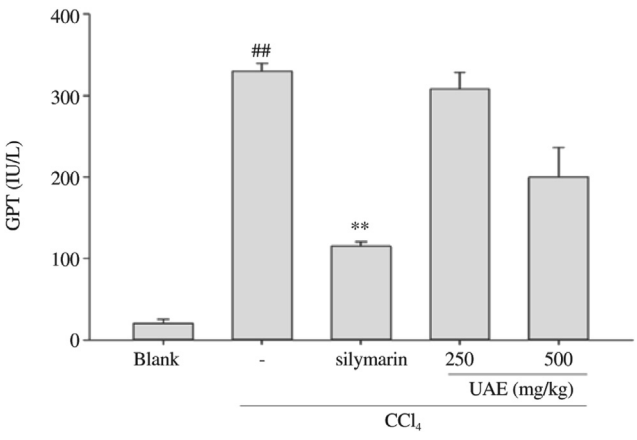

Figure 3. GPT values of CCl_4-induced mice after administration of *A. nipponensis* extracts. Each value presents mean ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$. Statically significant was calculated relative to the hepatotoxic control group. $^{\#\#}P < 0.01$. Statically significant was calculated relative to the normal group.

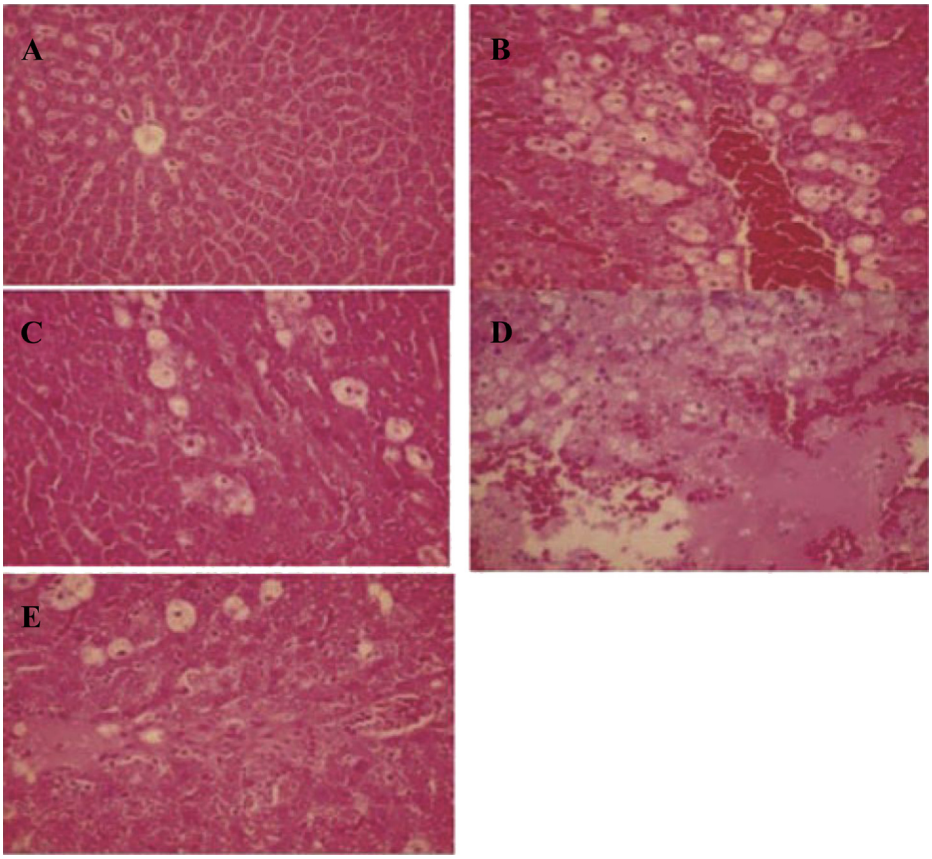

Figure 4. The pathological assessment (×200). A: Blank; B: CCl_4; C: CCl_4+Silymarin; D: CCl_4+*A. nipponensis* (250 mg/kg); E: CCl_4+*A. nipponensis* (500 mg/kg).

3.4. Histological analyses

As shown in Figure 4, hepatic cells of CCl_4-induced group were observed vacuole formation, necrosis and hemolysis around the central vein when compared to the control group. This phenomenon was significantly improved by treatment with *A. nipponensis* extracts (500 mg/kg).

4. Discussion

UAE functions creating bubbles or cavities close to a solid surface and causes a strong impact to the solid surface from its high-speed jets of liquid, thus resulting in the collapse of cavities and the erosion of the surfaces [16,17]. Moreover UAE has been used in extraction process for the food industry and processing in several researches for essential oil extraction of sage (*Salvia officinalis*); extraction of polyphenols from orange (*Citrus* sinensis L.) peel; extraction of antioxidants from *Rosmarinus officinalis*, rutin from *Sophora japonica*, *etc* [18–22]. Except flavonoids concerned as vital antioxidants in our initial experiments for the determination of extraction method used for *A. nipponensis* extracts, ecdysterones are unique active compounds contained in *Ajuga* plants. Beyond traditional usages like antifungal, anthelmintic against intestinal disorders and other functions, recent works have confirmed multiple biological activities related to ecdysterones, for example, hepatic function stimulations [23]. Despite lack of hydroxyl groups, the antioxidant activity of ecdysterones has been truly proved by some studies [9] and it might be contributed to their hepatoprotective abilities. As a result, we both took flavonoids and ecdysterones as active antioxidants for optimization of *A. nipponensis* extracts by UAE. In our previously study, the hypoglycaemic effects of UAE and SFE extracts from five common *Ajuga* species in Taiwan were investigated. The results indicated that UAE was a better extraction technique for getting higher flavonoid and ecdysterone contents than SFE. Furthermore, UAE prepared *A. nipponensis* extracts had α-glucosidase inhibitory activity and glucose uptake effect in vitro and showed the hypoglycaemic effect *in vivo*.

In the antioxidant system, oxidation is accompanied by reduction. Antioxidant index is the measurement of reducing power. Antioxidant capacity can be measured from absorbance values, the higher the absorbance the greater the antioxidant capacity. Figure 1 showed *A. nipponensis* extracts had slightly lowered free radical scavenging effect and reducing power than synthetic BHA, *A. nipponensis* extracts being a natural product and an efficient scavenging power has a higher commercial value.

GPT and GOT, also called alanine aminotransferase and aspartate aminotransferase, are important hepatic enzymes. GPT as well as GOT is important markers of liver injury, and their elevation after CCl_4-induction has been clarified [14]. In the hepatoprotective test, pretreatment of *A. nipponensis* significantly decreased GOT and GPT levels. These results demonstrated the hepatoprotective potentials of *A. nipponensis* extracts *in vivo*. On the other hand, histological biopsies

showed the hepatic cells were protected by treatment with *A. nipponensis*. The above-mentioned findings further illustrated that *A. nipponensis* extracts prevented CCl_4-induced liver injury and maintained hepatocellular membrane structural integrity.

Suitable extraction methods, extraction parameters optimization, and *A. nipponensis* antioxidant as well as hepatoprotective abilities were investigated in this study. This study determined that the UAE was the best extraction process for isolating flavonoids from *A. nipponensis*. Through the orthogonal test, the extraction time, extraction temperature and the ratio of sample to solvent factors for UAE of *A. nipponensis* were optimized. The antioxidant abilities, DPPH scavenging and reducing power abilities of the *A. nipponensis* extract under the defined extraction conditions were also clarified. Finally, this study found that *A. nipponensis* extracts treatment restored the serum GPT and GOT levels significantly and prevented the hepatocellular injury. Thus, the present study demonstrated that *A. nipponensis* extracts by UAE possess excellent antioxidant abilities which might contribute to its hepatoprotective potentials.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgment

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