

# ***In vitro* antioxidant and *in vivo* hepatoprotective effect on Ethanol-mediated liver damage of spray dried *Vernonia amygdalina* water extract**

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**Abstract:** *Vernonia amygdalina* is a strong natural antioxidant that possessed various medicinal properties. In this study, the spray-dried water extract of *V. amygdalina* was evaluated for its *in vitro* antioxidant capacity and *in vivo* hepatoprotective effect against alcoholic-mediated liver damage. Total phenolic and flavonoid content of spray-dried *V. amygdalina* water extract were determined. Liver enzyme profiles, liver antioxidant level and nitric oxide level were evaluated in alcohol-induced liver injured mice or co-supplement with spray-dried *V. amygdalina*. Water extract of spray-dried *V. amygdalina* that contained phenolic content of 24.8±1.5 mg/g gallic acid equivalent and total flavonoid content of 25.7±1.3 mg/g catechin equivalent was able to inhibit 50% of xanthine and tyrosinase oxidation at 170 µg/mL and 2 mg/mL, respectively. On the other hand, extracts at both 10 and 50 mg/kg body weight were able to reduce the levels of Alanine transaminase (ALT), Alkaline phosphatase (ALP), Aspartate transaminase (AST), triglyceride and total bilirubin content in the alcohol-mediated liver injury in mice. Furthermore, it also helped to increase levels of Superoxide dismutase (SOD), Ferric reducing ability of plasma (FRAP) and reduce the levels of Nitric oxide (NO) and Malondialdehyde (MDA) in the liver of the treated mice. These results suggested that water extract of spray-dried *V. amygdalina* exhibited liver protective effect, which could be contributed by its antioxidant properties.

**Keywords:** Spray-dried water extract; phenolic; flavonoid; alcohol; liver damage.

## **INTRODUCTION**

Antioxidants have long been known to protect biological systems and mankind through inhibition or prevention of oxidation stress induced by reactive oxygen substances generated during normal metabolic activities or environmental factors (Hwang *et al.*, 2010). Thus, it was believed that insufficient intake of antioxidants in human may lead to structural damage of DNA, lipids and proteins which may cause various diseases such as cancers. Plants including herbs and vegetables have been widely accepted as a source of natural antioxidants (Rahimi *et al.*, 2012). For example, *Camellia sinensis* (green tea extract) was shown to protect ethanol toxicity through its antioxidant and anti-inflammation activity (Balouchzadeh *et al.*, 2011).

*Vernonia amygdalina*. Del is a seedless soft wooden shrub with 2 to 5 meters height. Traditionally, it is used to treat stomach disorder, diarrhea and hepatitis (Yeap *et al.*, 2010; Yiniger *et al.*, 2007). This plant also possesses antidiabetic effect and was reported to be safe for use in

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ethno medicine (Ekpo *et al.*, 2007; Eteng *et al.*, 2008). Besides, *V. amygdalina* is identified as an excellent source of protein, which explains its wide consumption as green vegetable in Africa (Yeap *et al.*, 2010). Also, it has been reported as a good source of natural antioxidant with high ascorbic acid and phenolic content (Odukoya *et al.*, 2007). Its antioxidant activity was shown to be protective against potassium bromate-induced tissue damage (Josiah *et al.*, 2012). *V. amygdalina* produces a variety of flavonoids and bitter sesquiterpene lactones that contributed to its bioactivities such as anti-nociceptive and anti-phlogistic effects (Nangendo *et al.*, 2002; Favi *et al.*, 2008; Iroanya *et al.*, 2010). However, the plant is often avoided due to its bitter taste. Processes such as blanching and extraction are able to enhance the palatability of *V. amygdalina* but these processes could reduce its antioxidant activity (Obboh, 2005).

We have employed a common approach of herbal preparation i.e. via water extraction and sequential spray-drying to produce an extract of *V. amygdalina*. It is therefore crucial to examine the effects of these

processing on the antioxidant capacity effect of *V. amygdalina*. Additionally, the protective effect of *V. amygdalina* on ethanol-mediated liver damage was also of our interest. In this study, we examined the antioxidant capacity of the water extract of spray-dried *V. amygdalina*. The hepatoprotective potential of this extract and its influence on liver antioxidant level and on *in vivo* alcohol-mediated liver damage were also studied.

## MATERIALS AND METHODS

### Reagents and chemicals

Aluminium chloride, ascorbic acid, catechin, dopachrome (DOPA), folin-ciocalteu reagent, gallic acid, hypoxanthine, L-cysteine, L-tyrosine, mushroom tyrosinase, sodium nitrate, superoxide dismutase, xanthine oxidase, XTT (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide) were purchased from Sigma-Aldrich (USA). Wheatgrass (*Triticum aestivum* L.) was purchased from Easy Phamax (Malaysia), standardized *Phyllanthus niruri* extract (contains 4% corilagin and 18% Phyllanthus flavonoids) from Nova Laboratories (Malaysia) and silymarin from Biolife (Malaysia). All solvents were of analytical reagent grade (Merck, Germany).

### Plant material

*V. amygdalina* leaves were harvested from herbal farm located in Kuala Selangor, Malaysia and were assigned with voucher number FRIM 43216 by the Forestry Division of the Forest Research Institute Malaysia (FRIM). Leaves were air-dried in shade and ground into fine powder using grinder (HFM2413, Taiwan). *V. amygdalina* extract was prepared by heating 1g of ground leaves in 80 mL of deionised water (60°C, 2 hours) followed by filtration using Whatman filter paper no. 1 (Millipore, Malaysia) and spray drying at inlet temperature of 150°C and outlet temperature of 100°C. The native extract ratio obtained was 4:1 (yield 25%, w/w). The spray-dried powder with moisture content <5% was then stored at 4°C for further analysis.

### Xanthine oxidase superoxide scavenging assay

This assay was performed to evaluate the scavenging activity of the *V. amygdalina* spray-dried powder on superoxide free radical anions via indirect measurement of XTT reduction rate. *V. amygdalina* spray-dried extract was reconstituted in ultra pure-water and mixed with NaHCO<sub>3</sub> buffer (50mM, pH 9.4), hypoxanthine (0.5mM), EDTA (1mM) and XTT (0.25mM). Then, the mixture was added with xanthine oxidase (200 mU/mL) and subjected to absorbance reading at 480 nm every 20 seconds for 5 minutes. Superoxide dismutase (6 X 10<sup>-3</sup> U/mL) was used as a standard and ultra pure-water as negative control. The results were expressed as the concentration of the extract that scavenged free radicals by 50% (SC50).

### Total phenolic content (TPC) determination

The Folin-ciocalteu method adopted from Slinkard and Singleton (1977) was used to examine the total phenolic content of *V. amygdalina* extract. The samples were first oxidized with Folin-ciocalteu reagent followed by neutralization using sodium carbonate. Samples were left for 60 minutes before reading the absorbance at 760 nm wavelength. Standard curve was established using gallic acid. Results were expressed as milligram of gallic acid equivalents (GAE) per gram of *V. amygdalina* spray-dried extract.

### Total flavonoid content determination

The total flavonoid content of *V. amygdalina* spray-dried extract was determined using NaNO<sub>2</sub> and AlCl<sub>3</sub> based on aluminium chloride complex formation (Zhishen *et al.*, 1999). *V. amygdalina* extract was first added with 5% NaNO<sub>2</sub> followed by 10% of AlCl<sub>3</sub> at the 5<sup>th</sup> minute and 1M NaOH at the 6<sup>th</sup> minute. The absorbance was read at 510 nm. Catechin was used as the standard and the flavonoid content was reported as milligram catechin equivalent (CE) per gram of *V. amygdalina* extract.

### Tyrosinase-inhibition assay

Inhibition of tyrosinase activity was measured by using L-tyrosine as substrate. Mushroom tyrosinase (200 U/mL) was added with phosphate buffer (pH 6.8) and *V. amygdalina* extract. After 10 minutes of incubation at 37°C, 10mM of L-tyrosine was added and the mixture was further incubated for 15 minutes at 37°C. The amount of DOPA (dopachrome) was measured at 475 nm using ELISA plate reader. The results were expressed as percentage of tyrosinase activity inhibition. L-cysteine was used as the standard tyrosinase inhibitor in this assay.

### In vivo hepatoprotective test

Alcohol-induced hepatotoxicity in mice was used as a model to determine the hepatoprotective effect of spray-dried *V. amygdalina* water extract. ICR mice aged 8 weeks old (~18-22g) were purchased from Animal House, Faculty of Veterinary Medicine, University Putra Malaysia. Mice were housed under 12 hours dark/light cycle at 22°C and allowed for standard pellet diet and water *ad libitum*. This study was approved by the Animal Care and Use Committee, University Putra Malaysia (UPM/FPV/PS/3.2.1.551/AUP-R2).

The animals were randomly assigned into 8 treatment groups ( $n=7$ ). Liver damage was induced in treatment groups 2 to 5 through daily administration of 50% ethanol solution using in tragastric tube for 7 consecutive days. The study design is as stated below:

**Treatment group 1:** Mice p.o. (*per os*) with Normal Saline for 14 consecutive days (Normal control)

**Treatment group 2:** Mice p.o. with 50% of ethanol (9.4g/kg/day, p.o. for 7 days) followed by p.o. with 1 X PBS for 7 days

**Table 1:** *In vitro* antioxidant activity and total phenolic/flavonoid content of *V. amygdalina*

	Total phenolic (mg gallic acid equivalent/g extract)	Total flavonoid (mg catechin equivalent/g extract)	Superoxide Dismutase (SC <sub>50</sub> (IC <sub>50</sub> µg/mL)	Tyrosinase inhibition test (IC <sub>50</sub> mg/mL)
<i>V. amygdalina</i>	24.80±1.50	25.7±1.30	170±4.70	2±0.30
Wheat ( <i>Triticum aestivum</i> L.)	2.75±0.71	0.29±0.01	-	-
Vitamin C	-	-	60±5.20	-
L-cysteine	-	-	-	0.30±0.10

**Table 2:** Effect of *V. amygdalina* and positive controls on serum Alanine transaminase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Triglyceride (TG), total bilirubin and unconjugated bilirubin levels in alcohol induced acute liver toxicity in mice

Treatment	ALT (U/L)	ALP (U/L)	AST (U/L)	Triglyceride (µmol/L)	Total Bilirubin (µmol/L)	Unconjugated Bilirubin (µmol/L)
Group 1	76.3 ± 9.5*	81.7 ± 8.2*	207.5 ± 26.9*	1.02 ± 0.24*	194.1 ± 22.5*	86.4±4.5*
Group 2	168.5 ± 11.3 <sup>†</sup>	115.4 ± 6.9 <sup>†</sup>	602.4 ± 42.3 <sup>†</sup>	1.51 ± 0.05 <sup>†</sup>	215.3 ± 9.6 <sup>†</sup>	97.8±2.3 <sup>†</sup>
Group 3	48.7 ± 3.4 <sup>*,†</sup>	97.3 ± 5.6 <sup>*,†</sup>	196 ± 21.5*	1.14 ± 0.07*	174.2 ± 13.8*	77.3±5.1 <sup>*,†</sup>
Group 4	49.4 ± 3.1 <sup>*,†</sup>	138.8 ± 2.7 <sup>*,†</sup>	221.5 ± 14.9*	1.16 ± 0.37*	173.2 ± 12.2 <sup>*,†</sup>	77.6±4.7 <sup>*,†</sup>
Group 5	70.2 ± 11.1*	141.2±10.6 <sup>*,†</sup>	285.6 ± 37.4*	1.33 ± 0.18	139.2 ± 31.3 <sup>*,†</sup>	74.3±3.8 <sup>*,†</sup>
Group 6	52.5 ± 2.6 <sup>*,†</sup>	117.5 ± 7.2 <sup>*,†</sup>	243.5 ± 35.1*	1.02±0.28*	142.5 ± 23.4 <sup>*,†</sup>	73.6±4.2 <sup>*,†</sup>
Group 7	66.8 ± 7.2 <sup>*,†</sup>	80.3 ± 6.8*	182.4 ± 19.2*	1.04 ± 0.11*	138.3 ± 18.6 <sup>*,†</sup>	68.9±3.9 <sup>*,†</sup>
Group 8	49.3 ± 8.4 <sup>*,†</sup>	80.1 ± 3.5*	174.5 ± 23.6*	1.01 ± 0.08*	132.5 ± 19.2 <sup>*,†</sup>	66.4±3.6 <sup>*,†</sup>

Values are represented as means ± S.D. of 7 animals in each group. <Significant (P<0.05) of comparison between each groups vs group 2 (ethanol control) was labeled as\*; <while vs group 1 (normal control) was labeled as<sup>†</sup>. Group 1: Normal; Group 2: Untreated ethanol; Group 3: *Phyllanthus niruri* (15 mg/kg) treated ethanol; Group 4: silymarin (200 mg/kg) treated ethanol; Group 5: *V. amygdalina* (10 mg/kg) treated ethanol; Group 6: *V. amygdalina* (50 mg/kg) treated ethanol; Group 7: *V. amygdalina* (10 mg/kg) treated normal; Group 8: *V. amygdalina* (50 mg/kg) treated normal.

**Table 3:** Effect of *V. amygdalina* and positive controls on liver Superoxide dismutase (SOD), Malondialdehyde (MDA), Ferric reducing ability of plasma (FRAP) and Nitric oxide (NO) levels in alcohol induced acute liver toxicity in mice

Treatment	SOD (U/mg protein)	MDA (nmol/mg protein)	FRAP (U/mg protein)	NO (µM)
Group 1	8.71 ± 0.15	12.54 ± 0.63	1.44 ± 0.10	3.12 ± 0.63
Group 2	4.13 ± 0.64 <sup>†</sup>	26.51 ± 0.37 <sup>†</sup>	0.72 ± 0.02 <sup>†</sup>	9.52 ± 1.33 <sup>†</sup>
Group 3	7.88 ± 0.69*	18.73 ± 0.71 <sup>*,†</sup>	0.93 ± 0.11 <sup>*,†</sup>	4.87 ± 0.86 <sup>*,†</sup>
Group 4	7.62 ± 0.36*	19.64 ± 0.83 <sup>*,†</sup>	0.98 ± 0.26 <sup>*,†</sup>	5.12 ± 0.91 <sup>*,†</sup>
Group 5	6.33 ± 0.42*	24.49 ± 2.11 <sup>*,†</sup>	0.84 ± 0.13 <sup>*,†</sup>	6.37 ± 1.14 <sup>*,†</sup>
Group 6	7.97 ± 0.83*	21.38 ± 1.65 <sup>*,†</sup>	1.05 ± 0.27 <sup>*,†</sup>	5.04 ± 1.25 <sup>*,†</sup>
Group 7	16.34 ± 1.12 <sup>*,†</sup>	12.78 ± 1.03*	1.62 ± 0.41*	3.03 ± 0.92*
Group 8	25.87 ± 0.74 <sup>*,†</sup>	13.12 ± 0.62*	2.41 ± 0.38 <sup>*,†</sup>	2.97 ± 0.76*

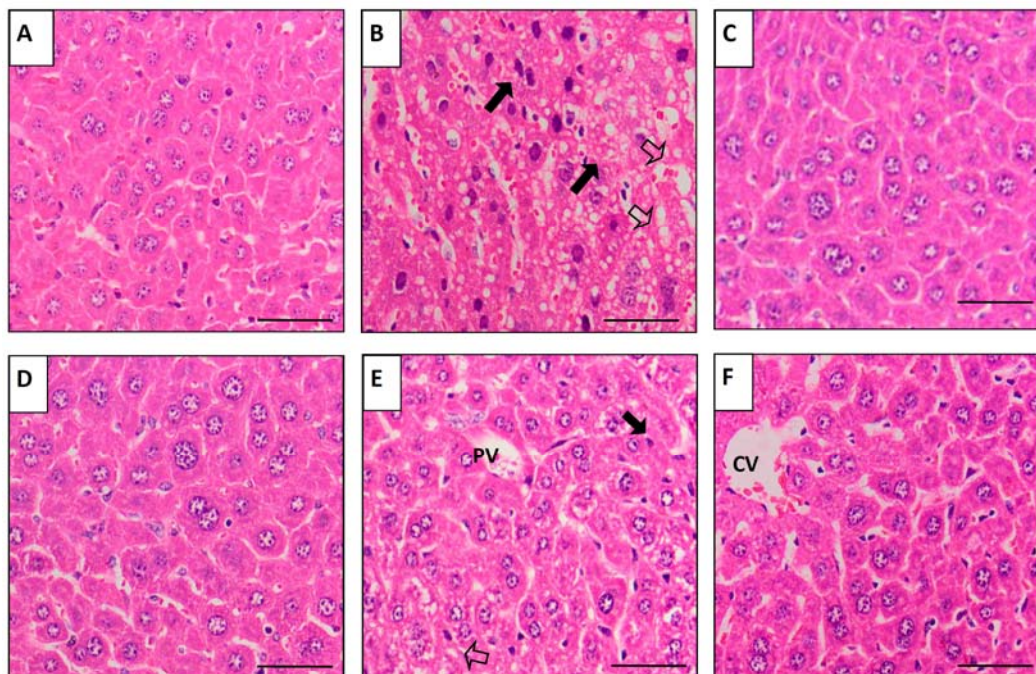
Values are represented as means ± S.D. of 7 animals in each group. Significant (P<0.05) of comparison between each groups vs group 2 (ethanol control) was labeled as\*; while vs group 1 (normal control) was labeled as<sup>†</sup>. Group 1: Normal; Group 2: Un-treated ethanol; Group 3: *Phyllanthus niruri* (15 mg/kg) treated ethanol; Group 4: silymarin (200 mg/kg) treated ethanol; Group 5: *V. amygdalina* (10 mg/kg) treated ethanol; Group 6: *V. amygdalina* (50 mg/kg) treated ethanol; Group 7: *V. amygdalina* (10 mg/kg) treated normal; Group 8: *V. amygdalina* (50 mg/kg) treated normal.

**Treatment group 3:** Mice p.o. with 50% of ethanol (9.4g/kg/day, p.o. for 7 days) followed by p.o. with *Phyllanthus niruri* (15 mg/kg) for 7 days.

**Treatment group 4:** Mice p.o. with 50% of ethanol (9.4g/kg/day, p.o. for 7 days) followed by p.o. with silymarin (200 mg/kg in 1% carboxymethyl cellulose) for 7 days

**Treatment group 5:** Mice p.o. with 50% of ethanol (9.4g/kg/day, p.o. for 7 days) followed by p.o. with *V. amygdalina* extract (10 mg/kg) for 7 days.

**Treatment group 6:** Mice p.o. with 50% of ethanol (9.4g/kg/day, p.o. for 7 days) followed by p.o. with *V. amygdalina* extract (50 mg/kg) for 7 days



**Fig. 1:** Representative photomicrographs of livers from mice with or without ethanol induction.

(A) Histologic appearance of normal liver in mice from the control group without ethanol induction, (B) liver section of mice given ethanol for 7 days as ethanol control (C-F) liver sections of mice treated with silymarin, *P. niruri*, 10 mg/kg of *V. amygdalina* and 50 mg/kg of *V. amygdalina*, respectively after 7 days of ethanol induction. Steatosis (block arrow), necrosis (filled arrow) and loss of normal cellular architecture were observed in liver of mice after ethanol induction (B). After treatment by silymarin or *P. niruri* (C, D), reversion of the histological appearance to normal was observed. Similarly, reversion to normal histological appearance was also observed in the group treated by 50 mg/kg of *V. amygdalina* (F). However, after treatment with lower concentration of *V. amygdalina* (E), a small amount of fat droplets were still observable in the mice liver section. (H&E staining, original magnification:  $\times 200$ ) Centrilobular vein (CV), peripheral vein (PV), hepatic artery (HA). Bar=50  $\mu$ m.

**Treatment group 7:** Mice p.o. with *V. amygdalina* extract (10 mg/kg) for 7 days

**Treatment group 8:** Mice p.o. with *V. amygdalina* extract (50 mg/kg) for 7 days

On day 13, the mice were fasted overnight, anesthetized with ether and sacrificed by decapitation. Serum was obtained for quantification of various marker enzymes including Alkaline phosphatase (ALP), Alanine transaminase (ALT), Aspartate aminotransferase (AST) (Kind and King, 1954), Triglyceride (TG) (Biovision, USA), Total bilirubin and conjugated bilirubin (Mallay *et al.*, 1969). Livers were collected, homogenized (0.1 g/mL of PBS) and subjected to *in vivo* antioxidant assays (SOD, MDA and FRAP) (Ho *et al.*, 2012). NO quantification was performed using Gries's method (Aydn *et al.*, 2001). Livers were also removed and subjected to pathological examinations.

#### **Histopathological evaluation**

Liver tissue processing for his to pathological evaluation was performed according to Ho *et al.* (2012). Briefly, after overnight fixation with 10% formalin, tissues were dehydrated in ethanol and processed using TP1020 automated tissue processor (Leica, Germany). Then, tissues were embedded in paraffin, sectioned using the

Jung Multicut 2045 microtome (Leica, Germany), and deparaffined in Xylol, followed by ethanol dehydration. Slides stained with haematoxylin (Sigma, USA) and Eosin (Sigma, USA) were then examined using the BX51 light microscope (Olympus, Japan).

#### **STATISTICAL ANALYSIS**

The results are presented as mean  $\pm$ S.D. One way analysis of variance (ANOVA) followed by Duncan test was used to determine the significance level of the results. P values  $<0.05$  were considered as statistically significant.

#### **RESULTS**

##### ***In vitro* antioxidant activities**

The total phenolics, total flavonoids, Xanthine oxidase superoxide scavenging activity and tyrosinase inhibition activity are summarized in table 1. Spray-dried *V. amygdalina* water extract was able to inhibit up to  $81.5 \pm 0.5\%$  of superoxide radicals at  $250 \mu\text{g/mL}$  (results not shown) and achieved 50% of scavenging inhibition (SC50) at  $170 \mu\text{g/mL}$ . The content of phenolics and flavonoids in *V. amygdalina* were  $24.8 \pm 1.5$  mg GAE/g extract and  $25.7 \pm 1.3$  mg CE/g extract, respectively. The total phenolic and flavonoid content of *V. amygdalina*

extract was higher when compared to wheat extract. However, its xanthine oxidase superoxide scavenging activity and tyrosinase inhibition activity were lower when compared to the positive control vitamin C and L-cysteine.

#### ***In vivo hepatoprotective effect***

The levels of ALT, ALP, AST, TG and total bilirubin in mice sera after the period of study were summarized in table 2. Significant differences between the levels of ALT, ALP, AST, TG and total bilirubin in the normal (p.o. with normal saline) and ethanol control groups were observed. *P. niruri* extracts exhibited the most significant hepatoprotective effect with the lowest level of ALT ( $48.7 \pm 3.4$  U/L), ALP ( $97.3 \pm 5.6$  U/L), AST ( $196 \pm 21.5$  U/L), TG ( $1.14 \pm 0.07$   $\mu$ mol/L), total bilirubin ( $174.2 \pm 13.8$   $\mu$ mol/L) and unconjugated bilirubin ( $77.3 \pm 5.1$   $\mu$ mol/L) among all groups. In comparison to 10 mg/kg BW of *V. amygdalina*, 50 mg/kg BW of the plant showed a more comparable effect to silymarin and *P. niruri* extracts on the regulation of AST, AST, TG and total bilirubin. While also showing the capability to restore marker enzymes to near normal level, 10 mg/kg of *V. amygdalina* extract however exhibited the lowest liver protective potential, with the greatest content of ALT ( $70.2 \pm 11.1$  U/L), ALP ( $141.2 \pm 10.6$  U/L), AST ( $285.6 \pm 37.4$  U/L) and TG ( $1.33 \pm 0.18$   $\mu$ mol/L) among all groups.

#### ***In vivo antioxidant capacity and NO level of liver***

The degree of oxidative stress in the liver was evaluated by measuring levels of SOD, MDA, FRAP and NO (table 3). *V. amygdalina* (group 7 and 8) was able to enhance the levels of SOD and FRAP in normal mice. On the other hand, ethanol significantly decreased the level of SOD and FRAP while elevating the levels of MDA and NO. Treatment with silymarin, *P. niruri* and *V. amygdalina* extracts significantly restored the level of FRAP and prevented SOD inhibition. Besides, reduction of MDA and NO levels indicated that all the treatments effectively inhibited lipid peroxidation and production of NO in alcohol-induced liver injured mice. Among all groups, 50mg/kg of *V. amygdalina* was able to enhance the SOD ( $7.97 \pm 0.83$  U/mg protein) and FRAP ( $1.05 \pm 0.27$  U/mg protein) levels while reducing MDA ( $21.38 \pm 1.65$  nmol/mg protein) and NO ( $5.04 \pm 1.25$   $\mu$ M) levels. On the other hand, 10mg/kg of *V. amygdalina* showed the weakest antioxidant effect with the lowest SOD ( $6.33 \pm 0.42$  U/mg protein) and FRAP ( $0.84 \pm 0.13$  U/mg protein) level and highest MDA ( $24.49 \pm 2.11$  nmol/mg protein) and NO ( $6.37 \pm 1.14$   $\mu$ M) content. These results indicated that the hepatoprotective effect of *V. amygdalina* was dosage dependent.

#### ***Histopathological evaluation***

After 7 days of ethanol induction, damage to the liver was observed whereby hepatocytes were rendered with undefined lining and loss of nucleus. Massive steatosis

(accumulation of fatty droplets) and necrosis were also observed (fig. 1B). Signs of cirrhosis were also observed where inflammatory cells were found to infiltrate fibrotic bands within the tissue. Treatment with silymarin and *Phyllanthus niruri* for 7 days against the ethanol-induced hepatotoxicity showed recovery of the histological appearance of liver section to although nucleus sizes were still bigger than the untreated control group (fig. 1C and 1D). As observed in fig. 1A, liver sections of mice that were not induced by ethanol treatment appeared normal where hepatocytes were with well-preserved cell lining, well-defined cytoplasm and nucleus structures. However, recovery of hepatocytes after treatment with 10 mg/kg of *V. amygdalina* was incomplete where a number of necrotic cells and lipid droplets were still observable after 7 days of continuous 10 mg/kg of extract treatment (fig. 1D). In contrast, treatment with 50 mg/kg of *V. amygdalina* was capable of recovering the hepatocytes to their normal structures with absence of steatosis (fig. 1E).

## **DISCUSSION**

Synthetic compounds such as butylatedhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT) are among the strong antioxidants that are commonly used as preservatives in food processing. However, usage of these compounds had been reported to produce adverse side effects such as liver damage and mutagenesis when consumed (Grice *et al.*, 1986). Hence, the search for natural antioxidants from tropical plants may allow us to discover more natural and non-toxic antioxidants that serve as better alternatives than the synthetic compounds.

From this study, spray-dried *V. amygdalina* water extract was shown to possess significant antioxidant activity even at a low concentration. This activity may be contributed by the high content of phenolics and flavonoids that were detected in the extract. Phenolics and flavonoids are the secondary metabolites synthesized in plants that possess strong anti-oxidizing properties (Rahimi *et al.*, 2012). Previous research suggested that the synergistic effects of antioxidant constituents in *V. amygdalina* exhibited high antioxidant activity (Erasto *et al.*, 2007). Luteolin 7-*O*- $\beta$ -glucuronoside (the most abundant compound in this plant), luteolin 7-*O*- $\beta$ -glucoside and luteolin are the flavonoids that contribute to the antioxidant activity of *V. amygdalina* and these active ingredients in *V. amygdalina* have also been proven to remain stable even after a strong heating process such as boiling (Ola *et al.*, 2009). Likewise in our study, we found that not only the total phenolic and flavonoid content of the extract, but the antioxidant activity (in xanthine oxidase and tyrosinase inhibition test) also remained high after the spray drying process.

Since many plants that show high antioxidant activities had also been associated with protective capability against

liver damage (Babu *et al.*, 2001; Harish and Shivanandappa, 2006; Jain *et al.*, 2008), the hepatoprotective potential of *V. amygdalina* against alcohol-induced liver damage was also evaluated in the present study. Alcoholic liver disease remains as one of the most common causes of chronic liver diseases. Ethanol administration can lead to oxidative stress which can further lead to lipid peroxidation, raised membrane permeability and eventually necrosis. Hepatic injury can be determined by measuring the leakage of cellular enzymes into plasma or serum. Increased serum enzyme levels, including ALT and AST had been associated with loss of hepatocyte membrane integrity, or more specifically necrosis in the case of ALT elevation (Navarro and Senior, 2006). On the other hand, bilirubin is a breakdown product of hemoglobin which will conjugate with glucuronic acid in hepatocytes to increase its water solubility. Thus, release of unconjugated bilirubin from damaged or dead liver cells is also one of the major markers for liver damage (Palanivel *et al.*, 2008). In this study, ethanol-treated mice were observed with higher total and unconjugated bilirubin while all the treatments were able to reduce both total and unconjugated bilirubin (table 2). Traditionally, plants or herbs that contain natural antioxidants have been used to prepare tonic for alcoholic liver diseases (Saravanan *et al.*, 2006). While being shown to be non-hepatotoxic itself (Ojiako *et al.*, and HU Nwanjo, 2006), the methanolic and aqueous extracts of *V. amygdalina* had also been shown to possess liver protective effects against radiation (Saravanan *et al.*, 2006) and carbon tetrachloride (CCl<sub>4</sub>) (Arhoghro *et al.*, 2009), respectively. In this study, we have demonstrated that the spray-dried water extract of *V. amygdalina* was able to restore liver damage induced by alcohol in a dosage dependent manner (table 2 and fig. 1F). Administration of *V. amygdalina* extract significantly decreased the serum levels of ALP, ALT and AST of ethanol-induced liver injured mice. Apart from the mice with liver damage, normal mice treated with the extract (group 7 and 8) also showed lower ALT level as compared to normal control (group 1). This result indicated that the extract helped to stabilize the plasma membrane and suppressed the leakage of enzymes through cellular membrane (Huang *et al.*, 2012) in both normal and mice with liver damage. This hepatoprotective effect may be contributed by its antioxidant capacity. A previous study reported that reduction of superoxide anion radical by xanthine oxidase inhibitors may remedy liver diseases (Lin *et al.*, 2000). From the *in vitro* antioxidant results, spray-dried *V. amygdalina* water extract that showed inhibitory effect to xanthine oxidase might revert the changes of liver damage induced by alcohol through its strong antioxidant capacity and high content of natural phenolics and flavonoids. This is because *V. amygdalina* extract was able to restore the liver *in vivo* antioxidant level of ethanol treated mice. SOD is an effective defense enzyme that protects biological macromolecules from

oxidative stress (Zhang *et al.*, 2003) while MDA and Noaresui table indicators to evaluate the levels of lipid peroxidation and inflammation during liver damage (Adaramoye *et al.*, 2008a). NO is a very reactive oxidant that is over-produced in liver during hepatic inflammation and this oxide can further intensify oxidative stress by reacting with reactive oxygen species. Thus, stimulation of NO may lead to oxidative stress due to depletion of antioxidant enzymes (Adaramoye *et al.*, 2008a). It was shown in a previous study that silymarin was able to protect rats from acute liver damage by inhibiting lipid peroxidation, pro-inflammation and oxidative stress via down regulation of iNOS, COX-2 and MMP-9. In this study, we compared the *in vivo* antioxidant, anti-inflammation and anti-lipid peroxidation effects of *V. amygdalina* extract with that of silymarin. Our results showed that mice treated with ethanol were found to be associated with reduction of SOD and FRAP levels and elevated levels of MDA and NO. Previously, we have demonstrated that *V. amygdalina* extract was capable of enhancing the content of liver SOD and FRAP after 14 days of administration (Ho *et al.*, 2012). Likewise in this study, administration of either positive control (silymarin or *P. niruri*) or *V. amygdalina* extract was able to increase the liver SOD and FRAP levels, suggesting that *V. amygdalina* extract was able to restore the antioxidant enzyme activities in ethanol-damaged liver. Also consistent with our previous findings (Ho *et al.*, 2012), *V. amygdalina* extract did not significantly alter MDA and NO levels in normal mice (Group 7 and 8, table 3) in this study. However, *V. amygdalina* extract was able to reduce the MDA level and suppress NO production in mice treated with ethanol. Overall, 50mg/kg BW of extract exhibited comparable liver protective and *in vivo* antioxidant activity when compared to both positive control silymarin and *P. niruri* extract.

Previously, Adaramoye *et al.* (2008b) demonstrated the potential of methanolic extract of *V. amygdalina* as the treatment for hyperlipidemia through reduction of cholesterol-induced low-density lipoprotein and triglyceride. In this study, we found that the spray-dried extract was also able to reduce the serum triglyceride level that was elevated after alcohol intoxication in a dosage-dependent manner where fat droplets that were present in the 10mg/kg BW of extract was not observed in 50 mg/kg BW of extract. This result was supported by the histopathology study where a number of fat droplets still appeared in the liver section of mice treated with a low concentration of the extract. Optimal concentration of spray-dried *V. amygdalina* water extract may elicit not only antioxidant and liver protective effects but could also offer other health benefits such as maintaining a low level of triglyceride in the body system.

Results from this study showed that spray-dried water extract of *V. amygdalina* possessed potent antioxidant

effect and this antioxidant activity could also be the major contributor to its hepatoprotective effect against alcohol-induced acute liver toxicity in mice. However, detailed mechanism on the regulation of cytochrome P450 2E1 pathway (Lu and Ai Cederbaum, 2008) by *V. amygdalina* is still unclear. Thus, pathway regulation of the extract in ethanol-induced liver injury would be a major focus for future studies.

## ABBREVIATIONS

2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT), Alanine transaminase (ALT), Alkaline phosphatase (ALP), Aspartate transaminase (AST), butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT), Carbon tetrachloride (CCl<sub>4</sub>), Cyclooxygenase-2 (COX-2), Dopachrome (DOPA), Ethylenediaminetetraacetic acid (EDTA), Ferric reducing ability of plasma (FRAP), Forest Research Institute Malaysia (FRIM), Gallic acid equivalents (GAE), Inducible nitric oxide synthase (iNOS), Malondialdehyde (MDA), Matrix metalloproteinase 9 (MMP-9), Nitric oxide (NO), Scavenged free radicals by 50% (SC50), Superoxide dismutase (SOD), Triglyceride (TG).

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