

Full Length Research Paper

***In vitro* and *in vivo* antioxidant activity of *Vernonia amygdalina* water extract**

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***Vernonia amygdalina* water extract was previously found as a potential *in vitro* antioxidant agent. In this study, the *in vitro* and *in vivo* antioxidant activity of *V. amygdalina* spray dried water extract were quantified by using DPPH radical scavenging assay, superoxide dismutase (SOD) activity, malondialdehyde (MDA) level and total antioxidant capacity (TAOC). *In vitro* DPPH assay showed that, *V. amygdalina* spray dried water extract was a moderate antioxidant agent when compared with vitamin C. For *in vivo* test, increased SOD and TAOC and reduced MDA levels were observed on the organs and blood of the animal treated with the extract. It was concluded that *V. amygdalina* spray dried water extract is a potential antioxidant agent that can protect oxidation stress of the cells in the organ.**

Key words: Antioxidant, *in vivo*, *Vernonia amygdalina*.

INTRODUCTION

Oxidation stress by free radicals is known to be one of the major mechanisms that lead to serious disease such as cancer. Consumption of supplements or foods with high antioxidant capacity such as vitamin C, E, co-enzyme Q₁₀, red wine and green tea are believed could help to prevent chronic disorders through repair of oxidative damage (Sung et al., 2000).

Vernonia amygdalina Del, originated from Africa and currently is available in various countries including Malaysia. It is a rapid regenerated soft wooded shrub or small tree of 2 to 5 m with petiolate leaf of about 6 mm diameter and has an elliptical shape from the genus *Vernonia* (Arene, 1972). It is commonly called bitter leaf which is due to its bitter taste (Igile et al., 1994). Researchers, who studied the zoopharmacology, dis-

covered the potential of this herb when they found that sick chimpanzee with empty stomach used succulent pith and juice from unsavoury plant. *Vernonia* stalk which they normally avoid to remove gastrointestinal parasites, enhance body fitness, increase strength or appetite and reduce constipation or diarrhoea within 20 to 24 h (Huffman and Seifu, 1989; Clayton and Wolfe, 1993). Previous study has reported that, *V. amygdalina* extract was able to reduce oxidation *in vitro* (Erasto et al., 2007; Odukoya et al., 2007). Various bioactives compounds including luteolin, luteolin 7-*O*- β -glucuronoside (the most abundant compound) and luteolin 7-*O*- β -glucoside have been isolated from *V. amygdalina*. These flavanoids are well known with strong antioxidant activity (Igile et al., 1994). Besides, other type of compounds such as steroid glucosides (Vernonioside A to E) and sesquiterpene lactones (Vernolide and Vernodaline) were also found in *V. amygdalina* (Jisaka et al., 1993). However, the study that deals with *in vivo* antioxidant effect of *V. amygdalina* extract is yet to be available. As there is no one reported for the *in vivo* antioxidant activity of this extract, this study

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was carried out to elucidate the dosage effect of *V. amygdalina* spray dried water extract on *in vivo* antioxidant activity.

MATERIALS AND METHODS

Chemicals

Sodium acetate trihydrate, glacial acetic acid, sodium cyanide, riboflavin, butylated hydroxytoluene (BHT), trichloroacetic acid, thiobarbituric acid, phosphate buffered saline (PBS), methanol, vitamin C, ethylenediaminetetraacetic acid (EDTA), sodium hydroxide (NaOH), iron reagent (TPTZ), ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), nitro blue tetrazolium (NBT) and hydrogen chloride (HCL) were all purchased from Sigma. Vitamin E was purchase from SimeDarby, Malaysia.

Plant material

Leaves of *V. amygdalina* were collected from *V. amygdalina* plantation in Kuala Selangor, Malaysia in June 2008 and were identified by science officer Lim Chung Lu (Kepong, Selangor) from Forestry Division, Forest Research Institute Malaysia (FRIM). The voucher number of *V. amygdalina* is FRIM 43216. The leaves of the plant were air-dried in shade and finely powdered. The leaf extract was prepared by the addition of 1 g of leaf powders soaked in 80 ml of deionised water (60°C) for 2 h. Then, the extract was filtered with Whatman filter paper no. 1 and subjected to spray drying at an air pressure of 1.4 bar and operating temperature of 145°C (yield 25%, w/w). The spray dried powder was store at 4°C. The moisture content of the spray dried powder was determined by the Karl Fisher method.

In vitro DPPH radical-scavenging activity

1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used to determine the free radical-scavenging activity of *V. amygdalina* spray-dried powder (Ebrahimzadeh et al., 2010). *V. amygdalina* spray-dried powder was dissolved in methanol at different concentrations (2000, 1000, 500, 250, 125, 62.5 and 31.25 µg/ml) and was added with an equal volume to methanolic solution of DPPH (10 µM) (Sigma, USA). After 15 min incubation at room temperature, the absorbance was read at 517 nm. The experiment was carried out in triplicate and vitamin C (Sigma, USA) and E (SimeDarby, Malaysia) were used as standard controls. IC_{50} values which represent concentration required to scavenge 50% of DPPH free radicals was compared among *V. amygdalina* extract, vitamins C and E.

Animals and experimental design

ICR mice (8 weeks old and 18 to 22 g) were provided by the animal house of the Faculty of Veterinary, UPM. The animals were maintained on a 12 h dark/12 h light cycle at approximately 22°C and allowed free access to standard laboratory pellet diet and water during the experiments. Mice were randomly divided into four groups, each consisting of 8 animals, which included: Group 1, mice (X8) p.o. with normal saline for 14 days; Group 2, mice (X8) p.o. with 200 mg/kg/0.1 ml of vitamin C for 14 days; Group 3, mice (X8) p.o. with 10 mg/kg/0.1 ml of Bittermin for 14 days; Group 4, mice (X8) p.o. with 50 mg/kg/0.1 ml of Bittermin for 14 days.

Group 1 fed with normal saline was used as untreated control, while group 2 fed with vitamin C was the positive control. 24 h after

the last administration, the animals were killed by decapitation. Blood sample was collected and processed to obtain serum and red blood cell (RBC) by centrifuging the whole blood at 1000 xg at 4°C for 10 min. Organs (liver, heart, spleen, thymus and lung) were excised from the animal and weighted immediately. The immune organs (thymus and spleen) indices were calculated base on the following formula:

$$\text{Thymus or spleen index} = \frac{\text{Weight of thymus or spleen}}{\text{Body weight}} \times 100$$

The organs (liver, heart, spleen and lung) were then homogenated in 0.1 g (wet weight/ml) of ice-cold phosphate buffer saline. Supernatants of the homogenated organs were then collected after centrifugation and subjected to superoxide dismutase (SOD), malondialdehyde (MDA) and total antioxidant capacity (TAOC) level measurement by spectrophotometric methods. TAOC was analysed by the ferric reducing power test.

SOD activity was tested by evaluating the inhibition of the reduction of nitro blue tetrazolium (NBT) of the serum or homogenates at 560 nm (Ilouno et al., 1996). Briefly, samples were added with 0.1 mol/L EDTA, 1.5 mg sodium cyanide/100 ml, 1.5 mmol/L NBT, 0.12 mmol/L riboflavin and 0.067 mol/L phosphate buffer, pH 7.8 at final volume of 300 µl. The reduction of NBT was quantified at 560 nm.

For MDA, samples were mixed with phosphate-buffered saline (8.1 g NaCl, 2.302 g Na_2HPO_4 and 0.194 g $\text{NaH}_2\text{PO}_4/\text{L}$, pH 7.4) and butylatedhydroxytoluene (BHT, 88 mg/10 ml absolute alcohol) solution follow by 30% trichloroacetic acid. The mixture was incubated on ice for 2 h and pelleted. The supernatant was then, added with 75 µl of 0.1 M EDTA and 250 µl of 1% thiobarbituric acid in 0.05 M NaOH and placed on boiling water for 15 min. After cooling to room temperature, the absorbance was measured at 532 and 600 nm.

Ferric reducing power test (FRAP) was used to quantified the total antioxidant capacity (TAOC) of the organs. Absorbance of the FRAP reagent (containing 300 mmol/L acetate buffer with pH 3.6; 10 mmol/L TPTZ in 40 mmol/L HCL and 20 mmol/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) with organ sample was taken every 1 min for total of 10 min. Fe(II) standard solution was tested in parallel.

Statistical analysis

Experimental results are expressed as mean \pm SD in triplicate. The data were analysed by analysis of variance (ANOVA) ($p < 0.05$) and means separated by Duncan's multiple range tests (by SPSS version 13 software). The IC_{50} values were calculated from linear regression analysis.

RESULTS

In vitro DPPH radical-scavenging activity

In this experiment, the antioxidant capacity of *V. amygdalina* spray-dried extract was compared with vitamin C and E. The spray-dried extract had moisture content of $4.8 \pm 0.2\%$. It was found that, the radical-scavenging activities of *V. amygdalina* and both vitamins increased with increasing concentration. IC_{50} for *V. amygdalina* spray dried extract was 600 ± 7.4 µg/ml, while for vitamin C and E were 5.2 ± 0.3 and 32 ± 3.8 µg/ml, respectively. This result suggests that *V. amygdalina*

Table 1. Effects of *V. amygdalina* extract on the thymus and spleen indices of mice.

Group	Spleen index	Thymus index
1 (Normal control)	0.34 ± 0.42	0.26 ± 0.28
2 (Vitamin C 200 mg/kg)	0.87 ± 0.37*	0.25 ± 0.29
3 (<i>V. amygdalina</i> 10 mg/kg)	0.50 ± 0.45*	0.22 ± 0.47
4 (<i>V. amygdalina</i> 50mg/kg)	0.44 ± 0.51*	0.26 ± 0.34

Table 2. Effect of *V. amygdalina* spray-dried extract on the activity of SOD in different organs of mice (U/mg protein).

Group	Lung	Liver	Spleen	Heart	Plasma	Red blood cell
1 (Normal control)	12.3 ± 0.31	9.10 ± 0.13	12.50 ± 0.57	16.00 ± 0.66	21.05 ± 0.57	25.00 ± 0.22
2 (Vitamin C 200 mg/kg)	14.76 ± 0.26	16.99 ± 0.64*	19.21 ± 0.61*	19.42 ± 0.29*	18.20 ± 0.43	29.92 ± 0.63
3 (<i>V. amygdalina</i> 10 mg/kg)	14.77 ± 0.22	20.82 ± 0.33*	17.86 ± 0.78*	22.13 ± 0.56*	29.54 ± 0.36*	42.01 ± 0.37*
4 (<i>V. amygdalina</i> 50 mg/kg)	16.19 ± 0.41*	31.23 ± 0.45*	17.90 ± 0.82*	25.51 ± 0.55*	43.59 ± 0.28*	51.44 ± 0.41*

The values were the means ± SD of eight animals. The differences between the control group and treated group were determined by one-way ANOVA (*P ≤ 0.05).

Table 3. Effect of *V. amygdalina* spray-dried extract on MDA levels in different organs of mice (nmol/mg protein).

Group	Lung	Liver	Spleen	Heart	Plasma	Red blood cell
1 (Normal control)	2.10 ± 0.24	13.14 ± 0.52	5.13 ± 0.66	3.52 ± 0.58	13.60 ± 0.31	12.45 ± 0.49
2 (Vitamin C 200 mg/kg)	1.93 ± 0.33*	12.26 ± 0.27*	4.98 ± 0.34*	3.51 ± 0.29	14.51 ± 0.46	11.14 ± 0.31*
3 (<i>V. amygdalina</i> 10 mg/kg)	1.87 ± 0.47*	13.22 ± 0.58	4.94 ± 0.46*	3.42 ± 0.22*	14.39 ± 0.35	11.29 ± 0.44*
4 (<i>V. amygdalina</i> 50 mg/kg)	1.67 ± 0.25*	13.28 ± 0.39	5.09 ± 0.71	3.40 ± 0.51*	16.88 ± 0.26	13.99 ± 0.57

The values were the means ± SD of eight animals. The differences between the control group and treated group were determined by one-way ANOVA (*P ≤ 0.05).

spray-dried extract showed potential antioxidant activity when compared with the standard control.

***In vivo* antioxidant activity of *V. amygdalina* extract**

V. amygdalina modulated the thymus and spleen indices of the mice. Compared with the non-treated and vitamin C treated mice, the spleen index of mice treated with *V. amygdalina* spray-dried extract and vitamin C showed significant change. *V. amygdalina* spray-dried extract did not influence the thymus index (Table 1).

SOD, commonly known as cytochrome, is a free radical-metabolising enzyme which protects the cell membrane from damage by the superoxide free radicals (Ilouno et al., 1996). Treatment of mice with both vitamin C and *V. amygdalina* spray-dried extract enhanced the SOD activity. For *V. amygdalina* spray-dried extract, the increase of SOD activity was in the dose-dependent manner. Among the tested organ, the highest increase of SOD activity by the extract was observed in the liver (Table 2).

Cell membrane damage is always associated with increase in MDA level (Ilouno et al., 1996). Table 3

shows the results of the MDA level after treatment with the extract, with vitamin C and also that of the control. Vitamin C significantly reduced the MDA level of the lung, liver, spleen and red blood cell. On the other hand, *V. amygdalina* spray-dried extract was able to reduce the MDA level of the lung, spleen, heart and red blood cell. Among the tested organ, MDA level in the liver and lung was most significantly reduced by 10 and 50 mg/kg of the extract, respectively (Table 3).

Total antioxidant capacity was measured by evaluating the ability of the organ or blood to reduce ferric by using FRAP assay (Benzie and Strain, 1996). It reflects the capacity of non-enzymatic antioxidant defense system. As shown in Table 4, both vitamin C and the extract enhanced the FRAP reducing capacity of the liver and lung in dose dependent manner but not on spleen and heart.

DISCUSSION

Oxidative stress which is caused by insufficient capacity of biological system to neutralize excessive free radical product, has been associated with all kind of human

Table 4. Effect of *V. amygdalina* spray-dried extract on total antioxidant capacity (TAOC) in different organs of mice (U/mg protein).

Group	Lung	Liver	Spleen	Heart
1 (Normal control)	1.23 ± 0.46	1.52 ± 0.28	1.40 ± 0.14	0.91 ± 0.16
2 (Vitamin C 200 mg/kg)	2.28 ± 0.28*	1.20 ± 0.25	1.49 ± 0.18	1.27 ± 0.27
3 (<i>V. amygdalina</i> 10 mg/kg)	4.40 ± 0.22*	1.96 ± 0.31*	1.34 ± 0.13	1.24 ± 0.22
4 (<i>V. amygdalina</i> 50 mg/kg)	9.91 ± 0.37*	2.85 ± 0.39*	1.43 ± 0.22	1.84 ± 0.25*

The values were the means ± SD of eight animals. The differences between the control group and treated group were determined by one-way ANOVA (*P≤0.05).

diseases and aging (Jensen et al., 2008). In this study, *V. amygdalina* spray-dried extract possessed *in vitro* antioxidant activity when tested with DPPH radical scavenging assay. The IC₅₀ for the extract was much higher (600 µg/ml) when compared with vitamins C and E. Thus, it can act as moderate radical-scavengers which can reduce the autooxidation in body system or even food product that contains unsaturated lipid when compared with vitamins C and E (Rao et al., 2007).

People believe that the antioxidant capacity of food base product such as herb can help to protect the oxidative stress not only *in vitro* but also *in vivo* (Jensen et al., 2008). In this study, the *in vivo* antioxidant capacity of this *V. amygdalina* spray-dried extract by checking the SOD capacity, MDA level and total antioxidant capacity of various organ and blood samples after treated with either vitamin C or extract for 14 days was evaluated.

SOD is one of the major antioxidant enzymes that prevent the biological macromolecules from oxidative damage (Zhang et al., 2003). In this study, SOD activity in all the organs was significantly increased after treated with either *V. amygdalina* spray-dried extract or vitamin C. This effect was associated with the decrease of MDA level as observed in Table 3. MDA was previously reported as the major marker of endogenous lipid peroxidation (Zhang et al., 2003). For the TAOC test which represent the non-enzymatic antioxidant defense system, *V. amygdalina* spray-dried extract only modulated the TAOC of lung, liver and heart (at 50 mg/kg) but not for spleen. This means that, *V. amygdalina* spray-dried extract reacted more toward the enzymatic antioxidant defense mechanism than the non-enzymatic antioxidant defense mechanism.

The results indicated that, the antioxidant capacity of *V. amygdalina* spray-dried extract that can enter into blood plasma and organ was much higher than vitamin C in this study. This may be due to the fact that vitamin C contains both pro-oxidant and antioxidant capacity which may further reduced its ability to help protect the cell from oxidation stress (Jensen et al., 2008). Unlike vitamin C, *V. amygdalina* has been reported to contain flavonoids (Luteolin, luteolin 7-*O*-β-glucuronoside and luteolin 7-*O*-β-glucoside) (Igile et al., 1994) which are more promising antioxidant agents when compared with vitamin C (Jensen et al., 2008).

Previous report had suggested that, the increase of spleen index can be indicated as the stimulation of immune function (Zhang et al., 2003). In this study, both vitamin C and *V. amygdalina* spray-dried extract was able to increase the spleen index when compared with the untreated mice. However, both vitamin C and the extract had no stimulation effect on thymus index. This result gives an idea that *V. amygdalina* spray-dried extract was able to stimulate the secondary immune organ but not the primary immune organ.

In conclusion, it can be said that *V. amygdalina* spray-dried extract possessed both *in vitro* and *in vivo* antioxidant activity and it was not only able to enhance the plasma and red blood cell antioxidant level, but was also able to enter into living cells in the organ and protected them from oxidative damage after 14 days of continuous consumption. It can be used in compensating the decrease in total antioxidant capacity in lung and liver and enhance the SOD level in organs and blood and thereby reduces the risks of lipid peroxidation.

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