The in vitro and in vivo antioxidant properties of seabuckthorn (Hippophae rhamnoides L.) seed oil

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\textbf{A B S T R A C T}

The antioxidant capacity of seabuckthorn (Hippophae rhamnoides L.) seed oil was investigated with a number of established in vitro assays and in an in vivo study of carbon tetrachloride (CCl\textsubscript{4})-induced oxidative stress in mice. The results showed that DPPH radical scavenging activity, ferrous ion chelating activity, reducing power and inhibition of lipid peroxidation activity all increased with increasing concentrations of seabuckthorn seed oil. Moreover, the EC\textsubscript{50} values of seabuckthorn seed oil from the hydrogen peroxide, superoxide radical, hydroxyl radical scavenging assays were 2.63, 2.16 and 0.77 mg/ml, respectively. In the in vivo study, seabuckthorn seed oil inhibited the toxicity of CCl\textsubscript{4}, as seen from the significantly increased activities of the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. The GSH content in the liver was also increased, whereas hepatic malondialdehyde was reduced. Taken together, these results clearly indicate that seabuckthorn seed oil has significant potential as a natural antioxidant agent.

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1. Introduction

Reactive oxygen species (ROS) including superoxide anions, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and hydroxyl radicals are known to play multiple important roles in the oxidative damage that is closely related to cardiovascular disease, cancer, liver disease and other chronic and inflammatory diseases (Halliwell, 1997). Several lines of evidence from both epidemiological and experimental studies have demonstrated that natural antioxidants, such as carotenoids, tocopherols and flavonoids, can effectively prevent and cure oxidative stress-related diseases (Vitaglione, Morisco, Caporaso, & Fogliano, 2004; Willett et al., 1984). Therefore, growing attention has been paid to phytochemicals that have been characterised as natural antioxidants.

Seabuckthorn (Hippophae rhamnoides L., Elaeagnaceae) is widely distributed throughout Eurasia and is found in India, China, Nepal, Russia, Britain, Germany, Finland and France. Seabuckthorn is a thorny deciduous bush, and it is a popular medicinal plant that has traditionally been used in its raw form as a health food and nutritional supplement (Yang & Kallio, 2002). The bark, leaves, berries and seeds of seabuckthorn are well known for their medicinal properties, and all parts of the plant contain high concentrations of various bioactive substances. Seabuckthorn leaf extracts contain plentiful flavonoids, which have been reported to have significant antioxidant, anti-inflammatory and hepatoprotective activities (Geetha, Sai Ram, Singh, Ilavazhagan, & Sawhney, 2002; Geetha et al., 2008). Yang and Kallio (2002) reported that seabuckthorn berries are rich in antioxidant substances, such as tocopherols, flavonoids and carotenoids that can improve the immune function and can also suppress certain risk factors for cardiovascular disease. Furthermore, Upadhyay et al. (2009) showed that seabuckthorn seed oil is safe and has a positive impact on human health. Seabuckthorn seed oil also contains large quantities of unsaturated fatty acids, tocopherols, carotenoids and flavonoids, which are known to have significant anti-bacterial, anti-atherogenic and cardioprotective activities (Basu et al., 2007; Negi, Chauhan, Sadia, Rohinishree, & Ramteke, 2005). In various experimental models, seabuckthorn seed oil has also been reported to protect against hyperoxia-induced transvascular fluid leakage and carbon tetrachloride (CCl\textsubscript{4})-induced toxicity in the liver (Hsu, Tsai, Chen, & Lu, 2009; Purushothaman et al., 2008).

Although seabuckthorn seed oil contains abundant phytocemicals, only limited information is available about its antioxidant properties. Therefore, the present study was designed to investigate the antioxidant properties of seabuckthorn seed oil in vitro and in vivo.
2. Materials and methods

2.1. Chemicals

Silymarin, gallic acid, thiobarbituric acid (TBA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), linoleic acid and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents used were analytical grade.

2.2. Seabuckthorn seed oil

The preparation of seabuckthorn (H. rhamnoides L.) seed oil from China Beijing Tongrentang Co., Ltd. (Taipei City, Taiwan) was collected exclusively by supercritical carbon dioxide extraction from seabuckthorn seeds. The quality of seabuckthorn seed oil was described and provided by the company. According to the company-provided data, the final content of the seabuckthorn seed oil was 98.29% crude fat, 0.93% crude protein and 0.78% total carbohydrate.

2.3. Determination of chemical composition

Fatty acid composition in the seabuckthorn seed oil were measured by gas chromatography with a flame ionisation detector (GC-FID) using a Perkin Elmer Autosystem Gas Chromatograph (Model N611-9000) as described previously (Morrison & Smith, 1964). Tocopherols in the seabuckthorn seed oil were estimated using a high performance liquid chromatography (HPLC) system (Jasco International Co., Ltd., Japan) comprising a Jasco PU-2080 Plus intelligent pump, a Jasco MD-2015 plus multiwavelength detector and a C18 column (250 × 4.6 mm, 5 μm, Luna) (Phenomenex®, USA), as described previously (Cunha, Amaral, Fernandes, & Oliveira, 2006). Beta-carotene was estimated using the HPLC system as described previously (Chen, Chuang, Lin, & Chiu, 1993).

2.4. In vitro antioxidant potential of seabuckthorn seed oil

2.4.1. DPPH radical scavenging activity

The method was based on that reported by Epsin, Soler-Rivas, and Wichers (2000) with some modifications. The reaction mixture contained 200 μl of 0.1 mM DPPH and 30 μl of dimethyl sulphoxide containing seabuckthorn seed oil at different concentrations (0.92–18.30 mg/ml). The mixture was shaken vigorously and then left to stand at room temperature for 60 min in the dark. Absorbance was measured by a UV/vis spectrophotometer (JASCO V-500 series, Jasco International Co., Ltd., Japan) at 517 nm. In this assay, lower absorbance indicates stronger scavenging activity. The effective concentration at which 50% of the DPPH radicals were scavenged (denoted by EC50; units: mg sample/ml) was obtained by plotting the scavenging activity against the sample concentration. All the tests were performed in triplicate, and the mean values were plotted.

2.4.2. Ferrous chelating ability

The chelation of ferrous ions by seabuckthorn seed oil was estimated as in Dinis, Maderia, and Almeida (1994), with some modifications. Briefly, 0.94 ml of seabuckthorn seed oil in ethanol at different doses was added to 0.02 ml of 2 mM FeCl2. The reaction was initiated by the addition 0.04 ml of 5 mM ferrozine, after which the mixture was shaken vigorously and left standing at room temperature for 10 min. After equilibrium had been reached, the absorbance of the solution at 562 nm was measured with a UV/vis spectrophotometer. All the tests were performed in triplicate, and the mean values were plotted.

2.4.3. Reducing power

Reducing power was measured according to the method reported by Hu, Lin, Lu, Chou, and Yang (2008). Aliquots of seabuckthorn seed oil solution (0.5 ml) at different concentrations were mixed with 0.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 0.5 ml of 1% K3Fe(CN)6, followed by a 20-min incubation at 50 °C. After adding 0.5 ml of 10% trichloroacetic acid, the mixture was centrifuged at 3750 g for 10 min. The upper layer of solution (0.5 ml) was mixed with 0.5 ml methanol and 0.1 ml of 0.1% ferric chloride for 10 min, and the absorbance was determined at 700 nm with a UV/vis spectrophotometer (higher absorbance indicated stronger reducing power). All tests were performed in triplicate, and the mean values were plotted.

2.4.4. Total antioxidant activity determination using the ferric thiocyanate method (inhibition of lipid peroxidation)

The reaction mixture contained 1.5 ml of 0.2 M phosphate buffer (pH 7.0), 1.0 ml of 0.02 M linoleic acid in ethanol and various amounts of seabuckthorn seed oil (0.60–3.00 mg/ml) in test tubes (5 ml volume) and were well shaken to form emulsion. The tubes were sealed tightly with silicon rubber caps and kept at 40 °C in the dark. At regular intervals, aliquots of the reaction mixtures were withdrawn with a micro-syringe. The oxidation of each sample was measured using a slight modification of the ferric thiocyanate method (Mitsuda, Yasumodo, & Iwami, 1966). A 0.6-ml aliquot of the reaction mixture was mixed with 0.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate, and 0.1 ml of 0.02 M ferrous chloride solution in 3.5% HCl. After 3 min, the absorbance of the coloured solution at 500 nm was measured with a UV/vis spectrophotometer. All tests were performed in triplicate, and the mean values were plotted.

2.4.5. Hydrogen peroxide scavenging activity

The reduction of hydrogen peroxide was determined as described by Yoshiki, Yamanaka, Satake, and Okubo (1999) with some modifications. To test hydrogen peroxide scavenging ability, a reaction mixture consisting of H2O2, seabuckthorn seed oil and acetaldehyde was added to phosphate-buffered saline (PBS) in the stainless steel container of a chemiluminescence analysis system (CLA 2100, Tokyo Electronic Industries, Tokyo, Japan), and the chemiluminescence intensity was recorded. The total chemiluminescence intensity was calculated as the integration of the area under the curve minus the background level. Gallic acid was used as the reference compound. All the tests were performed in triplicate, and the mean values were plotted.

2.4.6. Superoxide anion scavenging activity

Superoxide radicals were generated by the xanthine–xanthine oxidase system as described previously (Oosthuizen, Engelbrecht, Lambrechts, Greyling, & Levy, 1997), with some modifications. Briefly, xanthine oxidase (EC 1.1.3.22; grade I, from buttermilk, 0.25U; one unit converts 1 μmol of xanthine to uric acid per min at pH 7.5 at 25 °C), lucigenin, xanthine and various concentrations of seabuckthorn seed oil were added to PBS, pH 7.4, in the stainless steel container of a chemiluminescence analysis system, and the chemiluminescence intensity was measured. Superoxide dismutase (SOD) was used as the reference compound. All tests were performed in triplicate, and the mean values were plotted.

2.4.7. Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by the addition of ferrous iron to the buffer solution (Yildiz & Demiryürek, 1998). Freshly prepared FeSO4 (in 0.5% NaCl), luminol and various amounts of seabuckthorn seed oil were added to PBS, pH 7.4, in a stainless steel container, and the chemiluminescence intensity was measured. The total chemiluminescence intensity was calculated by
integrating the area under the curve and subtracting the background level. DMSO was used as the reference compound. All the tests were performed in triplicate, and the mean values were plotted.

2.5. In vivo antioxidant properties of seabuckthorn seed oil

2.5.1. Animals
Male ICR mice (20 ± 2 g) were obtained from the Animal Department of BioLASCO, Taiwan, and were quarantined and allowed to acclimate to the laboratory for a week prior to experimentation. The animals were handled under standard laboratory conditions with a 12-h light/dark cycle in a temperature of 25 ± 2 °C and a relative humidity of 55 ± 5% controlled room. The basal diet used in these studies, PMI Nutrition International, LLC, Certified Rodent LabDiet 5001, is a certified feed with appropriate analyses performed by the manufacturer and provided to WIL Research Laboratories, LLC. Food and water were available ad libitum. Our Institutional Animal Care and Use Committee approved all protocols for the animal study, and the animals were cared for in accordance with the institutional ethical guidelines.

2.5.2. Treatment
The animals were randomly divided into seven groups of 10 mice each. Group I served as the control and was given normal saline daily for a period of 8 weeks. Group II served as the vehicle control and was given olive oil daily for a period of 8 weeks. Oxidative stress was induced in Groups III, IV, V, VI and VII by oral administration of 1 ml/kg body weight of CCl4 (20% CCl4 in olive oil) twice a week for a period of 8 weeks. Group III served as the CCl4 control. Group IV served as the positive control and was given silymarin (200 mg/kg, orally) daily for a period of 8 weeks. Groups V, VI and VII were given seabuckthorn seed oil dissolved in olive oil at doses of 0.26, 1.30 and 2.60 mg/kg, respectively, by mouth daily for a period of 8 weeks. At the end of the experiment, the animals were sacrificed by cervical dislocation. Liver samples were dissected out, washed immediately with ice-cold saline to remove as much blood as possible, and immediately stored at −70 °C until further analysis.

2.5.3. Measurement of antioxidant enzyme activities and GSH in liver homogenate
Liver homogenates were prepared in cold Tris–HCl (5 mM containing 2 mM EDTA, pH 7.4) using a homogenizer. Unbroken cells and cell debris were removed by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatant was used immediately for SOD, catalase, glutathione peroxidase (GPx), glutathione reductase (GRd) and glutathione (GSH) assays. The activities of all of these enzymes were determined according to the instructions provided with the Randox Laboratories Ltd. kit.

2.5.4. Measurement of lipid peroxidation
The quantitative measurement of lipid peroxidation was done by determining the concentration of thiobarbituric acid reactive substances (TBARS) in the liver according to the method published by Buege and Aust (1978). The amount of malondialdehyde (MDA) formed was quantitated by reaction with thiobarbituric acid (TBA), and used as an index of lipid peroxidation. Briefly, samples were mixed with TBA reagent (0.375% TBA and 15% trichloroacetic acid in 0.25 N hydrochloric acid). The reaction mixtures were placed in a boiling water bath for 30 min and centrifuged at 1811 g for 5 min. The supernatant was collected, and its absorbance was measured at 535 nm. The results were expressed as nmol/mg protein using the molar extinction coefficient of the chromophore (1.56 × 10⁻⁶ M⁻¹ cm⁻¹).

2.6. Statistical analysis
All values are expressed as the means ± SD. Comparison between any two groups was evaluated using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests. Statistically significant differences between groups were defined as p < 0.05.

3. Results and discussion
The generation of excessive ROS, beyond the antioxidant defense capacity of a biological system, can cause oxidative stress, which plays a role in degenerative and pathological events (Halliwell, 1997). Several studies have suggested that natural antioxidants are helpful in treating diseases that are mediated by oxidative stress (Vitaglione et al., 2004). In the present study, we investigated the in vitro and in vivo antioxidant effects of seabuckthorn seed oil on the production of ROS with several different assays. In addition, we also analysed the chemical composition of seabuckthorn seed oil.

3.1. Chemical composition in seabuckthorn seed oil

Table 1 shows chemical composition in seabuckthorn seed oil. The amount of total fatty acid in seabuckthorn seed oil was 641.2 g/kg, and the weight percent of the total saturated fatty acid and the total unsaturated fatty acid were 12.2% and 85.3%, respectively. The major components of fatty acid in seabuckthorn seed oil were linoleic acid, linolenic acid and oleic acid. Further, the content of α-tocopherol and γ-tocopherol were 111 and 46.1 mg/100 g, respectively; they were 93.8% of total tocopherols in seabuckthorn seed oil. Further, the content of α-tocopherol and γ-tocopherol in seabuckthorn seed oil were 6.71 and 3.69 mg/100 g, respectively, while the total carotenoid was 12.6 mg/100 g. Basu et al. (2007) observed that seabuckthorn seed oil was rich in polyunsaturated fatty acids like oleic acid, linoleic acid and linolenic acid. Negi et al. (2005) pointed out that seabuckthorn seed oil content contained unsaturated fatty acids, tocopherols and carotenoids. Arimboor, Venugopalan, Sarinkumar, Arumughan, and Sawhney (2006) reported that α-tocopherol and γ-tocopherol were the major tocopherols in seabuckthorn seed oil whether using hexane or supercritical carbon dioxide extraction.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Results</th>
</tr>
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<tbody>
<tr>
<td>(A) Fatty acid composition (wt%)</td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>111</td>
</tr>
<tr>
<td>β-Tocopherol</td>
<td>6.71</td>
</tr>
<tr>
<td>γ-Tocopherol</td>
<td>46.1</td>
</tr>
<tr>
<td>δ-Tocopherol</td>
<td>3.69</td>
</tr>
<tr>
<td>(B) Total tocopherols (mg/100 g)</td>
<td>168</td>
</tr>
<tr>
<td>(C) Total carotenoids (mg/100 g)</td>
<td>12.6</td>
</tr>
</tbody>
</table>

Table 1
Chemical composition of seabuckthorn seed oil.
3.2. In vitro antioxidant properties

3.2.1. DPPH radical scavenging activity

The DPPH radical scavenging assay is regularly used for the relatively rapid evaluation of the antioxidant activity. DPPH is a stable free radical, even at room temperature, and shows strong absorbance at 517 nm. The DPPH radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule with a different colour. Thus, the degree of its discoloration from purple to yellow is attributed to the hydrogen donating ability of the added compound, which is indicative of its radical scavenging potential (Epsin et al., 2000). In the present study, the DPPH radical scavenging ability of seabuckthorn seed oil was calculated in a dose-dependent manner (Fig. 1A). Seabuckthorn seed oil proved to be an effective scavenger of DPPH radicals. At concentrations ranging from 0.92 to 18.3 mg/ml, the DPPH radical scavenging ability of seabuckthorn seed oil was measured at 13–92%. The EC50 value of seabuckthorn seed oil in the DPPH radical scavenging assay was 7.37 mg/ml; solutions of trolox (the reference compound used in this test) at the same concentrations had EC50 value of <0.92 mg/ml. Recently, our group demonstrated that the EC50 values of scavenging DPPH radicals of all-trans forms of zeaxanthin, lutein, β-carotene and α-carotene, were 22.8, 23.0, 24.2 and 24.5 mg/ml, respectively (Hu et al., 2008). Lower EC50 value indicates a higher DPPH free radical scavenging activity. By contrast, the present results showed that the activity of scavenging DPPH radicals by seabuckthorn seed oil is higher than that of all-trans forms of zeaxanthin, lutein, β-carotene and α-carotene but lower than that of trolox. Therefore, seabuckthorn seed oil is an outstanding free radical inhibitor and acts as a primary antioxidant. These results confirm those of other studies that reported the inhibition of DPPH radicals by the solvent extracts of seabuckthorn seed (Negi et al., 2005).

3.2.2. Ferrous chelating ability

The hydroxyl radicals are the most reactive species of ROS and are formed by the Fenton reaction of H2O2 with ferrous iron or the Haber–Weiss reaction of the superoxide anion with H2O2. Ferrous ions play an important role in the catalysis reaction of hydroxyl radical formation (Dinis et al., 1994). Several studies have demonstrated that the scavenging of hydroxyl radicals by antioxidants were effective mainly via metal ion chelation. Therefore, the estimation of metal ion chelating ability is important for appraising the free radical scavenging activity of natural antioxidants (Halliwell & Gutteridge, 1990). In the present study, the ferrous ion chelating ability was determined by the reduction of absorbance at 562 nm; this red colour is quantitatively formed by the reaction of ferrozine with ferrous ions. Fig. 1B shows the ferrous chelating abilities of seabuckthorn seed oil, and are compared with that of EDTA and trolox as reference compounds. At concentrations ranging from 0.92 to 18.3 mg/ml, the ferrous chelating ability of seabuckthorn seed oil was measured at 7.74–38.5%. The seabuckthorn seed oil exhibited much stronger metal chelating activity than trolox, which was found to have weak chelating ability for ferrous ions. However, the metal chelating activity of seabuckthorn seed oil was significantly lower than that of EDTA, which had the strongest chelating capacity, and reached 90.4% at concentration of 0.92 mg/ml. As described above, the ferrous chelating ability may be related to antioxidant activity and may modify the antioxidant activity by influencing other reactions. These results suggest that seabuckthorn seed oil has a beneficial effect on ferrous chelating ability and may thus exert protection against oxidative damage.

3.2.3. Reducing power

The reducing power assays are usually used to evaluate the capacity of natural antioxidants to donate an electron or hydrogen atom (Dorman, Peltokeeto, Hiltunen, & Tikkanen, 2003). Natural antioxidants are believed to break free radical chain reactions by donating an electron or hydrogen atom to free radicals, so, antioxidant activities should be reflected in the reducing power. In this assay, the yellow test solution becomes green or blue when the presence of reducers converts the Fe3+/ferricyanide complex to its ferrous form. Thus, higher absorbance at 700 nm indicates greater reducing power (Duh, 1998). The reducing power of seabuckthorn seed oil and trolox, as the reference compounds, were presented in Fig. 1C. As described in Fig. 1C, reducing power was increased with increasing concentrations of seabuckthorn seed oil and the reference compounds. All spectrophotometric
measurements were repeated twice with at least three replicates. At concentrations of 0.42, 0.83, 2.08, 4.18 and 8.32 mg/ml, the reducing power of seabuckthorn seed oil was 0.11, 0.12, 0.17, 0.23 and 0.37, respectively, while solutions of trolox at the same concentrations had reducing power values of 0.82, 1.55, 1.70, 1.69 and 1.69, respectively. These results showed that seabuckthorn seed oil has moderate electron donating abilities, which may be involved in its antioxidant activity.

3.2.4. Inhibition of lipid peroxidation

The ferric thiocyanate method is often used to measure peroxidation in the initial stages of lipid peroxidation. Increased lipid peroxidation is generally believed to be an important underlying cause of oxidative stress initiation upon various tissue injuries, cell death or the progression of many acute and chronic diseases (Halliwell, 1997). In this assay, the inhibition of lipid peroxidation was determined through the formation of a red ferric thiocyanate, which can be detected at 500 nm by spectrophotometry. Fig. 2 shows the results of seabuckthorn seed oil inhibition of linoleic acid peroxidation. At 72 h, seabuckthorn seed oil at doses of 0.60, 1.20, 1.80, 2.40 and 3.00 mg/ml inhibited lipid peroxidation in the linoleic acid system by 2.43%, 13.9%, 31.5%, 49.6% and 65.8%, respectively. The reference compound, trolox (3.00 mg/ml), inhibited lipid peroxidation at 6, 24, 48 and 72 h by 40.0%, 39.0%, 27.6% and 18.4%, respectively. These results showed that the seabuckthorn seed oil had significantly higher inhibition of lipid peroxidation than trolox.

Several studies have reported that seabuckthorn seed oil contains high concentrations of \( \alpha \)-tocopherol, \( \gamma \)-tocopherol, \( \beta \)-tocotrienol, carotenoids and flavonoids, which are known to have significant biological activities (Basu et al., 2007; Kallio, Yang, Peippo, Tahvonen, & Pan, 2002; Negi et al., 2005; Yang, Karlsson, Oksman, & Kallio, 2001). In the present study, we demonstrated that the major antioxidants in seabuckthorn seed oil included \( \alpha \)-tocopherol, \( \beta \)-tocotrienol, \( \gamma \)-tocopherol, \( \delta \)-tocotrienol and carotenoids (Table 1). Haraguchi (2001) reported that various types of phytochemicals, including tocophorins and carotenoids, are effective in preventing lipid peroxidation. Krinsky (1989) has also shown that \( \beta \)-carotene protects liposomes against lipid autoxidation mediated by superoxide and hydroxyl radicals as well as against lipid peroxidation and lysis caused by Fe\(^{2+}\)-generated radicals. Therefore, the tocotrienols and carotenoids enriched seabuckthorn seed oil might be responsible for their significant activities against lipid peroxidation.

3.2.5. \( \text{H}_2\text{O}_2 \), superoxide anion radicals and hydroxyl radicals scavenging activities

Superoxide anion radicals are normally formed in cellular oxidation reactions that can produce hydrogen peroxide and hydroxyl radical through dismutation and other chemical reactions. Hydrogen peroxide can also dissociate to form hydroxyl radicals in vivo. Hydroxyl radicals, the most toxic reactive oxygen metabolites, are mainly derived from the interaction of superoxide anion radicals, hydrogen peroxide and iron salts in the Haber–Weiss and Fenton reactions. These ROS are capable of binding to DNA, proteins or lipids, leading to membrane lipid peroxidation and finally cell necrosis (Halliwell & Gutteridge, 1990). Therefore, ROS scavengers are of great importance. In this study, chemical luminescence reaction systems were used to determine the superoxide anion radical, hydrogen peroxide and hydroxyl radical scavenging capacities of seabuckthorn seed oil and reference compounds. The chemiluminescence assays are very sensitive and convenient methods for determining antioxidant activities. Seabuckthorn seed oil (at doses of 0.76, 1.53, 3.05, 7.63 and 15.3 mg/ml) proved to be an effective scavenger of ROS, scavenging 32.1–82.7%, 20.3–89.4% and 49.6–90.4% of \( \text{H}_2\text{O}_2 \), superoxide anion radicals and hydroxyl radicals, respectively. The EC\(_{50}\) values of seabuckthorn seed oil in the hydrogen peroxide, superoxide radical and hydroxyl radical scavenging assays were 2.63, 2.16 and 0.77 mg/ml, respectively. Gallic acid, SOD and DMSO were used as reference compounds for scavenging \( \text{H}_2\text{O}_2 \), superoxide anion radicals and hydroxyl radicals with EC\(_{50}\) values of 12.5 mg/ml, 0.31 units and 11.7 mg/ml, respectively (Table 2). Recently, our co-author used the same methods to demonstrate that the EC\(_{50}\) values of lutein standard in the hydrogen peroxide, superoxide radical, hydroxyl radical scavenging assays were 6.23, 7.69 and 98.7 mg/ml, respectively, while solutions of a zeaxanthin standard at the same condition had EC\(_{50}\) values of 8.38, 7.52 and 85.6, respectively. With these results, this study confirmed that seabuckthorn seed oil was more effective in inhibiting \( \text{H}_2\text{O}_2 \), superoxide anion radicals and hydroxyl radicals than lutein and zeaxanthin, which are well-known antioxidants of xanthophylls.

3.3. In vivo antioxidant potential

3.3.1. Effect of seabuckthorn seed oil on antioxidant enzyme activities in mice

Several studies reported that antioxidant enzymes, such as SOD, catalase, GPx and GRd protect against oxidative stress-induced damage (Halliwell & Gutteridge, 1990). SOD is an exceedingly effective antioxidant enzyme that converts dismutated superoxide anions into \( \text{H}_2\text{O}_2 \). Catalase is a hemoprotein present in all aerobic cells that metabolizes \( \text{H}_2\text{O}_2 \) to oxygen and water. GPx catalyzes the reduction of \( \text{H}_2\text{O}_2 \) and hydroperoxides to nontoxic products and also helps to detoxify xenobiotics in the liver. GRd is a cytosolic hepatic enzyme that is involved in the conjugating reaction between xenobioc compounds and GSH (Szymonik-Lesiuk et al., 2003). In the present study, SOD, catalase, GPx, and GRd were measured as an index of the antioxidant status of tissues. The results shown in Fig. 3 revealed a significant reduction of liver SOD, catalase,

![Fig. 2. The lipid peroxidation inhibition of seabuckthorn seed oil by the ferric thiocyanate method. Each value is expressed as mean ± SD (n = 3).](image-url)

<table>
<thead>
<tr>
<th>Table 2</th>
<th>The EC(_{50}) values of seabuckthorn seed oil and reference compounds in hydrogen peroxide, superoxide radical and hydroxyl radical scavenging assays.</th>
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<tbody>
<tr>
<td></td>
<td>( \text{H}_2\text{O}_2 )</td>
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<tr>
<td>Seabuckthorn seed oil</td>
<td>2.63 ± 0.08</td>
</tr>
<tr>
<td>Gallic acid (mg/ml)</td>
<td>12.5 ± 0.78</td>
</tr>
<tr>
<td>SOD (unit)</td>
<td>–</td>
</tr>
<tr>
<td>DMSO (mg/ml)</td>
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</table>

Each value is expressed as mean ± SD (n = 3). \(^a\) EC\(_{50}\) means the effective concentration of sample that can decrease the ROS concentration by 50%.
and GRd activity in CCl4-treated mice compared to vehicle-control mice \((p < 0.05)\), indicating that CCl4 damaged the enzymatic antioxidant defence system. In contrast, significant increases in SOD, catalase, GPx, and GRd activities were observed in the seabuckthorn seed oil-treated groups at doses of 0.26, 1.30 and 2.60 mg/kg, respectively, compared to the CCl4-treated control group \((p < 0.05)\). Administration of silymarin, the positive control used in this test, significantly increased SOD, catalase, GPx and GRd activities relative to CCl4 treatment alone \((p < 0.05)\).

Szymonik-Lesiuk et al. (2003) indicated that antioxidant enzymes are inactivated by lipid peroxides or free radicals, which results in decreased activities of these enzymes, as seen by the CCl4 toxicity. On the other hand, the activities of antioxidant enzymes are important to protect cells or organs against oxidative damage. The results of the present study indicate that SOD, catalase, GPx and GRd activities were significantly elevated by the administration of seabuckthorn seed oil to CCl4-treated mice, suggesting that seabuckthorn seed oil has the ability to protect antioxidant enzyme activities in the CCl4-damaged liver. Similar results from our previous research confirmed that seabuckthorn seed oil played a protective role in the reduction of oxidative stress, and restored the activities of enzymes in the antioxidant defence system, such as SOD, catalase, GPx and GRd (Hsu et al., 2009).

### 3.3.3. Effect of seabuckthorn seed oil on lipid peroxidation

MDA is widely used as a marker of free radical-mediated lipid peroxidation injury. MDA is the major reactive aldehyde that appears during the peroxidation of polyunsaturated fatty acids in biological membranes. An increase in the MDA levels in the liver imply enhanced peroxidation leading to tissue damage and the formation of excess free radicals that overwhelm the antioxidant defence mechanisms (Buege & Aust, 1978). In this assay, TBA reacts with MDA to form a pink chromogenic adduct that can be detected spectrophotometrically at 532 nm. The results of the MDA assays

![Fig. 3.](H.-C. Ting et al. / Food Chemistry 125 (2011) 652–659)
in the present study are shown in Fig. 5. The MDA–TBA levels in the CCl4-treated group were significantly higher than those in the vehicle-control group ($p<0.05$), and treatment with seabuckthorn seed oil significantly reversed these changes. The administration of seabuckthorn seed oil caused a significant decrease in the MDA–TBA levels compared to the CCl4-treated group ($p<0.05$), suggesting that seabuckthorn seed oil can protect against CCl4-induced lipid peroxidation in mice. Silymarin also inhibited the elevation of the MDA–TBA levels upon CCl4 administration (Fig. 5). These results are consistent with previous research, which confirmed that the seabuckthorn seed oil could protect against MDA–TBA formation in the liver induced by acetaminophen, CCl4 and ethyl alcohol (Cheng, 1992).

### 4. Conclusions

The results of this study demonstrated that seabuckthorn seed oil contained abundant unsaturated fatty acids, tocopherols and carotenoids, which are known to have significant antioxidant activities. Furthermore, we also found that seabuckthorn seed oil exhibited remarkable antioxidant activity on the DPPH radical scavenging activity, ferrous ion chelating activity, reducing power and inhibition of lipid peroxidation activity, as well as high scavenging activities of superoxide anion radical, hydrogen peroxide and hydroxyl radical. The seabuckthorn seed oil also showed strong inhibition of oxidative damage induced by CCl4 on mice, increased the activities of SOD, catalase, GPx, GRd and GSH content, and decreased the MDA–TBA content in liver. The in vitro and in vivo elevations of antioxidant activities by seabuckthorn seed oil may be due to the presence of carotenoids and tocopherols. This phenomenon appears to be mediated primarily by the ability of carotenoids to quench excited sensitizer molecules and singlet oxygen (Krinsky, 1989). Furthermore, Basu et al. (2007) showed that $\alpha$-tocopherol, an efficient antioxidant, is the major form of vitamin E in seabuckthorn seed oil, and previous studies in our group also found that dietary seabuckthorn seed oil supplementation of CCl4-treated mice resulted in a significant reduction of lipid peroxidation and enhancement of the antioxidant defence system (Hsu et al., 2009). Therefore, the carotenoids and tocopherols content of seabuckthorn seed oil are reflected by its in vitro and in vivo antioxidant properties. Based on the assays presented here, it can be concluded that seabuckthorn seed oil is an accessible source of natural antioxidants that provides the expected health benefits.

### References
