AUTHOR QUERY FORM

ELSEVIER	**Journal: FOCH** **Article Number: 10620**	**Please e-mail or fax your responses and any corrections to:** **E-mail: corrections.eseo@elsevier.sps.co.in** **Fax: +31 2048 52799**

Dear Author,

Please check your proof carefully and mark all corrections at the appropriate place in the proof (e.g., by using on-screen annotation in the PDF file) or compile them in a separate list.

For correction or revision of any artwork, please consult http://www.elsevier.com/artworkinstructions.

Any queries or remarks that have arisen during the processing of your manuscript are listed below and highlighted by flags in the proof. Click on the 'Q' link to go to the location in the proof.

Location in article	Query / Remark: click on the Q link to go Please insert your reply or correction at the corresponding line in the proof
Q1	Please check the telephone number of the corresponding authors, and correct if necessary.

Thank you for your assistance.

Research highlights

▶ Leaves of five *Ligustrum* plants are described in Taiwan flora and used as health tea. ▶ *Ligustrum* plants leaves exhibit appreciate radical-scavenging capacity against ROS. ▶ *Ligustrum* plants leaves inhibit lipid peroxidation and haemolysis in cell-based models. ▶ *Ligustrum morrisonense* is a potential source of natural antioxidants.

Food Chemistry xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Evaluation of the antioxidant activity of five endemic *Ligustrum* species leaves from Taiwan flora *in vitro*

Chi-Rei Wu [a,*], You-Cheng Hseu [b], Jin-Cherng Lien [c], Yung-Ta Lin [d], Tzu-Ping Kuo [a], Hui Ching [e,*]

[a] Graduate Institute of Chinese Pharmaceutical Sciences, College of Pharmacy, China Medical University, No. 91, Hsueh Shih Road, Taichung 40402, Taiwan, ROC
[b] Taichung Hospital, Department of Health, The Executive Yuan, No. 199, San Min Rd., Taichung 40403, Taiwan, ROC
[c] Graduate Institute of Pharmaceutical Chemistry, College of Pharmacy, China Medical University, No. 91, Hsueh Shih Road, Taichung 40402, Taiwan, ROC
[d] Department of Pharmacy, Taichung Tzu Chi General Hospital, No. 66, Fongsing Rd., Tanzih Township, Taichung County 427, Taiwan, ROC
[e] Department of Cosmeceutics, College of Pharmacy, China Medical University, 91 Hsieh Shih Road, Taichung 40402, Taiwan, ROC

ARTICLE INFO

Article history:
Received 15 September 2010
Received in revised form 2 November 2010
Accepted 11 January 2011
Available online xxxx

Keywords:
Ligustrum
Antioxidant activity
Phenolics
Phenylpropanoids
DPPH method
Reactive oxygen species (ROS)
Lipid peroxidation

ABSTRACT

Leaves from the plant species belonging to the genus *Ligustrum* are widely used as tea or herbal medicine in Europe, China, and Japan. The antioxidant properties of five *Ligustrum* species from Taiwan were compared using *in vitro* antioxidant methods such as DPPH radical scavenging, TEAC, and FRAP assays. Cell-based antioxidant methods were used, including Fe^{2+}/ascorbate-induced lipid peroxidation on brain homogenate and AAPH-induced erythrocyte haemolysis. The amounts of major phenolic compounds from the *Ligustrum* species, including phenylpropanoids, flavonoids, and iridoids, were determined by spectrophotometric methods. The results showed that all *Ligustrum* species exhibited antioxidant, radical-scavenging, anti-haemolytic, and lipid peroxidation-inhibiting activities at different magnitudes of potency. A significant correlation was found between antioxidant activity and the amount of antioxidant components, in particular, total phenolics and phenylpropanoids. Among all *Ligustrum* species from Taiwan, *Ligustrum morrisonense* is presented as potential source of natural antioxidants.

1. Introduction

Reactive oxygen species (ROS), which are generated from many redox processes, are major free radicals in the human body that are capable of inducing damage to biomolecules such as carbohydrates, proteins, lipids, and DNA (Valko et al., 2007). Intracellular oxidative damage by ROS causes many chronic diseases, including neurodegenerative diseases and cardiovascular diseases (Butterfield & Sultana, 2008; Leopold & Loscalzo, 2009). Minimising the cellular redox imbalance may be one of the most important approaches to the prevention of these ageing-related diseases. Antioxidants scavenge free radicals by initiating and propagating oxidative chain reactions, and thus they can delay or prevent intracellular oxidative damage (Valko et al., 2007). Natural antioxidants such as phenolics and flavonoids have been shown to possess multiple pharmacological activities through their antioxidant properties, including neuroprotective and anti-ageing activities (Auddy et al., 2003; Uttara, Singh, Zamboni, & Mahajan, 2009).

In recent years, numerous plants belonging to the same genera have been reported to characterise antioxidant and radical-scavenging activities (Tepe, 2008; Tepe & Sokmen, 2007; Zhu et al., 2009). Some species from the genus *Ligustrum* (Oleaceae), including *Ligustrum lucidum*, *Ligustrum robustum*, and *Ligustrum purpurascens*, have been traditionally used as healthy tea or local medicine in European, Chinese, and Japanese communities for centuries. *L. lucidum* is commonly used by traditional Chinese physicians to cure hepatitis and ageing-associated symptoms. *L. robustum* and *L. purpurascens* are the major components of Ku-Ding-Cha, which is used to prevent cardiovascular diseases. Recent pharmacological studies indicate that these *Ligustrum* species possess antioxidative, anti-mutagenic, hepatoprotective, and neuroprotective activities (Lau, He, Dong, Fung, & But, 2002; Lin, Yen, Ng, & Lin, 2007; Nagy et al., 2006, 2009; Sung, Kim, Lee, Lee, & Kim, 2006; Zhu et al., 2009). Phytochemical characterisation of the end products of the general phenylpropanoid pathway such as phenylpropanoids, flavonoids, secoiridoids glycosides, and their aglycones, which possess antioxidant activities (He et al., 2001, 2003; Nagy et al., 2006, 2009; Wong, He, Huang, & Chen, 2001), is included. In the flora of Taiwan, the genus *Ligustrum* is represented by four species: *Ligustrum liukiuense* Koidz., *Ligustrum sinense* Lour., *Ligustrum pricei* Hayata and *Ligustrum morrisonense* Kaneh and Sasaki (Yang & Lu, 2000). They were often used as

* Corresponding authors. Tel.: +886 4 22053366 5506 (C.-R. Wu), +886 4 22053366 5507 (H. Ching).
E-mail addresses: crw@mail.cmu.edu.tw (C.-R. Wu), hui235911@mail.tbcnet.net (H. Ching).

0308-8146/$ - see front matter © 2011 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2011.01.041

herbal tea in the southeast regions of China and Taiwan. However, no scientific reports on the antioxidant components or *in vitro* properties of these *Ligustrum* species from Taiwan have ever been published.

In this paper, the results are presented as a comparative analysis of the antioxidant and radical-scavenging activities of methanol extracts from the four aforementioned *Ligustrum* species leaves in addition to *L. lucidum* leaves. Several different *in vitro* antioxidant test systems have been used, such as the 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay, the Trolox equivalent antioxidant capacity (TEAC) assay, the ferric-reducing antioxidant power (FRAP) assay, and the iron-chelating activity (ICA) assay. Since ROS play an important role in intracellular oxidative damage, effects of methanol extracts from the aforementioned *Ligustrum* leaves on the scavenging activities of superoxide anions were evaluated, hydrogen peroxide (H_2O_2) and hydroxyl radicals, the inhibition of lipid peroxidation induced by the Fenton reaction and the erythrocyte haemolysis caused by the peroxyl radical 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). Based on phytochemical reports on the genus *Ligustrum* (He et al., 2001, 2003; Nagy et al., 2006, 2009; Wong et al., 2001), the amounts of total phenolics, flavonoids, phenylpropanoids, and iridoids in the methanol extracts from the aforementioned *Ligustrum* leaves were determined by spectrophotometry.

2. Materials and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2-deoxyribose, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB), 6-hydroxy-2,5,7,8-tetramethy-chroman-2-carboxylic acid (Trolox), acetic acid, aluminium nitrate, ascorbic acid, (+)-catechin, copper sulphate ($CuSO_4$), dimethyl sulfoxide (DMSO), ferric chloride, ferrous sulphate, ferrozine, Folin–Ciocalteu's reagent, gallic acid, homovanillic acid (HVA), horseradish peroxidase (HRPase), hydrochloric acid (HCl), nitroblue tetrazolium (NBT), potassium ferricyanide, potassium persulfate, quercetin, sodium carbonate, sodium hydroxide, sodium molybdate, sodium nitrate, superoxide dismutase (SOD), thiobarbituric acid (TBA), trichloroacetic acid (TCA), xanthine, and xanthine oxidase were purchased from Sigma–Aldrich (St. Louis, MO). Acteoside and aucubin were purchased from Extrasynthese (France). Hydrogen peroxide (H_2O_2) and HPLC grade methanol were purchased from Merck (Darmstadt, Germany).

2.2. Apparatus

Microplate spectrophotometer (PowerWave$_X$, Bio-Tek instruments, Inc., USA) was used to record the absorbance of *in vitro* antioxidant test systems and phytochemical content assays. Fluorescence microplate reader (FLx800, Bio-Tek instruments, Inc., USA) was only used to record the fluorescence intensity in hydrogen peroxide-scavenging activity assay.

2.3. Collection of plant materials

The five *Ligustrum* species studied were obtained from the following sources. *L. morrisonense* was obtained from the Seed Improvement and Propagation Station, Taichung County. *L. pricei* was obtained from the Chi-Tou Forest Recreational Area, Nantou County. *L. sinense* and *L. liukiuense* were obtained from the Botanical Garden of National Museum of Natural Science, Taichung City. *L. lucidum* was obtained from the Herbal Garden of China Medical University, Taichung City. These species were identified by Professor Dr. Chung-Chuan Chen from the Department of Chinese Medicinal Resources, China Medical University.

2.4. Plant extraction

The air-dried leaves (10 g) obtained from the *Ligustrum* species were macerated with 100 ml of methanol and incubated at 37 °C for 24 h. After incubation, all sample solutions were sonicated for 90 min using a sonicated bath. The solutions were then filtered with a 0.45 m filter and the volume was adjusted to 100 ml with methanol. The concentration of all sample solutions was 100 mg of leaves of *Ligustrum* species per ml.

2.5. DPPH radical-scavenging effect

DPPH radical-scavenging capacity was determined as described in our previous paper (Wu, Huang, Lin, Ju, & Ching, 2007). First, 25 l of sample solution or catechin standard was pipetted into each well. Then 175 l of 300 M DPPH methanol solution was added. The absorbance of the mixture at 517 nm was read after 30 min of incubation at room temperature (RT). The inhibition percentage ($I\%$) of the radical-scavenging capacity was calculated using the following equation: $I\% = [(A_{DPPH} - A_{blank}) - (A_{s\text{-}DPPH} - A_{s\text{-}blank})]/(A_{DPPH} - A_{blank}) \times 100$, where A_{DPPH} is the absorbance of the DPPH· solution, A_{blank} is the absorbance of methanol instead of DPPH·, $A_{s\text{-}DPPH}$ is the absorbance of the DPPH· solution in the presence of sample, and $A_{s\text{-}blank}$ is the absorbance of methanol in the presence of sample. The results are expressed as mmol of (+)-catechin equivalents per gramme of sample.

2.6. TEAC assay

TEAC was measured by the ABTS radical-scavenging assay (Wu et al., 2007). Briefly, the ABTS radical was prepared from the reaction of aqueous 8 mM ABTS solution and 8.4 mM potassium persulfate solution at a ratio of 2:1. After storage in the dark at RT for 12–16 h, the radical solution was further diluted in ethanol until an initial absorbance value of 0.70 ± 0.05 at 734 nm was reached. One hundred and seventy-five microlitres of diluted ABTS solution was mixed with 25 l of sample solution or Trolox standards. The inhibition percentage ($I\%$) of the radical-scavenging capacity was calculated using the following equation: $I\% = [(A_{ABTS} - A_{blank}) - (A_{s\text{-}ABTS} - A_{s\text{-}blank})]/(A_{ABTS} - A_{blank}) \times 100$, where A_{ABTS} is the absorbance of the ABTS solution, A_{blank} is the absorbance of ethanol instead of ABTS, $A_{s\text{-}ABTS}$ is the absorbance of the ABTS solution in the presence of sample, and $A_{s\text{-}blank}$ is the absorbance of ethanol in the presence of sample. The results are expressed as TEAC values.

2.7. FRAP assay

The FRAP assay described by Zhu et al. (2009) was used with some modifications. Briefly, 25 l of sample solution or Trolox standards was mixed with 25 l of freshly prepared FRAP reagent, which consisted of 10 mM TPTZ solution in 40 mM HCl, 20 mM $FeCl_3$, and 50 mM acetate buffer (pH 3.6). The absorption of the reaction mixture was measured at 595 nm. The results were calculated on the basis of a standard curve obtained using Trolox, and are expressed as the relative Trolox equivalents per gramme of sample.

2.8. Superoxide anion-scavenging activity

Superoxide anion-scavenging activity was assessed using the NBT method (Valentão et al., 2002). Briefly, 25 l of sample solution or SOD standards was loaded into a 96-well plate. Seventy-five microlitres of 168 mM NBT in 50 mM phosphate buffer (pH 7.4)

Please cite this article in press as: Wu, C.-R., et al. Evaluation of the antioxidant activity of five endemic *Ligustrum* species leaves from Taiwan flora *in vitro*. *Food Chemistry* (2011), doi:10.1016/j.foodchem.2011.01.041

and 75 l of 800 M xanthine were added. The reaction was initiated by adding 25 l of 0.24 U/ml xanthine oxidase in 50 mM phosphate buffer (pH 7.4) to the above mixture. The reaction was incubated at RT, and the absorbance at 560 nm was determined every 1–5 min using the Microplate spectrophotometer. Absorbance values were corrected for radical decay using control solutions and percentage inhibition was calculated. The results were expressed as U of SOD equivalents per milligramme of sample.

2.9. Hydrogen peroxide-scavenging activity

Hydrogen peroxide-scavenging activity was evaluated as described in our previous paper (Wu et al., 2007). The reaction mixture contained 50 l of 25 mM phosphate buffer (pH 7.4), 50 l of 0.5 mM H_2O_2, and 50 l of sample solution or Trolox standards. After incubating for 5 min at RT, 25 l of 5 mM HVA and 25 l of 8 U/ml HRPase were added; the solution was then mixed and incubated for 25 min at RT. The fluorescence intensity was measured at an excitation of 315 nm and an emission of 425 nm, correcting for radical decay using control solutions. Percentage inhibition was calculated. The results were expressed as mol of Trolox equivalents per gramme of sample.

2.10. Hydroxyl radical-scavenging activity

Hydroxyl radical-scavenging activity was monitored using the 2-deoxyribose method. Briefly, the assay mixture contained 2.8 mM 2-deoxyribose, 20 M ferrous ion solution, 100 M EDTA, and sample solution in a total volume of 1 ml of 10 mM potassium phosphate buffer (pH 7.4). The ferrous iron solution and EDTA were premixed before they were added to the assay mixture. The reaction was initiated by the addition of a mixture of 1.42 M H_2O_2 and 100 M ascorbate. The mixture solution was incubated at 37 °C for 30 min. At the end of the incubation period, 1 ml of 1% (w/v) TBA in 50 mM sodium hydroxide and 1 ml of 2.8% (w/v) TCA were added and this solution was heated for 30 min in a boiling water bath, cooled, and measured at an absorbance of 532 nm (this corresponds to deoxyribose damage) (Wu et al., 2007). The reciprocal absorption values obtained for different concentrations were plotted against the concentrations of sample solution. From the graph, the second-order rate constants were calculated as described by Halliwell, Gutteridge, and Aruoma (1987), assuming that 2-deoxyribose reacts with the hydroxyl radical with a rate constant of $3 \times 10^9\ M^{-1}\ S^{-1}$.

2.11. ICA assay

This method is based on the formation of the coloured ferrozine–Fe^{2+} complex that has a strong absorbance at 562 nm. Reaction mixtures contained 25 l of sample solutions, 100 l of 50 M ferrous sulphate, and 100 l of 300 M ferrozine. After incubation at RT for 10 min, the absorbance was recorded (Wu et al., 2007).

2.12. Lipid peroxidation inhibition assay

Rat brain homogenate was used as a source of polyunsaturated fatty acids for determining the extent of lipid peroxidation. The entire brain was homogenised (100 mg/ml) in ice-cold 0.1 M phosphate buffer (pH 7.4), and the brain homogenate was then centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was divided into 1-ml aliquots and preserved at −80 °C until use. The reaction mixture consisted of 100 l of brain homogenate, 50 l of aqueous 1 mM ferrous sulphate, 100 l of aqueous 5 mM ascorbic acid and 50 l of sample solution. The reaction solution was incubated in a shaking water bath in an open tube. After the solution incubated at 37 °C for 30 min, the TBARS test was performed by rapidly adding 250 l of 1.2% (w/v) TBA in 50 mM sodium hydroxide and 50 l of 10% TCA. Then, the tubes were incubated at 90 °C for 60 min. After cooling, the system was centrifuged at 3000 rpm for 10 min and the absorbance of the supernatant was determined at 532 nm (Wu et al., 2007). Absorbance was recorded against blanks prepared in the same way as the experimental samples but without the homogenate.

2.13. Oxidative haemolysis inhibition assay (OxHLIA)

First, erythrocytes were prepared from rat blood by centrifugation (10 min, 2500 rpm). Erythrocytes were washed twice with 0.9% NaCl and once with phosphate-buffered saline (PBS, pH 7.4); they were then suspended in PBS to obtain approximately 10% haematocrit. Haemolysis of erythrocytes was carried out following the method described by Takebayashi, Chen, and Tai (2010) with minor modifications. Erythrocytes in 10% suspension were shaken gently while being incubated with AAPH (final concentration 50 mM) at 37 °C for 3 h. Then, the samples were centrifuged (10 min, 2500 rpm) and the extent of haemolysis was determined spectrophotometrically by measuring the absorbance of supernatants diluted with PBS (1:9, v/v) at 540 nm. The percentage of haemolysis was calculated in relation to 100% haemolysis caused by deionised water added in 9 parts per 1 part of erythrocyte suspension.

2.14. Determination of amount of antioxidant compounds

2.14.1. Determination of total phenolic content

The total phenolic content was determined as described in our previous paper (Wu et al., 2007). This method is based on the formation of blue-coloured products by the redox reaction with Folin–Ciocalteu's reagent. The absorbance of the coloured solutions at 725 nm is proportional to the total phenolic concentration. The total phenolic concentration of the samples was calculated on the basis of a standard curve obtained using gallic acid and is expressed as milligrammes of gallic acid equivalents (GAE) per gramme of sample.

2.14.2. Determination of flavonoid content

Flavonoid content was also assessed using the 96-well microtiter spectrophotometric method at 415 nm (Wu et al., 2007). This method is based on the formation of coloured products by flavonoids with aluminium salt. The absorbance of the coloured solutions is proportional to the total flavonoid concentration. The total flavonoid concentration of the samples was calculated on the basis of a standard curve obtained using quercetin and is expressed as milligrammes of quercetin equivalents per gramme of sample.

2.14.3. Determination of phenylpropanoid content

The total phenylpropanoid content was determined by a 96-well microtiter spectrophotometric method at 525 nm, modified from the method described by Arnow (1937). One-hundred and fifty microlitres of sample solution was pipetted into each well, and then 50 l of 0.5 N HCl, 50 l of Arnow reagent (contained 5% (w/v) sodium nitrate and 5% sodium molybdate), and 50 l of 2 N sodium hydroxide were added. After incubation at RT for 10 min, the absorbance was read. The absorbance of the coloured solutions is proportional to the total phenylpropanoid concentration. Total phenylpropanoid concentration of the samples was calculated on the basis of a standard curve obtained using acteoside and is expressed as milligrammes of acteoside equivalents per gramme of sample.

Please cite this article in press as: Wu, C.-R., et al. Evaluation of the antioxidant activity of five endemic *Ligustrum* species leaves from Taiwan flora *in vitro*. *Food Chemistry* (2011), doi:10.1016/j.foodchem.2011.01.041

2.14.4. Determination of total iridoid content

Total iridoid concentration was determined by a 96-well microtiter spectrophotometric method at 609 nm, modified from the method described by Trim and Hill (1952). Twenty-five microlitres of sample solution was pipetted into each well, and then 250 l of Trim–Hill reagent (acetic acid/0.2% $CuSO_4$/HCl at a ratio of 10:1:0.5) was added. After heating at 100 °C for 5 min, the absorbance was read. The absorbance of the coloured solutions is proportional to the total iridoids concentration. The total iridoid concentration of the samples was calculated on the basis of a standard curve obtained using aucubin and is expressed as milligrammes of aucubin equivalents per gramme of sample.

2.15. Statistical analysis

All results are expressed as the mean ± standard deviation (SD). The significance of difference was calculated by SPSS one-way ANOVA followed by Scheffe's test; values < 0.05 were considered to be significant.

3. Results and discussion

3.1. Antioxidant and radical-scavenging activity of Ligustrum leaves

DPPH and TEAC assays are simple, rapid methods commonly used to assess radical-scavenging activities *in vitro*. The methanol extracts of all collected *Ligustrum* leaves exhibited appreciative scavenging capacities against both radicals, and the inhibition percentage was proportional to the concentration of each sample solution. The IC_{50} values for *L. morrisonense*, *L. liukiuense*, *L. pricei*, *L. sinense*, and *L. lucidum* in the DPPH assay were 0.48, 0.94, 1.38, 1.47, and 2.06 mg/ml, respectively. The results obtained from the DPPH assay is similar to recent reports of other *Ligustrum* species, including *L. lucidum* and *L. robustum* (Chen, Wong, Leung, He, & Huang, 2002; Katsube et al., 2004; Nagy et al., 2006; She, Wang, Zeng, Yang, & Zhang, 2008; Zhu et al., 2009). For the TEAC assay, the IC_{50} values for *L. morrisonense*, *L. liukiuense*, *L. pricei*, *L. sinense*, and *L. lucidum* were 0.21, 0.24, 0.37, 0.52, and 1.40 mg/ml, respectively. The relative radical-scavenging potencies of the collected leaves from the *Ligustrum* species for (+)-catechin in the DPPH method and Trolox in the TEAC assay are shown in Fig. 1(A) and (B). *L. morrisonense* had the highest scavenging capacity against both radicals compared with the other *Ligustrum* species collected in Taiwan. The Trolox equivalent of *L. morrisonense* in the TEAC assay was higher than that of *L. robustum*, which is used as a source material of Ku-Ding-Cha (Zhu et al., 2009).

On the basis of the reports that showed the antioxidant activity of plants being closely associated with their reducing power (Wu et al., 2007; Zhu et al., 2009), we further evaluated the reducing powers of the collected leaves from the *Ligustrum* species using the FRAP assay. FRAP is a simple and speedy method that actually measures the reducing capability of antioxidants and screens for the ability to maintain the redox status in cells (Huang, Ou, & Prior, 2005). Our present results confirmed that *L. morrisonense* indeed has the highest reducing power (Fig. 1(C)), which is consistent with the free radical-scavenging capacity observed in the DPPH and TEAC scavenging assays. Our data also supported another finding that demonstrated that the FRAP values were lower than the TEAC values when low pH values decreased the ionisation potential and

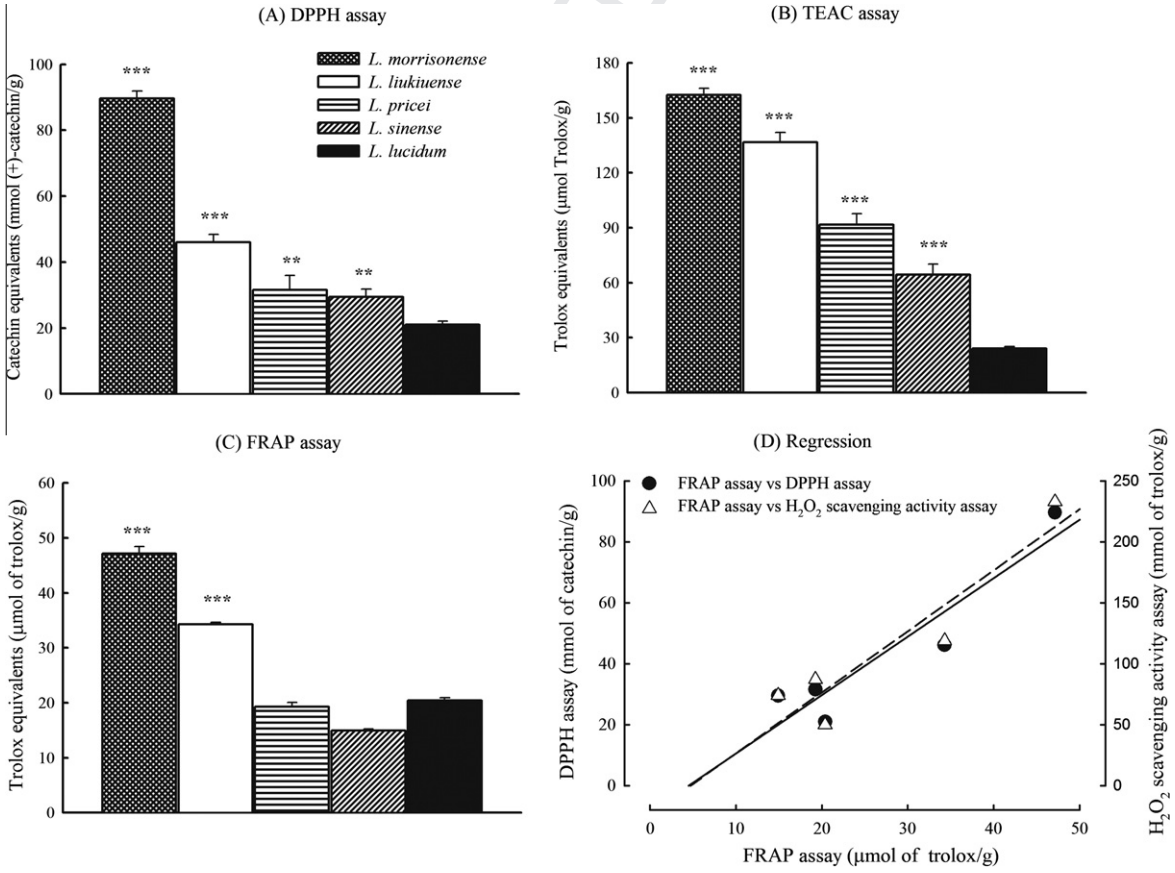

Fig. 1. Antioxidant activity of methanol extracts of *Ligustrum* species leaves on DPPH assay (A), TEAC assay (B), FRAP assay (C) and correlation between TEAC, total phenolic contents and reducing power (D). Data are expressed as mean ± SD ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$ compared with *L. lucidum*.

Please cite this article in press as: Wu, C.-R., et al. Evaluation of the antioxidant activity of five endemic *Ligustrum* species leaves from Taiwan flora *in vitro*. *Food Chemistry* (2011), doi:10.1016/j.foodchem.2011.01.041

increased the redox potential (Huang et al., 2005). This result further suggests that the antioxidant activities of the collected *Ligustrum* species leaves may be related to the modulation of the redox situation brought about by their reducing power.

3.2. ROS-scavenging activity of Ligustrum leaves

ROS, which mainly include the superoxide anion, hydrogen peroxide, and the hydroxyl radical, are major intracellular free radicals. The superoxide anion radical, a primary ROS, is produced within the mitochondrial of a cell and has been implicated in the pathophysiology of many diseases. Hydrogen peroxide, generated from the superoxide anion, readily crosses the cellular membranes and gives rise to the highly reactive hydroxyl radical by the Fenton reaction. The hydroxyl radical has the ability to react with biomolecules and ultimately damage cells. Thus, the above-mentioned ROS are largely involved in many serious diseases, including neurodegenerative and cardiovascular disorders (Valko et al., 2007). Because DPPH and ABTS do not exist *in vivo*, our present study further investigated the scavenging activities of the leaves against the superoxide anion, hydrogen peroxide, and hydroxyl radicals using the X/XO plus NBT spectrophotometric method, the HRPase plus HVA spectrofluorimetric method, and the 2-deoxyribose method. All sample solutions from the collected *Ligustrum* leaves also exhibited appreciable scavenging capacities against the superoxide anion and hydrogen peroxide. The IC_{50} values for *L. morrisonense*, *L. liukiuense*, *L. pricei*, *L. sinense*, and *L. lucidum* from the superoxide anion-scavenging assay were 95, 283, 252, 595, and 619 g of leaves/ml, respectively. The relative superoxide anion-scavenging potencies of these collected *Ligustrum* species for SOD are shown in Fig. 2(A). The highest superoxide anion-scavenging capacity was also seen in *L. morrisonense*, which was consistent with the free radical-scavenging capacity noted in the DPPH and TEAC assays. Furthermore, the highest H_2O_2-scavenging capacity was also observed in *L. morrisonense*. The IC_{50} values for *L. morrisonense*, *L. liukiuense*, *L. pricei*, *L. sinense*, and *L. lucidum* from the H_2O_2-scavenging assay were 0.42, 0.81, 1.11, 1.30, and 1.94 mg of leaves/ml, respectively. The relative H_2O_2-scavenging potencies of these collected *Ligustrum* species leaves for Trolox are shown in Fig. 2(B). The order of H_2O_2-scavenging activities for the leaves from the other collected *Ligustrum* species is as follows: *L. liukiuense* > *L. pricei* > *L. sinense* > *L. lucidum*. This study used the Fenton system (a mixture of ascorbic acid, hydrogen peroxide, and Fe^{2+}–EDTA) as a source of hydroxyl radicals to degrade its sugar target 2-deoxyribose. Among the other *Ligustrum* species used, *L. morrisonense* also had the highest second-order rate constants in scavenging the hydroxyl radicals (Fig. 2(C)). Since the reported correlations of some compounds with iron-chelating properties (Moran, Klucas, Grayer, Abian, & Becana, 1997; Rice-Evans, 1995; Wu et al., 2007), the iron-chelating activities of the *Ligustrum* species was further investigated. The leaves from the *Ligustrum* species showed a lack of iron-chelating activity (Fig. 2(D)). These results further revealed that all *Ligustrum* leaves collected from Taiwan possesses various degrees of scavenging activity against ROS and that *L. morrisonense* has the best ROS-scavenging activity among these *Ligustrum* species. Therefore, this antioxidant mechanism may not be related to iron chelation.

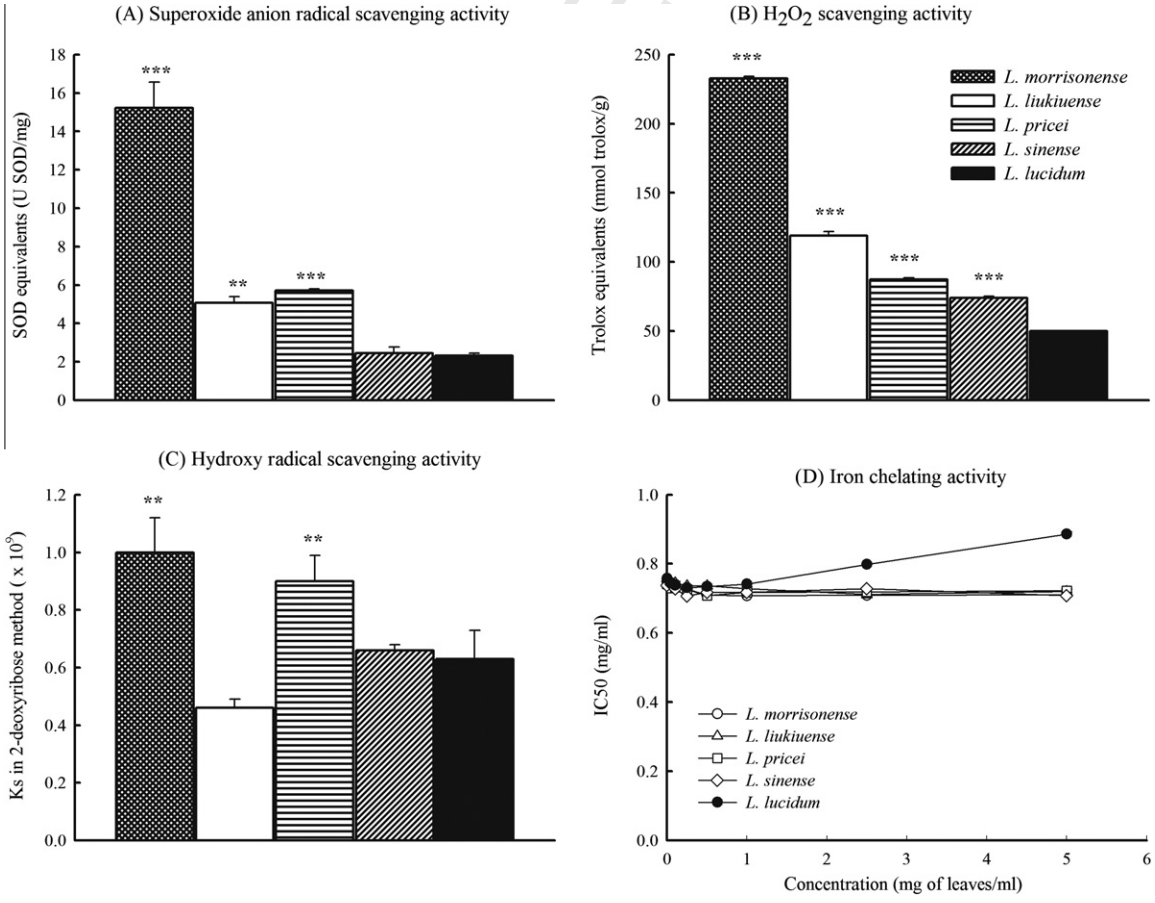

Fig. 2. IC_{50} values of scavenging superoxide anion (A), hydrogen peroxide (B), kinetic constant of scavenging hydroxyl radical (C) or iron chelating capacity (D) by methanol extracts of *Ligustrum* species leaves. Data are expressed as mean ± SD ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$ compared with *L. lucidum*.

Please cite this article in press as: Wu, C.-R., et al. Evaluation of the antioxidant activity of five endemic *Ligustrum* species leaves from Taiwan flora *in vitro*. *Food Chemistry* (2011), doi:10.1016/j.foodchem.2011.01.041

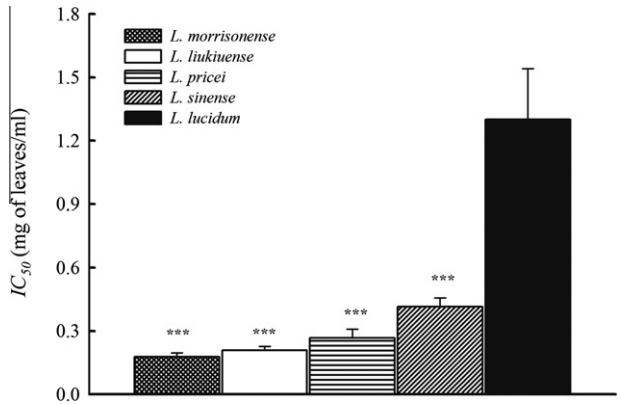

Fig. 3. IC_{50} values of lipid peroxidation inhibition on ferrous/ascorbate-treated rat brain homogenate by methanol extracts of *Ligustrum* species leaves. Data are expressed as mean ± SD ($n = 3$). $^{***}p < 0.001$ compared with *L. lucidum*.

3.3. Inhibition of lipid peroxidation by Ligustrum leaves

Hydroxyl radicals cause lipid peroxidation by attacking adjacent biomolecules, especially polyunsaturated fatty acids. Brain tissues – enriched polyunsaturated fatty acids in biological systems – easily carry out lipid peroxidation initiated by free radical chain reactions. Since the collected leaves from the *Ligustrum* species showed appreciable scavenging capacity against ROS, we further selected the rat brain homogenate as the oxidizable biomolecular target for the Fe^{2+}/ascorbate method to investigate the lipid peroxidation-inhibiting effects of the *Ligustrum* leaves. All samples showed a linear correlation between concentration and lipid peroxidation-inhibiting activity within the applied concentrations ($r^2 = 0.96–0.99$). Fig. 3 shows the IC_{50} values corresponding to the inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation. Again, the highest activity related to the inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation was also observed in *L. morrisonense*, followed by *L.pricei*, *L. sinense*, *L. liukiuense*, and *L. lucidum*. These results indicate that the collected *Ligustrum* leaves from Taiwan inhibit lipid peroxidation in brain homogenate systems by terminating the oxidative chain reaction through their radical-scavenging capacity.

3.4. Inhibition of erythrocyte haemolysis by Ligustrum leaves

The OxHLIA system is based on the property of erythrocytes that renders them susceptible to oxidative damage and utilises the biologically relevant radical source AAPH-derived peroxyl radicals to attack the erythrocyte membrane and cause erythrocyte haemolysis (Takebayashi et al., 2010). It is thus a good model for studying free radical-induced biomembranes and estimating "biologically relevant" antioxidant activity. The cell-based antioxidant bioassay method was used to compare the antioxidant activities of the collected *Ligustrum* leaves. The inhibition percentage of the leaves on AAPH-induced oxidative haemolysis was also dependent on concentration. All of the collected *Ligustrum* leaves were shown to protect erythrocytes from oxidatively-induced haemolysis in this order: *L. morrisonense* > *L. pricei* ≧ *L. sinense* ≧ *L. lucidum* > *L. liukiuense* (Fig. 4). From these cell-based data, it is suggested that the leaves from the *Ligustrum* species, especially *L. morrisonense*, may prevent ROS attack on biomolecules such as polyunsaturated fatty acids and lipoproteins in biological systems.

3.5. Quantification of phytochemical profiles in Ligustrum leaves

Phenolic compounds belong to the main class of natural antioxidants and have been shown to be closely correlated to the antioxidant activities of plants (Cai, Luo, Sun, & Corke, 2004; Huang et al., 2005; Wu et al., 2007). The most widespread and diverse phenolic compounds are the flavonoids (Hernandez, Alegre, Van Breusegem, & Munne-Bosch, 2009). Phenylpropanoids are the intermediate compounds in the biosynthesis of flavonoids and some phenolic compounds, and they also possess antioxidant activities (Korkina, 2007). Moreover, several phytochemical reports point to the antioxidant and anti-mutagenic activity of some phenylethanoids, flavonoids, and secoiridoids isolated from the genus *Ligustrum* (He et al., 2001, 2003; Nagy et al., 2006, 2009; Wong et al., 2001). Thus, the total phenolic, flavonoid, and phenylpropanoid

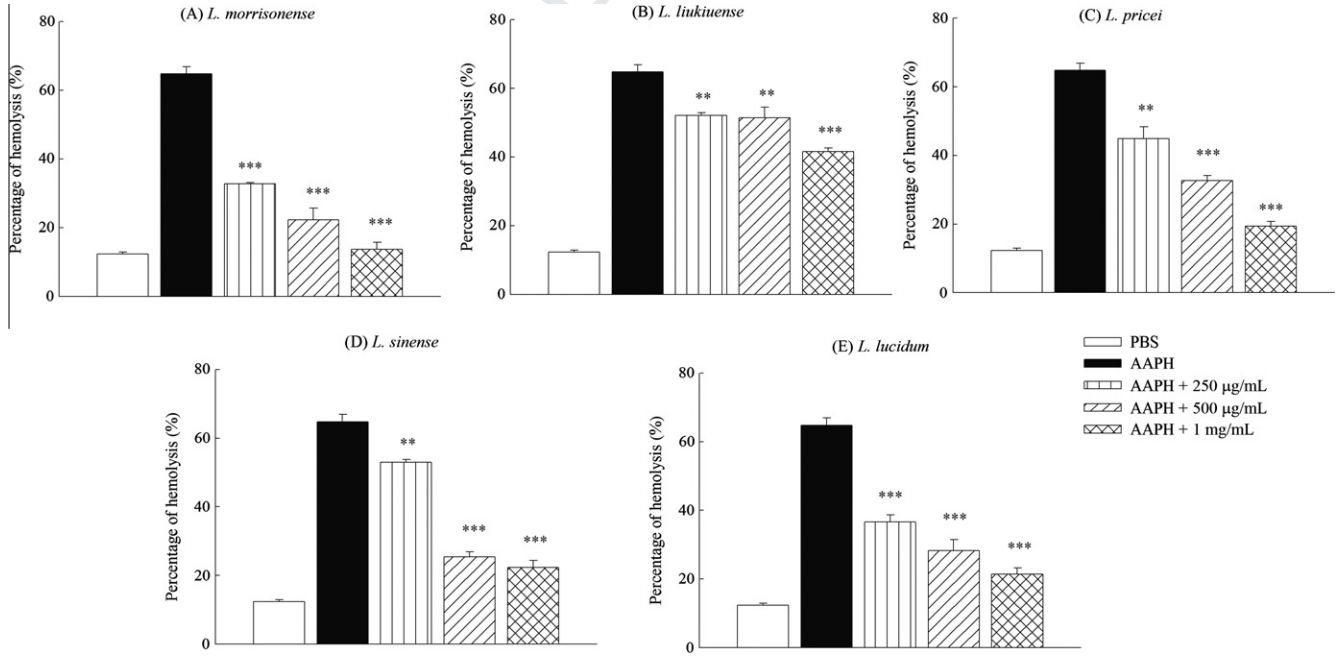

Fig. 4. Anti-haemolytic activity of methanol extracts of *Ligustrum* species leaves on AAPH-induced erythrocyte haemolysis *in vitro*. Data are expressed as mean ± SD ($n = 4$). $^{**}p < 0.01$, $^{***}p < 0.001$ compared with AAPH group.

Please cite this article in press as: Wu, C.-R., et al. Evaluation of the antioxidant activity of five endemic *Ligustrum* species leaves from Taiwan flora *in vitro*. *Food Chemistry* (2011), doi:10.1016/j.foodchem.2011.01.041

Table 1
The phytochemical composition of *Ligustrum* species leaves extracted with methanol.

Samples	Phenolic contents (mg of GAE/g)	Flavonoids contents (mg of quercetin/g)	Phenylpropanoids contents (mg of verbascoside/g)	Iridoids contents (mg of aucubin/g)
L. morrisonense	34.0 ± 1.22	7.45 ± 0.70	18.7 ± 0.61	1.63 ± 0.09
L. liukiuense	25.2 ± 0.43	5.70 ± 0.11	6.77 ± 0.94	0.42 ± 0.05
L. pricei	15.1 ± 0.06	0.43 ± 0.10	4.03 ± 0.12	1.01 ± 0.12
L. sinense	11.6 ± 0.32	3.16 ± 0.54	0.63 ± 0.14	5.17 ± 0.12
L. lucidum	8.04 ± 0.04	2.06 ± 0.11	3.90 ± 0.61	2.39 ± 0.28

Data were expressed as mean ± SD ($n = 3$).

Table 2
Pearson correlation coefficients (r) between parameters describing the amount of total phenolic (TP), flavonoids (TF), phenylpropanoids (TPP), and iridoids (TI) and different antioxidant activity of *Ligustrum* species leaves.

	DPPH	TEAC	FRAP	$O_2^{\cdot}$	H_2O_2	$OH^{\cdot}$	LPO	TP	TF	TPP
TEAC	0.87*									
FRAP	0.94**	0.86*								
$O_2^{\cdot}$	0.97**	0.81*	0.88*							
H_2O_2	0.999**	0.89*	0.93*	0.97**						
$OH^{\cdot}$	0.54	0.32	0.32	0.72	0.57					
LPO	0.83*	0.99**	0.80	0.79	0.85*	0.36				
TP	0.95**	0.97**	0.95**	0.81*	0.96**	0.36	0.94**			
TF	0.85*	0.77	0.89*	0.70	0.83*	0.05	0.68	0.86*		
TPP	0.96**	0.78	0.95**	0.97**	0.96**	0.57	0.73	0.90*	0.78	
TI	−0.31	−0.52	−0.53	−0.37	−0.33	−0.06	−0.53	−0.50	−0.18	−0.43

* $p < 0.05$.
** $p < 0.01$.

content in the aforementioned *Ligustrum* leaves was further measured. *L. morrisonense*, with the highest radical-scavenging capacity, also had the highest amount of phenolic, flavonoid, and phenylpropanoid compounds (Table 1). However, the highest amount of total iridoids was seen in *L. sinense*. The relationship between antioxidant potency parameters (DPPH, TEAC, FRAP, superoxide anion radical, H_2O_2, hydroxyl radical-scavenging activities, and LPO) and the amount of antioxidant components of the aforementioned *Ligustrum* leaves is shown in Table 2. These results indicate a significant positive correlation between various antioxidant potency parameters and antioxidant components, except for the hydroxyl radical-scavenging activity and total iridoids. All antioxidant assays (DPPH, TEAC, FRAP, superoxide anion radical, H_2O_2 and LPO) showed high correlation coefficients, suggesting the reliability and interchangeability between these spectrophotometer-based methods in predicting the antioxidant activities of natural plants. Among all phytochemical profiles, total phenylpropanoid content was positively and highly correlated with DPPH ($r = 0.96$), FRAP ($r = 0.95$), H_2O_2 ($r = 0.96$), and total phenolic content ($r = 0.90$) (Fig. 1(D) and Table 2). Total flavonoid content was also positively correlated with DPPH ($r = 0.85$), FRAP ($r = 0.89$), H_2O_2 ($r = 0.83$), and total phenolic content ($r = 0.86$) (Table 2). Some phenylpropanoid and flavonoid compounds such as acteoside, luteolin, quercetin and kaempferol glycosides isolated from *L. robustum*, *L. vulgare* and *L. purpurascens* are major active components involved in antioxidant activities (He et al., 2001, 2003; Nagy et al., 2006; Romani et al., 2000; Wong et al., 2001). Our own data and those of other reports on *Ligustrum* species indicate that phenolic compounds, especially phenylpropanoids and flavonoids but not iridoids, are major determinants of the antioxidant activities of the collected *Ligustrum* species, although some reports indicate that secoiridoids determine the antioxidant properties of Oleaceae spp. (Romani et al., 2000).

4. Conclusions

On the basis of the results obtained using *in vitro* antioxidant methods and cell-based antioxidant methods, the leaves from the *Ligustrum* species, especially *L. morrisonense*, *L. pricei*, and *L. sinense*, were found to be used as sources of natural antioxidants to prevent and/or cure oxidative stress-related diseases, exactly as these have been traditionally used for centuries. Their antioxidant components may be mainly phenylpropanoids and flavonoids but not iridoids. Further work can be carried out on the isolation, purification, and quantification of the active components from the leaves from these collected *Ligustrum* species.

Acknowledgements

We thank the financial support of the National Science Council (NSC97-2320-B-039-041 and NSC98-2320-B-039-041 MY3), the Chinese Medicine Committee (CCMP94-RD-013), and the China Medical University (CMU94-019 and CMU95-PH-02).

References

Arnow, L. E. (1937). Colorimetric determination of the components of 3,4-dihydroxyphenylalanin–tyrosine mixtures. *Journal of Biological Chemistry, 78*, 531–537.

Auddy, B., Ferreira, M., Blasina, F., Lafon, L., Arredondo, F., Dajas, F., et al. (2003). Screening of antioxidant activity of three Indian medicinal plants, traditionally used for the management of neurodegenerative diseases. *Journal of Ethnopharmacology, 84*, 131–138.

Butterfield, D. A., & Sultana, R. (2008). Redox proteomics: Understanding oxidative stress in the progression of age-related neurodegenerative disorders. *Expert Review of Proteomics, 5*, 157–160.

Cai, Y., Luo, Q., Sun, M., & Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences, 74*, 2157–2184.

Chen, Z. Y., Wong, I. Y., Leung, M. W., He, Z. D., & Huang, Y. (2002). Characterization of antioxidants present in bitter tea (*Ligustrum pedunculare*). *Journal of Agricultural and Food Chemistry, 50*, 7530–7535.

Halliwell, B., Gutteridge, J. M., & Aruoma, O. I. (1987). The deoxyribose method: A simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Analytical Biochemistry, 165*, 215–219.

He, Z. D., But, P. P. H., Chan, T. W., Dong, H., Xu, H. X., Lau, C. P., et al. (2001). Antioxidative glucosides from the fruits of *Ligustrum lucidum*. *Chemical and Pharmaceutical Bulletin, 49*, 780–784.

He, Z. D., Lau, K. M., But, P. P., Jiang, R. W., Dong, H., Ma, S. C., et al. (2003). Antioxidative glycosides from the leaves of *Ligustrum robustum*. *Journal of Natural Products, 66*, 851–854.

Please cite this article in press as: Wu, C.-R., et al. Evaluation of the antioxidant activity of five endemic *Ligustrum* species leaves from Taiwan flora *in vitro*. *Food Chemistry* (2011), doi:10.1016/j.foodchem.2011.01.041

Hernandez, I., Alegre, L., Van Breusegem, F., & Munne-Bosch, S. (2009). How relevant are flavonoids as antioxidants in plants? *Trends in Plant Science, 14*, 125–132.

Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*, 1841–1856.

Katsube, T., Tabata, H., Ohta, Y., Yamasaki, Y., Anuurad, E., Shiwaku, K., et al. (2004). Screening for antioxidant activity in edible plant products: Comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin–Ciocalteu assay. *Journal of Agricultural and Food Chemistry, 52*, 2391–2396.

Korkina, L. G. (2007). Phenylpropanoids as naturally occurring antioxidants: From plant defense to human health. *Cellular and Molecular Biology (Noisy-le-Grand, France), 53*, 15–25.

Lau, K. M., He, Z. D., Dong, H., Fung, K. P., & But, P. P. (2002). Anti-oxidative, anti-inflammatory and hepato-protective effects of *Ligustrum robustum*. *Journal of Ethnopharmacology, 83*, 63–71.

Leopold, J. A., & Loscalzo, J. (2009). Oxidative risk for atherothrombotic cardiovascular disease. *Free Radical Biology and Medicine, 47*, 1673–1706.

Lin, H. M., Yen, F. L., Ng, L. T., & Lin, C. C. (2007). Protective effects of *Ligustrum lucidum* fruit extract on acute butylated hydroxytoluene-induced oxidative stress in rats. *Journal of Ethnopharmacology, 111*, 129–136.

Moran, J. F., Klucas, R. V., Grayer, R. J., Abian, J., & Becana, M. (1997). Complexes of iron with phenolic compounds from soybean nodules and other legume tissues: Prooxidant and antioxidant properties. *Free Radical Biology and Medicine, 22*, 861–870.

Nagy, M., Krizkova, L., Mucaji, P., Kontsekova, Z., Sersen, F., & Krajcovic, J. (2009). Antimutagenic activity and radical scavenging activity of water infusions and phenolics from *Ligustrum* plants leaves. *Molecules, 14*, 509–518.

Nagy, M., Spilkova, J., Vrchovska, V., Kontsekova, Z., Sersen, F., Mucaji, P., et al. (2006). Free radical scavenging activity of different extracts and some constituents from the leaves of *Ligustrum vulgare* and *L. Delavayanum*. *Fitoterapia, 77*, 395–397.

Rice-Evans, C. (1995). Plant polyphenols: Free radical scavengers or chain-breaking antioxidants? *Biochemical Society Symposium, 61*, 103–116.

Romani, A., Pinelli, P., Mulinacci, N., Vincieri, F. F., Gravano, E., & Tattini, M. (2000). HPLC analysis of flavonoids and secoiridoids in leaves of *Ligustrum vulgare* L. (Oleaceae). *Journal of Agricultural and Food Chemistry, 48*, 4091–4096.

She, G. M., Wang, D., Zeng, S. F., Yang, C. R., & Zhang, Y. J. (2008). New phenylethanoid glycosides and sugar esters from ku-ding-cha, a herbal tea produced from *Ligustrum purpurascens*. *Journal of Food Science, 73*, C476–C481.

Sung, S. H., Kim, E. S., Lee, K. Y., Lee, M. K., & Kim, Y. C. (2006). A new neuroprotective compound of *Ligustrum japonicum* leaves. *Planta Medica, 72*, 62–64.

Takebayashi, J., Chen, J., & Tai, A. (2010). A method for evaluation of antioxidant activity based on inhibition of free radical-induced erythrocyte hemolysis. *Methods in Molecular Biology, 594*, 287–296.

Tepe, B. (2008). Antioxidant potentials and rosmarinic acid levels of the methanolic extracts of *Salvia virgata* (Jacq), *Salvia staminea* (Montbret & Aucher ex Bentham) and *Salvia verbenaca* (L.) from Turkey. *Bioresource Technology, 99*, 1584–1588.

Tepe, B., & Sokmen, A. (2007). Screening of the antioxidative properties and total phenolic contents of three endemic *Tanacetum* subspecies from Turkish flora. *Bioresource Technology, 98*, 3076–3079.

Trim, A. R., & Hill, R. (1952). The preparation and properties of aucubin, asperuloside and some related glycosides. *The Biochemical Journal, 50*, 310–319.

Uttara, B., Singh, A. V., Zamboni, P., & Mahajan, R. T. (2009). Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacology, 7*, 65–74.

Valentão, P., Fernandes, E., Carvalho, F., Andrade, P. B., Seabra, R. M., & de Lourdes Bastos, M. (2002). Studies on the antioxidant activity of *Lippia citriodora* infusion: Scavenging effect on superoxide radical, hydroxyl radical and hypochlorous acid. *Biological and Pharmaceutical Bulletin, 25*, 1324–1327.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry and Cell Biology, 39*, 44–84.

Wong, I. Y., He, Z. D., Huang, Y., & Chen, Z. Y. (2001). Antioxidative activities of phenylethanoid glycosides from *Ligustrum purpurascens*. *Journal of Agricultural and Food Chemistry, 49*, 3113–3119.

Wu, C. R., Huang, M. Y., Lin, Y. T., Ju, H. Y., & Ching, H. (2007). Antioxidant properties of Cortex Fraxini and its simple coumarins. *Food Chemistry, 104*, 1464–1471.

Yang, Y. P., & Lu, S. Y. (2000). Oleaceae. In T. C. Huang (Ed.). *Flora of Taiwan* (Vol. 4, pp. 137–140). Taipei, Taiwan: Editorial Committee of the Flora of Taiwan, Department of Botany, National Taiwan University.

Zhu, F., Cai, Y. Z., Sun, M., Ke, J., Lu, D., & Corke, H. (2009). Comparison of major phenolic constituents and *in vitro* antioxidant activity of diverse Kudingcha genotypes from *Ilex kudingcha*, *Ilex cornuta*, and *Ligustrum robustum*. *Journal of Agricultural and Food Chemistry, 57*, 6082–6089.

Please cite this article in press as: Wu, C.-R., et al. Evaluation of the antioxidant activity of five endemic *Ligustrum* species leaves from Taiwan flora *in vitro*. *Food Chemistry* (2011), doi:10.1016/j.foodchem.2011.01.041