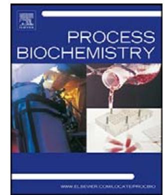
Process Biochemistry 45 (2010) 1865–1872

Contents lists available at ScienceDirect

Process Biochemistry

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



Identification of anti-lung cancer extract from *Chlorella vulgaris* C-C by antioxidant property using supercritical carbon dioxide extraction

Hui-Min Wang[a], Jian-Liang Pan[b], Chun-Yen Chen[c,d], Chien-Chich Chiu[e], Mu-Hoe Yang[b], Hsueh-Wei Chang[f], Jo-Shu Chang[c,d,g,*]

[a] Department of Fragrance and Cosmetic Science, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC
[b] Department of Chemical and Biochemical Engineering, Kao Yuan University, Kaohsiung 821, Taiwan, ROC
[c] Department of Chemical Engineering, National Cheng Kung University, Tainan 701, Taiwan, ROC
[d] Sustainable Environment Research Center, National Cheng Kung University, Tainan 701, Taiwan, ROC
[e] Department of Biotechnology, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC
[f] Department of Biomedical Science and Environmental Biology, Cancer Center, Kaohsiung Medical University Hospital, Graduate Institute of Natural Products, College of Pharmacy, Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC
[g] Center for Bioscience and Biotechnology, National Cheng Kung University, Tainan 701, Taiwan, ROC

ARTICLE INFO

Article history:
Received 26 March 2010
Received in revised form 29 April 2010
Accepted 19 May 2010

Keywords:
Chlorella vulgaris
Supercritical carbon dioxide extraction
Antioxidant
Lung cancer

ABSTRACT

In this study, supercritical carbon dioxide extraction (SC-CO_2) technology was developed to extract the active components (such as antioxidants) from a novel microalga, *Chlorella vulgaris* C-C, due to its superior advantages over conventional solvent or ultrasonic extraction methods. Using SC-CO_2 and ultrasonic extractions, the polyphenol contents of *C. vulgaris* C-C were 13.40 and 0.46 ($mg_{gallic\ acid}$/g lyophilized extract), respectively. The flavonoid content obtained from SC-CO_2 (3.18 $mg_{quercetin}$/g lyophilized extract) was also significantly higher than from ultrasonic extraction (0.86 $mg_{quercetin}$/g lyophilized extract). Investigation of *C. vulgaris* C-C extract from SC-CO_2 indicates strong antioxidant activities in radical scavenging, ferric reducing power and metal chelating abilities. In cell proliferation assay, the extract of *C. vulgaris* C-C inhibits human lung cancer H1299, A549, and H1437 cells in a dose-dependent manner. In addition, the treatment with extracts of *C. vulgaris* C-C effectively reduced the migration of tumor cells, suggesting the potential of using the *C. vulgaris* C-C extract to inhibit lung cancer metastasis.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

The free radicals and reactive oxygen species (ROS) generated by microsomal metabolism may augment an oxidative stress by the formation of hydrogen peroxide or superoxide anions in human beings [1]. Accumulating proofs indicate that free radicals or active oxygen species would assault on important biological molecules such as DNA, protein, or lipid leading to many degenerative disease conditions, such as cancer, gastric ulcers, Alzheimer's, arthritis and ischemic reperfusion [2]. For example, ROS make both nuclear DNA and mitochondrial DNA susceptible to damage and mutagenesis, and mutations in these two DNA pools were reported to lead to oxidative stress and both aging and carcinogenesis. Indeed, increased mutations in DNA have been observed in cancer cells of various tissue origins [3]. Fortunately, human normal cells possess antioxidant system, viz. enzyme-mediated system, such as superoxide dismutase, catalase, glutathione peroxidase, and non-enzymatic factors, glutathione, ascorbic acid (Vitamin C), a-tocopherol (Vitamin E) to protect them against the oxidative stress and consecutive lipid peroxidation.

The importance of diet in the prevention of some diseases is well recognized [4]. Natural antioxidant constituents are very significant in cosmetics or foods businesses because of the capacities to decrease free radical mediated degradations of cells and tissues in organisms of human [5–7]. Therefore, attention has shifted to nutritious or non-nutritive phytochemicals present in natural plant-based diet as potential chemopreventive agents. It is now estimated that more than 1000 different phytochemicals possess chemopreventive activities [8].

Traditionally, natural matrices have been obtained by means of conventional extraction with organic solvents. Nevertheless, possible changes in the physicochemical properties of extracts could alter their functionalities. Thus, these extraction processes should be performed at suitable and mild conditions [9]. Supercritical carbon dioxide extraction (SC-CO_2) is an advanced technology that has many advantages, including low environmental impacts due to no residue of harmful solvents, non-toxicity, non-corrosive, and easy separation from extracts [10,11]. Thus, they have been widely used

* Corresponding author at: Department of Chemical Engineering, National Cheng Kung University, No. 1 University Road, Tainan 701, Taiwan, ROC.
Tel.: +886 6 2757575x62651; fax: +886 6 2357146.
E-mail address: changjs@mail.ncku.edu.tw (J.-S. Chang).

1359-5113/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2010.05.023

in pharmaceutical and food industries within modern times [12].

In recent years, natural antioxidant activities of plants with the ingestion of fresh fruits, vegetables or teas that are rich in natural antioxidants have been claimed to have beneficial health functions for retarding aging and preventing cardiovascular, inflammatory, neurological diseases, as well as cancers [13]. In addition, more attention has been paid on natural antioxidative materials from marine resources (such as microalgae), acting as new and promising antioxidative alternatives [14]. *Chlorella vulgaris* is a unicellular green microalga that has been widely used for centuries as a food source with complete nutrients, such as carbohydrate, protein, vitamins and minerals [15]. *C. vulgaris* is marketed commercially as health supplement or incorporated in food such as oat or cereal [16]. Rodriguez-Garcia and Guil-Guerrero [17] applied ethanol to extract *C. vulgaris*, *Porphyridium cruentum* and *Phaeodactylum tricornutum*, and discovered that the extract from *C. vulgaris* contains the highest antioxidant activities. In animal studies, extracts from *C. vulgaris* displayed anti-atherogenic, anticholesterolemic, anti-inflammatory, and anti-tumor effects [18]. The *C. vulgaris* extract was also found to induce apoptosis and oxidative damage in HepG2 cells [19]. Therefore, microalgal species, such as *C. vulgaris*, could have the potential for the development of antioxidant and anti-cancer products.

In the present study, we attempted to assess the antioxidant and anti-cancer activity of our newly isolated indigenous *C. vulgaris* strain (designated as *C. vulgaris* C-C). This study examined the radical scavenging and antioxidant activities of *C. vulgaris* C-C extracts obtained from using SC-CO_2 or ultrasonic extraction. Different non-enzymatic antioxidant assay methodologies were used to determine the antioxidant activity of *C. vulgaris* extracts, including 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging, superoxide anion radical-generated scavenging, ferric thiocyanate assay and metal chelating activities. The inhibition effect of *C. vulgaris* C-C extract from SC-CO_2 on human lung cancer cells was also determined to evaluate its potential in producing chemopreventive agent for food or in pharmaceutical applications.

2. Materials and methods

2.1. Reagents and materials

Vitamin C, dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethylene diamine tetra-acetic acid (EDTA), 2,6-di-tert-butyl-4-methylphenol (BHT), nitro bluetetrazolium (NBT), 2,4,6-tripyridyl-S-triazine (TPTZ), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), potassium ferricyanide ($K_3Fe(CN)_6$), trichloroacetic acid, horseradish peroxidase (HRPase), $FeCl_3$ and $FeCl_2{\cdot}4H_2O$ were purchased from Sigma Chemical (St. Louis, MO). Fetal bovine serum (FBS) was obtained from GIBCO BRL (Gaithersburg, MD). Trypan blue dye, Dulbecco's modified Eagle's medium (DMEM) and nutrient mixture F-12 were acquired from Invitrogen (Technologies, Carlsbad, CA). All buffers and other reagents were of the highest purity commercially available.

2.2. Microalgae culture and medium composition

The microalgal species used in this work was isolated from fresh water located in southern Taiwan. This microalga isolate was identified as *C. vulgaris* C-C by its morphology as well as by 18S rDNA and 23S rDNA sequence matching. The medium used to cultivate the pure culture of *C. vulgaris* C-C consisted of (g/l): $NaHCO_3$, 1.5; KNO_3, 0.313; KH_2PO_4, 1.25; $MgSO_4{\cdot}7H_2O$, 1.0; $CaCl_2$, 0.0835; $FeSO_4{\cdot}7H_2O$, 0.0498; EDTA·2Na, 0.5; H_3BO_3, 0.1142; $ZnSO_4{\cdot}7H_2O$, 0.0882; $MnCl_2{\cdot}4H_2O$, 0.0882; $Na_2MoO_4{\cdot}2H_2O$, 0.01; $CuSO_4{\cdot}5H_2O$, 0.0157; $Co(NO_3)_2{\cdot}6H_2O$, 0.0049. *C. vulgaris* C-C was grown at 28 °C for 72 h under a light intensity of approximately 9.0 W/m^2 (illuminated by TL5).

2.3. Operation of photobioreactor

The microalga was cultivated in a photobioreactor (PBR), which was a 20-l glass-made vessel illuminated with six external light source (14 Watt TL5 tungsten filament lamps; Philips Co., Taipei, Taiwan) mounted on both sides of the reactor. The light intensity on the vessel wall of PBR was adjusted to ca. 9.0 W/m^2. The precultured *C. vulgaris* C-C was inoculated into the PBR with an inoculum size of 30 mg/l. The PBR was operated under 28 °C and pH 6.0. Sodium bicarbonate ($NaHCO_3$) was used as the sole carbon source. The liquid sample was also collected from the sealed glass vessel with respect to time to determine final microalgae cell concentration, overall biomass production rate and residual $NaHCO_3$ concentration.

2.4. Determination of microalgae cell concentration

The cell concentration of the culture was determined regularly by optical density measurement at a wavelength of 685 nm (i.e., OD_{685}) using a spectrophotometer (model U-2001, Hitachi, Tokyo, Japan) after proper dilution with deionized water. The dry cell weight of microalgae biomass was obtained by filtering 10 ml aliquots of culture through a cellulose acetate membrane filter (0.45 μm pore size, 47 mm in diameter). Each loaded filter was dried at 105 °C until the weight was invariant. The dry weight of blank filter was subtracted from that of the loaded filter to obtain the microalgae dry cell weight (DCW). The OD_{685} values were converted to biomass concentration via proper calibration between OD_{685} and dry cell weight (i.e., 1.0 OD_{685} approximately equals 350 mg DCW/l).

2.5. Measurement of light intensity

The light intensity on the reactor wall was measured with a LI-250 Light Meter with a LI-200SA pyranometer sensor (LI-COR, Inc., Lincoln, Nebraska, USA). This light meter gives a unit of W/m^2 for the measured light intensity.

2.6. Estimation of residual $NaHCO_3$ concentration

The residual $NaHCO_3$ concentration in the culture medium was measured by Liqui TOC apparatus (Elementar Analysensysteme GmbH Company, Germany) equipped with an infrared detector. Samples were injected into a 1-m-long quartz column. High-purity oxygen gas was used as the carrier gas with a flow rate of 200 ml/min and a controlled pressure of 0.95–1.00 bar. The temperature of injector was initially set at 35 °C, increased from 35 to 800 °C, and was then held at 800 °C. Liquid samples were centrifuged (6000 × *g* for 20 min) and filtered (0.22 μm membrane) prior to being injected into TOC for analysis.

2.7. Lyophilization procedures

The freeze-drying process was performed in a laboratory-scale lyophilizer FDU-2100 (EYELA Company, Japan). The chamber pressure, temperature and operation time of the lyophilizer were 1.33 Pa, −83 °C, and ca. 48 h, respectively, throughout the whole lyophilization cycle. The dried products were collected and stored in screw-capped glass bottles.

2.8. Supercritical fluid equipment

The supercritical CO_2 fluid extraction was performed on Jeoou-Rong supercritical fluid extractor (Jeoou-Rong Co., Ltd., Taiwan) with the extractor volume of 0.3 l. Schematic description of supercritical CO_2 fluid extraction equipment is given in Fig. 1. The liquid CO_2 from a cylinder was pressured to reach the supercritical state with a piston pump and cooling at −4 °C in a water bath before it passed into the extraction vessel (with 300 ml inner extraction container). The extraction pressure was adjusted by needle and micrometric valves. The extraction temperature was controlled by a thermostatic batch. The flow rate of CO_2 was regulated by a rotameter. The consumption volume of CO_2 was recorded by a gas meter.

After the temperature of the extraction vessel reached the set point, the micrometric valves were closed. Then, the microalgae (0.3 g) was loaded into a 300 ml stainless steel extraction container (59.0 mm ID) with stainless steel filters placed at both ends to prevent carry-over of the particles. The container was placed into the extraction vessel. Afterward, 50 ml of 50% aqueous ethanol as co-solvent was added in the extractor. The pressure of extraction vessel was controlled at 4500 psi by a needle valve and set a 20 min of static extraction time with a timer. The solute-rich fluid departing from the extractor was expanded through micrometric valves to atmospheric pressure. The flow rate was set 6 NL/min and the total volume of gas was measured with a gas meter. These extracts were analyzed on total antioxidant abilities and anti-cancer bioassays. The CO_2 discharge was recycled to cultivate microalgae to reduce CO_2 emission to the environment.

2.9. Ultrasonic extraction

A sample of the freeze-dried microalgal biomass (0.5 g) was suspended in 50% aqueous ethanol (60 ml). The sample was extracted under 15 h sonication in an ultrasonic batch (model S-300H, Elmasonic S, GmbH, Germany) at 10 °C. The supernatant liquid was recovered by centrifugation and stored at −21 °C prior to analysis.

2.10. Determination of total polyphenol contents

Total phenolic contents of testing samples were determined by modified Folin–Ciocalteu method [20]. Aliquots (1.0 ml) of the extract sample were transferred into the tubes and diluted with 4 ml of 95% ethanol, and then centrifuged at 1000 × *g* and 4 °C for 5 min. One ml of supernatant solution was mixed with 1 ml of 95% ethanol, 5 ml of distilled water 1 ml of 5% sodium carbonate (Na_2CO_3) and

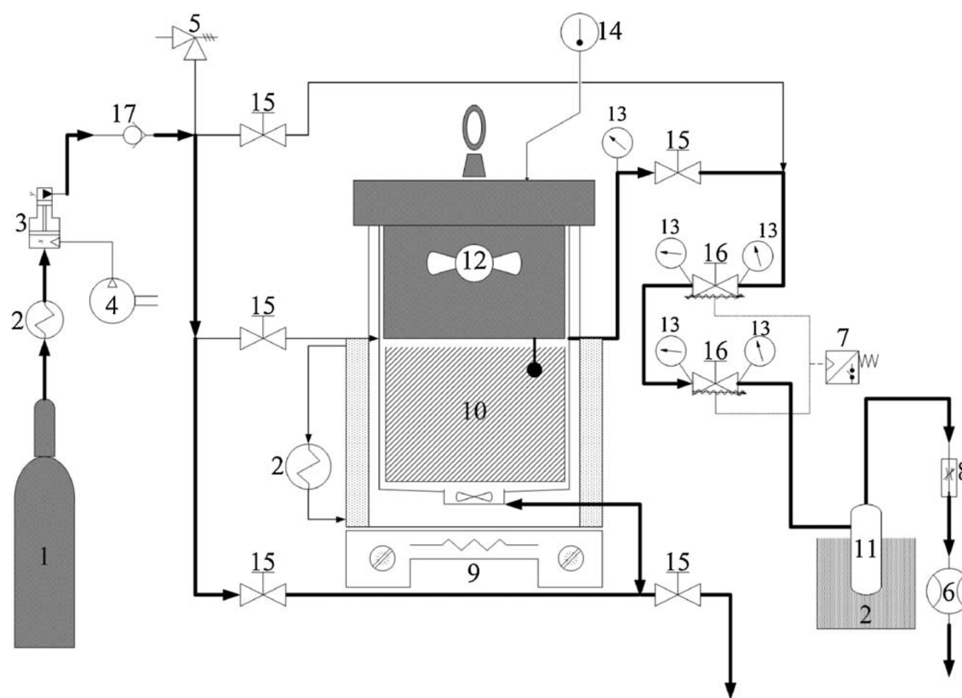


Fig. 1. Schematic diagram of the flow apparatus for supercritical batch extraction: 1, gas cylinder; 2, thermostatic bath; 3, pump; 4, air pump; 5, safety valve; 6, gas meter; 7, temperature controller; 8, rotameter; 9, stirrer; 10, extraction vessel; 11, collection vessel; 12, steel bolt; 13, manometer; 14, thermal couple; 15, needle valve; 16, micrometric valve; 17, check valve.

0.5 ml of 50% Folin–Ciocalteu reagent. After incubation at 35 °C thermostatic batch for 60 min in the dark, the absorbance of the reaction mixture was measured at 725 nm against a blank containing 4 ml of distilled water, 1 ml of 95% ethanol and 1 ml of 5% sodium carbonate on a spectrophotometer (model U-2001, Hitachi, Tokyo, Japan). Gallic acid was chosen as a comparative standard. The amount of polyphenols was expressed as milligram gallic acid equivalents/g lyophilized extract.

2.11. Determination of total flavonoid contents

Total flavonoid contents of testing samples were determined by modified the aluminum chloride colorimetric method [21]. Aliquots (1.0 ml) of the extract sample were transferred into the test tubes and diluted with 4 ml of 95% ethanol, and then centrifuged at $1000 \times g$ and 4 °C for 5 min. One ml of supernatant solution was mixed with 0.5 ml of 10% aluminum chloride hexahydrate ($AlCl_3{\cdot}6H_2O$), 0.5 ml of 1 M sodium acetate (CH_3COONa) and 2.0 ml of deionized water. After incubation at 35 °C thermostatic batch for 40 min in the dark, the absorbance of the reaction mixture was measured at 415 nm against a blank containing 2.0 ml of distilled water, 2.0 ml of 95% ethanol, 0.5 ml of 10% aluminum chloride hexahydrate and 0.5 ml of 1 M sodium acetate on a spectrophotometer (Hitachi, Model U-2001). Quercetin was chosen as a comparative standard. The data were expressed as milligram quercetin equivalents/g lyophilized extract.

2.12. Determination of DPPH radical scavenging capacity

Most cosmetics and food compounds have free radical scavenging ability. The antioxidant activity of testing compound was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH method as modified by Wang et al. [22]. Proper concentrations of the samples were added to 0.2 ml of DPPH (60 μM) solution. When DPPH reacts with an antioxidant compound that donates hydrogen, it is reduced, resulting in a decrease in the absorbance at 520 nm. The absorbance was recorded at 30 min using a UV–vis spectrophotometer. Vitamin C was used as a positive control. The percentages of remaining DPPH were plotted against the sample to obtain the amount of antioxidant required to reduce the initial concentration of DPPH. Scavenging activity (%) was determined as

$$\frac{100 \times (\mathrm{OD_{control}} - \mathrm{OD_{sample}})}{\mathrm{OD_{control}}} \quad (1)$$

2.13. Metal chelating activity

The ferrous ion-chelating potential of chlorophyll was investigated according to the method described by Decker and Welch [23]. Briefly, testing samples at suitable concentrations dissolved in DMSO were added to a solution of 2.0 mM $FeCl_2{\cdot}4H_2O$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixture was vigorously shaken and left standing at room temperature for 10 min. After the mixture reached equilibrium, the absorbance of the mixture was measured at 560 nm against a blank. EDTA was used as a positive control and chelating activity calculation formula was similar to Eq. (1).

2.14. Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power was determined using a modification of the FRAP assay described by Benzie and Strain [24]. The FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 20 mM ferric chloride and 10 mM TPTZ in 40 mM hydrochloric acid. All three solutions were mixed together in the ratio of 10:1:1 (v/v/v). The FRAP assay was performed using reagents preheated to 37 °C. Aliquots (1.0 ml) of the extract samples were transferred into the tubes and diluted with 4 ml of 95% ethanol, and then centrifuged at $1000 \times g$ and 4 °C for 5 min. 0.1 ml of supernatant solution was mixed with 3.0 ml of FRAP reagent and 0.3 ml of deionized water. After incubation at 37 °C thermostatic batch for 30 min in the dark, the absorbance of the reaction mixture was measured at 593 nm against a blank containing 3.0 ml of FRAP reagent and 0.4 ml of deionized water on a spectrophotometer (Hitachi, Model U-2001). Ferrous sulfate heptahydrate solution was used to perform the calibration curves. The data were expressed as milligram ferrous sulfate heptahydrate.

2.15. Superoxide anion radical scavenging capacity

The superoxide anion radical scavenging capacity was determined according to a modified protocol developed by Re et al. [25]. The PMS (120 μM), NADH (936 μM) and NBT (300 μM) solutions were prepared in 0.1 M sodium phosphate buffer (pH 7.4) and kept on ice for duration of the experiments. A 1.0 ml aliquot of the extract sample was pipetted into a test tube and diluted with 4.0 ml of 95% ethanol, and then centrifuged at $1000 \times g$ and 4 °C for 5 min. 1.0 ml of supernatant solution was mixed with solutions, followed by the addition of 1.0 ml of PMS, NADH and NBT, respectively. After incubation at 37 °C thermostatic batch for 5 min in the dark, the absorbance of the reaction mixture was measured at 560 nm against a blank on a spectrophotometer (Hitachi, Model U-2001). The superoxide anion radical scavenging activity calculation formula was similar to Eq. (1).

2.16. Cell culture

Three human NSCLC cell lines, H1299, A549, and H1437 were used as our model cells. All tested cells were maintained in a 1:1 ratio of DMEM/F12 supplemented with 8% FBS, 2.0 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2.

Table 1
The extraction yield, and polyphenol and flavonoid contents from SC-CO_2 and ultrasonic extraction of *Chlorella vulgaris* C-C.

	Extraction yield (dry extracts) (mg/g[a])	Total polyphenol contents ($mg_{gallic\ acid}$/g[a])	Total flavonoid contents ($mg_{quercetin}$/g[a])
SC-CO_2 extraction	87.05 ± 0.20[b]	13.40 ± 0.11[b]	3.18 ± 0.03[b]
Ultrasonic extraction	97.27 ± 0.21[b]	0.46 ± 0.00[b]	0.86 ± 0.01[b]

[a] 1.0 g lyophilized extract.
[b] Data were expressed as a mean value of three independent experiments.

2.17. Cell proliferation and cell viability assay

The cell proliferation rate and cell viability of cells was determined by Trypan blue dye exclusion assay combined with an automated cell counter performed according to the manufacturer's instruction (Invitrogen). Briefly, 1×10^5 cells were seeded and treated with vehicle or 200, 100, and 20 μg/ml dilutions of extracts in medium for 24 and 48 h, respectively. After incubation, cells exposed to 0.2% Trypan blue were counted in the Countess™ automated Cell Counter (Invitrogen).

2.18. Wound-healing assay

The potential of cellular migration was determined by wound-healing migration assays, which was performed according to the methods reported by Geback et al. [26]. In brief, 5×10^5 cells were treated with testing samples or PBS to seed in 12-well plates, and grown to complete confluence, respectively. A yellow 200 μl plastic pipette tip was used to create a clean 1-mm-wide wound area on a confluent culture of tested cells. After the indicated incubation time (i.e., 24 and 48 h), the wound gaps were photographed using an inverted phase-contrast microscopy (TE2000-U; Nikon, Tokyo, Japan) equipped with NIS-Elements (Nikon) Software. The migration and cell movement throughout the wound area were examined and calculated by the free software "TScratch" (www.cse-lab.ethz.ch/software.html). Magnification: 100×. Bars, S.D.

2.19. Analysis of plasmid DNA strand breaks

DNA breaks were measured by the conversion of supercoiled (S) double-stranded (ds) DNA to opened circular (OC) and linear (L) dsDNA. In 10 μl reaction mixture (distilled water), 150 ng plasmid pBR322 DNA was incubated in the presence of 0.05% H_2O_2 and 0.1 mM FeSO4 at 37 °C for 30 min with 0.15, 0.075, 0.038, 0.019, 0.010 mg/μl of extracts. The reaction was stopped by the addition of 2 μl of 6× gel loading dye. The samples were resolved on 1% agarose gel electrophoresis (1× TAE buffer) at 50 V for 1–2 h. Gel was stained with ethidium bromide. All studies were repeated three times under identical conditions.

2.20. Statistical analysis

All data were presented as mean ± S.D. and the Student's *t*-test was used to identify the mean difference between the two groups.

3. Results

3.1. Microalgal biomass production using sodium bicarbonate as substrate

The pure strain of *C. vulgaris* C-C was cultivated in a 20-l batch photobioreactor illuminated with TL5 filament lamps at a light intensity of ca. 9.0 W/m^2 using $NaHCO_3$ as the sole carbon source. $NaHCO_3$ is a product resulting from CO_2 capture with alkali method and was found to be a suitable carbon source for the growth of the *C. vulgaris* C-C strain in our preliminary tests. The time-course growth curve results show that the cumulative biomass production and overall biomass production rate of *C. vulgaris* C-C reached 630 mg/l and 78.8 mg/l/d, respectively (Fig. 2). Also, the pH value increased along with the cell growth due to the consumption of $NaHCO_3$ (Fig. 2). Moreover, a high sodium bicarbonate conversion of 98% was also observed, indicating that sodium bicarbonate was a suitable substrate for microalgal biomass production. After cell growth reached its stationary phase, the microalgal biomass was harvested and lyophilized according to the procedures mentioned earlier and the dry microalgae powder was then used for extraction with SC-CO_2 or ultrasonic methods.

3.2. Microalgal biomass extraction using SC-CO_2 and ultrasonication

Table 1 shows the amounts of polyphenol and flavonoid obtained from SC-CO_2 or ultrasonic extraction with 50% aqueous ethanol. After 0.3 g dry microalgae powder was extracted using SC-CO_2 extraction at 4500 psi, 50 °C, 6 NL/min with 50 ml of 50% aqueous ethanol modifiers, a yield of 87.05 mg/g lyophilized extract was obtained. The polyphenols and flavonoids contents were 13.40 $m_{gallic\ acid}$/g lyophilized extract and 3.18 $mg_{quercetin}$/g lyophilized extract, respectively. On the other hand, when 0.5 g microalgae was extracted from ultrasonic extraction at 10 °C, 60 ml of 50% aqueous ethanol, the contents of polyphenol and flavonoid were 0.46 $m_{gallic\ acid}$/g lyophilized extract and 0.86 $mg_{quercetin}$/g lyophilized extract, respectively. Obviously, SC-CO_2 extraction gave better performance in obtaining polyphenol and flavonoid contents. Even both extraction strategies gave similar production yields, SC-CO_2 extraction appeared to display much higher efficiency in extracting the functional components (i.e., polyphenol and flavonoid) attaining 30- and 4-fold higher polyphenol and flavonoid contents, respectively, than that obtained by using ultrasonic extraction.

3.3. DPPH free radical scavenging activity

DPPH free radical scavenge testing system is an acknowledged mechanism by which antioxidants act to inhibit oxidation products. Hence, this DPPH assay has been widely applied as one of the indicators for antioxidant activity. To investigate the antioxidant activity of *C. vulgaris* C-C extracts, a dosage of 0.83 mg/ml was used to determine the free radical scavenging activity. In this DPPH assay, antioxidants were able to decrease the stable radical DPPH to the

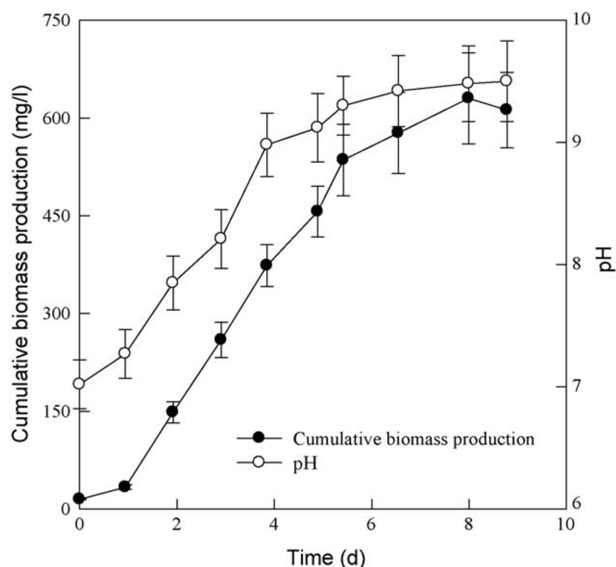


Fig. 2. Time course of cumulative biomass production and pH value by using $NaHCO_3$ as the carbon source.

Table 2
The antioxidant activities of *C. vulgaris* C-C extracts obtained from SC-CO_2 and ultrasonic extractions with a dosage of 0.83 mg/ml.

	DPPH (%)	Metal chelating (%)	FRAP (mg_{FeSO_4})	Superoxide anion radical (%)
SC-CO_2 extraction extract	47.24 ± 0.44[a]	91.38 ± 1.38[a]	12.14 ± 0.07[a]	18.08 ± 0.11[a]
Ultrasonic extraction extract	0.74 ± 0.01[a]	46.69 ± 0.52[a]	5.23 ± 0.26[a]	nd[b]
Gallic acid	100 ± 0.00[a]	nt[c]	10.71 ± 0.44[a]	100 ± 0.00[a]
EDTA	nt[c]	100 ± 0.00[a]	nt[c]	nt[c]

[a] Data were expressed as a mean value of three independent experiments.
[b] nd: not detectable.
[c] nt: not tested.

light yellow colored diphenyl-picrylhydrazine. The inhibition values of *C. vulgaris* C-C extracts at 0.83 mg/ml of DPPH are listed in Table 2. The gallic acid at 0.83 mg/ml as same as *C. vulgaris* C-C extracts was included as positive control for DPPH assay. It shows that SC-CO_2 extraction had much higher inhibition value (47.24%) in DPPH assay than that of ultrasonic extraction (0.74%), but lower than positive control (100%).

3.4. Ferrous ions chelating capacity

The ferrous ion-chelating activities of *C. vulgaris* C-C extracts are described in Table 2. The EDTA at 0.83 mg/ml as same as *C. vulgaris* C-C extracts was included as positive control for ferrous ion-chelating activities assay. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, such as *C. vulgaris* C-C extracts, the reagent complex formation was disrupted, resulting in a reducing in the dark red color of the complex. The extract from SC-CO_2 at the dosage of 0.83 mg/ml shows a high level of Fe^{2+} scavenging effect of 91.38%. However, extract from ultrasonic extraction at the same dosage only presents a moderate level of Fe^{2+} scavenging effect of 46.69%, but lower than positive control (100%).

3.5. Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power assay was a simple and reliable test, measuring the reducing potential of an antioxidant reacting with a ferric 2,4,6-tripyridyl-S-triazine [Fe(III)–TPTZ] complex, producing a dark blue colored ferrous Fe(II)–TPTZ complex by an adopted reductant. This complex has a conspicuous blue color that could be monitored at 593 nm. A higher absorbance at 593 nm indicates a higher ferric reducing power. In Table 2, *C. vulgaris* C-C extract from SC-CO_2 demonstrates a higher ferric reducing power, giving a FRAP value of 12.14 mg ferrous sulfate heptahydrate equivalent of the sample at the dosage of 0.83 mg in contrast to ultrasonic extraction (5.23 mg_{FeSO_4}) and positive control (10.71 mg_{FeSO_4}).

3.6. Superoxide anion radical scavenging capacity

The radical scavenging activity of *C. vulgaris* C-C extracts against superoxide anion radical was evaluated by the reduction of NBT. Superoxide anion radicals are generated in a PMS–NADH system by oxidation of NADH. The color variation between superoxide anion radicals and NBT was measured at 560 nm. The decrease of absorbance at 560 nm indicated the consumption of superoxide anion in the PMS–NADH–NBT system. Table 2 shows the superoxide anion radical scavenging capacity of extracts from SC-CO_2 extraction, ultrasonic extraction and gallic acid (positive control). At a dosage of 0.83 mg, the positive control exhibits a superoxide anion radical scavenging capacity of 100% higher than SC-CO_2 extraction (18.08%), whereas no superoxide anion radical scavenging capacity was detected from the extract of ultrasonic extraction.

3.7. Anti-proliferative effects of SC-CO_2 C. vulgaris C-C extract on NSCLC cells

The cell proliferation assay shows that the anti-cell proliferation of *C. vulgaris* C-C extract on all tested NSCLC tumor cells for 24 h treatment. Cells were incubated in the presence of serially diluted concentrations (i.e., 200, 100, and 20 μg/ml) of extracts from *C. vulgaris* C-C, and the cell growth rate was determined for each test. The proliferation of H1299, A549, and H1437 cells was inhibited by extract of *C. vulgaris* C-C in a dose-dependent manner from 20 to 200 μg/ml (Fig. 3). The inhibitory effect was in a dose-dependent manner.

3.8. Cell migration of NSCLC cells inhibited by extract of C. vulgaris C-C

The inhibition of NSCLC cell migration by *C. vulgaris* C-C extract was examined by a wound-healing assay. The results are shown in Fig. 4. Photographs in Fig. 4a present the migration of NSCLC cells (5×10^5 cells) with various concentrations of *C. vulgaris* C-C extract from SC-CO_2. In addition, the quantification analysis was shown in Fig. 4b. With the treatment of *C. vulgaris* C-C extract, cells were not permitted to migrate into the area of clearing for 24 h or 48 h at an extract concentration of 200 μg/ml or 100 μg/ml. The assay data show that a higher dosage (200 μg/ml) of extract from SC-CO_2 led to significant inhibitory effect on migration of all tested NSCLC tumor cells. In Fig. 4b, the migration quantitative assay demonstrates the migration potential of H1299, A549, and H1437 cells treated with a high dose (200 μg/ml) of *C. vulgaris* C-C extract at 24 and 48 h were 0.59- and 0.62-fold, respectively, compared to the vehicle control,

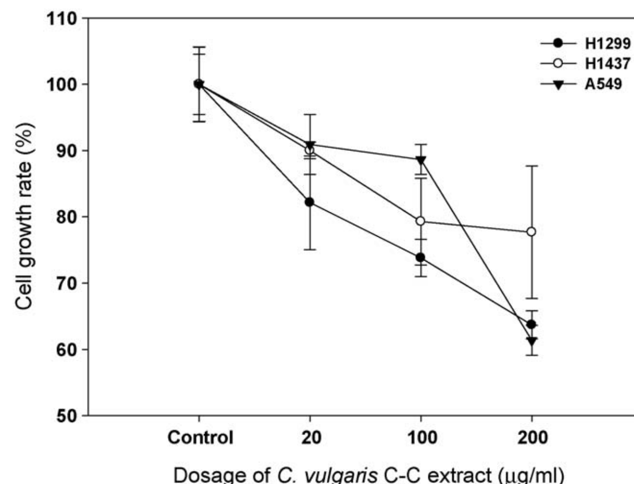


Fig. 3. Effects of *C. vulgaris* C-C on cell proliferation of H1299, A549, and H1437 cells. Cells were incubated in the presence of serially diluted concentrations (i.e., 200, 100, and 20 μg/ml) of extracts from *C vulgaris* C-C for 24 h. The cell growth rate was calculated as a ratio of *C. vulgaris* C-C-treated cells and control cells (PBS treatment).

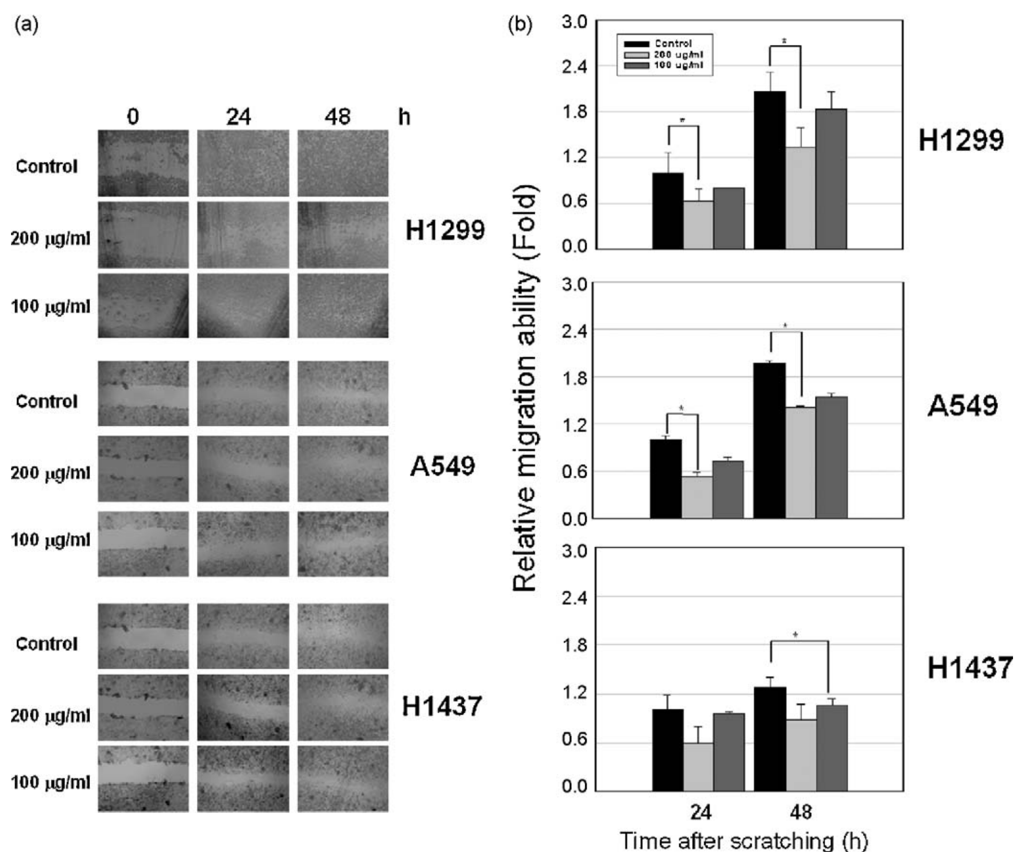


Fig. 4. Significant inhibition of migration potential *C. vulgaris* C-C-treated H1299, A549, and H1437 cells. The wound-healing assay was used to examine the effects of *C. vulgaris* C-C on migration potential. (a) 5×10^5 cells of tumor cells treated with *C. vulgaris* C-C or PBS (as control), respectively seeded in 12-well plates, and grown to complete confluence. Cells were then scraped with a 200 μl plastic tip to produce a clean ~1-mm-wide wound area. Afterward, cells were permitted to migrate into the area of clearing for 24 and 48 h, respectively, and photographed. (b) Quantification of the migration potential of *C. vulgaris* C-C- or PBS-treated cells. The control was in black color, and the 200 μg/ml was in light gray and 100 μg/ml was in dark gray. The migration and cell movement throughout the wound area were examined and calculated by the free software "TScratch" (www.cse-lab.ethz.ch/software.html). Magnification: 100×. Bars, S.D.

suggesting the efficient inhibitory effect of *C. vulgaris* C-C extract along with a dose-dependent mode.

3.9. H_2O_2-induced plasmid DNA strand breaks inhibited by extract of C. vulgaris C-C

As shown in Fig. 5, incubation of DNA with $H_2O_2/FeSO_4$ alone (lane 2) results in increasing in formation of open circular (OC) and linear (L) forms of DNA when compared to the control (lane 1) and extract treatment alone (lanes 8–12) which dominantly shows the supercoiled (S) form DNA. In comparison to DNA damage control (lane 2), the presence of different doses of extracts incubated with $H_2O_2/FeSO_4$ shows accumulation of more amount of S form DNA when the extract doses are increased although it is not completely converted from OC to S form (from lanes 7 to 3). This indicates the protective role of *C. vulgaris* C-C extract against DNA damage (lanes 5 and 6).

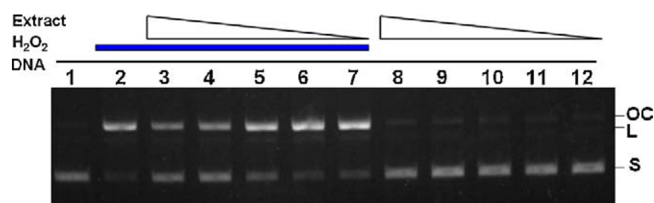


Fig. 5. Protective effect of the extract from *Chlorella vulgaris* C-C against H_2O_2-induced DNA damage. Plasmid DNA (150 ng) was incubated for 30 min in water at 37 °C. Lane 1 was normal DNA. Lanes 2–7 were treated with 0.1 mM $FeSO_4$ and 0.05% H_2O_2 in the presence of different concentration of extract ranging from 0.15, 0.075, 0.038, 0.019, and 0.010 mg/ml, respectively. Lanes 8–12 was treated with the extract alone (0.15, 0.075, 0.038, 0.019, and 0.010 mg/ml, respectively). O, L, and S indicate the open circle, linear, and supercoiled forms of plasmids.

4. Discussion

Human aging has been associated with free radical formation and consumers usually request for additive-free, fresher and more natural-making products. The use of natural antioxidants as preservatives in cosmetics and foods has a great potential. Antioxidant properties, especially in free radical scavenging capacities, are also vital because of the deleterious role of radicals in functional cares and healthy foods [27,28]. Excessive formation and accumulation of free radicals accelerates the oxidation of fats or lipids in foods and cosmetics, resulting in the decrease in the qualities and consumer acceptances [29]. We obtained the high amounts of polyphenol and flavonoid from *C. vulgaris* C-C, indicating that the extracts of *C. vulgaris* C-C exhibit strong antioxidant properties. In addition, when using SC-CO_2 extraction, much more of these active materials could be obtained when compared with ultrasonic extraction (Table 1). The antioxidant property of *C. vulgaris* C-C extracts might mainly be due to their redox properties, which could play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [30]. *C. vulgaris* C-C extracts might form a stable radical molecule to stop the radical

chain reaction by acting as chain-breaking active materials. The other possible pathway mechanism is that *C. vulgaris* C-C extracts could bind with the radical ions that were essential for the radical chain reactions to terminate the reaction.

Supercritical fluid possesses special physicochemical properties of liquid and gas, such as high diffusivity, high compressibility, low viscosity, and low surface tension. These features allow the supercritical fluid to diffuse through natural solid matrix, resulting in better extraction efficiency on the natural constitutes when compared with conventional organic liquid-solvent extraction methods. Furthermore, carbon dioxide had a low critical temperature and pressure (31.1 °C and 73.8 atm), making it an ideal solvent for extracting natural compounds due to the avoidance of thermal degradation reaction during the process. In contrast, the functional components might be damaged by harsh organic solvents in conventional extraction or by thermal degradation/physical disruption in ultrasonication. The antioxidant activity was greatly depended on the polyphenol and flavonoid contents. This indicates that SC-CO_2 with 50% aqueous ethanol is suitable for the extraction of polyphenols and flavonoids from the microalgal biomass (Table 1). Indeed, when the total antioxidant activities obtained from SC-CO_2 extraction were compared with those from ultrasonic extraction through the evaluation of four different assays (namely, DPPH radical, superoxide anion radical, metal chelating and FRAP methods), the results of all the four assays show that SC-CO_2 extraction was much better than ultrasonic extraction (Table 2). Comparing two extraction methods, the antioxidant activities acquired from SC-CO_2 extraction were greatly higher in terms of scavenging free radicals (DPPH and superoxide anion ions). In metal chelating and FRAP assays, SC-CO_2 extraction also demonstrated better performances when compared with traditional techniques. This SC-CO_2 extraction technique is harmless to the target biological molecule and easy for recycle usage, thereby retaining more active biomaterials in the extracts.

In normal mammalian physiological conditions, cell migration plays an important physiological role that involves in development and homeostasis maintenance. In addition, cell migration is a multiple process involved growth factors binding to membrane receptors, stimulating signaling pathways and resulting stimulation of cellular motility mechanism. However, the dysregulated cellular migration is responsible for the metastasis and invasion in malignant tumor cells. Recent studies reported that the interactions of tumor cells and extracellular matrix (ECM) are critical for accelerating cellular proliferation and migration [31]. In addition, the zinc-dependent endopeptidases and matrix metalloproteinases (MMPs) degrade ECM, allowing tumor cells to invade surrounding tissues, and intravasate into the circulation systems, including lymphatic and blood vessels, then travel to distant sites [32].

To date, the major cause of ineffective treatment resulting in death of malignant tumor patient is tumor metastasis. Hence, to improve the efficacy of cancer treatment, it is important and worth developing a novel medicinal component in the cancer therapy [33]. Many anti-cancer therapies killed cancer cells by generating high ROS [34]. However, accumulated ROS are often detected various cancer cells. It is well known that low concentration of intrinsic ROS induces activation and releases of MMP-2 and -9, which are responsible for cancer metastasis [35]. Moreover, a recent study showed a natural antioxidant, silybin inhibited cellular proliferation and metastasis of human hepatocellular carcinoma cell by eliminating ROS generation in the glucose-oxidase [36]. We demonstrated in this study that the proliferation and cell migration of H1299, A549, and H1437 cells was markedly inhibited by *C. vulgaris* C-C extract (Figs. 3 and 4). To our best knowledge, this is the first demonstration that *C. vulgaris* C-C extract from SC-CO_2 could inhibit proliferation and migration of human NSCLC cells. Our further study will shed light on the mechanism underlying the anti-proliferation and anti-metastasis of *C. vulgaris* C-C against human NSCLC cells. Nevertheless, the detail signaling pathway and the regulation of MMPs in *C. vulgaris* C-C pure compounds induced NSCLC tumor cells still need further investigation. Therefore, our results demonstrate that SC-CO_2 extract of *C. vulgaris* could become a potential and effective antioxidant as well as a potent candidate for inhibiting MMPs-mediated cancer progression, including cancer proliferation, invasion and metastasis.

5. Conclusions

Effective microalgal biomass production from the newly isolated indigenous *C. vulgaris* C-C was achieved using the inexpensive carbon and nitrogen sources of $NaHCO_3$ and KNO_3 along with proper light sources and light intensity. The *C. vulgaris* C-C extracts from SC-CO_2 exhibit significant antioxidant activities from various antioxidant assay systems *in vitro*. The *C. vulgaris* C-C extract obtained from SC-CO_2 presents dual inhibitions to lung cancer cell growth and migration ability, which is the index of cancer metastasis. Accordingly, *C. vulgaris* C-C might be a potential candidate for cancer chemoprevention. Moreover, these extracts could be used as easily available resource of natural antioxidants and as possible food supplements or in pharmaceutical applications.

Acknowledgements

The authors gratefully acknowledge financial supports from Taiwan's National Science Council under grant numbers: NSC 95-2221-E-006-164-MY3, NSC 96-2628-E-006-004-MY3, NSC 96-2218-E-006-295, NSC 97-2218-E-006-005, NSC97-2221-E-037-002- and NSC98-2221-E-037-005-, from Kaoshiung Medical University (grant no. KMU-EM-99-1.4), and from Ministry of Economic Affairs (grant no. 98-EC-17-A-10-S2-0066). The authors also would like to thank Min-Yii Julie Wang for English editing and Ya-Ling Yeh, Jung-Haung Yan and Chi-Chen Yeh for the assistances.

References

[1] Vuchetich PJ, Bagchi D, Bagchi M, Hassoun EA, Tang L, Stohs SJ. Naphthalene-induced oxidative stress in rats and the protective effects of vitamin E succinate. Free Radic Biol Med 1996;21:577–90.
[2] Suja KP, Jayalekshmy A, Arumughan C. Free radical scavenging behavior of antioxidant compounds of sesame (*Sesamum indicum* L.) in DPPH center dot system. J Agric Food Chem 2004;52:912–5.
[3] Singh KK, Kulawiec M, Still I, Desouki MM, Geradts J, Matsui S. Inter-genomic cross talk between mitochondria and the nucleus plays an important role in tumorigenesis. Gene 2005;354:140–6.
[4] Yao S, Tan H, Zhang H, Su X, Wei W. Bulk acoustic wave bacterial growth sensor applied to analysis of antimicrobial properties of tea. Biotechnol Prog 1998;14:639–44.
[5] Jin D, Hakamata H, Takahashi K, Kotani A, Kusu F. Determination of quercetin in human plasma after ingestion of commercial canned green tea by semi-micro HPLC with electrochemical detection. Biomed Chromatogr 2004;18:662–6.
[6] Szydlowska-Czerniak A, Karlovits G, Hellner G, Dianoczki C, Szlyk E. Effect of enzymatic and hydrothermal treatments of rapeseeds on quality of the pressed rapeseed oils. Part I: Antioxidant capacity and antioxidant content. Process Biochem 2010;45:7–17.
[7] Batista I, Ramos C, Coutinho J, Bandarra NM, Nunes ML. Characterization of protein hydrolysates and lipids obtained from black scabbardfish (*Aphanopus carbo*) by-products and antioxidative activity of the hydrolysates produced. Process Biochem 2010;45:18–24.
[8] Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3:768–80.
[9] Thana P, Machmudah S, Goto M, Sasaki M, Pavasant P, Shotipruk A. Response surface methodology to supercritical carbon dioxide extraction of astaxanthin from *Haematococcus pluvialis*. Bioresour Technol 2008;99:3110–5.
[10] Macias-Sanchez MD, Mantell C, Rodriguez M, de la Ossa EM, Lubian LM, Montero O. Supercritical fluid extraction of carotenoids and chlorophyll a from *Nannochloropsis gaditana*. J Food Eng 2005;66:245–51.
[11] Chun BS, Kishimura H, Kanzawa H, Klomklao S, Nalinanon S, Benjakul S, et al. Application of supercritical carbon dioxide for preparation of starfish phospholipase A_2. Process Biochem 2010;45:689–93.
[12] Mendes RL, Nobre BP, Cardoso MT, Pereira AP, Palavra AF. Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae. Inorg Chim Acta 2003;356:328–34.

[13] Lesniewski LA, Connell ML, Durrant JR, Folian BJ, Anderson MC, Donato AJ, et al. B6D2F1 mice are a suitable model of oxidative stress-mediated impaired endothelium-dependent dilation with aging. J Gerontol A-Biol 2009;64:9–20.

[14] Sheih IC, Fang TJ, Wu TK. Isolation and characterisation of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the algae protein waste. Food Chem 2009;115:279–84.

[15] VanderGheynst J, Dooley T, Guo HY, Scher H, Cheng YS. Storage and release of solutes and microalgae from water-in-oil emulsions stabilized by silica nanoparticles. Process Biochem 2010;45:1–6.

[16] Halperin SA, Smith B, Nolan C, Shay J, Kralovec J. Safety and immunoenhancing effect of a Chlorella-derived dietary supplement in healthy adults undergoing influenza vaccination: randomized, double-blind, placebo-controlled trial. Can Med Assoc J 2003;169:111–7.

[17] Rodriguez-Garcia I, Guil-Guerrero JL. Evaluation of the antioxidant activity of three microalgal species for use as dietary supplements and in the preservation of foods. Food Chem 2008;108:1023–6.

[18] Hasegawa T, Noda K, Kumamoto S, Ando Y, Yamada A, Yoshikai Y. *Chlorella vulgaris* culture supernatant (CVS) reduces psychological stress-induced apoptosis in thymocytes of mice. Int J Immunopharmacol 2000;22:877–85.

[19] Malaysian Journal of Pathology, Md. Saad S, Mohd Yusof YA, Wan Ngah WZ. Comparison between locally produced *Chlorella vulgaris* and *Chlorella vulgaris* from Japan on proliferation and apoptosis of liver cancer cell line, HepG2. Malays J Biochem Mol Biol 2006;13:32–6.

[20] Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. Food Chem 2005;91:571–7.

[21] Lin JY, Tang CY. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. Food Chem 2007;101:140–7.

[22] Wang KJ, Zhang YJ, Yang CR. Antioxidant phenolic constituents from *Fagopyrum dibotrys*. J Ethnopharmacol 2005;99:259–64.

[23] Decker EA, Welch B. Role of ferritin as a lipid oxidation catalyst in muscle food. J Agric Food Chem 1990;38:674–7.

[24] Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. AnBio 1996;239:70–6.

[25] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;26:1231–7.

[26] Geback T, Schulz MMP, Koumoutsakos P, Detmar M. TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. BioTechniques 2009;46:265–74.

[27] Garcia A, Toledano A, Andres MA, Labidi J. Study of the antioxidant capacity of Miscanthus sinensis lignins. Process Biochem 2010;45:935–40.

[28] Sun YX, Liu JC, Yang XD, Kennedy JF. Purification, structural analysis and hydroxyl radical-scavenging capacity of a polysaccharide from the fruiting bodies of *Russula virescens*. Process Biochem 2010;45:874–9.

[29] Gülçin I, Elmastas M, Aboul-Enein HY. Determination of antioxidant and radical scavenging activity of basil (*Ocimum basilicum* L. family Lamiaceae) assayed by different methodologies. Phytother Res 2007;21:354–61.

[30] Wang SY, Ballington JR. Free radical scavenging capacity and antioxidant enzyme activity in deerberry (*Vaccinium stamineum* L.). LWT-Food Sci Technol 2007;40:1352–61.

[31] Yang L, Zeng W, Li D, Zhou R. Inhibition of cell proliferation, migration and invasion by DNAzyme targeting MMP-9 in A549 cells. Oncol Rep 2009;22:121–6.

[32] Lin SS, Lai KC, Hsu SC, Yang JS, Kuo CL, Ling JP, et al. Curcumin inhibits the migration and invasion of human A549 lung cancer cells through the inhibition of matrix metalloproteinase-2 and -9 and Vascular Endothelial Growth Factor (VEGF). Cancer Lett 2009, doi:10.1016/j.canlet.2009.04.037.

[33] Wang Y, Yang H, Liu H, Huang J, Song X. Effect of staurosporine on the mobility and invasiveness of lung adenocarcinoma A549 cells: an *in vitro* study. BMC Cancer 2009;9:174.

[34] Ding H, Han C, Guo D, Chin YW, Ding Y, Kinghorn AD, et al. Selective induction of apoptosis of human oral cancer cell lines by avocado extracts via a ROS-mediated mechanism. Nutr Cancer 2009;61:348–56.

[35] Belkhiri A, Richards C, Whaley M, McQueen SA, Orr FW. Increased expression of activated matrix metalloproteinase-2 by human endothelial cells after sublethal H_2O_2 exposure. Lab Invest 1997;77:533–9.

[36] Huber A, Thongphasuk P, Erben G, Lehmann WD, Tuma S, Stremmel W, et al. Significantly greater antioxidant anticancer activities of 2,3-dehydrosilybin than silybin. Biochim Biophys Acta 2008;1780:837–47.