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Antioxidant activities of crude extracts from peel and seed of *Cinnamomum camphora.*

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Abstract

In this study, we aim to investigate the free radical scavenging, anti-melanogenic and antibacterial properties of crude extracts from peel and seed of *Cinnamomum camphora*. Results showed that methanol extract of peel was minor sensitive to *Staphylococcus aureaus* and *Escherichia coli.* The fractions obtained by n-hexane, ethyl acetate (EtOAc), n-butanol or water of peel and seed had significant antioxidant activity. The IC_{50} value of n-butanol, water and ethyl acetate fraction of the peel exhibited 48.82, 221.32 and 647.85 μg/ml scavenging activity in DPPH free radical. The IC_{50} value of n-butanol, water and ethyl acetate fraction of the peel exhibited 1135.47, 438.69 and 3127.09 μg/ml in metal chelating ability on ferrous ions assays. n-butanol fraction of peel displayed the best antioxidant activity in ABTS assay. Especially, IC_{50} of the mushroom tyrosinase inhibition at n-hexane extraction of the seed was 53.68 μg/ml. The results obtained in the present investigation suggest that the crude extracts of peel and seed of *C. camphora* had promising antibacterial and antioxidant activity against free radicals. The n-butanol or water extract of peel may be further study to isolate the active compounds in the future.

Keywords: *Cinnamomum camphora*, Antioxidant activity, Mushroom tyrosinase inhibition, Antibacterial efficiency.

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Introduction

Oxidants such as nitric monoxide ($NO^{\bullet}$), superoxide (O_2^-) and hydroxyl ($OH^{\bullet}$) are free radicals. Hydroxyl radicals, superoxides, and hydroperoxides are ROS (Reactive Oxygen Species) with characteristics according their reactivity. ROS is associated with cancer, diabetes, obesity and chronic inflammation [1-3]. The excess of ROS is harmful to cells and tissues. ROS can produce oxidative damage to lipid, proteins and DNA in living bodies and cause many chronic diseases such as cancer and diabetics. These diseases can be cured or slowed down by using natural or synthetic compounds. It is widely known that fruits and vegetables have potential to reduce the risk of oxidative stress related diseases. Antioxidants such as vitamins A, C, E and phytochemicals can prevent ROS production [4-6].

Ultraviolet ray (UV) is harmful to human health particularly in skin. UV stimulates tyrosinase activity and accelerates the dark spot formation [7,8]. Melanin plays an important role against UV in human skin. Tyrosinase is the rate-limiting enzyme in melanin formation. Inhibition of melanin can reduce melanin pigments in the skin tissue. A lot of pharmaceutical companies are searching novel compounds in preventing UV irritation [7,9]. Studies have shown that some phytochemicals such as caffeic acid and ferulic acid have tyrosinase inhibition [10,11].

Cinnamomum camphora is also known as camphor tree and native to China, Taiwan and Japan. *C. camphora* belonged to Lauraceae family. *C. camphora* has been used in tradition use such as bronchitis, asthma, indigestion and inflammation-related diseases. The previous studies have shown that *C. camphora* contains alkaloids and essential oil such as camphor and type II ribosome-inactivating proteins [12-15]. A study reported *C. camphora* extracts has anti-inflammatory and anti-oxidative effects [16]. The details mechanisms of the anti-inflammatory activity and active components, however, remain further examine.

Multidrug-Resistant (MDR) bacterial is a serious problem in the antibacterial therapy. β-lactamase-producing *Escherichia coli*, carbapenem-resistant Enterobacteriaceae, *Acinetobacter baumannii*, and methicillin-resistant *Staphylococcus aureus* (MRSA) are well known MDR bacterial in the world [17,18]. The therapeutic options for these pathogens are extremely limited. Researchers are searching the effective alternatives in the treatment of MDR bacterial. The phytochemicals for

antimicrobial therapy *in vitro* have been reported with pharmacological activities [19].

In the present work were studied the various extracts (n-hexane, water, methanol, compression, n-butanol and acetyl acetate) from different parts (seed and peel) of *C. camphora* in anti-melanogenic, antibacterial and anti-oxidation activities.

Materials and Methods

Collection of plant materials

The peel and seed of *C. camphora* were collected from Chiayi County, Taiwan, May 2009. Plant material was identified by Professor Fu-Yuan Lu (Department of Forestry and Natural Resources, College of Agriculture, National Chiayi University). A voucher specimen was deposited in the School of Medicial and Health Sciences, Fooyin University, Kaohsiung City, Taiwan.

Extraction and fractionation

In this study, we used four *C. camphora* organic solvent extracts. The air-dried peels of *C. camphora* (0.4, 0.6, 0.4, 0.5 kg) were extracted with n-butanol, MeOH, H_2O and EA (5 L × 5) at room temperature, respectively. The extracts from n-butanol, MeOH, H_2O and EA were 32.8, 29.7, 22.7 and 64.9 g through concentration under reduced pressure. The air-dried seeds of *C. camphora* (1.1 kg) were extracted with n-hexane (4 L × 3) at room temperature and the extract (42.5 g) concentrated under reduced pressure.

Test organisms and culture preparation

The bacterial and fungus strains of *Escherichia coli* (ATCC 605) and *Staphylococcus aureus* (ATCC 25923) were obtained from American Type Culture Collection. All the test organisms were grown in nutrient broth for overnight at 37˚C separately before performing assays.

Determination of antibacterial assay disc diffusion method

The antibacterial activity of extracts of the seed was analysed by disc diffusion method. Nutrient agar was prepared, sterilized by autoclaving and poured into the sterile petri plates. After solidification, the plates were swabbed with test organism (10^9 CFU/ml) by using sterile cotton swabs. Sterile paper discs of 8 mm diameter were concentrated with 40 µl of all extracts and air dried. The discs were placed on the surface of the nutrient agar plates and inoculated with test organisms. All the plates were incubated at 37°C for 48 h and the zone of inhibition was measured at the end of incubation period.

Metal chelating activity

Briefly, various different concentrations of 1 ml of samples were dissolved in DMSO and added to a solution of 0.05 ml of 2 mM $FeCl_2$•$4H_2O$. The reaction was initiated by addition 0.02 ml of 5 mM ferrozine. The mixture was vigorously shaken and incubated at room temperature for 10 min. The absorbance of the test sample was then measured at 562 nm. The metal chelating activity was determined as: $1-((A_{control}-A_{sample})/A_{control}) \times 100\%$. EDTA was used as a positive control. The amount of inhibition by the test samples was expressed as the percentage of concentration necessary to achieve 50% inhibition (IC_{50}).

Assay on mushroom tyrosinase activity

The test sample was dissolved in DMSO (60 µL) with 50 mM phosphate buffer (pH 6.8, 70 µL) and 125 U/ml of mushroom tyrosinase (10 µL). L-tyrosine (0.3 mg/ml, 70 µL) was added in the same buffer and incubated at 37°C for 30 min. The assays were conducted in a 96-well microplate and absorbance was determined at 490 nm. Percent inhibition of tyrosinase activity was calculated as the following equation: % tyrosinase inhibition=100% × ((A-B)-(C-D))/(A-B) where A is blank solution with tyrosinase; B is blank solution without tyrosinase; C is the test substance with tyrosinase; and D is test substance without tyrosinase. Kojic acid was used as a positive control. The amount of inhibition by the test samples was expressed as the percentage of concentration necessary to achieve 50% inhibition (IC_{50}).

Determination of DPPH• radical scavenging capacity

Briefly, various concentrations 0.2 ml of the samples were added to 3.8 ml of stable DPPH solution (final concentration, 0.1 mM). The mixture was vortexed for 1 min and incubated at room temperature in the dark for 30 min. The absorbance for test sample was measured at 517 nm. The analysed time interval was 10 min per point up to 30 min. Catechin was used as a positive control in this experiment. The DPPH• radical discolouration of the sample was determined as: $(1-(A_{sample}/A_{control})) \times 100$. The amount of inhibition by the test samples was expressed as the percentage of concentration necessary to achieve 50% inhibition (IC_{50}).

ABTS radical scavenging assay

The stock solution of ABTS is 7 mM and potassium persulfate is 2.45 mM. The two stock solutions were mixed equal volume and incubated overnight at room temperature in the dark. The working solution was then diluted with methanol to obtain an absorbance of 0.706 ± 0.01 units at 745 nm. The test samples were reacted with 1 ml of ABTS solution and the absorbance was measured at 745 nm after 7 min by double beam spectrophotometer. Results are presented as the ability of test samples was determined against a standard calibration curve TEAC (Trolox equivalent antioxidant capacity) and expressed as µM.

Statistical analysis

All data are the means ± SD from at least triplicate experiments.

Results

In vitro antimicrobial activity

The antibacterial activity of different spices used in this study is given in Table 1. The methanol extraction of camphor tree peel showed minor activity against the tested microorganisms compared with ampicillin by the disc diffusion method (Figure 1). The positive control ampicillin was extremely effective on *Escherichia coli* and *Staphylococcus aureus* with inhibition zones ranging from 9.04 to 8.66 mm. The antibacterial activity of methanol extraction showed more effective on *Staphylococcus aureus* than on *Escherichia coli*.

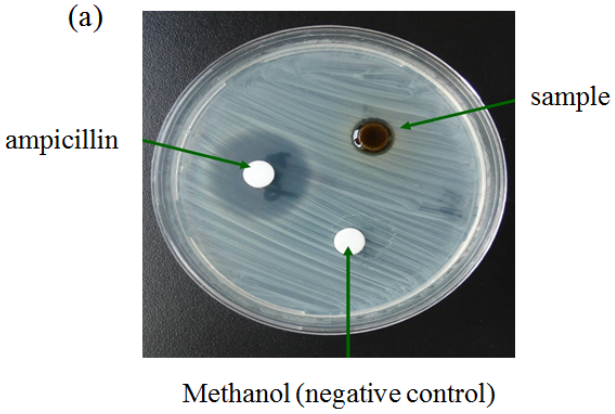

Figure 1. *The antibacterial activity of MeOH extraction of C. camphora (peel) on Staphylococcus aureus (a) and Escherichia coli (b).*

Antioxidant activities and mushroom tyrosinase inhibition of extracts and fractions from C. camphora

Table 1 showed the total antioxidant activity of different extractions expressed as TEAC (Trolox equivalent antioxidant capacity). The results indicated the compression and n-hexane fraction from the seed were barely detected antioxidant activity. 1-butylalcohol fraction of peel displayed the best antioxidant activity.

In the DPPH assay, antioxidants are able to reduce the stable DPPH radicals at room temperature. Results showed that 1-butylalcohol fraction of peel displayed the best radical scavenging activity. The IC_{50} values of 1-butylalcohol, water, ethyl acetate extraction of peel were 48.82 ± 4.32, 221.32 ± 7.63, 648.75 ± 5.48 μg/ml in the DPPH assay (Table 2).

The ferrous ion chelating activities of different fractions of the peel were shown in Table 2. Ferrozine quantitatively produced complexes with Fe^{2+}. Water extraction of peel of Fe^{2+} showed best scavenging effect. EDTA is the positive control. The IC_{50} values of 1-butylalcohol, water, ethyl acetate fraction of peel were 1135.47 ± 10.32, 438.69 ± 7.83, 3127.09 ± 9.87 μg/ml, in the chelating assay.

Further, the inhibitory effect of different fractions of seed was determined in an *in vitro* mushroom tyrosinase inhibition assay (Table 2). Kojic acid t is a commonly used as a positive control. Compression and n-hexane fraction of the seed showed inhibition in mushroom tyrosinase inhibition assay. The IC_{50} values of compression and n-hexane fraction were 83.52 ± 6.84 and 53.68 ± 7.30 μg/ml.

Table 1. *Antibacterial activity of MeOH extract of C. camphora (peel), ampicillin and methanol after 48 h treatment.*

Pathogens	Inhibition diameter (mm)		
	Including disk diameter of 8.0 mm		
	Peel	Ampicillin	Methanol
Staphylococcus aureus	8.18 ± 0.08	9.04 ± 0.11	8.00 ± 0
Escherichia coli	8.11 ± 0.03	8.66 ± 0.30	8.00 ± 0

Results are mean ± SD values of three replications. #The diameter of 8.0 mm is included in the inhibition zone disc diameter.

Table 2. *Antioxidant and anti-tyrosinase properties of different extracts.*

Fraction		Mushroom tyrosinase (μg/ml)	DPPH (μg/ml)	Chelating (μg/ml)	ABTS (μM)
Seed	n-hexane	53.68 ± 7.3	0	0	0.03 ± 0.001
	compression	83.52 ± 6.84	0	0	0
Peel	n-butanol	-	48.82 ± 4.32	1135.47 ± 10.32	13.20 ± 1.32
	H_2O	-	221.32 ± 7.63	438.69 ± 7.83	5.87 ± 0.87
	ethyl acetate	-	647.85 ± 5.48	3127.09 ± 9.87	2.79 ± 0.24
Kojic acid		47.39 ± 3.82	-	-	-
EDTA		-	-	1.39 ± 0.05	-
Catechin		-	3.94 ± 0.02	-	-

Data were expressed as a mean value of at least three independent experiments. Catechin was used as a positive control on DPPH assay; EDTA was used as a positive control on metal chelating; Kojic acid was used as a positive control of mushroom tyrosinase assay; - means no test.

Discussion

Traditional Chinese Medicine (TCM) has many bioactive substances including polyphenol and anthocyanins compounds with anti-oxidation and anti-inflammation effects [20]. It is well known that antioxidants are free radical scavengers. Free radicals are produced in several oxidative-reductive processes and free radicals may involve chronic diseases such as cancer and heart disease. Antioxidants neutralize free radicals, thus preventing them from causing damage. Antioxidants act through different possible mechanisms. In this study, we evaluated the extracts of antioxidant capacity by DPPH, metal chelating and ABTS assays. The antioxidant capacity of different plants gives the plant their significant nutritional and therapeutic values [21]. In this study, we found that the extracts from seed did not have antioxidation activity by compression and n-hexane extraction. These results indicated that the antioxidation activity mainly comes from the peel. While the water or n-butanol fraction of peel displayed the better antioxidant activity than ethyl acetate fraction.

Tyrosinase is produced only by melanocyte cells. Tyrosinase plays an important role in controlling melanogenesis. The extracts and isolated compounds from plants have tyrosinase inhibition. Agents or products that inhibit tyrosinase activity have been used in skin whitening cosmetics. Hydroquinone, arbutin, kojic acid, azelaic acid and L-ascorbic acid have tyrosinase inhibition as skin whitening agents [22-24]. In this study, we found that n-hexane extraction and compression of the seed has tyrosinase inhibition. We think the bioactive components could be degraded at high temperature in compression method.

Although a lot of *in vitro* studies demonstrate that extracts have antibacterial activity. In the current study, methanol extract from peel was minor sensitive to *Staphylococcus aureus* and *Escherichia coli*. The antibacterial activity is depending on the chemical composition, extraction and bacterial strains tested. At least, the current study indicated that methanol extract from peel exhibited limited antibacterial activity.

There are many ongoing studies for developing synthetic antioxidant compounds. Previous study has demonstrated that *C. camphora* total extractions displayed anti-inflammatory actions because of the modulation of cytokine, NO and PGE2 production and oxidative stress [16]. Additionally, camphor and camphor oil are an important source for perfume in this plant. Camphor also displays a lot of biological properties such as insecticidal and antimicrobial activities. Studies have shown essential oils from *C. camphora* aerial parts and stem barks displayed insecticidal activities. Few studies discussed the biological and pharmacological properties of *C. camphora* extracts from the seed and peel. In this study, we compared various fractions of seed and peel of *Cinnamomum camphora* in anti-melanogenic, antioxidation and antibacterial activity.

Conclusion

In this study, we first demonstrated different crude extracts of peel and seed of *C. camphora* displayed antioxidation, anti-melanogenic and antibacterial activity. In the future, it is necessary to further isolate the active constituents and determine their pharmacological effects.

Acknowledgements

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