DPPH Radical Scavenging and Semicarbazide-Sensitive Amine Oxidase Inhibitory and Cytotoxic Activities of *Taiwanofungus camphoratus* (Chang-Chih)


1National Research Institute of Chinese Medicine, Taipei, Taiwan
2Department of Internal Medicine, School of Medicine, Taipei Medical University, Taipei, Taiwan
3Department of Internal Medicine, Taipei Medical University Hospital, Taipei, Taiwan
4Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei, Taiwan
5Traditional Herbal Medicine Research Center, Taipei Medical University Hospital, Taipei, Taiwan

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Wild, liquid state culture and solid state culture of *Taiwanofungus camphoratus* (Chang-chih) were sequentially extracted with cold water, methanol, and hot water to get cold water soluble, methanol soluble, and hot water soluble extracts respectively. The extracts from three Chang-chih were used to determine 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, semicarbazide sensitive amine oxidase inhibitory, and cytotoxic activities against B16-F10 and HT-1080 cell lines. It was found that extracted fractions from three Chang-chih exhibited the different levels of biological activities.

**Key words:** 1,1-diphenyl-2-picrylhydrazyl (DPPH); liquid-state culture (LSC); semicarbazide-sensitive amine oxidase (SSAO); solid-state culture (SSC); *Taiwanofungus camphoratus*

The traditional name of the fungus Chang-chih (CC) literally means “fungus of fortune from the camphor tree.” Its scientific name is *Taiwanofungus camphoratus*. It belongs to the Polyporaceae, Basidiomycotina family and has a special host in the endemic perennial tree, *Cinnamomum kanehirai* (Bull camphor tree), in Taiwan. *Antrodia camphorata* (niu-chang-chih) has been widely applied to this fungus. CC is recognized as a folk medicine. A solid-state culture (SSC) of a CC extract exhibited both antioxidative activity against iron-induced lipid peroxidation and hepatoprotective activity against CCl4-induced hepatic injury. The fermented filtrate of a submerged culture of CC showed a protective effect against CCl4-induced hepatic injury. The polysaccharide from cultured mycelia and fruiting bodies of CC exhibited an anti-hepatitis B virus effect. The maleic and succinic acid derivatives of the mycelia of CC exhibited cytotoxic activities against the LLC tumor cell line from mycelia of CC, while the methanol extract of a CC submerged culture exhibited cytotoxic activity and induced apoptosis against the human hepatoma HepG2 cell line. Due to the growth of CC on specific tree in Taiwan, *C. kanehirai* (the bull camphor tree), it is difficult to find in the wild and very expensive to buy. SSC-CC and liquid state culture (LSC)-CC from mycelia were hence developed. Recently, W-CC and SSC-CC have been reported to have antihypertensive effects on spontaneously hypertensive rats.

Free radical-mediated reactions are involved in degenerative and pathological processes such as aging, cancer, coronary heart disease, and Alzheimer’s disease. Many epidemiological results have revealed an association between a diet rich in fresh fruits and vegetables and a decrease in the risk of cardiovascular diseases and certain forms of cancer in humans. Several reports have shown natural compounds in fruits and vegetables to have antioxidant activities, such as phenolic compounds, anthocyanin, echinacoside in the Echinacea root, and the storage proteins of yam tuber.

Semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.6) is the common name for a group of heterogenous enzymes widely distributed in nature, including plants, microorganisms, and organs of mammals (vasculature, dental pulp, eye, and plasma). SSAO converts primary amines into the corresponding aldehydes, generating hydrogen peroxide and ammonia. It has been...
found that endogenous compounds, aminoacetone and methylamine, are good substrates for most SSAOs. In recent research, it was found that plasma SSAO was raised in diabetes mellitus, inflammatory liver disease, hypertension, and heart failure, and was implicated in atherosclerosis, in respect of endothelial damage.

In this study, wild (W), LSC, and SSC of CC were sequentially extracted with cold water, methanol, and hot water to get, respectively, cold-water-soluble (CWS), methanol-soluble (MS), and hot-water-soluble (HWS) extracts. It was found that extracted fractions from the three CC exhibited different levels of biological activities.

**Materials and Methods**

**Materials.** Benzylamine, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid (ABTS), bovine plasma (P-4639, reconstituted with 10 ml deionized water), 1,1-diphenyl-2-picrylhydrazyl (DPPH), horseradish peroxidase (148 units/mg solid), phosphate buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and semicarbazide were purchased from Sigma Chemical (St. Louis, MO). B16-F10 and HT-1080 cells were purchased from the Bioresource Collection and Research Center and Food Industry Research and Development Institute (Hsinchu, Taiwan). Dulbecco’s modified eagle medium (DMEM), fetal bovine serum (FBS), and trypsin-EDTA were from GibcoBRL (Gaithersburg, MD). W-CC, LSC-CC, and SSC-CC were provided by Well Shine Biotechnology Development (Taipei, Taiwan). Other chemicals and reagents were from Sigma Chemical.

**Preparation of various Taiwanofungus camphoratus extracts.** The CWS, MS, HWS extracts from the three CCs were extracted by different solvents, as reported previously. Briefly, 50 g each of W-CC, LSC-CC, and SSC-CC was extracted first with 500 ml of cold water by stirring at room temperature overnight, and after being filtered, the residue was extracted with cold water under the same procedure twice. The filtrates were collected and lyophilized as CWS extracts. The residues were secondarily extracted with 500 ml of methanol by stirring at room temperature overnight. After being filtered, the residue was extracted with methanol under the same procedure twice. The filtrates were collected and concentrated as MS extracts. Finally, the residues were extracted with 500 ml of boiling water for 2 h twice, and the filtrates were collected and concentrated as HWS extracts. All fractions were lyophilized and stored at -20°C for further investigation.

**Scavenging activity against DPPH radical analyzed by spectrophotometry.** The scavenging activity of CWS, MS, HWS extracts from the three CCs against the DPPH radical was measured according to the method of Hou et al.18,19) CC extracts (0.3 ml for W-CC, CWS, 0–0.175 mg/ml; MS, 0–0.2 mg/ml; HWS, 0–0.125 mg/ml; for LSC-CC, CWS, 0–0.175 mg/ml; MS, 0–0.6 mg/ml; HWS, 0–1.2 mg/ml; for SSC-CC, CWS, 0–0.2 mg/ml; MS, 0–0.175 mg/ml; HWS, 0–0.125 mg/ml) were added to 0.1 ml of 1 mM Tris–HCl buffer (pH 7.9), and then mixed with 0.6 ml of 100 μM DPPH in methanol for 20 min under light protection at room temperature. The decrease of absorbance at 517 nm was measured and expressed as ΔA517. Means of triplicates were measured. Distilled water or methanol was used in blank experiments. The scavenging activity of DPPH radicals (%) was calculated by the equation: (ΔA517blank − ΔA517sample) ÷ ΔA517blank × 100%. IC50 stands for a concentration of 50% scavenging activity.

**SSAO inhibitory activities of Taiwanofungus camphoratus extracts.** SSAO inhibitory activity was determined by the spectrophotometric method according to Szutowicz et al.23) with some modifications. The total 200 μl reaction solution (containing 50 μl of 200 mM phosphate buffer, pH 7.4, 50 μl of 8 mM benzylamine, bovine plasma containing SSAO, 2.53 units and different amounts of CC extracts, for W-CC, CWS, 0–0.1875 mg/ml; MS, 0–0.1875 mg/ml; HWS, 0–0.1875 mg/ml; for LSC-CC, CWS, 0–1.875 mg/ml; MS, 0–0.5625 mg/ml; HWS, 0–0.4375 mg/ml; for SSC-CC, CWS, 0–0.5 mg/ml; MS, 0–0.1875 mg/ml; HWS, 0–0.4375 mg/ml) was placed at 37°C for 1 h and then heated at 100°C to stop reaction. After cooling and brief centrifugation, the 90 μl reaction solution was isolated and added to the 710 μl solution containing 200 μl of 200 mM phosphate buffer (pH 7.4), 100 μl of 2 mM ABTS solution, and 25 μl of horseradish peroxidase (10 μg/ml). Changes in absorbance at 420 nm were recorded during 1 min and expressed as ΔΔA420 nm/min. Means of triplicates were measured. The distilled water or methanol was used as a blank experiments. The SSAO inhibition (%) was calculated by the equation: (ΔΔA420 nm/minblank − ΔΔA420 nm/minsample) ÷ ΔΔA420 nm/minblank × 100%. IC50 stands for the concentration of 50% inhibitions.

**Effects of Taiwanofungus camphoratus extracts on cell viability.** B16-F10 and HT-1080 cells were cultured in DMEM supplemented with 10% FBS, 10,000 I.U. penicillin/ml, and 10,000 μg streptomycin/ml. The cell number was adjusted to 1 × 10⁵ cells/ml for B16-F10 and to 5 × 10⁵ cells/ml for HT-1080. CC extracts of 2 mg/ml were used for cytotoxicity screenings in the presence of polymyxin B (50 μg/ml), and cultured in a 5% CO₂ humidified incubator at 37°C for 48 h (for B16-F10) or 72 h (for HT-1080). Cell viability was assayed by MTT staining.24)

**Results and Discussion**

LSC-CC from mycelia and SSC-CC6) were developed
to substitute for W-CC, which is very expensive and not easy to find in nature. Therefore, crude extracts of LSC-CC and SSC-CC are frequently used in biological activity assays.\(^3\)\(^-\)\(^9\) In this study, extracts from LSC-CC and SSC-CC were used in the comparison with W-CC extracts in DPPH radical scavenging, SSAO inhibitory, and cytotoxic activities against B16-F10 and HT-1080 cells.

W-CCs, LSC-CCs and SSC-CCs were sequentially extracted with cold water, methanol, and hot water to get CWS, MS, and HWS extracts respectively. Recovery of the extraction in sequence was 6.64%, 23.4%, and 2.18% for W-CC; 60.37%, 3.23%, and 1.21% for LSC-CC; and 11.69%, 3.19%, and 2.29% for SSC-CC. LSC-CC had the highest extractable CWS contents, and W-CC had the highest extractable MS contents among the three CCs. The extractable HWS content was similar in W-CC and SSC-CC, and higher than in LSC-CC. Several triterpenoids were isolated and identified in methanolic (or ethanolic) extracts of W-CC,\(^2\)\(^5\)\(^-\)\(^2\)\(^8\) and Chen et al.\(^8\) have reported that HWS and ethanolic extracts in SSC-CC contained triterpenoids that are found in W-CC.

Each fraction from three CCs (W-CC, SSC-CC, LSC-CC) was analyzed for DPPH radical scavenging activities. The DPPH radicals were widely used in the model system to investigate the scavenging activities of several natural compounds. The color changed from purple to yellow, and its absorbance at wavelength 517 nm decreased. From the results shown in Fig. 1, it was found that the three fractions in the different CCs showed the dose-dependent scavenging activities against DPPH radicals. The orders of DPPH scavenging activities of CWS were W-CC \(\sim\) LSC-CC \(>\) SSC-CC (Fig. 1A); and of the MS were W-CC \(\sim\) SSC-CC \(\gg\) LSC-CC (Fig. 1B); and of the HWS were W-CC \(\sim\) SSC-CC \(\gg\) LSC-CC (Fig. 1C). From calculations on scavenging DPPH radicals, the IC\(_{50}\) (expressed as

<table>
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<tr>
<th>DPPH scavenging activity (mg/ml)</th>
<th>W-CC</th>
<th>SSC-CC</th>
<th>LSC-CC</th>
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<tr>
<td>CWS(^b)</td>
<td>0.093</td>
<td>0.143</td>
<td>0.090</td>
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<td>MS</td>
<td>0.114</td>
<td>0.113</td>
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<td>HWS</td>
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<th>LSC-CC</th>
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<td>CWS</td>
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<td>0.17</td>
<td>1.41</td>
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<tr>
<td>MS</td>
<td>0.08</td>
<td>0.09</td>
<td>0.37</td>
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<td>HWS</td>
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<th>Cytotoxic activity against B16-F10 (mg/ml)</th>
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<th>LSC-CC</th>
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<tr>
<td>CWS</td>
<td>0.99</td>
<td>0.90</td>
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<td>MS</td>
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<td>HWS</td>
<td>0.78</td>
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<th>Cytotoxic activity against HT-1080 (mg/ml)</th>
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<td>HWS</td>
<td>0.70</td>
<td>1.29</td>
<td>1.75</td>
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</table>

\(^a\)W, wild; LSC, liquid state cultures; SSC, solid state cultures; CC, chang-chih
\(^b\)CWS, cold-water-soluble extracts; HWS, hot-water-soluble extracts; MS, methanol-soluble extracts
\(^c\)expressed as the IC\(_{50}\) value.
mg/ml) of each fraction in three CC is shown in Table 1. In the W-CC fraction, it was found to be HWS > CWS > MS; in the SSC-CC fraction, it was found to be HWS > MS > CWS; and in the LSC-CC fraction, it was found to be CWS > HWS > MS. It was found that SSC-CC had scavenging DPPH capacity similar to W-CC, higher than LSC-CC. The antioxidant properties of different parts of submerged culture of CC have been reported, but the comparisons among W-, SSC-, and LSC-CC in their antioxidant properties are first reported.

In recent research, it was found that plasma SSAO was raised in diabetes mellitus, inflammatory liver disease, hypertension, and heart failure, and was implicated in atherosclerosis, in respect of endothelial damage. It was proposed that the increased methylglyoxal, formaldehyde, and hydrogen peroxide from the metabolic products of SSAO-catalyzed aminoacetone might be toxic to different tissues. Therefore, each fraction from the three CCs (W-CC, SSC-CC, LSC-CC) was analyzed for SSAO (from bovine plasma) inhibitory activities (Fig. 2). From the results shown in Fig. 2, it was found that the three fractions in different CCs showed dose-dependent SSAO inhibitory activities. In the same CWS fraction (Fig. 2A), it was found to be W-CC > SSC-CC > LSC-CC; in the same MS fraction (Fig. 2B), it was found to be W-CC > SSC-CC > LSC-CC; and in the same HWS fraction (Fig. 2C), it was found to be W-CC > SSC-CC > LSC-CC. From calculations on SSAO inhibitory activities, the $IC_{50}$ (expressed as mg/ml) of each fraction in the three CCs is shown in Table 1. W-CC exhibited the most potential SSAO inhibitory activities among the three CCs.

Submerged culture of CC extracts and purified maleic and succinic acid derivatives from the mycelia of CC have been reported to have cytotoxic activities against different tumor cell lines. In this report, each fraction from three CCs (W-CC, SSC-CC, LSC-CC) was used to treat two tumor cell lines, B16-F10 and HT-1080. The B16-F10 cell is a skin melanoma from mouse and the HT-1080 cell is a fibrosarcoma from humans. Cell viability was determined by MTT assay (Fig. 3). Under the same concentration of 2 mg/ml, it was found that CWS (except LSC-CC) and the HWS fractions of the three CCs, but not the MS fraction, had higher cytotoxic activities against B16-F10 (Fig. 3A) and HT-1080 cells (Fig. 3B). Therefore, the different amounts of CWS (except LSC-CC) and HWS from the three CCs (W-CC, SSC-CC, LSC-CC) were analyzed for cytotoxicity against B16-F10 (Fig. 4A) and HT-1080 (Fig. 4B) cells. From the results shown in Fig. 4, it was found that the tested HWS and CWS showed dose-dependent cytotoxicity against B16-F10 (Fig. 4A) and HT-1080 (Fig. 4B) cells, and had similar cytotoxic curves. For cytotoxic activities against B16-F10 cells (Fig. 4A), the order was W-CC-HWS > SSC-CC-CWS > W-CC-CWS > SSC-CC-HWS > LSC-CC-HWS; for cytotoxic activities against HT-1080 cells (Fig. 4B), the order was the same as in Fig. 4A. From calculations on cytotoxic activities, the $IC_{50}$ (expressed as mg/ml) of each fraction in the three CCs is shown in Table 1. W-CC-HWS exhibited the most potential cytotoxic activities against B16-F10 and HT-1080 cells among three CC.

In conclusion, this is the first report comparing different extractable fractions from the three CCs as to their DPPH scavenging activities, SSAO inhibitory activities, and cytotoxic activities against B16-F10 and HT-1080 cells. Isolation and identification of pure compounds in HWS fraction for investigation of cell cytotoxicity mechanism will be performed subsequently. SSC-CC and LSC-CC are easier to scale up than W-CC. Given its biological activities, tested in this report, SSC-CC or LSC-CC might someday be developed a healthy (or functional) food for daily use.
Fig. 3. Effects of 2 mg/ml of Cold-Water-Soluble (CWS), Methanol-Soluble (MS), and Hot-Water-Soluble (HWS) Extracts from Three Chang-Chih on the Cytotoxic Activities against (A) B16-F10 Cells and (B) HT-1080 Cells. W, wild; LSC, liquid state cultures; SSC, solid state cultures; CC, chang-chih. Cell viability was assayed by MTT staining.

Fig. 4. Effects of Different Amounts of Cold-Water-Soluble (CWS) and Hot-Water-Soluble (HWS) Extracts from Different Chang-Chih on the Cytotoxic Activities against (A) B16-F10 Cells and (B) HT-1080 Cells. W, wild; LSC, liquid state cultures; SSC, solid state cultures; CC, chang-chih. Cell viability was assayed by MTT staining.

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