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TISSUE CULTURE AND BIOAVAILABILITY OF TAIWAN'S "*ANTRODIA CAMPHORATA*"

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This study investigated how breeding conditions affected the culturing tissue of mycelia from *Antrodia camphorata* and evaluated its safety and cytotoxicity against HL-60 leukemia cancer cells. The effect of culturing medium, temperature, intensity of light, and pH value on the growth of mycelia were examined. The use of MEA medium, with no light, at 25℃ and pH 4.5 were found to yield mycelia with the largest diameter. The results indicated that the ethanolic extracts of mycelia from *A. camphorata* at concentrations of 200 μg/mL inhibited the growth of HL-60 cells while exhibiting under 500 μg/mL no significant cytotoxicity to normal cells (including Chang liver cell, Detroit 551 Human embryonic skin cell and WS-1 Human skin keratinocytes).

Keywords: *antrodia camphorata*, tissue culture, mycelia, bioavailability, cytotoxicity

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Ⅰ. Introduction

Antrodia camphorata of Polyporaceae family (Aphyllophorales), which is parasiticon the rotted trees of *Cinnamomum kanehirai*. Recent research had reported anticancer, antitumor and immunomodulating effects of the fruiting body of *A. camphorata.* Unlike *Ganoderma lucidum*, it was found to exist only on *Cinnamomum kanehirai* in Taiwan (Wu *et al*., 1997). Polysaccharides and triterpenoids were found to be two main types of active components of medicinal fungi (Tomoda *et al*., 1990; Kabir *et al*., 1988; Liu *et al*., 1997). *Antrodia camphorata* and *Ganoderma lucidum* are medicinal fungi (Lee *et al*., 2002). This pioneer research studied the tissue culture conditions of *A. camphorata* and evaluated their safety, cytotoxicity and bioavailability with 50% ethanolic extracts.

Ⅱ. Materials and Methods

2.1 Culture of mycelia

This research used 70% ethanol to initially sterilize wild fruit bodies and mycelia harvested from a tissue culture of *Antrodia camphorata.* The influence of various breeding conditions on the tissue culturing of the mycelia from *A. camphorata* was examined. *A. camphorata* was cultured using various medium components, temperatures, light intensities (1,500 Lux), and pH values on the growth of mycelia was examined (Fig. 1). Total five parameters such as carbon source, supplements, light, temperature and pH value were investigated. Carbon source and the supplement was first individually screened to obtain the best medium components (2% glucose and 2% maltose extracts). Respective examinations followed the lightness in the cultivated environment, temperature distribution of 15℃, 20℃and 25 ℃, and the pH value distribution of 4.5, 5.7 and 6.7 were conjugated.

2.2 Chemicals and reagents

Extracts of *Antrodia camphorata* were prepared by extracting fresh

mycelia powder using 50 % ethanol. Stock solutions of extracts were adjusted to 100 mg/ml by dilution in distilled water. For treatment, these extracts were further diluted in distilled water and added to cells in culture medium. Fetal bovine serum (GIBCO BRL, Grand Island, New York, USA), 100 U/mL penicillin, 10 μg/mL streptomycin, 2mM glutamine were purchased from GIBCO. Propidium iodide (PI) and other chemicals were obtained from Sigma Co. (St. Louis, MO). All of the chemicals were reagent grade.

2.3 Cells culture

Human promyelocytic leukemia HL-60 cells were obtained from Culture Collection and Research Center (CCRC) in Taiwan, having come originally from The American Type Culture Collection (ATCC). HL-60 cells, Chang liver cell,

Detroit 551 and WS-1 were cultured in Roswell Park Memorial Institutes medium (RPMI-1640), supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37℃, in a 5% CO_2 humidified atmosphere. Before *Antrodia camphorata* extract treatments were performed, cells were suspended at a final density of 2.5×10^5 cell/mL, and seeded in 24-well plates (1 ml/well).

2.4 Bioavailability

Cell viability was determined by PI exclusion method： The cells were treated with various concentration of mycelia extracts for 24 hours, washed once with phosphate buffered saline (PBS), resuspended in PBS containing 4 μg/mL PI, and then analyzed by flow cytometry (FACS Calibur™, Becton Dickinson, Mountain View, CA) within thirty minutes. All experiments were performed in triplicate (Jun et al., 1998).

MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)： Human promyelocytic leukemia HL-60 cells were plated at density of 2.5×10^4cells / well in 96-well plate. The stock of the extracts of the mycelia from *A. camphorata* were diluted with medium, then added to the wells for the desired final concentrations and the final volume is 100 μl/well. After 24 hours of exposure to the extracts of the mycelia from *A*.

camphorata, 10 μl of 5 mg/mL MTT was added to each well and incubated 4 more hours under 37℃, and the liquid in the wells was evaporated. Then, 110 μl of hydrochloric acid – isopropanol (0.02 N) was added. After dissolving the precipitate, the absorbance was detected in the ELISA reader at 570 nm (Mosmann, 1983).

Ⅲ. Results and Discussion

The influence of the growth medium of mycelia from *A. camphorata* was examined using several different components in the medium. The best conditions were found to involve MEA medium (including glucose 2%, maltose extract 2%, peptone 0.1%, agar 2% and pH 4.5), as shown in Fig. 1. Glucose was preferred over fructose in the carbon source, and addition of maltose extracts was preferred over yeast extracts in the supplement (Table 1), darkness was preferred over light for eight hours (1,500 Lux) (Table 1), 25℃ was the best temperature (Table 1), and pH 4.5 was optimal (Table 1). Figure 2 indicated that at 500μg/mL no significant cytotoxicity against three normal cells (50% ethanolic extracts of *A. camphorata*) exhibited. Chang liver cell, Detroit 551 and WS-1 came from dissimilar tissues of human body provided with the differential cell characteristics and the cytotoxicity capacity, were also shown in Figure 2. HL-60 cells were treated with 50 μg/mL or 200 μg/mL extracts of mycelia, and the cell-cycle distribution was measured after 24, 48 and 72 hours of incubation.

Experimental results indicated significant inhibition of HL-60 cell proliferation by 200 μg/mL of the 50% ethanolic extracts of *A. camphorata* after 72 hours of treatment. A dose- dependent decline in *A. camphorata*-treated cell proliferation was observed as shown in Figure 3. The rate inhibition reached 47.5%. Subsequent work will focus on separation, purification, and bioavailability testing of the active components of *A. camphorata.*

Ⅳ. Conclusions

This was the first successful trial to culture Taiwanese *A. camphorata* from wild fruit bodies. The best conditions for obtaining mycelia of *A. camphorata* with the largest diameter were MEA medium, no light, 25℃, and pH 4.5. Cell proliferation results showed that the 50% ethanolic extracts of *A. camphorata* at 200 μg/mL inhibited the growth of HL-60 cells after treatment for 72 hours (IC_{50} Inhibitory concentration was observed at 250 μg/mL). The extent of inhibition reached 47.5%. No conspicuous cytotoxicity to the normal cells was observed at 500μg/mL (including Chang liver cell, Detroit 551 Human embryonic skin cell and WS-1 human skin keratinocytes). These encouraging results prompted us to further investigate if *A. comphorata* might be a good anticancer drug.

Table.1 Influence of light, temperature and pH on mycelia formation from *Antrodia camphorata*

Cultivation conditions			*Diameter (cm)
MEA	Carbon source	Glucose (2%)	7.60 ±0.18[a]
		Fructose (2%)	6.20 ±0.23[a]
	Supplements	Maltose extract(2%)	7.55 ±0.39[a]
		Yeast extract (2%)	6.15 ±0.28[a]
Environment	No light (0 Lux)		7.70 ±0.12[c]
	Light of 8 hr (1,500 Lux)		3.00 ±0.21[c]
Temperature (℃)	15℃		2.50 ±0.18[c]
	20℃		5.48 ±0.25[c]
	25℃		7.30 ±0.26[c]
pH	4.5		7.55 ±0.29[a]
	5.7		5.83 ±0.39[a]
	6.3		5.22 ±0.63[a]

*: Diameter was measured to select an assumption of round of mycelia in the well plate by using A Vernier caliper (MITUTOYO 530-102 series). [a] $p<0.05$, [b] $p<0.01$[c], $p<0.001$

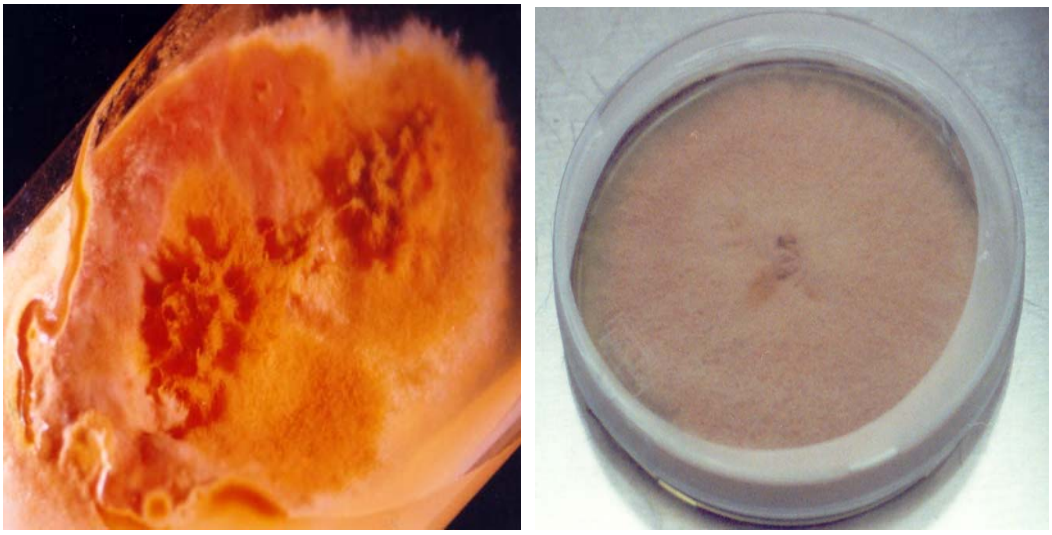

Fig. 1 Optical photography of growth mycelia of *Antrodia camphorata* cultured in MEA medium

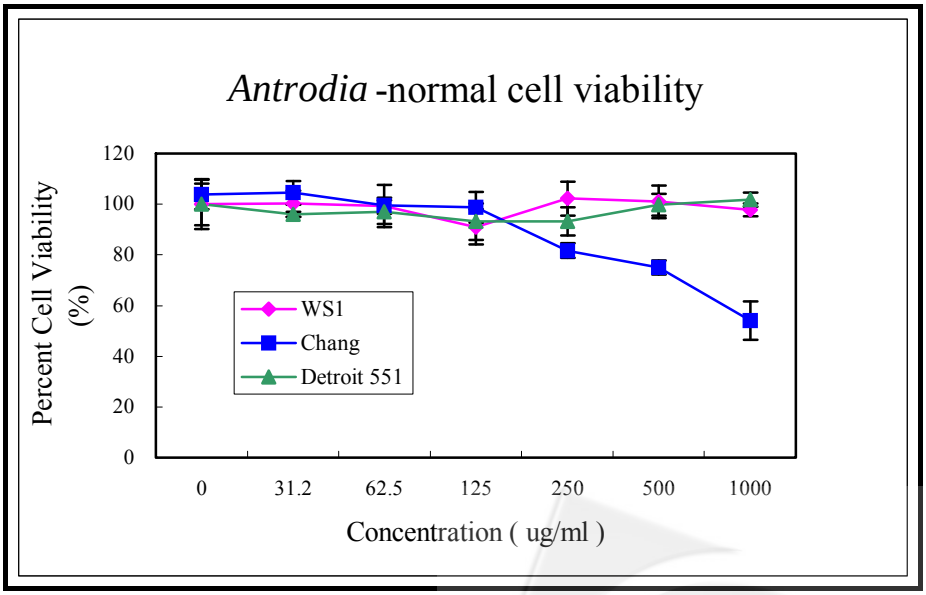

Fig. 2 Effect of *Antrodia camphorata* on normal cell viability (Chang cells, WS-1 and Detroit 551).

Note: Cellular viability was measured by MTT assay. Cells were exposed to indicated amounts of *Antrodia camphorata* for 24 hours. Viable cells in various treatments were expressed as a percentage of control. Data points represented means ± S.D. for three independent experiments.

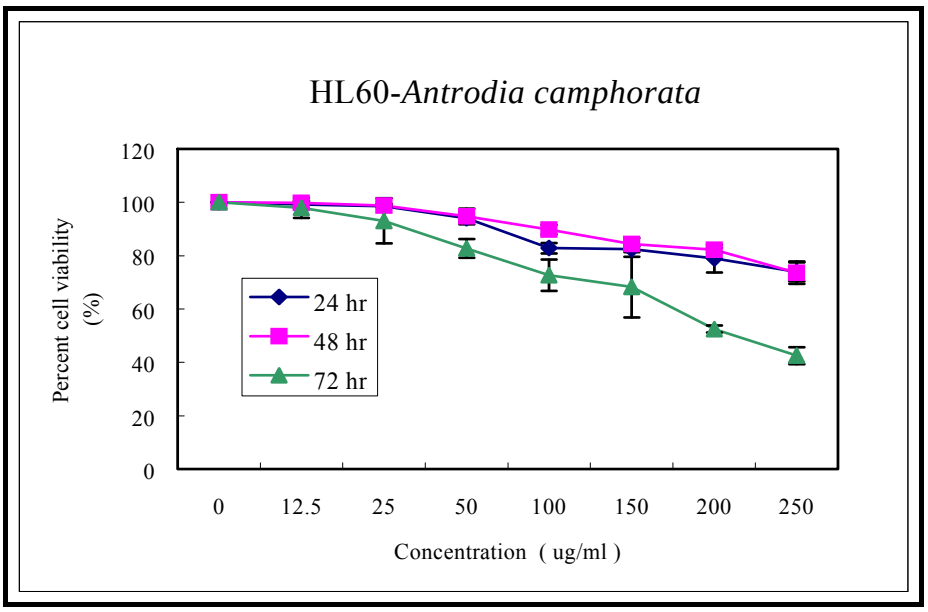

Fig. 3 Effect of ethanol extract of *Antrodia camphorata* on HL-60 cells viability

Note: Cellular viability was measured by PI exclusion. Cells were exposed to indicated amounts of extracts from *Antrodia camphorata* for 24, 48 and 72 hours. Viable cells in various treatments were expressed as a percentage of control. Data points represented means ± S.D. for three independent experiments.

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台灣樟芝之組織培養及生體可用性

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論文摘要

本研究以台灣產樟芝新鮮子實體為組織培養之先導樣品，循真菌類組織培養模式加以改良，探討培養基內容物及培養環境對菌絲體生長速率之影響(如：碳源、其他營養物質及溫度、光線、酸鹼度等環境因素)，並由所誘發生長之菌絲體，經傳統成分抽提法所得之粗抽物，對其進行癌細胞毒殺試驗及生體可用性評估。經實驗結果顯示，樟芝菌絲體之培養以 MEA 培養基、暗培養、25℃、pH 4.5 的條件下生長狀況較為良好。以 50% 酒精水溶液抽提之菌絲體於 200 μg/mL 劑量濃度下，對 HL-60 血癌細胞株有明顯的抑制及毒殺作用，且在 500 μg/mL 內不影響人類正常細胞（Chang liver cell, Detroit 551,and WS-1）的生長。

關鍵詞—樟芝、組織培養、生體可用性、細胞毒殺作用