行政院國家科學委員會補助專題研究計畫成果報告

牛樟芝各抗腫瘤有效成份的分離、純化，誘發腫瘤凋亡及其作用機轉的研究

Isolation and purification of the antitumor components from Antrodia camphorata and studies of their effect and mechanism on the induction of tumor apoptosis

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摘要

近年來食用菇類成為引人注目之機能性食品及藥物發展之來源，本計劃著重於樟芝（Antrodia camphorata; A. camphorata），一種寄生於台灣海拔450~1200公尺牛樟樹(Cinnamomum kanehirai Hay)上多孔科無褶菌目之新種擔子菌。由於坊間傳說其具有解毒、強身、治療肝病變、及各種癌症之作用，不過其功效僅止於口傳及偏方。由於對樟芝之成份分析、及生物醫學作用之文獻資料相當稀少，因此本研究旨在探討其免疫增強作用及抑瘤生長之功效。

首先從 A. camphorata 之菌絲體中抽取其多醣體(AC-PS), 再由健康人之週邊血液分離出免疫細胞— 單核細胞 (Mononuclear cells, MNC), 並以不同濃度之AC-PS 去刺激 MNC 以製備條件培養液 (AC-PS-MNC-CM)。然後，培養人類白血病 U937 細胞株，作為腫瘤標的細胞，以觀察 AC-PS 之免疫增強作用與抑瘤活性。此外，使用 ICR 品系的 小鼠，餵食樟芝菌絲多醣不同劑量後，觀察 AC-PS 對植有 Sarcoma 180 小鼠，其腫瘤抑制作用。

研究結果顯示，在體外(in vitro)實驗中，AC-PS 對血液單核細胞有明顯之活化作用。經由 AC-PS (100 μg/ml) 刺激而製備之 AC-PS-MNC-CM 具有明顯的抑制 U937 白血病細胞增生之作用，其抑瘤率高達 55-60 %。與 AC-PS-MNC-CM 相比，AC-PS (樟芝多醣體)本身或未經活化(靜態)之 MNC 的條件培養液 (Normal-MNC-CM) 則無抑瘤生長作用。此外，AC-PS-MNC-CM 尚可誘導 U937 白血病細胞分化為成熟的單核球~巨噬細胞，並具有吞噬活力及產生超氧化物之功能。在體內(in vivo)實驗中，Balb/C 小鼠在給予樟芝菌絲多醣 1 個月後，脾臟細胞之增生能力及非特異性自然殺手細胞活性有顯著的增加。此外，對植有 Sarcoma 180 的 ICR 小鼠，無論是經由腹腔亦或是經餵食樟芝菌絲多醣後，其腫瘤抑制作用具有 Dose-dependent 現象。

綜上所述，本研究證示樟芝多醣體有顯著的活化免疫細胞之功能，其抑瘤作用乃係經由提升免疫機能而達成。

關鍵詞：樟芝，多醣體，抗腫瘤活性，細胞凋亡
ABSTRACT

*Antrodia (A.) camphorata* is a popular folk medicine and has attracted great attention due to its fame for antitumor activity against several types of cancer. However, there is little biological information available about its action. The present study was to ascertain the immunomodulating and anti-tumor effects of *A. camphorata*. Polysaccharides (PS) composition of *A. camphorata* mycelia, harvested by submerged cultures, were isolated and used to study its effect on human mononuclear cells (MNCs). Our results had shown that the mononuclear cell-conditioned media (MNC-CM) treated with *AC-PS* (100 μg/ml) were found to suppress the proliferation of U937 leukemic cell line. However, *AC-PS* alone had no such effect 200 μg/ml. Since untreated mononuclear cells produced little or no cytokine, and normal MNC-CM did not suppress leukemic cell growth, it was suggested that the anti-tumor activity of *AC-PS* is derived from the activation of human mononuclear cells. The *in vivo* activity of splenocytes and nature killer cells also enhanced after orally administrated *AC-PS* for one month. The *in vitro* antitumor activity was substantiated by the *in vivo* therapeutical study of *AC-PS* in sarcoma 180-bearing mice. Intraperitoneal and oral administration of *AC-PS*, 100mg/kg and 200mg/kg significantly suppressed the tumor growth with inhibition rate 69.1% and 58.8%, respectively.

Keywords: *Antrodia camphorata*, polysaccharides, Antitumor activity, Apoptosis
Introduction:

Edible mushrooms have been used as flavorful foods and health nutritional supplements for several centuries. In Chinese, some mushrooms are especially treasured as medicine substances for health and longevity. However, systemic studies of its bio-function were not performed until the last third of past century when biochemical technology available for dissecting these traditional medicinal mushrooms and isolating their most active anticancer constituents. Although a number of bioactive molecules have been identified in numerous mushroom species (Mizuno, T. et al., 1995), polysaccharides have been established to be the most promising pharmacologically active antitumor compounds (Mizuno T. at al.,1992; Jong S.C. et al., 1993). In the last few decades, the biological activities of polysaccharides have attracted more and more attention in its immunomodulatory and antitumor effects. For example, in vitro, the study of Wang had shown that polysaccharides-rich fraction from *Ganoderma (G.) Lucidum* had strong stimulatory effects on both macrophages and T-lymphocytes in various cytokines releasing (Wang et al., 1997). Several investigators have demonstrated that partially purified polysaccharides of *G. Lucidum* could significantly inhibit the growth of implanted Sarcoma 180 in animal (5).

*Antrodia (A.) camphorata* (AC), a parasitical microorganism on the inner cavity wall of local evergreen *Cinnamomum kanchirai* Hay (Lauraceae), is a species known only from Taiwan. It was initially identified by Zang and Su as a new *Ganoderma* species in 1990 (Zang and Su, 1990), but was identified as a new basidiomycete *Antrodia camphorata* in the Polyporaceae lately (Wu et al, 1997). This species is well known in Taiwan under the name “niu-chan-ku” or “chang-chih”, and is also popular in Taiwan. However, because its host wood is a local species and is getting scarce, *A. camphorata* is difficult to find in the forest and is very expensive. Since the similarity of *A. camphorata* and *Ganoderma* species in several characteristics, *A. camphorata* is said to have many medicinal uses, such as food, alcohol, and drug intoxication, itching illness and especially cancer. Although phytochemical investigations have resulted in the isolation of a series of new steroid acids and triterpene acids (Chiang, H.C. at al, 1995; Cherng, I.H. et al, 1995; Cherng, I.H. et al, 1996;
Yang, S.W. et al, 1996; Shen, Y.C. et al, 1997), there are few reports about the biological activity of *A. camphorata*. Regarding its antitumor activity, only in the study of Chen and Yang has demonstrated that the crude extract of *A. camphorata* possessed *in vitro* cytotoxicity against P388 murine leukemia cells (13). In this study, we extracted polysaccharides enriched fraction from the wild air-dried *A. camphorata* mycelia, harvested from submerged cultures, and analyzed its effects on the functions of human blood mononuclear cells (MNCs) and on the growth of leukemic cell *in vitro*. Finally, we treated tumor-bearing mice with AC-PS to evaluate the *in vivo* antitumor activity of AC-PS against mouse sarcoma 180. Our results showed that cytokine production by blood MNCs was greatly increased after treatment with AC-PS and AC-PS-PK. The proliferation of leukemic cell was not affected by AC-PS directly, but was significantly inhibited by the conditioned medium from AC-PS activated blood mononuclear cells (PSG-MNC-CM). *In vivo*, the growth inhibition of sarcoma 180 was also observed by AC-PS administration.
Effects of AC-PS and AC-PS-MNC-CM on leukemic cell proliferation.

Table 1 demonstrated the growth of U937 cells in the presence or absence of various additives. After 5 days of incubation, the number of U937 cells in untreated culture gradually increased from $1 \times 10^5$ /ml up to $30.9 \times 10^5$ /ml with viability $\geq 98\%$. There was no change in cell proliferation in the culture treated with 100 $\mu$g/ml of AC-PS alone, yet, even at a higher dose of AC-PS (200 $\mu$g/ml) no significant effect on leukemic cell growth observed (Table 1). However, the proliferation of the U937 cells was inhibited by treatment with AC-PS-MNC-CM. As shown in Table 1, the inhibition of U937 cells growth by AC-PS stimulated MNC-CM is highly significant ($P<0.001$) and displays a dose-dependent manner. At the concentration of 100$\mu$g/ml of stimulants, 30% (vol/vol) stimulated-MNC-CM greatly suppress the leukemic cell growth, resulting in an inhibition rate of 55.3% for AC-PS. As negative control, normal MNC-CM had no significant suppressive effect on leukemic cell growth at comparable concentrations (Table 1).

Effect of AC-PS on Tumor Growth in Mice. In parallel with growth inhibition of leukemic cells, the antitumor activity against sarcoma-180 tumor in ICR mice model by AC-PS was also significant. As shown in Table 3, the mean tumor volume reached 2367.9 $\pm$ 395.4 mm$^3$ in the saline-treated control 4 weeks after tumor implantation. In contrast, a significant inhibition of tumor growth was observed in those mice orally administrated 50 mg/kg AC-PS (mean tumor volume was 1857.5 $\pm$ 300.2 mm$^3$). The potency of tumor growth inhibition by AC-PS increased as the administration dose increased, mean tumor volumes were 1429.0 $\pm$ 263.1 and 976.1 $\pm$ 119.8 mm$^3$ for 100mg/kg and 200mg/kg respectively. Intraperitoneal (IP) injection of AC-PS inhibited tumor growth even more effective than oral administration, thus a lower dose (25mg/kg) would achieve the same therapeutic efficacy as 50mg/kg by oral administration (Table 3). The inhibition rate for 50 and 100mg/kg of IP administration were 49.2% and 69.1%, respectively. Neither oral nor IP administration of AC-PS caused side effects such as reduced food intake or body weight gain change in tumor-bearing mice.
Table 1. Comparison of Growth Inhibition Effect of AC-PS Alone, Non-stimulated and Stimulated MNC-CM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell number (×10^5/ml)</th>
<th>Percent of inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>30.9 ± 0.6</td>
<td>-</td>
</tr>
<tr>
<td>AC-PS alone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>30.2 ± 0.7</td>
<td>1.8</td>
</tr>
<tr>
<td>200 µg/ml</td>
<td>29.4 ± 0.5</td>
<td>5.0</td>
</tr>
<tr>
<td>MNC-CM (30%; vol/vol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-stimulated</td>
<td>28.4 ± 1.2</td>
<td>8.4</td>
</tr>
<tr>
<td>AC-PS stimulated (100 µg/ml)</td>
<td>13.8 ± 0.6</td>
<td>55.3</td>
</tr>
</tbody>
</table>

*a* Leukemic U937 cell (1×10^5/ml initially) were incubated at 37℃ for 5 days in the presence or absence of AC-PS, non-stimulated or stimulated MNC-CM. Data are the mean ± S.E.M. of 5 separate experiments. *b* Percent of inhibition = (1—treated / untreated control)×100%. *c* p<0.001 compared with Non-stimulated MNC-CM.

Table 2 Effects of Intraperitoneal (IP) and Oral Administration AC-PS on Tumor Volume in Sarcoma 180–bearing Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor volume (mm^3)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP Dose, mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2249.5 ± 375.7</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>1486.0 ± 240.1</td>
<td>33.9</td>
</tr>
<tr>
<td>50</td>
<td>1143.2 ± 210.5</td>
<td>49.2</td>
</tr>
<tr>
<td>100</td>
<td>716.9 ± 140.3</td>
<td>69.1</td>
</tr>
<tr>
<td>Oral Dose, mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2367.9 ± 395.4</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>1857.5 ± 300.2</td>
<td>21.6</td>
</tr>
<tr>
<td>100</td>
<td>1429.0 ± 263.1</td>
<td>39.7</td>
</tr>
<tr>
<td>200</td>
<td>976.1 ± 119.8</td>
<td>58.8</td>
</tr>
</tbody>
</table>
Discussion

The antitumor activity of polysaccharides isolated from medical fungi were most attractive due to their low toxicity to normal cells and the apparent lack of side effects in clinical patients (14,15). *A. camphorata*, a new basidiomycete of the polyporaceae identified in 1990, is well known in Taiwan as a traditional Chinese medicine. This medical fungus has attracted great attention due to its fame for antitumor activity against several different cancers, including ovary, gastric, breast, and liver cancers. However, because its host wood, *Cinnamomum kanchirai* Hay, is a local species and is getting scarce, *A. camphorata* is becoming difficult to find in the forest and is very expensive for medical use. So far, there is little information available about antitumor activity of *A. camphorata*. Only Chen et al. had performed in vitro tests to demonstrate its antituomr activity (13). In the present study, we isolated the polysaccharides-rich fraction from submerged culture of *A. camphorata* and demonstrated its immuno-stimulatory effects. The observed MNC-stimulating effects of *AC-PS* was not due to the endotoxin contamination, since the effect was not blocked by the addition of polymyxin B to *AC-PS*-containing cultures. Polymyxin B is known to be a specific and potent inhibitor of LPS (Arend et al., 1989; Slagle et al., 1989).

Previously, many polysaccharides isolated from different medical fungi have been shown to have antitumor activity. Among them, Lentinan from *Lentinus edodes* (Berk.) Sing. (Suga et al., 1984), *PS-K/PS-P* from *Coriolus versicolor* (Mizushima et al., 1982; Yang et al., 1993) and *PS-G* from *Ganoderma lucidum* (Sone et al., 1985; Furusawa et al., 1992) are three important fungal polysaccharides that have been widely used in antitumor investigations. Many investigators have suggested the antitumor action of polysaccharides, instated of direct toxicity to tumor cells, is mediated by immunomodulating effect. For example, polysaccharides from *Ganoderma Lucidium* and *Coycydeps Sinensis* have been shown to be able to inhibit the proliferation of leukemic cells via stimulating the production of cytokines by activated MNCs (Wang S.Y. et al., 1997; Chen Y.J. et al., 1997). As showed in Table 1, *in vitro* study showed that the growth inhibition of U937 cells could only be induced by *AC-PS-MNC-CM*, but not by *AC-PS* alone even at doses up to 200 μg/ml. This is consistent to the previous reports that antitumor effects of polysaccharides may through the indirect pathway by activating the host immune response.

Although the correlation between composition of polysaccharides and its antitumor activity was not clear, the essential structures for the antitumor activity of polysaccharides were reported to be a branched glucan with a core involving (1 → 3)-β-, (1 → 4)-β- and (1 → 6)-β-linkages (Miyazaki and Nishijima, 1981; Chihara, 1992). The polysaccharides we used in the present study was a partial purified polysaccharides fraction. Its exact composition for the activation of blood MNCs remain to be further identified. However, other investigator has shown that glucan fraction of *A. camphorata* is composed of a backbone of (1 → 3)-linked β-D-glucopyranosyl residues (Huang, L. C., 2000).
addition to the core glucan, several experimental studies have also shown that protein residues, which consisted 20~40% of polysaccharides fraction, are responsible for part of their activity (Tsukagoshi, S. et al., 1984; Yang, Q.Y. et al, 1992). Therefore, it is important to further clarify whether protein residues are responsible for immuno-stimulation and antitumor activity of AC-PS or not. In parallel with growth inhibition of leukemic cells, the antitumor activity against sarcoma-180 tumor in ICR mice model by AC-PS was also statistically significant. Tumor volumes were significantly reduced by both intraperitoneal and oral administration of AC-PS (Table 3) without any reduction of body weight gain.

Although *A. camphorata* was a popular folk medicine and had attracted great attention due to its fame for antitumor activity against several types of cancer, there is little information available about its action. This is the first report described both *in vitro* and *in vivo* evidences about the therapeutical potential of this medical fungus against tumor. Our results suggested the antitumor activity of *A. camphorata* may through the activation of host immune response. However, further investigation about the relationship between immunomodulation and antitumor activity is needed.
Reference

25. Huang, L. C. Antioxidant properties and polysaccharide com-position analysis of *Antrodia camphorata* and *Agaricus blazei*. Thesis, National Chung-Hsing University, Taichung, Taiwan, **2000**; pp 63-76.
Contribution and Significance

1. In this report, we clearly demonstrated that AC-PS has an indirect antitumor effect by activating the host immune response.
2. It is the first time to demonstrated the in vivo antitumor activity of AC-PS through the systemic studies.