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ANTI-TUMOR METASTATIC COMPONENTS IN *TRAPA TAIWANENSIS* NAKAI AND *LIVISTONA CHINENSIS* R. Br.[1]

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Su-Ying Liu, Chao-Chin Hsu and I-Ching Ho (1986) Anti-tumor Metastatic Components in *Trapa taiwanensis* Nakai and *Livistona chinensis* R. Br. *Bull. Inst. Zool., Academia Sinica* **26**(2): 143-150. The hot water extracts of traditional Chinese medical herbs, *Trapa taiwanensis* Nakai and *Livistona chinensis* R. Br. showed significant blocking effects on the experimental metastatic behavior of B_{16} melanoma F_{10} cells in C57BL/6 mice. The active components extracted from the seeds of the plants consisted of proteins and carbohydrates. The anti-metastatic mechanism could be due to the activation effects of the components on the host immune system.

The lethality of most cancers could be attributed to their ability to spread to distant organs where they produce metastases. The present clinical treatments, including surgery removal, radiotherapy and chemotherapy limited in their regionality and undesirable side effects. To control metastasis by immunological means suggested an appropriate clinical reinforcement in cancer therapies.

Some of the traditional Chinese medical herbs did possess prominent therapeutic values in cancer treatments via unknown mechanisms. The well-known 1, 3-β-glucan purified from *Lentinus edodes*, *Poria cocos* Wolf and *Ganoderma lucidum* demonstrated immunological enhancing effects on cancer patients via r-IFN induction (Chihara *et al.*, 1970a, b; Cheng *et al.*, 1986). It was known that IFN could inhibit the growth of tumor *in vitro* (Paucker *et al.*, 1963) and enhance T-cell, macrophage and NK cell mediated cytotoxicities (Lindahl *et al.*, 1972; Farrar *et al.*, 1981; Rabinovitch *et al.*, 1977; Pace *et al.*, 1983; Djeu *et al.*, 1979; Fresa *et al.*, 1986; Shigeru, 1982). Therefore, it had been postulated that IFN inhibited the growth of tumor *in vivo* by direct inhibition of cell multiplication and augmentation of host immune responses. There were evidences demonstrated that linear 1, 3-β-glucan from *Alcaligenes foecalis var myxogenes* could activate polymorpho-nuclear leukocytes and exerted tumoricidal activities via Ca^{++}-dependent hydrogen peroxide mediator (Morikawa *et al.*, 1985 a, b; 1986). 1, 3-β-glucan with 1, 6-branches from culture of *Schizophyllum comonune* Fries showed nonspecific activation of macrophages (Yamamoto *et al.*, 1981). There required a certain molecular structure for the

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induction of cytotoxicities of different cell types by glcans. Levan, a polyfructose prepared from *Aerobacter levanicum*, induced a host-dependent tumor rejection (Leibovici *et al.*. 1979) and a direct effect on tumor cells (Leibovici *et al.*, 1980). BCG, *P. acnes*, Zymosan A and Nocardia CWS represented another group of immunomodulators which were microorganisms themselves or crude cell wall preparations. They also showed very potent activation effects on PMN (Morikawa *et al.*, 1984) and r-IFN induction (Izumi *et al.*, 1986).

The seeds of *Trapa taiwanensis* Nakai was a common dish in Chinese families and *Livistona chinensis* R. Br. was an ordinary Chinese medicine. The hot water extracts of the plants showed significant blocking effects on the experimental metastasis of murine B_{16} melanoma F_{10} cells in C57BL/6 inbred mice. The active components were also the members of the immunomodulators.

MATERIALS AND METHODS

Animals

Six to 8-week-old male C57BL/6 mice were obtained from Experimental Animal Center, National Taiwan University.

Tumors

Murine B_{16} melanoma cells were kind gifts of Dr. I. J. Filder and were maintained in DMEM containing 10% heat inactivated FCS.

Trapa taiwanensis Nakai

Seeds were purchased in the local market.

Livistona chinensis R. Br.

Seeds were kind gift of Taiwan Forestry Research Institute.

Preparations of *T. taiwanensis* and *L. chinensis*

Seventy-five grams of the seeds were chopped into pieces and soaked in 500 ml deionized water and boiled to the final volumes of 100 ml. The hot water extracts were then centrifuged at 10,000 rpm for 15 min at 4°C by Sorvall RC-5B Refrigerated Superspeed Centrifuge and the supernatants were filtered through Whatman #1 filter paper. The clear filtrates were then lyophilized.

Characterizations of the extracts

Spectroscanning: From 190 to 700 nm by a Hitachi 220 Spectrophotometer.

Total sugar content determination: By phenol-sulfuric acid method (Du Bois *et al.*, 1956).

Monosaccharide determination: The extracts were hydrolyzed in 2N H_2SO_4 at 105°C for 6h under vacuum, and the neutralized and concentrated hydrolysates were separated by TLC in butanol : pyridine : acetic acid : ethanol : water=10 : 3 : 3 : 3 : 4 system.

Total protein content determination: By Lowry's method.

Amino acid determination: The extacts were hydrolyzed in 6N HCl at 105°C for 24h under vacuum. The deacided hydrolysates were dissolved in citrate/sucrose buffer (pH 2.2) and subjected onto a Biotronik LC5000 amino acid autoanalyzer.

SDS PAGE: 10% SDS polyacrylamide gel electrophoresis was performed in the buffer of the Laemmli's system and stained by silver nitrate.

Cytotoxicities of the extracts on B_{16} melanoma F_{10} cells

Fifty of the B_{16} melanoma F_{10} cells were plated in wells of the 24 well multidish in 0.5 ml of DMEM (with 10% FCS) containing various concentrations of the lyophilized extracts, and cultured under humid air containing 10% CO_2 at 37°C. Seven days later, the dishes were washed with HBSS and stained in 0.01% Commassie blue in 50% methanol and 7% acetic acid. The colonies visualized were counted and expressed in percent of control.

Toxicities of the extracts in C57BL/6 mice

Mice were given 0.2 ml hot water extracts at various concentrations or 0.2 ml PBS in control group orally and the lethalities were observed for 48h.

Experimental metastasis

Mice were given 0.2 ml hot water extracts at the concentration of 5 mg/ml orally 24h before the injection of 5×10^4 F_{10} cells in 0.2 ml PBS into the lateral tail vein. Afterwards, same doses of the extracts were given every other day, and the mice were sacrificed by cervical dislocation 3 weeks later. The tumor nodules on the lung surfaces were counted under a stereomicroscope, and the spleens were weighed.

The stimulation of proliferation of the mouse spleen cells by the extracts

Mice were sacrificed by cervical dislocation and the spleens were dissected out of the body cavity aseptically, and washed with PBS and minced in RPMI-1640. The spleen cells were pelleted and red blood cells were lyzed.

Total splenocyte study: 1×10^6 spleen cells were cultured in each 24 wells of the mutidish in the presence of various concentrations of the extracts in RPMI-1640 containing 10% heat inactivated FCS for 96h. Then, 0.25 μCi H^3-thymidine (Amersham; Specific activity 86 Ci/m mole) were added in each well and labeled for 4h. The acid insoluble radioactivities were counted by a LKB 1211 Rackbeta liquid scintillation counter.

Nonadherent splenocyte study: Spleen cells were allowed to attach onto the plastic culture flasks for 2h and the nonadherent cells were collected and studied as the total splenocyte.

Cytostatic activities of the extracts activated spleen cells

Spleen cells were incubated with various concentrations of the extracts for 4 days. The washed spleen cells were then incubated with F_{10} cells in the ratio of 100 : 1, 50 : 1 and 10 : 1 for 2 days. The extensively washed, attached F_{10} cells were then labeled with 0.025 μCi H^3-thymidine (Amersham; Specific activity 86 Ci/m mole) per well for 2h. The acid insoluble radioactivities were counted by a LKB 1211 Rackbeta liquid scintillation counter.

RESULTS

The characteristics of the hot water extracts of *T. taiwanensis* and *L. chinensis* were shown in Table 1. Both were consisted of

TABLE 1
The physico-chemico properties of hot water extracts of *T. taiwanensis* and *L. chinensis*

		T. taiwanensis	*L. chinensis*
Nature (lyophilized)		yellowish powder	brown gum-like texture
Maximal optical absorbance (nm)		213.6, 263.7	213.8, 236.0, 238.2, 280.0
Content	carbohydrate	41.7%	59.1%
	protein	17.0%	50.0%
Amino acids		Asp. Ser. AsN. GlN. Pro. Gly. Ala. Val. Cys. Met. Ile. Leu. Tyr. Phe. Lys. His. Arg.	Asp. Thr. Ser. Glu. Pro. Gly. Ala. Val. Ile. Leu. Tyr. Phe. Lys. His. Trp. Arg.
Monosaccharides		Glu, Gal, Man, 1 unidentified spot	Glu, Gal, Man
Solubility		soluble in water, insoluble in organic solvents	soluble in water, insoluble in organic solvents
Molecular weights	carbohydrate	65.0 K	—
	glycoprotein	30.0 K	>100 K(2), 20 K
	protein	21.5 K	46 K, 42 K, 32 K

protein and carbohydrate. The absorption spectrum showed absorbance maximum of *T. taiwanensis* at 213 and 260 nm, and *L. chinensis* at 214, 230 and 280 nm. After acid hydrolysis and resolved by TLC, *T. taiwanensis* and *L. chinensis* were found to be composed of three monosaccharides in common, but there was one more unidentified monosaccharide in *L. chinensis*. Both SDS PAGE and Sephadex G-100 gel filtration chromatography (data not shown) demonstrated multiple components in each of the hot water extracts.

The hot water extracts were rather nontoxic both *in vitro* and *in vivo*. As shown in Table 2, orally given dose could be as high as 500 mg/ml and the cytotoxicities were at mg level.

These relatively nontoxic extracts did show significant blocking effects on experimental metastasis. The tumor nodules on lung surface decreased significantly by both treatments (Fig. 1), but the inhibitory activity was much more potent by the extract from *L. chinensis*.

The spleen weights of the mice of both treatments increased (Fig. 2). The trophic effects of the extracts could be due to their immunomodulating functions.

Murine spleen cells were actually stimulated to proliferate by the extracts *in vitro* (Fig. 3). The optimal dose was 0.003 mg/ml for 4 days incubation period, and the stimulations were declined significantly at the 7th day of incubation (data not shown).

Table 2
LD_{50} of the extracts on B_{16} melanoma cells and in C57BL/6 mice

A. LD_{50} for B_{16} melanoma F_{10} and F_1 cells *in vitro*

	F_{10} cells	F_1 cells
T. taiwanensis	1.00 mg/ml	1.80 mg/ml
L. chinensis	0.25 mg/ml	0.375 mg/ml

B. LD_{50} in C57BL/6 mouse (B. W.=20±2 g)

T. taiwanensis	>100 mg/mouse
L. chinensis	100 mg/mouse

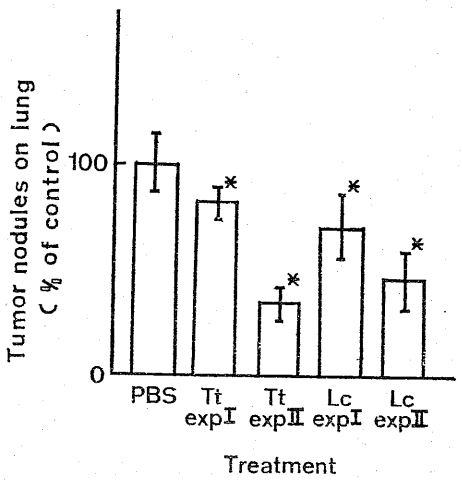

Fig. 1. Tumor nodule numbers on lung surface. 5×10^4 F_{10} cells were injected i. v. into C57BL/6 mice and 1 mg of the hot water extracts were given orally every other day. The mice (6 in each group) were sacrificed 3 weeks later and tumor nodules were counted and expressed as percent of control. Tt: *Trapa taiwanensis* Nakai, Lc: *Livistona chinensis* R. Br. The differences were significant at $p<0.01$ level by Students's T test.

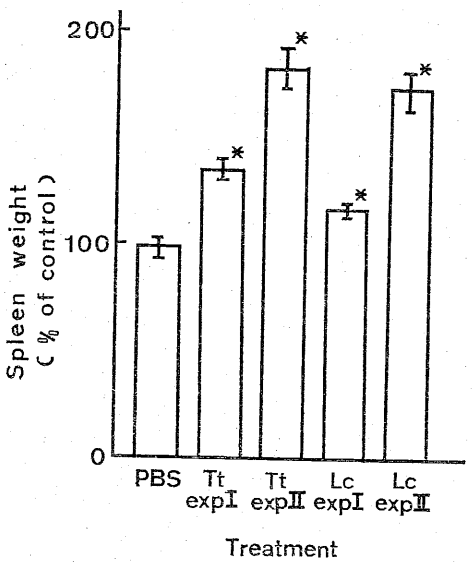

Fig. 2. Spleen weights of the mice after 3 weeks of treatments as which were depicted in Fig. 1. The differences were significant at $p<0.01$ level by Students' T test.

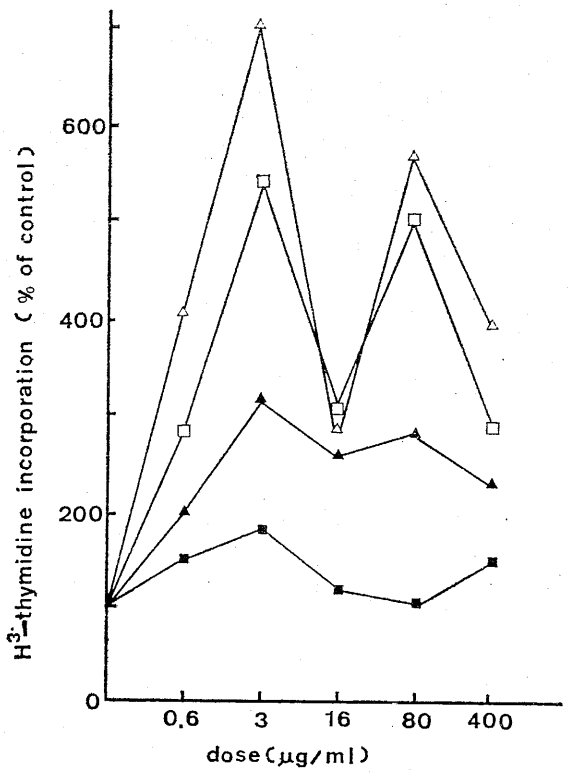

Fig. 3. Proliferation stimulations of the extracts on spleen cells after 4 days of incubation. H^3-thymidine (0.25 μci/well) was then added to measure the proliferation increases in spleen cells. △ *L. chinensis* extract on total spleen cells, □ *T. taiwanensis* extract on total spleen cells, ▲ *L. chinensis* on nonadherent cells, ▩ *T. taiwanensis* on nonadherent cells.

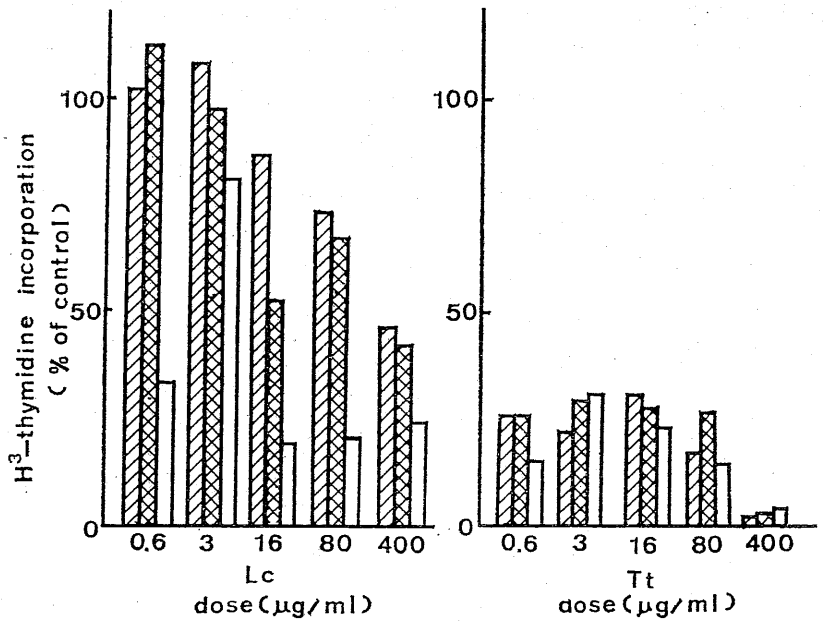

Fig. 4. Cytostatic effects of total spleen cells on F_{10} cells in culture. The spleen cells were preincubated with the extracts for 4 days and then incubated with F_{10} cells at various ratios for 2 days. Spleen cells were removed by extensive washing and the survived F_{10} cells were then measured by H^3-thymidine (0.025 μci/well) incorporations. ▨ Target: effector ratio of 1:10; ▩ Target: effector ratio of 1:50; □ Target: effector ratio of 1:100.

The activation of spleen cells by the extract of *L. chinensis* was dose related in terms of tumor cell killing activity *in vitro* (Fig. 4). When the effector: target ratio was 100 : 1, the extract at the dose of 0.6 mg/ml was sufficient to activate the effectors. When the dose increased to 80 mg/ml, the effector: target ratio of 10 : 1 was effective enough to kill the targets significantly. On the other hand, the extract from *T. taiwanensis* was much more potent, but the dose relationship was not significant.

DISCUSSION

The hot water extracts of *T. taiwanensis* and *L. chinensis* were composed of both carbohydrate and protein. The optical absorbance maximum at 214 nm, 230 nm and 280 nm could be due to the presence of polypeptide, but there was one absorbance maximum at 260 nm in *T. taiwanensis* which suggested the presence of nucleic acid.. The boiling process should have denatured all the proteins, therefore, the carbohydrate should play an important role in the anti-tumor metastatic effects of the extracts. The purification of the active component was currently underway in our laboratory.

The increased metastasis observed after generalized immunosuppression (Seshadri *et al.*, 1979) or suppression of T cell activity (Eccles *et al.*, 1979) and macrophages (Jones *et al.*, 1977) and in disfunction of NK cell activity (Talmadge *et al.*, 1980) indicated the roles of these components of the immune system in controls of metastases. The biological response modifiers (BRM) which were active in modifying the activities of these cells, such as BCG and *Corynebacterium parvum* did show significant values (Crowther *et al.*, 1978; Albert *et al.*, 1978) in clinical trials.

The relatively low tumoricidal activities shown by both extracts of *T. taiwanensis* and *L. chinensis* indicated that most of their *in vivo* anti-tumor metastasis effects resulted from the enhancement of the immuno-responses of the hosts. Indeed, they did stimulate the proliferation of the spleen cells much more potently than PHA (data not shown) and the stimulated spleen cells could kill the tumor cell efficiently.

When incubated the extracts with human peripheral blood leukocytes, the preliminary data showed that in normal persons, both the extracts of *T. taiwanensis* and *L. chinensis* increased OKT4, Tac and Leu 11b binding cell subsets and decreased OKT8 binding cell subset in a dose response manner. While in gynecological cancer patients, the Tac and Leu 11b binding cell subsets were stimulated by both the extracts. In the OKT8 binding cell subsets, *L. chinensis* extract increased it significantly but *T. taiwanensis* extract showed individual variation with no discernible pattern. There seemed to have a certain correlation of the patients' responses to the extracts with their prognosis. Due to the limited sample sizes, no definite conclusions could be made at present. However, there was a consistant tendency in increasing IL-2 stimulated cytotoxic T and NK cells by these extracts. Thus, they could be credible as immunomodulators. Whether the function of the extracts was via r-IFN induction was not known. The easy preparation and routine oral administration of these extracts could be relevant for their clinical applications.

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菱角及蒲葵種子內之抗癌轉移物質

劉素瑩 許朝欽 何宜靜

中國傳統中藥之菱角及蒲葵種子之熱水抽水物具有抑制 B_{16} 黑色素細胞癌 F_{10} 細胞在 C57BL/6 純種老鼠內實驗轉移的能力。此具有活性之抽出物含有蛋白質及碳水化合物，此抽出物之抗癌轉移作用之機制可能是經由其加强寄主免疫系統之能力。