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Growth Inhibition and Differential Induction of Human Leukemic U937 Cells by Chinese Medicinal Herbs

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ABSTRACT

Effects of boiling-water extracts and 80% (v/v) ethanol extracts from Chinese medicinal herbs on the antiproliferation and differentiation of human leukemic U937 cells were examined. Conditioned medium from peripheral blood monocytes (MNC-CM) stimulated with various levels (200-800 μg/mL) and types of herb extracts (MNC-CM mode) were used to screen the effective herbs. Results showed that among the herbs tested, MNC-CM from boiling-water extracts of Huang Gi (HG), Gan Cao (GC), Lian Zi (LZ), Da Zao (DZ), and Tang Shen (TS) showed remarkable (> 40%) growth inhibition on the U937 cells at high dosage, whereas mixtures of boiling-water extracts of tested herbs with culturing medium displayed insignificant results. Meanwhile, inferior inhibition on cell proliferation was observed when U937 cells were treated with ethanol extracts with MNC-CM or conventional mode. Assays for cytoplasmic superoxide production (NBT reducing ability) by U937 cells induced by ethanol precipitates of boiling-water extracts (100-400 μg/mL) from LZ indicated a dose-dependent positive percentage (31-49%), revealing the apparent differentiation-inducing effect of MNC-CM on U937 cells.

Key words: growth inhibition, differentiation, U937 cell, medicinal herb, condition medium, NBT reducing ability

INTRODUCTION

Use of medicinal herbs has been recorded in oriental countries and has recently attracted attention as one of the alternative, possibly more effective, cancer therapies. In immunologically oriented studies, proteins, polysaccharides and protein-bound lipopolysaccharides of herbs have been of interest for many years[1]. Juzen-Taiho-To showed mitogenic and complement-activating activities. Among the herbal components, polysaccharide fraction from Glycyrrhizae Radix contributed more than the other nine herbs[2]. Some Chinese medicinal herbs also display immunomodulating activity on cytotoxic T-lymphocyte (CTL) and enhancement of immunoglobulin (Ig) production by B-cells[3-4], interleukine (IL)-1 production by monocytes[3], IL-2 and tumor necrosis factor (TNF) production by macrophages[4].

Human peripheral blood mononuclear cells (MNC) contain macrophages, natural killer cells, T-lymphocytes, and B-lymphocytes. MNC is a model frequently used to study the immunomodulatory effect of bioactive components from food or herbal sources *in vitro* through the use of MNC-stimulated conditioned medium (MNC-CM) (MNC-CM mode)[5-7]. Wang *et al*. (1997) and Chen *et al*. (1997) observed that MNC-CM prepared with *Ganoderma lucidum* and *Cordyceps sinensis* was effective in inhibiting growth and differential induction of human leukemic U937 cells[5-6]. Cytokines such as IL-1β, TNF-α, and interferon (INF)-γ were present in MNC-CM and were responsible for the antiproliferative and differentiation-inducing effects of tumor cells[5-7]. Recently, apoptosis of U937 cells induced by *Coptidis chinensis* by presenting apparent nuclear fragmentation and DNA ladder was evident when tested by the MNC-CM mode[8].

In an attempt to evaluate the immunomodulating effect of medicinal herbs on human peripheral blood MNC, boiling-water extracts and ethanolic extracts were prepared to stimulate MNC. Then, U937 cells were incubated in the collected MNC-CM (MNC-CM mode) to observe and compare the growth inhibition and differentiation-inducing effect of the cultured U937 cells.

MATERIALS AND METHODS

I. *Preparation of Chinese Herbal Extracts*

Ten dried Chinese medicinal herbs, Huang Gi (HG), Gan Cao (GC), Lian Zi (LZ), Da Zao (DZ), Tang Shen (TS), Shan Yao, Gou Gi Zi, Huang Jing, Dang Gui, and Bai Zhu, were purchased from local Chinese herbal drugstores in Taipei City and were ground into powders (< 20 mesh) with a cycle blender (Osterizer Co., Berlin, Germany) before use. Distilled water (Milli-Q Ultrapure Water System, Millipore, Bedford, MA, USA) was added to each herbal powder (100 mg) at a ratio of 10:1(w/v), and then the mixture was refluxed in a boiling water bath for 2 hr. After centrifugation (10,000 × g, 30 min), the supernatant was concentrated to 150 mL by a rotary evaporator (A-3S, EYELA, Tokyo, Japan), followed by lyophilization (VD400F, Taitec, Tokyo, Japan). The ethanolic extracts were prepared with 80% (v/v) ethanol by the same proce-

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dures except the heating process and extraction time of 24 hr at room temperature (28 ± 2˚C).

On the other hand, the water extracts obtained from LZ, AR and GC were further treated overnight with 80% (v/v) ethanol or 100% saturated ammonium sulfate to obtain precipitates, which were dialyzed against 100 volumes of distilled water overnight before lyophilization. The powders were stored at -20˚C and dissolved in PBS (phosphate-buffered saline, 8 g NaCl/1.15 g Na_2HPO_4/0.2 g KH_2PO_4/0.2 g KCl/L) before the preparation of MNC-CM.

II. *Preparation of CM*

MNC of human peripheral blood donated by three healthy adults, aged between 22-25, with informed consent were separated by density centrifugation (400 ×g, 30 min) in a Ficoll-Hypaque solution (1.077 g/mL) (Pharmacia Fine Chemicals, Uppusala, Sweden). A concentration of 1.5 × 10^6 cells/mL was incubated in 1% glutamine (Gibco BRL, Gaitherburg, MD, USA)/10% fetal calf serum (FCS) (Hyclone, Logan, UT, USA)/RPMI 1640 medium (Gibco BRL, Gaitherburg, MD, USA). The MNC-CM was prepared by incubating the MNC with or without (PBS group) herbal extracts (200-800 μg/mL) or precipitates (100-400 μg/mL) in PBS at 37˚C in a humidified 5% CO_2 incubator for 1 day (24 hr)[6]. The cell-free supernatant was then collected by filtration through a 0.45 μm membrane and stored at -80˚C until use. PHA (phytohemagglutinin) (5 μg/mL) (Sigma, St. Louis, MO, USA) was also used to prepare CM (positive control).

III. *Cell Culture and Treatments*

The human myeloid leukemic U937 cells obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA) were used. The cells were cultured in 1% glutamine/10% FCS/RPMI 1640 medium and maintained in an exponential growth status. The cells were incubated in 35 mm petri dishes at an initial concentration of 1 × 10^5 cells/mL in the presence or absence (control group) of 20% (v/v) of MNC-CM prepared with herbal extracts or precipitates (MNC-CM mode)[6]. The numbers of viable and adherent cells in day 5 cultures were collected by gently rubbing the dishes with a rubber policeman (Bellco Glass, Vineland, NJ, USA) and counted using the trypan blue dye exclusion test[9] to determine the growth inhibition (%)[6]. In contrast, boiling-water or ethanolic extracts (200-800 μg/mL) were mixed with culturing medium, followed by the same treatment to observe the growth inhibition. Growth inhibition (%) = (1-number of cells treated with herb MNC-CM/cell number of control group) × 100%. PHA (5 μg/mL) was treated as positive control. Three separate experiments were each tested in duplicate.

IV. *Superoxide Assay*

The production of cytoplasmic superoxide by the differentiated myeloid cells was detected by the nitroblue tetrazolium (NBT) reduction test[10]. Cells collected from day 5 cultures were suspended in RPMI 1640 medium at a concentration of 1 × 10^6/mL and incubated at 37˚C for 30 min with an equal volume of NBT test stock solution (2 mg NBT/1 μM phorbol myristate acetate/mL PBS). Thus the obtained cell suspensions (80 μL) were cytocentrifuged onto glass slides with the aid of a Cytospin (Shandon Southern, England) (1,200 rpm, 5min), which were counter-stained with 0.5% Safranin O. The percentage of formazan-containing cells was assessed microscopically out of 200 cells. Three separate experiments were tested in duplicate.

V. *Maturation Profile*

Cells collected from day 5 cultures were harvested and cytocentrifuged onto a microscope slide using a Cytospin and then stained with Wright's stain. Morphological examination was performed by a microscope at a magnification of 1000 ×.

VI. *Statistical Analysis*

Results were analyzed for statistical significance using one-way analysis of variance (ANOVA) and Duncan's multiple range tests. $P < 0.05$ was accepted as statistically significant.

RESULTS

I. *Inhibition of U937 Cell Growth by Boiling-Water Extracts*

Table 1 shows the antiproliferation of U937 cells incubated for 5 days with 20% of various MNC-CMs prepared with various concentrations (200-800 μg/mL) and species of herb water extracts (MNC-CM mode). Growth inhibition was observed in a dose-dependent manner. Values of 45, 45, 43, 43, and 42% were obtained when U937 cells were treated with HG, GC, LZ, DZ, and TS extracts at a dosage of 800 μg/mL, respectively. The other herbal extracts displayed inferior growth inhibition effect, ranging from 37.5 to 21.7%, on U937 cells; however, these herbal extracts still showed promising inhibition on cell growth. In contrast to MNC-CM mode, the presence of corresponding level of herbal extracts in culturing medium apparently displayed insignificant inhibition (Table 2).

II. *Cell Growth Inhibition of U937 by Ethanolic Extracts*

Table 3 shows the growth inhibition of leukemic U937 cells of day 5 cultures by MNC-CMs prepared with herbal ethanolic extracts. Similar to those observed in Table 1, growth inhibition increased with the concentration of ethanolic extracts, reaching values of about 35, 34, and

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Table 1. Effect of boiling-water extracts on U937 cells by MNC-CM mode

Sample*	Growth inhibition (%)		
	Concentration (μg/mL)		
	200	400	800
Huang Gi	36.6 ± 1.7^{a}	34.7 ± 2.6^{bc}	44.7 ± 1.2^{a}
Gan Cao	34.9 ± 2.4^{a}	31.3 ± 1.8^{bc}	44.5 ± 2.8^{a}
Lian Zi	29.5 ± 1.2^{ab}	40.1 ± 0.4^{ab}	43.4 ± 1.2^{a}
Da Zao	17.2 ± 1.3^{c}	44.4 ± 0.4^{a}	42.6 ± 3.7^{a}
Tang Shen	29.6 ± 2.3^{ab}	37.0 ± 1.5^{abc}	42.3 ± 0.3^{ab}
Shan Yao	18.6 ± 1.2^{bc}	34.9 ± 2.2^{bc}	37.5 ± 1.1^{ab}
Gou Gi Zi	34.7 ± 2.1^{a}	40.1 ± 2.8^{ab}	36.6 ± 1.2^{ab}
Huang Jing	23.2 ± 2.6^{bc}	29.7 ± 0.2^{c}	34.2 ± 2.7^{bc}
Dang Gui	25.6 ± 0.3^{abc}	30.1 ± 1.4^{c}	28.3 ± 2.4^{cd}
Bai Zhu	19.2 ± 2.5^{bc}	14.9 ± 0.9^{d}	21.7 ± 1.6^{d}

U937 cells (1 × 10^5/mL) were incubated for 5 days in the presence of 20% MNC-CM or absence (control) of herb extracts. Viable and adherent cells were counted after 5 days of cultivation.
Growth inhibition of PHA (5 μg/mL) (positive control) was 50%.
Growth inhibition (%) = (1-number of cells treated with herb MNC-CM/ cell number of control group) × 100%.
[a-d]Means across column with different superscripts are different (p < 0.05). Results from three experiments are expressed as mean ± SEM.
*Medicinal herbs were heated in a boiling water bath for 2 hr in a reflex extraction system.

Table 2. Effect of boiling-water extracts on U937 cells

Sample*	Growth inhibition (%)		
	Concentration (μg/mL)		
	200	400	800
Huang Gi	4.5 ± 0.3^{b}	5.6 ± 1.5^{b}	6.4 ± 1.2^{abc}
Gan Cao	2.3 ± 1.2^{c}	4.3 ± 1.6^{bc}	3.3 ± 1.2^{c}
Lian Zi	2.3 ± 0.6^{c}	3.2 ± 0.6^{cd}	7.9 ± 1.5^{ab}
Da Zao	4.5 ± 0.5^{b}	4.5 ± 0.9^{bc}	5.6 ± 1.8^{bc}
Tang Shen	7.9 ± 1.6^{a}	8.1 ± 0.7^{a}	7.9 ± 0.5^{ab}
Shan Yao	1.5 ± 0.8^{cd}	5.6 ± 1.6^{b}	4.3 ± 0.2^{bc}
Gou Gi Zi	0.5 ± 0.4^{d}	2.8 ± 0.5^{cd}	9.7 ± 0.9^{a}
Huang Jing	8.9 ± 0.6^{a}	7.9 ± 0.8^{a}	4.9 ± 1.2^{bc}
Dang Gui	1.2 ± 0.0^{cd}	5.9 ± 0.5^{b}	7.3 ± 0.8^{ab}
Bai Zhu	1.2 ± 0.2^{cd}	2.3 ± 0.2^{d}	2.8 ± 1.0^{c}

U937 cells (1× 10^5/mL) were incubated for 5 days in the presence or absence (control) of herb extracts. Viable and adherent cells were counted after 5 days of cultivation.
Growth inhibition of PHA (5 μg/mL) (positive control) was 50%.
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[a-d]Means across column with different superscripts are different (p < 0.05). Results from three experiments are expressed as mean ± SEM.
*Medicinal herbs were heated in a boiling water bath for 2 hr in a reflex extraction system.

41% when MNC was stimulated with 800 μg/mL of GC, LZ, and HJ ethanolic extracts, respectively. However, herbal extracts spiked in culturing medium exhibited insignificant inhibition (< 10%) on U937 cells, as shown in Table 2 and Table 4.

III. *Growth Inhibition of Ethanolic Precipitates and Ammonium Sulfate Precipitates on U937*

Table 3. Effect of ethanolic extracts on U937 cells by MNC-CM mode

Sample*	Growth inhibition(%)		
	Concentration (μg/mL)		
	200	400	800
Huang Gi	3.8 ± 1.6^{ef}	16.3 ± 1.2^{c}	13.8 ± 1.5^{b}
Gan Cao	20.0 ± 1.2^{b}	21.3 ± 2.6^{bc}	35.0 ± 2.6^{a}
Lian Zi	19.5 ± 1.3^{bc}	31.0 ± 2.5^{a}	33.9 ± 0.8^{a}
Da Zao	15.2 ± 1.3^{bc}	15.2 ± 1.9^{e}	17.8 ± 5.8^{b}
Tang Shen	5.0 ± 1.2^{def}	2.5 ± 0.3^{e}	2.5 ± 0.2^{d}
Shan Yao	13.8 ± 1.9^{bcd}	12.6 ± 0.5^{cd}	19.0 ± 1.6^{b}
Gou Gi Zi	3.5 ± 0.8^{ef}	3.5 ± 0.9^{de}	10.9 ± 0.5^{bc}
Huang Jing	33.8 ± 2.2^{a}	30.0 ± 2.3^{ab}	41.3 ± 2.2^{a}
Dang Gui	10.3 ± 6.2^{cde}	12.1 ± 1.3^{cd}	16.7 ± 1.3^{b}
Bai Zhu	0.5 ± 0.2^{f}	2.5 ± 0.8e	3. ± 0.2^{cd}

U937 cells (× 10^5/mL) were incubated for 5 days in the presence of 20% MNC-CM or absence (control) of herb extracts. Viable and adherent cells were counted after 5 days of cultivation.
Growth inhibition of PHA (5 μg/mL) (positive control) was 50%.
Growth inhibition (%) = (1-number of cells treated with herb MNC-CM/cell number of control group) × 100%.
[a-d]Means across column with different superscripts are different (p < 0.05). Results from three experiments are expressed as mean ± SEM.
*Medicinal herbs were extracted with 80% (v/v) ethanol at room temperature for 24 hr.

Boiling-water extracts of each herb were precipitated by 80% ethanol and 100% saturated ammonium sulfate to obtain polysaccharide-rich portion and protein portion, respectively. Their effects on U937 cells by using MNC-CM mode were studied. As shown in Table 5, at a dose of 100 μg/mL, the polysaccharide-rich portion of LZ appeared stronger than the protein portion in inhibiting tumor cell growth. However, no difference in growth inhibition between those two portions was observed at higher dose (400 μg/mL). Similar trends in growth inhibition versus HG dose were also observed. However, protein portion of GC appeared to be remarkable in inhibiting U937 cells growth at low dose, while no difference was observed at the dose of 400 μg/mL.

IV. *Differentiation-inducing Effect (Superoxide Assay)*

As demonstrated in Figure 1, polysaccharide-rich portion of LZ not only suppressed the proliferation of U937 but also induced these cells to differentiate into mature monocytoid cells. NBT reduction test showed that these mature cells produced superoxide and formed blue-black formazan (Figure 2). NBT reduction test of polysaccharide-rich and protein portions were performed by using MNC-CM mode and the results were presented in Table 6. Among the samples tested, polysaccharide-rich portion of LZ displayed the most remarkable differentiation-inducing effect by showing 31, 37 and 49% of positive formazan formation when U937 cells were incubated with MNC-CM prepared with 100, 200 and 400 μg/mL, respectively. However, LZ protein portion was apparently insignificant (< 10%) in inducing differentiation of U937 cells.

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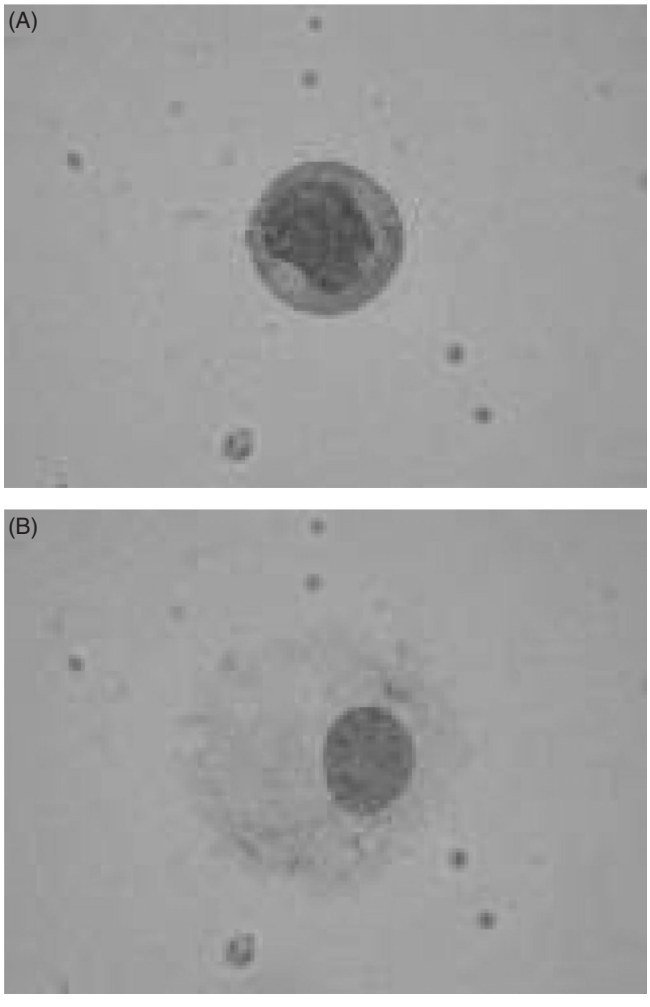

Figure 1. Morphological observation of U937 cells under a light microscope (1000 ×). Cells were stained with Wright's stain. (A) untreated cells; (B) cells treated with ethanol precipitates of boiling-water extracts (400 μg/mL) from NS (MNC-CM mode).

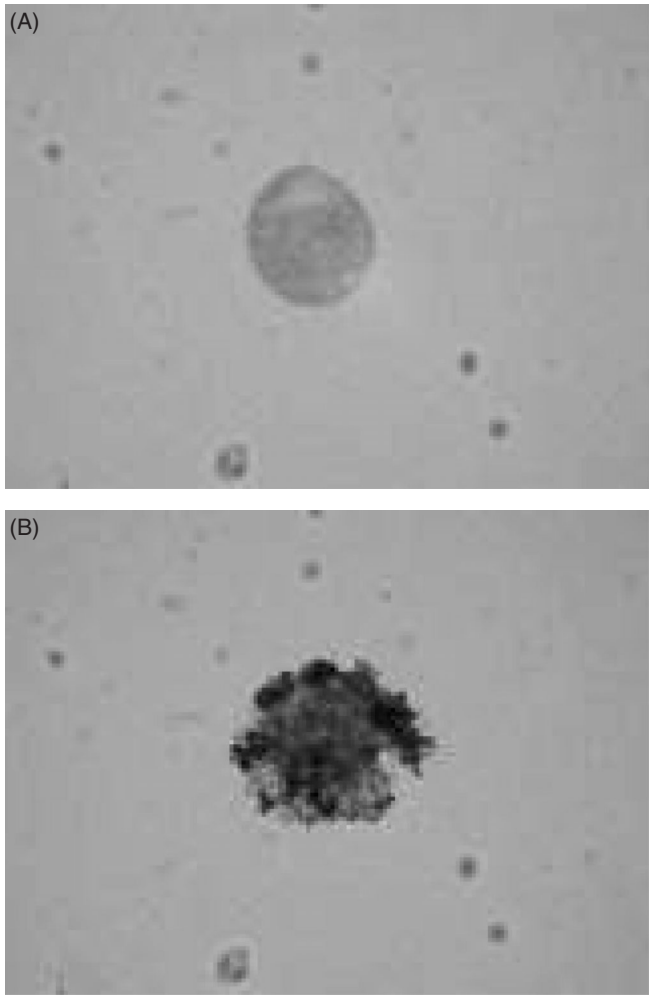

Figure 2. NBT reduction test of U937 cells observed under a light microscope (1000 ×). Cells were counterstained with Safranin O. (A) untreated cells; (B) cells treated with ethanol precipitates of boiling-water extracts (400 μg/mL) from NS (MNC-CM mode).

Precipitates of water extracts from HG and GC showed inferior differentiation-inducing effect for all doses used.

DISCUSSION

Growth inhibition on U937 was preliminary investigated for medicinal herbs to screen the effective herbs. It was found that boiling-water extracts from HG, GC, LZ, DZ and TS showed remarkable inhibitions (42-44%) on tumor cell growth at dose of 800 μg/mL by using the MNC-CM mode (Table 1). Chen *et al*.[6] indicated that isolated polysaccharide fraction of *Cordyceps sinensis* was a potent U937 cell suppressor, which decreased cell growth by about 80% at the dose of 10 μg/mL through MNC-CM mode. In addition, MNC-CM was examined to contain high levels of INF-γ, TNF-α, and IL-1β. In contrast, conventional mode through direct incubation of tumor cells with corresponding level of herb extracts in medium showed insignificant inhibition. *Ganoderma lucidum* presents antiproliferation effect on U937 and HL-60 cells by activating macrophages and T-lymphocytes and releasing cytokines, such as INF-γ, TNF-α, IL-1β, and IL-6, when it was incubated with peripheral blood MNC[5].

Compared to boiling-water extracts, the ethanolic extracts presented inferior growth inhibition on U937 cells especially for those by conventional mode (Tables 3 and 4). Polysaccharide-rich fractions contained higher bioactive ingredients in medicinal herbs such as *Ganoderma lucidum*[5], Astragali Radix (Huang Gi)[11], *Glycyrrhiza glabra* (Gan Cao)[12], and *Cordyceps sinensis*[6]. Boiling-water extracts, rather than ethanolic extracts, from Coptidis Rhizoma were also significantly effective (400 μg/mL) in suppressing tumor growth (82%) by induction to apoptosis and led to DNA fragmentation of human leukemic U937 cells through the MNC-CM mode[8]. Among the herbs tested, polysaccharide of HG was reported to enhance immunoglobulin M (IgM) production in aged mice[12]. HG extracts inhibited gastric cancer cells growth[13] and exerted an anticarcinogenic effect in carcinogen-treated mice through activation of cytotoxic activity and the production of cytokines such as IL-2 and INF-γ [14]. Activation of

macrophages by polysaccharide fraction from *Glycyrrhiza glabra* (Gan Cao) and *Glycyrrhiza uralensis* was also reported by Nose *et al*.[12]. In addition, GC was effective in inducing apoptosis of HL-60 cells[15]. From the tested herbs, only those displaying potent growth inhibition were used for further studies.

Table 4. Effect of ethanolic extracts on U937 cells

Sample*	Growth inhibition (%) Concentration (μg/mL)		
	200	400	800
Huang Gi	5.6 ± 61.2^{b}	4.5 ± 1.5^{bc}	7.9 ± 0.7^{ab}
Gan Cao	4.5 ± 1.3^{bc}	2.5 ± 0.3^{cd}	4.8 ± 0.9^{c}
Lian Zi	5.5 ± 0.5^{b}	5.6 ± 0.8^{b}	5.9 ± 0.5^{bc}
Da Zao	1.2 ± 0.5^{c}	5.6 ± 0.9^{b}	7.8 ± 0.9^{ab}
Tang Shen	4.5 ± 0.^{8bc}	2.1 ± 0.1^{d}	5.6 ± 0.7^{bc}
Shan Yao	1.5 ± 0.7^{c}	8.2 ± 0.9^{a}	5.9 ± 0.2^{bc}
Gou Gi Zi	1.2 ± 0.8^{c}	2.8 ± 0.5^{cd}	4.8 ± 0.4^{c}
Huang Jing	2.5 ± 0.2^{bc}	3.6 ± 0.7^{bcd}	8.9 ± 0.8^{a}
Dang Gui	8.9 ± 0.8^{a}	9.5 ± 0.8^{a}	9.8 ± 1.6^{a}
Bai Zhu	1.5 ± 0.8^{c}	2.5 ± 0.2^{cd}	7.8 ± 1.5^{ab}

U937 cells (1 × 10^5/mL) were incubated for 5 days in the presence or absence (control) of herb extracts. Viable and adherent cells were counted after 5 days of cultivation.

Growth inhibition of PHA (5 μg/mL) (positive control) was 50%.

Growth inhibition (%) = (1-number of cells treated with herb MNC-CM/cell number of control group) × 100%.

[a-d]Means across column with different superscripts are different ($p < 0.05$). Results from three experiments are expressed as mean ± SEM.

*Medicinal herbs were extracted with 80% (v/v) ethanol at room temperature for 24 hr.

Polysaccharide-rich fraction of LZ and HG displayed higher growth inhibition than the corresponding protein fraction at low dose of 100 μg/mL, revealing that the polysaccharides in ethanolic precipitates were relevant to the suppression of U937 cell growth (Table 5). However, either polysaccharide fraction or protein fraction of HG and GC showed relatively lower growth inhibition on U937 cells (Table 5), as compared to the corresponding boiling-water extracts at the same concentration (Table 1), suggesting that proteins and polysaccharides of those two herbs were synergistic in inhibiting U937 cell growth.

Polysaccharide-rich fraction of LZ presented remarkable differentiation-inducing effect on U937 cells (Table 6). The NBT positive percentage was as high as 49% when blast cells were incubated with MNC-CM prepared with 400 μg/mL LZ ethanolic precipitates. Macrophages produce superoxide, which is relevant to bactericidal effect, through the respiratory burst response as the results of the reaction of oxygen with NADPH (nicotiamide-adenine dinucleotide phosphate) to form superoxide anion and the following reactions with superoxide dismutase (SOD). Strong effect on producing superoxide of matured macrophages and monocytes, derived from immature U937 blast cells, suggested the remarkable differentiation-inducing effect of MNC-CN mode. Similar results were

Table 5. Effect of ethanolic and ammonium sulfate precipitates on U937 cells by MNC-CM mode

Sample*		Growth inhibition (%) Concentration (μg/mL)		
		100	200	400
LZ	Ethanolic precipitates	22.7 ± 1.5^{abc}	30.8 ± 6.3^{ab}	34.0 ± 2.6^{a}
	Ammonium sulfate precipitates	18.8 ± 1.4^{cd}	26.0 ± 1.2^{bcd}	34.2 ± 1.5^{a}
HG	Ethanolic precipitates	24.3 ± 0.8^{ab}	23.6 ± 1.5^{cd}	26.3 ± 2.7^{bc}
	Ammonium sulfate precipitates	18.9 ± 1.8^{cd}	13.8 ± 1.8^{e}	21.7 ± 1.4^{cd}
GC	Ethanolic precipitates	9.9 ± 1.8^{e}	15.3 ± 0.2^{f}	20.1 ± 1.2^{d}
	Ammonium sulfate precipitates	21.1 ± 0.9^{bc}	20.0 ± 1.5^{de}	19.7 ± 1.5^{d}

U937 cells (1 × 10^5/mL) were incubated for 5 days in the presence or absence (control) of 20% MNC-CM. Viable and adherent cells were counted after 5 days of cultivation.

Growth inhibition of PHA (5 μg/mL) (positive control) was 50%.

Growth inhibition (%) = (1-number of cells treated with herb MNC-CM/cell number of control group) × 100%.

[a-d]Means across column with different superscripts are different ($p < 0.05$). Results from three experiments are expressed as mean ± SE.

*Boiling water extracts of medicinal herbs were fractionated by 80% ethanol and 100% saturated ammonium sulfate to prepare precipitates.

Table 6. Effect of ethanolic and ammonium sulfate precipitates on NBT reduction activity of U937cells

Sample*		NBT reduction (%) Concentration (μg/mL)		
		100	200	400
LZ	Ethanolic precipitates	31.3 ± 0.5^{a}	37.1 ± 1.5^{a}	49.2 ± 2.1^{a}
	Ammonium sulfate precipitates	3.4 ± 0.8^{cd}	5.5 ± 1.3^{de}	7.2 ± 0.2^{fg}
HG	Ethanolic precipitates	3.6 ± 0.4^{cd}	4.1 ± 0.7^{e}	13.5 ± 1.8^{cd}
	Ammonium sulfate precipitates	3.5 ± 1.0^{cd}	3.0 ± 0.5^{e}	2.5 ± 0.8^{h}
GC	Ethanolic precipitates	5.2 ± 0.8^{c}	9.1 ± 1.3^{c}	16.3 ± 1.5^{c}
	Ammonium sulfate precipitates	2.9 ± 0.3^{cd}	3.2 ± 0.8^{e}	13.8 ± 0.8^{cd}

U937 cells (1 × 10^5/mL) were incubated for 5 days in the presence or absence (control) of 20% MNC-CM. Viable and adherent cells were counted after 5 days of cultivation.

[a-e]Means across column with different superscripts are different ($p < 0.05$). Results from three experiments are expressed as mean ± SEM.

*Boiling water extracts of medicinal herbs were fractionated by 80% ethanol and 100% saturated ammonium sulfate to prepare precipitates.

observed by Chen *et al*.[6], Liao *et al*.[16], and Chen and Chang[7] using *Cordyceps sinensis*, soybean polysaccharide, and fuling (*Poria cocos*), respectively, to stimulate MNC. On the other hand, media mixed with corresponding level of precipitates was ineffective in inducing U937 cells into differentiated monocytes (data not shown). Though inferior to LZ ethanolic precipitates in NBT positive percentage, HG and GC ethanol precipitates showed 13.5 and 16.3%, respectively.

Cytokines released from MNC upon stimulation with polysaccharide fraction of *Codyceps sinensis* were TNF-α, IL-1β, and INF-γ. These two cytokines contributed mainly to the growth inhibition and differentiation induction (NBT positive percentage) of U937 cells[5-7]. Fu-Ling extracts enhanced the secretion of immune stimulators (IL-1β, IL-6 and TNF-α), while suppressing the secretion of an immune suppressor (TGF-β) after *in vitro* cultivation of human peripheral blood monocytes[17].

In summary, HG, GC and LZ were all potent antitumor herbs through the MNC-CM mode by presenting suppression on U937 growth. In addition, tumor blast cells were also remarkably differentiated into mature cells with NBT reduction ability suggesting the potent immunomodulatory effect of LZ.

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