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Improving cachectic symptoms and immune strength of tumour-bearing mice in chemotherapy by a combination of *Scutellaria baicalensis* and Qing-Shu-Yi-Qi-Tang

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ABSTRACT

Background: Cancer cachexia is characterised by the loss of body mass and directly compromises immune response and the quality of life of cancer patients. In the present study, we set out to investigate the role of Chinese herbs as anticancer medicines and/or chemotherapeutic adjuvants to increase therapeutic efficacy and/or ameliorate given side-effects in animal model.

Methods: Twelve kinds of herbs were chosen from the ingredients of major Chinese herbal medicines, and their effects on the antioxidant activity were investigated. To obtain the anticancer effects of 5-fluorouracil (5-FU) when consumed with minimal side-effects, we investigated the combination effect of *Scutellaria baicalensis* and Qing-Shu-Yi-Qi-Tang that may enhance the anticancer activity of 5-FU on subcutaneous tumour growth in C57BL/6 mice challenged with Lewis lung carcinoma cells.

Results: Qing-Shu-Yi-Qi-Tang, a multiple-component herbal extract, was shown to have high anti-oxidation activity, while *S. baicalensis* (Chinese skullcap) was demonstrated to have high tumour-growth inhibition activity. Thus, *S. baicalensis* and Qing-Shu-Yi-Qi-Tang were evaluated for their combinaton effects on the cancer-induced cachectic murine upon receiving 5-FU chemotherapy. As a result, tumour masses and losses of carcass and/or gastrocnemius muscle were found to be significantly decreased. This combination otherwise increased both Th1/Th2 ratio and NK cytotoxicity. In the mice receiving with or without 5-FU, the serum levels of monocyte chemoattractant protein-1 (MCP-1) increased by all means but otherwise decreased when the herbal combination was administrated. Additionally, the expressions of nuclear factor-kappa B (NF-κB) and muscle RING finger protein-1 (MuRF-1) decreased in the gastrocnemius muscle when the herbal combination was applied.

Conclusion: Our results revealed that the combination of *S. baicalensis* and Qing-Shu-Yi-Qi-Tang is able to ameliorate cachectic symptoms and positively stimulate anti-tumour immunity while undergoing chemotherapy in animal model.

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1. Introduction

Lung cancer has high mortality and is one of the most common cancers in man.[1] Lung cancer killed more than 1 million victims in 2001; its global prevalence rate is increasing at 0.5% per annum.[2] Most patients die of disease progression. Current treatments include radiotherapy, chemotherapy and the combination of both, in which the chemotherapy is the mainstay treatment for small-cell and advanced non-small-cell lung cancers (stages III and IV).[3] The combination treatment, however, is extremely toxic to patients in stage III.[3]

Cachexia is one of the most devastating symptoms in cancers, manifesting with loss of weight, muscle atrophy, fatigue, weakness, loss of appetite, etc. Cachexia represents a complex metabolic state in close relation to late-stage cancers, which presents progressive weight losses and immunological malfunctions. Cachexia is known to be associated with given cancers, such as the gastrointestinal tract and lung cancers.[4] The pathomechanism of cancer cachexia is multifactorial, including a variety of pro-inflammatory cytokines to initiate cachectic promotion and progression.[5] In general, tumour-derived factors, therapeutic strategies, age, and nutritional and mental status all are associated with the development of cancer cachexia. Therefore, cancer cachexia is an ensemble result of chronic inflammation and impaired immune responsiveness.[6–8]

Chinese herbal medicines have long been used as a complementary or alternative medicine[9,10]; recently the cancer treatment by using the classical anticancer drugs along with the herbs has greatly been promoted. Although such a combination treatment has gradually been accepted by mainstream medicine,[11] scientific reports with regard to the effects by the co-administration remain few. Immune functions suppressed by anticancer drugs or tumour cells have long been an unsolved issue in cancer treatments.[7,8] Chinese herbal extracts, nevertheless, may possess some potentialities to modulate adverse immune responses to support classical cancer therapy.[12,13]

In this report, the study rationale is rooted on the hypothesis that a given combination of Chinese herbal extracts along with given anticancer drugs (5-fluorouracil (5-FU) was used here) may have synergistic anticancer effects. Nine single and three multiple components of Chinese herbal extracts were investigated in this study. A combination of Chinese herbal extracts was determined on the basis of their cancer-related biological activities. The typical dose of 5-FU (40 mg/kg) was halved (20 mg/kg) to contrast the pharmaceutical effects of the herbal combination examined. The immune responses and therapeutic effects by the co-administration in cancerous mice were reported here.

2. Materials and methods

2.1. Chinese herbal extracts

Nine single-composition Chinese herbal extracts (including *Astragalus membranaeus*, *Panax ginseng* C.A. Mey, *Scutellaria baicalensis*, *Morusalbal*, *Pueraria lobata*, *Trichosanthes kirilowii*, *Adenophoratetraphylla*, *Ophiopogon japonicas* and *Atractylodes macrocephala Koidz*) and three multiple-composition Chinese herbal extracts (include Liu-Jun-Zi-Tang, Bu-Zhong-Yi-Qi-Tang and Qing-Shu-Yi-Qi-Tang) were obtained from Chuang Song-Zong drugstore (Kaohsiung, Taiwan). These herbs have the same quality as the herbs used in commercially traditional herbal medicines, which were extracted by boiling water. The extracts were dried by spray drying.

2.2. Cell culture

Lewis lung carcinoma cells (LLC, C57BL/6 strain mice lung cancer cell line, ATCC CRL-1642) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum. Murine macrophage RAW264.7 cells were purchased from ATCC (TIB 71). They were cultured in DMEM containing 2 mM glutamine, 10 mM 4-[2-hydroxyethyl]-1-piperazineethanesulphonic acid (HEPES), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% faetal bovine serum. African green monkey kidney (Vero, ATCC CCL81) was maintained in RPMI-1640 medium supplemented with 5% faetal bovine serum. Cells were cultured at 37 °C in a humidified incubator with an atmosphere of 5% CO_2.

2.3. Cell viability assay

For cell viability study, 1×10^4 cells resuspended in 100 μl medium were plated into each well of a 96-well plate for 24 h. The cells were then treated with different concentrations (1.2, 0.6, 0.3, 0.15, 0.06, 0 μM) of the *S. baicalensis* extract (SBE) or the Qing-Shu-Yi-Qi-Tang extract (QSYQTE) or the combination (SBE + QSYQTE) for 48 h. Cell proliferation was monitored by incubation with resazurin (Alamar Blue, Invitrogen). The Alamar Blue assay was performed according to the method published earlier with modifications.[14] Briefly, after the treatment, a stock solution of 120 μg/ml resazurin in PBS was added to the cells at 1:20, incubated for 2 h at 37 °C and for another 15 min at room temperature, and then assayed for fluorescence (with an excitation wavelength of 520 nm and an emission wavelength of 590 nm).

2.4. Nitric oxide assay

The nitric oxide assay was performed as described previously with slight modifications.[15] After pre-incubation of RAW264.7 cells (8×10^5 cells/ml) with LPS (1 μg/ml) and in the presence of the *S. baicalensis* extract (SBE) or the Qing-Shu-Yi-Qi-Tang extract (QSYQTE) for 48 h, the quantity of nitrite in the culture medium was measured as an indicator of NO production. Amounts of nitrite, a stable metabolite of NO, were measured using Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Briefly, 50 μl of cell culture medium was mixed with 50 μL of Griess reagent. Subsequently, the mixture was incubated at room temperature for 10 min and the absorbance at 550 nm was measured in a microplate reader. Fresh culture medium was used as a blank in every experiment.

The quantity of nitrite was determined from a sodium nitrite standard curve.

2.5. Ferrous ion chelating activity

Ferrous ion chelating activities of Chinese herbal extracts were determined by the method of Dinis et al. with slight modifications.[16] 1.7 ml distiled water, 50 μl of 0.2 mM $FeCl_2 \cdot 4H_2O$, and 50 μl of 2.5 mg/ml sample solution were added into test tubes, and the mixture was left at room temperature for 1 min. 0.2 ml of 5 mM ferrozine was added, and final colour was monitored at 562 nm after 10 min of incubation. In control, water was used in place of samples. The inhibition percentage of ferrozine–Fe^{2+} complex formation against blanks containing $FeCl_2$ and ferrozine was calculated by the formula:

$$\% \text{ of metal chelating} = [1 - (A_{S:10}/A_{B:10})] \times 100$$

where $A_{S:10}$ is absorbance of sample and $A_{B:10}$ is absorbance of blank at 10 min reaction time.

2.6. DPPH radicals scavenging assay (DPPH assay)

The radical scavenging ability of Chinese herbal extracts was assessed by the method of Shimada et al. with slight modifications.[17] The radical scavenging ability of extracts was determined at concentrations of 10 mg/ml in ethanolic DPPH solution (0.1 mM). Single-composition and multiple-composition of Chinese herbal extracts were dissolved in water. In control, water was used in place of the sample in which the extract was prepared. Cuvettes were left in the dark at room temperature for 30 min and the resulting colour was measured spectrophotometrically at 517 nm against blanks. A decreasing intensity of purple colour was related to higher radical scavenging ability, which was calculated using the following equation:

$$\text{DPPH radical scavenging ability} = [1 - (A_{S:30}/A_{B:30})] \times 100$$

where $A_{S:30}$ is absorbance of the sample and $A_{B:30}$ is absorbance of the blank at 30 min reaction time.

2.7. Superoxide anion scavenging activity

The NADH/PMS/NBT system was used to determine the superoxide anion scavenging activity of Chinese herbal extracts.[18,19] Superoxide radicals are generated in PMS–NADH systems by oxidation of NADH and assay by the reduction of nitroblue tetrazolium (NBT). Briefly, 50 μl of NBT solution (300 μM in 100 mM phosphate buffer, pH 7.4) 50 μl NADH solution (936 μM in 100 mM phosphate buffer, pH 7.4) and 50 μl of sample solution (2.5 mg/ml in distilled water) were mixed. The reaction was started by adding 50 μl of phenazine methosulphate (PMS) solution (120 μM in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at room temperature for 5 min, and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The NADH/PMS/NBT solution without sample solution was used as the control. The percent inhibition of superoxide anion generation was calculated using the following formula;

$$\text{Scavenging activity}(\%) = [1 - (A_{S:5}/A_{B:5})] \times 100$$

where A_S is absorbance of sample and A_B is absorbance of blank at 5 min reaction time.

2.8. Animal model

Six- to eight-week-old Male C57BL/6 mice were obtained from National Laboratory Animal Centre (Taipei, Taiwan, ROC). Mice were divided into weight-matched groups: (1) control receiving water (con), (2) tumour-bearing administered with either PBS or 5-FU (20 or 40 mg/kg body weight) and (3) tumour-bearing administered with 5-FU (20 mg/kg) and oral high-dose (3 mg/g body weight) or low-dose (1.5 mg/g body weight) Chinese herbal complex. The Chinese herbal complex was a mixture of *S. baicalensis* extract (SBE) and Qing-Shu-Yi-Qi-Tang extract (QSYQTE). Tumours were induced with an injection of 1×10^5 LLC cells in 100 μl subcutaneously into the right foot dorsum of C57BL/6 mice. After one day, mice were daily given orally either water or low- or high-dose of Chinese herbal complex (SBE + QSYQTE) until sacrificed. 5-FU (20 or 40 mg/kg) was given by i.p. on days 7 and 14 after tumour implantation. Then, 24 days later, the mice were anaesthetised and killed, and the organs were removed and weighed, then stored at −20 °C for further analysis. Finally, the lungs were underwent histological analysis to measure metastasis.

2.9. Splenocyte isolation and flow-cytometric analysis

Splenocytes were isolated via centrifugation (300*g*), and red blood cells were lysed using Gey's reagent (0.829 g NH_4Cl, 0.1 g HCO_3 and 3.72 mg Na_2EDTA in 100 ml ddH_2O). Splenocytes were washed twice with complete RPMI and viability was determined by trypan blue exclusion. To determine the phenotypes of splenocytes isolated from spleen, cells were stained with the appropriate combination of FITC-labelled anti-mouse CD3ε (100306; Biolegend), allophycocyanin (APC)-labelled anti-mouse CD4 (100516; Biolegend), APC-Cy7-labelled anti-mouse CD8a (100714; Biolegend) and PE-labelled anti-mouse CD19 (115508; Biolegend) after blocking of the Fc receptor with anti-CD32/CD16 (BD Biosciences) at 4 °C. To determine the NK cytotoxicity, cells were isolated from mouse spleen, and regarded as effector cells. Stain the target cells (LLC) with DIOC18 (3,3′-dioctadecyloxacarbocyanine perchlorate) 10 μl per 1×10^6 cells. Incubation cells for 20 min at 37 °C, 5% CO_2. Wash the cells twice with buffer solution and resuspend in complete culture media at a concentration of 1×10^6 cells/ml. Prepare the target and effector cells (splenocytes) and make a co-culture of them with the desired ratio, i.e. E:T = 5:1; 20:1; 40:1. Incubate the co-culture for 4 h. Centrifuge the cell mixture at 250*g* for 5 min after washing, discard supernatant. Washed and resuspend the cultured cells. Label the cells with propidium iodide (2 μl/per test) incubate at room temperature in the dark. Analysis was performed using FACScan (BD Biosciences). For characterisation of cell types, a large gate was drawn to include the monocyte

and lymphocyte populations from forward scatter versus side scatter.

2.10. RNA extraction and real-time PCR

Total muscle RNA was extracted with mini kit (Qiagen) and cDNA was synthesised using M-MLV reverse transcriptase (Promega) and oligo-dT15-primer (Promega). Real-time PCR was performed in the Bio-Rad iCycler iQ system. Quantitative real-time PCR analysis was carried out in 25 μl reactions consisting of 12.5 μl iQ SYBR Green Supermix (Bio-Rad), 5 μl cDNA, RNase-free water, and 100 μM of each primer. Values were normalised to GAPDH mRNA amount. Real time PCR primer sequences are as follows:

mouse nuclear factor-kappa B (NF-κB)
F: 5′ TGTCCTCTCACATCCGATTTTTG 3′
R: 5′ CGGTTTACTCGGCAGATCTTG 3′
mouse muscle RING finger protein-1 (MuRF1)
F: 5′ CAAGTGCCAAGCAGCTAATCAA 3′
R: 5′ TCTCAAAGCCTTGCTCTGTCTTC 3′

2.11. Cytometric bead array (CBA) assay

Serum cytokine levels were detected by CBA assay. Mouse serum was collected and stored at −20 °C until the cytokine assay. The concentrations of TNF, IL-6 and monocyte chemoattractant protein-1 (MCP-1) were measured using cytometric bead array (CBA) with a series of anti-cytokine mAb-coated beads and PE-conjugated anti-cytokine mAbs, followed by FACScan flow cytometric analysis (BD Biosciences), using the CBA kit (BD Bioscience, San Jose, CA, USA) and software (BD).

2.12. Statistical analysis

Data were expressed as means ± standard deviation. Statistical significance was determined by one-way ANOVA. Significance was accepted at the level of $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

3. Results

3.1. Antioxidation activities of selected Chinese herbal extracts

In order to single out potential herbs that can ameliorate the cancer cachexia syndromes, 12 Chinese herbal extracts were examined for their antioxidation activities. First, the ferrous ion chelation assay was performed. These selected herbal extracts all possess the ferrous ion chelation activity but in various extents. Namely, nine out of 12 herbal extracts show >85% above the blank, while *O. japonicas*, *S. baicalensis* and *Morusalbal* were determined to be 77%, 75% and 61%, respectively (Fig. 1A). Second, the DPPH radical scavenging assay was performed; *S. baicalensis Georgi*, *P. lobata* and Qing-Shu-Yi-Qi-Tang showed relatively better radical scavenging ability (96%, 85% and 88%, respectively) (Fig. 1B). Third, the superoxide anion scavenging assay was conducted, in which *Adenophoratetraphylla* and Qing-Shu-Yi-Qi-Tang outweighed others (Fig. 1C). Taken together, Qing-Shu-Yi-Qi-Tang appeared to be the common intersection element from these three assays, so that the Qing-Shu-Yi-Qi-Tang was considered to be the overall best antioxidant out of the 12 herbs tested. On the other hand, although *S. baicalensis* was shown to have relatively low antioxidation activities (Fig. 1), it, however, was reported to have some superior biological activities, such as anti-inflammation[20] and anti-cancer.[21] As a result, the extracts of *S. baicalensis* (SBE) and Qing-Shu-Yi-Qi-Tang (QSYQTE) were chosen as a single or a combination to test their effects on cytotoxicity and LPS-induced nitric oxide production.

3.2. Inhibitory effect of the herbal extracts on LLC growth and NO production

S. baicalensis (SBE) was reported to be effective on suppressing tumour cell growth.[22–24] However, SBE alone or in combination with QSYQTE on the suppression of Lewis lung carcinoma (LLC) growth was not reported. Thus, SBE and QSYQTE as a sole component or as a combination of both (SBE + QSYQTE) were examined by the Alamar Blue assays for the anti-LLC activity. The African green monkey kidney (Vero) cells (normal cells) were treated with the extracts to serve as control. SBE is shown to be able to inhibit the proliferation of LLC in a dose-dependent manner. The growth of Vero cells was also inhibited but to a lesser extent, of which 30% of Vero cells was inhibited at a dose of 0.3 mg/ml as opposed to 50% of LLC at the same dose (Fig. 2A). Unlike SBE, the doses of QSYQTE as high as 1.2 mg/ml have no significant cytotoxicity against Vero or LLC cells (Fig. 2B). While, the tumour cell viabilities (cell survival rates) of LLC and Vero cells at the dose of 0.3 mg/ml in the combination of SBE and QSYQTE were 48% and 76%, respectively, which indicating that combination treatment has a slightly higher cytotoxic effect than *S. baicalensis* treatment alone, whereas to a much less extent in normal cells. Accordingly, a combination of *S. baicalensis* and Qing-Shu-Yi-Qi-Tang was shown to be effective in selectively killing cancer cells than *S. baicalensis* alone (Fig. 2C).

Next, the inhibitory effects of the extracts combination on NO production in the LPS-activated macrophages were examined. RAW 264.7 cells first were pre-induced by LPS; the NO production increased 3.9-fold in comparison to that of RAW 264.7 cells without the addition of LPS (Fig. 2D). The NO production in the RAW 264.7 cells with the LPS induction, however, was significantly reduced in the presence of QSYQTE extracts (66% inhibition at the dosage of 0.16 mg/ml, Fig. 2D). At higher doses (0.16 and 0.08 mg/ml), SBE was found to have a better NO inhibition effect than QSYQTE.

As a result, SBE is demonstrated to be more selective in killing the given cancer cells; QSYQTE otherwise is more effective in activities of anti-inflammation and anti-oxidation. According to those findings, we attempt to examine whether specific combination of SBE and QSYQTE enhances anti-cancer and anti-cachectic properties after chemotherapy.

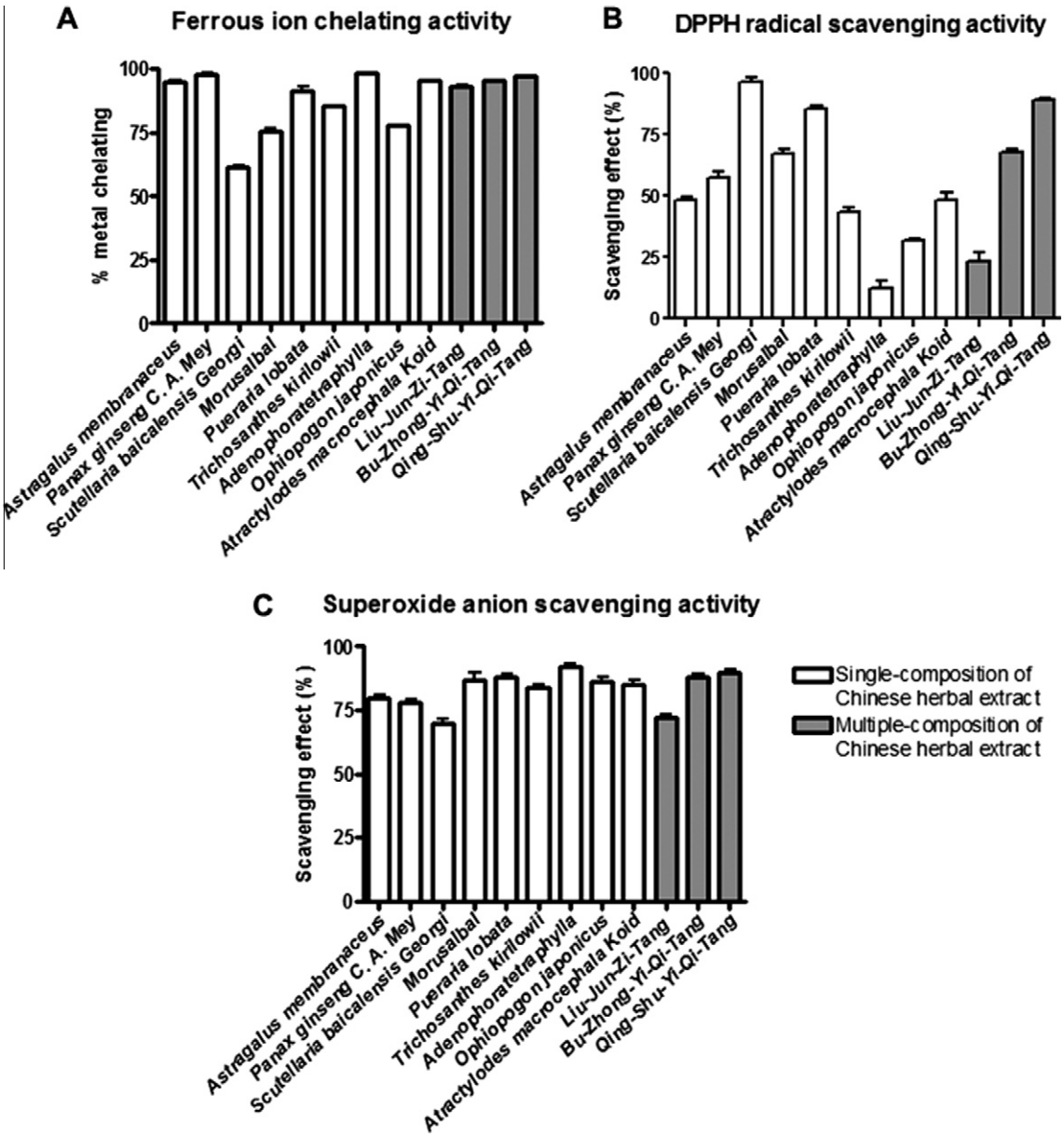

Fig. 1 – Antioxidation activities of 12 Chinese herbal extracts. The antioxidation activities for the extracts were determined by the ferrous ion chelating assay (A), the α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging assay (B) and the superoxide anion scavenging assay (C). Data are expressed as the means ± SD (n = 6).

3.3. *Effect of SBE and QSYQTE combination on organ mass change*

To ameliorate cancer cachectic symptoms and to understand the mode of action, the herbal-extracts combination (SBE + QSYQTE) in inhibiting tumour development was assessed *in vivo*. LLC cells were subcutaneously grafted into C57BL/6 mice, whereby solid tumours developed around the tumour-cells-injected site (Fig. 2E). After the tumour implantation, mice were fed orally with water or a low or high dose of the herbal combination (SBE + QSYQTE) each day until sacrifice; 5-FU was also administered in a high or low dose (F and F/2 for 20 and 40 mg/kg, respectively) at days 7 and 14 (Fig. 2E) (F/2 stands for the halved dosage of 5-FU (F) to contrast the effect of the herbal combination). Food uptakes were the same in all test groups (data not shown). At a dose of 3 mg/ml of Chinese herbal complex, there was no evidence of drug-related toxicity, suggesting that herbal-extracts combination (SBE + QSYQTE) is well tolerated by the animals.

At day 24, the macroscopic metastases in the livers for all test animals were examined; the numbers of metastatic foci (Fig. 3B) and the masses of the livers (Fig. 3A) in the mice administrated with 40 mg 5-FU/kg (T + F) were found to be significantly reduced (cf. those in the tumour-bearing (T) mice receiving no 5-FU). But, the mice groups receiving the herbal combination showed no significant difference from the tumour-bearing (T) mice (Fig. 3A and B). However, the final body weight of all tumour-bearing mice decreased significantly at day 24 when compared to the control (con) (Fig. 3D), likely as a result of losing gastrocnemius muscles (the opposite side

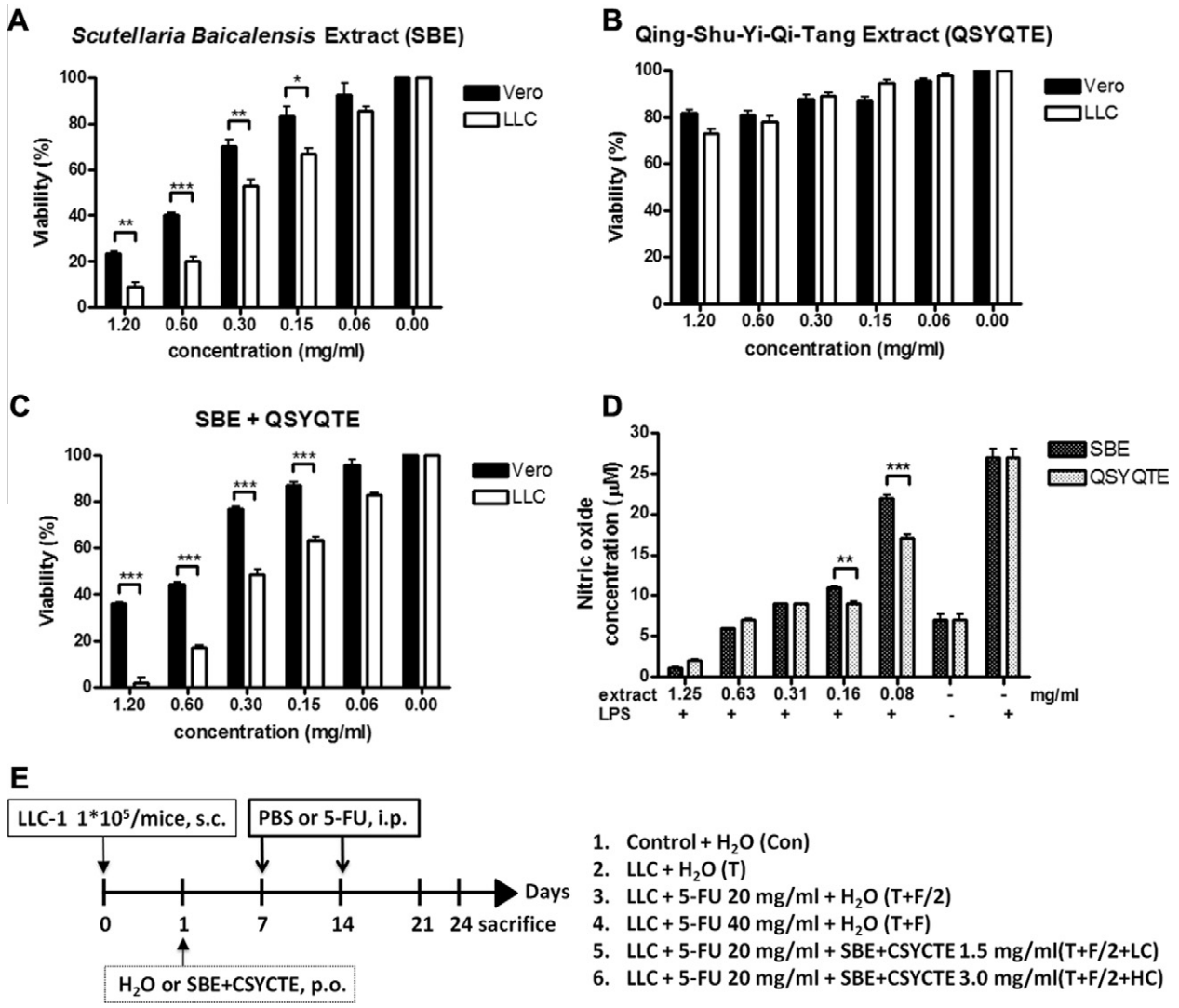

Fig. 2 – Cytotoxicity and LPS-induced nitric oxide production in the presence of Chinese herbal extracts. Cells were incubated in culture medium containing various concentrations of the *Scutellaria baicalensis* extracts (A) or the Qing-Shu-Yi-Qi-Tang extracts (B) or the combination (C) for 48 h. The growth rates of LLC and Vero cells in the presence of the selected Chinese herbal extracts were determined by the Alamar Blue assay. (D) The levels of nitric oxide in the LPS-stimulated RAW264.7 cells in the presence of the *Scutellaria baicalensis* extracts or the Qing-Shu-Yi-Qi-Tang extracts at 48 h. (E) The treatment protocol of the Chinese herbal extracts in tumour-bearing mice. Mice were fed orally with water or low- or high-dose of the Chinese herbal extracts (SBE + QSYQTE) till sacrifice. 5-FU (20 or 40 mg/kg) was given by i.p. on days 7 and 14 after tumour implantation. Data are expressed as the means ± SD (n = 6).

of tumour implantation, Fig. 3C). For the mice supplemented with a high dosage of the herbal-extracts combination (T + F/2 + HC), the body and muscle weights of the mice otherwise improved significantly. Such an improvement as a result of the dosage of 5-FU halved was ruled out because the tumour-bearing mice receiving also the halved-dosage of 5-FU (T + F/2) did not increase body weight or muscle masses.

The tumour sizes of the mice receiving 40 mg/kg of 5-FU were found to shrink significantly by 35% (tumour weight) in comparison to those of the mice receiving no treatment (Fig. 3E). Furthermore, the weights of tumours in the mice receiving 20 mg/kg 5-FU treatment were categorised into three levels, >6, 4–6 and <4 g, which corresponded to 20%, 60% and 20% of the mice subgroups (T + F/2, Fig. 3F). This distribution pattern however shifted in the mice groups supplemented with the herbal-extracts combination, in which 50% and 75% of the level of <4 g tumour weight accounted for the major distributions of the groups receiving low and high dosages of herbal-extracts, respectively (T + F/2 + LC and T + F/2 + HC, Fig. 3F). Taken together, the anti-tumour activity of the herbal-extracts combination plus the halved dosage of 5-FU is arguably equivalent to that of full-dosage 5-FU (Fig. 3E and F).

3.4. *Effect of the SBE and QSYQTE combination on immunity*

To know whether the reduction of the tumour size is in reference to immune responses, cell typing and the NK-associated

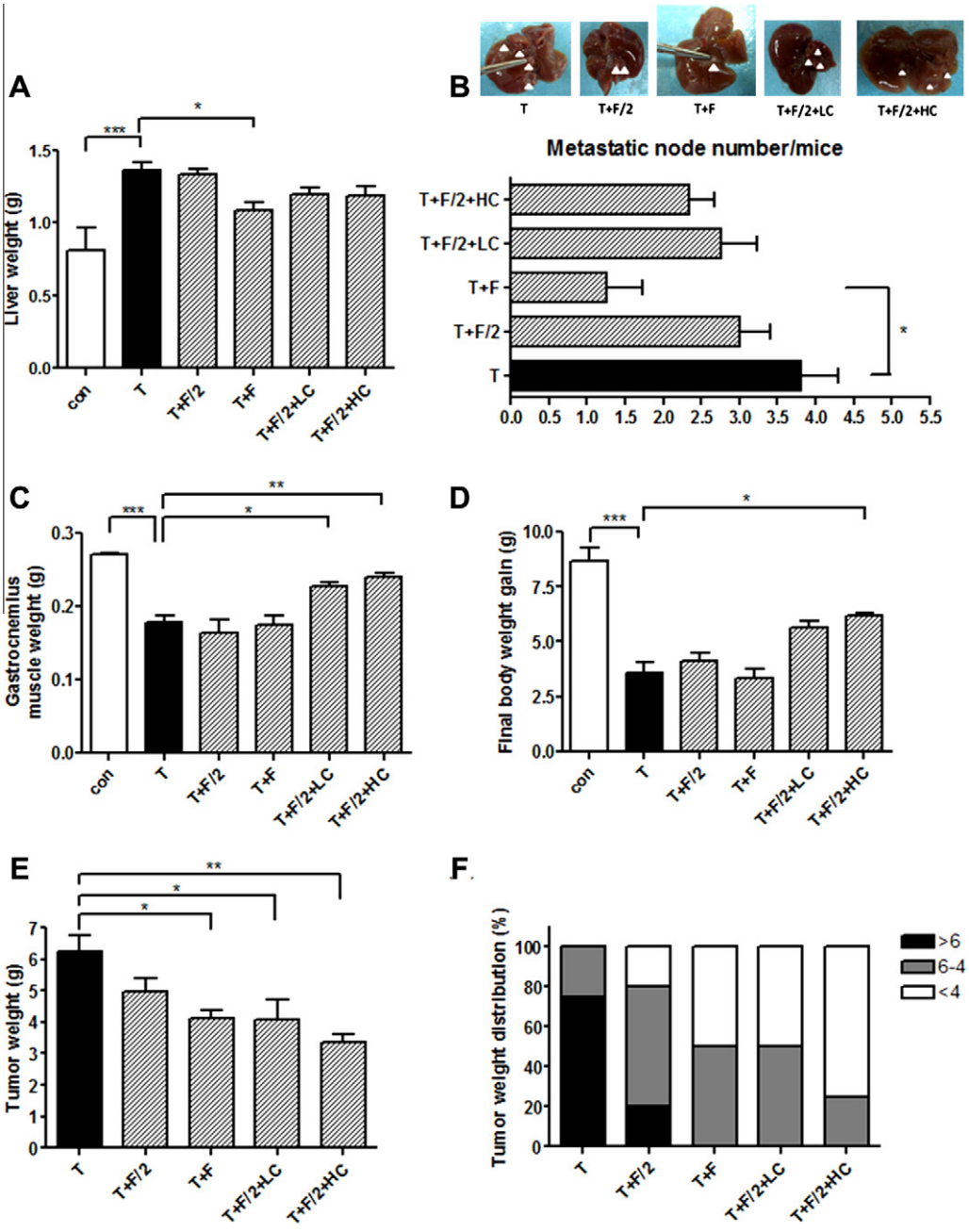

Fig. 3 – Effects of the SBE and QSYQTE combination on organ mass changes. At 24 day, mice were sacrificed and examined for livers (A), gastrocnemius muscles (C), tumour weights (E and F). The livers were subjected to histological analysis to measure metastasis (B). The gains of body weights (D) were determined by subtracting the tumour weight from the final body weight. Data are expressed as the means ± SD (*n* = 5–8 mice per group from two independent experiments).

cytotoxicity were thus examined. We first determined the cell numbers of $CD3^+$ T, $CD19^+$ B, $CD3^+CD4^+$ T and $CD3^+CD8^+$ T cells in the spleens of the control and the tumour-bearing mice at day 24 by flow cytometry. The results indicated that 50% of $CD3^+$ T cells depleted in the spleens of the mice implanted with tumour cells, while the $CD3^+$ T depletion reversed in the mice receiving high dosage of the herbal-extracts combination plus 5-FU (T + F/2 + HC). Additionally, there was no difference in the total numbers of $CD19^+$ B cells between the test and control groups (Fig. 4A).

Previous studies have reported that the occurrences of pre-malignant and malignant tissues are associated with the down-regulation of Th1 responses as well as the up-regulation of Th2 responses.[25–27] In fact, these findings are in line with our results; namely, the cell ratio of Th2 ($CD3^+CD4^+$ T cells) to Th1 ($CD3^+CD8^+$ T cells) increased in the group of tumour-bearing mice ($P < 0.01$, Fig. 4B). The Th2/Th1 ratios or the $CD3^+$ T cell numbers in the spleens of the mice administrated with halved- or full-dosage of 5-FU have no significant difference, despite the tumour growth

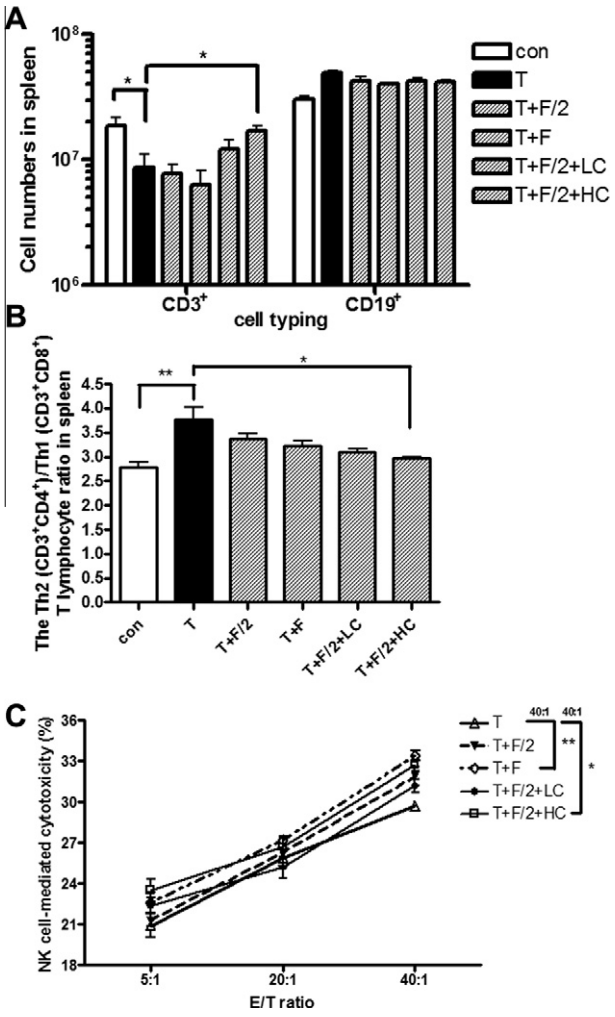

Fig. 4 – Alterations of immunological functions by the SBE and QSYQTE combination during cancer cachexia. (A) The total numbers of splenocytes in normal and tumour-bearing mice with or without 5-FU injection. The ratios of total Th2 ($CD3^+CD4^+$) to Th1 ($CD3^+CD8^+$) T cells (B), and NK cytotoxicity (C) in spleen cells. The splenocytes from C57BL/6 mice were used as the effector cells to determine the protective capability of the herbal extracts on the NK associated cytotoxicity against LLC. Data are shown as the mean ± SD (n = 3–6 mice per group from two independent experiments).

being partially or completely inhibited. In contrast, the ratio of Th2 to Th1 ($P < 0.05$, Fig. 4B) in the group administrated with the high dosage of the herbal-extracts combination plus 5-FU (T + F/2 + HC) levelled off to that of the control.

The SBE and QSYQTE combination then was examined for its effects on the NK-associated cytotoxicity. The E/T ratio of 40/1 corresponded to the maximal NK cytotoxicity, which may result from the administration of the high dose herbal extracts plus 5-FU (T + F/2 + HC). The spleens of the mice stained with propidium iodide (Fig. 4C) showed 33% of LLC cells, which should account for a significant decrease of the ratio (Th2/Th1).

3.5. *Effect of the combination of SBE and QSYQTE on cachexia*

Next, the immunological effectors in cancer cachexia were profiled. Tumours that can secrete given cytokines to cause chronic inflammation and to promote cachexia have been well documented.[5,28] In general, MCP-1 is an important mediator activating transcription factors, such as NF-κB and activated protein-1 (AP-1), in early inflammatory responses. The activation of NF-κB plays an important role by activating the E3 ubiquitin ligase genes (MuRF-1 and MAFbx) in the regulation of the proteasome-mediated protein degradation.[29,30] Serum cytokines were measured by the CBA assay. The levels of TNF and MCP-1 were found to increase 2-fold in tumour-bearing mice, while the levels of IL-6 have no significant difference in all test groups (Fig. 5A). The levels of MCP-1 were found to be down-regulated in the group receiving the high dose herbal extracts plus 5-FU (T + F/2 + HC) (Fig. 5A).

We further examined whether the herbal extracts plus 5-FU can modulate the immune signalling pathways in gastrocnemius muscle. The transcription of NF-κB mRNA was found to be significantly decreased in the groups receiving the high dose herbal extract plus 5-FU (T + F/2 + HC), while that of MuRF-1 mRNA has only modest decrease (Fig. 5B). Collectively, these results suggested that the high dose herbal extracts plus 5-FU can down-regulate the NF-κB signalling cascades, and thus attenuates cachectic symptoms to significant extents.

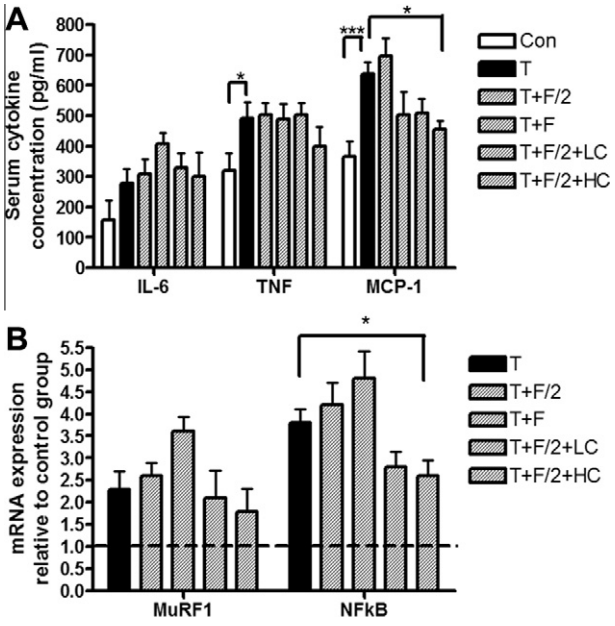

Fig. 5 – Effects of the SBE and QSYQTE combination on serum cytokines and gastrocnemius muscle gene expressions. (A) Serum cytokine levels (n = 5–8 mice per group from two independent experiments). (B) Gene expression levels of MurF1 and NF-κB in gastrocnemius muscles (n = 5 per group). Values are means of fluorescence signals, which are expressed as a percentage of healthy control mice and normalised by the mRNA amounts of GAPDH.

4. Discussion

Two major issues exist in modern cancer chemotherapies, which are: (1) drug toxicity, and (2) negative immune responses. Thus, to minimise side-effects and to maximise drug efficacy for any given medicine turn out to be the common goals in contemporary cancer therapeutic researches. In this study, the effects of the selected Chinese herbal extracts in combination with 5-FU on modulating immune functions and ameliorating cancer cachexia in murine LLC carcinoma model were investigated.

Tobacco smoke is the main cause for lung cancer and is responsible for 87% of all lung cancers in the United States.[31] Cigarette smoke, a major source of exogenous oxidants, leads to chronic airway inflammation with accumulation and activation of leucocytes which produce high levels of reactive oxygen species (ROS) and NO. Excessive or inappropriate production of endogenous and/or exogenous ROS and NO is implicated in the pathogenesis of lung cancer.[32,33] Among the Chinese herbal extracts tested, the Qing-Shu-Yi-Qi-Tang extracts (QSYQTE) were demonstrated to exhibit high anti-oxidation and anti-inflammation activities (Figs. 1 and 2D). Although the anti-oxidation activity of the *S. baicalensis* extracts (SBE) is not as high as that of QSAYQTE, in light of many reports[22,34,35] SBE otherwise possesses some desirable cytostatic effects.[36,37] In this report, we evidenced that the favourable immune responses and tumour-growth suppressions are result of the synergistic effect of SBE and QSYQTE (Fig. 2C).

SBE and QSYQTE were formulated and applied to the murine model upon 5-FU chemotherapy, wherein 5-FU is known to be effective against metastasis[38,39] and 40 mg/kg is in equivalence to the dose used in man.[40] However, 5-FU has deleterious side-effects, limiting their clinical application.[41,42] In order to minimise side-effects and maximise therapeutic effects, one-half dose of 5-FU (20 mg/kg) in combination with the herbal extracts was designed. The administration of the herbal combination was shown to regain body and muscle weights upon the 5-FU (half dose) chemotherapy, in addition to no change in metastatic foci (Fig. 3A and B). These effects may be attributed to the herbal combination which sensitise tumour cells to 5-FU or elicit some desired host immunity (Fig. 3E and F). Similarly, Takara et al. had independently demonstrated the *S. baicalensis* extracts can enhance the sensitivity of cancer cells to paclitaxel in HeLa cells.[43]

Immune suppressions as a result of chemotherapy often lead to disease aggravations, complications and delay suboptimal treatments (e.g. surgery, chemotherapy, radiotherapy) so as to decrease life quality and prognosis.[7,8,44,45] Cachexia is often associated with lymphopenia, and cachexia in C57BL/6 mice induced by LLC used in this study is no exception (Fig. 4A). An association between immunodeficiency and cachexia[46–48] has led us to speculate that a deficiency in the immune system might play a direct role in the development of cachexia, and that correcting or inhibiting the immune deficiency might allow protection. The dysfunction of the immune system is of multiple mechanisms and is often manifested with the reductions of monocyte-, macrophage- and dendritic-cell functions as well as NK-cell activity, which would increase risks of infections and the poor clinical outcome.[7,8,49,50] In addition, protection from wasting and muscle atrophy by infusion of $CD4^+$ T cells is associated with protection from lymphopenia.[51] Similarly, in our tumour model study the tumour-bearing mice exhibited an increased Th2 responses and an impaired NK-associated cytotoxicity (Fig. 4), while the tumour-bearing mice undergoing chemotherapy supplemented with the herbal combination showed an improved immune status. Our treatment strategy was based on some reports: Chui-Uren-Chien (CUC) is able to modulate non-specific immune responses.[52] Taxanes was shown to stimulate anti-cancer immune responses in humans; patients with breast cancer (stage II/III) under the treatment of taxanes can enhance T cell and NK cell functions.[53] In the study, the increased NK cell-mediated cytotoxicity was considered to be a synergistic effect from the herbal extracts as well as the drug.

The production of IL-6 in Th2 cells made no difference in tumour-bearing mice, which may be owing to that the assay used is less sensitive as diurinal fluctuations and/or a short half-life of IL-6. On the other hand, both serum levels of TNF and MCP-1 increase significantly after tumour implantation. However, the levels of MCP-1 significantly reduce in the group supplemented with the herbal combination plus 5-FU. MCP-1 has been known to play an important role in tumour progression.[54] Previous studies also revealed that both NFκ-B and AP-1 are subjected to TNF stimulation to regulate MCP-1 transcription.[55–57] For example, Wogonin, an active component in the root of *S. baicalensis Georgi*, was reported to down-regulate NF-κB in the signalling pathway of MCP-1-stimulated microglia.[58]

The activation of NF-κB would lead to an increased activity of the ubiquitin–proteasome proteolytic pathway, admittedly to be the principal protein degradation pathway in cancer cachexia.[59] Two muscle-specific ubiquitin ligases, MuRF-1 and atrogin-1/muscle atrophy F-box (MAFbx), are known to be up-regulated in catabolic conditions.[29] Here, the herbal combination is able to inhibit the NF-κB-mediated proteolytic pathway. In spite of no significant differences in the test groups, the expressions of MuRF-1 were found to be inhibited in the groups treated with the high-dose herbal extracts. We reasoned that MuRF-1 is a downstream effector of NF-κB, while the detailed mechanism of the NF-κB-mediated ubiquitin–proteasome proteolysis requires further study.

Taken together, our results showed that this given herbal-extracts combination, (Qing-Shu-Yi-Qi-Tang and *S. baicalensis*), is satisfactory in ameliorating cancer cachectic symptoms. The present findings suggest this formula can suppress tumourigenesis and tumour growth by exerting both anti-inflammation and -oxidation activities. With possessing a selective toxicity to some tumour cells, this formula would also be an ideal starting point for developing more effective treatments against cancers.

Conflict of interest statement

None declared.

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