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In vivo and *in vitro* anti-tumor and anti-metastasis effects of *Coriolus versicolor* aqueous extract on mouse mammary 4T1 carcinoma

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ABSTRACT

Coriolus versicolor (CV), a medicinal mushroom widely consumed in Asian countries, has been demonstrated to be effective in stimulation of immune system and inhibition of tumor growth. The present study aimed to investigate the anti-tumor and anti-metastasis effects of CV aqueous extract in mouse mammary carcinoma 4T1 cells and in 4T1-tumor bearing mouse model. Our results showed that CV aqueous extract (0.125–2 mg/ml) did not inhibit 4T1 cell proliferation while the non-cytotoxic dose of CV extract (1–2 mg/ml) significantly inhibited cell migration and invasion ($p < 0.05$). Besides, the enzyme activities and protein levels of MMP-9 were suppressed by CV extract significantly. Animal studies showed that CV aqueous extract (1 g/kg, orally-fed daily for 4 weeks) was effective in decreasing the tumor weight by 36%, and decreased the lung metastasis by 70.8% against untreated control. Besides, micro-CT analysis of the tumor-bearing mice tibias indicated that CV extract was effective in bone protection against breast cancer-induced bone destruction as the bone volume was significantly increased. On the other hand, CV aqueous extract treatments resulted in remarkable immunomodulatory effects, which was reflected by the augmentation of IL-2, 6, 12, TNF-α and IFN-γ productions from the spleen lymphocytes of CV-treated tumor-bearing mice. In conclusion, our results demonstrated for the first time that the CV aqueous extract exhibited anti-tumor, anti-metastasis and immunomodulation effects in metastatic breast cancer mouse model, and could protect the bone from breast cancer-induced bone destruction. These findings provided scientific evidences for the clinical application of CV aqueous extract in breast cancer patients.

Introduction

Coriolus versicolor (CV), also known as *Trametes versicolor* (L.) Lloyd, is a mushroom belonging to the species of Polyporaceae family, which is characterized by hymenium (fertile layer) in vertical pores on the underside of the caps (Hobbs 1995). Its medicinal value was recorded in the *Compendium of Chinese Materia Medica* and *Shen Non Compendium Medica* thousands of years ago in China. Nowadays, both preclinical and clinical evidences demonstrated that extracts obtained from CV displayed a wide array of biological activities, including stimulatory effects on immune system and inhibition of cancer growth (Zhou et al. 2007; Ng 1998; Sakagami et al. 1991; Tsukagoshi et al. 1984). It was demonstrated that the aqueous extract of CV was found to be effective in activating T lymphocytes, B lymphocytes, macrophages, natural killer (NK) cells, and lymphocyte-activated killer cells (Ng 1998; Sakagami et al. 1991), as well as promoting the production of antibodies and various cytokines such as IL-2 and IL-6, and tumor necrotic factor (TNF) *in vivo* (Tsukagoshi et al. 1984; Yang et al. 1999). Besides, the anti-tumor activities of CV extract have also been extensively studied both *in vitro* and *in vivo*. Our previous studies demonstrated that the ethanol-water extract of CV significantly increased the production of nucleosome in human leukemia (HL-60 and NB-4) and lymphoma (Raji) cells as well as induced apoptosis in breast cancer cells (MCF-7, MDA-MB-231, T-47D) (Lau et al. 2004; Ho et al. 2005).

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Similar findings were obtained by Yang et al. that an acidic fraction from CV inhibited the growth of the human leukemia (HL-60), liver cancer (SMMU-7721), and stomach cancer (SCG-7901) cells (Yang and Chen 2000; Dong et al. 1997). In addition, *in vivo* studies showed that prolonged administration of CV extract resulted in significant reduction of tumor size in mice with leukemia (Ho et al. 2006; Yang et al. 1992), lung (Zeng et al. 1993), or liver tumors (Dong et al. 1999). Furthermore, scientific evidences showed that CV prevented metastasis and improves survival rates in colorectal, gastric, lung, and breast cancers (Reigetsu et al. 2005; Eliza et al. 2012). The polysaccharides are believed to be responsible for the biological activities of CV (Chu et al. 2002). The largest and most biologically active group of CV is polysaccharide-K (PSK) and polysaccharopeptide (PSP). PSK and PSP are both protein-bound polysaccharides which are derived from the CM-101 and COV-1 strains of the mushroom CV by Japanese and Chinese researchers, respectively (Fisher and Yang 2002). PSK, also known as Krestin, is a unique protein-bound polysaccharide, which has been used as a chemo-immunotherapeutic agent in the treatment of cancer in Asia for over 30 years. Both polysaccharide preparations have documented anticancer activity *in vitro*, *in vivo* and in human clinical trials (Fisher and Yang 2002). Despite the various activities of CV extract and active ingredients, the aqueous extract of CV was seldom investigated in metastatic breast cancer with severe tumor metastasis to lung, liver and bone.

Breast cancer is the most common cancer among women worldwide, which ranks the top of most frequent cancer types in 2008, with a worldwide incidence of around one in eight women (Ferlay et al. 2010). In 2011, an estimation of about 300,000 new cases of breast cancer was expected to be diagnosed in women in United States, and about one in five women would die from the disease. The mortality usually results from the tumor metastatic spread to other organs. Breast cancer is characterized by a high potential to spread to almost any region of the body, and the most common sites are the bone, followed by the lung and liver. It is estimated that over 70% of patients dying of breast cancer get bone metastasis and associated with bone destruction (Suva et al. 2009). In order to mimic the high potential of tumor metastasis in humans, the mouse mammary carcinoma 4T1 cells were chosen. 4T1 cells are highly invasive and have metastatic characteristics displaying primary tumor metastasizes to lung, liver, bone and brain as early as 2 weeks after inoculation, resembling closely to the stage IV of breast cancer in humans (Heppner et al. 2000; Tao et al. 2008). In this study, we aimed to investigate the anti-migration and anti-invasion abilities of CV aqueous extract in 4T1 cells *in vitro*. Besides, the *in vivo* anti-tumor, anti-metastasis, anti-osteolysis and immunomodulatory activities of CV aqueous extract were also evaluated in a mouse 4T1-tumor bearing model.

Materials and methods

Aqueous extract preparation and chemical analysis

The dried fruiting bodies of *Coriolus versicolor* (CV) with origin of Yunnan Province of China, were purchased from the herbal supplier of Hong Kong and deposited in the museum of the Institute of Chinese Medicine, The Chinese University of Hong Kong with voucher specimen number as 2010–3291. One kilogram dried mushroom of CV was milled into powder and soaked in distilled water for 1 h, and then extracted with boiling water under reflux for another hour. The extract was filtered, and the extraction was repeated once. Subsequently, the filtrates were combined and evaporated under vacuum (EYELA N-1000, Tokyo Riakikai Co., Ltd., Tokyo, Japan) and then lyophilized with a freeze dryer (Ilshine Lab Co., Ltd., Dongduchun, Korea) into powder. The percentage yield of CV aqueous extract was 15.7% (w/w).

The major components of the CV aqueous extract are polysaccharides, and the polysaccharide content was analyzed in triplicates using the phenol sulfuric acid assay (Dubois et al. 1956). In order to determine the apparent molecular weight of CV aqueous extract, the Waters ACQUITY UPLC system (Waters, Milford, MA) equipped with a TSK-SW4000 column and an evaporative light scattering detector was employed (Han et al. 2012). Sample solution (10 μl of 1.0 mg/ml) was injected for each run, with water as the mobile phase at a flow rate of 0.3 ml/min. The linear regression was calibrated using T-series dextran standards (MW 2000, 410, 270, 150, 80, 50, 12, 5 kDa). The amino acid contents in CV aqueous extract was determined by ninhydrin colorimetric method (Oka et al. 1981).

Cells and reagents

The 4T1 cells, which were purchased from ATCC, USA, were cultured in RPMI-1640 medium containing 10% (v/v) fetal bovine serum (FBS) and 1% penicillin–streptomycin (Life technologies, USA) at 37 °C in 5% CO_2 humidified incubator. ELISA kits IL-2, IL-6, IL-10, IL-12, TNF-α, IFN-γ were purchased from BD Pharmingen, USA. Doxorubicin (DOX), heparin, and 3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma, USA. Transwell plates for transwell migration assay were from Corning Incorporated, USA. Creatine kinase (CK), alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) kits were purchased from Stanbio, USA. Ficoll-paque was purchased from GE Healthcare, UK. Coomassie brilliant blue and glycerol were obtained from USB, USA. Primary antibodies against MMP-2, MMP-9, β-actin, and secondary antibody conjugated to horseradish peroxidase were purchased from Abcam (Hong Kong).

Cytotoxicity assay

In order to determine the cytotoxicity of CV aqueous extract on 4T1 cells, the MTT assay was employed. The assay detects the reduction of MTT and reflects the normal functioning of mitochondria and hence cytotoxicity. Cells (1×10^4/well) were seeded in 96-well flat-bottomed plates and incubated with various concentrations of CV extract for 24, 48, and 72 h. Following incubation, 30 μl of MTT solution (5 mg/ml in PBS) was added to each well and the plate was incubated at 37 °C for another 4 h. Then, the medium was discarded and 150 μl of DMSO was added to dissolve the formazan crystals. The absorbance of each sample was read at 540 nm using a microplate reader (Biotek μ-Quant, USA). Results were expressed as percentage of cell viability with respect to untreated control cells (as 100%).

Cell migration and invasion assays

The effects of CV aqueous extract on 4T1 cell migration and invasion *in vitro* were assessed using scratch wound healing and transwell migration assays.

In the scratch wound healing assay, 4T1 cells (1×10^5/well) were seeded in 24-well plates and incubated at 37 °C for 24 h. After 24 h starvation in culture medium without FBS, the cells were then scraped with crosses in the middle of well using 200 μl pipette tips. The culture medium was changed with fresh medium with various concentrations of CV. After cells incubated for different time intervals (9 or 18 h), each well was photographed under a microscope (Nikon Eclipse TS100). The percentages of open wound area were measured and calculated using the TScratch software (Vigl et al. 2009).

In the transwell migration assay, 4T1 cells (5×10^4 in 100 μl culture medium) were added into transwell chambers. At the same time, 100 μl medium containing various concentrations of CV (with 1% (v/v) FBS) was added to the upper chambers. Then, 500 μl of complete culture medium (with 10% (v/v) FBS), served as chemoattractant media, was added in the lower chambers. After incubation for 4–5 h at 37 °C, cells were fixed with methanol and stained with hematoxylin. The non-migrated cells on the top surface of the filter membrane were scraped with cotton swab. Stained filters were photographed under microscope (Nikon Eclipse TS100). The migrated cells were quantified by manual counting in blinded manner (Xiao and Singh 2007). Changes in cell numbers are represented as a percentage of control values (as 100%).

Gelatin zymography

Degradation of the extracellular matrix and components of the basement membrane by matrix metalloproteinases MMP-2 and MMP-9, play a critical role in tumor invasion and metastasis. Cells (1×10^5/well) in 1 ml culture medium were seeded in 24-well plates and incubated with various concentrations of CV (0, 0.5 and 1 mg/ml) at 37 °C for 24 h. The supernatant was collected and stored at −80 °C. Protein sample (20 μg) from the supernatant was fractionated in 10% SDS–polyacrylamide gel with 0.1% gelatin substrate. Following electrophoresis, the gels were washed three times in 2.5% Triton X-100 in PBS for 30 min at room temperature. The gels were then incubated overnight at room temperature in developing buffer (50 mM Tris base, 200 mM NaCl, 0.005 mM $ZnCl_2$, 5 mM $CaCl_2 \cdot 2H_2O$, and 0.02% NaN_3, pH 7.5) and then stained with 0.125% (w/v) Coomassie brilliant blue for 20–30 min and destained in destain buffer (10% acetic acid and 5% ethanol in distilled water) for 1–2 days. Visualization of bands was performed on a Bio-RAD, XBS+ imaging system (Bio-Rad, USA).

Western blot

4T1 cells (5×10^5/well) were seeded in 90 mm dishes and incubated with various concentration of CV aqueous extract at 37 °C for 24 h. The cells were lysed in lysis buffer (0.02% aprotonin, 2% SDS, 10% glycerol, 62.5 mM Tris–HCl, pH6.8) on ice for 15 min. After the lysate was boiled, the protein samples (50 μg) were fractionated in 12% SDS-polyacrylamide gel. Proteins on the gel were transferred to PVDF membrane (Millipore, USA). Afterwards, the membrane was incubated with primary antibodies overnight at room temperature. After washing with PBS-T, the membrane was then incubated with secondary antibodies conjugated with phosphatase for 2 h. Visualization of protein bands was performed using the ECL substrate reagent kit (GE Healthcare, USA) on a ChemiDoc XBS imaging system (Bio-Rad, USA).

Mouse mammary tumor model

Female BALB/c mice (6–8 weeks of age) were provided by Laboratory Animal Services Center, The Chinese University of Hong Kong, and were housed under pathogen-free conditions. The experiments were approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong. 4T1 cells (4×10^5, resuspended in 200 μl PBS) were subcutaneously (s.c.) inoculated at the mammary fat pad of the mice. One week after 4T1 cells inoculation, the tumor-bearing mice were randomly assigned into three groups ($n = 10$): control group (distilled water, oral gavage everyday), CV aqueous extract group (1 g/kg, oral gavage everyday), doxorubicin (DOX) group (2 mg/kg, i.p. injected twice a week). All treatments lasted for 4 weeks. Body weight of each animal was measured once a week during treatment period. On day 28, mice were anaesthesized, blood was collected by cardiac puncture and the plasma was stored at −80 °C until plasma enzyme analysis. Lungs and livers were excised for the quantification of tumor burden. Tibias of mice were removed for micro-computed tomography analysis.

The effects of CV aqueous extract treatments on hemato-biochemical markers were assessed by measuring the activities of liver specific (AST, ALT), bone-related (ALP) and heart-related (CK) enzymes in the plasma using commercially available kits.

Histology

Lungs and livers of tumor-bearing mice were excised and fixed in 10% buffered formalin for 7 days at room temperature. Then, the tissue samples were paraffin embedded, sectioned longitudinally at 5 μm, and stained with hematoxylin and eosin (H&E). Stained sections were examined and photographed using an Olympus IX71 microscope (Japan) and SPOT advanced software (version 3.5.6). Tumor burden, defined as the tumor area to the total lung or liver area on the section, was measured using Image J (NIH, USA) and expressed as an average tumor area of 3 sections per mouse in absolute unit (mm^2).

Micro-computed tomography (μ-CT) analysis

Tibias removed from tumor-bearing mice were scanned with a high resolution microtomographic system, μ-CT 40 (Scanco Medical AG, Switzerland). Tibias were aligned perpendicularly to the scanning axis. The scanning was conducted at 55 kVp and 144 μA with a resolution of 8 μm per voxel. Each three-dimensional (3D) image data consist of approximately 500 micro-CT slide images starting from the growth plate of tibial interface and moving down the tibia. Bone volume (mm^3) was generated from μ-CT analysis and compared with the control tibia for each animal.

Proliferation and cytokines production of spleen lymphocytes of tumor-bearing mice

The spleen lymphocytes were isolated as described previously (Yue et al. 2008). The isolated spleen lymphocytes were resuspended in RPMI-1640 medium and seeded in 96-well flat-bottom culture plates at a concentration of 5×10^4 cells/well. The mitogen, lipopolysaccharide (LPS, Sigma, USA) or concanavalin A (ConA, Sigma, USA), was added at a final concentration of 10 μg/ml. After 72 h incubation, the cell-free supernatant was collected and stored at −80 °C for enzyme-linked immunosorbent assay (ELISA) experiments. The supernatants were analyzed for the concentrations of various cytokines such as IL-2, IL-6, IL-10, IL-12, TNF-α and IFN-γ by ELISA. The assays were carried out according to the manufacturer protocols. Results were expressed as the percentage of cytokine concentration with respect to untreated control cells as 100%.

To determine the proliferative response of spleen lymphocytes, the cells incubated with or without mitogens for 72 h were subjected to [methyl-^{3}H]-thymidine incorporation assay (Yue et al. 2012). Results were expressed as percentage of count per minute with respect to untreated control cells as 100%.

Statistical analysis

All data were expressed as mean ± SD for *in vitro* studies, or mean ± SEM for *in vivo* studies. Statistical analysis was performed using one-way ANOVA, with p-value <0.05 as regarded statistically significant.

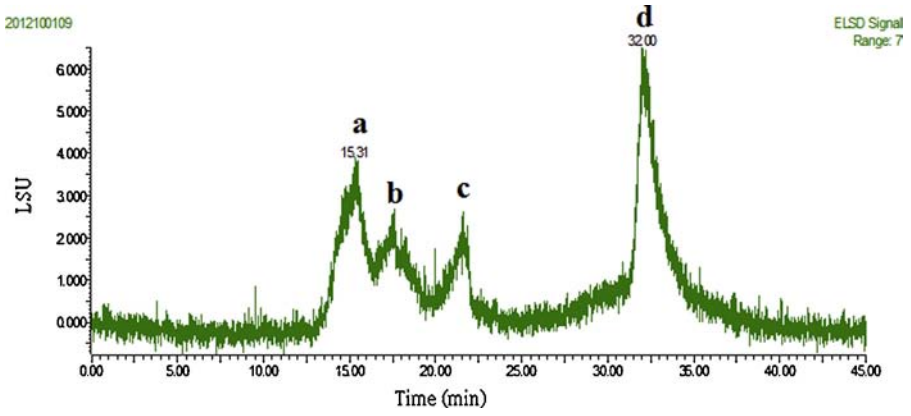

Fig. 1. UPLC chromatogram of CV aqueous extract with four fractions (a)–(d). For molecular weight evaluation, a Waters Acquity UPLC system was used. The molecular mass was calculated by referring to a series dextran molecular standards.

Results

Phytochemical analysis of CV aqueous extract

Using phenol sulfuric acid assay, the polysaccharide contents in CV aqueous extract was found to be 8.34 ± 0.11% (w/w). Using ninhydrin colorimetric method, the amino acid content in CV aqueous extract was 38.4 ± 0.9% (w/w).

The UPLC analysis showed that CV aqueous extract mainly composed of four fractions, according to the T-series Dextrans, fraction a is locating in the molecular weight range of 270–2000 kDa, with a peak of 376 kDa; fraction b is locating in the molecular weight range of 50–270 kDa, with a peak of 169 kDa; fraction c is locating in the molecular weight range of 12–50 kDa, with a peak of 41.9 kDa; fraction d is locating in the molecular weight range less than 12 kDa, with a peak of 1.1 kDa (Fig. 1).

Cytotoxicity of CV extract on 4T1 cells

The CV aqueous extract at 0.125–2 mg/ml did not show any obvious cytotoxicity on breast cancer 4T1 cells *in vitro* after incubated for 24 or 48 h (Fig. 2). Though the inhibition effect of CV extract on 4T1 cells was enhanced after incubated for 72 h, the cell viability was still about 85% even at the highest concentration of 2 mg/ml. Hence, the non-cytotoxic dose range (0.25–2 mg/ml) of CV aqueous extract was chosen for further investigation in migration and invasion assays.

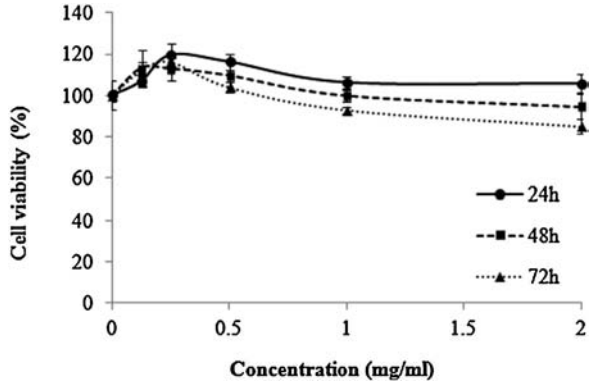

Fig. 2. Cytotoxicity of CV aqueous extract on 4T1 cells after 24, 48 or 72 h incubation. Data were expressed as mean ± SD.

Inhibitory effect of CV aqueous extract on 4T1 cell migration and invasion

Cell migration and invasion are essential components in cancer propagation (Perlikos et al. 2013). To determine the efficacy of CV aqueous extract against cancer cell migration and invasion *in vitro*, the scratch wound and transwell migration assays were employed. As shown in Fig. 3A and B, CV significantly inhibited cell migration of 4T1 from 1 to 2 mg/ml after 9 h incubation, and the inhibition was enhanced when the incubation lasted for 18 h (Fig. 3C). In Fig. 3D, CV aqueous extract inhibited 4T1 cells invasion efficiently with the increase in CV concentrations. In the presence of 0.5, 1 and 2 mg/ml, CV inhibited cell invasion of 4T1 cells significantly by 40.7%, 49.8% and 61.5%, respectively.

Effect of CV aqueous extract on the activities and protein levels of MMP-2 and MMP-9

Gelatin zymography was carried out to evaluate the effect of CV aqueous extract on the activity of matrix metalloproteinases (MMP)-2 and -9, which played an important role in extracellular matrix (ECM) and basement membrane degradation (Chaudhary et al. 2013). As shown in Fig. 4, MMP-9 and MMP-2 in 4T1 cell culture supernatant were detected in the gel at molecular weight of 92 and 72 kDa, respectively. The enzyme activity of MMP-9 was suppressed by CV dose-dependently, while the activity of MMP-2 was not affected by CV treatment. In order to further confirm the result of CV aqueous extract on MMP-2 and -9, the western blot analysis was performed. As shown in Fig. 5, the protein levels of MMP-9 was significantly inhibited after the cells were treated with CV aqueous extract, while no significant difference was shown in MMP-2, these findings were consistent with the zymography results.

CV aqueous extract inhibited in vivo growth of 4T1 tumors and metastasis to lung and liver

To investigate the activity of CV aqueous extract on breast tumor growth and metastasis, orthotopic breast tumor model was employed. It was observed that no significant body weight loss was found in mice in CV-treated group (Fig. 6A) during the treatment period. However, the western chemotherapeutic drug doxorubicin (DOX) significantly decreased the body weight of animals on days 21 and 28, indicating that DOX was toxic to the animal. Besides, there was no significant change on the activities of liver specific (AST, ALT), bone-related (ALP) and heart-related (CK) plasma

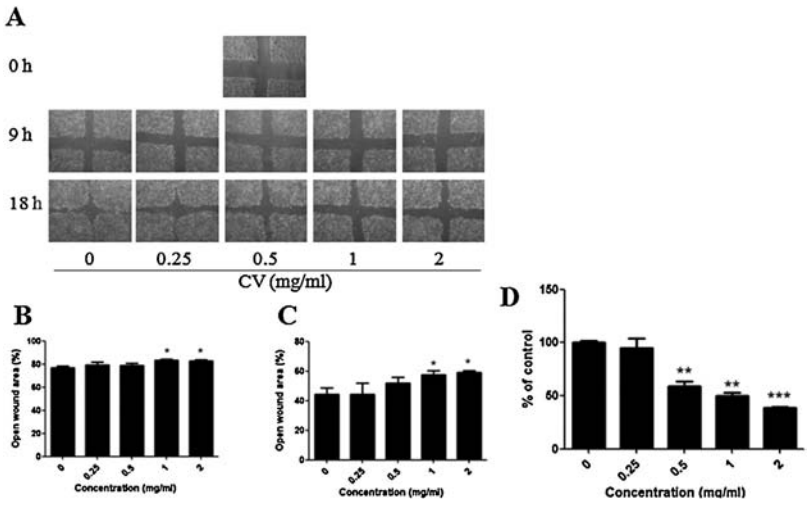

Fig. 3. Effect of CV on 4T1 cell migration and invasion activities in scratch wound and transwell migration assays, respectively. (A) Representative images of the wounded cell monolayers of 4T1 cells. (B) and (C) Quantitative analysis of the anti-migration activity of CV after 9 h (B) and 18 h (C) of incubation. Data were expressed as the percentage of open wound area from baseline cultures without treatment and presented as the mean + SD ($n = 4$). (D) Quantitative analysis of the anti-invasion activity of CV. Data were presented as mean + SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with control.

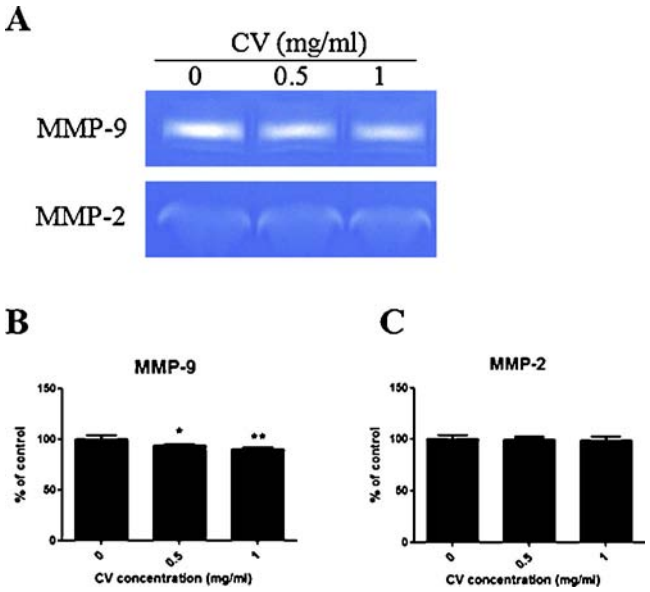

Fig. 4. Effect of CV aqueous extract on MMP-9 and MMP-2 activities. (A) Representative zymograms were shown on 4T1 cells after treated with CV aqueous extract (0, 0.5 and 1 mg/ml) for 24 h. (B)–(C) Statistical analysis of MMP-9 (B) and MMP-2 (C) activities after CV aqueous extract treatment. Data were shown as mean + SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with control.

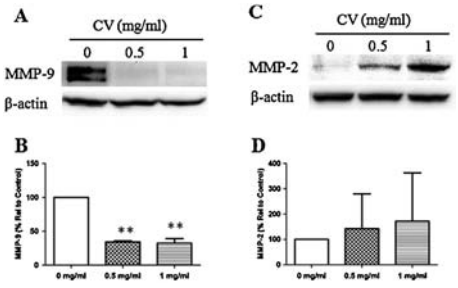

Fig. 5. Effect of CV aqueous extract on MMP-9 and MMP-2 proteins. (A, C) Representative images of Western blot of proteins after treated with CV aqueous extract (0, 0.5 and 1 mg/ml) for 24 h. (B, D) Statistical analysis of MMP-9 (B) and MMP-2 (D) on protein expressions after CV aqueous extract treatment. Data were shown as mean + SD ($n = 3$). $^{**}p < 0.01$ as compared with control.

enzymes (Fig. 6B) after 4-weeks of CV aqueous extract treatment. Nevertheless, tumor weights at the orthotopic site were significantly decreased in CV-treated group by 36.0% when compared with untreated control group (Fig. 6C and D). A significant difference was present between CV and DOX groups.

Lungs and livers from tumor-bearing mice were excised for assessment of metastasis. Fig. 6 showed the representative histological sections of lungs (Fig. 7A) or livers (Fig. 7C) from different groups. Tumor nodules were found in control group as the arrows pointed, indicating the successful establishment of metastasis to lungs and liver in 4T1 tumor-bearing mouse model. The tumor area in lungs was decreased significantly in CV- and DOX-treated groups. Tumor burden in lungs was found to decrease by 70.7% (from 6.19% to 1.81%) in CV-treated group, against untreated control group in which the tumor burden in lung was 6.2%. The results suggested that the CV aqueous extract were effective in decreasing metastasis to lung (Fig. 7A and B). However, there was no obvious change in the tumor burden in liver after CV treatment (Fig. 7C and D).

CV aqueous extract protected the bone from breast cancer-induced bone destruction

As shown in Fig. 8A, the 3D μ-CT images of representative tibia from control group demonstrated extensive bone destruction when compared to the corresponding tibia from naive group (normal mice group without tumor and treatment). Animals treated with CV aqueous extract showed preservation of the integrity of bone. To quantify the total bone volume (BV), the tibias of all the animals in each group were compared at a selected region beginning at the growth plate and extending downwards 500 × 8 μm slices, which encompassed all of the cancer lesions. The amount of bone volume in control group was 2.85 mm^3, lost nearly 30% of BV when compared to the tibia of naive group (Fig. 8B). In contrast, CV aqueous extract showed remarkable protection of bone against breast cancer-induced osteolysis, with a significant increase (10.3%) in BV of CV-treated mice when compared with control group.

CV aqueous extract promoted the cytokines production in tumor-bearing mice

In order to assess the immunomodulatory effects of the CV aqueous extract on tumor-bearing mice, spleen lymphocytes were

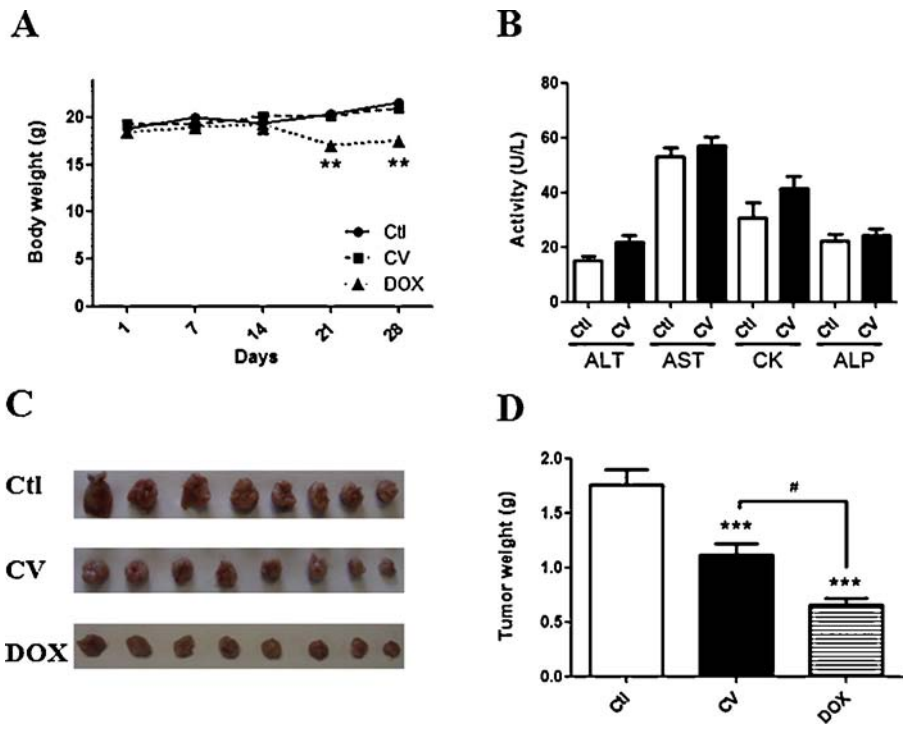

Fig. 6. *In vivo* anti-tumor effect of CV aqueous extract in 4T1 mouse mammary tumor model. (A) Body weight change during treatment periods. (B) Evaluation of the hemato-biochemical markers of mice plasma after treated with CV aqueous extract. (C) Representative tumor images from different groups at the end of experiment. (D) The anti-tumor effects of CV aqueous extract against mouse mammary tumors. Data were expressed as mean + SEM, $n = 10$. $^{**}p < 0.01$ and $^{***}p < 0.001$, as compared with control. "Ctl" means control group, in which the mice were bearing with 4T1 tumor and treated with distilled water.

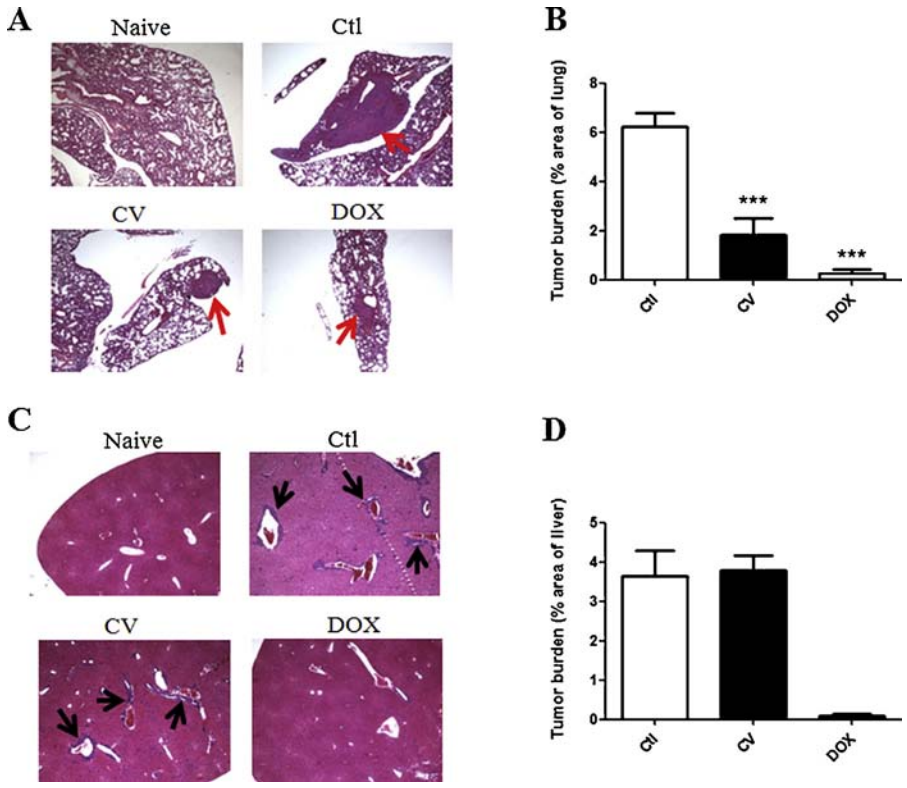

Fig. 7. Effects of CV aqueous extract on lung and liver metastasis in mice orthotopic breast cancer. (A) Representative H&E-stained sections of mouse lungs with arrows showing the 4T1 tumors in lungs. Histological sections of the lungs were used to assess tumor burden of lungs. (B) The graph represented the tumor burden in lungs according to the tumor area as a percentage of whole lung area. Data shown were an average from representative sections of each animal. Mean + SEM, $n = 10$. (C) Representative H&E stained sections of livers obtained from different groups. (D) Graph showed the tumor burden in liver expressed as an average per group. Data were expressed as mean + SEM, $n = 10$. $^{***}p < 0.001$, as compared with control. "Ctl" means control group, in which the mice were bearing with 4T1 tumor and treated with distilled water. "Naive" means normal mice without tumor and treatment.

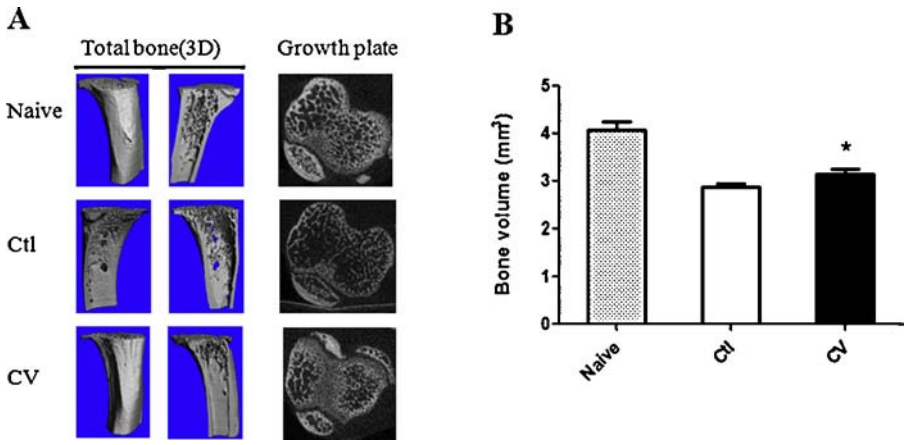

Fig. 8. Qualitative and quantitative assessment of bone structure in mice tibias after administration of CV aqueous extract. (A) Representative μ-CT 3D images, section-cut and growth plate of mice tibias obtained from different groups. (B) The graph showed the bone volume of tibias from different groups. Data were expressed as mean + SEM, $n = 10$. $^{*}p < 0.05$, as compared with control. "Ctl" means control group. "Naive" means normal mice without tumor and treatment.

isolated from 4T1 tumor-bearing mice treated with or without CV aqueous extract and cultured *ex vivo*. As shown in Fig. 9A, the proliferative responses of spleen lymphocytes from CV-treated group were significantly higher than that from control group ($p < 0.05$). Similarly, the cytokines productions, such as IL-2, IL-6, IL-12, IFN-γ, and TNF-α from splenic lymphocytes were higher in CV treatment group when compared with untreated control group (Fig. 9B–E), while no significant change was shown in IL-10 (Fig. 9G).

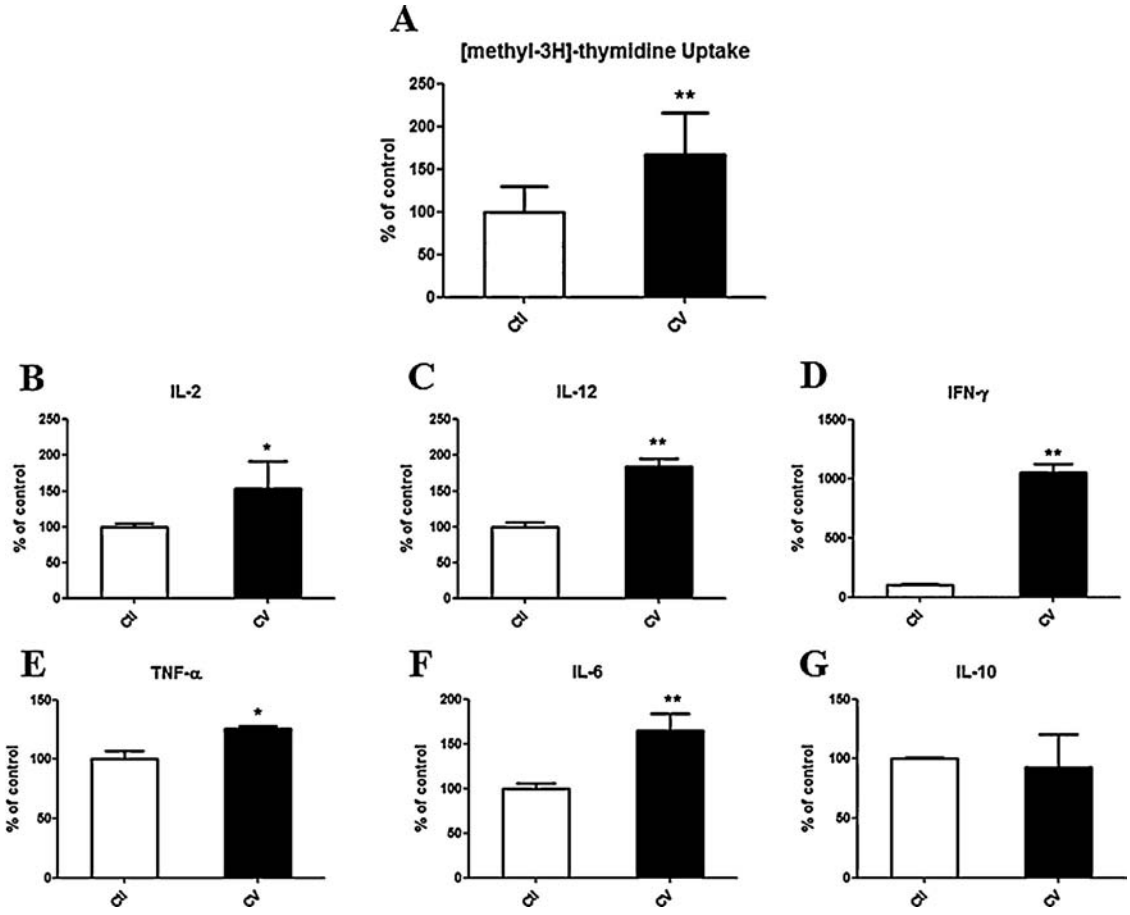

Fig. 9. Proliferative response and cytokines productions of mouse splenic lymphocytes after CV aqueous extract treatment. (A) Proliferative response of mouse splenic lymphocytes after CV treatment. (B)–(G) Cytokines productions of mouse lymphocytes after CV treatment. (B) IL-2, (C) IL-12, (D) IFN-γ, (E) TNF-α, (F) IL-6 and (G) IL-10 productions were assessed. The resting lymphocytes (A, B, C) and LPS-activated lymphocytes (D)–(G) from tumor-bearing mice after treated with CV aqueous extract were seeded in 96-well plates and incubated for 72 h. Culture supernatants were collected and the cytokines productions were specifically determined by ELISA. Data were expressed as mean + SD. $^{*}p < 0.05$ and $^{**}p < 0.01$, as compared to control. "Ctl" means control group.

Discussion

Throughout the world, Chinese medicinal mushroom *Coriolus versicolor* (CV) has been widely consumed as health-promoting food supplements and medicine. CV enjoyed a long history in China as food especially used in soups, to boost the immune system and supply a comprehensive protection for human beings. Given the increasing popularity and commercial promotion of CV for adjuvant cancer treatment, there is an interest to study the potential protection of CV against cancer metastasis, especially breast tumor metastasis to lung, liver and bone. In the present study, we aimed to evaluate the anti-tumor, anti-metastasis and immunomodulatory effects of CV aqueous extract in a mouse mammary tumor model using the highly invasive mouse mammary carcinoma 4T1 cells.

Our results demonstrated that the aqueous extract of CV did not show any obvious cytotoxicity in 4T1 cells even at the high concentration of 2 mg/ml (Fig. 2). Previous studies have shown that CV extracts possessed selective cytotoxic activity against certain tumor cells (Lau et al. 2004; Yang and Chen 2000). Besides, the non-cytotoxic dose of CV aqueous extract was found to be effective in inhibiting 4T1 cell migration and invasion (Fig. 3). Growing evidence showed that CV prevented metastasis in colorectal, gastric, esophageal, lung, and breast cancers *in vitro* (Ho et al. 2006; Yang et al. 1992; Zeng et al. 1993; Dong et al. 1999). In addition, results from gelatin zymography revealed that the enzyme activity of MMP-9 was suppressed by CV in 4T1 cells (Fig. 4), suggesting that CV could inhibit the migration of cancer cells. The Western blot analysis further confirmed the findings of zymography that CV aqueous extract significantly inhibited the protein levels of MMP-9, while it did not affect the activity and protein levels of MMP-2 (Fig. 5). The mRNA expressions of MMP-2 and MMP-9 have been evaluated in CV aqueous extract-treated 4T1 cells; however, there was no significant change observed (data not shown). It is possible that the CV extract might affect the expression of MMP-9 at translation level but not at transcription level. MMP-9 was shown to play an important role in ECM and basement membrane degradation, and strong clinical and experimental evidence demonstrated association of elevated levels of MMPs and u-PA with cancer progression, metastasis and shortened patient survival (Chaudhary et al. 2013). It was previously demonstrated that PSK in CV extract down-regulated MMP-9 and MMP-2 in pancreatic cancer (NOR-P1) and gastric cancer (MK-1P3) cells (Zhang et al. 2000).

In our animal study, the anti-tumor and anti-metastasis effects of CV aqueous extract were investigated in the 4T1 tumor-bearing mouse model, which closely mimics stage IV of breast cancer in humans (Tao et al. 2008). No significant difference between CV treated and untreated control group was shown in body weights and plasma enzyme (AST, ALT ALP and CK) activities, indicating low/no toxicity of CV to the hosts (Fig. 6A and B). Besides, the anti-tumor effect of CV aqueous extract was firstly investigated in BALB/c mice bearing 4T1 tumors. Previous reports showed that the ethanol-water extract of CV significantly reduced tumor size in mice with leukemia, lung, or liver tumors (Reigetsu et al. 2005; Eliza et al. 2012). 4T1 cells are documented to be highly invasive and primary tumors typically metastasize to the lungs and livers after establishment for 2 to 3 weeks in BALB/c mice (Tao et al. 2008). The present study showed that the daily administration of CV aqueous extract resulted in significant decrease in lung metastasis. This is the first time that the aqueous extract of CV was demonstrated to be effective not only in decreasing the tumor weight, but also in inhibiting breast cancer-induced lung metastasis (Fig. 7). The results were in line with the findings that PSK from CV was effective in inhibition of pulmonary metastasis in nude mice bearing with prostatic 145M or PC-3M tumors (Zhang et al. 2000). However, no obvious effect of CV was shown on inhibition of liver metastasis.

The polysaccharide contents in our CV aqueous extract may be responsible for the effectiveness of CV extract on anti-tumor and anti-metastasis. The polysaccharide content in CV aqueous extract was 8.34% (w/w) and UPLC analysis further confirmed that CV aqueous extract composed of several high molecular weight fractions (Fig. 1). Previous report demonstrated that CV contains many bioactive components, including PSK and PSP in which the mean molecular weight of PSK was about 94 kDa (Kobayashi et al. 1995), and the molecular weight of PSP was about 100 kDa (Ng 1998). In our study, the UPLC analysis of CV extract showed that several fractions were included, and fraction **b** is locating in the molecular weight range of 50–270 kDa, with a peak of 169 kDa. The result suggested that the CV aqueous extract is likely to contain the PSK and PSP, and they might have located in fraction b. Furthermore, the fraction **a** with a peak of 376 kDa was present, indicating other macro-molecular components are also contained in CV aqueous extract. The macro-molecular components in fraction a will be further evaluated in future.

Polysaccharides especially PSK have documented anticancer activity *in vitro*, *in vivo* and in human clinical trials (Fisher and Yang 2012; Roomi et al. 2012; Mickey et al. 1989; Jiménez-Medina et al. 2008). PSK induced apoptosis in leukemia, melanoma, fibrosarcoma and cervix, lung, pancreas and gastric cancer cells (Jiménez-Medina et al. 2008). PSK has been demonstrated to have inhibition on MMP-2 and MMP-9 protein expressions (Zhang et al. 2000). Nevertheless, the activity and expressions of MMP-2 were not affected by CV aqueous extract in the present study. The possibly reason may be that our CV aqueous extract contained PSK, but the ratio might not be high enough to exert inhibitory activity. In other animal study, treatment with PSK resulted in inhibition of tumor growth against MethA fibrosarcomas in immunodeficient NOD/SCID mice (Hoshi et al. 2011). In Japan, PSK was an antitumor agent used in combination with other chemotherapeutic agents as postoperative adjuvant therapy for gastrointestinal cancer and small-cell lung carcinoma (Hirahara et al. 2012).

At the late stage of cancer propagation, breast cancer cells usually metastasize to bone and produce osteolytic bone destructions, and such breast cancer bone metastasis eventually promotes a vicious cycle of bone destruction and tumor expansion (Azim et al. 2012). In order to determine the breast cancer-induced bone destruction, μ-CT analysis was employed. The 3D μ-CT images from untreated control group showed extensive bone destruction and significant bone volume loss when compared to the corresponding tibiae from naive group, indicating the successful establishment of breast cancer-induced osteolysis (Fig. 8). Treatment with CV aqueous extract resulted in significant protection of bone structure, with remarkable increases in the bone volume by 10.2%. The anti-osteolysis effect of CV aqueous extract was firstly investigated in mice bearing 4T1 tumors and demonstrated to be effective in bone protection against breast cancer-induced osteolysis. The anti-osteolysis effect of CV may be due to the anti-tumor effect by decreasing tumor cells metastasis to bone and eventually protected the bone structure. Further investigation on the *in vitro* osteoclast functions should be assessed to determine whether the aqueous extract of CV has direct anti-osteolysis effect.

The proliferative responses and cytokines productions of spleen lymphocytes isolated from 4T1 tumor-bearing mice have been determined by thymidine incorporation assay and ELISA, respectively (Fig. 9). The proliferative responses and cytokines productions (TNF-α, IFN-γ, IL-2 and IL-12, and IL-6) of spleen lymphocytes from CV-treated mice were significantly higher than those from untreated control mice. However, no significant effect of CV aqueous extract was shown to IL-10. The increased cytokine productions of spleen lymphocytes were not likely due to the presence of LPS in CV aqueous extract since the extract was orally administered to the mice. The observed immune responses

were expected to be the effects of the bioavailable components of CV aqueous extract. The immunostimulating effect of CV was widely investigated. PSK has been found to be effective in activating T-lymphocytes and promoted the production of antibodies and various cytokines such as IL-2, IL-6 and TNF (Tsukagoshi et al. 1984). PSP in CV extract was also found to be effective in inducing the productions of IFN-γ and IL-2, increase T-cell proliferation (Ng 1998). Our previous results also showed that CV ethanol-water extract enhanced the production of Th1-related cytokines in mouse lymphocytes *in vitro* (Ho et al. 2004). These findings were completely in line with our current results. The potent anti-tumor and anti-metastasis effects of CV may be due to the immunomodulatory effects. It was widely demonstrated that immunomodulation in host system may result in inhibition of tumor growth, and immunotherapy is broadly accepted as an adjuvant therapy for cancer treatment (Murugaiyan and Saha 2013). More detailed molecular mechanisms, for instance, genomic and proteomic responses underlying the CV-induced anti-metastasis effects remain to be elucidated.

In conclusion, our results presented the first evidence of CV aqueous extract on the anti-tumor and anti-metastasis, and bone protection against breast cancer-induced osteolysis, as demonstrated by *in vitro* 4T1 cell migration and invasion inhibition and *in vivo* tumor weight and lung metastasis reduction in BALB/c mice bearing orthotopic 4T1 tumors. Our studies provided scientific evidence in supporting the use of CV aqueous extract as health supplement in breast cancer patients.

Conflict of interest

The authors have no conflicts of interest.

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