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Green tea (*Camellia sinensis*) extract inhibits both the metastasis and osteolytic components of mammary cancer 4T1 lesions in mice

Ke-Wang Luo[a,b,1], Chun-Hay Ko[a,b,1], Grace Gar-Lee Yue[a,b], Julia Kin-Ming Lee[a,b], Kai-Kai Li[a,b], Michelle Lee[c], Gang Li[d,e], Kwok-Pui Fung[a,b,e], Ping-Chung Leung[a,b], Clara Bik-San Lau[a,b,*]

[a]*Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong*
[b]*State Key Laboratory of Phytochemistry and Plant Resources in West China, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong*
[c]*Discipline of Orthopaedics and Trauma, University of Adelaide, Adelaide, SA, Australia*
[d]*Department of Orthopaedics and Traumatology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong*
[e]*School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong*

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Abstract

Green tea (*Camellia sinensis*, *CS*), a kind of Chinese tea commonly consumed as a healthy beverage, has been demonstrated to have various biological activities, including antioxidation, antiobesity and anticancer. Our study aims to investigate the antitumor, antimetastasis and antiosteolytic effects of *CS* aqueous extract both *in vitro* and *in vivo* using metastasis-specific mouse mammary carcinoma 4T1 cells. Our results showed that treatment of 4T1 cells with *CS* aqueous extract resulted in significant inhibition of 4T1 cell proliferation. *CS* extract induced 4T1 apoptosis in a dose-dependent manner as assessed by annexin-V and propidium iodide staining and caspase-3 activity. Western blot analysis showed that *CS* increased the expression of Bax-to-Bcl-2 ratio and activated caspase-8 and caspase-3 to induce apoptosis. *CS* also inhibited 4T1 cell migration and invasion at 0.06–0.125 mg/ml. In addition, *CS* extract (0.6 g/kg, orally fed daily for 4 weeks) was effective in decreasing the tumor weight by 34.8% in female BALB/c mice against water treatment control (100%). Apart from the antitumor effect, *CS* extract significantly decreased lung and liver metastasis in BALB/c mice bearing 4T1 tumors by 54.5% and 72.6%, respectively. Furthermore, micro-computed tomography and *in vitro* osteoclast staining analysis suggested that *CS* extract was effective in bone protection against breast cancer-induced bone destruction. In conclusion, the present study demonstrated that the *CS* aqueous extract, which closely mimics green tea beverage, has potent antitumor and antimetastasis effects in breast cancer and could protect the bone from breast cancer-induced bone destruction.

Keywords: *Camellia sinensis* (*CS*); Green tea; 4T1 cells; Antitumor; Antimetastasis; Bone destruction

1. Introduction

Cancer is a major health problem worldwide, with an upward incidence and mortality rate. Breast cancer ranks the top of most frequent cancers in 2008, with a worldwide incidence of around one in eight women [1]. 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 with a high potential to spread to almost any region of the body, and the most common sites are the bone, followed by the lungs and liver. It is estimated that over 70% of patients dying of breast cancer get bone metastasis and associate with bone destruction [2]. Breast cancer bone metastasis frequently produces osteolytic bone lesions by activating local osteoclasts [3]. Although many significant advances on the frontline breast cancer research and chemotherapy have been developed, the efficacies of current therapies are limited by a range of adverse effects, toxicity and drug resistance. Therefore, novel therapeutic strategies and more effective agents for advanced disease are still urgently needed.

Green tea is the most popular beverage consumed worldwide, especially in China and Asian countries. It is obtained from the dried leaves of the plant *Camellia sinensis* (*CS*), which contains as many as 200 bioactive compounds, including tea polyphenols (catechins and flavonols), caffeine, theanine, vitamins and minerals. The largest and most active group of tea components is tea polyphenols, including epicatechin (EC), EC gallate (ECG), epigallocatechin (EGC), and EGC gallate (EGCG) [4]. To date, the use of extracts and polyphenols from *CS* for biomedical purposes is increasing for the treatment of various diseases such as obesity, cardiovascular diseases and diabetes mellitus [5]. Epidemiological studies have shown that green tea extract intake was associated with increased weight loss due to diet-induced thermogenesis [6]. A cohort study of more than 40,000

* Corresponding author. E305, Science Centre East Block, Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong. Tel.: +852 39436109.
E-mail address: claralau@cuhk.edu.hk (C.B.-S. Lau).
[1] Authors with equal contribution.

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Japanese adults found that green tea consumption was inversely associated with cardiovascular disease mortality [7]. There are also a number of literatures reporting the antitumor effects of *CS* and its polyphenolic components. It was demonstrated that the green tea polyphenol (GTP) could induce apoptosis in a variety of cancer cells including leukemia, breast cancer, prostate cancer, lung and liver cancer cells, through production of H_2O_2, inhibition of cell-cycle progression, or activation of the mitogen-activated protein kinase cascade [8,9]. Clinical study also showed that treatment with tea polyphenol catechin in prostate cancer patients result in significant inhibition of tumor growth [10]. Intake of green tea and breast cancer risk have been investigated in three case–control studies in Los Angeles County, Shanghai and Zhejiang Province, China. These three studies showed a significant reduction of breast cancer risk with regular green tea intake after adjustment for soy and other potential confounding factors [11]. However, for the case of breast cancer metastasis, only few preclinical examples are available. For instance, *in vitro* studies indicated that GTPs could reduce the metastasis in breast cancer MDA-MB231, MCF-7 and 4T1 cells by down-regulating matrix metalloproteinase-2 and -9 [12,13]. In animal study, the tumor growth and lung metastasis were reduced significantly in 4T1 tumor-bearing mice upon the treatment of GTPs or EGCG [14,15]. However, there is no report available for liver or bone metastasis in breast cancer upon *CS* treatment. Besides, the *CS* water extract that closely mimics Chinese tea beverage was seldom investigated in animal study with severe tumor metastasis to the lungs, liver and bone.

The present study aimed to investigate the antimetastasis and antiosteolytic effects of *CS* extracts in breast cancer 4T1 lesions mice. Also, the role of *CS* extracts in different mechanisms of action would be discussed. In this regard, the mouse mammary tumor cell line 4T1 was employed, due to the high capacity of metastasis to sites affected in human breast cancer and resembling exactly to stage IV of breast cancer in humans [16]. Here, we assessed the apoptosis induction, antimigration and anti-invasion abilities of *CS* extract *in vitro*, and then further evaluated the antitumor and antimetastasis activities of *CS* extract in a mouse mammary tumor model. Besides, the antiosteolysis effect of *CS* extract was also evaluated both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Aqueous extract preparation and high-performance liquid chromatography analysis

The dried leaves of green tea (*CS*), with origin of Hainan province China, were purchased from the herbal supplier of Hong Kong, and a voucher specimen was kept in the museum of the Institute of Chinese Medicine, The Chinese University of Hong Kong, with voucher specimen number 2011-3336. One kilogram of dried leaves of *CS* was soaked in boiled water for 15 min each for two times. Following the filtration, the water extracts were combined and evaporated under reduced pressure at 60°C to dryness to give a powder. The extraction yield was 19.5% (weight of extract/weight of raw herb). The *CS* extract powder was stored in a dry storage box inside a desiccator at room temperature before use.

The chemical composition of *CS* extract was analyzed by high-performance liquid chromatography (HPLC; Agilent, Santa Clara, CA, USA) as described [17]. Two-gradient elution system including mobile phase A [85% orthophosphoric acid and water (0.05:99.95, vol/vol)] and mobile phase B (acetonitrile) was introduced. The gradient used was as follows: 0–4 min, 2% B; 4–21 min, linear gradient from 2% to 9% B; 21–32 min, linear gradient from 9% to 23% B; and 32–45 min, 23% B. The concentrations of catechins and alkaloid in *CS* extract are listed in Fig. 1 as determined by HPLC.

2.2. Cells and reagents

The 4T1 cells were cultured in RPMI-1640 medium containing 10% (vol/vol) fetal bovine serum and 1% penicillin–streptomycin (Life Technologies, Carlsbad, CA, USA) at 37°C in 5% CO_2 humidified incubator. Annexin V–FITC kit was purchased from BD Pharmingen, San Jose, CA, USA. Doxorubicin (DOX) and 3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma, St. Louis, MO, USA. Transwell plates for transwell migration assay were from Corning Incorporated, Corning, NY, USA. Caspase-3 activity assay kit was purchased from Invitrogen, Carlsbad, CA, USA. Creatine kinase (CK), alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) kits were purchased from Stanbio, Boerne, TX, USA.

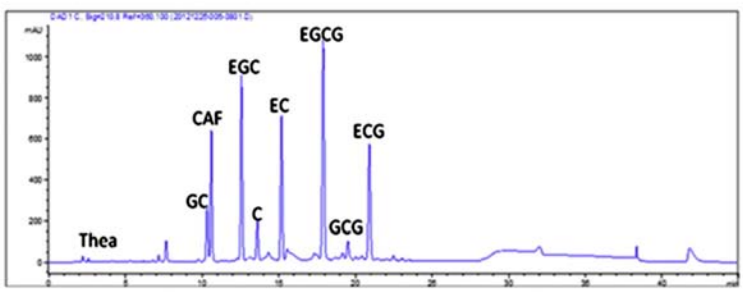
2.3. Cell viability assay

The effect of *CS* extract on the growth inhibition of 4T1 cells was measured by MTT assay. Cells (1×10^4/well) were seeded in 96-well flat-bottomed plates (Corning) and incubated with different concentrations of *CS* for 24, 48 and 72 h. Following incubation, 30 μl of MTT solution [5 mg/ml in phopshate-buffered saline (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 dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance of each sample was read at 540 nm using a microplate reader (Biotek μQuant, Winooski, VT, USA).

2.4. Annexin V–FITC/propidium iodide double staining

Flow cytometry was used to determine the phosphatidyl serine exposed apoptotic cells by Annexin V–FITC and propidium iodide (PI) double staining. Briefly, after treatment with various concentrations of *CS* for 24 or 48 h, 4T1 cells were collected and washed with ice-cold PBS and then stained in solution containing Annexin V–FITC and PI for 15 min in the dark at room temperature. Subsequently, the fluorescent signal in the cells was detected by flow cytometry (FACSCanto; Becton Dickinson). Cell debris, characterized by a low forward scatter/side scatter, was excluded from the analysis. Positioning of quadrants on annexin-V/PI plots was performed to distinguish living cells (FITC−/PI−), early apoptotic cells (FITC+/PI−) and late apoptotic or necrotic cells (FITC+/PI+). The separation of the quadrants was adjusted by gating more than 95% cells of negative control (i.e., 0 mg/ml) into the left lower quadrant.

A



B

Components	Thea	CAF	EGC	C	EC	EGCG	ECG
Pre-treatment Content (mg/ml)	1.59 ± 0.19%	6.12 ± 0.03%	5.57 ± 0.04%	1.43 ± 0.07%	4.95 ± 0.18%	8.54 ± 0.09%	5.17 ± 0.04%
Post-treatment Content (mg/ml)	1.63 ± 0.08%	6.16 ± 0.17%	5.45 ± 0.11%	1.62 ± 0.16%	5.47 ± 0.39%	8.28 ± 0.09%	5.38 ± 0.03%

Fig. 1. Quantitative analysis of *CS* aqueous extract (both pretreatment and posttreatment) by HPLC. (A) Representative HPLC chromatogram of *CS* aqueous extract. (B) Quantitative analysis of ingredients in *CS* aqueous extract (both pretreatment and posttreatment). Abbreviations: Thea, theanine; CAF, caffeine; C, catechin.

2.5. Caspase-3 activity assay

Cells treated with various concentrations of *CS* for 48 h were collected and lysed in lysis buffer to determine the caspase-3 activity, using the caspase-3 activity assay kit (Invitrogen). After 10 min in lysis buffer on ice, insoluble material was pelleted after centrifugation and an aliquot of the lysate was collected and stored at −80°C. After tested for protease activity, normalized sample lysate was added to microplate together with reaction buffer and substrate solution, the microplate was then incubated at 37°C for 2–4 h. The plate was then read at 405 nm in a microplate reader.

2.6. Efficacy of CS against cell migration and invasion in vitro

The efficacy of *CS* extract against 4T1 cell migration and invasion *in vitro* was assessed using scratch wound and transwell migration assays.

In the scratch wound assay, 4T1 cells (1×10^5/well) were seeded in 24-well plates and incubated at 37°C for 24 h. After starving in a medium without fetal bovine serum (FBS) for 24 h, 4T1 cells were scraped with crosses using 200-μl pipette tips. The medium was then replaced with a fresh medium with different concentrations of *CS*. 4T1 cells were incubated for different time intervals (9 or 18 h), and each well was photographed under a microscope (Nikon Eclipse TS100). The percentages of open wound area were measured and calculated using the TScratch software [18]. Motility was determined by the decrease in open wound area.

During the transwell migration assay, 4T1 cells (5×10^4/well) were added into transwell filter chambers with 1% vol/vol FBS. At the same time, 100 μl medium containing various concentrations of *CS* (with 1% vol/vol FBS) was added to the upper chambers. Then 500 μl complete RPMI-1640 medium (with 10% vol/vol FBS), served as chemoattractant medium, was added into the lower well. 4T1 cells were allowed to migrate through the chamber membrane to the lower well for 4–5 h at 37°C, 5% CO_2. After incubation, cells were fixed with methanol and stained with hematoxylin. Stained filters were photographed under a microscope (Nikon Eclipse TS100). The migrated cells were quantified by manual counting, and the results were presented as a percentage of control values [19].

2.7. Western blot analysis

4T1 cells (5×10^5/well) were seeded in 90-mm dishes and incubated with various concentrations of *CS* at 37°C for 24 h. The cells were lysed in lysis buffer [0.02% aprotonin, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 62.5 mM Tris–HCl, pH 6.8] on ice for 15 min. After the lysate boiled, the protein samples (50 μg) were fractionated in 12% SDS-polyacrylamide gel. Proteins on the gel were transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Afterward, a machine named Snap i.d. from Millipore was introduced for Western blot. The membrane was placed on the Western blot holder and blocked with 0.5% milk for 1 min, and then incubated with primary antibodies for 10 min at room temperature. After washing with PBS–Tween 20, the membrane was incubated with secondary antibodies conjugated with phosphatase for 10 min. Visualization of protein bands was performed using the ECL substrate reagent kit (GE Healthcare) on a ChemiDoc XBS imaging system (Bio-RAD, Hercules, CA, USA).

2.8. 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, approved by Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. 4T1 cells (4×10^5) resuspended in 0.2 ml PBS, were subcutaneously inoculated at the mammary fat pad of each mouse. Treatments were initiated 1 week after cancer cell implantation and lasted for 4 weeks. The *CS* powder was redissolved in water and orally administered to mice intragastrically for 28 days. After 4T1 cell implantation, the tumor-bearing mice were randomly assigned into three groups ($n=10$): control group (distilled water, orally fed everyday), *CS* group (0.6 g/kg, orally fed everyday), DOX group (2 mg/kg, i.p. injected twice a week). The dose of *CS* was derived from the usual health supplement dose of 2 g per day [20], while the DOX treated group was set up as a positive control group in animal study. In order to control the quality of *CS* water extract, HPLC analysis was performed both pretreatment and posttreatment. During *CS* treatment, the body weight of each mouse was measured once a week during the treatment period. At day 28, mice were sacrificed, and the lungs and livers were removed for quantification of tumor burden. Tibias of mice from different groups were removed for micro-computed tomography (micro-CT) analysis. The effects of *CS* on hematobiochemical markers were assessed by measuring the activities of liver- or heart-related enzymes (ALP, ALT, AST, and CK) in the plasma using assay kits purchased from Stanbio Co. Ltd.

2.9. Histology

Lungs and livers were fixed in 10% buffered formalin for 7 days at room temperature. Then 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 were analyzed using SPOT advanced (version 3.5.6) software. Tumor burden, defined as the tumor area, was calculated from the section of the lung or liver and expressed as an average tumor area per group in absolute units (mm^2).

2.10. μ-CT analysis

Tibias removed from mice were scanned with a high-resolution microtomographic system, μ-CT 40, Scanco Medical AG, Zürich, Switzerland. Each three-dimensional image data consist of approximately 500 micro-CT slide image (8 μm/slide) starting from the growth plate of tibial interface and down to the tibia. Bone volume (BV; mm^3) was generated from μ-CT analysis and compared with the control tibia for each animal [21].

2.11. Osteoclast staining

To determine whether *CS* extract had direct antiosteoclastogenesis effect *in vitro*, the osteoclast staining assay was employed. After treatment with RANKL (receptor activator of nuclear factor kappa-B ligand) and *CS* extract for 4 days, the RAW 264.7 cells differentiated osteoclasts were fixed and stained for tartrate-resistant acid phosphatase (TRAP), an osteoclast enzyme marker, by using an acid phosphatase kit (Sigma), according to the manufacturer's instructions. TRAP-positive multinucleated cells showing more than three nuclei were counted as osteoclasts. Photomicrographs were taken with an inverted microscope at 40× magnification. The number of osteoclasts per field was calculated by averaging the counting from eight separated views.

2.12. Statistical analysis

All data were expressed as mean±S.D./S.E.M. Statistical analysis was performed using one-way analysis of variance, with $P<.05$ as considered statistically significant.

3. Results

3.1. HPLC analysis of polyphenols in CS aqueous extract

The chemical composition of tea polyphenols in *CS* extract was analyzed by HPLC. As shown in Fig. 1A, *CS* extract contained a large amount of tea polyphenols of about 25%, with EGCG as the most abundant, followed by EGC, ECG and EC. Besides, *CS* extract contained caffeine (6.12%) and trace amount of gallocatechin (GC; 0.4%) and gallocatechin gallate (GCG; 0.58%). In order to ensure the quality of *CS* water extract, HPLC analysis was performed before and after the study. Our HPLC analysis showed that no significant component loss was found in *CS* water extract before and after the experiment (Fig. 1B).

3.2. Inhibitory effect of CS extract on 4T1 cell proliferation

The effect of *CS* extract on cell proliferation was tested using MTT assay. Treatment with *CS* extract for 24, 48 and 72 h resulted in inhibition of cell proliferation in a time- and dose-dependent manner. As shown in Fig. 2, *CS* extract inhibited the growth of 4T1 cells with an IC_{50} of 0.36, 0.21 and 0.17 mg/ml at 24, 48 and 72 h, respectively. Also,

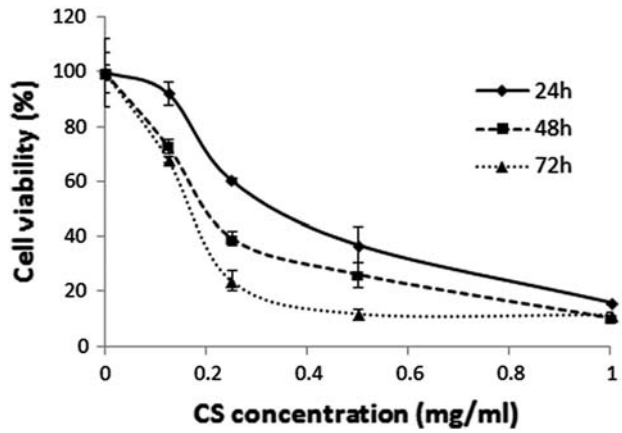


Fig. 2. The antiproliferative effect of *CS* extract on 4T1 cells after 24, 48 or 72 h incubation. Data were expressed as mean±S.D.

the dose range of *CS* extract from 0 to 0.125 mg/ml was selected for subsequent cell migration and invasion assays.

3.3. Apoptosis induction of CS extract on 4T1 cells

Annexin-V–FITC/PI staining showed that when 4T1 cells were incubated with increasing dose of *CS* extract from 0 to 0.5 mg/ml, cells at early or late stages of apoptosis were increased in a dose- and time-dependent manner. The percentages of apoptotic cells upon treatment with 0.125, 0.25 and 0.5 mg/ml of *CS* extract were found to be 10.7%, 31.3% and 37.7% for 24 h and 13.9%, 60.5% and 75.1% for 48 h, respectively (Fig. 3A). Furthermore, *CS*-induced 4T1 cell apoptosis was concomitant with a dose-related increase in caspase-3 activity. Caspase-3 activity was increased significantly with the increase of *CS* concentration (Fig. 3B). Besides, significant differences were also shown among the various doses of *CS* extract on apoptotic cell induction and caspase-3 activity.

3.4. Effect of CS extract on protein expression

Treatment with various concentrations of *CS* for 24 h resulted in the change of protein expression level (Fig. 4A). As shown in Fig. 4B and C, the *CS*-treated cells increased the expression of proapoptotic protein Bax and decreased the expression of antiapoptotic protein Bcl-2. In addition, *CS* activated caspase-8 in a dose-dependent manner, indicating the involvement of death-receptor pathway for apoptosis (Fig. 4D). *CS* also induced the increased expression of caspase-3, which was further evidenced by the increased level of cleaved PARP (poly ADP ribose polymerase) (Fig. 4E–F). Besides, significant differences were also shown between the low dose (0.1 mg/ml) and high dose (0.2 mg/ml) of *CS* extract on Bcl-2, Bax, PARP and caspase-8 and caspase-3 protein expression.

3.5. Inhibitory effect of CS extract on 4T1 cell metastasis

Cell migration and invasion are essential components in cancer propagation. To determine the efficacy of *CS* extract against cancer cell metastasis *in vitro*, the scratch wound and transwell migration assays were introduced. As shown in Fig. 5A, *CS* could significantly inhibit cell migration of 4T1 from 0.06 to 0.125 mg/ml after a 9-h incubation, and the inhibition was enlarged when the incubation lasted for 18 h (Fig. 5B). In Fig. 5C, *CS* extract inhibited 4T1 cell invasion efficiently with the increase of *CS* extract concentration. In the presence of 0.06 and 0.125 mg/ml, *CS* inhibited cell invasion of 4T1 significantly by 41.8% and 57.9%, respectively. Significant differences were also shown between the low (0.03 mg/ml) and high dose (0.125 mg/ml) of *CS* extract on 4T1 cell migration and invasion *in vitro*.

3.6. CS extract inhibited in vivo growth of 4T1 breast cancer cells and decreased metastasis to the lungs and liver in BALB/c mice

To investigate the activity of *CS* extract on tumor growth and metastasis, a mammary fat pad model was employed, in which cells were injected into the mammary fat pad of female BALB/c mice. It was observed that no significant body weight loss was found in the *CS*-treated group during the treatment (Fig. 6A). Hematobiochemical marker test also showed that no significant difference was shown in the *CS*-treated group on plasma activities of liver-related (AST, ALT and ALP) and heart-specific (CK) enzymes (Fig. 6C). At the end of the experiment, tumors were excised from each animal for examination of tumor weight and assessment of the antitumor effect of *CS* extract. Tumor weight at the orthotopic site was found to decrease significantly in the *CS*-treated group (by 34.8%) when compared with control (Fig. 6B).

Lungs and livers from each mouse were removed for histological analysis. Tumor burden was defined as the tumor area, calculated from the section of the lungs or livers and expressed as an average tumor area per group in absolute units (mm^2). Huge 4T1 tumors were found in the control group, while the tumor area and nodules were decreased in the *CS*-treated group (data not shown). Tumor burden in the lungs was found to decrease by 54.5% (lung metastasis decreased from 6.2% to 2.8%) in the *CS*-treated group when compared with the nontreated group, indicating that the *CS* extract was effective in slowing down metastasis to lungs (Fig. 7A). In addition, the tumor burden of the livers was also assessed, and consistent result was found. Data from H&E staining showed that the tumor burden in the livers was 3.63% in the nontreated control group, while the *CS*-treated group occupied only 0.97% of tumor area in the liver, translating to a significant decrease by 72.6% against control as 100% (Fig. 7B).

3.7. CS extract protected the bone significantly from breast cancer-induced bone destruction

At the late stage of cancer propagation, once breast cancer cell metastasizes to bone, it may cause severe bone destruction [22]. To

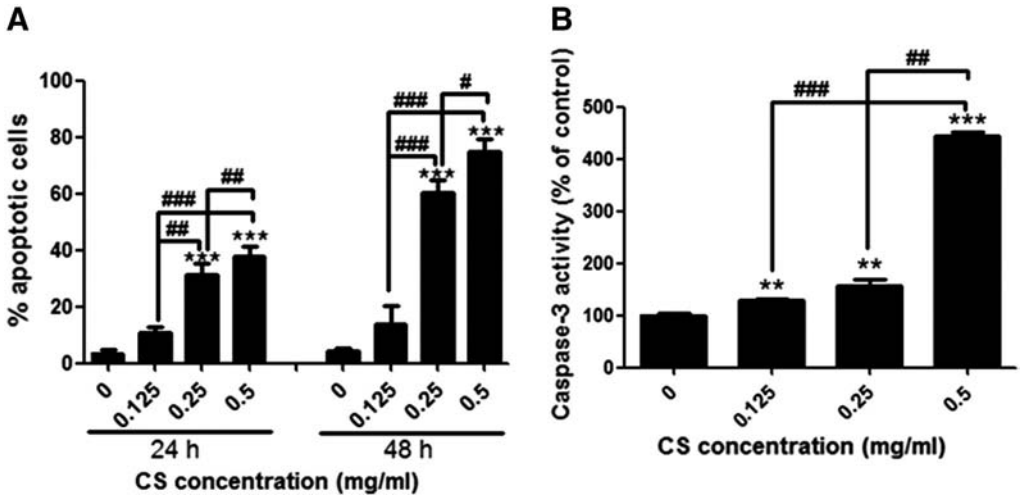


Fig. 3. Induction of apoptosis on 4T1 cells by *CS* extract. (A) Quantitative analysis of the percentage of apoptotic cells of *CS* after 24- and 48-h incubation. Data were presented as mean ±S.D. (n=3). (B) Caspase-3 activity test of 4T1 cells after incubated with *CS* extract. The data showed a dose-dependent increase in caspase-3 activity and presented as mean±S.D. (n=3). $^{**}P<.01$ and $^{***}P<.001$, as compared with control; $^{\#}P<.05$, $^{\#\#}P<.01$ and $^{\#\#\#}P<.001$, as compared among the doses of *CS* extract.

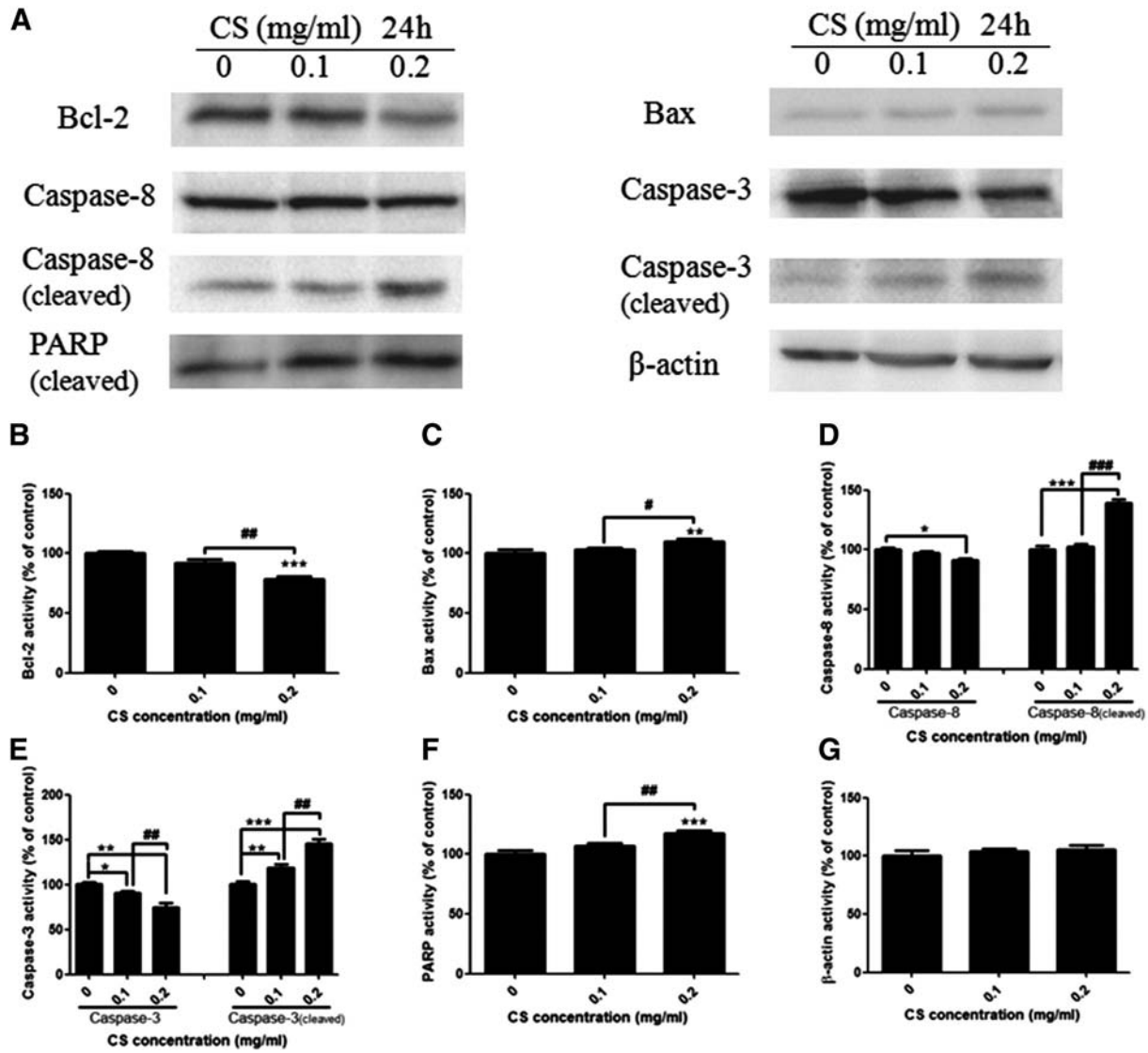


Fig. 4. Effect of *CS* extract on protein expression. (A) Representative images of Western blot of proteins after treated with 0.1 and 0.2 mg/ml of *CS* extract for 24 h in 4T1 cells. (B–G) Statistical analysis of Bcl-2, Bax, caspase-8, caspase-3, PARP and β-actin protein expressions after *CS* water extract treatment. Data were presented as mean±S.D. (n=3). $^{*}P$<.05, $^{**}P$<.01 and $^{***}P$<.001, as compared with control; $^{\#}P$<.05, $^{\#\#}P$<.01 and $^{\#\#\#}P$<.001, as compared between the low (0.1 mg/ml) and high doses (0.2 mg/ml) of *CS* extract.

evaluate the efficacy of *CS* extract against breast cancer-induced bone destruction, μ-CT was introduced and the tibias of mice from different groups were removed for μ-CT analysis. The three-dimensional (3D) μ-CT images of tibiae from the control group demonstrated extensive osteolysis when compared to the corresponding tibiae from the naïve group (data not shown). However, animals treated with *CS* extract showed preservation of the integrity of bone. To quantify the total BV, we compared the tibiae of all the animals in each group at a selected

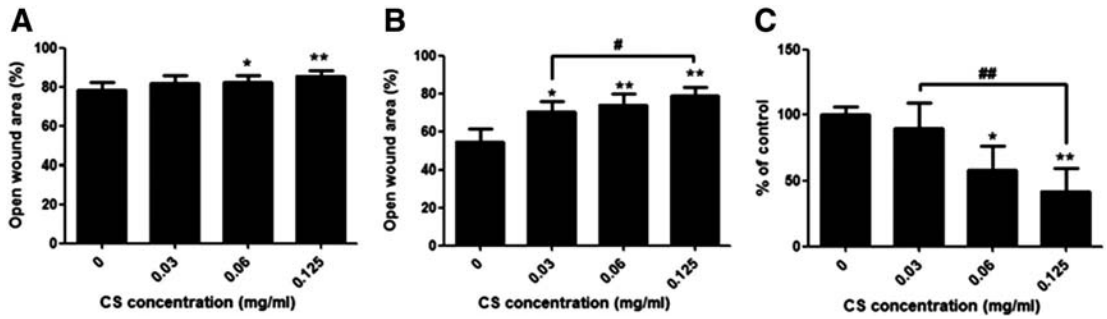


Fig. 5. Effect of *CS* on 4T1 cell migration and invasion activities in scratch wound and transwell migration assays. (A and B) Quantitative analysis of the antimigration activity of *CS* after 9-h (A) and 18-h (B) of incubation. Data were expressed as the percentage of open wound area from baseline cultures without treatment and presented as mean±S.D. (n=4). (C) Quantitative analysis of the anti-invasion activity of *CS*. Data were presented as mean±S.D. (n=3). $^{*}P$<.05 and $^{**}P$<.01 as compared with control; $^{\#}P$<.05 and $^{\#\#}P$<.01, as compared among the doses of *CS* extract.

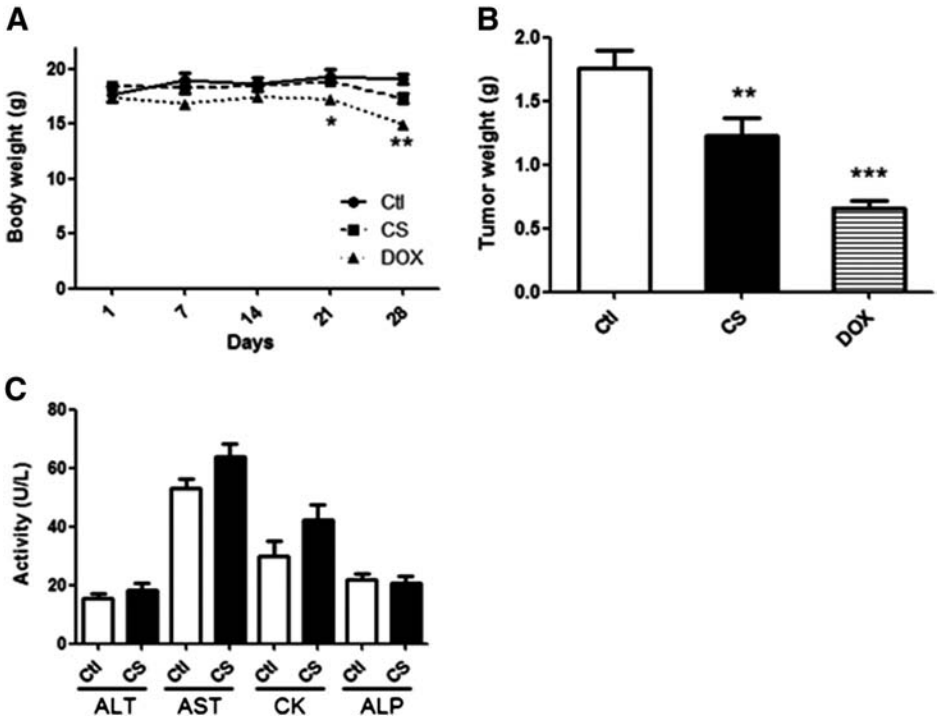


Fig. 6. *In vivo* antitumor effect of *CS* in 4T1 mouse mammary tumor model. (A) No significant body weight loss of mice was found during *CS* treatment period. During *CS* treatment, body weight of mouse was measured once a week. Data were expressed as mean±S.E.M., $n=10$. (B) The antitumor effects of *CS* against mouse mammary tumors. Data were expressed as mean±S.E.M., $n=10$. (C) Evaluation of the hematobiochemical markers of mice plasma after treated with *CS*. Data were expressed as mean±S.E.M., $n=10$. $^{*}P<.05$, $^{**}P<.01$ and $^{***}P<.001$, as compared with control.

region beginning at the growth plate and extending downward as 500×8-μm slices, which encompassed all of the cancer lesions. As shown in Fig. 8B, the amount of BV in the control group was 2.85 mm^3, lost nearly 30% of BV when compared to the tibiae of the naïve group. In contrast, *CS* extract showed remarkable protection of bone against breast cancer-induced osteolysis, translating to a significant increase (18%) in BV in mice when compared to the control group (Fig. 8A).

In order to determine whether *CS* extract had a direct antiosteolysis effect *in vitro*, the osteoclast staining assay was employed. As shown in Fig. 8B, a dose-dependent inhibitory response (25–100 μg/ml) was observed when compared with non-*CS*-treated cells with RANKL induction. At 100 μg/ml, the number of TRAP-positive osteoclasts was significantly decreased by 88% when treated with *CS*. Our data indicated that *CS* could effectively regulate RANKL-induced osteoclastogenesis on RAW 264.7 cells.

4. Discussion

Throughout the world, green tea (*CS*) has been widely consumed as tea beverage and health-promoting food ingredients. Given the increasing popularity and commercial development of green tea and related tea polyphenol supplements in cancer treatment, there is an urgent need to study the comprehensive protection of *CS* water extract against cancer metastasis, especially tumor metastasis to the lungs, liver and bone. In this study, we aim to investigate the

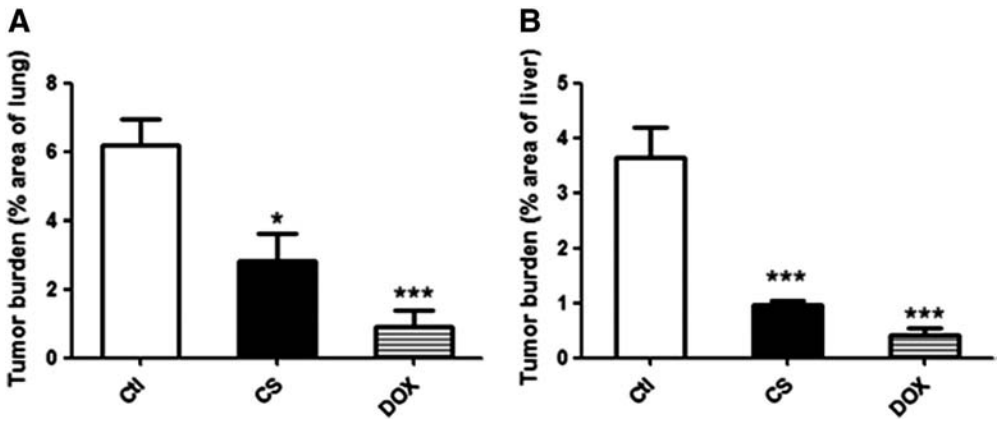


Fig. 7. Effects of *CS* extract on lung and liver metastasis in mice orthotopic breast cancer. (A) The graph represented the tumor burden in lungs according to the tumor area as a percentage of whole lung area. Data shown were an average from representative sections of each animal. mean±S.E.M., $n=10$. (B) Graph showed the tumor burden in liver expressed as an average per group. Data were expressed as mean±S.E.M., $n=10$. $^{*}P<.05$ and $^{***}P<.001$, as compared with control.

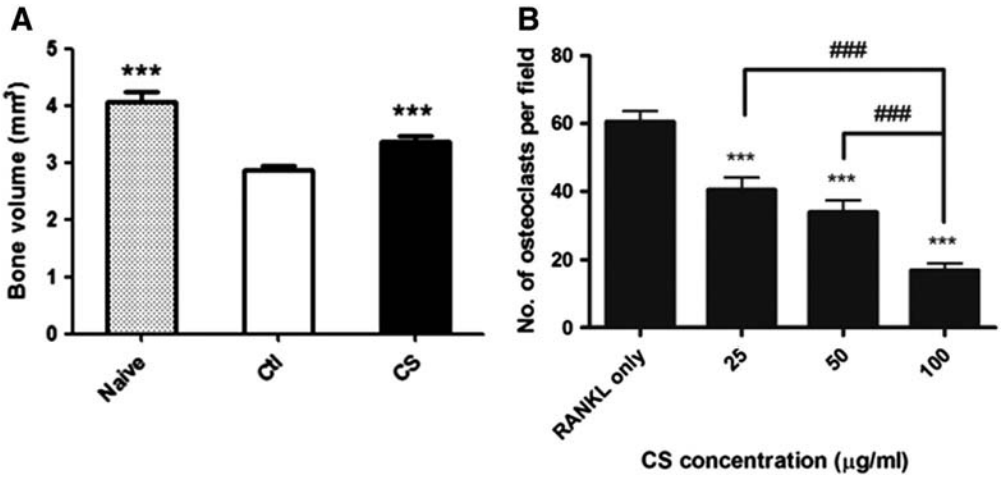


Fig. 8. Qualitative and quantitative assessment of bone structure in mice tibiae after administration of *CS* and *in vitro* investigation of the antiosteolysis effect of *CS*. (A) The chart showed the BV of tibias from different groups. Data were expressed as mean±S.E.M., $n=10$. (B) Direct antiosteolysis effect of *CS in vitro*. The graph presented the effect of *CS* on RANKL-induced osteoclastogenesis. The number of osteoclasts per field was calculated by averaging the counting from eight separated views. The *x*-axis represented the concentration of *CS* extract in μg/ml. Data were expressed as mean±S.D., $n=3$. $^{***}P<.001$, as compared with control; $^{###}P<.001$, as compared among the doses of *CS* extract.

antitumor, antimetastasis and antiosteolysis effects of *CS* aqueous extract (which closely mimics the tea beverage) in a mouse mammary tumor model using the metastasis-specific mouse mammary carcinoma 4T1 cells.

In the present study, we found that the treatment of *CS* extract resulted in dose- and time-dependent inhibition of cell viability on 4T1 cells *in vitro* (Fig. 2). To determine whether the antiproliferative effect of *CS* extract was associated with apoptosis induction, AV/PI double staining and caspase-3 assays were employed. The results showed that *CS* extract induced apoptosis in 4T1 cells in a dose-dependent manner (Fig. 3). These findings were in line with the effect of GTPs on 4T1 cells, which inhibited 4T1 cell proliferation and induced apoptosis in this cell line by activation of caspase-3 and PARP [14]. The involvement of apoptosis in cancer propagation plays a crucial role, which was considered as a protective mechanism against the development and progression of cancer. Apoptosis is modulated by a large number of antiapoptotic and proapoptotic effectors. To gain insight into the underlying mechanism of *CS*-induced apoptosis, several proteins were tested, including Bax, Bcl-2, caspase-8, caspase-3, and PARP. *CS*-treated cells increased the expression of Bax, while decreasing the Bcl-2 expression level (Fig. 4). This result was in complete agreement with the finding of Baliga et al. [14] that GTP EGCG treatment resulted in down-regulation of antiapoptotic protein Bcl-2 with a concomitant up-regulation in proapoptotic protein Bax in 4T1 cells. Same findings that GTPs increased the ratio of Bax-to-Bcl-2 were also found in human breast cancer MDA-MB231 and MCF-7 cells [23,24]. In addition, *CS* activated caspase-8, suggesting that the *CS*-induced apoptosis may be related to the death-receptor pathway. Furthermore, *CS* induced a significant increase of caspase-3 and cleaved PARP in a dose-dependent manner. The results were in line with the effect of GTPs shown in 4T1, MDA-MB231 and MCF-7 cells [14,23,24].

Apart from the antiproliferation and apoptosis induction effects of *CS* extract, the noncytotoxic dose of *CS* extract was found to be effective in inhibiting 4T1 cell migration and invasion in a dose-dependent manner as assessed by wound healing and transwell migration assays, respectively (Fig. 5). The results were comparable to previous reports that GTPs could reduce the metastasis in breast cancer MDA-MB-231, MCF-7 and 4T1 cells [12,13]. Besides, tea polyphenol EGCG inhibited proliferation and migration of human colon cancer SW620 cells [25]. In animal study, the antitumor and antimetastasis effects of *CS* aqueous extract were investigated in a mouse mammary tumor model, which closely mimics stage IV of breast cancer in humans [16]. After treatment, no significant difference was shown on body weight and hematobiochemical markers (AST, ALT ALP and CK), indicating no obvious toxicity of *CS* to the hosts (Fig. 6A and C). The administration of *CS* extract was able to inhibit the tumor weight significantly (Fig. 6B), which was in line with the results of Baliga et al. [14] that administering BALB/c mice with GTPs in drinking water significantly inhibited 4T1 tumor growth. Clinical study also demonstrated that treatment with tea polyphenol catechin in prostate cancer patients resulted in significant inhibition of tumor growth [10]. Three case–control studies in the United States and China have illustrated that intake of green tea regularly could significantly decrease the breast cancer risk [11]. Besides, 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 [26]. The present study demonstrated that administration of *CS* extract resulted in a significant decrease in lung and liver metastasis. The tumor area in the lungs and liver were decreased significantly in the *CS*-treated group. This is the first time that the water extract of *CS* was demonstrated to be effective not only in decreasing the tumor weight but also in inhibiting lung and liver metastasis (Fig. 7). The findings were in line with the study of GTPs or EGCG that metastasis of tumor cells to lungs was decreased in tea polyphenols or EGCG-treated groups in 4T1 tumor-bearing mice [14,15]. At the late stage of cancer propagation, breast cancer cells usually metastasize to the bone, and it may promote a vicious cycle of bone destruction and tumor expansion [22]. In order to determine the bone metastasis and breast cancer-induced bone destruction, micro-CT analysis was introduced. The 3D μ-CT images from the control group showed extensive bone destruction and significant BV loss when compared to the corresponding tibiae from the naïve group, indicating the successful establishment of breast cancer-induced osteolysis. Treatment of *CS* extract resulted in significant protection of bone structure, with a remarkable increase in the BV by 18% (Fig. 8A). The antiosteolysis effect of *CS* extract may arise from the antitumor effect, which decreased the tumor cells metastasis to the bone and protect the bone structure, and/or from the direct protection of the bone by antiosteoclast. In order to determine whether *CS* extract had a direct antiosteolysis effect or not, the *in vitro* osteoclast staining assay was employed. The results showed that *CS* extract decreased the number of TRAP-positive osteoclasts significantly (Fig. 8B), indicating that *CS* extract had direct antiosteoclast effect. Consistent

results were found in the study of Ko et al. [27], which illustrated that the tea polyphenol EGC had positive effects on bone metabolism through a double process of promoting osteoblastic activity and inhibiting osteoclast differentiations. Recent evidence also showed that tea polyphenol EGCG has a significant effect in inhibiting osteoclasts formation and differentiation by down-regulating c-Fos expression and suppressing the nuclear factor-κB signal [28]. The animal study further confirmed the findings and showed that EGCG decreases ovariectomy-induced bone loss in mice via inhibition of osteoclasts [29].

We reported for the first time that the water extract of *CS* was effective in decreasing tumor weight and metastasis to the lungs, liver and bone in the 4T1 mouse mammary tumor model. The effectiveness of *CS* water extract on antitumor and antimetastasis may be due to the GTPs. The chemical analysis by HPLC showed that total tea polyphenols in *CS* extract were around 25% (wt/wt), with EGCG being the most abundant, followed by EGC, ECG and EC (Fig. 1), and no significant difference was shown among the chemical components of *CS* extract between pretreatment and posttreatment. GTPs were demonstrated to be the largest and most biologically active group of *CS* [4]. GTP was effective in inhibiting carcinogenesis in both *in vitro* and *in vivo* studies [8–10]. In the present study, the *CS* extract contains 25% of GTP and 8.5% of EGCG, which means that the effective *in vivo* doses of GTP and EGCG are 2.77 mg/mouse/day and 0.92 mg/mouse/day, respectively. Similar results were found in the animals inoculated with less aggressive human breast cancer cells MDA-MB-231, 3 mg/mouse/day of GTP and 1 mg/mouse/day of EGCG could reduce the tumor size and incidence after 10 weeks of treatment [23]. Baliga et al. [14] reported that treatment with purified GTP (with 60% EGCG content) in drinking water in BALB/c mice could significantly inhibit 4T1 tumor growth and decrease metastasis of tumor cells to the lungs. They demonstrated that 0.2% (wt/wt) and 0.5% (wt/wt) of GTP for treating the animals were effective doses, n which were equivalent to 6 and 15 mg of EGCG in 5 ml daily water per mice, which were much higher than what was used in our study. In fact, our study demonstrated better result that *CS* water extract was effective in decreasing tumor weight, lung metastasis and liver metastasis at lower concentration of EGCG. It may imply that there are some synergistic effects among tea polyphenols and other water-soluble tea components. Increasing evidence reveals that other tea polyphenols such as theanine, ECG and EGC could also possess some antitumor effects [30–32]. Gross green tea extract may possess more potent effect than the single tea polyphenol; however, more in-depth experiments are needed to support this claim.

In our study, the dose of *CS* extract was 0.6 g/kg for mice study, which was equivalent to a single dose of 2.92 g *CS* water extract for a 60-kg adult. This dosage of *CS* extract was effective in antitumor, antimetastasis and antiosteolysis in tumor-bearing mice. Besides, the treatment of *CS* did not result in body weight loss in mice, which was further confirmed by hematobiochemical markers (AST, ALT ALP and CK) test that no significant differences were shown between the control and *CS*-treated groups, indicating no obvious toxicity of *CS* to the liver and heart. However, this high concentration of *CS* extract was unlikely to be consumed as tea beverage in human. It was demonstrated by Henning et al. [20] that a single dose of green tea extract (618 mg of EGCG) or purified EGCG (518 mg) was healthy for individuals and had significant antioxidant activities. The total polyphenols in *CS* water extract were about 25%, and hence, the animal dose in our study was translated to about 730 mg polyphenol for a 60-kg adult. In this regard, the effective dose of *CS* water extract in our study would be achievable for human beings as a consumption of several powder capsules in concentrated form.

In summary, our results present the first evidence on the antitumor, antimetastasis and antiosteolytic effects of *CS* water extract as demonstrated by *in vitro* 4T1 growth and metastasis inhibition and *in vivo* tumor weight and metastasis reduction in BALB/c mice bearing orthotopic breast cancer tumors. More detailed molecular mechanisms, for instance, genomic and proteomic responses underlying the *CS*-induced breast cancer cell apoptotic cell death, antimetastasis and antiosteolysis, remain to be elucidated. Besides, further investigation is needed to determine the clinical efficacy and safety of *CS* in human subjects with breast cancer metastasis. The herb–drug interaction between *CS* and conventional anticancer agents also remains to be explored. Although it is highly unlikely that the plasma concentration of *CS* extract would reach a therapeutic level under normal consumption, it is anticipated that various highly purified, enrich *CS* products, dietary health supplements and pharmaceutical products will soon be made available in response to increasing evidence of the beneficial effect of green tea. Our observation holds promise for further studies to examine the chemopreventive efficacy of green tea and to develop *CS* as a potential anticancer supplement against breast cancer.

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