# Quercetin is increased in heat-processed *Cuscuta campestris* seeds, which enhances the seed's anti-inflammatory and anti-proliferative activities

Meng-Shiou Lee[1], Chao-Jung Chen[2,3,4], Lei Wan[4,5,6], Ayano Koizumi[7,8], Wen-Te Chang[1], Meng-Ja Yang[1], Wen-Hsin Lin[9], Fuu-Jen Tsai[4,5,6] and Ming-Kuem Lin[1]

[1] School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University. No 91, Hsueh-Shih Road, Taichung 40402, Taiwan

[2]Graduate Institute of Integrated Medicine, China Medical University, Taichung, Taiwan

[3]Proteomics Core Laboratory, Office of Research and Development, China Medical University, Taichung, Taiwan

[4]Department of Medical Research, China Medical University Hospital, Taichung, Taiwan

[5]School of Chinese Medicine, China Medical University, Taichung, Taiwan

[6]Department of Health and Nutrition Biotechnology, Asia University, Taichung, Taiwan

[7]School of Pharmacy, Nihon Pharmaceutical University, Saitama 362-0805, Japan.

[8]Tsuzuki Institute for Traditional Medicine, China Medical University, Taichung 404, Taiwan

[9]School of Pharmacy Undergraduate Program, Master Degree Program, Ph.D Program, China Medical University, Taichung, Taiwan

Corresponding author:
Dr. Ming-Kuem Lin
No.91 Hsueh-Shih Road, Taichung 40402, Taiwan
Tel.: +886-4-22053366 ext. 5212
Fax: +886-4-22078083
E-mail: linmk@mail.cmu.edu.tw

**Abstract**

*Cuscuta* seeds have been used as a Chinese medicine for many years. In this study, the chemical constituent profile and pharmacological bioactivities of extracted *Cuscuta campestris* seeds after a plain stir-baking process were investigated. Using a LC/MS-based metabolomics strategy, the compounds that changed significantly after plain stir-baking of the seeds were identified. Compared with unprocessed seeds, LC-MS/MS and HPLC chromatography analysis demonstrated that the compound that increased most significantly in the processed seeds was quercetin (23 fold). Moreover, the stir-baked seeds exhibited higher anti-inflammatory activity against RAW264.7 cells and higher antiproliferative activity against MDA-MB-231 and MCF-7 cells. Here for the first time it is shown that *Cuscuta* seeds have anti-inflammatory and antiproliferative activity. In addition, it was found that processed seeds had higher levels of these two activities than unprocessed seeds. Taken together, these results suggest that quercetin in *Cuscuta campestris* seeds plays an important role in these two bioactivities.

**Keywords:** *Cuscuta campestris*, plain stir-baking, LC-MS/MS, quercetin, anti-inflammation, antiproliferative

## 1. Introduction

Processing Chinese materia medica (CMM) is a conventional method to clean the collected material, enhance the pharmaceutical efficacy of the CMM, detoxify or reduce toxic substances within the raw material, inactivate enzyme activities that may hydrolyze glycosidic bonds in active glycosidic constituents of the CMM and make formulation with other herbal material easier [1]. The techniques used to process CMM are generally classified into seventeen categories and include heating, scalding, steaming, processing with liquor, butter and honey, stewing, calcination, filing and blanching. Among the above methods, plain stir-heating is most often employed as processing method for CMM [1].

*Cuscuta* seeds, also named Semen Cuscutae, mainly refer to the seeds of *Cuscuta chinensis*, which is a commonly used Chinese medical plant. It was first recorded in the famous book "Shen Nong's Herbal" (http://en.tcm-china.info/culturehistory/literature/75831.shtml) as one of the best Chinese medical plants. *Cuscuta* seeds have been widely prescribed by Chinese medicinal practitioners to nourish the liver and kidney. Extracts of *Cuscuta* seeds include various flavonoid compounds, a range of polysaccharides, a number of different alkaloids and various other chemicals [2-5]. Total flavones and ethanolic

extracts of *Cuscuta* seed have been shown to help defective kidneys in an animal model [6] and to have hepatoprotective activity [7]. In addition to antioxidative activity, crude polysaccharides prepared from *Cuscuta* seeds have also been shown to have a potent stimulating effect on lymphocyte proliferation and antibody production [8]. Traditionally, *Cuscuta* seed is process by plain stir-baking in accordance with "Lei's Treatise on the Processing of Drugs", which is a guideline book on the processing of CMM to give active medicines [9]. The process involves placing clean *Cuscuta* seeds in a pot and stir-baking them using slow fire until the color of the seeds become slight brown [9]. However, as far as we know, the phytochemical changes that occur within the Cuscuta seeds during processing and whether this affects the bioactivity levels of the seeds have not been investigated.

Metabolomic profiling approaches based on LC/MS techniques have been shown to be both sensitive and rapid; this approach has emerged in recent years as the method of choice when comparing samples of this type [10-12]. Therefore, in this study we used a LC/MS-based metabolomic approach to investigate the metabolite profile in *Cuscuta campestris* seeds after heat processing for various times. We also explored the effects on the seeds of the processing in terms of their anti-inflammatory activity using RAW264.7 cells and in terms of their anti-proliferative activity using MDA-MB-213 and MCF-7 cells. To the best of our knowledge, the metabolic changes

identified after heat processing and changes in pharmaceutical activity after heat-processing, are the first demonstrated to date.

## 2. Materials and methods

### *2.1. Chemicals*

Quercetin, hyperoside and kaempferol were purchased from Sigma-Aldrich, Germany. Acetonitrile (ACN) with 0.1% formic acid (FA) and water with 0.1% formic acid (LC-MS grade, J. T. Baker, Phillipsburg, NJ) were used as the mobile phase for the LC-MS analysis. Deionized water (18 MΩ·cm) from a Milli-Q system (Millipore, Bedford, MA) was used throughout this study. All other reagents were of analytical grade.

### *2.2. Seeds*

Three types of *Cuscuta* seeds were used in this study. First, raw seeds, which are seeds collected from *Cuscuta campestris* plants grown on *Bidens pilosa* L. var. radiata Sch. Bip in the wild (Taichung, Taiwan). Second, plain stir-baked seeds, that are the raw seeds plain stir-baked in a pot with low heat for 15 or 30 minutes. Finally, marketed seeds, obtained from a Chinese Medicine Hospital (Union Traditional Medical Hospital, Taichung, Taiwan), which are seeds of *Cuscuta chinensis*, that have usually been plain stir-baked before purchase.

*2.3. Extraction*

The three types of *Cuscuta* seeds were ground into fine powder with a pulverizer and the powder was then passed through a 50-mesh sieve. 1 gram of the powder from each type of seeds was extracted with 50 ml of methanol by sonication for 1 hour at 60℃. The supernatant was separated by centrifugation at 3000 rpm for 15 min and collected. Then, 40 ml of methanol was added to the residue and the powdered seeds processed again in a similar manner to that described above. The two supernatants were pooled and dried out using a rotary evaporator to give methanol extracts of the various samples. These methanol extracts were redissolved in methanol and DMSO for analysis by HPLC and LC-MS/MS and for bioassays, respectively.

*2.4. LC-MS and LC-MS/MS*

HPLC separation was carried out on an Atlantis T3 $C_{18}$ 5µm 2.1 x 150 mm, analytical column (Waters, Millford, MA). A linear gradient, at a flow rate of 0.25 mL/min, was used to elute the extracted metabolites of the *Cuscuta* seed. The mobile phase consisted of water containing 0.1% (v/v) formic acid (A) and acetonitrile containing 0.1%(v/v) formic acid (B), with a gradient from 10% (v/v) B to 40% B in the first 25 min, and to 70% B over 5 min. It was then held at 70% B for another 5 min at the flow rate of 0.25ml/min, which was followed by a return to the starting conditions and re-equilibration of the column for 3 min with 10% B (v/v) prior to the next injection.

The HPLC was performed on a Dionex Ultimate 3000 HPLC system (Dionex, Germany) equipped with a pump (DGP 3600M), an auto sampler (WPS-3000T). HCTultra PTM Discovery (Bruker Daltonics, German) and an ESI source. The mass spectrometer was suitable for LC-MS full scan measurements and LC-MS/MS experiments. The ESI source was operated in the negative ion mode. Nitrogen was used as a nebulizing (50 psi) and drying gas (10 L/min, 350$^{o}$C). MS scan measurements were carried out in the mass range of 100-800 m/z using the ultra-scan mode. For the MS/MS settings, the most eight intense ions from each MS spectrum were automatically selected as the precursor ion peaks for the following auto MS/MS experiments. Helium was used as the collision gas. Profile Analysis software 2.0 (Bruker Daltonics, Germany) was used to perform the multivariate data analysis. In the principal components analysis (PCA) settings, the "find molecular feature" function was selected for chemical compound finding with the following parameters: minimum retention time 1 min, maximum retention time 25 min, minimum spectral peak width 0.2 amu, minimum retention time peak width 5 scans, retention time tolerance 0.5 min and mass tolerance 0.3 amu.

*2.5. HPLC* for quantification

All samples were filtered through a 0.45 μm pore-size membrane filter and then

analyzed by HPLC. The chromatographic conditions used were as follows: column: Inertsil ODS-3V (5 μm, 4.6 I.D. × 150 mm); detection wavelength: 360 nm; mobile phase: Methanol(30%)+0.4%$H_3PO_4$(70%) → Methanol(70%)+0.4%$H_3PO_4$(30%); injection volume: 20 μl; flow rate: 1 ml/ min; and run time: 30 min. Quantification was obtained by comparing the sample peak with the corresponding standard compound.

*2.6. Macrophage cell culture*

The murine macrophage cell line RAW264.7 (BCRC, Hsinchu, Taiwan) was cultured with GIBCO™ Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA ) containing 10% (v/v) fetal bovine serum supplemented at 37 °C under 5% $CO_2$. The RAW264.7 cells were seeded onto a 96 well plate at a density of $1.5 \times 10^6$ cell/mL. After one hour of pre-incubation, 1 μg/mL lipopolysaccharide (Sigma, Louis, MO) and various concentrations of the test samples (methanol extracts of the processed, unprocessed, and marketed seeds (0 - 250 μg/ml), hyperoside (0 - 25 μg/ml) and quercetin (0 - 25 μg/ml) were added and the plate was then incubated for 20 h.

*2.7. MTT cell viability assay*

MTT assays were performed to measure the cytotoxicity of the LPS and the test

compounds using the RAW264.7 cells. After 20 h of incubation with LPS (1 μg/mL) and the test samples as described above, 10 μL of 5 mg/mL of MTT (3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyl tetrazolium bromide) solution was added to each well. After 4 h of incubation, the medium was discarded and the formazan blue, which had formed in the cells, was dissolved using 100 μl DMSO. The optical density was then measured at 590 nm.

*2.8. Nitrite determination*

After 20 h of incubation with LPS (1 μg/mL) and tested samples as described above, 100 μL of medium was collected and the amount of nitrite present was determined using Griess reagent (1% sulfanilamide, 0.1 % N-1-naphthyl ethylenediamine dihydrochloride, and 2.5 % phosphoric acid) as described in an earlier study [13]. Briefly, the same volume of medium and Griess reagent were mixed and absorbance at 550 nm was measured.

*2.9. Human breast cancer cell culture and determination of cell viability*

The human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from the American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine

serum (Invitrogen) and 1% penicillin–streptomycin solution (Invitrogen) at 37 $^{0}$C in a 5% $CO_2$ incubator. After the cells had been cultured separately overnight on 96-well plates, cells were incubated with various concentrations of the methanol extracted test samples, hyperoside, kaempferol, and quercetin (*Sigma-Aldrich*) for 72 h. Next 20 µl of the staining dye WST-1 was added to each sample and the mixture incubated for an additional 2.5 h. The absorbance was then read at 450 nm on a Bio-Rad model 550 microplate reader and the results used to calculate the half maximal inhibitory concentration (IC50).

## 3. Results and Discussion

### The metabolite profile of *Cuscuta campestris* seeds changes during processing by plain stir-baking

To investigate the chemical profile of *Cuscuta* seeds after the plain stir-baking, the seeds of *Cuscuta campestris* were used. First, the seeds were stir-baked in a pot over a low heat for 15 min or 30 min. After extraction, LC/MS was used to analyze the changes in metabolite profile between the unprocessed and processed seeds. Each sample was run four or five times and this was followed by data processing and principal components analysis (PCA). The PCA was designed to extract, display and rank the variance in the data matrix using a mathematical projection technique. The

PCA is able to reduce the dimensionality of the dataset while simultaneously retaining the information present in the data. The principal components were calculated using the LC/MS data obtained from the unprocessed and processed samples. The first two components, PC1 and PC2, were found to be sufficient to account for most of the variation in the data. Therefore, based on PC1 and PC2, the PCA results consist of a scores plot and a loadings plot, which are invariably linked together.

As shown in Fig. 1A, the scores plot represented the sample distribution in the new multivariate space and three distinct clustering were observed for the unprocessed, processed for 15 min and processed for 30 min samples. The increasing distance of the 15 min and 30 min processed samples from the unprocessed sample for PC1 indicated that the metabolite profiling changes are increased as the processing time increases. This finding supports the hypothesis that the chemical components of the *Cuscuta* seeds do indeed change after the heat processing.

**Quercetin increased significantly in the stir-baked *Cuscuta campestris* seeds**

Next, the corresponding "loadings plot" analysis was used to visualize which metabolites are responsible for the sample clustering scores plot. As shown in Fig. 1B, the result showed that several compounds located on the right region (PC1>0.1) had a major impact on the sample clustering. Among them, the far right one (PC1 ~0.7), had

the greatest impact on the clustering of the processed and unprocessed samples; this has a MS peak (300.9 m/z) that eluted at 19.6 min. To identify this compound, additional LC-MS/MS analysis was applied to generate product ion information. Based on the parent ion mass, LC retention time and product ion spectrum, the high impact metabolite eluted at 19.6 min with a 300.9 m/z was identified as quercetin (Fig.2). The fact that the amount of quercetin increased significantly during the heat-processing was confirmed by observing the ion signals for this peak (300.9 m/z, 19.6 min) in the two samples (Fig. 1C). In parallel, conventional HPLC chromatography was also conducted to assess the metabolite changes during the heat-processing. The chromatograms showed that quercetin peak was significantly increased during the heat-processing (Fig. 3A) and subsequently HPLC chromatography was used to quantify the amount of quercetin present in the samples. Marketed seed was included in the comparison too and Fig. 3B shows that the metabolite profiles of the marketed *Cuscuta* seeds and the test *C. campestris* seeds are slightly different. The chromatogram showed that hyperoside was the major compound in the marketed seeds and therefore the amount of hyperoside present was included in the study with the amount and bioactivity of this compound also being compared in the following studies. By comparing with commercial standard compounds (Fig. 3A), the quantification showed that hyperoside decreased from

0.8998 mg/g to 0.6463 mg/g after heat-processing (Fig. 4A), while quercetin increased 23 fold (from 0.0939 to 2.1398 mg/g) (Fig. 4B). This result is in agreement with the LC-MS/MS results (data not shown for hyperoside, Fig 1B and 1C). Overall, heat treatment to give processed seeds seems to slightly decrease the amount of hyperoside in the seeds while dramatically and significantly increasing the amount of quercetin present.

Isoflavone glucosides have been shown to be sensitive to heat and are usually hydrolyzed to aglycones during heating process [14]. In contrast, quercetin glucosides have been shown to be heat stable under different heating process and only minor amounts of thermal degradation [15-17]. In this present study, our results showing that hyperoside (quercetin-3-O-galactoside) decreases slightly in the processed seeds agrees with this previous result. This consistent result reveals that the significant increasing of quercetin aglycone in the processed seeds was not mainly caused by the thermal instability of quercetin glucosides. Therefore, it is interesting to further investigate whether the underling mechanism of the increasing of quercetin aglycone is due to the enhancement of production of quercetin aglycone or enzymatic hydrolysis of quercetin glucosides.

**Processed *C. campestris* seeds show stronger anti-inflammatory activity**

To investigate the effect of the heat-processing on seed in terms of bioactivity, anti-inflammatory activity of the seeds was examined. Methanolic extracts of the marketed seeds, the 30 minute processed seeds and the unprocessed seeds were examined as to their effect on macrophage RAW264.7 cells. Nitrite production was used to represent nitric oxide production, which is indicator of the level of inflammation. As shown in Fig. 5A, nitrite production by the LPS stimulated macrophages was significantly reduced when the cells were treated with 50-125 μg/mL of the three extracts. In contrast, the nitrite was almost undetectable in macrophage cells that had not been exposed to LPS. This result reveals for the first time that *Cuscuta* seeds possess anti-inflammatory activity as assessed by LPS-activated macrophage RAW264.7 cells.

When RAW264.7 cell was treated with more than 50 ug/mL of the extract from heat-processed *C. campestris* seed, the nitrite decrease was significant. Importantly, the reduction caused by the heat-processed seed extract was more significant than that produced by the unprocessed or marketed seeds. Thus it seems that the anti-inflammatory activity of the heat-processed seeds was greater than that of the unprocessed seeds or the marketed seeds. It is important to note that these three samples did not exhibit cytotoxicity at the concentrations tested (Fig. 5B). However, when the cells treated with 125 μg/ml of extract, there was no further significant reduction in nitrite production and this could be an effect of other chemicals in the extracts that increase nitrite production.

Quercetin was tested to determine whether it contribute to the anti-inflammatory activity. As shown in Fig 6A, when up to 6 μg/mL of quercetin was used, the production of nitrite in activated macrophages was markedly decreased. There was an about 50% reduction in nitrite production when macrophage cells were treated with

20 μg/mL of quercetin. Furthermore, quercetin also did not exhibit cytotoxicity at the concentrations tested (Fig. 6B). Thus quercetin is able to significantly decrease the production of nitrite in activated macrophages and this effect is consistent with earlier studies [18-20]. Our chromatographic analysis showed that hyperoside was also a major compound found in *Cuscuta* seeds, but that heat treatment slightly decreased this compound. To test whether hyperoside also contributed to the extracts' anti-inflammatory activity, this compound was also examined. When hyperoside was added up to a concentration of 25 μg/mL, the production of nitrite by RAW264.7 cells was not changed (Fig. 6A). This indicates that hyperoside does not have any anti-inflammatory activity. Furthermore, hyperoside was not cytotoxic when added to macrophage RAW264.7 cells (Fig. 6B). These results suggest that quercetin is the main contributing chemical to the heat-processed *C. campestris* seeds' anti-inflammatory activity and this effect is enhanced when the *C. campestris* seeds are heat treated because this process increases the amount of quercetin in the seeds.

**Processed *C. campestris* seeds possess stronger antiproliferative activity**

We also examined whether heat-processed *C. campestris* seeds exhibited antiproliferative activity. The extracts of the marketed seeds, 30 min-processed and unprocessed seeds were used and the extracts' antiproliferative activities against MCF-7 and MDA-MB-231 human breast cancer cells were examined. These two cell lines were treated with various amounts of the extracts and IC50s were calculated. Fig. 7A shows that the IC50 of the extracts of the marketed seeds, processed *C. campestris*

seeds and unprocessed *C. campestris* seeds for MCF-7 cells were 0.28 μg/uL, 0.23 μg/ul and 0.43 μg/ul, respectively. Similarly, the IC50s of the extracts of the marketed seeds, the processed *C. campestris* seeds and unprocessed *C. campestris* seeds for MDA-MB-231 cells were 0.22 μg/uL, 0.15 μg/ul and 0.29 μg/ul, respectively. These results indicate that the antiproliferative activity of the heat-processed *C. campestris* seed extract was significantly higher than that of the unprocessed *C. campestris* or marketed seed extracts when tested against both cancer cell lines. To the best of our knowledge, this is the first time that *Cuscuta* seeds have been shown to exhibit antiproliferative activity against MCF-7 and MDA-MB-231 human breast cancer cells.

To further characterize whether the antiproliferative activities of the extracts against MCF-7 and MDA-MB-231 human breast cancer cells were related to quercetin or to hyperoside present in the samples, pure quercetin and hyperoside were assayed and their IC50s against the two cancer cells tested. In addition, because kaempferol is also present in the marketed seed (Fig. 3), this compound was also included in the study at this point. The results showed that hyperoside did not show antiproliferative activity (data not shown) and it was observed that the antiproliferative activity of quercetin against both MCF-7 and MDA-MB-231 cells was higher than that of kaempferol (Fig. 7B). Chromatographic analysis showed that

the marketed seeds contained kaempferol and low amounts of quercetin (Fig. 3B); the extract of these seeds exhibited a lower activity in all assays. However, the processed *C. campestris* seed extract contained higher amounts of quercetin and the extract of these seeds exhibited a higher activity in all assays. Taken together, these results strongly suggest that quercetin plays a critical role in the antiproliferative activity of processed *C. campestris* seeds. Similar conclusions can be drawn from both the MDA-MB-231 and MCF-7 cell assays, with the former cell type showing greater sensitivity.

Quercetin have antiproliferative activity against different cell lines [21-24]. The identification of quercetin's antiproliferative activity in this study was in accordance with these previous studies. The results of the anti-proliferative study also support the hypothesis that heat-processing increases the amount of quercetin present, which in turn enhances anti-proliferative cell activity.

*Cuscuta* seed is a commonly used Chinese materia medica (CMM) and the findings in this study demonstrated for the first time that quercetin in the seeds of *C. campestris* plays a critical role in terms of the seeds' anti-inflammatory and anti-proliferative activities. Finally, it is important to note that this study has demonstrated that heat processing of one CMM is able to modify its chemical constituents and affect its bioactivity profile; this might be true for other CMMs that

1 are treated in specific ways before use.

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3 **Acknowledgments**

4 This work was supported by CMU grant CMU99-N1-01-1 and NSC grant NSC

5 98-2311-B-039-001-MY3 of Taiwan.

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## Figure Legends

**Figure 1, LC-MS and PCA analysis.** The scores plot (A) and corresponding loadings plot (B) for the PCA of the LC-MS data derived from all metabolite extractions of *Cuscuta* seeds. The scores plot shows distinct clustering of the control group (0 min), the treatment for 15 min group (15 min) and the treatment for 30 min group (30 min). On the loadings plot, the ions most responsible for the variance in the scores plot are represented by their distance from the origin. The peak at 300.9 was eluted at 19.6 min and can be recognized as the most responsible for the differences between the three samples. (C) The bucket statistic figure shows the ion signals of the peak (300.9 m/z, 19.6 min) in the samples from replicated runs.

**Figure 2, LC-MS/MS analysis.** Extraction ion chromatography and MS/MS spectrum of (A) the peak (300.9 m/z, 19.6 min) and (B) of quercetin.

**Figure 3. HPLC analysis.** HPLC chromatograms of the extracts of unprocessed and processed *C. campestris* seeds after heat treatment for 15 minutes and 30 minutes, (A), and of marketed *C. chinensis* seeds, processed *C. campestris* seeds and unprocessed *C. campestris* seeds as well as three flavonoid standards (hyperoside, quercetin, and kaempferol) (B).

**Figure. 4. Hyperoside (A) and quercetin (B) contents in the extracts of marketed *C. chinensis* seeds, unprocessed and processed *C. campestris* seeds.**

**Figure 5. Effect of the extracts on RAW264.7 cells.** Extracts of the marketed *C. chinensis* seeds (M), the processed *C. campestris* seeds (P) and unprocessed (U) *C. campestris* seeds were used to examine the effect on LPS-induced NO production in RAW264.7 cells (A) and on the cell viability (B). The cells were incubated with various concentrations (0, 0.5, 5, 50, 125, 250 μg/ml) of the three extracts in the absence (P; □, U; ◇, M; ○) or presence of 1 μg/ml LPS (P; ■, U; ◆, M; ●) for 20 hours. Each dot represents mean ± SEM.

**Figure 6. Effect of hyperoside and quercetin on RAW264.7 cells.** Hyperoside and quercetin were used to examine the effect on LPS-inducted NO production in RAW264.7 cells (A) and on cell viability (B). The cells were incubated with various concentrations (0, 0.00156, 0.0156, 0.156, 1.56, 6.25, 25 μg/ml) of hyperoside and quercetin in the absence (hyperoside; □, quercetin; ◇) or presence of 1 μg/ml LPS ((hyperoside; ■, quercetin; ◆) for 20 hours. Each dot represents mean ± SEM.

**Figure 7 Effects of the extracts, hyperoside and quercetin on MCF-7 and MDA-MB-231 cells.** Extracts of the marketed *C. chinensis* seeds, the processed *C. campestris* seeds, unprocessed *C. campestris* seeds (A), together with quercetin and kaempferol (B) were examined to determine their effect on the proliferation of MCF-7 and MDA-MB-231 breast cancer cells. These two types of cells were treated with increasing amounts of the three extracts (A) and pure kaempferol and quercetin (B), and their IC50s were calculated and plotted.