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*Full Length Research Paper*

# Anti-infection effects of buckwheat flavonoid extracts (BWFEs) from germinated sprouts

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**Buckwheat has been used in Chinese traditional medicine or dietary treat against various kinds of cancer for several decades. Germination method was used to elevate the nutritional value of flavonoids in tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn) sprouts. To examine the growth inhibitory effects of tartary buckwheat flavonoid extracts (BWFEs), MTT assay was applied to detect the cell viability of mammary cancer MCF-7 cells. Several sample of BWFEs revealed strong inhibitory activity, and the 50% inhibitory concentrations ($IC_{50}$s) were separately determined. For further verification of the cytotoxic action of BWFEs on MCF-7 cells, cell apoptosis and cell cycle progression were measured by flow cytometry. An *in vivo* chick chorioallantoic membrane (CAM) assay was also tested, BWFEs showed obvious angiogenesis inhibitory activity on the formation of micro blood vessels. According to these data, we conclude that BWFEs extracted from malting tartary buckwheat seeds demonstrate significant inhibitory activity on the growth of MCF-7 cancer cells, therefore, BWFEs can be further explored for antitumor therapy or functional food additives.**

**Key words:** Buckwheat flavonoid extracts (BWFEs), anti-infection, germinated sprouts, mammary cancer, antitumor activity, cell apoptosis.

## INTRODUCTION

Buckwheat is a kind of cereal well-known for its immunity-improvement function and anti-inflammatory effect, in case of its unsavory flour and other food products, buckwheat extract has been found to be able to treat cancer long time ago (Gao and Meng, 1993). In China, extracts from different fractions of buckwheat plant (such as root, stem, seed hull and so on) have been proved to possess anti-tumor capacity (Chan 2003; Kim et al., 2007; Lin, 2004). Yet, most of these researches were carried out by *in vitro* study , mainly upon various tumor cell lines originated from different organs, including Hela (from uterine cevix), SGC (from stomach), KB (from mouth epiderm), GLC, H460 and A549 (from lung), HepG2 and Hep3B (from liver), HCT116 (from colon), K562 (from leukocytes), U2OS (from bone) and MCF-7 (from breast). The ingredients of those anti-tumor extracts were identified as buckwheat protein extract (BWP), buckwheat protease inhibitor (BTI), flavonoid, tannin, etc.

According to animal experiments, Kayashita et al. (1999) reported that dietary of BWP retarded the mammary cancer by lowering serum estradiol, then Liu et al. (2001) proved that intake of BWP decrease colon carcinogenesis by reducing cell proliferation. MTT assay revealed that an anti-tumor protein (named TBWSP31, with molecular weight of 57,000) could inhibit cell proliferation of human mammary cancer Bcap37 cell line in a time- and dose-dependent way (Guo et al., 2007). Besides of anti-tumor activity, some multifunctional BWPs still had other biologic activities, such as antifungal activity (Leung and Ng, 2007) and protease inhibitory activity (Park and Ohba, 2004).

Due to the research result of Park and Ohba (2004), with Arg residue(s) structural modified in buckwheat protease inhibitor, both trypsin inhibitory activity and anti-tumor activity were abolished at the same time. There were still many other reports revealing the relationship between protease inhibitory activity and anti-tumor activity of recombinant buckwheat trypsin inhibitor (rBTI), for example, the apoptosis of multiple myeloma cell line IM9 (Zhang et al., 2007), leukemia cell line HL60 (Gao et al., 2007) and K562 (Wang et al., 2007) could be induced

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by rBTI. Though the precise action mechanism of rBTI anti-proliferation of tumor cells has not been made clear yet, recent research by Li et al. (2009) proved that human solid tumor cell lines (EC9706, HepG2 and HeLa) apoptosis induced by rBTI might be caused by mitochondrial transmembrane potential loss and caspase activation.

Similar to rBTI, tartary buckwheat flavonoid was found to be able to induce HL-60 cell apoptosis via caspase activating pathway (Ren et al., 2003), and buckwheat flavonoids were also found to be able to up-regulate anti-inflammatory cytokines tumor necrosis factor (TNF-α) inside colon carcinoma cell line CoLoTC cells (Ishii et al., 2008). Though buckwheat protein had been proved to be effective in suppress mammary cancer by both *in vivo* and *in vitro* detection (Kayashita et al., 1999; Guo et al., 2007), the question whether buckwheat flavonoids could inhibit mammary cancer was not clear. Therefore, we investigated the anti-proliferation effect of buckwheat flavonoid extracts (BWFEs) against mammary cancer cell line MCF-7. In order to improve BWFE ingredients in tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn) sprouts, seed malting method was applied.

## MATERIALS AND METHODS

Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) was cultivated in Shanxi Province of China in 2007, then the seeds were collected and dried at harvested festival.

### Preparation of buckwheat flavonoid extracts from malting seeds

Buckwheat seeds were cleaned and washed in 0.9% (w/v) NaCl solution, and then marinated in deionized water for 10 h. The soaked seeds were put into 8-layers sterile gauze, and malted for 1 to 7 days in SPX-300 IC microcomputer artificial climate chamber (30°C, 80% humidity). The malted sprouts were dehulled and dried by lyophilization, and then crushed into powder. The powder from malted buckwheat seeds was dissolved in 70% ethanol for flavonoid extraction. After extracting in 70°C water bath for 6 h, buckwheat flavonoid extracts (BWFEs) were filtered, and the filtrates were stored for further use (Watanabe et al., 1997).

### Cell culture

The mammary carcinoma cell line MCF-7 was purchased from cell bank in Shanghai Institutes for Biological Sciences (SIBS) of Chinese Academy of Sciences (CAS). MCF-7 cells were cultured in RPMI-1640 medium (37°C, 5% $CO_2$), containing 10% (v/v) fetal calf serum (FCS) and supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin. Culture medium was replaced every 2 to 3 days. After cells grew to near confluence, they were treated with 0.25% (w/v) trypsin and 0.02% (w/v) EDTA for 5 min and replaced to new flask.

### Cell-proliferation inhibitory action assay

The anti-proliferation assay of MCF-7 was measured with MTT assay (Mosmann, 1983; Hongrapipat et al., 2008). MCF-7 cells (2.5 × $10^4$ cells) were transferred to 96-well microtiter plates, after 48 h incubation, 10 µl of 0.5% (w/v) MTT solution was added to each well. Dying for 4 h at 37°C in a 5% $CO_2$ environment, and absorbance was measured with a microtiter plate reader (Tacan GENios Pro, Germany) at 570 nm. All experiments were carried out in three independent repeats, and the 50% inhibitory concentration (IC50) was also detected.

### Analysis of cell apoptosis and cell cycle progression

MCF-7 cells were incubated with buckwheat flavonoid extracts (BWFEs) or medium (blank control) for 48 h, then cells were collected and washed by cooled PBS. After fixtion by 70% ethanol for 16 h, the cells were washed two times by PBS buffer. The cells were stained for 30 min in the dark by PI (50 µg/ml) binding buffer, containing RNase A (100 µg/ml), and then cell apoptosis and cell cycle progression were analysed by flow cytometry (Yan et al., 2009).

### Angiogenesis inhibitory effects assayed by chick chorioallantoic membrane (CAM) model

The fertilized chick eggs were incubated at 37°C for 10 days, then the chorioallantoic membrane (CAM) assay was carried out (Sasaki et al., 2000). Each egg was opened a window to expose the CAM, then BWFEs together with basic fibroblast growth factor (bFGF) were added to the center of coverslips, air dried for 1 h. Then the coverslips were placed onto the CAM and incubated for another 2 days. The angiogenesis inhibitory effects were analysed by comparing the density and amount of newly-growing micro-blood vessels.

### Statistical analysis

The statistical analysis of the data was carried out by SPSS 17.0 statistical software. The results were expressed as the mean ± SEM of at least three experiments. While comparing the change, the data was analyzed by one-way ANOVA, followed by the LSD to detect significant difference between different groups. The level of significance was set at $P<0.05$.

## RESULTS

### Levels of buckwheat flavonoid extracts from tartary buckwheat during seed germination

Buckwheat flavonoid extracts (BWFEs) from tartary buckwheat seeds were extracted and designated as BWFE-T0-7 respectively, and the contents of total flavonoids at different malting days were detected. According to data examined (Figure 1), the contents of total flavonoids tartary from buckwheat seed were certainly up-regulated by germination methods. On day 3, the level of total flavonoids reached 21.66 mg/g, exhibited nearly 1.5 fold increase than that of day 0 (14.33 mg/g). The total flavonoid level continued to rise during the following germination day, then got to a maximum at day 6 (26.56 mg/g). In all, total flavonoid contents on day 3 to 7 of seed malting period were obviously increased, compared with that of day 0 ($p<0.05$). The ingredients of

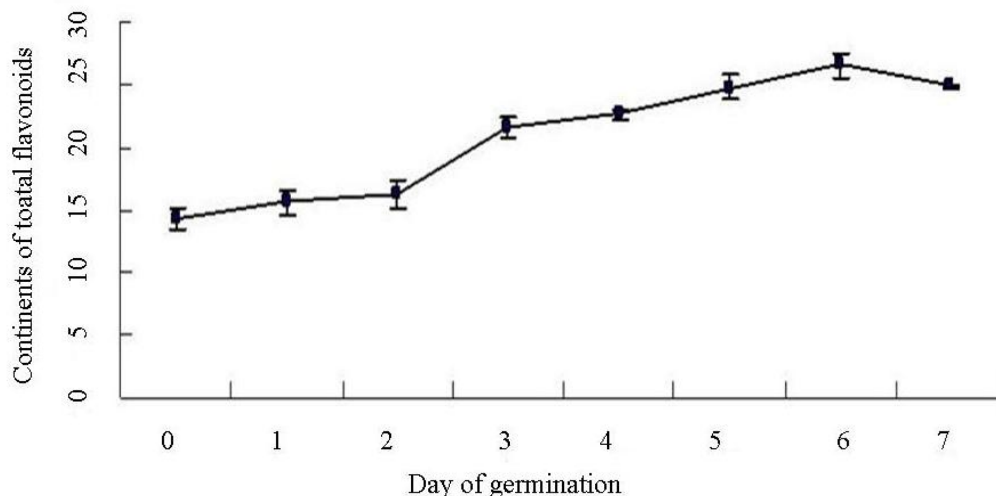


**Figure 1.** The content of total flavonoid extracts in tartary buckwheat at different malting days. All the data demonstrated are the mean of three independent experiments. Indicated $p<0.05$ analyzed by SPSS.

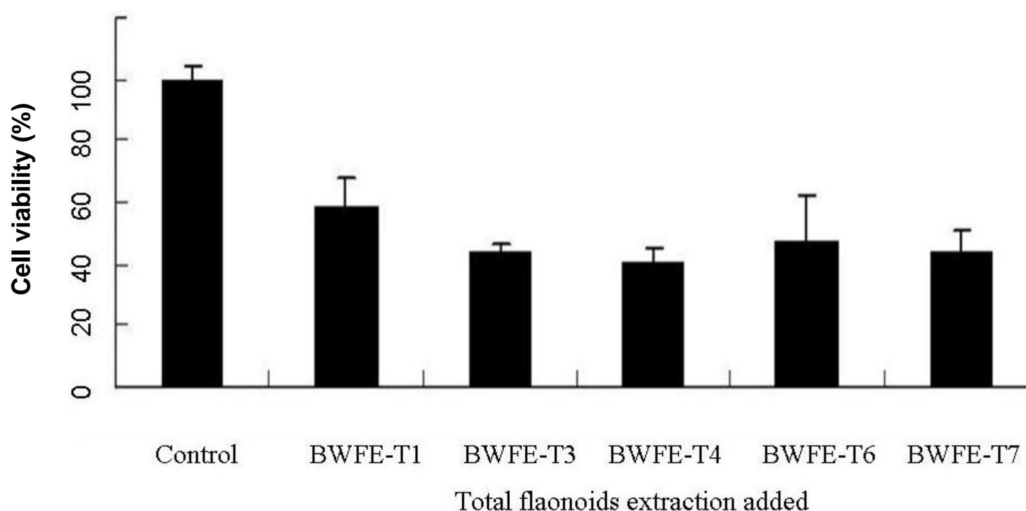


**Figure 2.** Anti-proliferation properties of BWFEs against human marmary cancer cell line MCF-7 tested by MTT assay. All data demonstrated are the mean of three independent experiments. Indicated $p<0.05$ analyzed by SPSS 17.0 software.

buckwheat seed flavonoids were mainly rutin and quercetin, analyzed by high performance liquid chromatography (HPLC). However, the rate between rutin and quercetin was different due to different germination days.

## Anti-proliferation properties of BWFEs against MCF-7 Cells

The cell growth inhibition of BWFEs against human marmary cancer cell line MCF-7 was tested by MTT assay. Compared with untreated cell group (Figure 2), Sample BWFE-T1, 3, 4, 6 and 7 exhibited obvious anti-proliferation property ($p<0.01$). The cell viability of MCF-7 cells treated by BWFE-T3 and T4 were 43.82 and 40.49% respectively, therefore, the anti-tumor activity of BWFE-T3 and T4 were much more effective than other samples ($p<0.01$).

The 50% inhibitory concentrations (IC50) of BWFEs were determined as shown in Table 1, the $IC_{50}$ values of BWFE-T1, 3, 4, 6 and 7 were 0.346, 0.269, 0.216, 0.323

**Table 1.** $IC_{50}$ of BWFEs inhibited cell proliferation on MCF-7 cancer cell line.

| Favonoids extracts | $IC_{50}$ values (mg/ml) |
|---|---|
| BWFE-T1 | 0.346 |
| BWFE-T3 | 0.269 |
| BWFE-T4 | 0.216 |
| BWFE-T6 | 0.323 |
| BWFE-T7 | 0.289 |

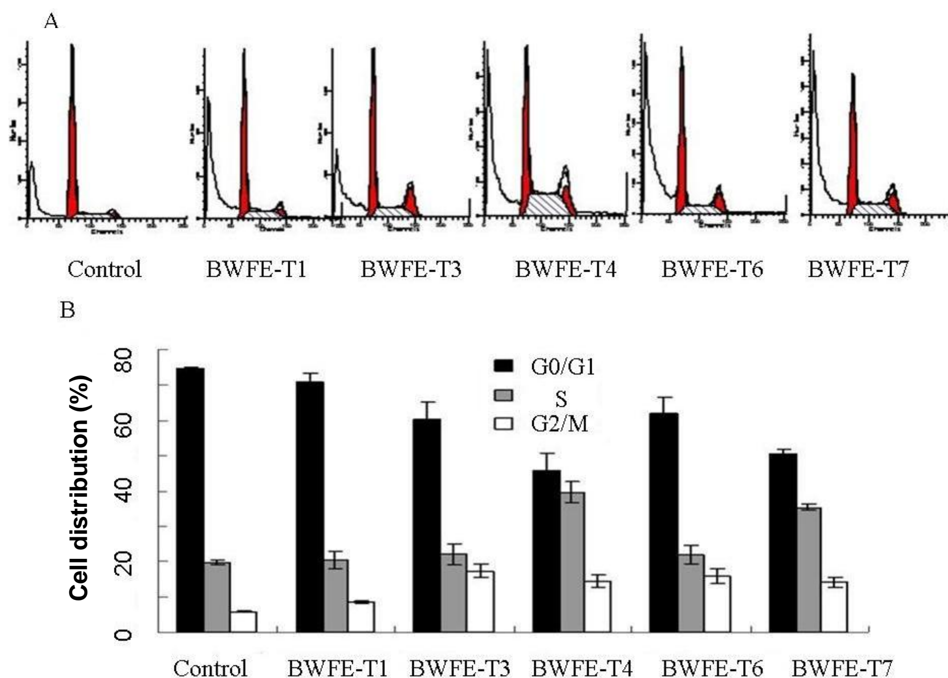


**Figure 3.** Cell apoptosis and cell cycle progression of MCF-7 cell line treated by BWFEs. All the data demonstrated are the mean of three independent experiments.

and 0.289 mg/ml respectively.

### The effects of BWFEs on cell apoptosis and cell cycle progression

Flow cytometry (FCM) were used to study the effects of BWFEs on cell apoptosis and cell cycle distribution (Figure 3). The apoptosis rate of cells treated by BWFE-T1, 3, 4, 6 and 7 were detected as 40.25, 41.04, 61.14, 41.02 and 49.43% respectively, while the apoptosis rate of control group was 3.74% (Figure 3A). These data illustrated that treatment of BWFEs could obviously cause MCF-7 cell apoptosis. Our results also revealed that the percent of G0/G1 phase cells decreased after incubation with BWFEs, however, the cell population of G2/M phase ascended (Figure 3B). Hence, we could conclude that BWFEs evoked cell cycle arrest at G2/M phase.

### Angiogenesis inhibitory action of BWFEs

The CAM assay was explored as *in vivo* model to examine the angiogenesis inhibitory effect of BWFEs. Tumor growth depended badly on the nutrition substance applied through blood vessels, so the inhibition of newly-growing microvessels districted the growth of tumor. As data showed in Figure 4, BWFE-T1, 3 and 4 exhibited obvious angiogenesis inhibitory effect.

## DISCUSSION

Buckwheat has been increasingly focused on in many countries of the world recently, for its special dietary

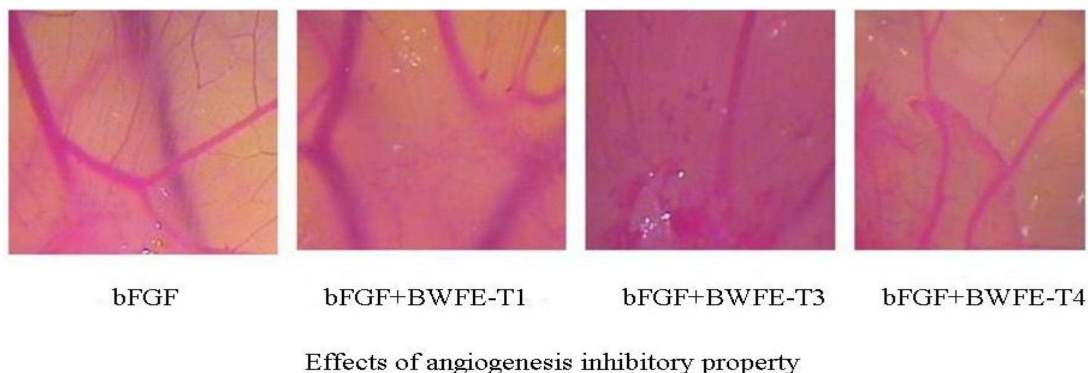


**Figure 4.** Angiogenesis inhibitory effects of BWFEs. All the data demonstrated are the mean of three independent experiments.

therapy effects including anti-tumor property, antioxidative activity, antibacterial activity, etc. Flavonoids are thought to be able to play critical role in those healing activity, and the main functional molecules are rutin, quercitrin, hyperin and so on (Watanabe et al., 1997). Buckwheat rutin considered to be a major source of dietary flavonoids, and rutin are found to be stored in buckwheat root, hull, immature seed and seedlings (Oomah and Mazza, 1996).

The aim to enhance the nutrition value and flavonoids of buckwheat seed, there are several choices that can be applied: selecting native buckwheat cultivars which produce much more flavonoid content is a proper method, and altering environmental conditions (such as day length, sunshine radiation) during cropping season is also available (Oomah and Mazza, 1996; Jovanovic et al., 2006), moreover, malting seeds to improve the nutrional compounds in seedlings is effective, too. Our research in this paper proved that the content of total flavonoids was distinctly elevated during seed germination (Figure 1), beginning at day 4, the increase of total flavonoids is of significant difference ($p<0.05$).

The point that buckwheat ingredient could inhibit mammary cancer by *in vivo* and *in vitro* test has already been assured (Kayashita et al., 1999; Guo et al., 2007), but the functional component was proved to be buckwheat protein. Therefore, we investigated the antitumor activity of buckwheat flavonoids against mammary cancer, using MTT assay. Results demonstrated that 5 samples of BWFEs BWFE-T1, 3, 4, 6 and 7) showed obvious antiproliferation activity against MCF-7 cells (Figure 2), especially BWFE-T3 and T4, their cell viability of MCF-7 cells were merely 43.82 and 40.49%, compared with control group. The 50% inhibitory concentrations of BWFE-T1, 3, 4, 6 and 7 were respectively 0.346, 0.269, 0.216, 0.323 and 0.289 mg/ml. For further examination of antitumor activity of BWFEs, flow cytometry was used to test the effects on cell apoptosis and cell cycle arrest. Data expressed that BWFE-T1, 3, 4, 6 and 7 induced cell apoptosis and caused cell cycle arrest at G2/M phase (Figure 3). Chick chorioallantoic membrane was used to detect angiogenesis inhibitory action of BWFEs, however, only BWFE-T1, 3 and 4 exhibited inhibitory effects on the growth of microvessels, while BWFE-T6 and 7 did not exhibit angiogenesis inhibitory activity (Figure 4) .Therefore, we can conclude that BWFEs with angiogenesis inhibitory activity might retard marmary carcinoma by preventing blood vessel nutrition transportation.

In total, BWFEs extracted from malted tartary buckwheat seed reveal obvious cytotoxic properties and angiogenesis inhibitory activity, so we suggest that BWFEs can be explored as effective food additives. However, for pharmic application, there will be still too much further research to carry out, for example, the total flavonoids should be isolated and purified to characterize the structure of buckwheat rutin and quercetin, and the pharmaceutical properties and clinical test is also worthy to be investigated.

## Conclusions

According to the experiment data, we found that 5 samples of buckwheat flavonoid extracts (BWFE-T1, 3, 4, 6 and 7) showed obvious growth inhibitory effects on mammary cancer cell line MCF-7 cells, while the cell viability of MCF-7 cells treated by BWFE-T3 and T4 were merely 43.82 and 40.49% respectively. The 50% inhibitory concentrations ($IC_{50}$s) of BWFE-T1, 3, 4, 6 and 7 were 0.346, 0.269, 0.216, 0.323 and 0.289 mg/ml respectively. By flow cytometry assay, BWFE-T1, 3, 4, 6 and 7 induced cell apoptosis and caused cell cycle arrest at G2/M phase. Moreover, BWFE-T1, 3 and 4 exhibited inhibitory effects on the growth of microvessels chick chorioallantoic membrane (CAM) assay, so we can deduce that BWFEs might retard marmary carcinoma by preventing blood vessel nutrition transportation. Therefore, BWFEs can be further explored for antitumor

therapy or as functional food additives.

## ACKNOWLEDGEMENT

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