# Effect of Citrus Waste Substrate on the production of Bioactive Component, and Antioxidant and Antitumor Activity of *Grifola frondosa*

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**Abstract:** The contents of bioactive compounds, and antioxidant and anticancer activities of *Grifola frondosa* cultivated on citrus waste substrates such as citrus peel and premature Hallabong fruit drop were investigated in the present study. The total phenolics and naringin were 80- and 500-folds higher in the extracts from *Grifola frondosa* mycelia cultivated on citrus waste than those in the extracts of *Grifola frondosa* mycelia cultivated on the sawdust, which was reported in the previous findings. Moreover, $IC_{50}$ values of the extracts from *Grifola frondosa* mycelia cultivated on citrus waste in the DPPH radical scavenging assay were much lower than that of mushroom cultivated on the conventional substrate. The premature Hallabong mushroom extract showed the highest phenolic content (126.7 mg/g) and the best antioxidant activity. The $IC_{50}$ values of the premature Hallabong mushroom extract were 0.52, 0.38 and 0.46 mg/mL in the DPPH radical scavenging, superoxide radical scavenging and xanthine oxidase inhibition assays, respectively. Anticancer effects of *Grifola frondosa* cultivated on citrus waste in human colon and breast cancer cells were analyzed. Results demonstrated that *Grifola frondosa* cultivated on citrus waste exhibited antiproliferative effects in both HT-29 and MCF-7 cancer cells. Taken together, citrus waste can be utilized as a viable substrate to improve the antioxidant and anticancer activities as well as to increase total phenolic content of *Grifola frondosa*.

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**Keywords:** *Grifola frondosa*; citrus waste; Bioactive component; Antioxidant activity; Anticancer activity

## 1. Introduction

Globally citrus fruit production was estimated as 120 million tons pert year (Terol et al., 2010). Almost half of these fruits is squeezed to juice, and the remainder including peel, segment membranes and other by-products is considered as citrus wastes. Traditional use for these residues is as cattle feed which currently does not have sufficient value to cover the production and transportation costs. Therefore, a large fraction of citrus waste is still deposited, leading to economical and environmental disadvantages, and health problem to human beings.

*Grifola frondosa*, a traditional and edible mushroom, has gained in popularity among consumers, not only because of its taste and flavor, but also because of its reported medicinal value (Borchers et al., 2004; Masuda et al., 2009). Extensive research has been carried out on the most efficient cultivation methods for mushrooms because its optimal growing conditions exist within a limited range of temperature, moisture, humidity and other environmental factors (Garibay-Orijel et al, 2007; Zhong and Tang, 2004; Sanchez, 2010; Chen et al., 2010). As one of the greatest challenges to mushroom cultivation, investigators have recently exerted their efforts to optimize existing cultural techniques for edible and medicinal mushrooms using cheap and locally sourced substrate materials such as agricultural and food wastes (Gregori et al., 2009; Xiaoke and Shunxing, 2005; Chiu et al., 2000). Moreover, these studies done on mushrooms that have nutritional or pharmacological value focuses on the development of cultivation strategies that make it easy to obtain target compounds. Accordingly, our objective was to determine the potentiality of using citrus wastes such as peels and premature fruit drops as a basic raw material for growing *Grifola frondosa* mycelia. In addition, to investigate the health-promoting values of *Grifola frondosa* mycelia cultivated on citrus wastes, namely their bioactive compounds, antioxidant and anticarcinogenic properties were evaluated.

## 2. Material and Methods

### 2.1. Substrate

The peel waste of citrus fruit (Citrus *unshiu* Marc) after juice extraction was obtained from a local food processing company (Jeju Provincial

Development Co., Jeju, Korea). The fruit peels were dissected, weighed, lyophilized and then ground into a fine powder using a blender. Premature Hallabong ([C. *unshiu* Marcov × C. *sinensis* Osbeck] × C. *reticulate* Blanco) fruit drops, kindly supplied by commercial orchards (Seogwipo-si, Jeju, Korea), were washed, dried in an oven with air circulation at 40 ºC, and ground with a mortal mile. The powered substrates were stored at -20 ºC prior to use.

**2.2. Microorganism, inoculum and sample preparation**

*Grifola frondosa* (KACC 50027), obtained from the RDA-Genebank Information Center, Suwon, Korea, was maintained on potato dextrose agar (PDA, Difco, Detroit, MI, USA) slant at 4 ºC. Fugal inoculum was prepared from mycelia grown on PDA for 5 days at 24 ºC in the dark and agar plugs taken from the periphery of the growing colony were used to inoculate media. An agar disk of the strain was inoculated (2%, v/v) in a 50 mL of a medium (pH 5) containing 50 mg of powdered substrate, 100 mg of $CaCO_3$ and 1 g of agar. After incubation for 10 days at 27 ºC, the 50 mL culture was added to 1 L media which consisted of powdered substrate (1 kg) and $CaCO_3$ (50 g). The fermentations were implemented in a 5-liter jar fermenter for 15 days at a 25 ºC. One gram of free-dried mycelia was ground into powder, extracted with 60 mL of 80% ethanol solution by ultrasonication at room temperature for 6 h, and then purified by using a Sep-Pak $C_{18}$ cartridge and a 0.45 μm membrane filter (Waters, Milford, MA, US), were used directly for analysis of their bioactive components, antioxidant and antitumor activities.

**2.3. Analysis of total phenolic content**

The amount of total phenolics was determined by the modified method described previously (Zhang et al., 2010). Briefly, an aliquot of filtrate (20 μL) was diluted with double-distilled water (30 μL). Folin-Ciocalteu reagent (100 μL) was dissolved in diluted solution, shaken, and then incubated for 5 min at room temperature. One hundred microliters of 20% $Na_2CO_3$ solution and double-distilled water until a total volume of 2 mL were added to the mixture. After incubation for 2 h at 23 °C, the absorbance *versus* a blank was determined at 760 nm. Three replicates were measured per sample class.

**2.4. Analysis of naringin and hesperidin contents**

The naringin and hesperidin contents of samples were determined using a modification of the procedure described previously (Abeysinghea et al., 2007; Kanaze et al., 2003). The mobile phase consisted of 75 mM citric acid and 25 mM ammonium acetate in methanol/double-distilled water (40:60, v:v). The filtrate (20 μL) of each sample was injected onto a C-18 symmetry (5 μm, 3.9 mm × 150 mm) column of the Waters HPLC system equipped with a 626 pump, a 486 UV detector fixed at 280 nm plus autosampler (Waters, Milford, MA) with the flow rate of 1 mL/min. The retention times and spectra were compared to those of authentic standards.

**2.5. 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay**

The radical scavenging assays was conducted by the modified method described previously (Cavin et al., 1998). Briefly, 100 μL of each test compound with various concentrations was prepared in 96-well plates and equal volume of 0.4 mM DPPH in methanol was added to each well. The solution was kept in the dark for 10 min at room temperature and absorbance of the solution was measured at 517 nm using an ELISA reader (EL340, Bio-Tek). A metanolic solution of DPPH was used as control, whereas L-ascorbic acid, qurcertin and curcumin were used as reference compounds. Percent inhibition was calculated according to the formula: Inhibition percentage = [(Control absorbance –Test absorbance) / Control absorbance] × 100. A dose response curve was plotted to determine the $IC_{50}$ values. $IC_{50}$ is defined as the concentration sufficient to obtain 50 % of a maximum scavenging capacity. All tests were performed in triplicate.

**2.6. Superoxide scavenging activity**

The superoxide anion scavenging capacity of the test extracts was analyzed by estimation of the reduction product of nitroblue tetrazolium (NBT), as described previously (Chang et al., 1996; Furuno et al, 2002). The reaction mixture contained 50 mM $Na_2CO$ buffer, 3 mM xanthine, 3 mM ethylenediamine tetraacetic acid, 0.5 mM NBT and bovine serum albumin solution. Test extracts were added to the reaction mixture and incubated at 25 °C for 10 min. The reaction was started with the addition of xanthine oxidase (XO) (0.25 units/mL). After further incubation at 25 °C for 25 min, absorbance was recorded at 560 nm using an ELISA reader, against blank samples, which did not contain the enzyme. The superoxide production was confirmed by superoxide dismutase, which inhibited the reactions of NBT reduction in a concentration-dependent manner (data not shown). Each experiment was performed at least in triplicate.

**2.7. XO inhibitory activity**

XO activity was measured based on the procedure explained by Nguyen et al. (2004) with minor modifications. Assays were performed in 96-well plates with 115 µL reaction mixtures containing 35 µL of 200 mM phosphate buffer (pH 7.5), 30 µL of XO (0.05 units/mL in 200 mM phosphate buffer), and 50 µL of test plant extracts in deionized water. After preincubation at 25 °C for 15 min, the reaction was started by adding 60 µL of 0.5 mM xanthine in 200 mM phosphate buffer (pH 7.5) to the mixture. The reaction mixture was incubated at 25 °C for 30 min and then the absorption increments at 290 nm, which indicated the formation of uric acid, were determined with an ELISA reader. The control group contained no test agent and allopurinol was used as a reference compound. Three replicates were made for each test sample to calculate $IC_{50}$ values.

**2.8. Determination of the cell viability by using MTT assay**

The human fibroblast cells (HS-68), colon cancer cells (HT-29) and breast cancer cells (MCF-7) were obtained form the Korean Cell Line Bank (Seoul, Korea). HS-68 and MCF-7 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) (GIBCO/BRL, NY, US) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine at 37 ºC with 5% $CO_2$ in a humidified atmosphere. HT-29 cell lines were maintained in RPMI 1640 medium supplemented as above with L-glutamine, antibiotics and 10% heat-inactivated horse serum (Lonza, Walkersville, MD, US).

The inhibitory effect of samples on the proliferation of human normal and cancer cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, HS-68, HT-29 and MCF-7 cells ($2\times10^3$/well) were loaded into 96-well culture plates and treated with fresh medium containing various concentrations (0-1,000 µM) of each extract for 24 h. Cells were washed once with phosphate-buffered saline and reacted with the MTT solution (Boehringer Manheim, Indianapolis, IN) at 37 ºC for 4 h to produce the formazan salt. Finally, the formazan salt formed in each cultured cells was dissolved in DMSO, and the optical density (OD) value of each solution was measured at 540 nm using an ELISA reader (Bio-Tek, Winnoski, VT, US). The OD value detected for the control (cells without treatment with any samples) from the treated cells was plotted on the *x*-axis, designated as proliferation (% control), to demonstrate the effect of each sample on the viability of the related cells.

**2.9. Statistical analysis**

All analyses were replicated three times. Each data presented as means ± standard deviation. The data were statistically analyzed one-way analyses of variance followed by Duncan's multiple range tests (SPSS 12.0). Difference with *p* value less than 0.05 was considered statistically significant. After multiple comparisons, the means in the following table and figures were followed with different small letter "a-d" based on their values and statistical differences. In the case that a mean was followed with "ab", this mean was not significantly different from a mean with "a", and was not significantly different from another mean with "b". However, means with different letters were significantly different at the level of 0.05.

**3. Results and Discussion**

Phenolic compounds, aromatic secondary plant metabolites, which mainly include flavonoids, phenolic acids, stilbenes, courmarins and tannins (Robbins, 2003). In addition to vitamin C and carotenoids, a variety of phenolic compounds are present in fruit and vegetables, and exhibit a wide range of physiological and flavoring properties (Robbins, 2003). Until now, only a few authors have reported the content and composition of phenolic compounds of *Grifola frondosa*: It has been shown to contains high amounts of phenolic compounds (Lee et al., 2008) and 18 phenolic compounds including flavonoids being reported (Lee et al., 2010a). We, therefore, examined the total phenolic content of *Grifola frondosa* mycelial extracts cultivated on citrus waste substrate (Table 1).

**Table 1.** Total phenolic, naringin and hesperidine composition of *Grifola frondosa* mycelial extract cultivated on citrus waste substrate

| Content (mg/g dry matter) | Citrus peel substrate | Premature Hallabong substrate | Citrus peel mushroom | Premature Hallabong mushroom |
|---|---|---|---|---|
| Total Phenolics | 57.0±0.62*,a | 97.7±1.22b | 74.5±1.60c | 126.7±1.07d |
| Naringin | nd** | 2.7±0.05b | nd | 3.3±0.53b |
| Hesperidin | 5.8±0.10a | 13.3±0.07b | 6.2±0.06c | 13.7±0.10d |

*Mean ± S.D. for n=3; **Not detected; a~dValues with different superscripts in a row are significantly different ($p < 0.05$)

The content of total phenols was significantly higher for *Grifola frondosa* mycelial extract cultivated on premature Hallabong substrate (premature Hallabong mushroom,126.7 mg/g) than that for *Grifola frondosa* mycelial extract cultivated

on citrus peel substrate (citrus peel mushroom, 74.5 mg/g) ($p < 0.05$). These contents were 47- to 80-fold higher than previous result reported by other authors, who evaluated content of total phenols was 1.59 mg/g in the extract from *Grifola frondosa* mycelia cultivated on the sawdust (Mau et al., 2004). In addition, mushroom extracts using citrus peel and premature Hallabong as substrate shows more total phenolic contents than those of their substrates (57.0 and 97.7 mg/g), respectively ($p < 0.05$) (Table 1). This indicates that citrus waste substrate may be responsible for the total phenolic content as well as the growth of mushroom.

The extracts of *Grifola frondosa* mycelia cultivated on citrus waste were analyzed by a modified reversed-phase HPLC system to determine the content of naringin and hesperidin, which are two major flavonoids present in citrus fruits (Kanaze et al., 2003; Choi et al., 2007). There was significant variation in the contents of naringin and hesperidin in all mushrooms and their substrates (Table 1). Naringin was detected only in premature Hallabong mushroom (3.3 mg/g) and its substrate (2.7 mg/g), whereas hesperidin was found in the extracts of all mushrooms and their substrates. As expected from the result of total phenolic content, naringin and hesperidin were significantly higher in the extracts of citrus peel and premature Hallabong mushrooms than that in their substrates ($p < 0.05$), with the highest heperidin content found in premature Hallabong mushroom (13.7 mg/g) (Table 1). Recently, Lee *et al.* reported that the naringin content of the extract from *Grifola frondosa* fruit body cultivated on the sawdust was 0.0278 mg/g (Lee et al., 2010a), which is 500-fold lower than those of mushroom cultivated on premature Hallabong fruit drops. This implies that citrus waste substrate might contribute to the increase in the contents of naringin as well as of total phenolics of *Grifola frondosa* mycelia as compared with conventional substrate. Flavonoids have been associated with the health benefits derived from their antioxidant activity (Heim et al., 2002), and could be a natural source of antioxidants as well as a major determinant of antioxidant potentials of foods (Parr and Bolwell, 2000). Therefore, our results may provide important information on utilization of citrus wastes as primary substrate for *Grifola frondosa* cultivation as a medicinal mushroom. Since the key role of phenolic compounds to scavenge free radicals has been emphasized in several reports (Rauha et al., 2000; Archana and Dasgupta, 2005), antioxidant properties of *Grifola frondosa* mycelial extracts cultivated on citrus waste substrates were investigated in the following experiments.

Scavenging activity for free radicals of DPPH has been widely used to evaluate the antioxidant activity of natural products from plant sources (Huang et al., 2005; Zhu et al., 2004). As shown in Figure 1, dose-response curves for the DPPH radical scavenging activity were observed in all mushrooms and substrates. The most potent scavenging DPPH radical activity was observed in premature Hallabong mushroom (0.5 mg/mL $IC_{50}$), which possess significantly higher DPPH free radical scavenging activity than that of citrus peel mushroom (0.9 mg/mL $IC_{50}$) ($p < 0.05$). These $IC_{50}$ values were much lower than that of extracts of *Grifola frondosa* mycelia cultivated on the sawdust (4.95 mg/mL) (Mau et al., 2004; Mau et al., 2002), indicating that mushrooms cultivated on citrus waste substrate were more effective in scavenging effects than mushroom cultivated on conventional substrate. However, citrus peels (1 mg/mL $IC_{50}$) and premature Hallabong fruit drops (0.6 mg/mL $IC_{50}$) showed the similar activity with mushroom extracts using it as a substrate, respectively, even though they showed lower $IC_{50}$ values (Figure 1).

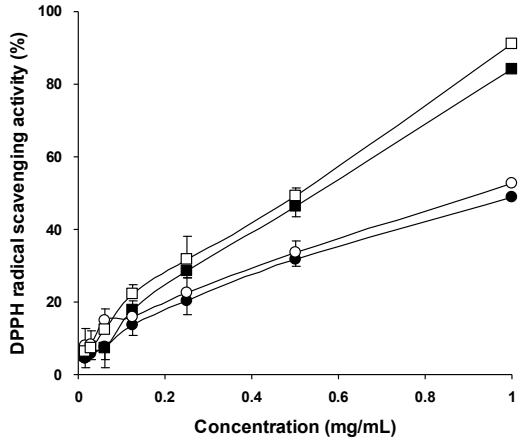
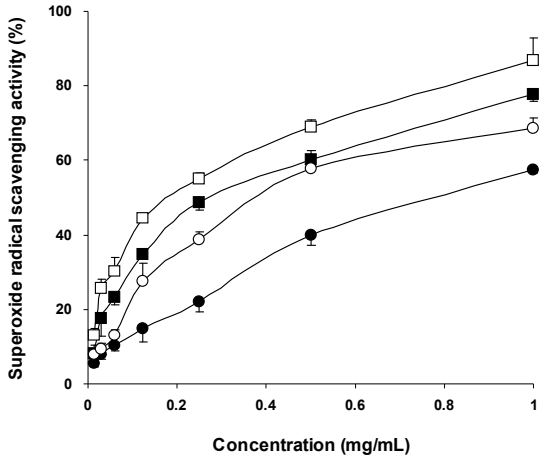



**Fig. 1.** DPPH radical scavenging activity of citrus peel substrate (●), premature Hallabong substrate (■) and the extracts of *Grifola frondosa* mycelia cultivated on citrus peel substrate (○) and premature Hallabong substrate (□). Values are means ± S.D. of three separated experiments. [a~b]Values with different superscripts in a row are significantly different ($p < 0.05$).

| Sample | Citrus peel substrate | Premature Hallabong substrate | Citrus peel mushroom | Premature Hallabong mushroom |
|---|---|---|---|---|
| $IC_{50}$ (mg/mL) | 1.0±0.07[a] | 0.6±0.03[b] | 0.9±0.09[a] | 0.5±0.01[b] |

Reactive oxygen species, including superoxide anions, hydrogen peroxide, hydroxyl radical, nitric oxide and peroxinitrite, play an

important role in oxidative stress related to the pathogenesis of various diseases such as inflammation, heart disease, diabetes, gout and cancer (Slater, 1984). In the present work, the activities were dose-dependent, and more effective antioxidant activity was found in premature Hallabong mushroom extracts (0.4 mg/mL $IC_{50}$) compared with that of citrus peel mushroom extracts (0.6 mg/mL $IC_{50}$), which exhibited superoxide scavenging activity using the NBT dye reduction assay (Figure 2).

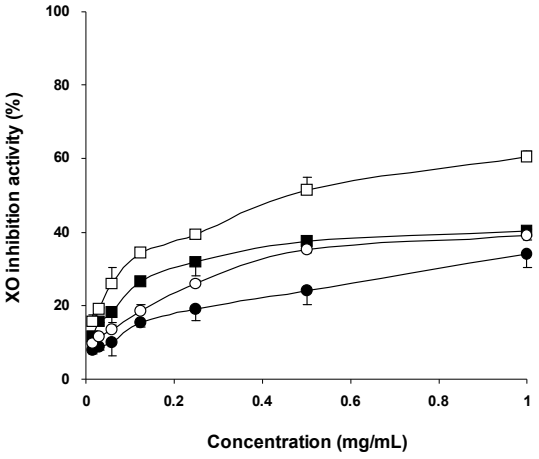



| Sample | Citrus peel substrate | Premature Hallabong substrate | Citrus peel mushroom | Premature Hallabong mushroom |
|---|---|---|---|---|
| $IC_{50}$ (mg/mL) | 0.8±0.02[a] | 0.4±0.08[b] | 0.6±0.03[c] | 0.4±0.02[b] |

**Fig. 2.** Superoxide radical scavenging activity of citrus peel substrate (●), premature Hallabong substrate (■) and the extracts of *Grifola frondosa* mycelia cultivated on citrus peel substrate (○) and premature Hallabong substrate (□). Values are means ± S.D. of three separated experiments. [a~c]Values with different superscripts in a row are significantly different ($p < 0.05$).

When this method is used, the effect of the extract on the XO activity must be checked, because an inhibitory effect on the enzyme itself would also lead to a decrease of NBT reduction (Halliwell et al., 1995). In this regard, we evaluated the effect of *Grifola frondosa* mycelial extracts cultivated on citrus waste substrates on the XO activity by the metabolic conversion of xanthine to uric acid (Figure 3).

Addition of the extracts from all mushrooms and substrates to the reaction mixture (from 0.125 to 2.0 mg/mL) resulted in a dose-dependent inhibition. The premature Hallabong mushroom extracts (0.6 mg/mL $IC_{50}$) showed a statistically higher XO inhibition activity than that of citrus peel mushroom extracts (1.2 mg/mL $IC_{50}$) ($p < 0.05$) (Figure 3). All the extracts from mushroom using citrus peel and premature Hallabong as substrate showed significantly higher ($p < 0.05$) activities in XO inhibition than those of their substrates (Figure 3). Mau et al. (2001) found that the metabolic extracts from mycelia of *Grifola frondosa* cultivated on the sawdust exhibited an $IC_{50}$ values of 3.63, 3.67 and 3.49 mg/mL for antioxidant activity, reducing powder and chelating effect on ferrous ions, respectively, whereas no scavenging effect on hydroxyl radicals. All $IC_{50}$ values in our study were below 1 mg/mL, indicating that utilization of citrus wastes as substrate enhanced antioxidant activity achieved by the scavenging of DPPH radical, superoxide radical, and XO inhibition, possibly because of the correlation between antioxidant activity and the content of total phenolics as mentioned above (Table 1).




| Sample | Citrus peel substrate | Premature Hallabong substrate | Citrus peel mushroom | Premature Hallabong mushroom |
|---|---|---|---|---|
| $IC_{50}$ (mg/mL) | 1.5±0.14[a] | 1.1±0.11[a] | 1.2±0.06[b] | 0.6±0.04[c] |

**Fig. 3.** XOD inhibition activity of citrus peel substrate (●), premature Hallabong substrate (■) and the extracts of *Grifola frondosa* mycelia cultivated on citrus peel substrate (○) and premature Hallabong substrate (□). Values are means ± S.D. of three separated experiments. [a~c]Values with different superscripts in a row are significantly different ($p < 0.05$).

Previous studies demonstrated that the polysaccharide extracted from *Grifola frondosa* strongly enhances anticancer effect, suggesting that some component of *Grifola frondosa* extract may represent potential cancer chemopreventive substances (Adachi et al., 1987; Hishida et al., 1988; Nanba, 1995; Fullerton et al., 2000; Shi et al., 2007; Shomori et al., 2009). Fullerton et al. (2000) reported that a bioactive β-glucan from *Grifola frondosa* has a cytotoxic effect, through oxidative stress, on prostatic cancer PC-3 cells, leading to apoptosis. Furthermore, both water-insoluble polysaccharide and waster-soluble extract from *Grifola frondosa* inhibited the proliferation of human gastric cancer cell lines (SGC-7901, TMK-1, MKN28, MKN45 and MKN74) (Shi et al., 2007; Shomori et al., 2009). However, there are few reports on the effects of *Grifola frondosa* extract on human cancer cell lines. Thus, it is necessary to perform the cell viability assay with *Grifola frondosa* extracts to investigate their antiproliferative effects in other types of cancer cells.

In the present study, colon cancer cells (HT-29) were treated with various concentrations (0-1 mg/mL) of extracts of mushrooms cultivated on citrus waste and their substrates separately for 24 h, and then analyzed by MTT cell viability assay for the cell viability, proliferation (% control), as shown in Figure 4A.

The formazan product of MTT assay was analyzed for quantification of the viability of cells. The extracts of all mushrooms and their substrates were found to exhibits significantly growth inhibitory effect in HT-29 cells, and premature Hallabong mushroom exhibited the most significant antiproliferative effect in HT-29 cells: it led to a maximum decrease of 41% at 1 mg/mL (Figure 4A). The antiproliferative effects of the mushrooms cultivated on citrus waste and their substrates in breast cancer MCF-7 cells were also investigated (Figure 4B). The number of viable cells slightly decreased after treatment with extracts of all mushrooms and their substrates. Premature Hallabong mushroom showed the most antiproliferative effect in MCF-7 cells, and their cell viability decreased by 27% at 1 mg/mL (Figure 4B). Based on the results obtained, the extracts of mushroom cultivated on citrus waste were effective in inhibiting colon (HT-29) and breast (MCF-7) cancer cell proliferations.

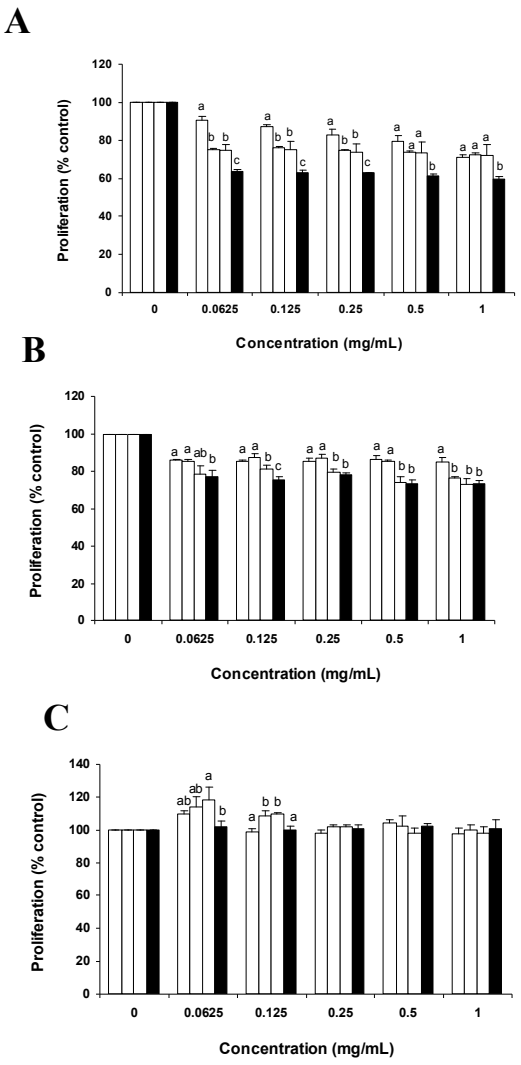



**Fig. 4.** Cell viability of human colon HT-29 cancer cells (A), breast MCF-7 cancer cells (B) and fibroblast HS-68 cells (C). Symbols: □, citrus peel; ▨, premature Hallabong; ▥, *Grifola frondosa* mycelial extract cultivated on citrus peel; ■, *Grifola frondosa* mycelial extract cultivated on premature Hallabong. Values are expressed as percentages compared to the baseline (0 mg/mL) value in the control (cells without treatment with any samples, considered to be 100%). Values are means ± S.D. of three separated experiments. [a~c]Mean values not sharing the same letter above the bars are significantly different at $p < 0.05$ by one-way analyses of variance followed by Duncan's multiple range tests.

For the development of anticancer agents from natural products, it is important not only to screen the selectivity (or specificity) of the natural products among several cancer cells but also to assay if natural products exhibited any cytotoxicity in non-

cancer cells. Thus, the effect of extracts of mushrooms cultivated on citrus waste and their substrates on non-cancer cells (human fibroblast HS-68 cells) was tested. The HS-68 cells did not exhibit cytotoxic effect with any extract, indicating the potential specificity of the mushroom extracts against target cancer cells (Figure 4C).

In conclusion, citrus waste can be utilized as a practical substrate to improve the antioxidant and anticancer activities as well as to increase total phenolic content of *Grifola frondosa* and it, therefore, may be developed as natural antioxidant for food industry and other fields.

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