

# Water extract of brewers' rice induces antiproliferation of human colorectal cancer (HT-29) cell lines via the induction of apoptosis

B.L. TAN<sup>1</sup>, M.E. NORHAIZAN<sup>1,2</sup>, S.K. YEAP<sup>3</sup>, K. ROSELINA<sup>4</sup>

<sup>1</sup>Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

<sup>2</sup>Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

<sup>3</sup>Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

<sup>4</sup>Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

**Abstract. – OBJECTIVE:** Brewers' rice, a mixture of broken rice, rice bran, and rice germ, is a rice by-product in the rice industry. The present study was designed to investigate the *in vitro* cytotoxicity of the water extract of brewers' rice (WBR) against colorectal cancer (HT-29) cells.

**MATERIALS AND METHODS:** The cytotoxicity activity was determined using the lactate dehydrogenase (LDH) assay. The morphological changes of the HT-29 cells were observed using inverted light and fluorescence microscope. Cell cycle and apoptotic cell death analyses were performed using flow cytometer. Besides that, the selected polyphenolic compounds in WBR were also analyzed using ultra performance liquid chromatography (UPLC).

**RESULTS:** The cytotoxicity results showed that WBR was more cytotoxic (but not significantly different) in HT-29 cells compared to the MBR, with  $IC_{50}$  value of  $21.88 \pm 12.43 \mu\text{g/mL}$  and  $34.50 \pm 5.92 \mu\text{g/mL}$  for WBR and MBR, respectively ( $p > 0.05$ ). WBR-treated HT-29 cells displayed the typical characteristics of apoptosis, as visualized using inverted light and fluorescence microscope. WBR also significantly increased the number of early and late apoptotic HT-29 cells compared to control cells ( $p < 0.05$ ). Results from UPLC analysis demonstrated that ferulic acid ( $36.42 \pm 2.97 \mu\text{g/g}$ ) was found the highest level in WBR, followed by gallic acid ( $26.09 \pm 2.01 \mu\text{g/g}$ ) and p-coumaric acid ( $7.13 \pm 0.36 \mu\text{g/g}$ ). These phenolics are speculated to partially contribute to apoptotic cell death.

**CONCLUSIONS:** Our results suggested that WBR derived from natural sources might represent a potential chemopreventive agent against colon cancer.

#### Key Words:

Brewers' rice, Colon cancer, Cytotoxicity, Apoptosis, Polyphenolic compound.

## Abbreviations

AO = acridine orange; BALB/c 3T3 = mouse fibroblast; DMEM = Dulbecco's Modified Eagle Medium; FBS = fetal bovine serum; FITC: Fluorescein isothiocyanate; HT-29 = colorectal cancer; LDH = lactate dehydrogenase; MBR = methanol extract of brewers' rice; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI = propidium iodide; RPMI: Roswell Park Memorial Institute; SD = standard deviation; SPSS = Statistical Package for Social Science; UPLC = ultra performance liquid chromatography; WBR = water extract of brewers' rice.

## Introduction

Colorectal cancer is the second most common cancer after breast cancer and it becomes first among males and second among females in Peninsular Malaysia at 2006<sup>1</sup>. The treatment of cancer is highly expensive and which involves drugs that have adverse side effects or toxicity complications<sup>2</sup>. To overcome existing cancer treatments that have adverse side effects in normal cells, it is necessary to identify a therapy that is directly targeted to the apoptosis machinery of cancer cells without destroying normal cells. Thus, identifying novel or non-toxic anti-cancer agents from natural products that selectively destroy tumor cells or have a high potential to enhance the treatment of colon cancer, particularly for late-stage colon cancer patients, has become a promising approach in cancer control.

Rice has become one of the most important staple foods for approximately half of the world's

population<sup>3</sup>. Rice paddy milling yields nearly 70% of rice (endosperm) as its major product; where there are some of the unconsumed portions of rice by-products, such as brewers' rice, which consists of broken rice, rice bran, and rice germ. Brewers' rice, which is known locally as *temukut*, is usually used as animal feed and brewing adjunct<sup>4</sup>. Rice bran and rice germ, which are present in brewers' rice, have been reported to contain phenolic base compounds and have a high amount of vitamins, minerals, and fiber<sup>5</sup>. The production of brewers' rice in rice milling is explained in more detail in Esa et al<sup>6</sup>. To date, no specific study has demonstrated the *in vitro* cytotoxicity effects of brewers' rice on colorectal cancer (HT-29) cells. Thus, the objective of this study was to determine the *in vitro* cytotoxicity induced by the water extract of brewers' rice (WBR) in colorectal cancer (HT-29) cells.

## Materials and Methods

### Chemicals and Reagents

Reference standards, such as gallic acid, caffeic acid, vanillic acid, syringic acid, p-coumaric acid, and ferulic acid, with purity greater than 95% were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The Lactate Dehydrogenase (LDH) Assay mixture was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), RPMI-1640 medium, Mycoplex<sup>TM</sup> fetal bovine serum (FBS), penicillin and streptomycin (100×), and trypsin EDTA (1×) were purchased from PAA Laboratories GmbH (Pasching, Austria). A Cycle TEST PLUS DNA Reagent Kit and Annexin V-FITC Apoptosis Detection Kit I were purchased from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA). All other chemicals and reagents used were of analytical grade and purchased from Sigma-Aldrich.

### Cell Culture and Treatment

Colorectal cancer (HT-29) and mouse fibroblast (BALB/c 3T3) cell lines from American Type Culture Collection (ATCC; Rockville, MD, USA) were cultured in DMEM and RPMI-1640 medium, respectively, and supplemented with 10% (v/v) FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained and incubated at 37°C in a humidified atmosphere and 5% CO<sub>2</sub> atmosphere. HT-29 cells were treated with different concentrations of WBR (16, 32, and 64 µg/mL) for 72 hours.

### Stabilization of Brewers' Rice

Immediately after reaching the laboratory, 500 g of brewers' rice was stabilized by heating for 2 minutes in a microwave oven at 2450 MHz. The sample was mixed homogeneously and microwaved for an additional 2 minutes. Stabilized samples were cooled at room temperature and stored at -20°C until further analyses.

### Preparation of Water and Methanol Extracts of Brewers' Rice

Stabilized brewers' rice was extracted with water or methanol as previously described by Yu et al<sup>7</sup>.

### Lactate Dehydrogenase Release Assay

Cytotoxicity was measured by the release of lactate dehydrogenase (LDH) using an *in vitro* Toxicology Assay Kit, which was based on lactic dehydrogenase, according to the manufacturer's protocols.

### Morphological Changes

HT-29 cells were treated with different concentrations (16, 32, and 64 µg/mL) of WBR for 72 hours. The morphological changes of the HT-29 cells were observed using an inverted light microscope (Olympus, Center Valley, PA, USA).

### Quantification of Apoptosis Using Acridine Orange (AO) and Propidium Iodide (PI) Double Staining

HT-29 cells were treated with different concentrations (16, 32, and 64 µg/mL) of WBR and incubated for 24, 48, and 72 hours. After incubation, HT-29 cells were stained with 1 mg/mL of AO and PI each, at 1:1 mixture. The glass slide was viewed using a fluorescence microscope (Olympus, Center Valley, PA, USA) at 400× magnification. The percentage of viable, apoptotic, and necrotic cells was determined in more than 200 cells for every sample and expressed as a proportion of the total cell number (%).

### Cell Cycle Distribution Analysis

The Cycle TEST PLUS DNA Reagent Kit was used to detect cell cycle arrest, according to the manufacturer's protocols. Data acquisition and analysis were performed using flow cytometry FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with Cell Quest 3.3 software.

### **Detection of Apoptotic Cell Death Using Annexin V-Propidium Iodide (PI)**

The Annexin V-FITC Apoptosis Detection Kit I was used to detect early and late apoptotic activity, according to the manufacturer's protocols. The fluorescence of the cells was analyzed immediately using flow cytometry FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

### **Identification of Polyphenols by Ultra Performance Liquid Chromatography (UPLC)**

UPLC analysis was performed using the Agilent Technologies 1290 Infinity model G4220A equipped with a diode array detector setup wavelength of 280 nm and 320 nm. Chromatographic separations were performed on a LiChroCART® 250-4, 6 C-18 column (5 µm, 250 mm × 4.6 mm). The mobile phase consisted of solvent (A) water-acetic acid (94:6, v/v, pH 2.27) and solvent (B) acetonitrile. These solvent composition and gradient elution conditions have been previously described by Chirinos et al<sup>8</sup>. The solvent gradient was as follows: 0-15% B for 40 min, 15-45% B for 40 min, and 45-100% B for 10 min. A flow rate of 0.5 mL/min was used and a 20-µL sample was injected. The sample and mobile phase were filtered through a 0.22-µm Millipore filter, type GV prior to UPLC injection.

### **Statistical Analysis**

Statistical analyses were performed according to the Statistical Package for Social Science version 17.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as the mean ± standard deviation (SD). An Independent-Samples *t*-test was used for the comparison of the yield of brewers' rice extracts and LDH between WBR and MBR. The results obtained from other analyses were analyzed using a one-way analysis of variance (ANOVA). A *p*-value < 0.05 was considered significant.

## **Results**

### **Yield of Brewers' Rice Extracts**

The yield of MBR extract (3.214 ± 0.228%) was higher than WBR (2.99 ± 0.001%), but this difference was not significant (*p* > 0.05).

### **Cytotoxicity Activity of WBR and MBR on Colorectal Cancer (HT-29) Cells**

The cytotoxic effect of WBR and MBR ranged from 1.56-200 µg/mL in HT-29 cells and

BALB/c 3T3 cell lines were measured using the LDH release assay. The growth inhibitory activity (IC<sub>50</sub>) from the LDH value of WBR and MBR in HT-29 cells was 21.88 ± 12.43 µg/mL and 34.50 ± 5.92 µg/mL, respectively. However, this difference was not significant (*p* > 0.05). Moreover, no toxicity was found in BALB/c 3T3 cells upon the completion of the incubation as no IC<sub>50</sub> was determined, which indicated that WBR and MBR were not cytotoxic against normal cell lines. Due to this result, the morphological changes, mode of cell death, cell cycle arrest, and apoptosis in response to WBR were further analyzed in HT-29 cells.

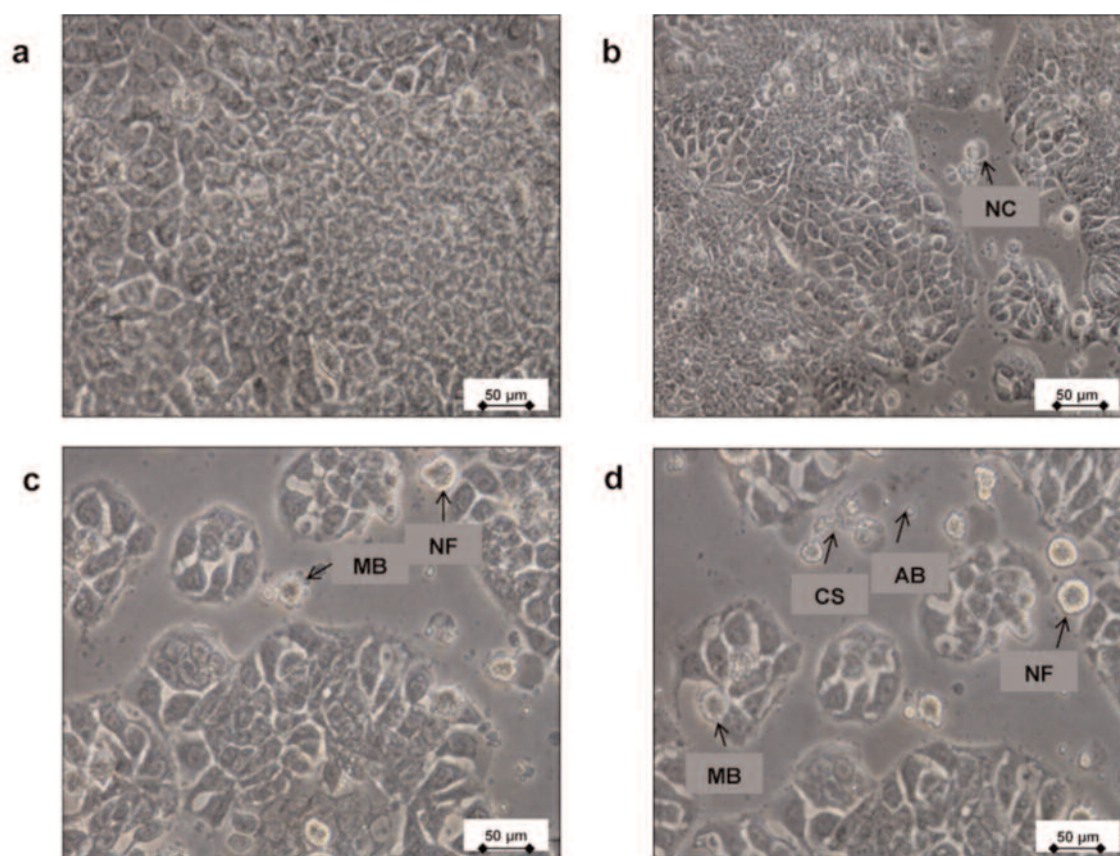
### **Morphological Changes Induced by WBR Treatment in HT-29 Cells**

The most significant characteristic changes of apoptosis included cell shrinkage, nuclear condensation, and membrane blebbing with the formation of apoptotic bodies, as shown in Figure 1 (b-d). Decreased cell viability was also observed with increasing concentrations of WBR as viewed using an inverted light microscope. In contrast, the untreated control cells (Figure 1a) were evenly distributed on the substratum. These findings were further analyzed using fluorescence analysis, where AO/PI fluorescence dye was used to stain the cells.

### **Mode of Cell Death with Treatment of WBR in HT-29 Cells**

As shown in Figure 2b, no prominent changes were observed in HT-29 cells after treatment with 16 µg/mL of WBR for 72 hours. However, HT-29 cells exhibited chromatin condensation, nuclear fragmentation, and nuclear margination after a 72-hour treatment with 32 µg/mL and 64 µg/mL of WBR, which is associated with apoptosis. This was observed by the fluorescent bright-green color (Figure 2c-d). In contrast, untreated HT-29 cells appeared healthy, had a round morphology and similar sizes, and exhibited a green color with an intact nucleus (Figure 2a). Besides apoptotic cells, necrotic cells were also observed in red color (Figure 2c-d).

Data from Figure 3 furthermore, shows that 32 µg/mL of WBR significantly reduced the population of viable HT-29 cells in a time-dependent manner compared to untreated cells (control) (*p* < 0.05). In contrast, the apoptotic cells were significantly increased in a time-dependent manner after treatment with 32 µg/mL of WBR (*p* < 0.05). However, no significant difference was ob-



**Figure 1.** Close-up views of untreated HT-29 cells (**a**) and HT-29 cells treated with water extract of brewers' rice at concentration of 16  $\mu\text{g/mL}$  (**b**), 32  $\mu\text{g/mL}$  (**c**), and 64  $\mu\text{g/mL}$  (**d**) for 72 hours; viewed under an inverted light microscope. The cells showed characteristics of apoptosis, such as nuclear compaction (NC), apoptotic bodies (AB), membrane blebbing (MB), cellular shrinkage (CS), and nuclear fragmentation (NF) (magnification 400 $\times$ ).

served in the necrotic counts between 24 and 48 hours, between 48 and 72 hours ( $p > 0.05$ ) (Figure 3). Thus, further study was performed to determine the potential cell cycle arrest of WBR.

#### **Effect of WBR on Cell Cycle Kinetics**

As shown in Figure 4, untreated HT-29 cells during the exponential growth period were characterized by cell populations in the sub- $G_0$  ( $6.76\% \pm 0.44$ ),  $G_0/G_1$  ( $73.26\% \pm 0.95$ ), S ( $6.46\% \pm 0.08$ ), and  $G_2/M$  ( $13.52\% \pm 0.58$ ) phase of the cell cycle. After 72 hours of incubation with three different concentrations of WBR (16, 32, and 64  $\mu\text{g/mL}$ ), there was a significant increase in the cell population at the sub- $G_0$  phase compared to the control ( $p < 0.05$ ), indicating cell death. Furthermore, the level of cell death was significantly higher when the concentration of WBR increased ( $p < 0.05$ ), indicating that WBR increased the cell population in the sub- $G_0$  phase in a dose-dependent manner. In contrast,

the proportion of cells in the phase of  $G_0/G_1$ , S, and  $G_2/M$  significantly decreased ( $p < 0.05$ ) in a dose-dependent manner compared to control without cell cycle arrest.

#### **Treatment with WBR Induced Apoptosis in HT-29 Cells**

To further investigate the apoptosis-inducing activity of WBR, HT-29 cells were incubated with WBR at different concentrations (16, 32, and 64  $\mu\text{g/mL}$ ) for 72 hours. As shown in Figure 5, WBR significantly increased the number of early and late apoptotic HT-29 cells compared to control cells ( $p < 0.05$ ). Collectively, incubation with WBR resulted in a significant increase in the total number of apoptotic cells, which can be observed after 72 hours, with a maximum effect marked at a WBR concentration of 32  $\mu\text{g/mL}$  ( $p < 0.05$ ).

After 72 hours of incubation with WBR in HT-29 cells, a significant increase in the total number of apoptotic in HT-29 cells was also observed at con-

centrations of 16 and 64  $\mu\text{g/mL}$  ( $p < 0.05$ ) compared to control cells. The anti-proliferative effect of WBR may potentially be due to the presence of bioactive polyphenolic compounds. Thus, the content of polyphenols in WBR was also investigated.

### Bioactive Polyphenolic Constituents

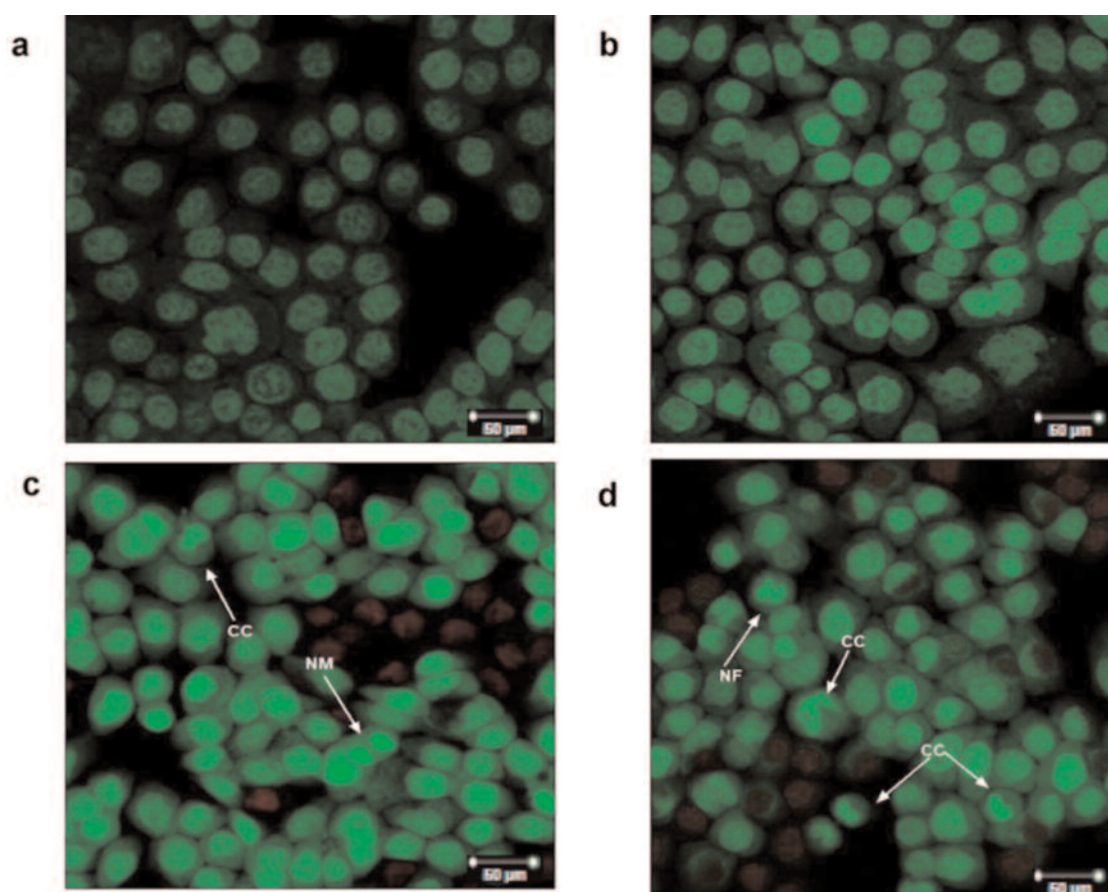
The results showed that WBR contained the highest levels of ferulic acid ( $36.42 \pm 2.97 \mu\text{g/g}$ ), followed by gallic acid ( $26.09 \pm 2.01 \mu\text{g/g}$ ), p-coumaric acid ( $7.13 \pm 0.36 \mu\text{g/g}$ ), syringic acid ( $5.87 \pm 1.71 \mu\text{g/g}$ ), and caffeic acid ( $5.32 \pm 2.48 \mu\text{g/g}$ ). The lowest amount of vanillic acid ( $2.87 \pm 0.15 \mu\text{g/g}$ ) was found in WBR.

### Discussion

In the present study, the yield of MBR extract was higher than WBR was consistent with the re-

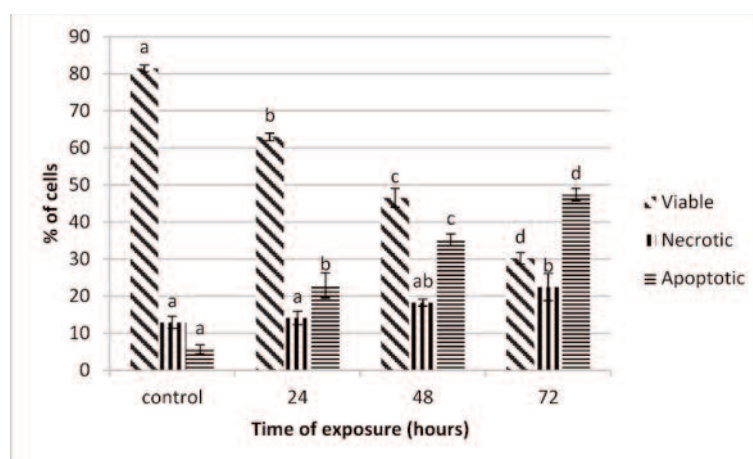
sults obtained by Liu et al<sup>9</sup>, which showed that a high polarity solvent, such as water, does not provide a good yield. Although methanol can produce a higher yield, ethanol and water were selected as the extraction medium because they are safer<sup>10</sup>.

The loss of intracellular LDH in the culture medium indicated that irreversible cell death was caused by damage of the cell membrane<sup>11</sup>. Our previous results<sup>12</sup> using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as a cytotoxicity index showed that the  $\text{IC}_{50}$  of WBR in the MTT assay in HT-29 cells was  $38.33 \pm 6.51 \mu\text{g/mL}$ , whereas MBR was  $54.00 \pm 5.29 \mu\text{g/mL}$ . According to the National Cancer Institute Guidelines (USA), extracts with  $\text{IC}_{50} < 100 \mu\text{g/mL}$  were regarded as anti-proliferative agents<sup>13</sup>. Thus, WBR may potentially be used as an anti-proliferative agent for colon cancer. In addition, given the broad cyto-



**Figure 2.** Morphological characterization of cells undergoing apoptotic cell death using acridine orange and propidium iodide staining. Colorectal cancer (HT-29) cells were either untreated (**a**) or induced with different concentrations of water extract of brewers' rice at 16  $\mu\text{g/mL}$  (**b**), 32  $\mu\text{g/mL}$  (**c**), and 64  $\mu\text{g/mL}$  (**d**) for 72 hours. Treated cells (**c** and **d**) showed the typical characteristics of apoptosis, such as chromatin condensation (CC), nuclear fragmentation (NF), and nuclear margination (NM) (magnification 400 $\times$ ).

**Figure 3.** Percentages of viable, necrotic, and apoptotic cells after treatment with water extract of brewers' rice at a concentration of 32  $\mu\text{g}/\text{mL}$  in HT-29 cells for 24, 48, and 72 hours. The values are expressed as the mean  $\pm$  standard deviation of three determinations.



toxicity range between the MTT and LDH assay in WBR against HT-29 cell lines, only three concentrations (16, 32, and 64  $\mu\text{g}/\text{mL}$ ) were selected.

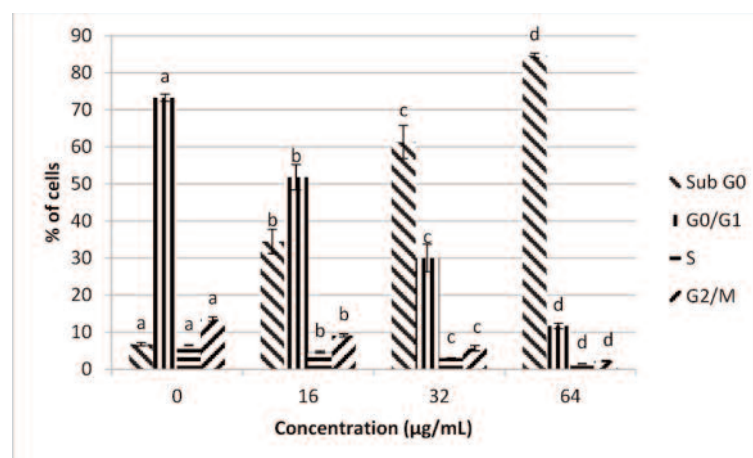
The most prominent changes of apoptosis such as cell shrinkage, nuclear condensation, and membrane blebbing with the formation of apoptotic bodies, which are packed densely of cellular organelles and nuclear fragments<sup>14,15</sup>. Moreover, induction of apoptosis was more favorable than necrosis because it did not stimulate inflammatory responses<sup>16</sup>.

Cell cycle analysis was performed to confirm whether the WBR effect was mediated by an alteration of a specific phase in cell cycle progression. The cells accumulated at the sub-G<sub>1</sub> phase due to the cleavage of nuclear DNA into multiple fragments<sup>17</sup>, which indicate induced cell death via apoptosis<sup>18</sup>. The percentage of total apoptotic cells was more pronounced compared to necrotic cells in HT-29 cells treated with WBR (< 6%). This in-

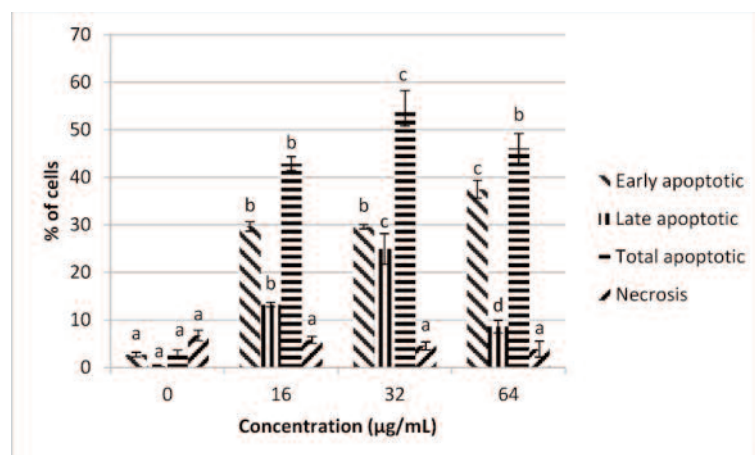
dicated that WBR might be used as a therapeutic agent for human colorectal cancer. In the present study, a majority of HT-29 cells treated with WBR died via induction of the apoptotic pathway, and only a small number of cells died via the necrotic pathway. Thus, it can be suggested that WBR markedly induced apoptosis, but to a much lesser extent, to cause necrotic cell death. The development of a benign to malignant phenotype is strongly associated with apoptosis and the related signaling pathways and cellular events that control this process. Thus, apoptosis is predominantly targeted for therapy in different malignancies, such as colon cancer<sup>19</sup>. Our results provide strong evidence that apoptotic cell death is induced via WBR in colorectal cancer (HT-29) cells.

The major phenolic acids found in whole grains include ferulic acid, p-coumaric acid, vanillic acid, caffeic acid, and syringic acid<sup>20</sup>. In the present study, WBR contained the highest lev-

**Figure 4.** Cell cycle kinetics of water extract of brewers' rice treated HT-29 cells at different concentrations (16, 32, and 64  $\mu\text{g}/\text{mL}$ ). After 72 hours of exposure to the water extract of brewers' rice, the cell cycle kinetics were analyzed using flow cytometry. The values are expressed as the mean  $\pm$  standard deviation of three determinations, and values with different superscript letters demonstrate a significant difference as assessed using the Tukey test ( $p < 0.05$ ).



**Figure 5.** Apoptotic cell death of HT-29 cells treated with water extract of brewers' rice at different concentrations (16, 32, and 64  $\mu\text{g/mL}$ ). After 72 hours of exposure to the water extract of brewers' rice, apoptosis was evaluated using the Annexin assay. The values are expressed as the mean  $\pm$  standard deviation of three determinations, and values with different superscript letters demonstrate a significant difference as assessed using the Tukey test ( $p < 0.05$ ).



els of ferulic acid was consistent with a study reported by Huang and Ng<sup>21</sup>, who found that rice bran rich in ferulic acid in which the ferulic acid contents in the rice bran of different commercial rice varieties from Taiwan were reported to be in the range of 7.32-68.0  $\mu\text{g/g}$ . This result was further supported by Zhou et al<sup>22</sup>, who reported that ferulic acid was abundantly present in rice grains. The amount of ferulic acid in brewers' rice demonstrated in our present study was even higher compared to several types of brown rice from Taiwan, which ranged from 1.62-15.14  $\mu\text{g/g}$ <sup>21</sup>.

Previous studies have demonstrated that the biological activities in cereal grains are strongly correlated with their polyphenol content<sup>23</sup>, which is known to exhibit potent antioxidant activities<sup>24</sup>. Polyphenolic compounds have also been associated with the scavenging of free radicals and the enhancement of immune systems, which can reduce the risk to develop cancer<sup>25</sup>, can affect the process of carcinogenesis by counteracting the occurrence of oxidative stress<sup>26</sup>, and can prevent the onset and development of cancer<sup>27</sup>. Thus, polyphenolic compounds found in brewers' rice are speculated to be partially responsible for apoptotic cell death in addition to other active constituents, such as vitamin E, oryzanol, and phytic acid, which were reported in our previous study<sup>12</sup>.

## Conclusions

WBR is cytotoxic in HT-29 cells via the induction of apoptosis. This study provided evidence that WBR fulfills two basic criteria as an anti-cancer agent, including tumor specificity and minimal toxicity against normal cells. Our results sug-

gest that WBR originating from natural sources may become a potential chemopreventive agent against colon cancer. However, further studies are required to elucidate the molecular mechanisms underlying the WBR-mediated induction of apoptotic cell death in colon cancer cells.

## Acknowledgements

We thank BERNAS, Seri Tiram Jaya, Selangor, Malaysia, for supplying the brewers' rice sample. We also acknowledge the Ministry of Education, Malaysia, for providing research funding (RUGS-2 project no. 05-02-12-1862RU).

## Conflict of Interest

The authors declare that they have no conflict of interests.

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