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ORIGINAL ARTICLE

Neuroprotective Effect of Rice and Corn Extracts Against H_2O_2-Induced Neurotoxicity in HT22 Murine Hippocampal Neuronal Cells

CHUMPIYA S[1], PLAINGARM W[1], TENCOMNAO T[2]

[1]M.Sc. Program in Clinical Biochemistry and Molecular Medicine, [2]Ph.D. Program in Clinical Biochemistry and Molecular Medicine, [3]Center for Excellence in Omics-Nano Medical Technology Development Project, Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, 154 Rama I Road, Pathumwan, Bangkok 10330, Thailand.

ABSTRAK

Kerosakan neuron aruhan stress oksidatif yang disebabkan oleh akumulasi hydrogen peroksida (H_2O_2) dalam sel telah ditunjukkan terlibat dalam penyakit neurodegeneratif. Beras dan jagung boleh menjadi sumber anti-oksidan dengan kehadiran fitokimia seperti melatonin dan triptofan untuk melawan sepsis reaktif oksigen (ROS). Melatonin telah diketahui boleh meningkatkan ekspresi gen brain-derived neurotrophic factor (BDNF) untuk merangsang fungsi sel saraf dan mengawal-atur kesan anti-penuaan oleh sel otak. Kajian ini bertujuan untuk menentukan kesan beras putih, beras perang, pulut hitam, jagung manis dan 'baby corn' terhadap neurotoksisiti aruhan H_2O_2 dan mekanisme terlibat dalam sel hipokampus HT22 mencit. Selepas sel HT22 menerima prarawatan ekstrak individu selama 24 jam dan seterusnya menerima cabaran dengan H_2O_2 selama 24 jam, kebolehhidupan sel, apoptosis, ROS intrasel dan ekspresi gen BDNF diukur. Ekstrak beras dan jagung dinilai untuk aktiviti anti-oksidan dan aras triptofan dan melatonin. Ciri-ciri neurotoksisiti aruhan H_2O_2 termasuk penurunan kebolehhidupan sel, peningkatan selular ROS dan penurunan ekspresi BDNF. Pra-rawatan dengan ekstrak beras dan jagung mengurangkan neurotoksisiti aruhan H_2O_2 secara signifikan, dan juga mengurangkan pengeluaran ROS secara signifikan. Pra-rawatan dengan ekstrak-ekstrak tersebut berupaya juga untuk meningkatkan ekspresi mRNA dan protein BDNF yang mungkin disumbang oleh kandungan melatonin dan triptofan. Oleh itu, ekstrak beras dan jagung mungkin melindungi sel HT22 dari neurotoksisiti aruhan H2O2 dengan merencat pengeluaran ROS dan modulasi ekspresi BDNF.

Kata kunci: jagung, HT22, sel neuron hipokampus murin, hidrogen peroksida, neurotoksisiti, kesan perlindungan neuro, beras

Address for correspondence and reprint requests: Tewin Tencomnao. Center for Excellence in Omics-Nano Medical Technology Development Project, Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, 154 Rama I Road, Pathumwan, Bangkok 10330, Thailand. Tel: +6622181081 ext. 313 Fax: +6622181082 Email: tewin.t@chula.ac.th

ABSTRACT

Oxidative stress-induced neuronal damage, mediated by the cellular accumulation of hydrogen peroxide (H_2O_2), was reported to be involved in neurodegenerative diseases. Rice and corn may serve as sources of antioxidants with the presence of phytochemicals such as melatonin and tryptophan, for fighting against reactive oxygen species (ROS). Melatonin are known to upregulate the expression of brain-derived neurotrophic factor (BDNF) gene for enhancement of the nerve cell function and mediation of the anti-aging effect of the brain cells. The present study aimed at determining the effect of white rice, brown rice, black glutinous rice, sweet corn and baby corn extracts against H_2O_2-induced neurotoxicity and their underlying mechanisms in the mouse hippocampal HT22 cells. Following pretreatment of the HT22 cells with individual extract for 24 hrs and subsequent challenge with H_2O_2 for 24 hrs, cell viability, apoptosis, intracellular ROS and BDNF gene expression were measured. Rice and corn extracts were evaluated for their antioxidant activities and the levels of tryptophan and melatonin. Characteristics of H_2O_2-induced neurotoxicity included decreased cell viability, increased cellular ROS and decreased BDNF expression. Pretreatment with rice and corn extracts significantly attenuated H_2O_2-induced neurotoxicity, and also significantly decreased ROS production. Pretreatment with these extracts could also upregulate the expression of BDNF mRNA and protein, which may be contributed by their melatonin and tryptophan contents. Therefore, rice and corn extracts may protect HT22 cells against H_2O_2-induced neurotoxicity by inhibition of ROS production and modulation of BDNF expression.

Keywords: corn, HT22, murine hippocampal neuronal cells, hydrogen peroxide, neurotoxicity, neuroprotective effect, rice

INTRODUCTION

Neurodegenerative diseases are diseases caused by the loss of neuronal function in the brain. If the loss due to cell damage, the brain region cannot function properly and this will result in reduced brain volume. The contributing risk factors include both genetic and environmental impacts. In particular, environmental factors such as toxic exposure, stress and unhealthy food were recognized to play a crucial role in modifying the risk of neurodegenerative diseases. Several known neurodegenerative diseases are Alzheimer's, Parkinson's, encephalitis, epilepsy, genetic brain disorders, stroke, multiple sclerosis, Huntington's and depression (Prilipko et al. 2004). Moreover, oxidative stress induced neuronal cell damage was reported to be involved in neurodegenerative diseases such as Alzheimer's disease and depression (Kim et al. 2015; Xie & Chen 2016). The damage is mediated by the accumulation of free radicals include reactive oxygen species (ROS), mainly superoxide anion (O_2^-) and

hydrogen peroxide (H_2O_2) in cells. However, several studies showed that antioxidants can inhibit ROS (Andersen 2004; Fatokun et al. 2008).

Presently, there are no ideal drugs for neurodegenerative diseases. The desired drugs should possess high efficacy, no side effects and low cost. Development of preventive and therapeutic approaches for neurodegenerative diseases should be ongoing. According to previous studies, rice and corn, our fundamental foods, are beneficial, not only as our main carbohydrate source, but also preventive and therapeutic effect with respect to neurodegenerative effects (Torre et al. 2008; Ismail et al. 2012). This might be due to the chemical known as melatonin (N-acetyl-5-methoxytryptamine) found in rice and corn since melatonin can function as an antioxidant (Mamiya et al. 2007). Melatonin was also shown to help reducing sleeplessness in depressive patients (Lewy et al. 1998; Trotti & Karroum 2016). Furthermore, it was demonstrated to exhibit the favorable effects for patients with Alzheimer's (Ng et al. 2010) and Parkinson's diseases (Miller et al. 1996; Breen & Barker 2016).

Melatonin is a chemical compound derived from the metabolism of L-tryptophan (Cardinali & Pévet 1998). Melatonin is not only found in mammals, but also in various herbs (Hattori et al. 1995; Dubbels et al. 1995). Rice and corn are considered to be the main agricutural product cultivated in Thailand, and they are reported to contain melatonin (Hernandez-Ruiz & Arnao 2008). Regarding its molecular meachanism of action, several lines of evidence showed that melatonin exterted its effects by upregulating the expression of brain-derived neurotrophic factor (BDNF) gene (Imbesi & Manev 2008; Imbesi et al. 2008; Zhang et al. 2013). This particular molecule mediates its effects by enhancing both the function of the nerve cells and the anti-aging activity of the brain cells via kinase signaling pathway (Mizuno et al. 2003). In the present study, we examined the effect of white rice, brown rice, black glutinous rice, sweet corn and baby corn extracts against H_2O_2-induced neurotoxicity and their underlying mechanisms in the mouse hippocampal HT22 cells.

MATERIALS AND METHODS

CHEMICALS

Chemicals such as 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium persulfate, quercetin, Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Other chemicals such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Bio basic (Toronto, Canada). Ascorbic acid (vitamin C), dimethyl sulfoxide (DMSO) and Hydrogenperoxide (H_2O_2) were purchased from Merck (Darmstadt, Germany). Methanol, ethanol, hexane were purchased from RCI Labscan (Bangkok, Thailand). Penicillin-streptomycin solution was obtained from Corning Inc. (Corning,

Table 1 : Details of five Thai plants in this study

Scientific name	Common name	Source	Part used	Herbarium number
Oryza sativa L.	White glutinous rice (RD6)	Market Chiangrai	Seed	HN : A013730 (BCU)
Oryza sativa L.	Brown glutinous rice (RD6)	Market Chiangrai	Seed	HN : A013730 (BCU)
Oryza sativa L.	Black glutinous rice (RD6)	Market Chiangrai	Seed	HN : A013731 (BCU)
Zea mays var.rugosa	Sweet corn (Star plus)	Market Chiangrai	Seed	HN : A015135 (BCU)
Zea mays var.rugosa	Baby corn (Star plus)	Market Chiangrai	Seed	HN : A015135 (BCU)

NY, USA). Annexin V FITC and PI kit were purchased from Biolegend (San Diego, CA, USA) and H_2DCF-DA was purchased from Life technology (Carlsbad, CA, USA).

CELL CULTURE

The HT22 cells (a generous gift from Prof. Dr. David Schubert at the Salk Institute, San Diego, CA, USA) were cultured in DMEM supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin in a 5% CO_2 humidified incubator at 37˚C. The cells were passaged by trypsinization once they reached approximately 80% confluence.

COLLECTION OF RICE AND CORN SAMPLES

Five types of Thai plants (rice and corn samples) in this study were collected from the market at Chiangrai, the Northern Province, Thailand. All of them were identified and deposited at the Prof. Dr. Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. Their details such as scientific names, parts used and herbarium numbers for this study were presented in Table 1.

PREPARATION OF RICE AND CORN EXTRACTS

All glutinous rice and corns were ground with a mortar and pestle. Ground samples were add with solvent water, ethanol and hexane (ratio 1:10). Samples with water were extracted by heating up. Briefly, each sample was mixed with distilled water and then kept in water bath at 70°C for 30 mins. The supernatants were collected, passed through Whattman no. 1 filter paper, and then dried using Moduly OD freeze Dryer. For samples extracted using ethanol and hexane, they were extracted by maceration. Briefly, samples were shaking in the incubator shaker at 25 °C and 48 hrs. The supernatant was filtered through Whattman no. 1 filter paper, and then pooled and dried using rotatory evaporator to concentrate the extract. Finally, all of the plant extracts were dissolved in DMSO in the concentration of 100 mg/mL as stocks,

and stored with protection from light at -20 °C until use.

MEASUREMENT OF CELL VIABILITY USING MTT ASSAY

To determine the ability of rice and corn extracts to protect HT22 cells from H_2O_2, MTT assay was performed. Briefly, HT22 cells were seeded into 96-well culture plates at density of 1 x 10^4 cells/mL and were allowed to attach. After 24 hrs, the cells were treated with rice and corn extracts individually in a concentration range of 1.56–100 µg/mL for 24 hrs. Similarly, cells were treated with H_2O_2 in a concentration range of 31–2000 µM up to 24 hrs. For the determination of neuroprotective effect, cells were pretreated each rice and corn extract diluted in medium for 24 hrs and then challenged with H_2O_2 for another 24 hrs. Rice and corn extracts were dissolved in DMSO which was maintained at 0.1% as this concentration showed no toxicity to the cells. MTT was added to all wells and allowed to incubate in dark at 37˚C for 4 hrs. The amount of MTT formazan product was determined by measuring absorbance at 550 nm using a microplate reader. The results were expressed as % cell survival, assuming as 100% the absorbance in control untreated cells. All the MTT assays were performed in triplicate.

ASSESSMENT OF APOPTOSIS USING FLOW CYTOMETRY

To detect the effects of rice and corn extracts on number of apoptotic cells induced by H_2O_2. The HT22 cells were stained with FITC-conjugated Annexin V and propidium iodide. Briefly, HT22 cells were seeded in 6-well plates at density of 2 x 10^5 cells/mL. The cells were pretreated with rice and corn extracts with water, ethanol and hexane at concentration 100 µg/mL for 24 hrs before being exposed to 250 µM H_2O_2 for another 24 hrs. The cells were trypsinized and cell pellets were resuspended in ice-cold 1 x binding buffer. Annexin V-FITC solution 5 µL and propidium iodide 10 µL were added to 100 µL of cells suspension. The tube was incubated on ice for 15 mins in the dark followed by addition of 400 µL ice-cold 1 x binding buffer and mixing gently. The samples were analyzed using flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA).

MEASUREMENT OF FREE RADICAL SCAVENGING USING 1,1-DIPHENYL-2-PICRYLHYDRAZYL (DPPH ASSAY)

To investigate the antioxidant property of rice and corn extracts. Generating the stable free radical DPPH (DPPH·), DPPH was dissolved in absolute ethanol. DPPH solution was prepared daily for fresh, and absorbance of the DPPH solution was measured at 517 nm. Then, 20 µL of the extracts (1 mg/mL) or Ascorbic acid (serving as a standard) (125 – 1 µg/mL) was mixed with 180 µL of DPPH solution and incubated for 30 mins in the dark at room temperature. The absorbance of the reaction mixture was measured at a wavelength of 517 nm. The results are expressed as mg ascorbic acid equivalent per g fresh weight of sample.

The % scavenging activity (% SC) was calculated using the following formula:

% SC = {[Abs. control - (Abs. sample - Abs. blank sample)]/Abs. control} * 100

Control included 180 µL of DPPH solution and 20 µL of absolute ethanol; whereas, blank sample included 180 µL of absolute ethanol and 20 µL of extracts.

MEASUREMENT OF FREE RADICAL SCAVENGING USING 2,2-AZINOBIS (3-ETHYLBENZOTHIAZOLINE-6-SULFONIC ACID) RADICAL (ABTS ASSAY)

To investigate the antioxidant property of rice and corn extracts. The working solution or ABTS·+ solution was freshly prepared by mixing 7 mM ABTS stock solution and 2.45 mM $K_2S_2O_8$ at ratio 8:12, and was subsequently incubated for 16–18 hrs at room temperature in the dark. Thereafter, ABTS·+ solution was diluted by mixing with absolute ethanol at ratio about 1:20 to obtain an absorbance of 0.700 ± 0.020 at 734 nm. Then, 20 µL of the extracts (1 mg/mL) or ascorbic acid (serving as a standard) (60–1 µg/mL) was mixed with 180 µL of ABTS·+ solution for 45 mins in the dark. The absorbance was measured at a wavelength of 734 nm. The results are expressed as mg ascorbic acid (vitamin C) equivalent per g fresh weight of sample. The % SC was calculated using the following formula:

% SC = {[Abs. control - (Abs. sample - Abs. blank sample)]/Abs. control} * 100

Control included 180 µL of ABTS·+ solution and 20 µL of absolute ethanol; whereas, blank sample included 180 µL of absolute ethanol and 20 µL of extracts.

DETERMINATION OF TRYPTOPHAN AND MELATONIN CONTENT IN RICE AND CORN EXTRACTS BY HPLC

To determine tryptophan and melatonin content in rice and corn extracts. A 20 µL samples were injected into an Eclipse XDB C18 column (15 cm x 45; 5 µm) No. 1. A gradient elution program was used with two mobile phases: A (1% acetic acid in water) and B (absolute methanol). The applied gradient was as follows: (time, solvent B): 0.1 mins, 10% ; 15 mins, 40% ; 20-30 mins, 70% ; 32-50 mins, 10%. The flow rate of tryptophan and melatonin were fixed at 1 mL/min. The contents of tryptophan and melatonin were detected using DAD UV detector at 280 nm.

MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS) USING FLOW CYTOMETRY

To determine the level of intracellular ROS, HT22 cells were seeded in 6-well plates at density of 2 x 10^5 cells/mL. The cells were pretreated with black glutinous rice extract with ethanol, baby corn extract with water and baby corn extract with ethanol 100 µg/mL for 48 hrs before being exposed to 250 µM H_2O_2 and H_2DCF-DA for another 30 mins at 37˚C under 5% CO_2. The cells were subsequently harvested and washed with phosphate-buffered

saline (PBS). Fluorescence intensity was measured using flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA), with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

MEASUREMENT OF BDNF MRNA EXPRESSION BY REAL-TIME PCR

To investigate the mRNA level expression of BDNF mRNA, total RNA was isolated with Trizol (Invitrogen, Carlsbad,CA). An amount of 1 μg of RNA were reverse transcribed using Super-Script™ II Reverse Transcriptase (Invitrogen) and 0.5 μg OligodT (Bio basic, Toronto, Canada) in a 20 μl final volume. Real-time PCR was carried out using the Exicycler Real Time Quantitative Thermal Block (Bioneer, Daedeok-gu, Korea) and SYBR Green was used for the detection of double-strand DNA. The PCR reaction was set up into microtubes in a volume of 25 μl with 1 μl of cDNA and 24 μl of Master mix SYBR Green I (Bioneer). For BDNF quantitation, fwd AACCATAAGGACGCGGACTTG and rev TTGACTGCTGAGCATCACCC primers were used, while for β-actin quantitation, used as internal control, fwd GGCTGTATTCCCCTCCATCG and rev CCAGTTGGTAACAATGCCATGT primers were used. The PCR program included an initial denaturation step during 10 mins at 95°C, followed by 35 cycles of a 15 s melting step at 95°C, a 15 s annealing step at 57°C, and a 30 s elongation step at 72°C. At the end of each cycle, the fluorescence emitted by SYBR Green was measured. At the end of PCR reaction, samples were subjected to a temperature ramp (from 60°C to 94°C, 1°C/s) with continuous fluorescence monitoring for melting curve analysis. For each PCR product, a single narrow peak was obtained by melting curve analysis at the specific temperature. The analysis was performed with Light Cycler Relative Quantification Software. Samples containing no template were used as negative controls in each experiment.

MEASUREMENT OF BDNF PROTEIN EXPRESSION BY WESTERN BLOT ANALYSIS

To investigate the protein level expression of BDNF protein, HT22 cells were seeded in 6-well plates at density of 2 x 10^5 cells/mL. The cells were pretreated with black glutinous rice extract with ethanol, baby corn extract with water and baby corn extract with ethanol 100 μg/mL for 24 hrs before being exposed to 250 μM H_2O_2 for another 24 hrs. The cells were lysed with lysis buffer (50 mM Tris [pH8.0], 150mM NaCl, 1% NP40, 1mM PMSF, 1mM DTT). The total protein concentrations of the lysates were determined using the Bradford protein assay and proteins (20 μg) were loaded on 12% Sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (SDS-PAGE gels), transferred onto polyvinylidene fluoride (PVDF) membranes (Biorad, Hercules, CA, USA) and incubated overnight with primary and 1 hr with appropriate secondary antibodies. Primary antibodies were diluted as follows: rabbit anti-β actin (Cell signalling, Danvers, Massachusetts, USA, #4967) 1:16,000; rabbit anti-BDNF (Abcam, Cambridge, UK, ab72439) 1:1,000. Secondary horseradish peroxidase-

Table 2 : Extraction yield (%) of five Thai plants extracted successively in water, ethanol and hexane

Types of plant	% Yield of plant extractions (w/w)		
	Water	Ethanol	Hexane
White glutinous rice	7.63	0.17	0.63
Brown glutinous rice	6.40	3.36	3.95
Black glutinous rice	12.87	2.58	2.99
Sweet corn	7.53	7.08	4.57
Baby corn	18.23	9.59	4.82

conjugated antibodies (Cell signalling, Danvers, Massachusetts, USA, #7074) were diluted 1:16,000. The expression of β-actin, a housekeeping gene, was used for normalization. Western blotting data were reproduced three times independently. Intensity of the bands was estimated using the Quantity One software (Biorad).

STATISTICAL ANALYSIS

All experiments were performed independently at least three times and in triplicates or quadruplicates as indicated. Data was expressed as the mean ± SE of the mean (SEM). The statistical probability for correlation coefficients was calculated using Statistical Package for Social Science (SPSS) version 17 (IBM Corporation, Armonk, NY, USA). Statistical analysis was conducted by one-way ANOVA and Student's *t*-test. $P < 0.05$ was considered as significant.

RESULTS

EXTRACTION YIELD OF RICE AND CORN EXTRACTS

The percentage yield of all five Thai plants that were successively extracted with water, ethanol and hexane ranged from 0.17% to 18.23%. Furthermore, the highest percentage yields were obtained from water fractions in Table 2.

EFFECT OF RICE AND CORN EXTRACTS ON CELL VIABILITY INDUCED BY H_2O_2 IN HT-22 CELLS

We investigated the effects of white rice, brown rice, black rice, sweet corn and baby corn on H_2O_2-induced cell death in HT-22 cells. HT-22 cells exposed to H_2O_2 in the absence and presence of pretreated with white rice, brown rice, black rice, sweet corn and baby corn was evaluated using MTT assay. The results showed that exposure of HT-22 cells to white rice, brown rice, black rice, sweet corn and baby corn extract with water, ethanol and hexane separately up to 24 hrs over concentration range of 1.56-100 g/mL produced no alteration in cell viability as compared to untreated control. So, all of these plant extracts showed no toxicity to HT-22 cells (Figure 1A), (Figure 1B) and (Figure 1C).

On the other hand, exposure of cells to 250 μM H_2O_2 for 24 hrs resulted in approximately 60% cell cytotoxicity in

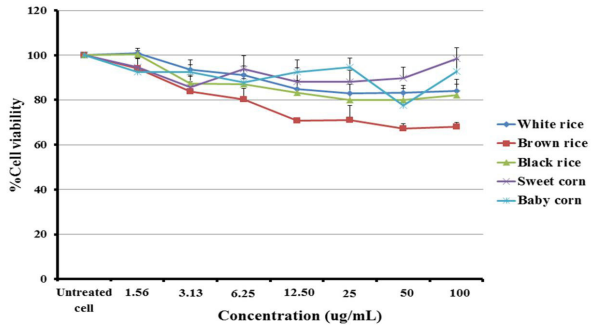

Figure 1A: Effect of white rice, brown rice, black rice, sweet corn and baby corn extract with water on cell viability in HT-22 cells.

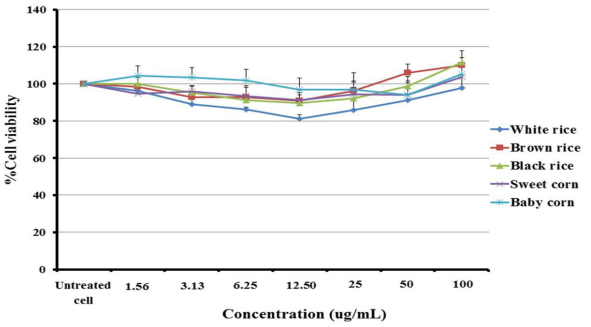

Figure 1B: Effect of white rice, brown rice, black rice, sweet corn and baby corn extract with ethanol on cell viability in HT-22 cells.

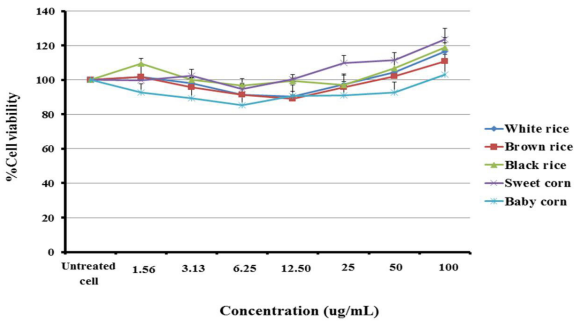

Figure 1C : Effect of white rice, brown rice, black rice, sweet corn and baby corn extract with hexane on cell viability in HT-22 cells.

comparison to control cells (Figure 2). Therefore, 250 μM H_2O_2 was chosen for incubation of HT-22 cells for 24 hrs to induced cell death in all subsequent experiments.

In contrast, pre-treatment with Rice and Corn extract with water, ethanol and hexane after that exposure to 250 μM H_2O_2, the result showed that the protective effect of white rice, brown

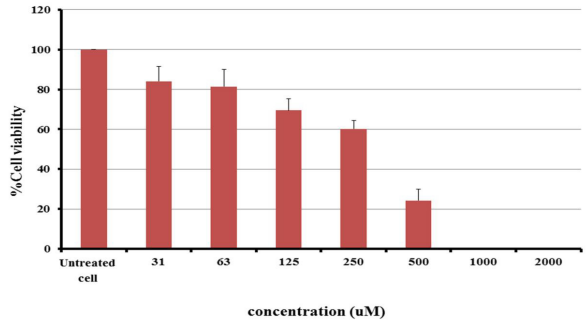

Figure 2: Exposure of HT-22 cells to H2O2 over concentration range 31-2000 M for 24 h was evaluated using MTT assay.

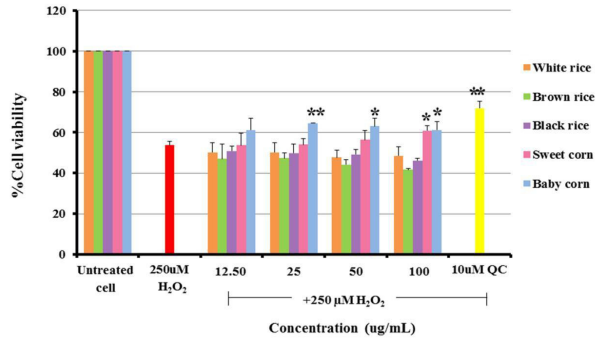

Figure 3A: Protective effect of white rice, brown rice, black rice, sweet corn and baby corn extract with water against 250 M H2O2. The significance is between the indicated group and the group treated with hydrogen peroxide only. (* = $p < 0.05$) and (** = $p < 0.01$)

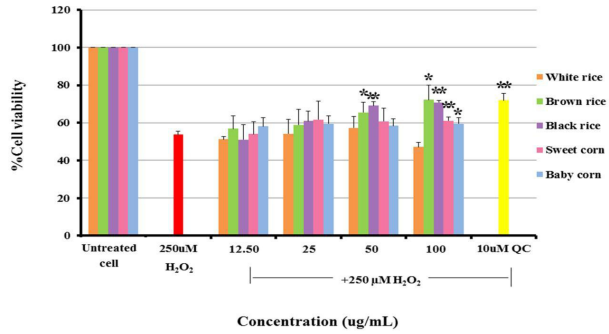

Figure 3B: Protective effect of white rice, brown rice, black rice, Sweet corn and Baby corn extract with ethanol against 250 M H2O2. The significance is between the indicated group and the group treated with hydrogen peroxide only. (* = $p < 0.05$) and (** = $p < 0.01$)

rice and black rice extract with water were no significant, but baby corn extracts with water at 25, 50, 100 µg/mL and sweet corn extracts with water at 100 µg/mL were significantly ($p < 0.05$) increased the viability of HT-22 cells against H_2O_2-induced cytotoxicity (Figure 3A). In addition, exposure of HT-22 cells to white rice extracts with ethanol were no significant, while brown rice

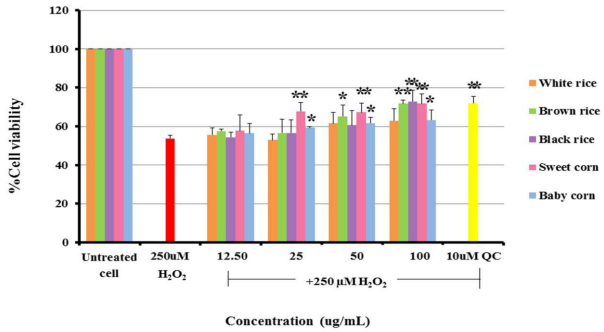

Figure 3C: Protective effect of white rice, brown rice, black rice, sweet corn and baby corn extract with hexane gainst 250 µM H_2O_2. The significance is between the indicated group and the group treated with hydrogen peroxide only. (* = $p < 0.05$) and (** = $p < 0.01$)

and black rice extract with ethanol at concentration 50 and 100 µg/mL, sweet corn and baby corn at concentration 100 µg/mL were significantly ($p < 0.05$) increased the viability of HT-22 cells against H_2O_2-induced cytotoxicity (Figure 3B). Furthermore, exposure of HT-22 cells to white rice extracts with hexane were no significant, however sweet corn and baby corn extract with hexane at concentration 25, 50 and 100 µg/mL and brown rice extracts with hexane at concentration 50, 100 µg/mL and black rice extracts with hexane at concentration 100 µg/mL were significantly ($p < 0.05$) increased the viability of HT-22 cells against H_2O_2-induced cytotoxicity. The result show that brown rice, black rice, sweet corn and baby corn extract with hexane (Figure 3C) were able to neutralize the effect of 250 µM H_2O_2. Quercetin (10 µM) was used as sample control.

EFFECT OF RICE AND CORN EXTRACTS ON H_2O_2 INDUCED APOPTOSIS IN HT22 CELLS

In this study, we evaluated the protective effect of rice and corn extracts on apoptosis of the HT-22 cells using flow cytometry with Annexin V-FITC/PI double staining. The H_2O_2-treated cells (Pos) significantly increased the number of the apoptotic cells (Figure 4A,4B). The percentages of the apoptotic cells at concentration 100 µg/mL of pretreatment with brown rice extracts with hexane (BrHe), black rice extracts with ethanol (BlE) and hexane (BlHe), baby corn extracts with water (BaH) and ethanol (BaE), sweet corn extracts with water (SH) and hexane (SHe) were 48.2%, 31.27%, 35.66%, 42.52%, 49.37%, 47.67% and 43.325% respectively and were found to be significantly ($p < 0.05$) lower when compared to the percentages of the cells treated with H_2O_2 alone at 51.47% (Figure 4A, 4B). While, brown rice extracts with ethanol (BrE), baby corn extracts with hexane (BaHe) and sweet corn extracts with ethanol (SE) were not significant. These results indicate that brown rice extracts with hexane (BrHe), black rice extracts with ethanol (BlE) and hexane (BlHe), baby corn extracts with water (BaH) and ethanol (BaE), sweet corn extracts with water (SH) and hexane (SHe) prevented neuronal cell apoptosis.

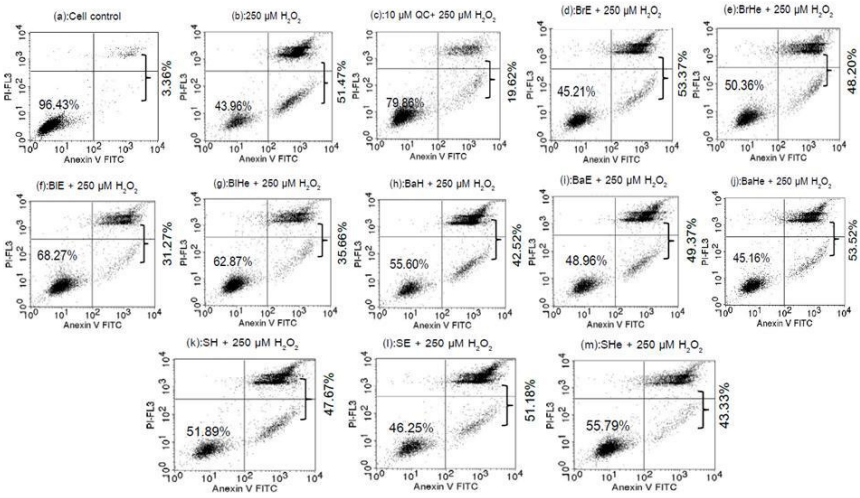

Figure 4A: Flow cytometric analysis type of cell death in H_2O_2-treated HT-22 cells. Cell, untreated HT-22 cells; 250 μM H_2O_2,HT-22 cells treated with 250 μM H_2O_2 for 24 h; QC, exposure of HT-22 cells to 250 μM H_2O_2 over 24 h in the 24h pre-treated 10 μM QC, 100 μg/mL BrE, BrHe, BlE, BlHe, BaH, BaE, BaHe, SH, SE and SHe. The significance is between the indicated group and the group treated with hydrogen peroxide only. (* = $p < 0.05$) and (** = $p < 0.01$) (BrE; Brown rice extract with ethanol, BrHe; Brown rice extract with hexane, BlHe; Black rice extarct with hexane, BlE; Black rice extract with ethanol, BaH; Baby corn extract with water, BaHe; Baby corn extract with hexane, BaE; Baby corn extract with ethanol, SH; Sweet corn extract with water, SHe; Sweet corn extract with hexane SE; Swet corn extract with ethanol, QC; Quercetin

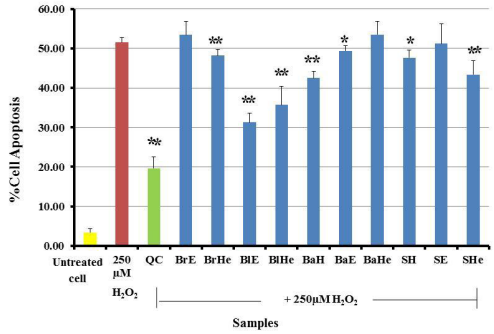

Figure 4B : Flow cytometric analysis type of cell death in H2O2-treated HT-22 cells. Cell, untreated HT-22 cells; 250 M H2O2,HT-22 cells treated with 250 M H2O2 for 24 h; QC, exposure of HT-22 cells to 250 M H2O2 over 24 h in the 24h pre-treated 10 M QC, 100 g/mL BrE, BrHe, BlE, BlHe, BaH, BaE, BaHe, SH, SE and SHe. (* = $p < 0.05$) and (** = $p < 0.01$)

ANTIOXIDANT ACTIVITY OF RICE AND CORN EXTRACTS

To determine the antioxidant activities, DPPH and ABTS assay were used. DPPH assay is based on hydrogen donor property of antioxidants and is widely used in natural antioxidant studies because of its sensitivity and simplicity. ABTS assay has also widely used to evaluate antioxidant activities

Table 3: Antioxidant activities of rice and corn extracts derived from different solvents by DPPH assay

Types of plant	% Scavenging activity (%SC)			mg Vit C g^{-1} fresh weight of sample		
	Water	Ethanol	Hexane	Water	Ethanol	Hexane
White glutinous rice	0.62 ± 2.17	5.67 ± 2.62	4.73 ± 2.51	0.32 ± 1.56	4.46 ± 1.28	3.61 ± 1.56
Brown glutinous rice	4.28 ± 3.74	4.89 ± 2.84	4.46 ± 3.00	3.60 ± 2.18	3.82 ± 1.76	3.68 ± 1.65
Black glutinous rice	6.34 ± 1.25	13.44 ± 1.99	5.76 ± 2.80	4.39 ± 0.76	9.54 ± 1.21	3.47 ± 1.94
Sweet corn	2.21 ± 2.66	5.28 ± 2.98	8.40 ± 3.01	1.45 ± 2.11	3.61 ± 2.33	6.38 ± 1.73
Baby corn	4.27 ± 4.13	5.42 ± 3.93	4.12 ± 2.59	3.55 ± 2.88	4.44 ± 2.53	3.07 ± 1.94

Table 4: Antioxidant activities of rice and corn extracts derived from different solvents by ABTS assay

Types of plant	% Scavenging activity (%SC)			mg Vit C g^{-1} fresh weight of sample		
	Water	Ethanol	Hexane	Water	Ethanol	Hexane
White glutinous rice	2.57 ± 0.99	6.02 ± 2.09	2.22 ± 1.93	2.77 ± 0.12	4.93 ± 0.85	2.56 ± 0.71
Brown glutinous rice	2.05 ± 2.06	4.49 ± 0.46	2.34 ± 0.94	2.46 ± 0.79	3.97 ± 0.20	2.64 ± 0.13
Black glutinous rice	8.97 ± 1.22	26.73 ± 0.42	8.06 ± 1.49	6.75 ± 0.41	17.80 ± 0.65	6.19 ± 1.13
Sweet corn	5.72 ± 1.14	9.83 ± 0.64	4.79 ± 0.87	4.74 ± 0.89	7.28 ± 0.86	4.16 ± 0.42
Baby corn	23.42 ± 0.10	27.63 ± 0.93	2.62 ± 0.85	15.73 ± 0.35	18.36 ± 0.42	2.80 ± 0.59

caused its can detect in both aqueous and lipid phase. The result of the DPPH and ABTS assay are listed in Table 3 and 4, respectively. In both of antioxidant activity assays, black rice extract from ethanol fraction had the richest antioxidant activity, DPPH assay (% Scavenging 13.44 ± 1.99 and mg Vit C g^{-1} fresh weight of sample 9.54 ± 1.21) and ABTS assay (% Scavenging 26.73 ± 0.42 and mg Vit C g^{-1} fresh weight of sample 17.80 ± 0.65). In addition, ABTS assay showed more extracts different DPPH assay including baby corn extracts with water and ethanol. Baby corn extracts with water had % Scavenging 23.42 ± 0.10 and mg Vit C g^{-1} fresh weight of sample 15.73 ± 0.35 and baby corn extracts with ethanol had % Scavenging 27.63 ± 0.93 and mg Vit C g^{-1} fresh weight of sample 18.36 ± 0.42 in Table 4.

TRYPTOPHAN AND MELATONIN CONTENT IN RICE AND CORN EXTRACTS

Tryptophan and Melatonin content in rice and corn extracts were determined by HPLC. Data of the tryptophan and melatonin level in rice and corn extracts are listed in Table 5. Baby corn extracts from water and ethanol fractions had the highest tryptophan contents at 2898.44 and 1303.09 µg/kg, respectively. In addition, black rice

Table 5: Tryptophan and Melatonin content in rice and corn extracts determined by HPLC.

Types of plant	Tryptophan (µg/Kg)			Melatonin (µg/Kg)		
	Water	Ethanol	Hexane	Water	Ethanol	Hexane
White glutinous rice	34.88	486.89	1.97	6.62	161.12	6.99
Brown glutinous rice	3.40	59.73	1.29	18.10	166.26	15.17
Black glutinous rice	317.56	103.25	3.64	19.16	396.38	23.24
Sweet corn	344.40	448.99	37.74	4.39	13.36	8.97
Baby corn	2898.44	1303.09	103.22	19.70	26.36	12.42

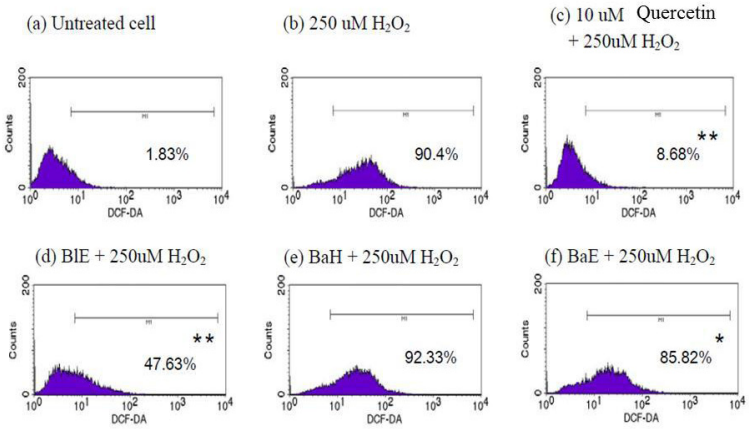

Figure 5A: (a) : Cell control, (b) : Cell + 250 uM H_2O_2 , (c) : Cell + 10 uM Quercitin + 250uM H_2O_2, (d) : Cell + 100 ug/mL Black rice/EtOH + 250uM H_2O_2, (e) : Cell + 100 ug/mL Baby corn/H_2O + 250uM H_2O_2, (f) : Cell + 100 ug/mL Baby corn/EtOH + 250uM H_2O_2. The significance is between the indicated group and the group treated with hydrogen peroxide only. (* = $p < 0.05$) and (** = $p < 0.01$) (BlE; Black rice extract with ethanol, BaH; Baby corn extract with water, BaE; Baby corn extract with ethanol)

extracts from ethanol fraction had the highest level of melatonin content at 396.38 µg/kg.

EFFECT OF RICE AND CORN EXTRACTS ON H_2O_2 INDUCED PRODUCTION OF REACTIVE OXYGEN SPECIES (ROS) IN HT22 CELLS

To investigate the protective effect of black glutinous rice extracts with ethanol (BlE), baby corn extracts with water (BaH) and ethanol (BaE) against H_2O_2 in the production of ROS, the cells were pretreated with 100 µg/mL of black glutinous rice extracts with ethanol (BlE), baby corn extracts with water (BaH) and ethanol (BaE) for 24 hrs before being treated with 250 µM H_2O_2 together with DCFDA for 30 mins. The results show that treated with H_2O_2 alone significantly ($p < 0.05$) increased the level of ROS by approximately 90.4%, in comparison with the control group. Pretreatment with 100 µg/mL of black glutinous rice extracts with

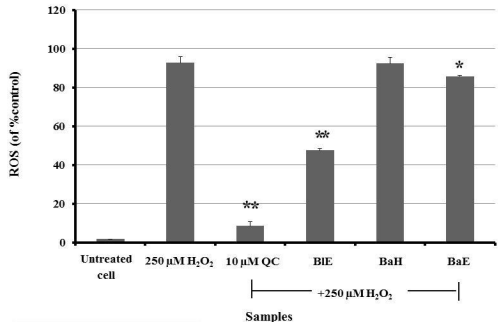

Figure 5B : The protective effect of black glutinous rice extracts with ethanol (BlE), baby corn extracts with water (BaH) and ethanol (BaE) on H_2O_2 induced production of ROS in HT-22 cells. The cells were pretreated with 100 µg/mL of black glutinous rice extracts with ethanol (BlE), baby corn extracts with water (BaH) and ethanol (BaE) for 24 h before being treated with 250 µM H_2O_2 together with DCFDA for 30 min. The significance is between the indicated group and the group treated with hydrogen peroxide only. (* = $p < 0.05$) and (** = $p < 0.01$)

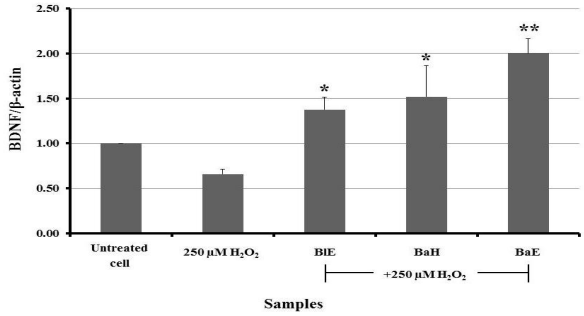

Figure 6 : Real-time PCR for quantification of BDNF expression levels following exposure to 250 µM H2O2, 100 µg/mL black glutinous rice extracts with ethanol (BlE), baby corn extracts with water (BaH) and ethanol (BaE) + 250 µM H_2O_2. The fold change was calculated based on normalization with β-actin gene expression. The significance is between the indicated group and the group treated with hydrogen peroxide only. (* = $p < 0.05$) and (** = $p < 0.01$)

ethanol (BlE) and baby corn extracts with ethanol (BaE) significantly reduced the level of the ROS production. In contrast, baby corn extracts with water (BaH) was no significant (Figure 5A,5B).

EFFECT OF RICE AND CORN EXTRACTS ON H_2O_2 INDUCED EXPRESSION OF BDNF MRNA IN HT22 CELLS

To determine the protective effect of black glutinous rice extracts with ethanol (BlE), baby corn extracts with water (BaH) and ethanol (BaE) on the mRNA level expression of BDNF mRNA. The gene was monitored by quantitative real-time RT-PCR assay. Cell treated with 250 µM H_2O_2 alone resulted in decreased expression of the BDNF genes expression. Pretreated with 100 µg/mL of black glutinous rice extracts with ethanol (BlE), baby corn extracts with water (BaH) and ethanol (BaE) for 24 hrs before being

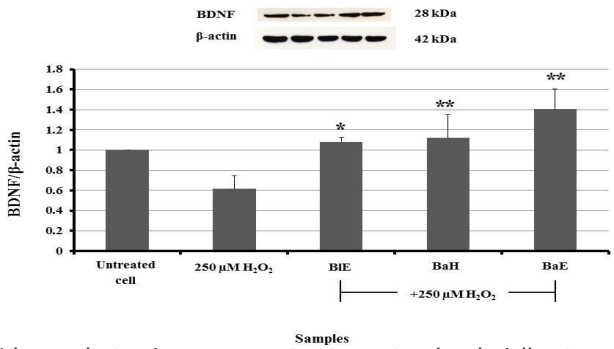

Figure 7: Western blot analysis of BDNF protein expression levels following exposure to 250 μM H_2O_2, 100 μg/mL black glutinous rice extracts with ethanol (BlE), baby corn extracts with water (BaH) and ethanol (BaE) + 250 μM H_2O_2. The fold change was calculated based on normalization with β-actin protein expression. The significance is between the indicated group and the group treated with hydrogen peroxide only. (* = $p < 0.05$) and (** = $p < 0.01$)

treated with 250 μM H_2O_2 the result show that (BlE), (BaH) and (BaE) were significantly ($p < 0.05$) increased in BDNF expression levels 1.37, 1.52 and 2.00-folds respectively (Figure 6).

EFFECT OF RICE AND CORN EXTRACTS ON H_2O_2 INDUCED EXPRESSION OF BDNF PROTEIN IN HT22 CELLS

To determine the protective effect of black glutinous rice extracts with ethanol (BlE), baby corn extracts with water (BaH) and ethanol (BaE) on the expression of BDNF protein. Cell treated with 250 μM H_2O_2 alone resulted in decreased expression of the BDNF protein. Pretreated with 100 μg/mL of black glutinous rice extracts with ethanol (BlE), baby corn extracts with water (BaH) and ethanol (BaE) for 24 hrs before being treated with 250 μM H_2O_2 the result show that (BlE), (BaH) and (BaE) were significantly ($p < 0.05$) increased in BDNF expression levels 1.08, 1.11 and 1.40-folds respectively, as compared to H_2O_2 group (Figure 7).

DISCUSSION

The H_2O_2-induced cell damage has been shown to be involved in neurodegenerative diseases (Kim et al. 2015; Xie & Chen 2016). Several studies have demonstrated the H_2O_2-induced neurotoxicity since it can directly induce apoptosis in neuronal cell by several mechanisms such as oxidative stress (Rao et al. 2013; Zhao et al. 2013). The present study demonstrated that rice and corn extracts may exhibit the neuroprotective effect since they contain melatonin and the precursor for its biosynthesis (tryptophan) and antioxidants. Also, the induced expression of BDNF level by our extracts demonstrated in this work is of great interest since this protein is believed to play a functional role against neurodegenerative diseases. Rice and corn were revealed to have the beneficial effects to the HT22 cells, and this might be due to the presence of such antioxidants as tryptophan and melatonin (Marshall et al. 1996; Pandi-Perumal et al. 2005).

As we first utilized the MTT assay for determining the neuroprotective of rice and corn extracts against H_2O_2 toxicity in cultured HT-22 cells, our current results demonstrated that rice and corn extracts significantly improved the cell viability after H_2O_2 exposure. In contrast, white rice extracts could not improve the cell viability after the presence of H_2O_2. To find the related mechanisms, we evaluated the protective effects of rice and corn extract against H_2O_2-induced neuronal apoptosis. The number of apoptotic cells was quantitatively analyzed using flow cytometry. We found that rice and corn extracts significantly protected the HT-22 cells from H_2O_2-induced cytotoxicity through the apoptosis pathway. Nevertheless, brown rice and sweet corn extracted using ethanol and baby corn extracted using hexane did not significantly protect the HT-22 cells from H_2O_2-induced cytotoxicity through the apoptosis pathway.

Numerous studies have demonstrated the antioxidant activities of rice and corn (Torre et al. 2008; Ismail et al. 2012; Soi-ampornkul et al. 2012; Thummayot et al. 2014). Our study actually demonstrated that our local rice and corn extracts possess varying degrees of antioxidant activities, depending on the extraction solvent types. We demonstrated that black rice extracts with ethanol, baby corn extracts with water and ethanol were shown to possess the highest content of antioxidants. We also determined the levels of melatonin and tryptophan in rice and corn extracts. Previously, there was a study revealing the level of melatonin content in black rice at 140 μg/kg (Manchester et al. 2000; Hernandez-Ruiz & Arnao 2008; Setyaningsih et al. 2014). Our results demonstrated that the highest levels of melatonin content were detected in black rice extracts from ethanol fraction at 396.38 μg/kg. In addition, the highest levels of tryptophan content were detected in baby corn extracts from water and ethanol. These data demonstrated the contents of melatonin and tryptophan in line with the results of antioxidant activities.

We selected the best three fractions for further analyses, black rice extracts from ethanol fraction, baby corn extracts from water and baby corn extracts from ethanol fractions. Numerous studies, both in vitro and in vivo, revealed that H_2O_2 treatment significantly increased ROS production, which resulted in apoptosis (Andersen 2004; Fatokun et al. 2008; Rao et al. 2013; Zhao et al. 2013; Kim et al. 2015; Xie & Chen 2016). Moreover, consistent with the findings from previous studies, we found that the levels of intracellular ROS had markedly increased after the treatment of H_2O_2 at 250 μM. Pretreating cells with black rice extracts from ethanol fraction and baby corn extracts from ethanol fraction significantly suppressed H_2O_2-induced ROS accumulation, thereby suggesting that the antioxidant activity of black rice extracts from ethanol fraction and baby corn extracts from ethanol fraction may be useful for attenuating and preventing apoptosis in neurodegenerative disease (Ismail et al. 2012; Thummayot et al. 2014).

We finally explored the effect of black rice extracts from ethanol fraction, baby corn extracts from water

and baby corn extracts from ethanol fraction on the expression of BDNF mRNA and protein in H_2O_2-induced HT-22 cells using real time PCR and Western blot. BDNF, recognized as a member of the "neurotrophin" family of growth factors, is a secreted protein encoded by the BDNF gene (Binder & Scharfman 2004). Several studies have demonstrated that BDNF has a mechanism related to H_2O_2 toxicity. Cells exposed to H_2O_2 resulted in the decreased BDNF level (Huang & McNamara 2012; Ghaffari et al. 2014). Moreover, several lines of evidence showed the preventive effect of BDNF on neurodegenerative diseases. In depression and Alzheimer's disease, when the hippocampus was also damaged, the reduced levels of the neurotrophic factor were documented. Therefore, antidepressants were applied to raise the levels of BDNF to protect and increase the volume of hippocampal and other cells (Mattson 2008). In the present study, we found that H_2O_2 is significantly decreased BDNF mRNA and protein levels in line with the previous findings. Interestingly, we found that pretreating HT22 cells with black rice extracts from ethanol fraction, baby corn extracts from water and baby corn extracts from ethanol fraction increased BDNF mRNA and protein levels. Pretreated cells with black rice extracts from ethanol fraction, baby corn extracts from water and baby corn extracts from ethanol fraction reversed this H_2O_2- induced neurotoxicity, suggesting the beneficial effects of rice and corn in vitro.

Our limitations for this investigation included plant samples collected one occasion only, cell-based assay (not systemic approach), plant extractions using few solvent types and limited and only BDNF gene determined. However, it was the first study demonstrating the advantageous effects of rice and corn extracts against H_2O_2-induced neurotoxicity, particularly the impact on BDNF expression in HT22 cells, thus highlighting their nutraceutical properties and health benefits.

CONCLUSION

Rice and corn including black rice extracts from ethanol fraction, baby corn extracts from water and baby corn extracts from ethanol fraction protected the neurotoxicity of HT-22 cells from H_2O_2. Inhibition of ROS production in conjunction with modulation of BDNF expression, by their endogenous contents of melatonin and tryptophan, may contribute to the underlying mechanisms.

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