Biological Properties of Extracts from Riceberry and Black rice for Inhibition of Herpes Simplex Viruses, Phytochemical compounds and Antioxidant activities

Boonpa Suantai[1,2] and Yingmanee Tragoolpua[2*]

[1]The Graduate School, Chiang Mai University, Chiang Mai, Thailand
[2]Division of Microbiology, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand
[*]Corresponding Author, E-mail: yingmanee.t@cmu.ac.th

Abstract

Rice (*Oryza sativa*) is the most important food for the population in the world. Rice contains a bioactive compound which shows anti-aging and anti-cancer properties. Therefore, this study aimed to investigate biological properties on anti-herpes simplex viruses, phytochemical compounds and antioxidant activities of extracts obtained from riceberry and black rice. The highest nontoxic concentrations of extracts were used for treatment with both type of herpes simplex virus (HSV). The results showed that aqueous extract of black rice showed the highest anti-HSV-2 when treated during viral attachment with inhibition of 80.45 ± 1.28% and ED_{50} = 1,957.55 ± 13.73 µg/ml. Ethanolic extract of black rice inhibited HSV-2 after viral attachment with 54.26 ± 1.06% and ED_{50} = 2,492.89 ± 109.72 µg/ml. Moreover, the cytotoxicity of rice extracts was determined on Caco-2 colon cancer cells. It found that the extract of riceberry showed the most toxicity on Caco-2 cells. The aqueous extract of riceberry showed high antioxidant activity when measurement by both the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays. Besides, the aqueous extract of black rice contained the highest amount of phenolic compound and the level of total anthocyanin content was the highest in ethanolic extract of black rice.

Keywords**:** *black rice, riceberry, phytochemical, antioxidant, herpes simplex viruses*

1. Introduction

Viruses are pathogenic intracellular microorganisms in human. Some viruses are capable of causing cancer such as human papillomavirus, hepatitis B virus, Epstein-Barr virus, and herpes simplex virus (Cantalupo et al., 2018; Schiller and Lowy, 2014). Moreover, an approximate 15% of all human cancers worldwide may be attributed to viruses and 10 million new death are expected in 2020 (Bray et al., 2018; Jemal et al., 2011). Herpes simplex virus (HSV) is one of pathogenic and endemic in human. They are major opportunistic infections in immunodeficiency patients. HSVs are divided into two types, HSV-1 and HSV-2. While HSV-1 is the primary agent of orolabial disease, HSV-2 involves genital herpes. Both types of HSVs remain latent within sensory neural ganglia. Thus, HSV reactivation is generated by stress, hormonal change and radiation. Acyclovir is widely used as a routine treatment of HSV infection, which targets viral polymerase. However, the antiviral agent is expensive, some side effect and drug-resistant strain of HSV may occur in patients who use the drug for a long time. Nowadays, the finding of natural products against the herpes simplex virus and human disease are increasing (Son et al., 2013). Drugs from the herbal plants are safe and show low side effect. Rice is one of the most common foods for human, especially in Asia. It contains a variety of tannin, flavones, γ-oryzanols, cyanidin 3-glucoside, and anthocyanin, which are found to possess many bioactive properties, including antioxidant, anti-allergic, anticancer and anti-atherosclerosis (Deng et al., 2013). Previous research reported that rice and rice by-products inhibited colon cancer cell, liver cancer and human prostate cancer (Yoon et al., 2014; Banjerdpongchai, Wudtiwai & Sringarm, 2013). Besides, anthocyanins are widely present in many plants. Previous studies reported that anthocyanins inhibited proliferation and reduced inflammation in a cell line (Zhao et al., 2019; Li et al., 2017; Lin et al., 2017). However, the anti-HSV in this plant was not reported. Therefore, the objective of this study was to investigate the effect of riceberry and black rice extracts on herpes simplex viruses. Toxicity of the rice extracts on Caco-2 cancer cell and antioxidant activity were also determined. The new knowledge from this study may be developed in the future, which increases the value of rice.

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2. Objective

The objective of the study was to determine the anti-herpes simplex viruses and antioxidant activity of the extracts from riceberry and black rice.

3. Materials and Methods

3.1 Samples and preparation of extracts

Riceberry and black rice were purchased from an organic farm in Chiang Mai, Thailand. Rice samples were blended and extracted with 95% ethanol for 3 days at room temperature and soaked in distilled water for 3 hours at 45 °C with the ratio of rice powder and solvent of 1:10 (w/v). The extract was filtered and evaporated by rotary evaporator. Then, the crude extract was dried by lyophilizer. The dried crude rice extract was dissolved in dimethyl sulfoxide (DMSO) and stored at -20 °C until analysis (Yoon et al., 2014).

Percentage of yield = [extracts from black bean (g)/ Initial weight of black bean (g)] ×100

3.2 Cell culture and viruses

Vero cells and Caco-2 cell line were cultured in Dulbecco's Modified Eagle medium (DMEM; Gibco, UK) supplemented with 10% (v/v) heated fetal bovine serum (FBS), penicillin-streptomycin solution at 37 ºC in a humidified atmosphere of 5% CO_2. Herpes simplex virus type 1 strain F and type 2 strain G (HSV-1F and HSV-2G) were used and propagated in Vero cells. The infected cells were maintained at 37°C in 5% CO_2 until 80-90% of the infected cells showed a cytopathic effect. Infected cells were frozen and thawed twice, and virus stocks were collected and stored at -80°C for future use.

3.3 Cytotoxicity on Vero cells and Caco-2 cancer cells

The cytotoxicities of riceberry and black rice extracts on Vero and Caco-2 cell lines were determined. Cell viability was determined using a colorimetric MTT assay. Both cells were plated in 96- well plate (1.5×10^4 cells/well) and rice extracts at various concentrations (0.156 – 5 mg/ml) were added into each well. Then, the 96-well plate was incubated at 37 ºC in 5% CO_2 for 48-72 h. MTT solution was added and incubated for 4 hours. Then, the supernatant was removed, and dimethyl sulfoxide (DMSO) was added to dissolve formazan. The optical density of the solution was measured at a wavelength of 540 and 630 nm. The results were expressed as the percentage of cell survival compared to untreated control (Tan, Norhaizan, Yeap & Roselina, 2015).

3.4 Plaque titration assay

Vero cells were seeded into 24-well plate using 1.5×10^5 cells/well in DMEM supplemented with 10% FBS and incubated for 24 hours to form monolayers before treated with each dilution of HSV, which was serially ten-fold diluted in DMEM. After viral adsorption for 1 hour, the cells were washed with FBS and overlaid with 1.5% carboxymethylcellulose to each well. Plaques were formed after 2-3 days of incubation and stained with 0.1% crystal violet in 1% ethanol. The viral titers were expressed as plague forming unit/ml.

3.5 Anti-Herpes simplex activities

The inhibitory effects of extracts from riceberry and black rice were investigated when treating HSV before, during, and after viral attachment. The monolayer of Vero cells on a 24-well plate was treated with extracts for 1 hour before viral attachment. After that, the suspension was removed and washed with PBS before infection with HSV. Then, the overlay medium was added at the same time and incubated at 37°C in 5% CO_2 for 2-3 days.

During viral attachment. HSV and extracts were added at the same time on Vero cells cultured in 24-well plate. After that, the mixture was incubated at room temperature for 1 hour. The mixture was removed and washed with PBS. And then, overlay medium was added and incubated at 37ºC in 5% CO_2 for 2-3 days.

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Moreover, HSV was also added into Vero cells and incubated at room temperature for 1 hour. After viral adsorption, the cells were washed with PBS, and the rice extracts were added into the infected cell. Then, the overlay medium was added and incubated at 37ºC in 5% CO_2 for 2-3 days. The number of plaques was stained with 0.1% crystal violet in 1% ethanol. (Cantatore, Randall, Traum & Adams, 2013).

The number of the plaque was counted and compared to untreated virus control. Also, 50% effective dose (ED_{50}) was calculated dose-response curves.

3.6 Antioxidant activities

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical- Scavenging activity

The free radical scavenging capacity of the riceberry and black rice extracts were measured using the DPPH method. The extracts at various concentration were mixed with DPPH reagent. Then, the mixture was incubated in the dark at room temperature for 20 min. The mixture was measured at a wavelength of 517 nm. DPPH radical scavenging of activity was expressed as mg of gallic acid / g of extract (Jun et al., 2012).

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation scavenging assay

The extracts at various concentration were mixed with ABTS reagent. Then, the mixture was incubated in the dark at room temperature for 10 min and measured at a wavelength of 725 nm. The results were expressed as mg Trolox equivalents (TEAC)/g of extract (Jun et al., 2012).

3.7 Determination of total phenolic content

Total phenolic content was determined using the Folin-Cioalteu colorimetric method. The extracts at various concentration were mixed with Folin-Cioalteu reagent and incubated in the dark at room temperature for 5 min. Then, the reaction was neutralized with 5% sodium carbonate and incubated in the dark at room temperature for 1 hr. The mixture was measured at a wavelength of 725 nm. Total phenolic content was expressed as mg of gallic acid equivalent / g of extract (Xue et al., 2016).

3.8 Determination of total anthocyanin determination

Total anthocyanin content was determined by a modified pH- differential method. The extracts at various concentration were mixed in potassium chloride buffer, pH 1.0 and sodium acetate buffer, pH 4.5 (1:100 v/v) and incubated at room temperature for 20 min. After incubation, the mixture was measured at a wavelength of 510 nm and 700 nm, respectively. Total anthocyanin content was expressed as mg of cyanidin 3 glucoside equivalent / g of extract (Sutharut & Sudarat, 2012).

4. Results and Discussion

After extraction, percentage yields of aqueous extracts of riceberry and black rice were 9.29% and 6.84%, and ethanolic extracts of riceberry and black rice were 1.90% and 3.33%, respectively (Table 1).

Table 1 Percentage yield of crude extracts from riceberry and black rice

Type of extracts	Percentage yield (%)
Ethanolic extract	
Riceberry	1.90
Black rice	3.33
Aqueous extract	
Riceberry	9.29
Black rice	6.84

The cytotoxicity test of Vero cell showed that CD_{50} values of the ethanolic extracts of riceberry and black rice were 2,561.00 ± 45.59, 2,675.19 ± 42.95, respectively. Moreover, CD_{50} values of and aqueous extracts of riceberry and black rice were 2,197.50 ± 105.97 and 3,239.31 ±126.81 µg/ml, respectively. This results showed that the aqueous extract from riceberry showed the highest toxicity on Vero cells.

Moreover, the CD_{50} values of the ethanolic extract of riceberry and black rice on Caco-2 cell were 1,397.37 ± 35.58 and 1,698.56 ± 55.99 µg/ml. Aqueous extracts of riceberry and black rice were 1,742.14 ±

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24.54 and 3,349.89 ± 117.63 µg/ml, respectively. These result suggested that the ethanolic extract of riceberry showed the highest toxicity on Caco-2 cells (Table 2). The toxicity might come from the compound in the rice extract such as cyanidin-3-O-glucoside. In riceberry, cyanidin-3-O-glucoside has been reported to be one of the major anti-Caco-2 cells compound (Liang et al., 2017). Moreover, the previous study reported that the extract of Thai riceberry was shown to inhibit human cancer cell (Caco-2, MCF-7 and HL-60) (Leardkamolkarn et al., 2011).

Besides, the morphology of Caco-2 cells treated with the extracts was observed by inverted microscope. The Caco-2 cells treated with the ethanolic extract of riceberry and black rice showed morphological alterations, including cell rounding, shrinkage and loss of the attachment with the well plate when compared to the untreated control. These morphological changes increased after treating the extract by dose-dependent manner (Fig 1).

In the previous study, LNCaP cells (human prostate adenocarcinoma cells) and HepG2 cells (human hepatoma cells) were treated with the purple rice extract. The highest inhibitory effect was found on HepG2 cells, which were induced apoptosis via the mitochondrial pathway with the loss of mitochondrial transmembrane potential and activation of caspase-3 and -9 (Banjerdpongchai et al., 2013). When HepG2 cell line was treated with the black rice, HepG2 Cells were protected from oxidative stress by increasing the activation of pro-survival signal proteins (ERKs and Akt) (Yoon et al., 2014). Moreover, HT1080 cells (fibrosarcoma cells) and MDA-MB-231 cells (human breast cancer cells) treated with red jasmine rice demonstrated that the red jasmine rice could reduce cancer cells invasion. Matrix metalloproteinase-2 and 9 secretions in the cells were inhibited by proanthocyanidin, γ-oryzanol and γ-tocotrienol fraction from red jasmine rice extract (Pintha, Yodkeeree, Pitchakarn & Limtrakul, 2014). After treating Jurkat cells (human T lymphocyte cells) line with the fermented brown rice, these rice extract could reduce cell proliferation and induced cancer cell death by apoptotic pathway (Horie, Nemoto, Itoh, Kosaka & Morita., 2016). Thus, a high level of γ-oryzanol in these rice may involve as a bioactive compound for inhibition of cell proliferation (Huang et al., 2020; Kim, Kang, Nam & Friedman, 2012).

Table 2 Summary of the CD_{50} value of Vero cells and Caco-2 cell

Rice extracts	CD_{50} (µg/ml)	
	Vero cell (72 h)	**Caco-2 cell (48 h)**
Ethanolic extract		
Riceberry	2,561.00 ± 45.59	1,397.37 ± 35.58
Black rice	2,675.19 ± 42.95	1,698.56 ± 55.99
Aqueous extract		
Riceberry	2,197.50 ± 105.97	1,742.14 ± 24.54
Black rice	3,239.31 ±126.81	3,349.89 ± 117.63

Data are represented as mean ± SD from three independent experiments.

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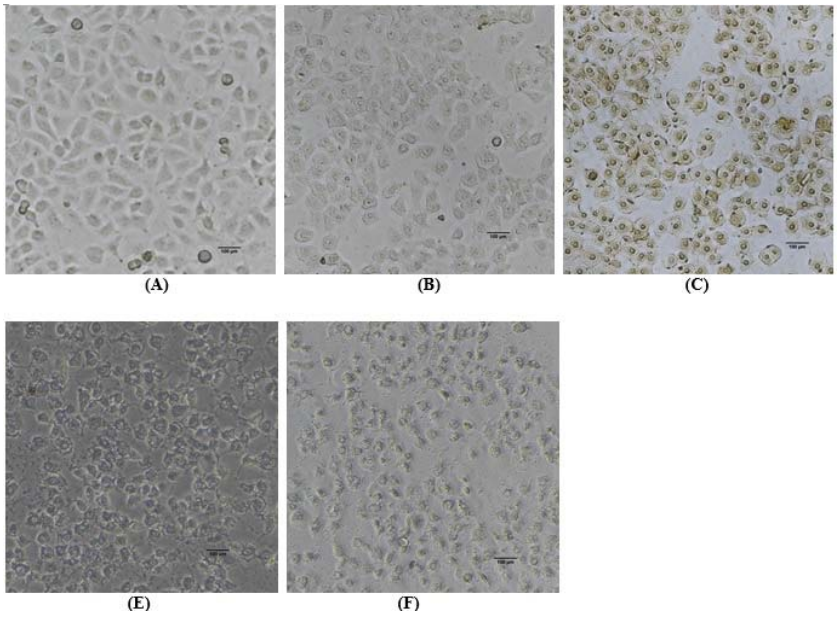

Figure 1 Morphology of Caco-2 cells after treating with various concentrations of the ethanolic extract of riceberry and black rice for 24 h. (A) = Cell control, (B) = Caco-2 cell grown with 2500 µg/ml of the ethanolic extract of riceberry, (C) = Caco-2 cell grown with 5000 µg/ml of the ethanolic extract of riceberry**,** (E) = Caco-2 cell grown with 2500 µg/ml of the ethanolic extract of black rice, Caco-2 cell grown with 5000 µg/ml of the ethanolic extract of black rice.

Cytotoxicity of Vero cells was used for the anti-HSV assay, which was selected from the highest nontoxic with Vero cells. The inhibition effects of extracts with HSV infection were determined and observed by plaque reduction assay. The results found that the aqueous extract of black rice showed the highest anti-HSV-2 of 80.45 ± 1.28% and ED_{50} = 1,957.55 ± 13.73 µg/ml when treated during viral attachment. After viral attachment, the virus was when treated with the ethanolic extract of black rice showed 54.26 ± 1.06% and ED_{50} = 2,492.89 ± 109.72 µg/ml. Moreover, when treated before and during viral attachment, ethanolic extract of black rice inhibits on HSV-2 was 41.82 ± 1.45% and, 46.05 ± 1.00%, respectively. Also, inhibition of the HSV-1 after viral attachment was 43.56 ± 0.75, which was treated with the ethanolic extract of black rice. In contrast, the aqueous and ethanolic extract of riceberry showed weak anti-HSV-1 and anti-HSV-2 activity of less than 40 % of inhibition (Table 3). From the result, ethanolic extract of black rice showed the highest anti-HSV-2 activity when treated after viral attachment to the cells. Therefore, the black rice extract should be used to treat HSV infection. The anti-HSV activity might be from cycoartenol or 24-methylenecycloartanol, which is found in rice extract (Akihisa et al., 2001). These compounds were also found to have anti-HIV-1 activity (Akihisa et al., 2001).

Currently, the study of Japanese rice- koji miso extract inhibited hepatitis A virus replication in human hepatocytes (Win et al., 2018). Moreover, HSV was inhibited by extracts from many plants. HSV-1 was inhibited when treated with extracts of mung bean sprouts (Hafidh et al., 2015), aloe vera (Rezazadeh et al, 2016), polyphenol fraction from *Pistachios Kernels* (Musarra-Pizzo et al., 2020) and olive leaf (Altindis et al., 2020). Besides, monogalactosyl diacylglyceride fraction from green microalgae extracts inhibited HSV-2 infection both *in vitro* and *in vivo* (Hayashi et al., 2019).

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Table 3 Inhibitory effect of aqueous and ethanolic extracts of riceberry and black rice on herpes simplex virus.

Rice extracts	Inhibition effect of extracts on HSV (%)					
	Before viral attachment		During viral attachment		After viral attachment	
	HSV-1	HSV-2	HSV-1	HSV-2	HSV-1	HSV-2
Ethanolic extract						
Riceberry	22.91 ± 0.92	13.51 ± 0.33	30.93 ± 0.87	34.18 ± 0.62	35.29 ± 0.62	27.41 ± 1.00
Black rice	38.22 ± 3.97	41.82 ± 1.45	37.21 ± 1.96	46.05 ± 1.00	43.56 ± 0.75	54.26 ± 1.06
Aqueous extract						
Riceberry	38.77 ± 0.79	9.78 ± 0.34	42.36 ± 1.42	23.59 ± 0.89	39.05 ± 0.35	20.10 ± 0.39
Black rice	37.13 ±0.67	38.12 ± 0.72	27.54 ± 0.59	80.45 ± 1.28	37.93 ± 0.93	42.01 ± 0.93

Data are represented as mean ± SD from three independent experiments.

Antioxidant activities of the extracts were determined in two different models, including DPPH and ABTS method. It found that the aqueous extract of riceberry showed the highest antioxidant activity of both DPPH and ABTS methods with 31.58 ± 0.05 and 45.25 ± 2.15 mg Trolox equivalents/g of extract, respectively. The ethanolic extract of black rice and riceberry and the aqueous extract of black rice showed antioxidant activities of 40.73 ± 1.75, 34.67 ± 1.44, 8.00 ± 0.70 mg Trolox equivalents/g of extract when using ABTS method. Whereas, the ethanolic extract of riceberry and black rice, the aqueous extract of black rice showed anti-oxidation of 22.59 ± 0.03, 4.69 ± 0.69 and 2.79 ± 0.69 mg gallic/g of extract, respectively when using DPPH method (Table 4).

Table 4 Antioxidant of extracts from riceberry and black rice

Rice extracts	Assay	
	DPPH (mg gallic/g of extract)	ABTS (mg trolox equivalents/g of extract)
Ethanolic extract		
Riceberry	22.59 ± 0.03	34.67 ± 1.44
Black rice	4.69 ± 0.69	40.73 ± 1.75
Aqueous extract		
Riceberry	31.58 ± 0.05	45.25 ± 2.15
Black rice	2.79 ± 0.69	8.00 ± 0.70

Data are represented as mean ± SD from three independent experiments.

Total anthocyanin and total phenolic content of rice extracts were assessed. The ethanolic extract of black rice contained high anthocyanin of 3,188.37 ± 40.81 mg of cy-3-glc equivalent/g extract followed by the aqueous extract of riceberry, the ethanolic extract of riceberry and the aqueous extract of black rice that showed anthocyanin of 1,367.08 ± 7.71, 1,327.05 ± 15.43, 389.64 ± 10.20 mg of cy-3-glc equivalent/g extract, respectively. Aqueous extract of black rice showed the highest total phenolic content of 51.67 ± 1.22 mg of gallic acid/ g extract followed by the ethanolic extract of riceberry, the aqueous extract of riceberry and the ethanolic extract of black rice with phenolic contents of 46.08 ± 0.15, 39.16 ± 0.08 and 35.74 ± 0.89 mg of gallic acid/ g extract, respectively (Table 5). The previous study demonstrated that anthocyanin from purple rice showed an inhibitory effect on cardiac inflammation and hypertrophy. The purple rice also attenuated cardiac fibrosis to protect cardiac function in the rat (Chen et al., 2016).

Table 5 Total anthocyanin and phenolic content of the extracts from riceberry and black rice

Rice extracts	Total anthocyanin content (mg cy-3-glc/g extract)	Total phenolic content (mg gallic acid/ g extract)
Ethanolic extract		
Riceberry	1,327.05 ± 15.43	46.08 ± 0.15
Black rice	3,188.37 ± 40.81	35.74 ± 0.89
Aqueous extract		
Riceberry	1,367.08 ± 7.71	39.16 ± 0.08
Black rice	389.64 ± 10.20	51.67 ± 1.22

Data are represented as mean ± SD from three independent experiments.

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5. Conclusion

Results from this study suggested that the aqueous extract of black rice showed the highest efficacy against HSV-2 infection when treated during viral attachment. Also, after viral attachment, the ethanolic extract of black rice showed the highest inhibition of HSV-2 infection. The ethanolic extract of black rice also contains high total phenolic and anthocyanin. Moreover, the ethanolic and aqueous extract of riceberry showed the greatest anti-oxidant activity using DPPH and ABTS assays. The ethanolic extract of riceberry also caused toxicity on Caco-2 cells. Besides, the aqueous extract of black rice contained the highest amount of phenolic compound, while ethanolic extract of black rice showed the highest level of total anthocyanin content. Therefore, the extracts from black rice should be developed as anti-HSV agents and provided anti-oxidation from phenolic and anthocyanin compounds. Moreover, the riceberry extracts should be applied as health supplementary product that provides anti-oxidant and causes colon cancer cell toxicity.

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