



Cytotoxicity Activity of Crude Extracts of Leum Phua Khaow-Mak (*Oryza sativa* L. variety Leum Phua) against Fibroblast Cell

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Abstract

Leum Phua Khaow-Mak is a fermented food, which provides high amounts of nutrients and antioxidant bioactive compounds such as phenolic compounds. Leum Phua Khaow-Mak extract compounds also have potential for applications in cosmetics and function food formulations. The objective of this research was to investigate cytotoxic activity of crude extracts from Leum Phua Khaow-Mak to human dermal fibroblast cells using sulforhodamine B (SRB) colorimetric assay method. Leum Phua glutinous rice (*Oryza sativa* L. variety Leum Phua) was fermented with a starter (Look Pang) at room temperature for 5 days. Fermented rice samples were extracted with 95% ethanol for 24 hours. The solvent was evaporated at 45°C to obtain dried crude extracts. The antioxidant capacity of crude extracts was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging. Butylated hydroxytoluene (BHT) was used as a standard antioxidant. The EC_{50} value of the extract was 0.367 mg/ml, as similar to that of BHT ($EC_{50} = 0.239$ mg/ml). A dried sample was dissolved with 10% (v/v) dimethyl sulfoxide (DMSO) in the cell culture media. Cytotoxic activity of crude extracts to human dermal fibroblast cell was evaluated. The cell cultures were fixed in ice-cold 40% trichloroacetic acid (TCA) and were dyed using 0.05% sulforhodamine B for 30 min. Sodium lauryl sulfate was used as the positive control. The optical density was measured at 510 nm in a microplate reader. The percentage of viability of human dermal fibroblast cell were calculated and compared with sodium lauryl sulfate. The results showed that the concentrations of crude extracts from Leum Phua Khaow-Mak at 0.0001-1.0 mg/ml have the percentage of viability at 105.17-102.29 respectively. The percentage of viability of sodium lauryl sulfate at 0.0001-1.0 mg/ml have the percentage viability at 99.43-4.91 respectively. The percentage of viability at the concentrations of 0.1 and 1.0 mg/ml were low. These obtained results indicated that the concentrations of crude extracts from Leum Phua Khaow-Mak in the range between 0.0001 and 1 mg/ml were non-toxic to human dermal fibroblast cells.

Introduction

Leum Phua glutinous rice is a colored rice known to be used for over 100 years by the Hmong people. It is a local variety found in the mountains of the North. Khao Rai Leum Pua, is still planted in Phop Phra District, Tak Province, in Thailand. Main pigment in both seed coat and whole grain of Leum Phua glutinous rice is purple black color containing phenolic compounds e.g. anthocyanin (Suttajit et al., 2006). These bioactive compounds show high nutritive values, such as Omega-3, 6 & 9, anthocyanin, gamma oryzanol, vitamin E and good minerals for health. Leum Phua rice has been identified as a source of natural antioxidants and reported to have antioxidant activity higher than other black rice varieties (Srisuwan et al., 2013). The antioxidant was contributed to inhibit the oxidation reaction and also to terminate the free radical chain reaction. The strength skin cell wall was reduced and collagen of cells was degraded leading to the decreasing of moisture and skin elasticity. Then, the wrinkles of skin cells were generated. By the addition of antioxidant compounds, the skin damage and wrinkle were protected and may slow-down the cell degeneration (Fereidoon & Chi-Tang, 2005). Moreover, The effect of high concentration of antioxidants in Luem Phua rice could improve learning and memory in mice (Srisuwan et al., 2013).

Khaow-Mak is known as one of the famous traditional Thai foods. It is produced from the fermentation process of rice using microorganisms. The starter or Look Pang is a semi-circular starch ball that incorporates various fermentative biological species such as molds and yeast. In the production process of Look Pang, Thai herbs such as pepper, garlic and galangal are added as antibacterial agents (Taechavasonyoo et al., 2013). Khaow-Mak has been documented as a rich source of probiotics and bioactive compounds, offering various food properties that influence human health. Several studies showed that the fermentation can increase bioactive compounds, such as minerals, vitamins, phenolic compounds and proteins digestibility. The degradation of antinutritional compounds as phytic acid, fermentation can lead to improved nutritional quality of food (Zhai et al., 2015; Plaitho et al., 2013; Michela et al., 2019). In addition, previous studies have examined the amount of phenolic compounds, flavonoid and antioxidant activities of Khaow-Mak crude extracts from colored rice varieties, such as Red Brown Jasmine Rice, Mun Poo Brown Rice, Sangyod Brown Rice, Black

Jasmine Rice (Hom-nin), Rice Berry, Luem Pua Glutinous Rice, Muser Purple Rice and Black Glutinous Rice. It was found that the extract from Luem Pua Glutinous Rice obtained highest amount of phenolic content, flavonoids content and antioxidant efficiency (EC_{50}). According to previous results (Wattanuruk et al., 2018; Semsang et al., 2016), the rice varieties at after fermentation had the antioxidant activity higher than before fermentation. Therefore, there is a potential to further develop the extracts as an active ingredient in cosmetic and food for health and beauty products.

Fibroblast cells is a component of connective tissue in the skin. In recent years, various studies have been conducted to verify the capacity of fibroblasts to regenerate the skin structure. Collagen is the most common form of extracellular envelope, which forms fibers in an extracellular environment; therefore, it determines the shape of tissues. Collagen is stored as a precursor in fibroblastic cells and is secreted to the extracellular medium. This extracellular matrix and collagen form the structural framework of tissues in animals and plays an important role in tissue repair. These connective tissue are very important to the skin and can also combine with the water in the skin cells to maintain the skin moisture. In addition, elastin proteins are typically spindle-shaped can arrange adjacent collagen. Main function of elastin proteins is to anchor the skin cells to increase both strength and elasticity. If the fibroblast cells are damaged, causing from an ultraviolet radiation (UVR) and environmental insults, the skin can be broken down. This results in the loss of skin elasticity, reduction of the number of dermal fibroblasts, loss of skin thickness and can be wrinkles and hyperpigmentation in the skin (Nilforoushzhadeh et al., 2017). Several studies have reported that the phenolic compounds of rice extracts showed immune and inflammatory cell function as well as antioxidant activity, so compounds with antioxidant properties will be useful against oxidative damage of connective tissue (Havsteen, 2002; Palungwachira et al., 2019).

The objective of this research was to check the antioxidant activity of Leum Phua Khaow-Mak by measuring DPPH free radical scavenging assay. The cytotoxicity studies carried out by using sulforhodamine B (SRB) colorimetric assay of crude extracts from Leum Phua Khaow-Mak against the fibroblast cell. The method is most widely used for in vitro cytotoxicity screening of natural compounds on cultured cells (Skehan et al., 1990). The results will be further used in the production

of skin cosmetic to create new value of fermented rice.

Materials and methods

1. Preparation of crude extracts from Leum Phua Khaow-Mak

Leum Phua glutinous rice (Tak province) was washed by water for 5 min. The rice was soaked in water for 6 hours before mixing with distilled water (1:3 w/v) and cooked with the ordinary rice cooker. Cooked rice was cooled at room temperature and fermented with 0.5% Look-Pang (a mixed culture of yeasts and molds) at room temperature for 5 days in a glass container (0.5g/100 g of raw rice). The fermented rice was dried in an oven at 60°C for 24 hours. Dried rice samples were extracted with 95% ethanol under stirring condition using an orbital shaker at 120 rpm for 24 hours. The ethanol extracts were separated using the centrifugation at 6,000 rpm for 10 min and were filtered through a filter paper (Whatman No.1, Whatman International, England). The remaining soluble extracts were reprocessed by the same method and the extracts were combined and transferred to a flat-bottomed flask. The solvents were evaporated by a rotary evaporator at 45°C until dry. The sample was kept at -10°C until used (Plaito, 2016).

2. DPPH radical scavenging activity

DPPH free radical scavenging activity was carried out according to (Fukumoto & Mazza, 2000) with some modifications. 0.02 g of Khaow-Mak extracts was diluted with 40 ml of 99.99% ethanol. A series of concentrations of the extract sample at 31.25, 62.50, 125, 250 and 500 mg/ml was prepared. BHT (butylated hydroxytoluene) was used as standard (positive-control) (Prabnok et al., 2016). Briefly, 1 ml of each extract was allowed to react with 2 ml of 0.1 mmol/l DPPH solution for 30 min in the dark before the absorbance was read at 517 nm. The radical scavenging activity was calculated as

$$\% \text{ Inhibition} = [(AB - AA)/AB] \times 100$$

where AA was the absorption of tested extract solution and AB was the absorption of blank sample.

The sample concentration providing 50% effective concentration (EC₅₀) was calculated from the graph plotting inhibition percentage against sample concentration.

3. Cultivation of human dermal fibroblast cell

Human dermal fibroblast cell lines (passage 50:

ATCC®PCS-201-012, USA) were cultivated in a culture medium containing Dulbecco' modified Eagle medium (DMEM) and supplemented with 10% Fetal bovine serum (FBS) (v/v) and 1% Penicillin-Streptomycin antibiotics (v/v) (Gibco, USA). The cells were mixed to obtain a homogeneous cell suspension and transferred to a sterilized tissue-culture bottle and incubated at 37°C in a humidified incubator with 5% CO₂ for 24 hours. Cell concentration was adjusted with a growth medium to obtain a seeding density of 10⁵ cells/ml by hematocytometer chamber under a microscope (Vichai & Kirtikara, 2006).

4. Cytotoxicity of crude extracts to fibroblast cell by SRB

Dried sample of crude extract from Leum Phua Khaow-Mak was weighed and dissolved in 10% (v/v) dimethyl sulfoxide (DMSO) (Labscan, Thailand) and culture media. The samples were then sterilized using a membrane filter (0.2 μ) and were diluted to obtain five concentrations of (mg/ml) 0.0001, 0.001, 0.01, 0.1 and 1 in 10% (v/v) DMSO in sterilized cultured media. Sodium lauryl sulfate were prepared in a sterilize cultured media to obtain the concentration of 0.0001, 0.001, 0.01, 0.1 and 1 mg/ml. Ten microliters of the sample was added into each well of a 96 well tissue culture plate. Negative control was performed by adding 10 μl of 10% (v/v) DMSO into a well of test sample. Sodium lauryl sulfate (10 μl) was added into a well for a positive control. Added 10 μl of sterilized cultured media to negative control well of sodium lauryl sulfate. Added 100 μl cells suspension to each well already containing compounds. Set aside a plate contain only cells suspension for a no-growth (day 0). The cell culture was incubated at 37°C in a humidified incubator with 5% CO₂ for 72 hours.

Cytotoxic activity of crude extracts to human dermal fibroblast cell was evaluated. Sulforhodamine B (SRB) is used for cell density determination, which performed to assess growth inhibition by a colorimetric assay by staining total cellular protein with the dye SRB. This assay cell cultures were fixed with 100 μl of ice-cold 40% (w/v) Trichloroacetic acid (99.0% TCA: Merck, Germany) per well, incubated at 4°C for 1 hour. The plates were washed with distilled water to wash non-viable cells. Then, and dried at room temperature. SRB solution (Sigma-Aldrich, USA) in 50 μl (0.05% w/v in 1% glacial acetic acid, Sigma, USA) was added to each well and left at room temperature for 30 min. The plates

were quickly rinsed with 1% (v/v) acetic acid to remove an unbound dye. Then, the plates were dried at room temperature for 30 min before added in 200 µl of 10 mM Tris base solution (Sigma, USA) pH 10.5 [Tris (hydroxyl methyl) aminomethane]. The plate was then shaken to solubilize the protein-bound dye. The optical density (OD) was measured at 510 nm in a microplate reader. Percentage of viability of fibroblast cell was calculated and compared with sodium lauryl sulfate using the equation below (Skehan et al., 1990 ; Vichai & Kirtikara, 2006). Morphology of fibroblast cell was studied by compound light microscope (100x).

$$\% \text{ of control cell growth} = \frac{\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{day 0}} \times 100}{\text{mean OD}_{\text{neg control}} - \text{mean OD}_{\text{day 0}}}$$

5. Statistical analysis

All values were expressed as means of each treatment group. Student's unpaired t-tests were used to compare between the two groups. $P < 0.01$ was considered statistically significant.

Results and discussion

DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, is reduced in the presence of an antioxidant molecule, giving rise to colorless ethanol solution. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized (Asgarpanah et al., 2011), which can be quantitatively measured from the changes in absorbance. In this experiment, the EC_{50} value of the Leum Phua Khaow-Mak extracts was 0.367 mg/ml, as similar to that of BHT ($EC_{50} = 0.239$ mg/ml). BHT was used as standard antioxidant in the performed experiments. It also known as dibutylhydroxytoluene, is a lipophilic organic compound, chemically a derivative of phenol, that is useful for its antioxidant properties. These obtained results indicated that the crude extract was able to scavenge DPPH radicals. Leum Phua is one of the aromatic and indigenous purple sticky rice, enriched with flavonoids, especially anthocyanins, and have total antioxidant higher than other varieties black rices of Thai rice namely, Black Rose, Hawm Nil, and Klam (Suwannalert & Rattanachitthawat, 2011). In addition, our previous studies have examined the amount of phenolic compounds, flavonoids, antrocyanins and

antioxidant activities of Khaow-Mak crude extracts from colored rice varieties around Thailand. The results showed that Leum Phua glutinous rice showed the highest antioxidant activity of DPPH and it contains high amount of phenolic compounds. The results indicated crude extracts from Leum Phua Khaow-Mak might be used as a natural antioxidant (Wattanuruk et al., 2020). Moreover, Rice Research and Development Division (2019) (Rice Department, Ministry of Agriculture and Cooperatives of Thailand) also reported that Leum Pua rice has higher antioxidant than other colored rice. Boonsit et al., (2010) reported that Leum Phua glutinous rice contains higher antioxidants and bioactive compounds such as anthocyanins, γ -oryzanol and phenolic compounds than white rice. In other research, Leum Phua cultivar exhibits high antioxidant content and activity of bran extracts from Thai rice cultivars. The bran extract obtained from the black rice cultivar Leum Phua exhibited a potent scavenging effect towards DPPH radical, correlated with its high content in phenolic compounds (Peaparkdee et al., 2019; Pansiri et al., 2019).

The SRB assays performed similarly, exhibiting moderate to excellent correlation in the evaluation of the cytotoxicity of chemicals on cultured cells. The assays are used for cell density determinations, based on the measurement of cellular protein content (Vajrabhaya & Korsuwannawong, 2018). The method was used to test the toxicity of compounds for adherent cell by dyeing. The assay relies on the ability of SRB for the binding of protein components with the cells and has been fixed with a tissue culture plate. SRB is a bright-pink aminoxanthene dye with two sulfonic groups to bind with the basic amino acid residues under mild acidic conditions for the survive cellular protein contents. Strong intensities of SRB staining indicates number of cell viabilities (Thongdeeying et al., 2007). Cell viability was expressed as a percentage of the control values.

The crude extracts from Leum Phua Khaow-Mak at concentration between 0.0001 and 1 mg/ml were focused. The results show the human dermal fibroblast cells with stained SRB are similar as the control group. The standards, sodium lauryl sulfate were prepared. When the concentration of sodium lauryl sulfate increases, the stains of SRB dyed were reduced (Fig. 1). The concentrations of Leum Phua Khaow-Mak extracts were prepared at the concentrations of 0.0001, 0.001, 0.01, 0.1, and 1 mg/ml and their percentages of viabilities were 105.17, 105.82, 103.54, 103.49, and 102.29, respectively.

Whereas, the concentrations of sodium lauryl sulfate standards were 0.0001, 0.001, 0.01, 0.1, and 1 mg/ml cell and their percentages of viabilities were 99.43, 96.06, 94.32, 9.85, and 4.91, respectively. Then, the concentrations of 0.1 and 1.0 mg/ml had the lower survival cell percentage (Table 1). In addition, the study of cells appearance under the microscope method was found that the testing cells with Leum Phua Khaow-Mak crude extracts displayed the similar appearance with group of control cells, with no alteration of the typical spindle-shape cell morphology. On the other hands, the exposure cells with sodium lauryl sulfate were exhibited the different appearance from the group of control cells. Because of the cell division, the irregular shapes and the tissue were indistinguishable from the control (Table 2). Many researcher groups reported that sodium lauryl sulfate that are known to produce irritation when applied topically to skin. The contact irritants were cytotoxic for keratinocytes and fibroblasts and suppressed growth at lower concentrations than the contact sensitizers. The contact irritants also produced histological changes (hyperplasia, incomplete keratinization, loss of the granular layer, acantholysis and necrosis) in organ-cultured skin (James et al., 2007; Corinne et al., 1998).

The results showed that the crude extracts from Leum Phua Khaow-Mak with all concentrations do not affect the toxicity of the cell components. Due to the extracts containing with polyphenol, flavonoid and anthocyanin, these bioactive compounds in the outer membrane of rice as seed coat or rice bran with either black or red with high amounts of antioxidant (Lee et al., 2007 ; Shen et al., 2009). Based on literature (Chen et al., 2018), no obvious cytotoxic effect was found with the increase of the concentration of the alcohol free rice bran fermentation solution. Moreover, the fermented alcohol-free rice bran did not cause irritation. However, fermented alcohol-free rice bran had also antiaging, melanin prohibition, whitening and moisturizer. Moreover, the fermented purple plain sap samples showed high antioxidative, the high amounts of tyrosinase inhibition, the high MMP-2 inhibition activities with low cytotoxicity to normal human skin fibroblast by SRB assay in comparing to other rice sap samples (Aranya et al., 2011). On the other hand, it is well accepted that several plants are the richest source of antioxidants. In other studied, the rice cell extracts (at 25–100 µg/ml) were not cytotoxic to the human dermal fibroblasts and keratinocytes. Furthermore, significantly enhanced the migratory ability of the two cell types (Kim

et al., 2016). The hydroglycolic crude extract of Thai red Hom–Kularb–Drice rice bran on UVB induced photoaging of human skin fibroblast. The extract showed a no cytotoxic response was showed in human skin fibroblast (Yakaew et al., 2020). Black Rice Extract (BRE) treatment did not affect cell morphology and viability of HaCaT and human dermal fibroblasts. These findings suggest that BRE contained antioxidative flavonoid components such as cyanidin 3-O-β-D glycoside and taxifolin 7-O -glucoside (Han et al., 2018).

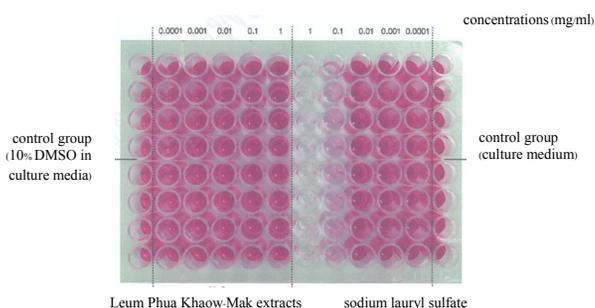


Fig. 1 The strong intensity of SRB staining is show that number of cell viability

Table 1 The percentage of viability of human dermal fibroblast cell

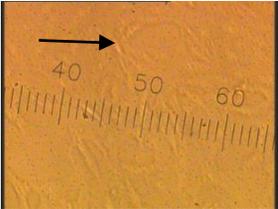
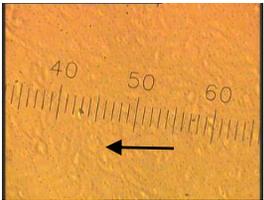
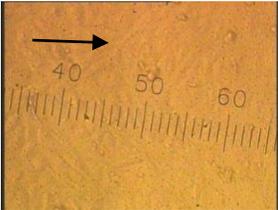
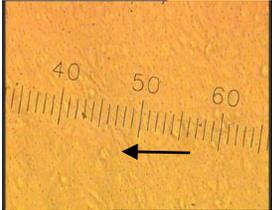
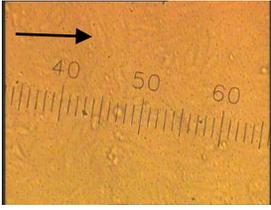
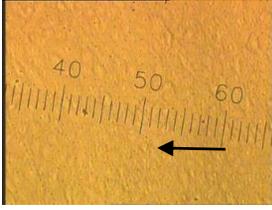
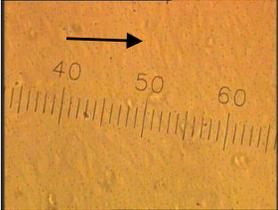
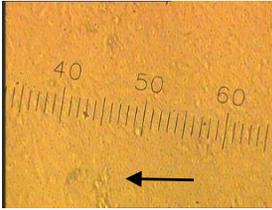
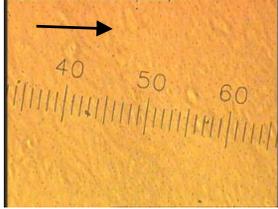
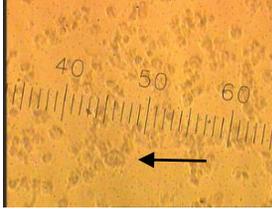
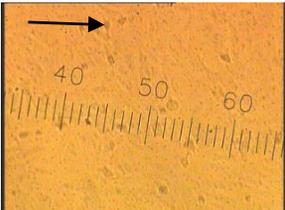
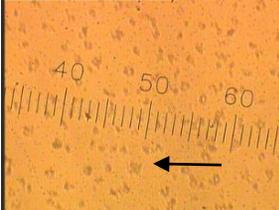
Test sample	Viability of human dermal fibroblast cell (%)				
	0.0001 (mg/ml)	0.001 (mg/ml)	0.01 (mg/ml)	0.1 (mg/ml)	1 (mg/ml)
Leum Phua Khaow-Mak extracts	105.17±3.93	105.82±2.69	103.54±2.69	103.49±2.76	102.29±4.42
Sodium lauryl sulfate	99.43±2.42	96.06±1.07	94.32±0.89	9.85±1.94	4.91±0.37

Remark: Each value are expressed as four replicate (mean±S.D.), (*p < 0.01)

Conclusion

In conclusion, the extract of Leum Phua Khaow-Mak exhibits good antioxidant and cytotoxic activities. The results can be summarized that EC_{50} value of the extract was 0.367 mg/ml, as similar to that of BHT ($EC_{50} = 0.239$ mg/ml). The concentrations of crude extracts from Leum Phua Khaow-Mak at 0.0001-1.0 mg/ml have the percentage of viability at 105.17-102.29 respectively. The percentage of viability of sodium lauryl sulfate at 0.0001- 1.0 mg/ml have the percentage viability at 99.43-4.91 respectively. The percentage of viability at the concentrations of 0.1 and 1.0 mg/ml were low. These obtained results indicated that the concentrations of crude extracts from Leum Phua Khaow-Mak in the range between 0.0001 and 1 mg/ml were non-toxic to human dermal fibroblast cells. The pure compounds of this strain might be further applied

Table 2 Characteristic of human dermal fibroblast cell under compound light microscope (100x) after was tested with Leum Phua Khaow-Mak extracts and sodium lauryl sulfate

concentrations (mg/ml)	Leum Phua Khaow-Mak extracts	Sodium lauryl sulfate
0 (control group)		
0.0001		
0.001		
0.01		
0.1		
1		

as antioxidant or anti-aging ingredients in cosmetic, food and other industries, including prevention of cellular oxidative damage.

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