Potential Anti-oxidative Activity of Crude Rice Oil Extracted from Cadmium-contaminated Rice as Determined Using an *In Vitro* Primary Human Fibroblast Cell Model

Thitinan Kitisin[1,4], Pahol Kosiyachinda[2] & Natthanej Luplertlop[3]

[1] Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand

[2] Center for Catalysis, Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand

[3] Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

[4] Center for Environmental Health, Toxicology and Management of Chemicals, Faculty of Science, Mahidol University, Bangkok, Thailand

Correspondence: Natthanej Luplertlop, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Tel: 66-894-807-337. E-mail: natthanej.lup@mahidol.ac.th

Abstract

Cadmium-contaminated rice was habitually consumed by local residents in Mae Sot Districk, Tak Province, Thailand. This study aimed to investigate the potential anti-oxidative activity of crude rice oil extracted from cadmium-contaminated rice as an alternative for utilizing cadmium-contaminated rice without compromising their health with hazard risks. The effects of crude rice oil extracted from cadmium-contaminated rice were determined in an *in vitro* primary human fibroblast (PHF) cell model. Results indicated that crude rice oil extracted from cadmium-contaminated rice exhibited anti-oxidative activities, in terms of preventing oxidative damage on cellular mitochondrial activity, down-regulation of *SIRT1* mRNA, and up-regulation of MMP-2 in PHF cells *in vitro*. It might be possible that *SIRT1* down regulation is related to MMP-2 up-regulation, which could delay aging and promote tissue remodeling in PHF cells. However, specific mechanisms for the regulation of these processes remain to be identified. This study demonstrates crude rice oil extracted from cadmium-contaminated rice exhibited the potential of anti-oxidative activity, which can be used for pharmaceutical and cosmeceutical use.

Keywords: crude rice oil, cadmium-contaminated rice, primary human fibroblast cell, SIRT1, MMP

1. Introduction

Plants absorb numerous elements from soil. Some of the absorbed elements are referred to as essentials because they are required for plants to complete their life cycle. However, plants also absorb elements which have no known biological functions and are even known to be toxic to the plants. These are arsenic, cadmium, chromium, mercury, and lead (Peralta-Videa et al., 2009).

Cadmium is one of important toxic heavy metals, which found contamination in soil and rice in Thailand and was reported in 1988 (Simmons et al, 2005). Mae Sot District in Tak Province is located on the Thailand-Myanmar border has zinc mining activities and rice paddy field. The cadmium contamination from what it co-exist naturally with zinc could show adverse effects on rice and other crops grew in the area and pose health hazard to local resident who habitually consumed cadmium-contaminated agricultural products (Shimada et al., 1977).

Rice (*Oryza sativa* L.) is one of the most important crops in Thailand. Cadmium toxicity in rice may induce rice self-defense mechanisms and trigger productions of a variety of natural products known as secondary metabolites (Jwa et al., 2006; Namdeo, 2007). Recently, many studies have reported several phytochemicals from rice, e.g. gamma-oryzanol (γ-oryzanol) (Xu & Godber, 1999), tocopherols, and tocotrienols (Shin & Godber, 1994). Previous reports showed that the phytochemicals from rice has potentials of anti-oxidative activities (Xu & Godber, 2001; Sasaki et al., 1990; Yasukawa et al., 1998).

In this study, we aimed to investigate the potential anti-oxidative activity of crude rice oil extracted from cadmium-contaminated rice as an alternative for utilizing cadmium-contaminated rice without compromising their health with hazard risks. The effects of crude rice oil extracted from cadmium-contaminated rice were determined in an *in vitro* primary human fibroblast (PHF) cell model. The expression of silent mating type information regulation 2 homolog 1 (*SIRT1*) gene and matrix metalloproteinase (MMPs) of PHF cells after being treated with crude rice oil was examined.

2. Materials and Methods

2.1 Crude Rice Oil Samples

Cadmium-contaminated rice grain was obtained from Mae Sot District, Tak Province, Thailand. Rice grain obtained from Pathumthani Province, Thailand was used as a non-contaminated control. The rice grain was ground with a mortar and stored in sealed plastic bags during the experiment, at $25\pm2°C$.

The crude rice oil extraction was performed, according to AOAC (1995) with some modifications. Exactly 40 g of grain sample was weighed and extracted with ethanol absolute (Merck) in a Soxhlet for 6 h at a condensation rate of 2-3 drops/s. The solvent was evaporated to dryness in a rotary evaporator. The remaining crude rice oil was weighed, diluted to 4 ml with ethanol absolute, and stored at -20°C before subsequent analysis.

2.2 Cell Preparation

Primary human fibroblast (PHF) cells (primary cells) were kindly provided by Asst. Prof. Dr. Natthanej Luplertlop from Applied Laboratory Unit, Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. PHF cells were maintained in Dulbecco's modified minimum essential medium (DMEM, Gibco, USA) supplemented with 10% heat inactivated (56°C for 30 min) fetal bovine serum (FBS, Hycone, USA), 1% L-glutamine, and 1% antibiotics (200 U/ml penicillin and 100 µg/ml of streptomycin, Gibco, USA) at 37°C with 5% concentration of CO_2 incubator. The cells were detached from surface using trypsin/EDTA (PAA Laboratories GmbH, Austria).

2.3 Anti-oxidative Activity: An In Vitro Assay

Cells for anti-oxidative activity experiments were cultured in 96 wells (12 x 8) cell culture plate. After subculture as described above, cell suspension was diluted in new medium to at least 1.0×10^5 cells/ml and 200 µl of the diluted cell suspension was transferred to each well. If not all 96 wells were used, only the middle wells of the plate would be used to prevent edging effect due to uneven evaporation and the unused wells were filled with water to maintain humidity. The plates were kept at 37°C in the 5% CO_2 incubator until they reached confluences (24 h) for subsequent processes.

2.4 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) Assay for Cell Viability

The colorimetric MTT assay used was essentially similar to that originally described by Mosmann (1983). A stock solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma-Aldrich, USA) was prepared by dissolving 7.5 mg MTT/ml in phosphate buffered saline (PBS) solution at pH 7.5 (Sigma-Aldrich, USA) and filtered through a 0.22 µm filter. After 24 h incubation of the test solutions were removed and the cells were washed two times with PBS. MTT solution was added at 100 µl of MTT in each well, which is yellow color. After incubation at 37°C in the dark for 2-3 h, the mitochondrial succinate dehydrogenases of viable cells cleaved the tetrazolium ring and yielded purple formazan crystals, which are insoluble in aqueous solution. The MTT solvent from each well was removed and washed with PBS. Then 100 µl of dimethyl sulfoxide (DMSO, Amresco, USA) was added in each well. The plate was spectrophotometrically measured at 570 nm on a microplate reader (Sunrise[TM] TECAN, Switzerland) correspond to a change in the amount of purple formazan formed or in the optical density and at 690 nm for background. The corrected readings from triplicate were averaged and expressed as the percentage of cellular mitochondrial activity. The highest concentrations of each test compound without cytotoxicity would be used to study its anti-oxidative activity. The percentage of cellular mitochondrial activity was calculated with the equation below:

$$\% \text{ cellular mitochondrial activity} = [(A_{570} - A_{690})/(A°_{570} - A°_{690})] \times 100$$

Whereas; A_{570}=Absorbance$_{570nm}$ of sample

A_{690}=Absorbance$_{690nm}$ of sample background

$A°_{570}$=Absorbance$_{570nm}$ of control

$A°_{690}$=Absorbance$_{690nm}$ of control background

2.5 Cytotoxicity Study of 2% Ethanol in DMEM

Since ethanol was required to dissolve γ-oryzanol in crude rice oil samples, the cytotoxicity of 2% ethanol, which was the final concentration after diluting in DMEM without FBS containing crude rice oil samples, was also considered. After PHF cells reached confluence, PHF cells were treated with 200 µl of 2% ethanol in DMEM without FBS was incubated with cells at 37ºC for 24 h in triplicate, followed by cell washing and MTT assay. The PHF cells treated with DMEM without ethanol and FBS was used for comparison.

2.6 Cytotoxicity Study of Crude Rice Oil Samples

PHF cells were treated with 200 µl of crude rice oil samples in the different concentrations of 250 mg/ml, 200 mg/ml, 150 mg/ml, 100 mg/ml, 50 mg/ml, mock (0%), and vitamin C served as positive control diluted with DMEM without FBS at 37ºC for 24 hours in triplicate, followed by cell washing and MTT assay.

The lethal dose of cytotoxic concentration from crude rice oil samples was expressed as 50% cellular mitochondrial activity, LD_{50} (mg/ml) and was obtained by interpolation from linear regression analysis. Vitamin C was used for comparison.

2.7 Primary Human Fibroblast Cells Treatment for Expression Analysis

In order to analyze the expression of *SIRT1* gene and MMPs, primary human fibroblast (PHF) cells seeded at a density of 1.0×10^5 per well, were grown in 24 wells plate and 1 ml of the diluted cell suspension was transferred to each well. The plates were kept at 37°C in the 5% CO_2 incubator until they reached confluence. After 24 h, cells was washed with PBS and added 1 ml of diluted crude rice oil samples at LD_{50} concentration that determined by MTT assay and added 1 ml of 2% ethanol in DMEM without FBS for control. The commercial γ-oryzanol available in the market was used to compare with crude rice oil samples. The commercial γ-oryzanol was supplied as a gift from a well-known manufacturer and kindly provided by Asst. Prof. Dr. Natthanej Luplertlop from Applied Laboratory Unit, Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. The vitamin C was used as a positive control for anti-oxidative agent. The cells were incubated in time series (1 h, 6 h, 12 h, 24 h, 48 h, and 72 h). The morphology of PHF cells were observed under the inverted microscope (Motic® AE21, USA). The supernatant was collected in each time series and cells in each time were used for mRNA extraction.

The supernatants were measured by using a NanoDrop® meter (ND-1000 UV-Vis Spectrophotometer V3.5, NanoDrop® Technologies Inc., USA) to determine the concentration of total proteins. The pH of supernatants was measured by using a pH meter (IQ240 pH meter, IQ Scientific Instruments, USA). The pH meter was standardized against pH buffers over the pH range of 6.0 to 8.5 and with the pH buffer at 7.0. Readings were reproducible to±0.01 pH unit.

2.8 Determination of SIRT1 mRNA Expression by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

After inoculated with crude rice oil samples in time series and the supernatant was removed, the total RNA of PHF cells was extracted with Trizol Reagent (Invitrogen, USA). According to the manufacturer's instructions, the total RNA was reverse-transcribed to a complementary DNA (cDNA) then cDNA amplified by using the one step reverse transcriptase (RT-PCR) method. The process of reverse transcription and cDNA amplification was done in one tube assay by using QIAGEN One-Step RT-PCR Kit (QIAGEN, USA). The extracted RNA was added to RT-PCR mixture. In this protocol, 100 ng of total RNA was amplified in 25 µl volumes.

All PCR primers are shown in Table 1 The PCR primer for *SIRT1* was designed according to the human *SIRT1* gene sequence (NM_012238) from GenBank (Bai et al., 2008). *HUGAPDH* was determined as a control for loading (Zhang & Sun, 2010).

PCR condition was as follows: cDNA strand was synthesized at 50ºC for 30 min, pre-denature (95ºC for 15 min), 30-35 cycles of denaturation (94ºC for 30-45 s), primer annealing (52-53.5ºC for 30 s), primer extension (72ºC for 1 min), final extension (72ºC for 10 min), cool down for stop reaction at 4ºC, and stored at -20 ºC. RT-PCR products will be analyzed by agarose gel electrophoresis.

2.9 Gelatinolytic Activity Assay by SDS-PAGE Gelatin–embedded Enzymography (zymogram) for MMPs Detection

The primary human fibroblast (PHF) cells were used to study the expression of MMPs with zymogram technique after crude rice oil treatments. The PHF cells were inoculated with crude rice oil samples and the supernatants were collected at 1-72 h post stimulation as mentioned previously. In zymogram technique, gelatin zymography was used to detect MMP-2 (72 kDa gelatinase A) as well as MMP-9 (92 kDa gelatinase B) according to the method of Heussen and Dowdle (1980) with some modifications. The vertical stab minigel apparatus (Bio-Rad

Laboratories, USA) of polyacrylamide gel was used with a separating gel (1.5 mm thickness) containing acrylamide 7% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) embedded with 0.1% gelatin and a stacking gel containing 5% (w/v) acrylamide. The amount of 100 µg of total proteins was loaded with sample buffer. The supernatant from primary human fibroblast cells induced by 10 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, USA) was used as a positive control.

Table 1. Primers for RT-PCR reaction

Genes	Primers (5′-3′)	Product size (bp)	Annealing temperature (°C)	Cycle times
SIRT1	F: 5′-GAACAGGTTGCGGGAATC-3′	553	53.5	30
	R: 5′-AACATGAAGAGGTGTGGGTG-3′			
HUGAPDH	F: 5′-GTGGACCTGACCTGCCGTCT-3′	323	52	35
	R: 5′-CTTCCTCTCGTGCTCTTGCT-3′			

2.10 Data Analysis

All data were obtained from one independent experiment carried out in triplicate. Results are expressed as means ±standard deviation. Analysis of variance was performed by a nonparametric Kruskal-Wallis test, and then a Mann-Whitney U test was performed for comparison between groups. The results in each experiment were analyzed by using SPSS (statistical package version 16, SPSS Inc., Chicago, Illinois) software. Differences were considered statistically significant at $p<0.05$.

3. Results

3.1 Cytotoxicity Study of 2% Ethanol in DMEM

To measure cell viability under oxidative stress, an MTT assay was used to measure the activity of mitochondrial dehydrogenase in living cell (Mosmann, 1983). Since ethanol was one of the few solvents that could be used to dissolve γ-oryzanol in crude rice oil samples and was used for cell culture experiments, the effect of 2% ethanol in DMEM without FBS was studied with MTT assay. The results showed in Figure 1.

The cellular mitochondrial activity of primary human fibroblast cells incubated from 2% ethanol treated group (98.52±1.24%) was not significantly different from the control (100%) (DMEM without 2% ethanol and FBS) when incubated with PHF cells for 24 h ($p>0.05$). Therefore, in the further experiments, 2% ethanol in DMEM without FBS was always performed and referred to as the control for comparison.
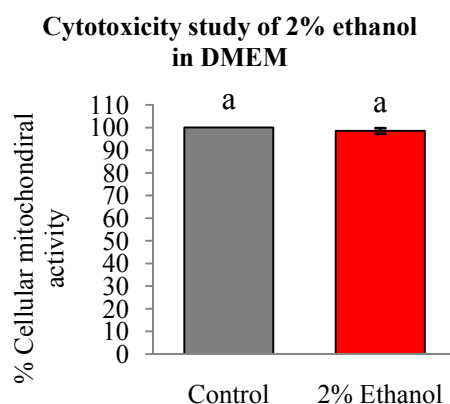

Figure 1. Cytotoxicity study of 2% ethanol in DMEM without FBS incubated with primary human fibroblast cells at 37ºC for 24 h

The number of viable cells was determined by MTT assay in triplicate; Data are expressed as means±standard deviation; Significant differences ($p<0.05$) are expressed by different letters.

3.2 Cytotoxicity Study of Crude Rice Oil Samples

In order to assess cytotoxicity of crude rice oil samples on PHF cells, their capability to reduce MTT was determined. Crude rice oil samples (dissolved with 2% ethanol in DMEM without FBS) in a serial concentrations up 250 mg/ml was added to confluent PHF cells (200 µl/well) for 24 h at 37°C. The cytotoxicity results showed that, when incubated the PHF cells with crude rice oil samples for 24 h, the effects of crude rice oil extracted from cadmium-contaminated rice and control rice from Pathumthani Province on cellular mitochondrial activity were not the same when averaged out for concentration effect (Figure 2).
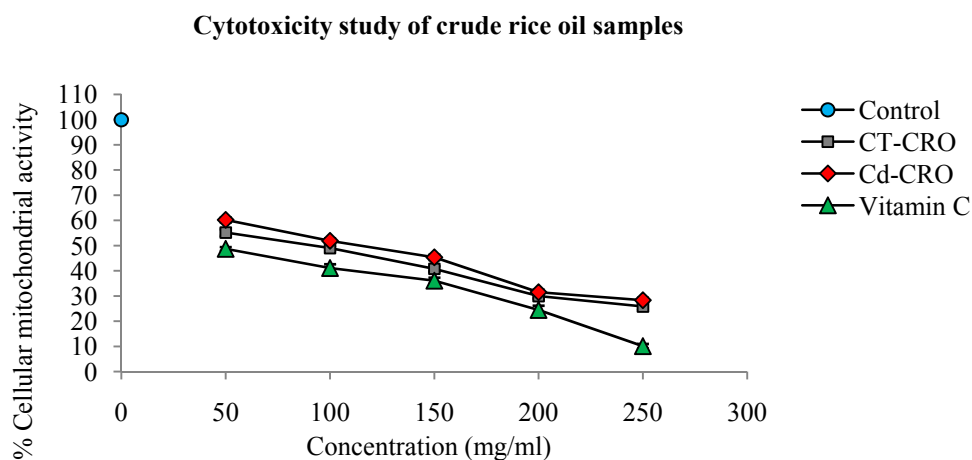
Cytotoxicity study of crude rice oil samples

Figure 2. Cytotoxicity study of crude rice oil extracted from control rice (CT-CRO), cadmium-contaminated rice (Cd-CRO), and viamin C incubated with primary human fibroblast cells at 37ºC for 24 h

The number of viable cells was determined by MTT assay in triplicate. Data are expressed as means±standard deviation.

From the Figure 2, treatments with crude rice oil extracted from cadmium-contaminated rice and control rice from Pathumthani Province reduced the cell viability in a dose-dependent manner. Results from vitamin C were similar to crude rice oil samples in MTT assay. The result of cell viability from crude rice oil samples and vitamin C was significantly different ($p<0.05$) when compared with the control. However, the cellular mitochondrial activity of all crude rice oil samples was higher than that of vitamin C at the same concentration.
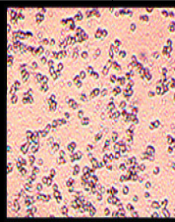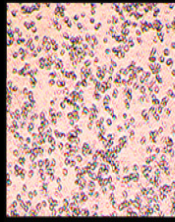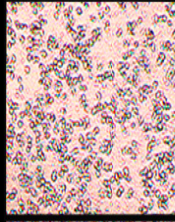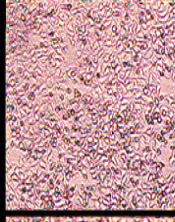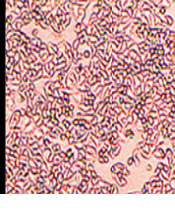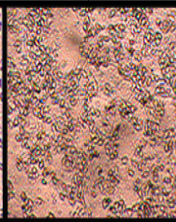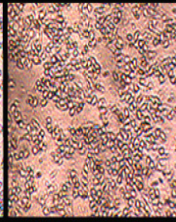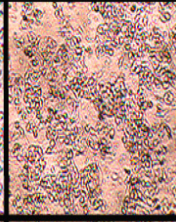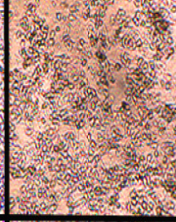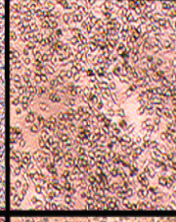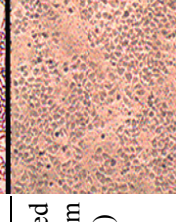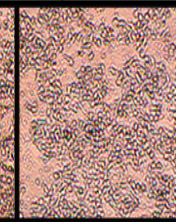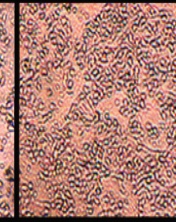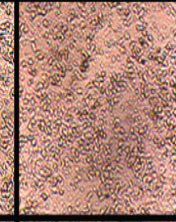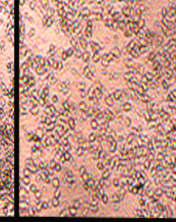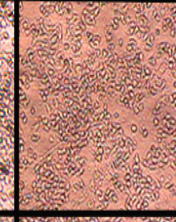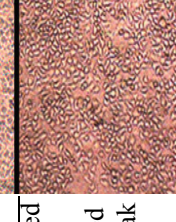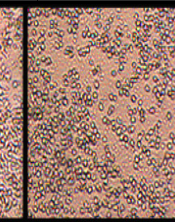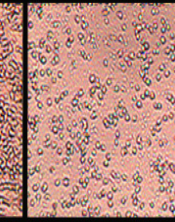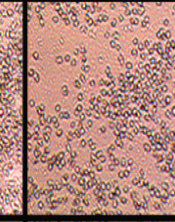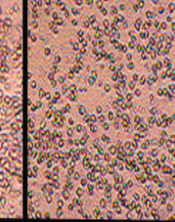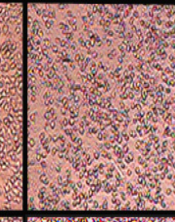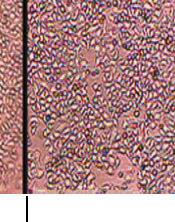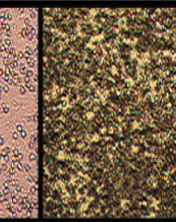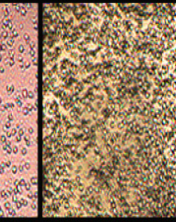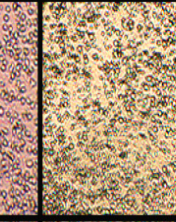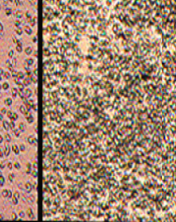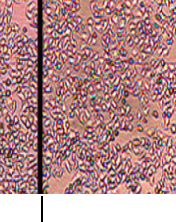
The lethal dose at LD_{50} values of crude rice oil extracted from cadmium-contaminated rice and control rice from Pathumthani Province determined by MTT assay are shown in Table 2. With regard to LD_{50} values of cytotoxic concentration, the maximum concentration of crude rice oil samples without affecting cell viability extracted from cadmium-contaminated rice was found (111.37 mg/ml) higher than crude rice oil extracted from control rice from Pathumthani Province (86.97 mg/ml). All crude rice oil samples gave higher LD_{50} than standard vitamin C (LD_{50}=54.76 mg/ml).

Table 2. LD_{50} values (mg/ml) of cytotoxic concentration on 50% cellular mitochondrial activity of crude rice oil extracted from cadmium-contaminated rice and control rice

Type of Samples	LD_{50} (mg/ml)[*]
Control rice (rice from Pathumthani Province)	86.97
Cadmium-contaminated rice (rice from Mae Sot area)	111.37
Vitamin C	54.76

[*]LD_{50} values were calculated by interpolation from linear regression analysis.

Figure 3. Morphology of primary human fibroblast cells post stimulation from various treatments at 1-72 h under phase contrast microscope, 40X magnification

Treatments	Morphology of primary human fibroblast cells post stimulation at various times					
	1 h	6 h	12 h	24 h	48 h	72 h
Mock						
Crude rice oil extracted from control rice (from Pathumthani Province)						
Crude rice oil extracted from cadmium-contaminated rice (from Mae Sot, Tak Province)						
Commercial γ-oryzanol						
Vitamin C						

3.3 Cell morphology of Primary Human Fibroblast Cells after being Treated with Crude Rice Oil Samples

The primary human fibroblast cultured cells were examined under an inverted microscope for their different morphologies after being treated with crude rice oil samples, commercial γ-oryzanol, vitamin C, and mock in various time post stimulations (Figure 3). The physical changes of PHF cells after being treated with various treatments could be used to show the effect of anti-oxidant activity to maintain cells integrity under oxidative stress.

Figure 3 showed the morphology of primary human fibroblast cells in mock from first hour post stimulation. The cells in the culture plate were adherent cells. They tended to cluster together and appeared as a form of continuous sheet. A very small intercellular space could be observed. Imaging the cells under an inverted microscope (40X magnification) showed a polygonal appearance when viewed from above. At first hour post stimulation, morphology of PHF cells in all treatment groups showed a similar appearance to mock. PHF cells in all treatment groups and mock were observed for their morphological changes over time. PHF cells started to change their morphology from polygonal shape to round shape as incubation time increased (Figure 3). During 24-72 h post stimulation, some of PHF cells were detached from culture plates. The commercial γ-oryzanol treated group and mock showed the most cells detached and fewer detached cells were observed in vitamin C treated group. The accumulations of cellular debris were found in all treated group, which was found most in the vitamin C treated group.

3.4 pH of Primary Human Fibroblast Cell Supernatants after Being Treated with Crude Rice Oil Samples

The pH values in primary human fibroblast cell culture supernatants were measured after being treated with crude rice oil samples, commercial γ-oryzanol, vitamin C, and mock in various time post stimulations (Figure 4). Changes of pH in PHF cell supernatants after being treated with various treatments could be used to study the effect of anti-oxidative activity to cell culture supernatants under oxidative stress. From the result, the pH of cell culture in mock from first hour post stimulation represented a normal pH of the cell supernatant. The pH values in cell culture supernatant from both crude rice oil samples and commercial γ-oryzanol treated groups showed the pH values in the range of 6.9-7.0. The results showed similar pH values to mock group at first hour post stimulation. The pH value in cell culture supernatant of vitamin C treated group at first hour post stimulation was 6.60±0.15, which showed higher acidity compared to other groups. Moreover, as the incubation time increased, the acidity in cell supernatants increased in all treated groups and mock. At 72 h post stimulation, the pH value in cell culture supernatant from vitamin C treated group showed highest acidity (5.87±0.15) and crude rice oil extracted from cadmium-contaminated rice showed lowest acidity (6.8±0.1).
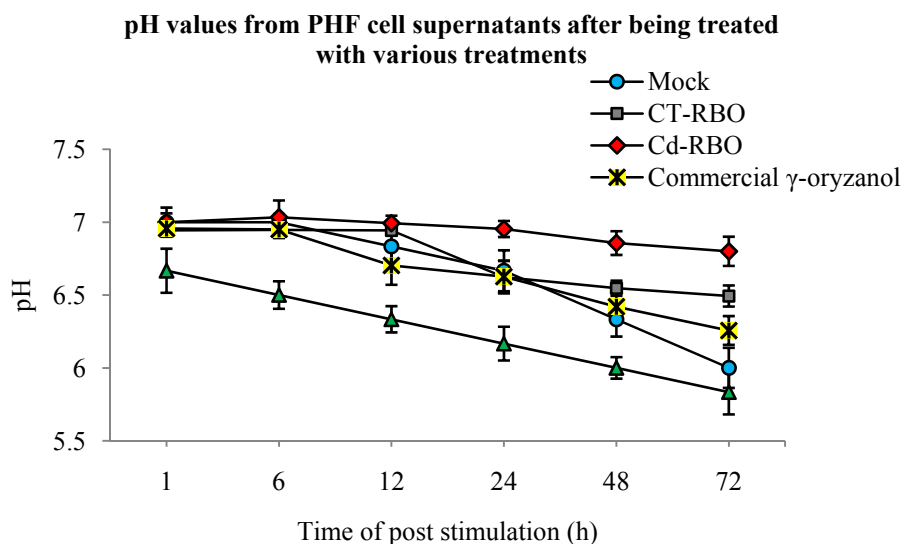

Figure 4. pH values from primary human fibroblast cell supernatants after being treated with crude rice oil extracted from control rice (CT-CRO), cadmium-contaminated rice (Cd-CRO), commercial γ-oryzanol, and vitamin C at 1-72 h post stimulation in triplicate. Data are expressed as means±standard deviation.

3.5 Determination of SIRT1 mRNA Expression by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The study of *SIRT1* mRNA expression after primary human fibroblast cells were treated with crude rice oil samples, One-Step RT-PCR technique was performed. Total RNA was extracted from PHF cells at various times under normal (mock) and post stimulated conditions. The electrophoresis bands of *SIRT1* and *HUGAPDH* (the housekeeping gene) were changed into OD value by a densitometer. The ratio of *SIRT1* and *HUGAPDH* OD value was termed as *SIRT1* relative mRNA expression. The results showed an amplicon band of *SIRT1* PCR product at 553 bp and *HUGAPDH* PCR product at 323 bp (Figure 5).
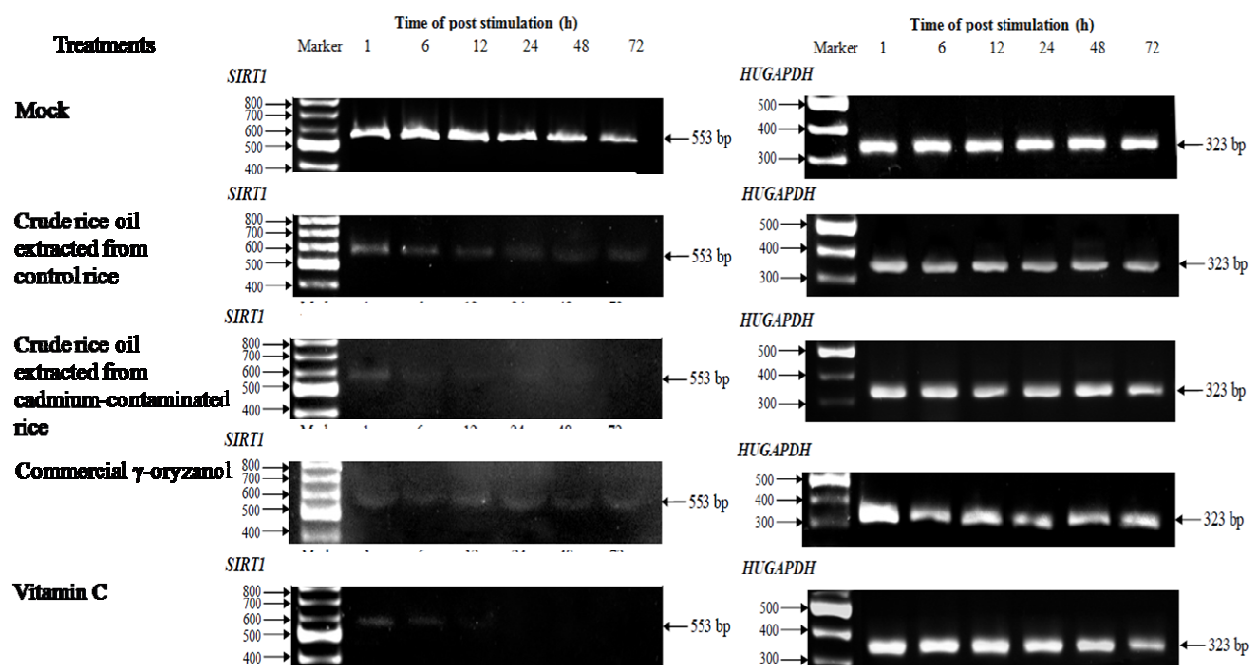
Figure 5. Time-spatial expression of *SIRT1* in PHF cells after being treated with various treatments at 1-72 h post stimulations

Total RNA was isolated and subjected to RT-PCR analysis. The results were represented as ethidium bromide stained gels of target products (*SIRT1*) as well as *HUGAPDH* in the same sample.

Time-spatial expressions of *SIRT1* mRNA in primary human fibroblast cells under normal and post stimulated conditions were investigated. Analysis indicated *SIRT1* mRNA was differently expressed in PHF cells after being treated with crude rice oil samples (extracted from cadmium-contaminated rice and control rice from Pathumtani Province), commercial γ-oryzanol, and vitamin C when compared to mock. *SIRT1* mRNA was most expressed in mock and least in vitamin C treated group (Figure 5). However, the level of *SIRT1* mRNA expression did not affect the level of *HUGAPDH*.

To determine the difference of expression of *SIRT1* mRNA in primary human fibroblast cells after being treated with various treatments, the densitometer was used to obtain the quantity of the electrophoresis band. The expression of *SIRT1* by PHF cells from various treatments post stimulation is shown in Figure 6 expressed as a fold change in comparison to levels found in the control cells.

From the results, *SIRT1* mRNA expression was significantly different between non-treated group (mock) and treated groups ($p < 0.05$) (Figure 6). When compared with the expression profile observed in the treated group, *SIRT1* mRNA highest peak expression from PHF cells after being treated with crude rice oil samples (extracted from cadmium-contaminated rice and control rice) and vitamin C was detected at first hour post stimulation (0.177 ± 0.014 fold, 0.127 ± 0.015 fold, and 0.030 ± 0.017 fold, respectively). The level of *SIRT1* mRNA expression in crude rice oil extracted from control rice was significantly higher than crude rice oil extracted from cadmium-contaminated rice ($p < 0.05$) in different time of post stimulation. Moreover, *SIRT1* mRNA expression

in most treated groups was down-regulated in a post stimulation time dependent manner except only in commercial γ-oryzanol treated group, which showed slight up-regulating of *SIRT1* mRNA expression.

Relative fold expression of SIRT1 mRNA in PHF cells after being treated with various treatments
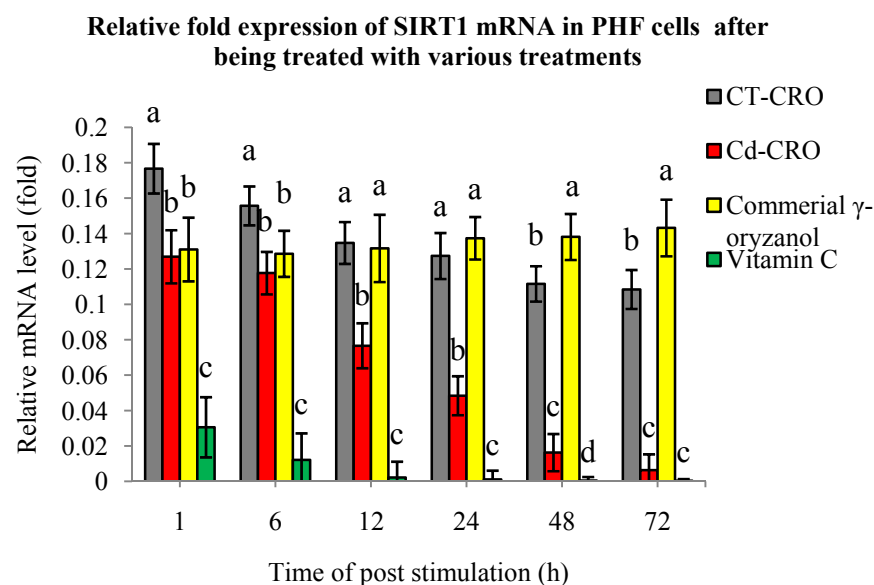
Figure 6. Relative fold expression of *SIRT1* mRNA in primary human fibroblast cells after being treated with crude rice oil extracted from control rice (CT-CRO), cadmium-contaminated rice (Cd-CRO), commercial γ-oryzanol, and vitamin C at 1 h, 6 h, 12 h, 24 h, 48 h, and 72 h post stimulations

The effect of each treatment on *SIRT1* mRNA expression assigned as fold expression of *SIRT1* from treated group to those in mock. The results represented as means±standard deviation of three independent determinations. Different letters indicate significant differences ($p<0.05$).

3.6 Gelatinolytic Activity Assay by SDS-PAGE Gelatin–embedded Enzymography (zymogram) for MMPs Detection

In order to investigate the effects of crude rice oil samples on expression of MMP-2 and MMP-9, gelatinolytic activity assay was performed using the supernatant of cell after being treated with different treatments and PMA to stimulate positive expression of MMPs in primary human fibroblast cells. PHF cell culture supernatants were collected at 6 different time periods (1 h, 6 h, 12 h, 24 h, 48 h, and 72 h post, respectively), lyophilized, and assayed for their MMPs content. All samples show zymolytic band of MMP-2 (72 kDa gelatinase A) but no MMP-9 (92 kDa gelatinase B) bands appeared on SDS-PAGE gel (Figure 7).

Time-spatial expressions of MMP-2 in primary human fibroblast cell culture supernatants after normal and stimulated conditions were investigated. Analysis indicated MMP-2 was differently expressed in PHF cell culture supernatants after being treated with crude rice oil samples (extracted from cadmium-contaminated rice and control rice from Pathumtani Province), commercial γ-oryzanol, and vitamin C when compared to mock (Figure 7). MMP-2 was most expressed in vitamin C and least in commercial γ-oryzanol treated group (Figure 7). It is important to note that, of the two principal gelatinases, only MMP-2 was detected in the zymograms. No other gelatinase activity bands were observed on the gels, suggesting that the other major gelatinease, MMP-9, was not present in detectable amounts in these experiments. The unique detection of MMP-2 in cell culture supernatant of PHF cells may indicate the important role of this collagen-degrading enzyme in the remodeling of ECM in PHF cells *in vivo*.
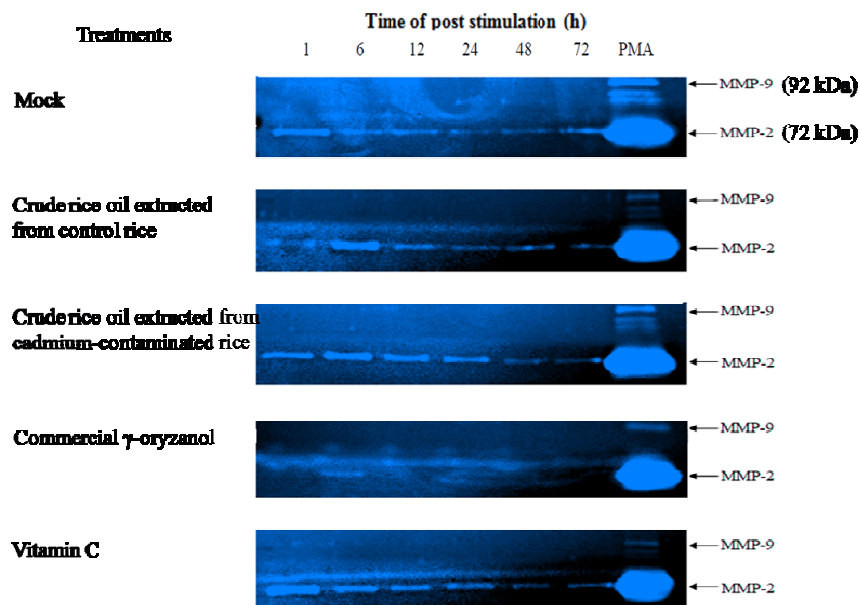
Figure 7. Time-spatial expression of MMP-2 in primary human fibroblast cell culture supernatants after being treated with various treatments at 1 h, 6 h, 12 h, 24 h, 48 h, and 72 h post stimulations

MMP-2 activities in cell culture supernatants were determined by gelatin zymography.The supernatant from PHF cells induced by PMA was used as a positive expression of MMP-9 and MMP-2. The results are representative of three independent experiments.

To determine the difference of enzymatic activity of MMP-2 in cell culture supernatants from various treatments, the densitometer was used to obtain the quantity of the zymolytic band. The expression of MMP-2 by primary human fibroblast cells from various treatments post stimulation is shown in Figure 8. The results are expressed as a fold change in comparison to levels found in the control cells.

Under normal conditions, the expression of MMP-2 from primary human fibroblast cell culture supernatants reached a peak at first hour post stimulation, and then fluctuated until at 48 hours post stimulation the expression of MMP-2 was again increased. For treated groups, the expression of MMP-2 showed similar phenomenon to the non-treated group.

Furthermore, MMP-2 expression of PHF cells after being treated with crude rice oil samples (extracted from cadmium-contaminated rice and control rice) and vitamin C was produced in high level of more than 2 folds at 6 h post stimulation, compared to the non-treated group at the same time of collection (Figure 8). After PHF cells was treated with crude rice oil extracted from cadmium-contaminated rice, the expression of MMP-2 was significantly higher than other treatments at 6 h post stimulation ($p<0.05$). The results showed no significant difference in the amount of MMP-2 expression of PHF cells after being treated with crude rice oil extracted from cadmium-contaminated rice and vitamin C ($p>0.05$) at 12 and 24 h post stimulation. However, both treatments exhibited significantly higher MMP-2 expression than crude rice oil extracted from control rice and commercial γ-oryzanol treated groups at the same time of post stimulation ($p<0.05$). The MMP-2 expression of PHF cells after being treated with crude rice oil extracted from control rice at 48 and 72 h post stimulation was significantly increased and higher than crude rice oil extracted from cadmium-contaminated rice treated group ($p<0.05$). The level of MMP-2 expression after being treated with vitamin C was significantly higher than non-treated group ($p<0.05$) at different time of post stimulation. It was observed that most of the treatments up-regulated the MMP-2 expression in a time dependent manner when compared the fold expression to non-treated group. However, commercial γ-oryzanol could not efficiently up-regulated the MMP-2 expression in PHF cells.

Relative fold expression of MMP-2 in PHF cell culture supernatants after being treated with various treatments
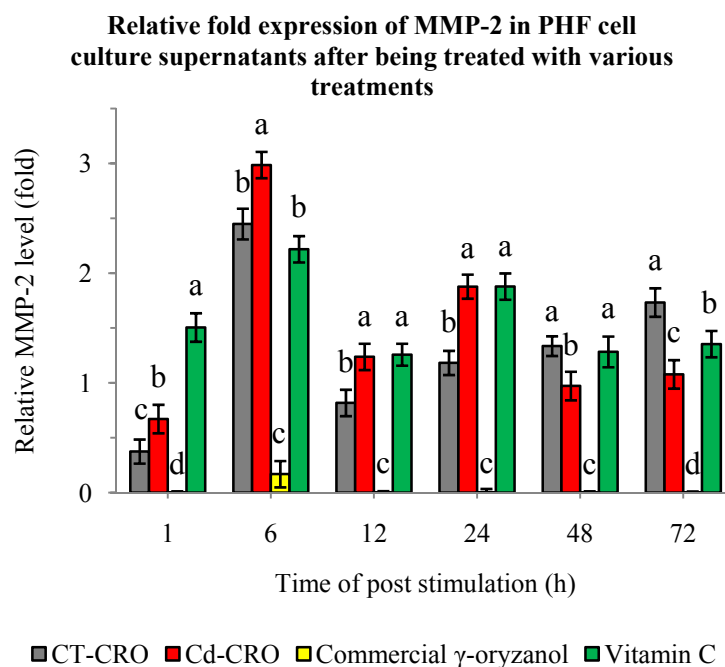

Figure 8. Relative fold expression of MMP-2 in primary human fibroblast cell culture supernatants after being treated with crude rice oil extracted from control rice (CT-CRO), cadmium-contaminated rice (Cd-CRO), commercial γ-oryzanol, and vitamin C at 1 h, 6 h, 12 h, 24 h, 48 h, and 72 h post stimulations

The effect of each treatment on MMP-2 expression assigned as fold expression of MMP-2 from treated group to those in mock. The results represented as means±standard deviation of three independent determinations. Different letters indicate significant differences ($p<0.05$).

When considering the relationship between *SIRT1* and MMP-2 expression, the expression level of *SIRT1* mRNA was decreased in PHF cells after being stimulated with crude rice oil samples (extracted from cadmium-contaminated rice and control rice) and vitamin C. In contrast, the level of MMP-2 expression was increased. From the results, it indicated that MMP-2 expression was influenced by expression of *SIRT1* under these stimulated conditions.

4. Discussion

4.1 Cytotoxicity Study of 2% Ethanol in DMEM

Since γ-oryzanol the most anti-oxidant found in rice bran oil is not water soluble (Patel & Naik, 2004), it has been a challenge to prepare crude rice oil solution that γ-oryzanol could be dissolved for cell culture studies. Ethanol is a solvent that can dissolve the highest amount of γ-oryzanol from rice bran as reported by Chen & Bergman (2005). Therefore, in primary human fibroblast cell culture experiment, the cytotoxicity of 2% ethanol in DMEM without FBS was studied. The results showed that, the cellular mitochondrial activity of PHF cells incubated with 2% ethanol in DMEM without FBS (98.52±1.24%) was not significant different from the control (100% DMEM without FBS) after 24 h incubation ($p>0.05$) (Figure 1). This indicated that no cytotoxicity of 2% ethanol on the PHF cells was observed. The result has also anticipated the alternative way to solubilize the crude rice oil extract for cosmetic and pharmaceutical applications.

4.2 Cytotoxicity Study of Crude Rice Oil Samples

The crude rice oil samples extracted from both cadmium-contaminated rice and control rice from Pathumthani Province were investigated for PHF cells cytotoxicity by MTT assay. The cytotoxicity results showed that when incubated with PHF cells for 24 h, the effects of crude rice oil extracted from cadmium-contaminated rice was significantly higher the cellular mitochondrial activity of PHF cells than crude rice oil extracted from control rice and vitamin C ($p<0.05$) in every tested concentration (Figure 2). The LD_{50} of crude rice oil extracted from cadmium-contaminated rice and control rice from Pathumthani Province and standard vitamin C were

significantly different ($p<0.05$) at 111.37 mg/ml, 86.97 mg/ml, and 54.76 mg/ml, respectively. This indicated that crude rice oil samples have lower cytotoxicity effect to PHF cells than vitamin C. Therefore, we used LD_{50} concentration of crude rice oil samples and vitamin C as the optimal concentration.

Gamma-oryzanol is a mixture of ferulate esters with triterpene alcohol and plant sterols (Xu & Godber, 2001; Banthorpe, 1991; Rogers et al., 1993). In 1992, Kahlon et al. reported that phytosterols from plant helps to lowering the cholesterol in animal cells. It has been reported that γ-oryzanol is an anti-oxidative compound and is associated with decreasing serum cholesterol (Sasaki et al., 1990). Gamma-oryzanol has also been used to protect the skin from oxidative damage due to environmental influence or chemical treatment (Brigitte, 1995). From the previous studies reported on the protective role of γ-oryzanol in oxidative damage, therefore, the less cytotoxicity from PHF cells after being treated with crude rice oil extracted from cadmium-contaminated rice and control rice from Pathumthani Province may result from γ-oryzanol in crude rice oil samples.

Thus, it may be speculated that association with PHF cells, probably on cell membrane, is necessary for γ-oryzanol in crude rice oil to protect cells from oxidative damage. When consider the structure of γ-oryzanol, the presence of the sterol or triterpene alcohol part in γ-oryzanol seems to alter the anti-oxidative activity (Goad, 1991), which may enhance the mechanical stability of the PHF cell membrane.

4.3 Determination of SIRT1 mRNA Expression by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Sirtuin 1 (SIRT1), a mammalian homolog of silencing information regulator 2 (Sir2) in yeast, belongs to the nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase family and had been implicated in aging metabolism, and stress resistance (Michan & Sinclair, 2007). Therefore, the effect of crude rice oil with anti-oxidative activity on the *SIRT1* mRNA expression in primary human fibroblast cells was investigated. From the results, *SIRT1* mRNA expression was significantly higher under normal condition than post stimulated condition ($p<0.05$) (Figure 5 and Figure 6). The level of *SIRT1* mRNA expression in PHF cells after being treated with crude rice oil extracted from cadmium-contaminated rice was significantly lower than in PHF cells after being treated with crude rice oil extracted from control rice ($p<0.05$) at different time of post stimulation. In most treated groups, *SIRT* mRNA expression in PHF cells was down-regulated in a post stimulation time dependent manner. Especially after PHF cells were treated with vitamin C, *SIRT1* mRNA expression was significantly down-regulated when compared to others treated groups ($p<0.05$). But after PHF cells were treated with commercial γ-oryzanol, *SIRT1* mRNA expression was slightly up-regulated in a time-dependent manner.

Previous reports have studied on the SIRT1 function on cell survival and aging. Cell culture experiments showed that SIRT1 can prevent cell apoptosis and senescence (Zhao et al., 2008). As SIRT1 promote longevity, one would expect that SIRT1 activation or increased expression should protect against cancer. SIRT1 could mediate this protective effect by limiting replicative lifespan, by protecting against DNA damage and oxidative stress or by guarding against accumulation of mutations and against genomic instability. In contrast, SIRT1 over expression allows rapid proliferation and loss of checkpoints to promote continued propagation in the presence of accumulating mutations and leading to cancer (Saunders & Verdin, 2007). In 2008, Han et al. showed that SIRT1 promotes p53 (tumor suppressor) transcription-independent apoptosis. When Reactive oxygen species (ROS) activate cytoplasm SIRT1 protein, SIRT1 binds to and deacetylates p53. SIRT1 can redirect p53 from the cytosol to the mitochondria in response to increased ROS. The biological consequence of which is transcription-independent p53 induced apoptosis.

From the morphology of primary human fibroblast cells under normal and post stimulated conditions, during 24-72 h post stimulations, the PHF cells after treated with commercial γ-oryzanol showed most cells detached similar to normal group (Figure 3). The highest *SIRT1* expression from normal group and slightly up-regulation of *SIRT1* expression from commercial γ-oryzanol treated group may have resulted from the oxidative stress induced by ROS which could lead to cell death. The results suggested that commercial γ-oryzanol produced from manufacturer may not be able to reduce the oxidative stress in PHF cells. This suggested that commercial γ-oryzanol was poor anti-oxidative agent.

The PHF cells after treated with vitamin C showed down-regulation of *SIRT1* significantly differences from normal condition and other stimulated condition. The high anti-oxidative activity of vitamin C may have decreased the oxidative stress by lowering the ROS of PHF cells. However, the morphology of PHF cells after treated with vitamin C in all post stimulation times were found most cell debris than other groups. The result showed that pH in cell culture supernatant from vitamin C treated group was decreased to 5.87 at 72 h post stimulation (Figure 4). This indicated that cell debris may have resulted from acidosis caused by vitamin C. Mackenzie et al. (1961) reported that acidification of the medium could caused a striking increase in the number of cytoplasmic granules present in the HeLa liver cell line, increase in cell debris, and inhibit cell growth. Thus,

after PHF cells treated with vitamin C, the acidosis of culture medium could inhibit cell growth as incubation time increased. Moreover, previous report demonstrated that vitamin C induced apoptosis in HCT116 cell line (Camins et al., 2009). From the experiment, the result suggested that vitamin C processes the ability to scavenge oxidatively generated free radicals but also could inhibit cell growth caused by acidosis from its acidic property.

On the other hand, the PHF cells after treated with crude rice oil samples showed their morphology slightly changed when incubation time increase compared with all groups (Figure 3). From the present study, it seemed that crude rice oil samples could decrease the oxidative stress from their anti-oxidative activity. The PHF cells after treated with crude rice oil samples showed down-regulation of *SIRT1*, which may have resulted from inactivation of ROS. However, the specific mechanism for the regulation of these processes remains to be identified.

Therefore, the anti-oxidative activity of crude rice oil samples may probably help to delay aging in PHF cells by inactivation of p53, thus, leading to cell survival. The results indicated that crude rice oil samples exhibit the anti-oxidative activity and could provide anti-aging property. Crude rice oil extracted from cadmium contaminated rice showed higher anti-oxidative activity and exhibited lower down-regulation of *SIRT1* mRNA expression after stimulated in PHF cells than crude rice oil extracted from control rice ($p<0.05$). The anti-oxidative activity of this crude rice oil may help to delay aging, which provide an evidence for further alternative approach of using cadmium-contaminated rice without compromising with health hazard risks.

4.4 Gelatinolytic Activity Assay by SDS-PAGE Gelatin–embedded Enzymography (Zymogram) for MMPs Detection

Matrix metalloproteinases (MMPs) comprise a family of extracellular matrix degrading enzymes that are believed to play pivotal roles not only as a structural support but also as a biological regulator of cell growth and differentiation (Shapiro, 1998). Thus, the MMPs are important for tissue remodeling during aging, morphogenesis, wound healing, angiogenesis, and pathological processes, such as tumor invasion (Stetler-Stevenson et al., 1993; Tryggvason et al., 1993). However, turnover of collagen is rapid in young human skin and unlike other proteins, falls to a very low level as human aged or their growth rates decline (Varani et al., 2004). To increase collagen turnover and hence affect positively tissue degradation caused by aging, factors that increase the expression and activation of MMPs can be sought to trigger the degradation of older collagen, which may stimulate the production of more new collagen (Keller et al., 2009). In this study, a primary human fibroblast cell model was used to examine the effects of crude rice oil extracted from cadmium-contaminated rice may have on the activity of MMPs comparison with crude rice oil extracted from control rice from Pathumthani Province. From the results, the MMP-2 expression in PHF cells after being treated with crude rice oil extracted from cadmium-contaminated rice was significantly higher than crude rice oil extracted from control rice from Pathumthani Province ($p<0.05$) (Figure 7 and Figure 8). However, it was found that MMP-2 expression in PHF cells after being treated with crude rice oil extracted from control rice was significantly higher than crude rice oil extracted from cadmium-contaminated rice ($p<0.05$) after 48-72 h post stimulation. The up-regulation of MMP-2 expression in PHF cells after being treated with vitamin C was significantly higher than other groups ($p<0.05$) only at first hour post stimulation. Moreover, the effect of treatments on MMPs in PHF cells has shown no effect of commercial γ-oryzanol in the expression of MMP-2.

Similarly, findings in the present study regarding the effect of vitamin C in MMPs contrast with past studies where in addition of vitamin C suppressed MMP activity in various cell types (Ho et al., 2007; Pfeffer et al., 1998). On the contrary, different studies have found an increase in mRNA levels and secretion for collagens type I and type III in tissue cultures of different kinds of cells after treatment with vitamin C, which supports our findings (Wardas & Jurczak, 2002). Vitamin C, apart from being a cellular anti-oxidant, it is also a critical co-factor for collagen metabolism (Niki, 1987). Therefore, it is *a priori* possible that activated collagen synthesis in response to attachment of fibroblasts to matrix is initiate the expression of a tissue remodeling program, and may result the over-expression of MMPs to degrade over production of substrates (Kennedy et al., 2008). In addition, the positive relationship between collagen production and MMP-2 activity, which further suggest that vitamin C may increase collagen synthesis by fibroblasts and repair tissue damage from oxidative stress. However, from the results, acidic property of vitamin C could affect the acidosis to cell culture, which may inhibit cell growth. This could be explained the lower expression of MMP-2 after primary human fibroblast cells were treated with vitamin C than other treatments as post simulation time increased. Moreover, vitamin C is water-soluble which instability and ease of oxidation in aqueous solutions. Thus, the problem of vitamin C makes its use in cosmetic products difficult (Gallarate et al., 1999).

Crude rice oil extracts from both cadmium-contaminated rice and control rice from Pathumthani Province showed the similar results of MMP-2 expression in PHF cells post stimulation as vitamin C. This suggested that crude rice oil extracts may provide a potential role to up-regulate the MMP-2 and may promote tissue remodeling in PHF cells. Unlike vitamin C, γ-oryzanol is water insoluble and stable at extrusion temperature 110°C in rice bran oil process (Shin et al., 1997). Moreover, it has been proposed as a UVA filter in sunscreen cosmetics to prevent photoaging (Coppini et al., 2001). Additionally, the degradation rate of γ-oryzanol was constant during storage after extrusion. It seems reasonable to assume that γ-oryzanol is stabilized and can also be used as an anti-oxidant for pharmaceutical purposes.

The signaling mechanism by which treatments may increase MMP activity in PHF cells is not clear. At the level of transcription, it has been reported that activation of motigen-activated protein kinease (MAPK) plays an important role in the regulation of MMP expression (Phillips et al., 1997). Additionally, MT1-MMP has also been implicated in the collagen-induced MMP-2 activation, suggesting that the presence of a considerable amount of active MT1-MMP protein on the fibroblast surface may be essential for MMP-2 activation (Guo & Piacentini, 2003). However, additional research is necessary to clarify the signaling mechanisms by which treatments may increase MMP activity specifically in primary human fibroblasts.

In regards to post stimulation time effect, there was an interesting in the re-expression of MMP-2 from PHF cells after 24 h and again in 72 h post stimulation (Figure 8). As previously described, the up-regulation of MMP-2 may be response to collagen production, which more MMP-2 needed to denature the substrates during tissue-remodeling and turnover. On the other hand, due to the fact that the anti-oxidant itself, the 24 h pre-incubation, and cell metabolic waste production, may cause a stress to cell. The results may be relevant to the inflammatory response to tissue replenishment following tissue damage due to oxidative stress, which lead to MMPs expression (Ito et al., 2005). However, more research is needed to further understand the mechanism of anti-oxidant on MMPs expression.

A previous study found that MMPs expression was related to *SIRT1*. Lee and Kim (2011) reported that activation of *SIRT1* could inhibit MMP-2 expression in human fibrosarcoma cell. Ohguchi et al. (2010) reported that SIRT1 may control MMPs expression through directly targeting the AP-1 and NF-κB components. In general, the expression of MMPs in fibroblasts is regulated by highly complex processes, which include several factors such as mitogen-activated protein kinease (MAPK), activator protein-1 (AP-1), and nuclear factor (NF-κB) (Kida et al., 2005). Previous reports demonstrated that several human *MMP* gene promoter sequences revealed an important role of the activator protein-1 (AP-1)-binding site for transcriptional activation, which could be SIRT1 targets (Westermarck & Kahari, 1999). Therefore, it suggested a possibility that regulation of *SIRT1* could inhibit the gene expression of *MMPs* by deacetylation of AP-1 binding site and down-regulate the AP-1 transcriptional activity. However, it was interesting due to the fact that MMP-2 promoter does not contain an AP-1 binding site (Benbow & Brinckerhoff, 1997). Zhang et al. (2009) reported strong evidence that AP-1 and c-Jun/ c-Fos heterodimers are somehow involved in regulating MMP-2 activation. Moreover, AP-1 plays a key role in regulating the synthesis and secretion of MMPs including MMP-2. Nevertheless, the precise molecular mechanisms by which *SIRT1* modulates MMP-2 expression remain to be elucidated.

After PHF cells were stimulated with commercial γ-oryzanol, the expression of MMP-2 was very low when detected with gelatinolytic zymography. This implied that PHF cells after stimulated with commercial γ-oryzanol, it may not be able to produce MMP-2 in a detectable amount, which may not enough to enhance the tissue-remodeling as well as tissue turnover. The slightly up-regulation of *SIRT1* in commercial γ-oryzanol treated group compared to other treated groups indicated that *SIRT1* may induced by oxidative stress, thus, down-regulated the expression of MMP-2. The up-regulation of *SIRT1* could be possible activate p53, which caused cell growth arrest in response to oxidative stress (Yi & Luo, 2010). This suggested that commercial γ-oryzanol exhibit negative potential as an anti-oxidative agent compared to crude rice oil extracts.

Furthermore, in this study, the down-regulation of *SIRT1* in primary human fibroblast cells after being treated with crude rice oil extracted from both cadmium-contaminated rice and control rice showed up-regulation of MMP-2 expression similar to vitamin C. The MMP-2 expression was higher in PHF cells after being treated with crude rice oil extracted from cadmium-contaminated rice when compared to crude rice oil extracted from control rice. Considering the anti-oxidative activity in crude rice oil extracts, which may resulted in scavenging the ROS, crude rice oil may reduced *SIRT1* expression and thereby promoted MMP-2 expression. It is possible that activation of MMP-2 induced by this crude rice oil extracts after stimulated in PHF cells would favor to tissue remodeling and may leading to synthesis the extracellular matrix (ECM), which may support the reorganization of the newly deposited collagen and other constituents of the ECM in a primary human fibroblast.

These findings have been obtained by deliberate comparison of crude rice oil extracted from cadmium-contaminated rice and control rice from Pathumthani Province. This may be a ground for an alternative use of the cadmium-contaminated rice by extracting its crude rice oil. This crude rice oil could have potential as novel therapeutic agents for attenuation of aging, which can be exploited to produce high value products for pharmaceutical and cosmeceutical use.

5. Conclusion

The significant effects on the anti-oxidative activity of the crude rice oil extracted from cadmium-contaminated rice may have resulted from its significant quantity of anti-oxidative compounds. The *in vitro* cell model using primary human fibroblast cells showed that crude rice oil extracted from cadmium-contaminated rice was found to possess anti-oxidant activity in preventing cellular mitochondrial damage. After PHF cells were stimulated with crude rice oil extracted from cadmium-contaminated rice, the PHF cells showed down-regulation of *SIRT1* mRNA expression and up-regulation of MMP-2. It might be possible that *SIRT1* down regulation related to MMP-2 up-regulation, which could delay aging and help promote tissue remodeling in PHF cells. However, the specific mechanism for the regulation of these processes remains to be identified. From the results, we suggested that crude rice oil extracted from cadmium-contaminated rice exhibited the potential anti-oxidantive activity of as an alternative for utilizing cadmium-contaminated rice in pharmaceutical and cosmeceutical use without compromising their health with hazard risks.

Acknowledgements

References

Association of Official Analysis Chemists. (1995). *Official Method of Analysis of the AOAC International* (16th ed.). Arlington, VA: Association of Official Analysis Chemists.

Bai, L., Pang, W. J., Yang, Y. J., & Yang, G. S. (2008). Modulation of Sirt1 by resveratrol and nicotinamide alters proliferation and differentiation of pig preadipocytes. *Mol. Cell Biochem., 307,* 129-140. http://dx.doi.org/10.1007/s11010-007-9592-5

Banthorpe, D. V. (1991). Classification of terpenoids and general procedures for their characterisation. In B. V. Charlwood & D. V. Banthorpe (Eds.), *Methods in Plant Biochemistry* (vol. 7, pp. 1-42). London, England: Elsevier Science.

Benbow, U., & Brinckerhoff, C. E. (1997). The AP-1 site and MMP gene regulation: What is all the fuss about? *Matrix Biol., 15*, 519-526. http://dx.doi.org/10.1016/S0945-053X(97)90026-3

Brigitte, K. (1995). Cosmetic sunscreen composition containing feruric acid and gamma-oryzanol. *Chem. Abstr.*, *123*, 296-297.

Camins, A., Junyent, F., Verdaguer, Beas-Zarate, C., Rojas-Mayorquín, A. E., Ortuño-Sahagún, D., & Pallàs M. (2009). Resveratol: An antiaging drug with potential therapeutic applications in treating diseases. *Pharmaceuticals, 2*, 194-205. http://dx.doi.org/10.3390/ph2030194

Chen, M. H., & Bergman, C. J. (2005). A rapid procedure for analyzing rice bran tocopherol, tocotrienol, and γ-oryzanol contents. *J. Food Comp. Anal., 18*, 319-331. http://dx.doi.org/10.1016/j.jfca.2003.09.016

Coppini, O., Paganizzi, D., Santi, P., & Ghirardini, A. (2001). Capacità protettiva nei confronti delle radiazioni solari di derivati di origine vegetale. *Cosmetic News, 136*, 15-20.

Gallarate, M., Carlotti, M. E., Trotta, M., & Bovo, S. (1999). On the stability of ascorbic acid in emulsified system for tropical and cosmetic use. *Int. J. Pharm., 188*, 233-241. http://dx.doi.org/10.1016/S0378-5173(99)00228-8

Goad, L. J. (1991). Phytosterols. In B. V. Charlwood & D. V. Banthorpe (Eds.), *Methods in Plant Biochemistry* (vol. 7, pp. 369-434). San Diego, CA: Academic Press Inc.

Guo, C., & Piacentini, L. (2003). Type I collagen-induced MMP-2 activation coincides with up-regulation of membrane type 1-matrix metalloproteinase and TIMP-2 in cardiac fibroblasts. *J. Biol. Chem., 278,* 46699-46708. http://dx.doi.org/10.1074/jbc.M307238200

Han, M., Song, E., Guo, Y., Ou, X., Mantel, C., & Broxmeyer, H. E. (2008). SIRT1 regulates apoptosis and Nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization. *Cell Stem Cell*, *2,* 241-251. http://dx.doi.org/10.1016/j.stem.2008.01.002

Heussen, C., & Dowdle, E. B. (1980). Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal. Biochem., 102*, 196-202. http://dx.doi.org/10.1016/0003-2697(80)90338-3

Ho, F., Liu, S., Lin, W., & Liau, C. (2007). Opposite effects of high glucose on MMP-2 and TIMP-2 in human endothelial cells. *J. Cell Biochem., 101*, 442-450. http://dx.doi.org/10.1002/jcb.21192

Ito, K., Ito, M., Elliott, W. M., Cosio, B., Caramori, G., Kon, O. M., … Barnes, P. J. (2005). Decreased histone deacetylase activity in chronic obstructive pulmonary disease. *N. Engl. J. Med., 352*(19), 1967-1976. http://dx.doi.org/10.1056/NEJMoa041892

Jwa, N. S., Agrawal G. K., Tamogami, S., Yonekura, M., Han, O., Iwahashi, H., & Rakwal, R. (2006). Role of defense/stress-related marker genes, proteins and secondary metabolites in defining rice self-defense mechanisms. *Plant Physiol. Biochem., 44,* 261-273. http://dx.doi.org/10.1016/j.plaphy.2006.06.010

Kida, Y., Kobayashi, M., Suzuki, T., Takeshita, A., Okamatsu, Y., Hanazawa, S., … Hasegawa, K. (2005). Interlukin-1 stimulates cytokines, prostaglandin E2 and matrix metalloproteinase-1 production via activation of MAPK/AP-1 and NF-kappaB in human gingival fibroblasts. *Cytokine, 29*, 159-168. http://dx.doi.org/10.1016/j.cyto.2004.10.009

Kahlon, T. S., Saunders, R. M., Sayre, R. N., Chow, F. I., Chiu, M. M., & Betschart, A. A. (1992). Chloresterol-lowering effects of rice bran and rice bran oil fractions in hyperchlorestrolemic hamters. *Cereal Chem., 69*(5), 485-489.

Keller, K. E., Aga, M., Bradley, J. M., Kelley, M. J., & Acott, T. S. (2009). Extracellular matrix turnover and outflow resistance. *Exp. Eye Res., 88*, 676-682. http://dx.doi.org/10.1016/j.exer.2008.11.023

Kennedy, L., Shi-wen, X., Carter, D. E., Abraham, D. J., & Leask, A. (2008). Fibroblast adhesion results in the induction of a matrix remodeling gene expression program. *Matrix Biol., 27*, 274-281. http://dx.doi.org/10.1016/j.matbio.2008.01.004

Lee, S. J., & Kim, M. M. (2011). Resveratrol with antioxidant activity inhibits matrix metalloproteinase via modulation of SIRT1 in human fibrosarcoma cells. *Life Sci., 88*, 465-472. http://dx.doi.org/10.1016/j.lfs.2011.01.005

Mackenzie, C. G., Mackenzie, J. B., & Beck, P. (1961). The effect of pH on growth, protein synthesis, and lipid-rich particles of cultured mammalian cells. *J. Biophys. Biochem. Cytol., 9*, 141-156.

Michan, S., & Sinclair, D. (2007). Sirtuins in mammals: insights into their biological function. *Biochem. J., 404*, 1-13. http://dx.doi.org/10.1042/BJ20070140

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods, 65*, 55-63. http://dx.doi.org/10.1016/0022-1759(83)90303-4

Namdeo, A. G. (2007). Plant cell elicitation for production of secondary metabolites: a review. *Phcog. Rev., 1*(1), 69-78.

Niki, E. (1987). Interaction of ascorbate and α-tocopherol. *Ann. N. Y. Acad. Sci., 498*, 186-199. http://dx.doi.org/10.1111/j.1749-6632.1987.tb23761.x

Ohguchi, K., Itoh, T., Akao, Y., Inoue, H., Nozawa, Y., & Ito, M. (2010). SIRT1 modulates expression of matrix metalloproteinases in human demal fibroblasts. *Br. J. Dermatol., 163*, 689-694. http://dx.doi.org/10.1111/j.1365-2133.2010.09825.x

Patel, M., & Naik, S. N. (2004). Gamma-oryzanol from rice bran oil – A review. *J. Sci. Ind. Res., 63*, 569-578.

Peralta-Videa, J. R., Lopez, M. L., Narayan, M., Saupe, G., & Gardea-Toressdey, J. (2009). The biochemistry of environmental heavy metal uptake by plants: Implications for the food chain. *Int. J. Biochem. Cell Biol., 41*, 1665-1667. http://dx.doi.org/10.1016/j.biocel.2009.03.005

Pfeffer, F., Casanueva, E., Kamar, J., Guerra, A., Perichart, O., & Vadillo-Ortega, F. (1998). Modulation of 72-kilodalton type IV collagenase (matrix metalloproteinase-2) by ascorbic acid in cultured human amnion-derived cells. *Biol. Reprod., 59*, 326-329. http://dx.doi.org/10.1095/biolreprod59.2.326

Phillips, A. O., Steadman, R., Morrisey, K., Martin, J., Eynstone, L., & Williams, J. D. (1997). Exposure of human renal proximal tubular cells to glucose leads to accumulation of type IV collagen and fibronectin by decreased degradation. *Kidney Int., 52*, 973-984.

Rogers, E. J, Rice, S. M., Nicolosi, R. J., Carpenter, D. R., McClelland, C. A., & Romanczyk, L. J. Jr. (1993). Identification and quantitation of γ–oryzanol components and simultaneous assessment of tocols in rice bran oil. *J. Am. Oil Chem. Soc., 70*(3), 301-307. http://dx.doi.org/10.1007/BF02545312

Sasaki, J., Takada Y, Handa K, Kusuda, M., Tanabe, Y., Matsunaga, A., & Arakawa, K. (1990). Effect of gamma-oryzanol on serum lipids and apolipoproteins in dyslipidemic schizophrenics receiving major tranquilizers. *Clin. Ther., 12*(3), 263-268.

Saunders, L. R., & Verdin, E. (2007). Sirtuins: critical regulators at the crossroads between cancer and aging. *Oncogene, 26*, 5489-5504. http://dx.doi.org/10.1038/sj.onc.1210616

Shapiro, S. D. (1998). Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr. Opin. Cell Biol., 10*, 602-608. http://dx.doi.org/10.1016/S0955-0674(98)80035-5

Shimada, M., Piscator, T., & Nishino, H. I. (1977). Urine analysis for detection of cadmium-induced renal changes, with special reference to β_2-microglobulin. *Environ. Res., 13*, 407-424. http://dx.doi.org/10.1016/0013-9351(77)90021-4

Shin, T. S., & Godber, J. S. (1994). Isolation of four tocopherols and four tocotrienols from variety of natural sources by semi-preparative high-performance liquid chromatography. *J. Chromatogr. A, 678*, 49-58. http://dx.doi.org/10.1016/0021-9673(94)87073-X

Shin, T. S., Godber, J. S., Martin, D. E., & Wells, J. H. (1997). Hydrolitic stability and changes in E vitamers and oryzanol of extruded rice bran during storage. *J. Food Sci., 62*, 704-708. http://dx.doi.org/10.1111/j.1365-2621.1997.tb15440.x

Simmons, R. W., Pongsakul, P., Saiyasitpanich, D., & Klinphoklap, S. (2005). Elevated levels of cadmium and zinc in paddy soils and elevated levels of cadmium in rice grain downstream of a zinc mineralized area in Thailand: Implications for public health. *Environ. Geochem. Health, 27*, 501-511. http://dx.doi.org/10.1007/s10653-005-7857-z

Stetler-Stevenson, W. G., Aznavoorian, S., & Liotta, L. A. (1993). Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell Biol., 9*, 541-573. http://dx.doi.org/10.1146/annurev.cb.09.110193.002545

Tryggvason, K., Hoyhtya, M., & Pyke, C. (1993). Type IV collagenases in invasive tumors. *Breast Cancer Res. Treat., 24*, 209-218. http://dx.doi.org/10.1007/BF01833261

Varani, J., Schuger, L., Dame, M. K., Leonard, C., Fligiel, S. E. G., Kang, S., … Voorhees, J. J. (2004). Reduced fibroblast interaction with intact collagen as a mechanism for depressed collagen synthesis in photodamaged skin. *J. Invest. Dermatol., 122*, 1471-1479. http://dx.doi.org/10.1111/j.0022-202X.2004.22614.x

Wardas, M., & Jurczak, T. (2002). Effects of fluoride and ascorbic acid on collagen biosynthesis in mouse liver fibroblast cultures. *Fluoride, 35,* 104-109.

Westermarck, J., & Kahari, V. (1999). Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB. J., 13*, 781-792.

Xu, Z., & Godber, J. S. (2001). Antioxidant activities of major components of γ-oryzanol from rice bran using a linoleic acid model. *J. Am. Oil Chem. Soc., 78*(6), 645-649. http://dx.doi.org/10.1007/s11746-001-0320-1

Xu, Z., & Godber, J. S. (1999). Purification and identification of components of γ-oryzanol in rice bran oil. *J. Agric. Food Chem., 47*, 2724-2728. http://dx.doi.org/10.1021/jf981175j

Yasukawa, K., Akihisa, T., Kimura, Y., Tamura, T., & Takido, M. (1998). Inhibitory effect of cycloartenol ferulate, a component of rice bran, on tumor promotion in two-stage carcinogenesis in mouse skin. *Biol. Pharm. Bull., 21*(10), 1072-1076.

Yi, J., & Luo, J. (2010). SIRT1 and p53, effect on cancer, senescence and beyond. *Biochim. Biophys. Acta., 1804*, 1684-9. http://dx.doi.org/10.1016/j.bbapap.2010.05.002

Zhang, H. W., Wang, X., Zong, Z. H., Huo, X., & Zhang, Q. (2009). AP-1 Inhibits expression of MMP-2/9 and its effects on rat smooth muscle cells. *J. Surg. Res., 157*, 31-37. http://dx.doi.org/10.1016/j.jss.2009.02.015

Zhang, H. Y., & Sun, H. (2010). Up-regulation of Foxp3 inhibit cell proliferation, migration and invesion in epithelial ovarian cancer. *Cancer Lett., 287*, 91-97. http://dx.doi.org/10.1016/j.canlet.2009.06.001

Zhao, W., Kruse, J. P., Tang, Y., Jung, S. Y., Qin, J., & Gu, W. (2008). Negative regulation of the deacetylase SIRT1 by DBC1. *Nature, 451*(7178), 587–590. http://dx.doi.org/10.1038/nature06515