Anti-invasive Activity against Cancer Cells of Phytochemicals in Red Jasmine Rice (*Oryza sativa* L.)

Komsak Pintha, Supachai Yodkeeree, Pornsirit Pitchakarn, Pornngarm Limtrakul*

Abstract

Red rice contains pharmacological substances including phenolics, oryzanol, tocotrienol and tocopherol. Recently, red rice extract has been employed as a source of antioxidants for inhibition of tumor growth. This study was carried out to evaluate the anti-invasion effects of red rice extract fractions on cancer cells. It was found that at 100 μg/ml of crude ethanolic extract (CEE), hexane fraction (Hex) and dichloromethane fraction (DCM) could reduce HT1080 and MDA-MB-231 cancer cell invasion. Hex and DCM revealed higher potency levels than CEE, whereas an ethyl acetate fraction (EtOAc) had no effect. Gelatin zymography revealed that Hex decreased the secretion and activity of matrix metalloproteinase-2 and -9 (MMP-2 and-9). In contrast, the DCM fraction exhibited slightly effect on MMPs secretion and had no effect on MMPs activity. Collagenase activity was significantly inhibited by the Hex and DCM fractions, High amounts of γ-oryzanol and γ-tocotrienol were found in the Hex and DCM fractions and demonstrated an anti-invasion property. On the other hand, proanthocyanidin was detected only in the CEE fraction and reduced MDA-MB-231 cells invasion property. These observations suggest that proanthocyanidin, γ-oryzanol and γ-tocotrienol in the red rice fractions might be responsible for the anti invasion activity. The red rice extract may have a potential to serve as a food-derived chemotherapeutic agent for cancer patients.

Keywords: Matrix metalloproteinase - red rice extract - invasion - phytochemicals

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Introduction

Excess extracellular matrix (ECM) degradation by proteolytic enzymes is an important step in cancer cells metastasis. The key proteases involved in degrading the components of the ECM are matrix metalloproteinases (MMPs) (Yadav et al., 2014). Therefore, inhibition of ECM degradation was used as the target for anti cancer metastasis.

Many studies have shown that the pigmented rice, such as black and red rice, are the most nutritious of the rice and also a good source of bioactive phytochemicals, including tocopherols, tocotrienols, γ-oryzanol, and phenolic compounds (Zeng et al., 2013). Recently, several studies have shown that γ-oryzanol, γ-tocotrienol and anthocyanidin from pigmented rice exert anti-carcinogenesis and inhibit cancer cell growth (Banjerdpongchai et al., 2013; Zeng et al., 2013; Summart and Chewonarin, 2014). Among those phytochemicals, γ-tocotrienol, protocatechuic, ellagic acid, ferulic acid and chlorogenic acid have proven to exhibit anti invasion in cancer cells (Liu et al., 2010; Lin et al., 2011; Weng and Yen, 2012; Pitchakarn et al., 2013; Tsai et al., 2013; ). However, there have been no reports on the anti-invasion activity of red rice extract in human cancer cells. Here, we investigated the anti invasion effect of red rice extract fractions on the HT1080 fibrosacroma and MDA-MB-231 breast human cancer cells.

Materials and Methods

Chemicals

Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin, and trypsin-EDTA were purchased from GIBCO-BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). MTT, gelatin, hydroxybenzoic acid, and catechins were purchased from Sigma Aldrich (St Louis, MO, USA). Matrigel was purchased from Becton Dickinson (Bedford, MA, USA). Protocatechuic acid, chlorogenic acid, p-coumaric acid, vanillic acid, ferulic acid, gallic acid and vitamin E analogs including tocopherol and tocotrienol (α-, β-, γ- and δ-) were purchased from ChromaDex (Irvine,CA,USA), and γ-Oryzanol was purchased from Wako Pure (Osaka, Japan). The solvents for red rice extraction and the mobile
Plant material
Whole grains of red rice (Oryza sativa L.) were harvested from Phayao Province, Thailand. A voucher specimen number was certified by the herbarium at the Flora of Thailand, Faculty of Pharmacy, Chiang Mai University (voucher specimen no. 023108) which was kept for future reference.

Preparation of red rice fractions
The air-dried and powdered specimens of red rice grains (1.0 kg) were extracted with 70% ethanol by being shaken at room temperature for 12 h. After evaporation and lyophilization, the red rice crude ethanolic extract (CEE) was suspended in water and then sequentially extracted with n-hexane, dichloromethane and ethyl acetate to give hexane (Hex), dichloromethane (DCM) and ethyl acetate (EtOAc) fractions, respectively.

Determination of total proanthocyanidin content
Total proanthocyanidin concentration was determined using the vanillin assay (Gunaratne et al., 2013). The red rice extract was mixed with sulfuric acid/methanol solution and 1% vanillin in methanol (w/v). A control mixture of the sample was prepared by adding 100% methanol instead of the vanillin solution to correct the absorbance by the non-vanillin reactive compounds. After incubation for 15 min in a 30°C water bath, the absorbance of the sample and the control mixtures was measured at 490 nm against a reagent blank and their difference was used to determine total proanthocyanidin concentrations of the sample, which was expressed as milligram catechin/gram extract (mg CE/g extract).

HPLC analysis for phenolic, vitamin E analogs and γ-oryzanol in red rice fractions
The phenolic compounds and γ-oryzanol were determined by HPLC using an Inertsil ODS-3-C18 column (250 x 4.6 mm, 5 μm particle diameter, GL Science Inc., Japan). Gradient elution using two solvents: A (0.1% trifluoroacetic acid in water) and B (100% methanol) for detection phenolic compounds (Shao et al., 2014) and isocratic elution (methanol:acetonitrile; 65:35) were used for detection γ-oryzanol (Cho et al., 2012). Ten micro liters of the samples were injected to the column with a flow rate 1.0 ml/min. Standard phenolic compounds and γ-oryzanol were monitored at 280 and 325 nm, respectively. The vitamin E analogs were determined by HPLC C30 column (250 x 4.6 mm, 5 μm particle) (Pegg and Amarowicz, 2009) using isocratic elution with methanol:H₂O (93:7) as the mobile phase. An injection volume of 10 μl was used. The standards for the tocopherols and tocotrienols were monitored at 292 nm.

Cell lines and culture conditions
MDA-MB-231 human breast carcinoma cells, HT1080 human fibrosarcoma cells, and NIH3T3 fibroblasts cells were obtained from the American Type Culture Collection (ATCC). The cells were grown in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) of FBS. Cultures were maintained at 37°C in an atmosphere consisting of 5% CO₂. Before the cell treatment, the red rice extract fractions were dissolved in DMSO and were then diluted with the culture medium for the final concentration of DMSO at less than 0.1%.

Cell viability assay
Cytotoxicity of red rice extracts on HT1080 and MDA-MB-231 cells were determined by MTT assay as described previously (Banjerdpongchail et al., 2013). HT1080 and MDA-MB-231 cells were plated at 1.5x10³ cells per well in 96-well plates and cultured in DMEM with 10% FBS. After being cultured for 24 h, various concentrations (0-200 μg/ml) of red rice extracts were added and the samples were incubated for 48 h. At the end of the treatment, 15 μl of MTT solution (5 mg/ml) were added and the samples were incubated for 4 h. The MTT formazan was dissolved with DMSO and absorbance was measured using a microplate reader at 540 nm with a reference wavelength of 630 nm.

Cell invasion assay
The invasive behavior of MDA-MB-231 and HT1080 cells was tested using the modified Boyden chamber assay as previous described (Pitchakarn et al., 2013). Polyvinylpyrrolidone-free polycarbonate filters (Millipore, Co Cork, Ireland) (8 μm pore sized) were coated with Matrigel (12 μg/filter). The lower chamber contained serum-free conditioned medium of NIH 3T3 fibroblast cells, which acted as a chemoattractant. MDA-MB-231 or HT1080 cells, at 1.25x10³ cells, were plated onto the upper chamber with or without the tested compounds and the samples incubated for 24 h at 37°C, under 5% CO₂. After incubation, the non-invading cells were removed from the upper surface of the membrane. The invading cells on the lower surface of the membrane were fixed with methanol for 1 min and stained with toluidine blue for 5 min. The cells that had actively migrated to the underside surface of the filter were dissolved with 20% acetic acid and they were indirectly quantified by measuring the absorbance at 570 nm.

Gelatin zymography assay
The secretions of MMP-2 and MMP-9 of the treated cells were analyzed by gelatin zymography as previously described (Pitchakarn et al., 2013). HT1080 and MDA-MB-231 cells were treated with various concentrations (0-100 μg/ml) of the red rice fractions for 24 h in DMEM serum-free medium, and the culture supernatant was collected in equal amounts of the cells. The culture supernatant was separated by 10% polyacrylamide gels containing 0.1% w/v of gelatin under non-reducing conditions. After electrophoresis, gels were washed twice with 2.5% v/v of Triton X-100 for 30 min at room temperature to remove SDS. The gel was then incubated at 37°C for 18 h in an activating buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl₂, pH 7.4). Gels were stained with Coomassie Brilliant Blue R (0.1%w/v) and destained in 30% methanol, 10% acetic acid. MMPs activity appeared
as a clear band against a blue background. Digestion bands were quantitated by Bio 1D software (Viber Lourmat).

To analyze the effect of red rice extract on MMP-2 and MMP-9 activities, the culture supernatant of HT1080, which contained MMP-2 and MMP-9, was subjected to 10% polyacrylamide gels containing 0.1% w/v of gelatin, as described above. After being washed with Triton X-100, the gel slab was cut into slices corresponding to the lanes and then put in to different tanks containing various concentrations of the red rice extract fractions suspended in activating buffer and were then incubated at 37°C for 24 h. The strip of gel was stained with Coomassie Brilliant Blue R and the MMPs activity was quantitated as described above.

Collagenase activity assay

The proteolytic activity of collagenase was measured using EnzChek Collagenase Assay kit (Molecular Probe). Briefly, 1 U/ml of collagenase was mixed with 10 µg/ml of fluorescein-conjugated gelatin (DQ gelatin) containing various concentrations of the red rice fractions suspended in reaction buffer and put on 96 well microplates. The rate of proteolysis was determined by measuring the fluorescence intensity at 3 min intervals for 30 min with fluorometer. The fluorescence values were measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Enzyme inhibitory activity was estimated by linear regression of the fluorescent intensity recorded during that time.

Statistical analysis

All experiments were performed in duplicate. Quantifications are defined as mean±SD of three independent experiments and expressed as percentage of the control. Statistically significant differences throughout this study were calculated by Student’s t-test. In all cases, a value of p<0.01 or p<0.05 was considered significant.

Results

Effect of red rice extract fractions on viability of HT1080 and MBA-MB-231 cells

The cytotoxicity of the red rice extract fractions on human cancer cells were evaluated by MTT assay. As shown in Figure 1A and 1B, at high concentrations (100 µg/ml) of the CEE, Hex, DCM and EtOAc fractions for 48 h had no affect on the MDA-MB-231 and HT1080 cell viabilities.

Effect of red rice extract fractions on HT1080 and MDA-MB-231 cell invasion

To investigate the anti-invasive property of red rice fractions on HT1080 and MBA-MB-231 cells, the Boyden chamber assay was used. The results displayed in Figure 2A and 2B demonstrate that the CEE, Hex and DCM fractions at 100 µg/ml significantly reduced the invasion of both HT1080 and MDA-MB-231 cells to 73%, 59%, 66% and 80%, 36%, 34%, respectively. However, the treatment with EtOAc fraction did not affect the cell invasion. Moreover, the invasion of the MDA-MB-231 cells was inhibited with the Hex and DCM fractions in a dose-dependent manner with IC_{50} of 50 and 45 µg/ml, respectively (Figure 2C and 2D).

Effect of red rice active fractions on MMPs secretion in HT1080 and MBA-MB-231 cells

MMP-2 and -9 are the important enzymes involved in ECM degradation and cancer metastasis. To explore...
the suppressive potential of the active fractions that was associated with MMPs secretion, the HT1080 and MDA-MB-231 cells were treated with the Hex and DCM red rice fraction. The result from gelatin zymography indicate that treatment of the HT1080 cells with the Hex fraction at 100 μg/ml reduced MMP-2 and MMP-9 levels to 30% and 56%, respectively (Figure 3A). Moreover, DCM at 100 μg/ml also decreased the MMP-9 secretion from the cells to 61%, but had no effect on MMP-2 secretion (Figure 3B). In addition, at 100 μg/ml of the Hex and DMC fractions reduced the MMP-9 secretion from MDA-MB-231 cells to 65 and 70%, respectively when compared with the untreated cells (Figure 3C and 3D).

Inhibition of collagenase and MMPs activities by red rice active fractions

To determine whether red rice active fractions directly inhibit collagenase, MMP-2 and MMP-9 activities, the

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Fractions (mg/g extract)</th>
<th>CEE</th>
<th>Hex</th>
<th>DCM</th>
<th>EtOAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-tocotrienol</td>
<td>1.05±0.42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tocotrienol</td>
<td>0.24±0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-tocopherol</td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-oryzanol</td>
<td>70.82±1.63</td>
<td>127.5±2.57</td>
<td>94.8±6.49</td>
<td>16.1±0.76</td>
<td></td>
</tr>
</tbody>
</table>

*Values are means±S.D. (n=3), nd not detectable


The contents of tocopherols, tocotrienols and oryzanol in red rice extract fractions and their activities on cancer cells invasion

Tocopherols, tocotrienols and γ-oryzanol in rice bran oil have been demonstrated to possess anti-cancer properties. From the results above, Hex and DCM exhibited anti invasion activity in cancer cells. The content of vitamin E analogs in red rice fractions revealed in Table 1 that the Hex fraction had the highest concentration of γ-tocotrienol, followed by DCM and CEE. In contrast, vitamin E analogs could not be detected in the EtOAc fraction. In addition, tiny amounts α-tocotrienol and γ-tocopherol were only found in the Hex fraction. Gamma-oryzanol is the major sterol in rice bran. In this study, γ-oryzanol was identified in all fractions of red rice. However, the Hex fraction presented the highest amount of this sterol, followed by DCM, CEE and trace amounts were found in EtOAc. Next, we evaluated the anti-invasion of γ-oryzanol and γ-tocotrienol, which possessed the major phytochemical content in the non-
The contents of phenolic compounds and proanthocyanidin in red rice extract fractions and their activities on cancer cells invasion

Phenolic compounds have been reported to inhibit invasion of cancer cells. The results from Table 2 indicated that EtOAc had high content of proanthocyanidin and catechins, followed by ferulic acid and vanillic acid. The DCM fraction contained high content of vanillic acid and ferulic acid and low amount of chlorogenic acid and protocatechuic acid. In contrast, low concentrations of these phenolic compounds were detected in the CEE and Hex fractions.

Next, we determined the anti-invasion properties of the phenolic compounds represented in the red rice extracts. The results showed that 50 μg/ml of ferulic acid and vanillic acid, but not protocatechuic acid, coumaric acid and catechins could reduced MDA-MB-231 cell invasion to 60 and 65% (Figure 5B), respectively. Although, vanillic and ferulic acid were detected in the EtOAc fraction, 1 μg/ml of vanillic acid and ferulic acid, which were present in 100 μg/ml of the EtOAc fraction, were not sufficient to promote anti-invasion (Figure 5C).

On the other hand, high amounts of proanthocyanidin were only observed in the CEE fraction, as shown in Table 2. In contrast, the level of proanthocyanidin in the Hex, DCM and EtOAc fractions could not be detected. Our findings show that standard proanthocyanidin derived from grape seed extract could reduce cancer cell invasion in a dose dependent manner, for which 5 μg/ml of proanthocyanidin reduced cancer invasion to 70% of the control (Figure 5D).

Table 2. The Contents of Phenolic Compounds and Proanthocyanidin in Red Rice Extract Fractions

<table>
<thead>
<tr>
<th>Phenolic compounds (mg/g extract)</th>
<th>CEE</th>
<th>Hex</th>
<th>DCM</th>
<th>EtOAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photocatechuic 0.45±0.011</td>
<td>0.17±0.01</td>
<td>0.64±0.06</td>
<td>56.6±7.85</td>
<td></td>
</tr>
<tr>
<td>Catechin 0.48±0.16</td>
<td>0.49±0.23</td>
<td>21.2±0.36</td>
<td>65.7±4.33</td>
<td></td>
</tr>
<tr>
<td>Chlorogenic 1.18±0.141</td>
<td>0.40±0.09</td>
<td>0.15±0.003</td>
<td>0.91±0.64</td>
<td></td>
</tr>
<tr>
<td>Vanillic 0.14±0.003</td>
<td>0.09±0.01</td>
<td>4.57±0.14</td>
<td>12.1±2.07</td>
<td></td>
</tr>
<tr>
<td>Coumaric 0.03±0.001</td>
<td>nd</td>
<td>0.11±0.07</td>
<td>7.76±0.63</td>
<td></td>
</tr>
<tr>
<td>Ferulic 0.12±0.006</td>
<td>1.48±0.18</td>
<td>13.73±2.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proanthocyanidin 59.56±5.08</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

*Values are mean±S.D. (n=3), nd not detectable

Figure 5. Anti-invasion Effect of γ-tocotrienol and γ-oryzanol. A) catechin;Cat, protocatechuic;Pro, chlorogenic; Chl, vanillic;Val and ferulic; Fer B), 1 μg/ml of ferulic and vanillic C) and grape seed proanthocyanidin on MDA-MB-231 cell invasion D). The data represent the mean ±SD with *p<0.01

Discussion

Several studies have shown that pigmented rice extracts exhibit a number of beneficial bioactivities. Specifically, red rice extract has exhibited potent antioxidative, anti-inflammatory, and anti-cancer cell proliferation. The biological properties of the red rice extract were attributed, in part, to their phytochemical content, mainly found in phenolics, flavonoids, vitamin E analogs and γ-oryzanol (Tammasakchai et al., 2012; Gunaratne et al., 2013; Niu et al., 2013). To date, there is no evidence to show that red rice extract exerts an effect on cancer cell metastasis. Here, we investigated the anti-metastasis potential of red rice extract fractions on human cancer cells and identified the bioactive constituents in their fractions.

The metastatic progression of malignant tumors requires the proteolytic degradation of ECM components in the basement membrane, which allowed cancer cells to invade the blood or the lymphatic vessel and travel to other tissues or organs. In this study report for the first time that the CEE, Hex and DCM fractions of red rice extract reduced HT1080 and MDA-MB-231 human cancer cell invasion, for which the DCM and Hex fractions showed a higher potential than the CEE fractions.

Considerable evidence has accumulated which suggests that the inhibition of MMPs expression or enzyme activity can be used as an early target in preventing cancer metastasis.

Our results from the gelatin zymography showed that the Hex fraction had the highest potential to reduce the secretion of MMP-2 and-9 from HT1080 cells. However, the cells that were treated with the DCM fraction significantly decreased only the MMP-9 secretion, but not that of the MMP-2. On the other hand, the secretions of MMP-9 from MDA-MB-231 cells were reduced by Hex and DCM fractions. Moreover, the effects of red rice extract fractions on MMP-2 and-9 activity were examined by gelatin zymography. The Hex fraction reduced MMP-9 activity, but not MMP-2. In addition, the Hex fraction strongly inhibited collagenase activity better than the DCM fraction. From these results, it is suggested that the Hex and DCM fractions reduced cancer cell invasion, at least partly in the inhibition of the ECM degradation enzymes.

Red rice contains a variety of bioactive components with chemopreventive and chemotherapeutic activities, including phenolic compounds, γ-oryzanol, tocopherols and tocotrienols (Henderson et al., 2012; Tammasakchai et al., 2013). To date, there is no evidence to show that red rice extract exerts an effect on cancer cell metastasis.
The contents of γ-oryzanol and γ-tocotrienol in red rice extract fractions appeared highest in the Hex fraction, followed by the DCM fraction, while fewer amounts or none at all were detected in the EtOAc fraction. Specifically, γ-oryzanol is the major component contained in the Hex and DCM fractions. Gamma oryzanol is a mixture of phytosteryl ferulates found in rice bran. It is particularly important to note that γ-oryzanol exhibits antioxidant, anti-carcinogenesis and anti-inflammatory properties (Henderson et al., 2012; Klongpityapong et al., 2013). Here, we report for the first time that γ-oryzanol reduced MDA-MB-231 cell invasion, which corresponded with the finding that Hex and DCM fractions reduced cancer cell invasion. On the other hand, several studies have reported that γ-tocotrienol showed anti-tumor effects, as well as the potential to induce apoptosis, and to inhibit angiogenesis and metastasis (Henderson et al., 2012; Manu et al., 2012). Our results confirm that at 10 μg/ml of γ-tocotrienol reduced MDA-MB-231 cell invasion. This result is consistent with the findings of Liu et al in 2010. They reported that γ-tocotrienol reduced SGC-7901 gastric adenocarcinoma invasion via a down regulation of MMP-2 and -9 expressions (Liu et al., 2010). These data collectively support the acknowledgement of the inhibitory effect of the Hex and DCM fractions on cancer invasion, which might be the effect of γ-oryzanol and γ-tocotrienol.

Our data indicates that catechins are the major phenolic found in the EtOAc and DCM fractions. Moreover, the EtOAc fraction contained high concentrations of photocatechuic acid, and the minor phenolic constituents were vanillic acid, ferulic acid and coumaric acid. In this report, we demonstrated that vanillic acid and ferulic acid at a concentration of 50 μg/ml, reduced MDA-MB-231 cell invasion. Whereas, the other phenolic compounds in the EtOAc fraction could not reduce cancer cell invasion. These results are similar to those of several studies, which have reported on the anti-metastasis property of vanillic and ferulic acid (Lirdprapamongkol et al., 2009; Tsai et al., 2013). However, the concentration of the vanillic acid and ferulic acid were present in 100 μg/ml of the EtOAc fraction did not to be sufficient doses to promote anti-invasion.

Proanthocyanidin is a class of polymeric phenolic compound consisting mainly of catechin, epicatechin, gallocatechin, and epigallocatechin units. The degree of polymerization and galloylation affects their bioactivity and proanthocyanidin profiles differently depending on the food sources (Akagi et al., 2011; Praphasawat et al., 2011; Feng et al., 2014). Our results showed that proanthocyanidin was detected only in the CEE fraction, but not in the Hex and DCM fractions. We also demonstrated that proanthocyanidin reduced cancer cell invasion, which was similar to previous findings which have stated that proanthocyanidins derived from various kinds of medicinal plants, including grape seeds, have been known to exert an anti-metastasis effect on cancer cells (Sun et al., 2011). Thus, it is likely that the CEE fraction reduced cancer cell invasion, which is part of the proanthocyanidin.

In conclusion, our study demonstrated that the Hex and DCM fractions of red rice extract highly exhibited anti-invasion potential via the inhibition of MMPs secretion and activity which could be attributed to the high content of γ-oryzanol and γ-tocotrienol. On the other hand, the anti-invasion activity of CEE fraction may be partly due to the effect of the proanthocyanidin. This suggested that red rice extract containing γ-oryzanol, γ-tocotrienol and proanthocyanidin possess anti-invasion potential in cancer cells and might be used as a food supplement for cancer patients.

Acknowledgements

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References


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