CHAPTER 2

Materials and methods

2.1 Chemicals and materials used

Lists of the chemicals, reagents, and instruments were shown in Appendix A and B respectively. All reagents and/or buffers were shown in Appendix C.

2.2 Elucidation and characterization of the active component(s) from red rice extract

2.2.1 Plant material

The red rice whole grains (*Oryza sativa* L.) were collected from North of Thailand, Phayao province, which has been certified by Faculty of Pharmacy, Chiang Mai University (Herbarium at Flora of Thailand, specimen number 023108).

2.2.2 Extraction procedure

One kilogram of whole grain red rice was ground and soaked in 70% ethanol by gently shaking at room temperature for 12 h. The mixtures were then filtered to remove rice residue using filter paper and pooled with re-extracted samples. Subsequently, a rotary vacuum evaporator system (BUSHI, Switzerland) was used to evaporate the filtrated solution for obtaining the ethanolic fractions. The ethanolic fractions were further freeze-dried to obtain the crude ethanolic extract (CEE) powder. The CEE was kept in -20° C and protected from light (164).

2.2.3 Fractionated extraction based on liquid-liquid partition

The 50g of CEE was re-dissolved in hexane:water (1:1) and sequentially liquid-liquid partitioned with hexane, dichloromethane and ethyl acetate to

obtain hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and water fractions, respectively. Each fraction was dried under reduced pressure to obtain powder and then submitted for the bioassays. The active components in each fraction of red rice extract were further analyzed (164).

2.2.4 Determination of total phenolic content

Principle

Folin–Ciocalteu is a combination reagent containing phosphomolybdate and phosphotungstate that generally used to determine the concentration of phenolic and polyphenolic compounds. Tyrosine reacts with Folin-Ciocalteu's reagent at various concentrations which can be detected at 765 nm. Not only phenolic and polyphenolic compounds that can be measured using this reagent, but also reducing substances that can react to this reagent as well. The total reducing capacity of a sample then can be measured using this colorimetric assay. Other substances such as thiols and other sulfur-containing molecules, vitamins, the guanine nucleotide, amines and dihydroxyacetone, as well as some inorganic ions can also react with this reagent. Although Folin's reagent can react with variety substances, it is especially the phenols that can form a complex with copper and thus increase the reactivity towards this reagent.

Procedure

Modified Folin Ciocalteu method was performed to analyze total phenolic content (TPC) in the rice extracts. Each fraction of red rice extracts were added into test tubes and then Folin Ciocalteu reagent and deionized water was add and the resulting solution mixed by vortex mixer. After incubation for 2 min, 15% sodium carbonate was added into the mixture. The mixture was then incubated at room temperature for 30 min. The absorbance of the blue complex was measured using a UV-visible spectrophotometer at 765 nm. A standard curve was prepared with various concentrations of gallic acid (GA). The TPC result was shown as milligrams of GA equivalents per gram of red rice extract (mg GAE/g) (165).

2.2.5 Determination of total flavonoid content

Principle

The principle of aluminum chloride colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl. The amount of product was determined by UV-visible spectrophotometer at 435 nm.

Procedure

Total flavonoid content was determined by the aluminium colorimetric method. Briefly, each fraction of red rice extract was individually dissolved in DMSO. Then, the sample solution was mixed with 2% $AlCl_3$ and incubated for 10 mins at room temperature. After 10 mins, the mixture was measured at 435 nm compared with the standard catechin using a UV-visible spectrophotometer. Three replicates were performed for each test sample. The total flavonoid content was expressed as milligram catechin equivalents per gram extract (mg CE/g extract) (166).

2.2.6 Determination of total proanthocyanidin content

Principle

The vanillin reaction involves reaction of an aromatic aldehyde, vanillin, with the metasubstituted ring of flavanols to yield a red adduct. Although the vanillin reaction has been widely used to estimate condensed tannin (proanthocyanidin), the reaction is not specific for condensed tannins. Any appropriately substituted flavanol should react in the assay. Thus the formal monomer of the condensed tannin, cathechin, also reacts to yield a red colored adduct.

Procedure

Total proanthocyanidin content was measured using the vanillin assay. Briefly, each fraction of red rice extract was reconstituted in sulfuric acid/methanol solution and then mixed with of 1% vanillin in methanol (w/v). A control and the mixture of the samples were prepared by adding methanol

instead of the vanillin solution for correcting the absorbance by non-vanillin reactive compounds. The mixture was incubated in water bath at 30 °C for 15 min. After that, the absorbance of the samples and control mixture was measured at 500 nm and compared with standard cathechin using UV-visible spectrophotometer. A reagent blank was subtracted from the sample absorbance and their difference presented total proanthocyanidin content in the samples. The amount of total proanthocyanidin content was expressed as milligram catechin equivalents per gram extract (mg CE/g extract) (33, 167).

2.2.7 Determination of total anthocyanin content

Principle

The pH differential spectrophotometric method was used to determine the concentration of anthocyanin pigment. Specifically, pigmentation of anthocyanin depends on pH levels. The color of anthocyanins at pH 1 is variable between orange to purple but it has colorless at pH 4.5. The absorbance of a reaction was determined using visible-light spectrophotometry at 510 nm and 700 nm. Changing of C-ring structure influences on pigmentation of anthocyanins (Figure 2.1).

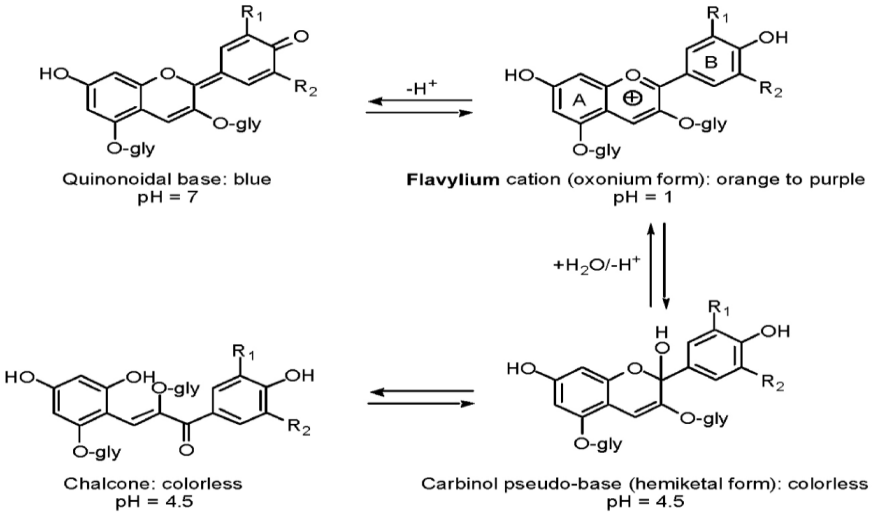

Figure 2.1 The reaction of anthocyanin in pH dependent (168)

Procedure

Total anthocyanin was determined using a pH differential method (166). Each fraction of red rice extract was diluted with buffer pH 1.0 (0.025 mM KCl) and incubated at room temperature for 15 min, whereas the samples diluted with the buffer pH 4.5 (0.4 mM CH_3COONa) were incubated for only 5 min. The absorbance of each sample was determined at wavelength 510 and 700 nm by using UV-visible spectrophotometer for elimination of interference from the background. The total anthocyanin content in the red rice extract was expressed as milligrams of cyanidin-3-glucoside equivalents per gram of rice extract (mg/g extract). Total anthocyanin content was calculated using the following equation:

$$\text{Total anthocyanin content} = \frac{A \times Mw \times DF \times 103}{\varepsilon \times 1}$$

$Abs = (Abs_{520nm}-Abs_{700nm})\ pH_{1.0} - (Abs_{520nm}-Abs_{700nm})\ pH_{4.5}$

MW =Molecular Weight of cyanidin-3-glucoside

DF = Dilution Factor

ε = Molar extinction coefficient = L $\times mol^{-1} \times cm^{-1}$

L = Length of cell path (1 cm)

2.2.8 High Performance Liquid Chromatography (HPLC) analysis of phenolic compounds, vitamin E derivatives and γ-oryzanol component from red rice extract

Principle

HPLC is a technique used in analytic chemistry to separate the components in a compound mixture, to identify and quantify each component. One of the most important steps in the separations is the pumping of sample mixtures in liquid solvent under high pressure through a column filled with an appropriate solid adsorbent material. The separation occurs depending on the ability of each component that can differently be interacted with the adsorbent material,

causing a delay of each component on a different portion of the column. Therefore the components are separated as they flow out the column.

Procedure

1) Phenolic compounds analysis

The profile of phenolic compounds in red rice extract was determined using HPLC analysis (Agilent 1200 series, Germany) compared with standard gallic acid, protocatechuic acid, catechin, chlorogenic acid, vanillic acid, coumaric acid and ferrulic acid as modified method described by Tian S and *et.al* (169).

Measurement of phenolic compounds was performed using an Inertsil ODS-3-C18 columns (250 x 4.6 mm, 5 mm particle diameter, GL Science Inc., Japan) coupled to a guard column. 20 µl of sample was subjected to the column. The chromatographic separation was carried out at room temperature with a flow rate 1.0 ml/ min of gradient elution using two mobile phase; A (0.1% trifluoroacetic acid in water) and B (100% methanol:MeOH). The detection wave length was set at 280 nm and linear gradient elution was used: 90% A (0-35 min), 10% A (35-40 min) and 90% A (40 min). The peak area was calculated and compared with the standard to obtain the concentration of all the compounds (mg/g extract).

2) Tocopherol and tocotrienol analysis

The eight derivatives of vitamin E (α-, β-, γ-, and δ-tocopherol /tocotrienol in each fraction of red rice extract were established using HPLC analysis as previously described by Pegg *et al* and Britz *et al* with slightly modified (170, 171). A YMC (Tokyo, Japan) C30 column (250 × 4.6 mm, 5 µm particle) with YMC carotenoid guard cartridge (23 x 40 mm, 5 µm particle) were used. Twenty µl of each sample was subjected to HPLC system compared with standard tocopherols and tocotrienols where the detection wave length was set at 280 nm. An

isocratic elution was employed with a mobile phase of methanol:H_2O (93:7) at a flow rate of 1.0 ml/min. The peak area was calculated to obtain the levels of vitamin E derivatives when compared to standard (mg/g extract).

3) Gamma (γ)-oryzanol analysis

The γ-oryzanol was analyzed using HPLC analysis as previously described with slightly modified (163). Measurement of the γ-oryzanol was performed using an Inertsil ODS-3-C18 columns (250 x 4.6 mm, 5 mm diameter, GL science Inc., Japan) coupled to a guard column. Twenty µl of each sample was subject to HPLC system and detection wavelength was set at 325 nm at room temperature with isocratic mobile phase composed of MeOH:acetonitrile (65:35) at a flow rate 1.0 ml/ min. The peak area was calculated to obtain the levels of γ-oryzanol concentration (mg/g extract).

Anti-chemoinvasive and ECM degradation enzymes inhibition from Red Jasmine Rice (*Oryza sativa* L.)

2.3 Cell lines and cell cultures

Human fibrosarcoma (HT-1080), Human breast cancer (MDA-MB-231), murine embryonic fibroblast (NIH-3T3) and RAW 264.7 mouse macrophage cells were obtained from the American Type Culture Collection (ATCC, USA). All of cell lines were cultured and maintained in RPMI, 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified incubator (37°C, 5% CO_2) until reaching 70-80% confluency. Cells were harvested and subsequent studies were performed.

2.4 Effect of the CEE, Hex, DCM, EtOAc and water fractions on cell viability

Principle

MTT (3-9,4,5 dimethylthiazol-2yl)-2,5 diphenyltetrazolium bromide) assay was used to analyze cell viability. In principle, viable cells can cleave the tetrazolium rings out from the MTT by the activity of a mitochondrial succinate dehydrogenase enzyme. The pale yellow MTT is then turned to form insoluble purple formazan crystals, which is impermeable to cell membranes. The formazan crystal is then accumulated inside the living cells. The purple formazan can be solubilized from cells with DMSO and absorbance was read at 570 nm and 630 nm (reference wavelength).

Procedure

$1.5x10^3$ cells of MDA-MB-231 and HT-1080 were seeded in 96-well microtitre plates and cultured in DMEM medium containing 10% FBS. After 24 h incubation, 0-200 µg/ml of PRFR were added into each well and incubated for a further 24-48 h. After 24-48 incubation, MTT solution (15 µl) was added and samples were incubated for a further 4 h. DMSO was added to dissolve MTT formazan. Absorbance was measured at 570 nm with a reference wavelength of 630 nm on 96-well plate reader.

2.5 Effect of the CEE, Hex, DCM, EtOAc and water fractions on MDA-MB-231 and HT-1080 cells invasion

Principle

Invasion of cancer cells to extracellular matrix (ECM) is one of the key steps for successful of metastasis. The invasion behavior of cancer cells was determined by modified Boyden chamber assay (172, 173). The chambers with a microporous membrane can assess the invasive capacity of tumor cells. Cells will be transferred to the upper compartment precoated with Matrigel and cells can degrade ECM and invade through the membrane pores. The invading cells will be determined from the lower part of the membrane.

Procedure

MDA-MB-231 and HT-1080 invasive capacity cells were determined by the modified Boyden chamber assay. Cell culture inserts with 8 μm pore sized (Millipore, Co Cork, Ireland) were coated with Matrigel (12 μg/insert). Conditioned medium was added into the lower chamber to act as a chemoattractant. 1.25×10^5 cells were seeded to the inserts and incubated with or without the tested compounds for 24 h. After incubation at 37°C and 5% CO_2, cells in the upper surface of the membrane were removed, whereas the invading cells (lower surface of the membrane), were then fixed with methanol and stained with toluidine blue. The cells that have invasive ability can actively migrated to underneath of the filter membrane. These cells were eluted with 20% acetic acid and absorbance was measured at 570 nm which is proportional directly to the number of invading cells (174). Gamma-tocotrienol and gamma-oryzanol**,** catechin, protocatechuic, chlorogenic (Chl), vanillic (Val), ferulic (Fer), grape seed and proanthocyanidin were used as a control compared with CEE, Hex, DCM, EtOAc and water fractions.

2.6 Effect of the CEE, Hex, DCM, EtOAc and water fractions on MMP-2 and MMP-9 secretions from HT-1080 cells

Principle

Method of zymography is an electrophoretic technique, which commonly used to analyze the activity of MMPs and uPA. The unfolding of proteins including MMPs under denaturing (SDS) and non-reducting conditions could be separated by a polyacrylamide gel containing enzymes substrate (gelatin or casein). Exchanging of SDS with nonionic detergent (Triton X-100) allows renaturation of resolved proteins. After removing of SDS, the gel is incubated with activation buffer and stained with Coomassie brilliant Blue. Both of proenzyme and active MMPs activities can be detected as clear bands against a blue background of undegraded enzyme substrates due to autocatalytic cleavage, denaturation and renaturation processes.

Procedure

MMP-2 and MMP-9 secretion from HT-1080 cells was performed by gelatin zymography (175). HT-1080 cells were treated with 0-100 μg/ml red rice extract fraction in serum-free DMEM medium for 24 h. The culture supernatant was collected and equal amounts of protein were subjected to gel electrophoresis (10% polyacrylamide gels) containing 0.1% w/v gelatin under non-reducing conditions. After twice washing with 2.5% (v/v) Triton X-100 for 30 min, gels were then incubated in an activation buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 200 mM NaCl, pH 7.4) at 37°C for 18 h. Gels were stained with Coomassie Brillant Blue R (0.1% w/v) and destained with destain solultion (30% methanol/ 10% acetic acid). Proteolytic activity of MMP-9 appeared as clear bands against a blue background. The digested bands were quantitated using Bio 1D software (Viber Lourmat).

2.7 Effect of the CEE, Hex, DCM, EtOAc and water fractions on MMP-9 secretion from MDA-MB-231 cells

MMP-9 secretion from MDA-MB-231 cells was determined using gelatin zymography. MDA-MB-231 cells were incubated with 0-100 μg/ml red rice extract in serum-free DMEM medium for 24 h. The culture supernatant was collected and equal amounts of protein were subjected to gel electrophoresis (10% polyacrylamide gels) containing 0.1% w/v gelatin under non-reducing conditions. After twice washing with 2.5% (v/v) Triton X-100 for 30, gels were then incubated in an activation buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 200 mM NaCl, pH 7.4) at 37°C for 18 h. Gels were stained with Coomassie Brilliant Blue R (0.1% w/v) and destained with destain solution (30% methanol/ 10% acetic acid). Proteolytic activity of MMP-9 appeared as clear bands against a blue background. The digested bands were quantitated using Bio 1D software (Viber Lourmat) (175).

2.8 Effect of the CEE, Hex, DCM, EtOAc and water fractions on MMP-2 and MMP-9 activities from HT-1080 cells

The culture supernatant of HT-1080 containing MMP-2 and MMP-9 was collected and subjected to gel electrophoresis (10% polyacrylamide gels) containing 0.1% w/v gelatin.

After twice washing with Triton X-100, gels were cut in single lane and incubated in activating buffer containing various concentrations of red rice extract at 37ºC for 24 h. Gel strips were stained with Coomassie Brilliant Blue R (0.1 % w/v) and proteolytic activities of MMP-2 and MMP-9 appeared as clear bands against a blue background. The digested bands were quantitated using Bio 1D software (Viber Lourmat) (176).

2.9 Effect of the CEE, Hex, DCM, EtOAc and water fractions on MMP-9 activity from MDA-MB-231 cells

The culture supernatant of MDA-MB-231 cells containing MMP-9 was collected and subjected to gel electrophoresis (10% polyacrylamide gels) containing 0.1% w/v of gelatin. After twice washing with Triton X-100, gels were cut in single land and incubated in activating buffer containing various concentrations of red rice extract at 37ºC for 24 h. Gel strips were stained with Coomassie Brilliant Blue R (0.1 % w/v) and proteolytic activity of MMP-9 appeared as clear bands against a blue background. The digested bands were quantitated using Bio 1D software (Viber Lourmat) (177).

2.10 Effect of the CEE, Hex, DCM, EtOAc and water fractions on collagenase activity

Principle

EnzChek Gelatinase/Collagenase Assay kit (fluorometric assay) was performed for analysis of collagenase activity. The kit contains substrate, DQ gelatin, fluorescein conjugated gelatin. Collagenase digestion of this substrate produces fluorescent peptides. The increase in fluorescence is proportional to Proteolytic activity of collagenase is directly proportional to fluorescence intensity, which measures with fluorescence plate reader.

Procedure

To investigate whether CEE, Hex, DCM, EtOAc and water fractions can directly inhibit collagenase activity *ex vivo*, Enz Chek Gelatinase/Collagenase Assay kit was performed to determine collagenase activity. Collagenase was incubated with various concentrations

of CEE, Hex, DCM, EtOAc and water fractions in the present of fluorescent substrated, DQ gelatin, the proteolytic activity was measured by fluorescence microplate reader.

Briefly, fluorescein-conjugated gelatin (10 μg/ml) (DQ gelatin) and collagenase (1 U/ml) were mixed and incubated with red rice extract in 96 well plates. Fluorescence intensity was recorded at 3 min intervals for 30 min with excitation wavelength 485 nm and emission wavelength 528 nm. Inhibitory effect of red rice extract on collagenase activity was calculated from the linear regression analysis of fluorescence intensity (178).

2.11 Effect of CEE, Hex, DCM, EtOAc and water fractions on NO production from LPS-induced RAW 264.7 mouse macrophage cells

Principle

The Griess reaction is an indirect colorimetric method for assay NO. The NO can be oxidized by CrO_3 to form nitrite, NO_2. The reaction between nitrites and a reagent containing sulphanilic acid and α-naphthylamine under acidic conditions then generates an azo dye which appears in a red pink color. The resulting stable water-soluble azo dye was measured using spectrophotometer at 540 nm. Although NO_2 can be further oxidized to NO_3, the oxidation is relatively slow and does not be converted to azo dye. Therefore autooxidation of NO_2 occurs in a very slow rate and can be considered to be insignificant to the measurement of NO.

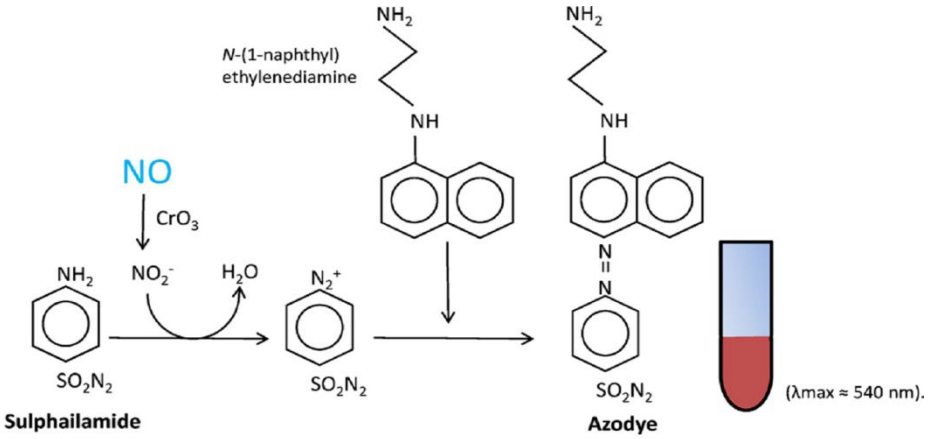

Figure 2.2 The NO detection method by Griess reagent (179)

Procedure

NO production was investigated by indirect measurement of the nitrite concentration in cultured medium using the Griess reagent system (180). 5.0×10^5 cells/well of RAW 264.7 mouse macrophage cells were seeded in a six-well plate for 24 h. Cells were pre-incubated with or without various concentrations of each fraction of red rice extract for 2 h. After 2 h incubation, cells were further treated with or without1 μg/mL LPS for 24 h. Culture supernatant was collected for Griess reaction for the determination of nitrite production. The absorbance was read at 540 nm using a 96 well plate reader. Concentration of nitrite in the cultured medium was calculated and compared with a standard curve of sodium nitrite ($NaNO_2$).

2.12 Effect of CEE, Hex, DCM, EtOAc and water fractions on IL-1, IL-6 and TNF-α production from LPS induced RAW 264.7 mouse macrophage cells

Principle

The enzyme-linked immunosorbent assay (ELISA) is a widely method based on a solid-phase enzyme immunoassay (EIA) to investigate unknown substances (antigen) by using specific antibodies. Multiple steps are required to provide a condition for the binding between the antigen-antibody pair. In brief, firstly, the antigens or unknown substances from the sample are binding to a solid surface. Secondly, an enzyme linked specific antibody can bind to the surface of antigen. The incubation is carried out for a certain period of time to allow a complete binding between the pair. Finally, the signal, which

commonly presented as a color change, generates through the activity of attached enzyme and can be analyzed using simple spectrophotometric method.

Procedure

The secretions of interleukin 1, 6 (IL-1 and IL-6) and tumor necrotic factor-alpha (TNF-α) in cultured medium were analyzed using an ELISA kit (Biolegend, USA). 5.0×10^5 cells/well of RAW 264.7 mouse macrophage cells were seeded in a six-well plate for 24 h. Cells were incubated with or without various concentrations of each fraction of red rice extract and LPS (1 μg/mL) for 24 h. The cultured supernatant was harvested for ELISA and the absorbance was read at 450 and 570 nm using a 96 well plate reader. The secretion of IL-1, IL-6 and TNF-α in the cultured medium was calculated and compared with a standard curve of IL-1, IL-6 and TNF-α (181).

Proanthocyanidin from red rice extract decreases invasiveness of MDA-MB-231 breast cancer cells via inhibition of the invasive proteins expression

2.13 Isolation of proanthocyanidin-rich fraction from red rice (PRFR)

The water fraction from red rice extract was further used for isolation of proanthocyanidin by Sephadex LH20 chromatography as previously described with slightly modified (167). Five gram water extract fraction was dissolved in methanol and loaded onto a Sephadex LH-20 Column (20 g hydrated in methanol and water). Each fraction was eluted with methanol/acetone (30:70). Proanthocyanidin content of fractions was determined by vanillin assay. Proanthocyanidin-rich fraction from red rice (PRFR) were collected, pooled and freeze-dried for further studies.

2.14 Determination of proanthocyanidin concentration by acid/butanol assay

Proanthocyanidin content in PRFR was determined using acid/butanol assay with slightly modified method (182, 183). Briefly, PRFR (1 mg) was dissolved in 1 ml of butanol/HCl (97.5:2.5 v/v) containing 2% (w/v) $NH_4Fe(SO_4)_2.12H_2O$ in 2 N HCl. This solution was boiled in hot water for 30 min and undissolved material was removed by centrifugation at 2,500 g for 5 min. The absorbance of proanthocyanidin from red rice was analyzed by

UV-visible spectrophotometer at 550 nm compared with standard proanthocyanidin from grape seeds.

2.15 Identification of proanthocyanidin types in PRFR using HPLC

Proanthocyanidin constituents in PRFR were investigated using HPLC analysis according to previously described method (167). PRFR (10 mg) was dissolved in 1.0 M HCl (1 ml) and subsequent hydrolysis by boiling the mixture for 30 min. 1.0% (v/v) trifluoroaetic acid (TFA) solution was added to hydrolysate solution. The ultra HPLC H class analysis was performed and. Sample was loaded to a C18-EPS Rocket column (53 mm x 7 mm, GRACE) and eluted for 5 min with isocratic of 0.05% TFA:acetonitrile (87:13), with flow rate 1.0 ml/min and detection of proanthocyanidin was measured at 210 nm.

2.16 Cell lines and cell culture

Human breast cancer cell lines (MDA-MB-231), human fibrosarcoma cell lines (HT-1080), human fibroblast, mouse fibroblast cells (NIH3T3) and human ovarian carcinoma cells (SKOV-3) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained under standard conditions (humidified atmosphere, 95% air and 5% CO_2 at 37°C).

2.17 Cytotoxicity of PRFR on MDA-MB-231, HT-1080, SKOV-3 and human skin fibroblast cells

MDA-MB-231, HT-1080, SKOV-3 ($2x10^3$ cells/well) and human skin fibroblast cells ($3.5x10^3$ cells/well) were seeded in 96-well plates and cultured in completed DMEM medium. After 24 h incubation, cells were treated with 0-200 μg/ml PRFR. After 24-48 h incubation, MTT solution was added and a further incubated for 4 h. DMSO was added to dissolve MTT formazan. Absorbance was measured at 570 nm with a reference wavelength of 630 nm on 96-well plate reader (184).

2.18 Anti-invasion and migration effects of PRFR on MDA-MB-231 cells

The inhibitory effect of PRFR on MDA-MB-231 cells invasion and migration was determined using a modified Boyden chamber assay as previously described (174). Cell culture inserts with 8 μm pore sized (Millipore, Co Cork, Ireland) were coated with Matrigel for invasion assay and coated with gelatin for migration assay. Conditioned medium was added into the lower chamber to act as a chemoattractant. 1×10^5 cells were seeded to the inserts and incubated with or without 0-20 μg/ml PRFR for 24 h. After incubation at 37°C and 5% CO_2, cells in the upper surface of the membrane were removed, whereas the invading cells (lower surface of the membrane), were then fixed with methanol and stained with toluidine blue. The cells that have invasion or migration abilities can actively migrated to underneath of the filter membrane. These cells were eluted with 20% acetic acid and absorbance was measured at 570 nm which is proportional directly to the number of invading/migrating cells.

2.19 Effect of PRFR on the secretion of MMP-9

MMP-9 secretion from MDA-MB-231 cells was determined using gelatin zymography. MDA-MB-231 cells were incubated with 0-20 μg/ml PRFR in serum-free DMEM medium for 24 h. The culture supernatant was collected and equal amounts of protein were subjected to gel electrophoresis (10% polyacrylamide gels) containing 0.1% w/v gelatin under non-reducing conditions. After twice washing with 2.5% (v/v) Triton X-100 for 30, gels were then incubated in an activation buffer (50 mM Tris-HCl, 10 mM CaCl2, 200 mM NaCl, pH 7.4) at 37°C for 18 h. Gels were stained with Coomassie Brilliant Blue R (0.1% w/v) and destained with destain solution (30% methanol/ 10% acetic acid). Proteolytic activity of MMP-9 appeared as clear bands against a blue background. The digested bands were quantitated using Bio 1D software (Viber Lourmat) (175).

2.20 Effect of PRFR on the MMP-9 activity in MDA-MB-231 cells

The effect of PRFR on MMP-9 activity was analyzed using gelatin zymography. The culture supernatant of MDA-MB-231 containing MMP-2 and MMP-9 was collected and subjected to gel electrophoresis (10% polyacrylamide gels) containing 0.1% w/v gelatin. After twice washing with Triton X-100, gels were cut in single lane and incubated in activating buffer containing various concentrations of PRFR at 37ºC for 24 h. Gel strips were stained with Coomassie Brilliant Blue R (0.1 % w/v) and proteolytic activity of MMP-9 appeared as clear bands against a blue background. The digested bands were quantitated using Bio 1D software (Viber Lourmat) (177).

2.21 Effect of PRFR on the activity of collagenase type IV activity

EnzChek Gelatinase/Collagenase Assay kit (fluorometric assay) was performed for analysis of PRFR on collagenase activity. Briefly, Fluorescein-conjugated gelatin (10 µg/ml) (DQ gelatin) and collagenase (1 U/ml) were mixed and incubated with 0-2 µg/ml PRFR in 96 well plates. Fluorescence intensity was recorded at 3 min intervals for 30 min with excitation wavelength 485 nm and emission wavelength 528 nm. Inhibitory effect of PRFR on collagenase activity was calculated from the linear regression analysis of fluorescence intensity (178).

2.22 Effect of PRFR on the production of IL-6 from MDA-MB-231 cells

The IL-6 secretion was determined using commercial kit (Biolegend, San Diego, CA, U.S.A.) as previously described. Briefly, 0-20 µg/ml proanthocyanidin (PRFR) were added to MDA-MB-231 cells. After 24 h incubation, the supernatant was harvested and centrifuged at 5,000 rpm for 5 min. IL-6 in culture supernatant was analyzed by ELISA and the absorbance was read at 450 and 570 nm using a 96 plate reader. IL-6 levels were calculated from IL-6 standard curve (181).

2.23 Effect uPA-plasminogen secretion by PRFR

The inhibitory effect of PRFR on uPA secretion from MDA-MB-232 cells was examined by casein-plasminogen zymography (185). The supernatant of PRFR-treated cells was

subjected to 10% PAGE which was copolymerized with human plasminogen (10 μg/ml) and β-casein (1 mg/ml) under non-reducing conditions. After twice washing with 2.5% Triton X-100 for 30 min, gels were incubated in an activation buffer. After 24 h incubation, gels were stained and followed by destaining as previously described.

2.24 Inhibitory effects of PRFR on the expression of MT1-MMP, uPAR, PAI-1 and ICAM-1 using western blot ananlysis

Principle

Western blot analysis is one of widely used analytical method to determine proteins on a gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. In this technique, proteins can be separated in different bands depends on theirs size, molecular weight. Protein bands are then transferred to a membrane which is later submerged in a solution containing labeled-specific antibodies to each protein. After adding antibodies in each step, the excess or unbound antibodies are removed, leaving only the bound antibodies to the protein. To determine the binding, the images of protein bands are exposed onto a film. If one specific antibody is used, a single band should be visible on the film. The thickness of the band or band intensity would correspond to the amount of protein present. Semi-quantitative determination of the amount of protein can also be performed by providing a set of standard amount of protein running on the gel in parallel with the sample.

Proceture

Protein extracts were prepared as previously described (174, 185). MDA-MB-231 cells were treated with 0-30 μg/mlf PRFR for 24 h. Cells were washed with ice cold PBS and scraped from 6 well plate in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 10 mM, EDTA, 10 μg/ml aprotinin, 1 mM PMSF, 10 μg/ml leupeptin, 1 % Triton X-100) containing a protease inhibitor . Cell lysate was centrifuged at 12,000 rpm for 10 min at 4 °C, and supernatant was collected. The protein concentrations were assessed using Bradford protein assay. MT1-MMP, uPAR, PAI-1 or ICAM-1 expressions in MDA-MB-231 cells were evaluated by Western blot as previously described. Protein extracts were then subjected to 10% SDS-PAGE. Resolved proteins were transferred onto

nitrocellulose membranes. Membranes were blocked with 5% defatted-milk in 0.1 % tween-PBS for 1 h. Membrane were probed with specific antibodies against MT1-MMP, uPAR, PAI-1 or ICAM-1 at 4°C, overnight and followed by incubation with secondary antibody consisting of horseradish peroxidase (HRP)-conjugated IgG. The HRP signals of bands were detected using an enhanced chemiluminescence and normalized against β-actin (102).

2.25 Effect of PRFP on the NF-κB DNA binding activity

Principle

DNA binding activity of NF-KB was investigated using a Cayman's NF-kappaB (p65) Transcription Factor Assay. It is also known for its high sensitivity that can be used with either nuclear extracts or whole cell lysates. Double stranded DNA (dsDNA) sequence containing the NF-κB responsive element are binding to the wells of a 96 well plate. The NF-κB responsive element can specifically bind to the NF-κB in a nuclear extract. The NF-κB (p65) can be detected by adding the primary antibody against NF-κB (p65), followed by the addition of a secondary anti-antibody conjugated to HRP. Once the HRP substrate is added, the yellow color is generated and can be readout at 450 nm. Specifically, this assay is specific to human NF-κB (p65) and does not cross-react with NF-KB (p50).

Procedure

The effect of PRFR on DNA-binding activity of NF-κB was determined using NF-κB (p65) Transcription Factor Assay Kit (Cayman Company, Ann Arbor, MI, USA) which was slightly modified (176). MDA-MB-231 cells were treated with 0-20 µg/ml PRFR. After 24 h incubation, cells were collected and washed with ice cold PBS. Nuclear protein fraction was extracted using the Nucbuster proteins extraction kit (Novagen) according to the manufacturer's instructions. Then, 10 µg/ml of nuclear extract were incubated in well-coated with dsDNA templates carrying NF-κB response elements at 4°C, overnight. Positive control and nonspecific binding samples as well as blanks were also incubated on the plate. Primary antibody (anti-NF-κB) was added in the well follow by adding of secondary antibody (goat anti-rabbit HRP). Then, the developing solution was added and

followed by stopping solution. Absorbance was read at 450 nm using microplate reader. Data were subtracted from nonspecific binding.

2.26 Statistical analysis

All assays were performed at least in duplicate. Data represent as mean ± S.D. of three independent experiments and express as percentage of control. Statistical analysis was analyzed by one-way ANOVA with Dunnett's multiple comparison test (Prism version 6.0 software). Statistical * $P < 0.05$ was considered significant.