CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Chemicals and instruments are listed in Appendices A-B.

2.2 Purple rice extraction

2.2.1 Plant material

Purple rice (*Oryza sativa* L.*indica*) variety Kum Doisaket used in this study was provided from Purple Rice Research Unit, Faculty of Agriculture, and Chiang Mai University, Thailand.

2.2.2 Preparation of purple rice extract

One kilogram of purple rice was blended and stirred overnight in 5 L of 80% ethanol. The ethanolic extract solution was filtered through filter paper (Whatman No.1) and evaporated by vacuum rotary evaporator. Then, concentrated extract solution was lyophilized to obtain purple rice extract (PRE) powder and stored at 4 ºC before use. The extract was dissolved in water to 100 and 500 mg/ml for animal experiment and dissolved in DMSO to 100 mg/ml for stock solution in cell culture experiment. A summary of the procedure of purple rice extract is shown in Figure 2.1.

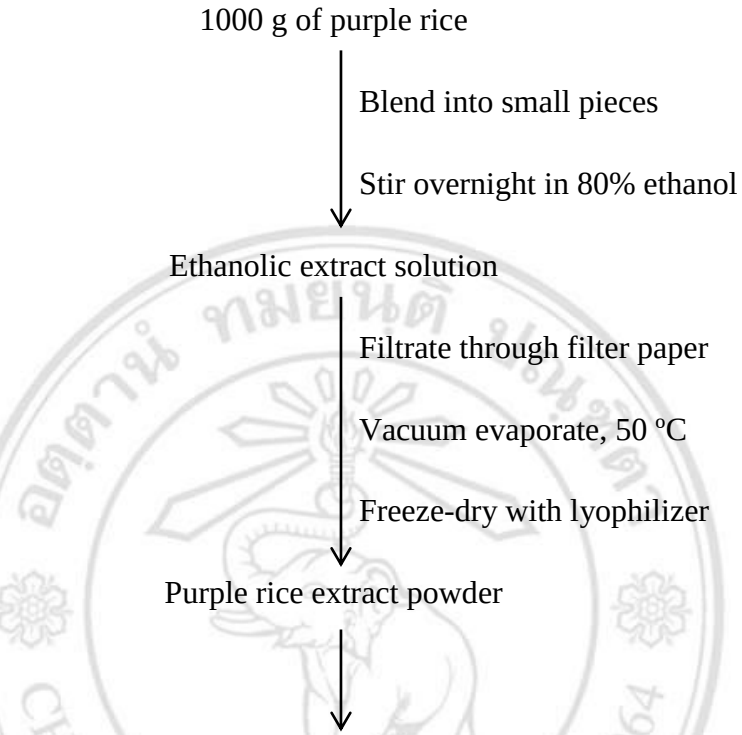

Figure 2.1 Schematic protocols for preparing purple rice extract

2.3 Determination of chemical composition and total antioxidant capacity of PRE

2.3.1 Determination of total phenolic compounds

The total phenolic content of PRE was determined by the Folin–Ciocalteu method. Twenty microliters of PRE (1 mg/ml) dissolved in DMSO, were mixed thoroughly with 100 µl of Folin–Ciocalteu reagent, following by the addition of 80 µl of 75 g/L sodium carbonate. After 30 minute of reaction at room temperature, the absorbance at 765 nm was measured. The total phenolic content was calculated from the gallic acid calibration curve, and the results were expressed as milligram of gallic acid equivalent (GAE) per gram weight of PRE.

2.3.2 Determination of anthocyanin

The anthocyanin content of PRE includes cyanidin-3-glucoside (C-3-G) and peonidin-3-glucoside (P-3-G). They were measured by reversed phase high performance liquid chromatography (RP-HPLC) for batch extraction control at central laboratory, Faculty of Agriculture, Chiang Mai University, Thailand.

One gram of PRE was soaked with 0.5% trifluoroacetic acid (TFA) in 20 ml of 95% ethanol. The extraction was shaken for 9 hours at room temperature. Then, the extracted solution was filtrated using Whatman filter paper No.4. After filtering extract was separated by C18 cartridge and then filtrated again passing filter 0.45 micron. RP-HPLC system has been used for the analysis of the sample. The extract 10 µl was subjected a HPLC instrument prepared with the column Allure C18 (reversed phase ODS C18); 25 cm × 4.6 mm diameter of particle size. Solvents used for the separation were 0.1% TFA in water and 0.1% TFA in methanol as the mobile phase A and B, respectively. The changing the mobile phase A to mobile phase B used a linear of water to methanol in 30 minutes at flow rate 1.0 ml/min. The separated molecules were monitored by UV-vis diode array detector at 280 nm. The anthocyanin contents were calculated by HPLC peak area compared with standard calibration curve.

2.3.3 Determination of antioxidant activity by DPPH assay

The antioxidant activity of PRE was determined on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhyorazyl (DPPH) free radical. In brief, 0.2 mM solution of DPPH in methanol was prepared. This solution (180 µl) was mixed with 20 µl of PRE in 96 well-plates. Corresponding reagent blank of test containing methanol with various concentrations of extract were prepared while of methanol with DMSO was used as a reagent blank of control. After the reaction was performed in dark at room temperature for 30 minutes and the absorbance was then measured at 517 nm using a microplate reader. The percentage of scavenging inhibition was calculated using the following formula:

% of DPPH scavenging activity =

$$\frac{((OD_{control} - OD_{blank}) - (OD_{test} - OD_{blank})) \times 100}{OD_{control} - OD_{blank}}$$

The results of scavenging inhibition presented as the concentration of the extracts which scavenged free radicals by 50% (SC_{50}).

2.4 Animal experimental protocol

2.4.1 Animals

The 8-week old male Wistar rats (300-350 g), from National Laboratory Animal Center of Mahidol University of Thailand, were housed in separated cages of each group. All experimental rats were performed in air conditioned room of 25-30 ºC under a 12 hours light-dark cycle, and were received free access to tap water and food. Experimental protocols described in this study and animal caring were approved by the Animal Ethic Committee of faculty of Medicine Chiang Mai University (19/2557).

2.4.2 Testosterone-depletion method (Castration)

The testosterone in rat circulation was depleted by castration, which caused undeveloped prostate gland. Male rats were neutered by the surgical removing of the testicles by open castration method with, sterilized instrument and environment. Firstly, rats were anesthetized by intraperitoneal injection with 25 mg/kg Zolentil-100. Then the sterilization was done by wiping 70% ethanol and providone iodine over the shaved skin. After rat's scrotum was ripped and spermatic cords were tided tightly, removing both testes and several layers of fascia was performed as shown in the Figure 2.2. After bleeding termination, the tunica vaginalis and scrotum were closed with suture. The unconscious rats were placed in cage under the warming lamp. Once conscious, the rats were relocated into a cage with clean litter and an ample supply of water and food. For disinfection, rats were wiping with povidone iodine for 7 days and administrated 20 mg/kg Enrofloxacin for 5 days. Finally, the rats were divided into designed groups.

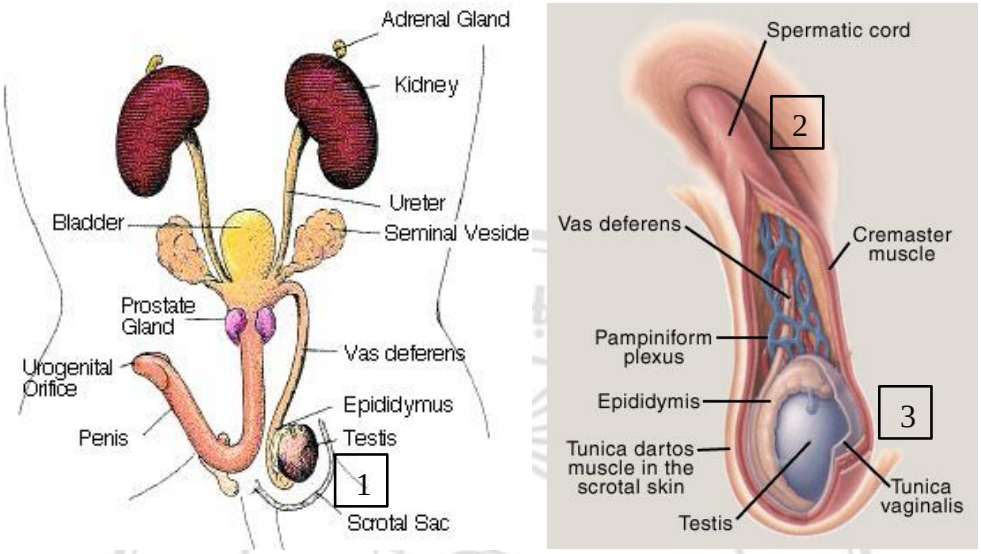

Figure 2.2 Castration in rat. 1) Ripe scrotum, 2) Tide spermatic cords tightly, 3) Remove both testes and several layers of fascia (derived from https://www.biologycorner.com/worksheets/rat_urogenital.html and http://malecontraceptives.org/methods/heat_biology.php)

2.4.3 Experimental protocol for testosterone-induced prostatic hyperplasia

Adult male Wistar rats were divided into four groups, with five rats per each group (Figure 2.3). Group 1 and group 2 were normal rats while group 3 and group 4 were castrated rats. In addition, group 2 and group 4 were subcutaneously administrated 3 mg/kg testosterone propionate in corn oil once a day for 30 days to induce prostatic growth. Control group (group 1 and group 3) received injections of corn oil. After 30 days, the rats were sacrificed by diethyl ether overdose. Blood samples were taken from portal vein to measure testosterone level. Prostate tissue was collected and weighted separately.

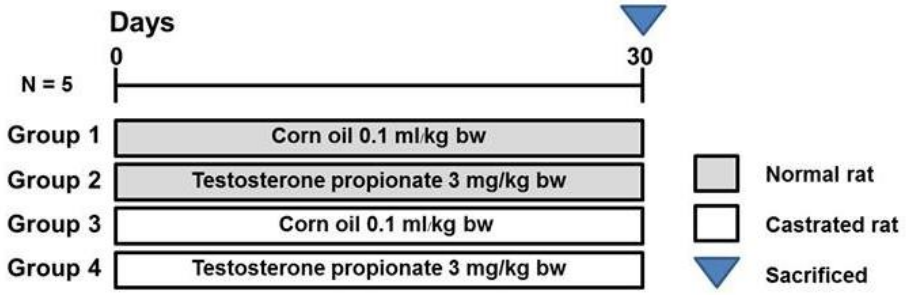

Figure 2.3 Preliminary experimental model of benign prostatic hyperplasia (BPH)

2.4.4 The study of PRE on testosterone-induced prostatic hyperplasia in rat

Male rats were divided into eight groups, with ten rats per group. Group 1 was normal rats and group 2-8 were castrated rats to reduce the endogenous testosterone following procedure in 2.4.2. In group 4-8, 3 mg/kg testosterone propionate was daily injected (s.c) to increase the prostatic growth for 30 days, as shown in Figure 2.4. Group 2 and 3 were non-treated group and the corn oil injection group for control. Group 4 was fed water while group 5 and 6 were fed 0.1g/kg and 1.0 g/kg crude extract of purple rice (PRE) respectively. Group 7 was administered 0.5 mg/kg finasteride in saline and group 8 was given 10 mg/kg bicalutamide (Casodex) in 0.5% Tween-80 phosphate buffered saline. The finasteride (5α-reductase inhibitor) and bicalutamide (anti-androgen receptor signaling) were used as positive drugs for inhibiting prostatic hyperplasia. After 30 days, rats were sacrificed with diethyl ether. Blood samples were taken from portal vein for measurement of testosterone level. Prostates were collected and weighed. Then, we divided prostate to ventral prostate and dorsolateral prostate (Figure 2.5) and determined the size. Part of the tissue (n=4) was fixed by 10% formaldehyde in PBS to evaluate in histological analysis. The remaining tissue (n=6) was immediately stored at -80 ºC for protein extraction.

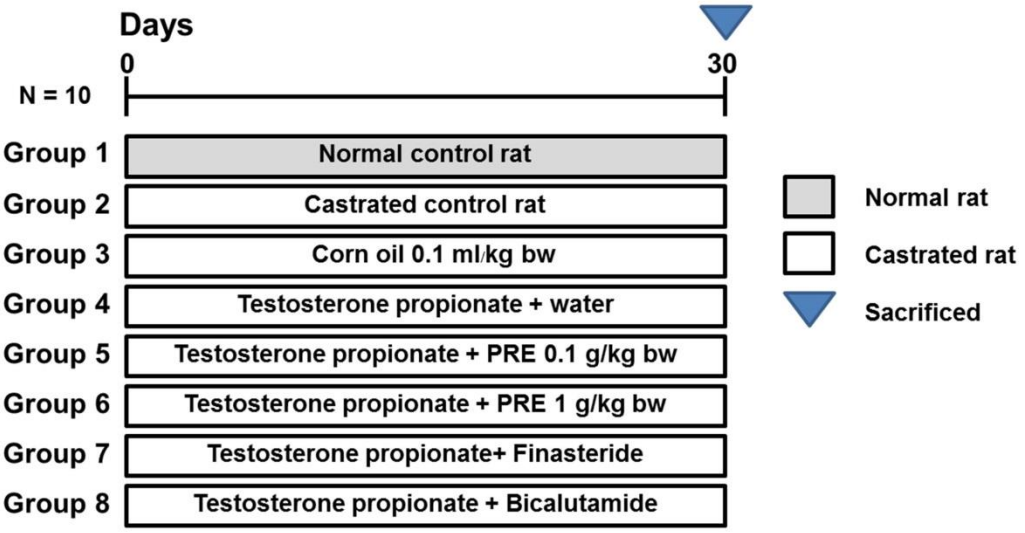

Figure 2.4 Experimental protocols for determination the effect of PRE on testosterone-induced prostatic hyperplasia

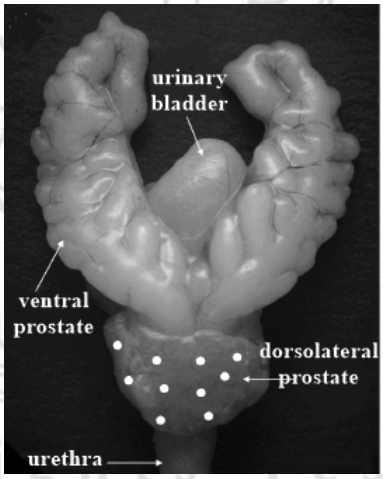

Figure 2.5 Gross appearance of the rat prostate, with a part of the ventral prostate and dorsolateral prostate (derived from http://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0716-97602011000300007)

2.4.4.1 Determination of testosterone levels in serum

Blood samples were collected and centrifuge at 3,000 rpm for 10 minutes. The separated serum was measured for testosterone level by central laboratory of Maharaj Nakorn Chiang Mai Hospital, Faculty of medicine, Chiang Mai University.

2.4.4.2 Histopathological examination

The prostates in each group were fixed with 10% formaldehyde in PBS for 24 hours. Subsequently, each part of prostate were cut and portioned in plastic cassette. Tissue sectioned slide preparation and hematoxylin and eosin staining were performed at department of pathology, Faculty of medicine, Chiang Mai University. The tissues were evaluated in histological analysis under the light microscope.

2.4.4.3 The study of PRE on androgen receptor expression in prostate

1) Protein extraction from prostate

The frozen rat prostates were defrosted on ice and then separated into ventral and dorsolateral prostate lobes. After weighting, 300 mg of dorsolateral prostate and 1 ml the tissue lysis buffer (Bio Basic Inc., Canada) were mixed and homogenized 10-15 seconds on ice-cold bucket. The homogenate was centrifuged at 20,000 g for 30 minutes at 4 ºC to separated non-solubilized particle. The supernatant were collected and stored at -20 ºC. Protein concentration was quantified using Bradford protein assay kit (BioRad, USA). The AR and PSA were determined by Western blot analysis with specific antibodies against rat AR and PSA.

2) SDS-PAGE and Western blot analysis

Ten microgram of total protein was separated by 8% gel SDS-PAGE under 90V for 2 hours. Separated proteins were then transferred onto HybondTM-ECL nitrocellulose membrane with 10 V for 1 hour. The membrane was incubated in 5 % skim milk for 1 hour at room temperature following incubated overnight with anti-AR antibody (1:500; Merck Millipore, USA) at 4 ºC or anti-β-actin (1:1000; Sigma, USA) for 1 hour at room temperature. After washing 5 times with TBS-Tween buffer, the membrane was incubated with HRP-goat anti-rabbit IgG antibody (1:10,000; Invitrogen, USA) for 2 hours at room temperature following washing 5 times with TBS-Tween buffer. The membranes were incubated with ECL substrate (Bio-Rad, USA) at room temperature for 5 minute. Chemiluminescent bands were visualized by exposing to X-ray films. The density of specific bands was analyzed using Image-J software. Relative of target protein in treatment group was normalized against β-actin expression and then compared to non-treatment group.

2.5 The effect of purple rice extract on growth of prostate cancer cell line

2.5.1 Cell Culture

Androgen-dependent human prostate cancer cell line (LNCaP) and androgen-independent human prostate cancer cell line (DU-145) were purchased from Cell Lines Service GmbH. LNCaP and DU-145 were grown in Roswell Park Memorial Institute medium (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100U/ml penicillin, 100 µg/ml streptomycin) in a humidified incubator with 5% CO_2 at 37 ºC.

2.5.2 Determination of cell viability by MTT assay

The human prostate cancer cell line (5×10^3 cells/well) was cultured in 96-well plates for 24 hours and then the culture media was changed by RPMI containing various concentration of PRE (0, 50, 100 and 200 µg/ml). Cells were continuously cultured at 37 °C in 5% CO_2 for 0, 24 and 48 hours. The cell viability was determined by using MTT assay. The fifteen microliters of sterilized stock MTT solution was added to each well and then the mixtures were incubated for 4 hours. After this, the solution was removed and then DMSO (100 µl/well) was added to dissolve formazan crystals. The absorbance was measured at 450/630 nm using a microplate reader. The percentage of cell viability was calculated at 24 and 48 hours.

2.5.3 Determination of gene expression by RT-PCR

2.5.3.1 RNA extraction

The prostate cancer cells (2.5×10^5 cells/well) were cultured in 6-well plates for 24 hours at 37 °C in 5% CO_2 and then treated with PRE of 0, 50, 100 and 200 µg/ml for 24 hours. Total RNA was extracted using Direct-zol™ RNA MiniPrep (Zymo Research, USA) according to the manufacturer's instructions. Briefly, the culture media was removed and 1000 µl TRI Reagent solution was added directly to remaining cell in each well. After well mixing, cell lysate was transferred into an RNase-free tube and 95% ethanol was added following by mixing again. The mixture was transferred into a Zymo-Spin™ IIC Column in a collection tube and centrifuged at 12,000 g, 4 ºC for 1 minute. The column was transferred a new collection tube and 400 µl Direct-zol™ RNA PreWash were added and then were centrifuged at 12,000 g, 4 ºC for 1 minute. RNA wash buffer (700 µl) was added to the column and the column was centrifuged at 12,000 g, 4 ºC for 1 minute. The column was repeated centrifugation at 12,000 g, 4 ºC for 2 minutes to assure complete removal of wash buffer. To elute RNA, the column was transferred into an RNase-free tube and 50 µl DNase/RNase-free water was added to middle of the membrane. Finally, the column was centrifuged at 12,000 g, 4 ºC for 1 minute to collect RNA solution. The concentration of RNA was measured by NanoDrop Spectrophotometer and then stored at -80 ºC. A summary of the procedure of RNA extraction is shown in Figure 2.6.

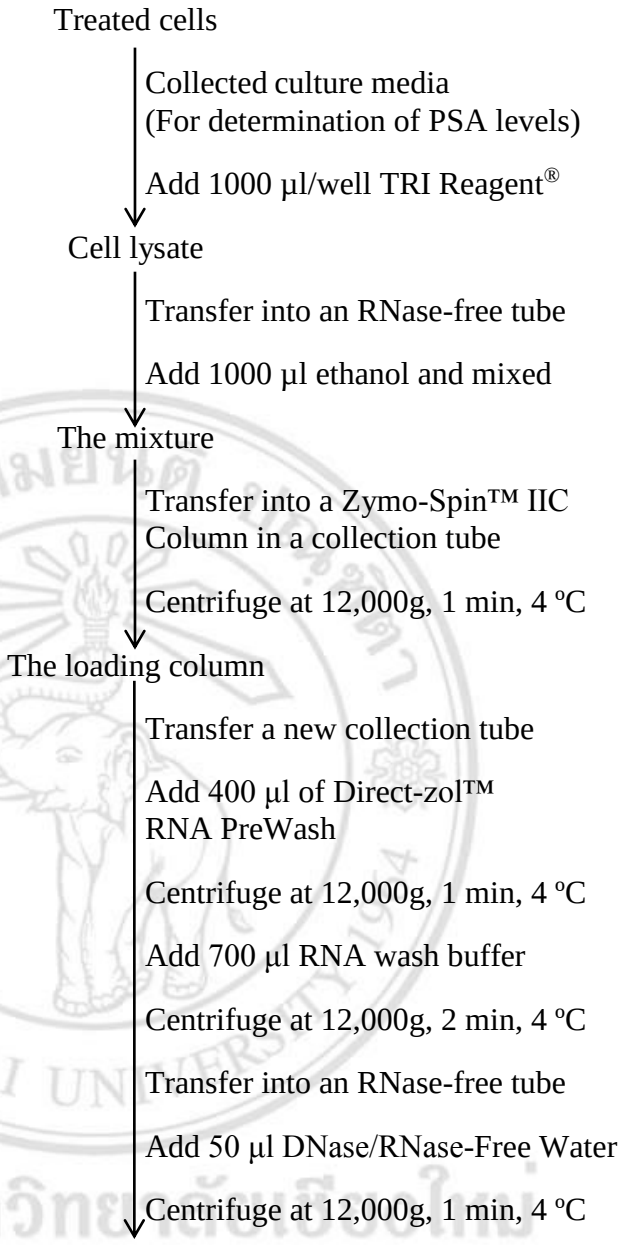

Figure 2.6 Schematic protocols for RNA extraction

2.5.3.2 cDNA synthesis

The total RNA was measured for concentration by NanoDrop Spectrophotometer. One microgram of RNA was subjected to cDNA synthesis using ReverTra Ace® qPCR RT Master Mix (Toyobo, Japan) according to the manufacturer's instructions. Firstly, RNA was denatured by heating to 65 ºC for 5 minute and then shield on ice. Then, the reaction solution was prepared following reagents in PCR tube on ice;

5x RT Master Mix	2	µl
RNA template	1	µg
Adjust volume by Nuclease-free Water		
Total Volume	10	µl

Finally step, the mixture was subjected to thermal cycler following the temperature program;

37 ºC	15	minute
50 ºC	5	minute
98 ºC	5	minute

After the reaction has finished, the PCR products were stored at 4 ºC.

2.5.3.3 Reverse transcription polymerase chain reaction (RT-PCR)

One microgram of cDNA was amplified by PCR using primers for specific genes as shown in Table 2.1. The reaction mixture was prepared as followed;

cDNA	1	µg
2x Quick Taq HS DyeMix (Toyobo, Japan)	10	µl
Forward primers (10 µM)	1	µl
Reverse primers (10 µM)	1	µl

Adjust volume by RNase free water to 20 µl

The reaction mixture was run by thermal cycler with cycling the condition:

Initial denaturation	94 °C	2 minutes	
Denaturation	94 °C	1 second	PCR cycle
Annealing	X °C	30 seconds	
Final extension step	68 ºC	1 minute	

The PCR products were separated by 2% agarose gels electrophoresis in 0.5M TAE buffer and gel was stained with ethidium bromide. The light bands were recorded by gel documentation and analysis (gel doc). The intensity of bands was analyzed using Image-J software. Relative of target mRNA in treatment group was normalized against GAPDH expression and then compared to non-treatment group

Table 2.1 Primer sequences, annealing temperature and PCR cycle for RT-PCR

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature	PCR cycle
AR	TGTCAACTCCAGGATGCTCTACTT	TTCGGACACACTGGCTGTACA	52	40
5α-reductases	CCTGTTGAATGCTTCATGACTTG	TAAGGCAAAGCAATGCCAGATG	55	30
GAPDH	ACCACAGTCACTGCCATCAC	TCCACCACCCTGTTGCTGTA	55	25

2.5.4 Determination the expression of AR and PSA

2.5.4.1 Cell treatment and Western blot analysis

LNCaP cells (2.5×10^5 cells/well) were cultured in 6-well plates for 24 hours and then treated with PRE at 0, 50, 100 and 200 µg/ml for 24 hours. After treatment, the cells were homogenized in RIPA buffer (1 M Tris pH 7.4, 5 M NaCl, 10% triton X-100, 1% sodium deoxycholate, 0.5 M EDTA) containing 200 µl protease inhibitor cocktail. The supernatant were collected and protein was quantified. Twenty microgram of total protein was measured AR and PSA expression by western blot analysis according to 2.4.4.3. The PSA expression was detected specific protein by using Anti-prostate-specific antigen antibody (1:1000; Thermofisher scientific, USA) and followed by HRP-goat anti-mouse IgG antibody (1:30,000; Merck Millipore, USA) to bind with primary anti-PSA.

2.5.4.2 Determination of PSA secretion into culture supernatant by ELISA

The PSA levels in cell media from 2.5.4.1 were determined by RayBio® Human PSA-total ELISA Kit (RayBiotech, USA) according to the manufacturer's instruction. Firstly, the reagents, samples and standards were prepared before use. One hundred microliters of standard or sample was added into appropriate wells. The well was cover and incubated for 2.5 hours at room temperature with gentle shaking. After the solution discarding, each well was washed 4 times with 1X wash solution (300 µl). Plate was inverted and put on clean paper towels. Biotinylated antibody (100 µl) was loaded to each well and plate then was incubated for 1 hour at room temperature with gentle shaking. After washing (4 times), Streptavidin solution (100 µl) was added to each well and plate was incubated for 45 minutes at room temperature with gentle shaking. The solution was discarded. Each well was washed 4 times with 1X wash solution (300 µl). Then, substrate reagent (100 µl) was added and plate was then incubated for 30 minutes at room temperature in the dark with gentle shaking. Finally, 50 µl stop solution was added to each well. The absorbance at 450 nm was measured by microplate reader immediately.

2.6 Statistical analysis

All calculation were done using Microsoft Excel and SPSS statistic 17.0. Data are expressed as mean ± standard deviation (SD). Results were analyzed and assessed by One-Way ANOVA and LSD. $P<0.05$ was considered statistically significant.