CHAPTER 3

RESULTS

3.1 The properties of purple rice extraction

3.1.1 Phytochemical constituents of PRE

One kilogram of Kum Doi Saket rice grain was extracted by 80% ethanol to produce crude ethanol purple rice extract (PRE). After lyophilization, the yield of PRE was 1.49 g/100 g rice grain.

Total phenolic content and anthocyanin content including cyanidin-3-glucoside and peonidin-3-glucoside of PRE extract were summarized in Table 3.1. In purple rice extract, phenolic was present in abundance in 62.35±4.26 mg GAE/g of extract. Additionally, the level of cyaniding-3-glucoside and peonidin-3-glucoside were 2.44±0.06 and 0.33±0.01 mg/g of extract respectively. The HPLC chromatogram of cyanidin-3-glucoside and peonidin-3-glucoside shows peaks with good resolution and magnitude in Figure 3.1.

Table 3.1 Phytochemical constituents of PRE

Phytochemicals	Total amounts* (mg/g of extract)
Phenolic content (mg GAE)	62.35±4.26
Cyanidin-3-glucoside	2.44±0.06
Peonidin-3-glucoside	0.33±0.01

*Mean±SD

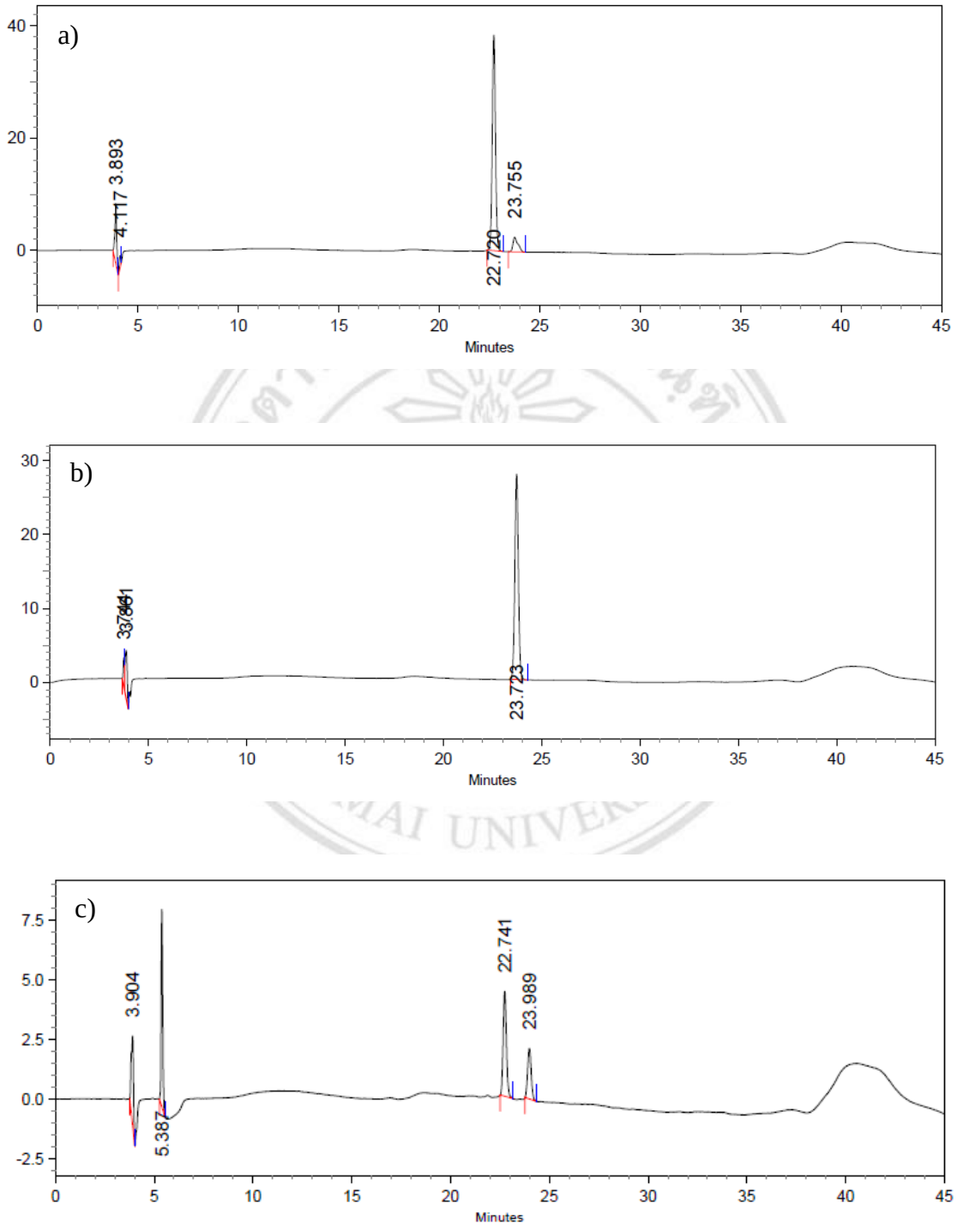

Figure 3.1 HPLC chromatogram of standard a) C-3-G, b) P-3-G and c) sample PRE 1 mg

3.1.2 Radical scavenging activity of PRE

The efficacy of PRE on DPPH free radical scavenging was determined compared to vitamin C. The PRE extract showed the maximum percent inhibition at 78.79% by concentration of 200 μg/ml. The SC_{50} of PRE was 124.75 μg/ml (R^2 = 0.990; Figure 3.2). One milligram of PRE showed the antioxidant activity equivalent to 56.5 μg vitamin C (Figure 3.3). Thus, PRE exhibited good antioxidants that might be the activity of its phenolic contents especially C-3-G or P-3-G.

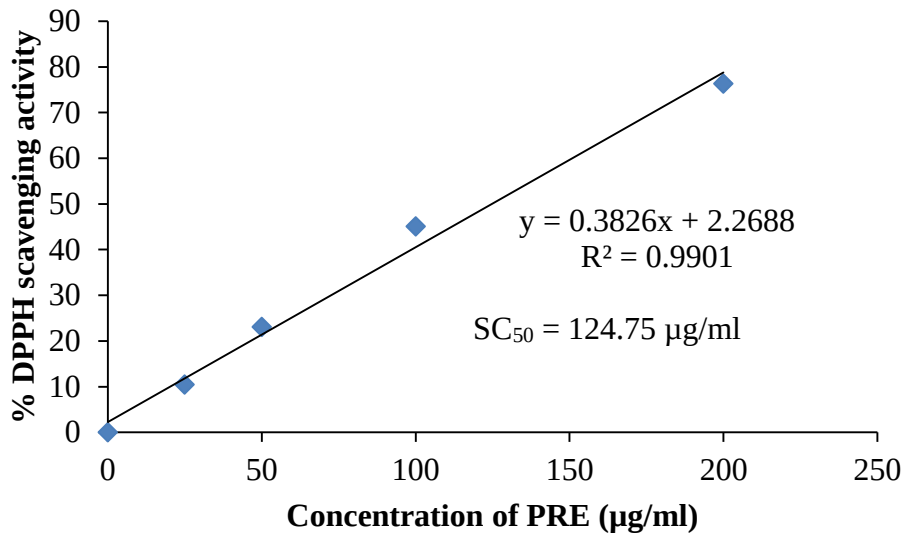

Figure 3.2 The linear equation of PRE concentration and free radical scavenging activity. SC_{50} of PRE obtained by linear equation: y = 0.3826x+2.2688.

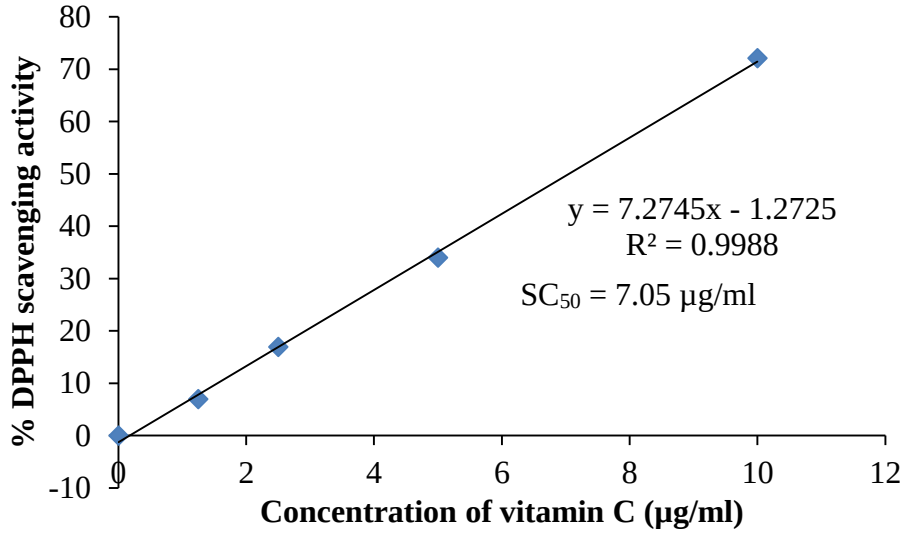

Figure 3.3 The linear equation of Vitamin C concentration and free radical scavenging activity. SC_{50} of vitamin C obtained by linear equation: y = 7.2745x-1.2725.

3.2 Effect of testosterone on rat benign prostatic hyperplasia

3.2.1 Prostate weight and prostate weight ratio

Prostate weight of experimental rats is shown in Table 3.2. Normal rats treated with testosterone showed the increasing of prostate weight about 15.6% compared to normal rat. In comparison with the normal rat group, the castrated rats showed smaller prostate which 82.6% lower prostate weight. However, the castrated rat treated with testosterone group significantly increased prostate weight by 79% compared to castrated rat group, but not significantly different when compare to normal rat.

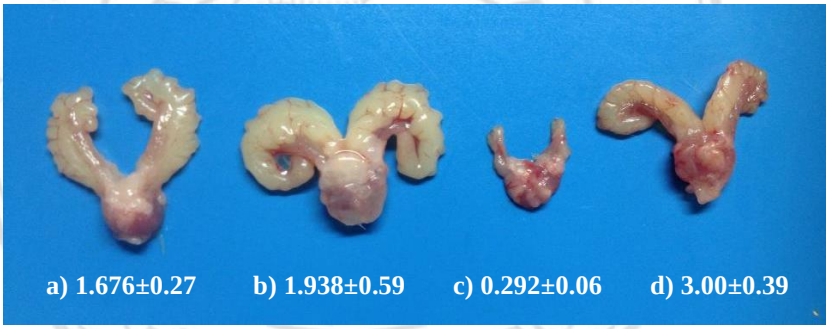

Figure 3.4 The gross observation and prostate weight (g) a) normal rat group, b) normal rat and testosterone injection group, c) castrated rat group, d) castrated rat and testosterone injection group

3.2.2 Serum on testosterone concentration

The serum testosterone (Table 3.2) in the castrated rat group was not detectable by using the automatic machine from hospital service (< 0.025 ng/ml). Castrated rat treated with testosterone group increased the testosterone levels (1.235 ± 0.19 ng/ml) compared to castrated control group which resembled to normal rat group (1.420 ± 1.32 ng/ml). While, the testosterone levels in the normal rats treated with testosterone was lower than the castrated rats treated with testosterone.

Table 3.2 Prostate weight and testosterone levels in serum of each group

Group	Prostate weight (g)	Testosterone (ng/ml)
1	1.676 ± 0.27	1.420 ± 1.32
2	1.938 ± 0.59	0.307 ± 0.24
3	0.292 ± 0.06[a,b]	< 0.025
4	3.000 ± 0.39[b,c]	1.235 ± 0.19

All values are mean ± SD (N=5) using one-way ANOVA (post hoc: LSD).
a: statistically significantly compared with group 1 (P<0.05)
b: statistically significantly compared with group 2 (P<0.05)
c: statistically significantly compared with group 3 (P<0.05)
Group 1: normal rat group
Group 2: normal rat and testosterone injection (3 mg/kg) group
Group 3: castrated rat group
Group 4: castrated rat and testosterone injection (3 mg/kg) group

3.3 Effect of PRE on testosterone-induced prostatic hyperplasia in rat

3.3.1 Effect of PRE on the growth of prostate

As shown in Table 3.3, the castrated rats showed a significant decrease in prostate weight by 87.4% compared to the normal rats which were similar result to the preliminary study. Rats treated with testosterone showed prostate weight about 14.65 fold higher compared to the castrated rats and 1.84 fold compared to normal rats. When compared to testosterone-treated alone, rats received PRE at 0.1 and 1.0 g/kg body weight showed slightly lower prostate weight about 8.31% and 9.50%, respectively. Then, to indicate the part of prostate which might be affected by PRE, dorsolateral and ventral were separately determined. The result showed that ventral prostates weight were not different between the testosterone treated alone rats and PRE treated rats, while rats treated by PRE at 0.1 and 1.0 g/kg body weight showed a significant decrease in dorsolateral prostate weight about 11.43% and 20.0%, respectively. In addition, finasteride and bicalutamide significantly reduced the prostate weight by 22.55% and 59.94% from testosterone treated alone rats respectively.

Table 3.3 Prostate weight of each experimental group

Group	Prostate weight (g)	Ventral prostate weight (g)	Dorsolateral prostate weight (g)
1	1.83 ± 0.14	1.12 ± 0.19	0.66 ± 0.08
2	0.23 ± 0.03[a]	0.08 ± 0.02[a]	0.15 ± 0.01[a]
3	0.24 ± 0.03[a]	0.09 ± 0.02[a]	0.14 ± 0.02[a]
4	3.37 ± 0.42[a,b]	2.25 ± 0.29[a,b]	1.05 ± 0.12[a,b]
5	3.09 ± 0.24[a,b]	2.10 ± 0.12[a,b]	0.93 ± 0.15[a,b,c]
6	3.05 ± 0.30[a,b,c]	2.15 ± 0.25[a,b]	0.84 ± 0.08[a,b,c]
7	2.61 ± 0.22[a,b,c]	1.65 ± 0.22[a,b,c]	0.86 ± 0.06[a,b,c]
8	1.35 ± 0.30[a,b,c]	0.81 ± 0.24[a,b,c]	0.05 ± 0.10[a,b,c]

All values are mean ± SD (N=10) using one-way ANOVA (post hoc: LSD).
a: statistically significantly compared with group 1 (P<0.05)
b: statistically significantly compared with group 2 (P<0.05)
c: statistically significantly compared with group 4 (P<0.05)

Group 1: normal rat
Group 2: castrated rat
Group 3: castrated rat and corn oil injection
Group 4: castrated rat treated with testosterone and water orally treatment
Group 5: castrated rat treated with testosterone and PRE (0.1 g/kg) orally treatment
Group 6: castrated rat treated with testosterone and PRE (1 g/kg) orally treatment
Group 7: castrated rat treated with testosterone and finasteride orally treatment
Group 8: castrated rat treated with testosterone and bicalutamide orally treatment

3.3.2 Effect of PRE on the testosterone levels in the serum

Mean serum testosterone level (ng/ml) of each experimental group is summarized in Table 3.4. By the normal range of rat serum testosterone (0.6-2.47 ng/ml (46)), castrated rats showed the undetectable level of testosterone by this measurement (<0.025 ng/ml). Whereas, rats treated with 3 mg/kg body weight of testosterone showed about 5 times higher level of testosterone than normal rat. There was no significant difference of testosterone level between groups of testosterone administration. Therefore, the PRE treatments have not affected rat the testosterone level.

Table 3.4 Testosterone levels in serum of each experimental group

Group	Testosterone (ng/ml)
1	0.979 ± 1.39
2	<0.025[a]
3	<0.025[a]
4	4.430 ± 1.97[a,b]
5	7.036 ± 3.58[a,b]
6	4.916 ± 1.91[a,b]
7	8.650 ± 2.42[a,b,c]
8	6.170 ± 3.47[a,b]

All values are mean ± SD (N=10) using one-way ANOVA (post hoc: LSD).
a: statistically significantly compared with group 1 (P<0.05)
b: statistically significantly compared with group 2 and 3 (P<0.05)
c: statistically significantly compared with group 4 (P<0.05)
Group 1: normal rat
Group 2: castrated rat
Group 3: castrated rat and corn oil injection
Group 4: castrated rat treated with testosterone and water orally treatment
Group 5: castrated rat treated with testosterone and PRE (0.1 g/kg) orally treatment
Group 6: castrated rat treated with testosterone and PRE (1 g/kg) orally treatment
Group 7: castrated rat treated with testosterone and finasteride orally treatment
Group 8: castrated rat treated with testosterone and bicalutamide orally treatment

3.3.3 Effects of PRE the histomorphology of the prostate tissue

To observe the histological changes of the prostate, the dorsolateral prostate tissue section were stained by H&E and the histological feature were determined under light microscope and photographed (Figure 3.6-3.8). The normal rat (group 1) maintained the size and shape of the acinus well without atrophy. The epithelium cells were arranged as a single layer and the secretory lumen was filled with acidophilic material (Figure 3.5). In castrated rat the prostate gland (group 2 and 3) showed underdeveloping feature with less secretory material filling in the gland. On the other hand the epithelium cells in testosterone treated group (group 4) showed the proliferation to develop excessive glands and multiple unorganized layers. The histopathological change in testosterone treated rat was slightly ameliorated by treatment with PRE at 1.0 mg/ml (group 6) and clearly observed in finasteride treated group (group 7).

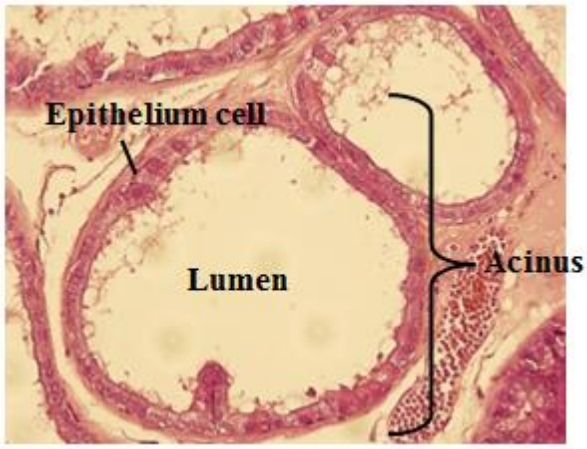

Figure 3.5 Dorsolateral prostate of normal rat was composed of epithelium cell and secretory lumen, are observed by H&E

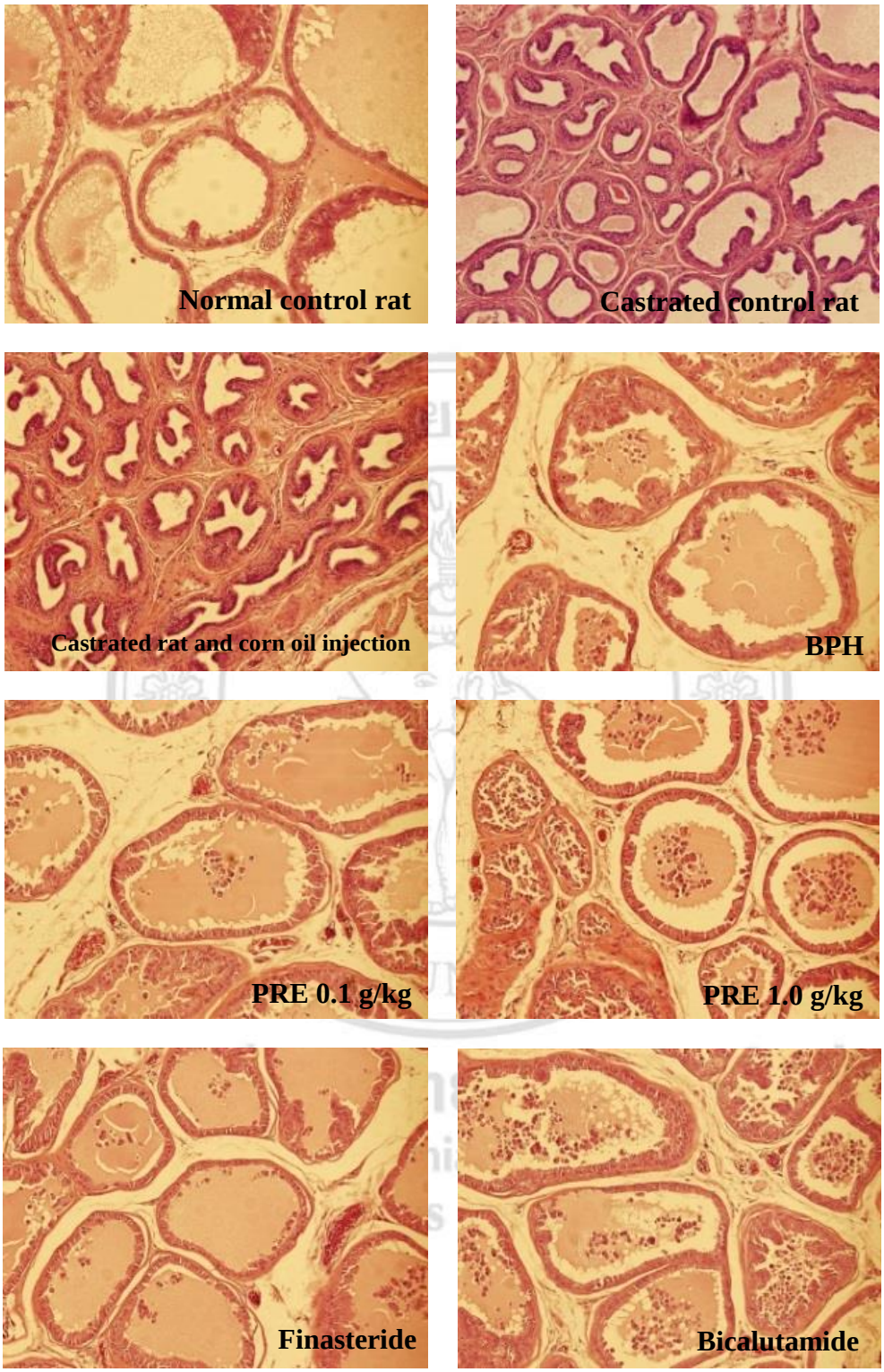

Figure 3.6 Histological examination of hematoxylin-eosin sections of rat dorsolateral prostate (x 10)

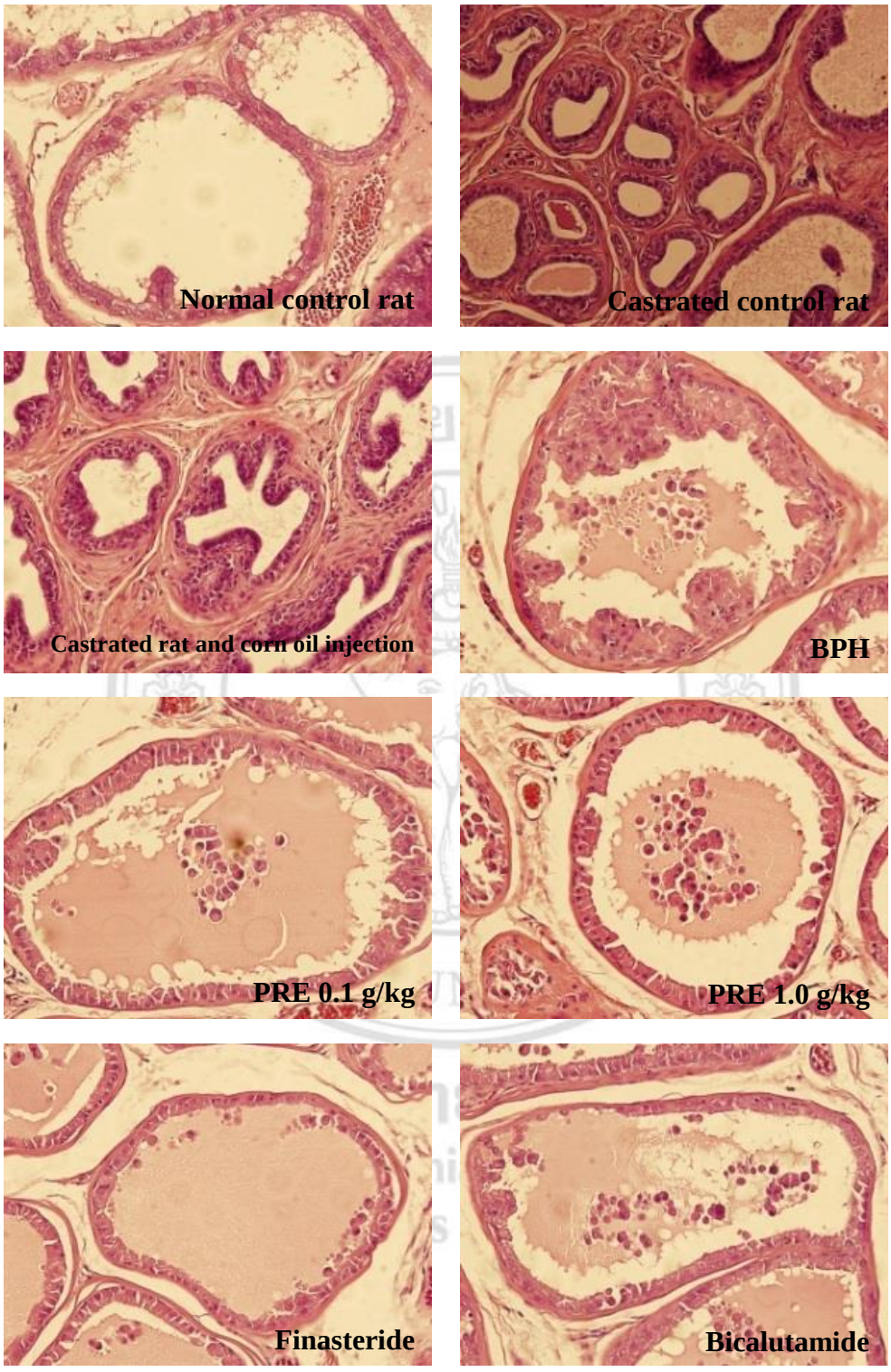

Figure 3.7 Histological examination of hematoxylin-eosin sections of rat dorsolateral prostate (x 20)

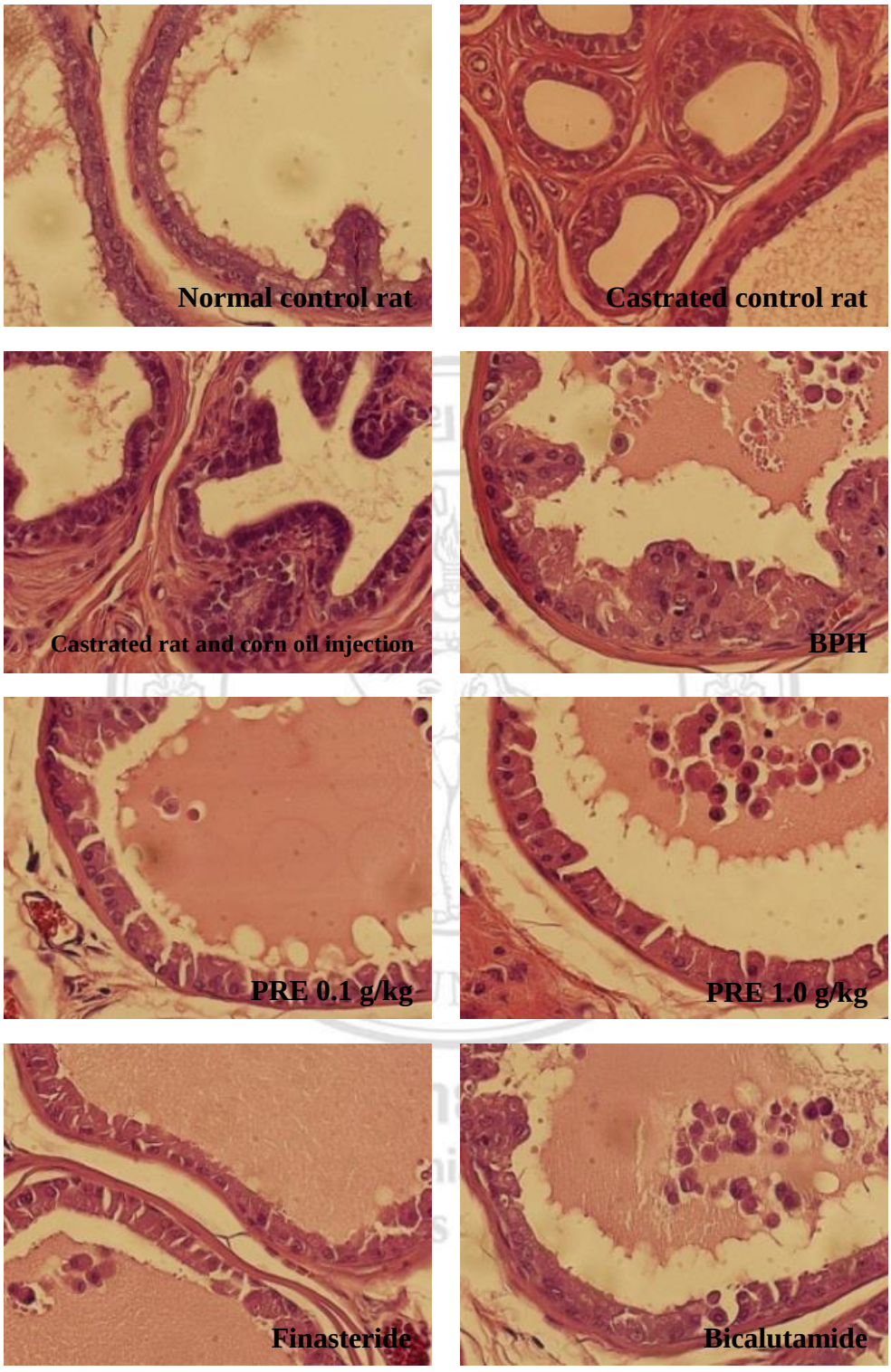

Figure 3.8 Histological examination of hematoxylin-eosin sections of rat dorsolateral prostate (x 40)

3.3.4 Effect of PRE on expression of AR in rat prostate

Western blot analysis was used for semi-quantifying the levels of androgen receptor in prostate tissue extracts. The expression of AR in each group is shown in Figure 3.9 (a and b) and the relative all AR band density was quantified and normalized with each β-actin band density. By the normalized band density, AR expression is summarized in Figure 3.8c. AR expression of the castrated rat group (0.905±0.21) was lower than that of the normal rat group (1.024±0.54). Castrated rat treated with testosterone (Group 4, 1.586±0.87) showed higher level of AR than that of the castrated rats and also the normal rats (Group 1). Meanwhile, the expression of AR in rat administration with PRE at 1 mg/kg (1.331±0.31, 16.1%), finasteride (1.252±0.30, 21.1%) and bicalutamide (1.177±0.13, 25.8%) were slightly decreased when compared to rat treated with testosterone alone, whereas the PRE at 0.1 mg/kg (2.489±1.55) was not effect.

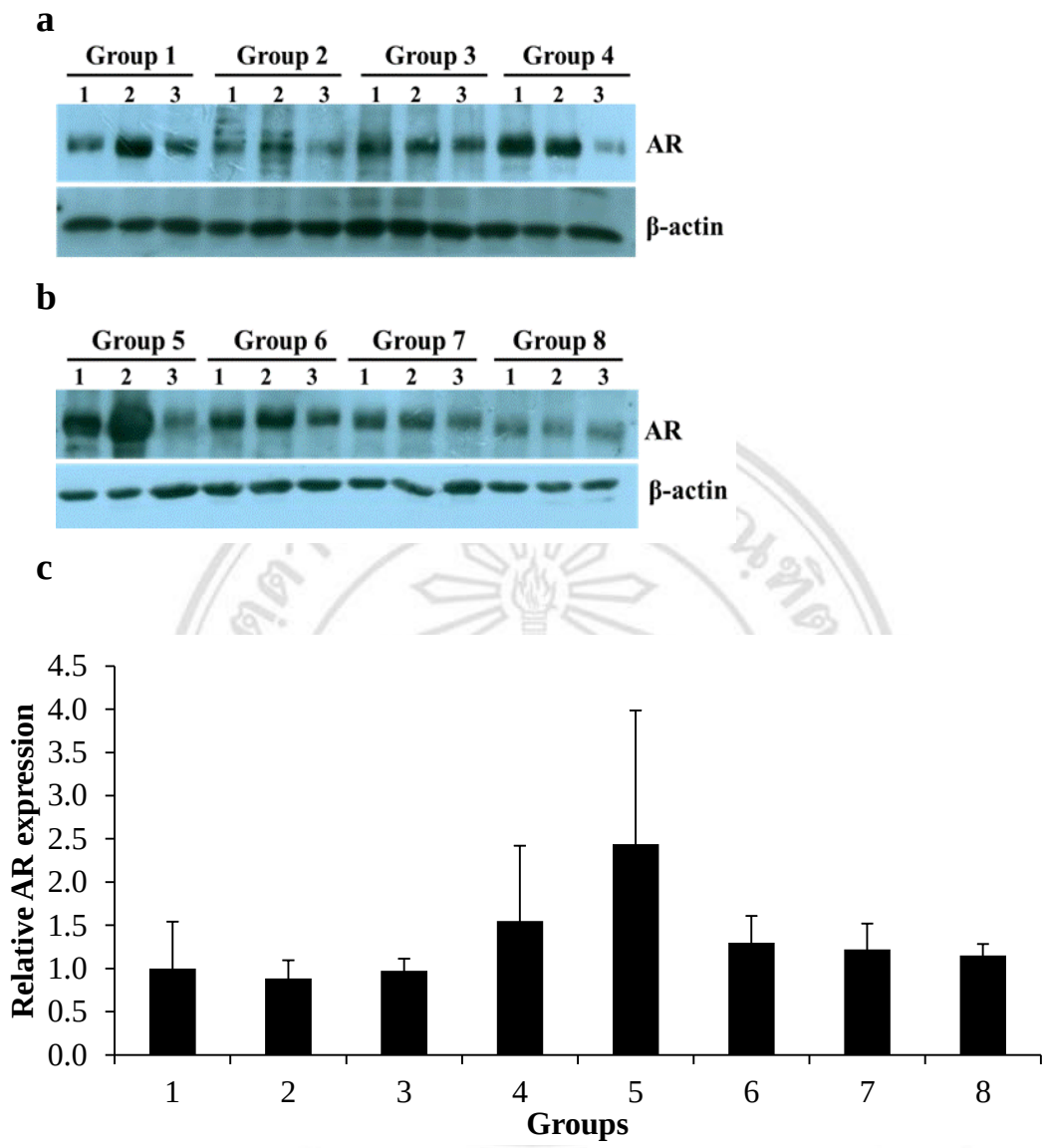

Figure 3.9 Western blot analysis of androgen receptor in dorsolateral prostate from rats. Experimental results for androgen receptor (upper band) and β-actin (lower band) represent three rats in each group. The relative level of androgen receptor expression is calculated and summarized in histogram. All values are mean ± SE (N=3), * P<0.05.

Group 1: normal rat
Group 2: castrated rat
Group 3: castrated rat and corn oil injection
Group 4: castrated rat treated with testosterone and water orally treatment
Group 5: castrated rat treated with testosterone and PRE (0.1 g/kg) orally treatment
Group 6: castrated rat treated with testosterone and PRE (1 g/kg) orally treatment
Group 7: castrated rat treated with testosterone and finasteride orally treatment
Group 8: castrated rat treated with testosterone and bicalutamide orally treatment

3.4 Effect of purple rice extract on the proliferation of prostate cancer cell

The effect of PRE on cell viability of androgen-responsive LNCaP was evaluated using MTT assay and showed in Figure 3.10. PRE tend to reduce cell viability of LNCaP both at 24 hours and 48 hours but not significantly. On the other hand, there is no effect of PRE on cell viability of androgen-independent DU145 human prostate cancer cells (Figure 3.11). From cell viability result, the growth rate of LNCaP was determined compare with 100% cell growth of each treatment at 0 hour. The results showed that growth rate of LNCaP at 24 hours in each treatment was similar (Figure 3.12). However, when LNCaP was treated with PRE 200 µg/ml the proliferation was slightly decrease by 3.85% compared to non-treatment group. At 48 hours, the proliferation rate of LNCaP was decrease when treated with PRE 100 and 200 µg/ml by 1.32% and 10.99%, respectively compared to non-treatment group. Therefore, PRE at dose 200 µg/ml could retard the proliferation of LNCaP cells at 24 and 48 hours.

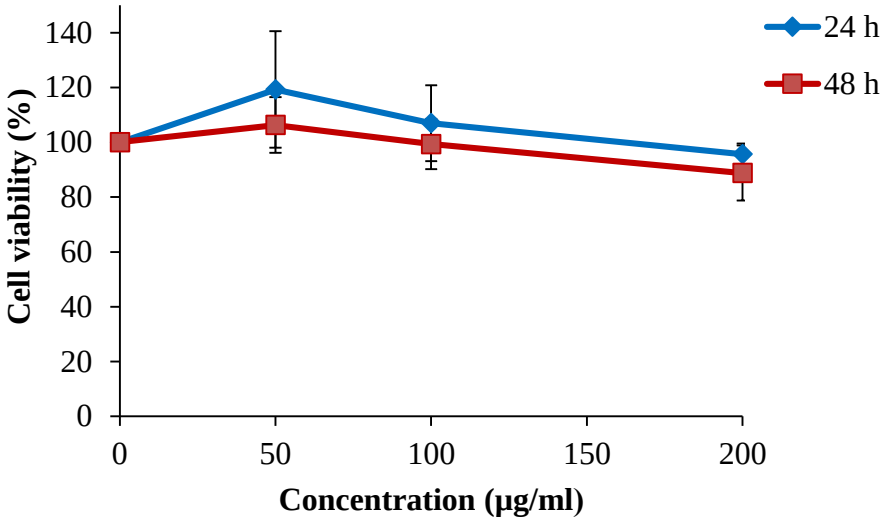

Figure 3.10 Effect of PRE on the proliferation of LNCaP. LNCaP cells were grown in RPMI media containing 50, 100 and 200 µg/ml of PRE then cell viability was measured at 24 and 48 hours. (The picture represents 3 independent experiments)

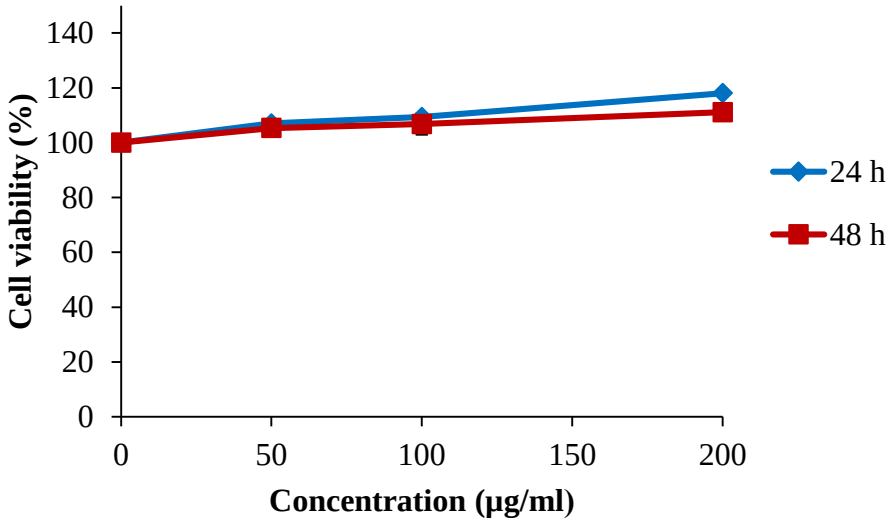

Figure 3.11 Effect of PRE on the proliferation of DU145. DU145 cells were grown in RPMI media containing 50, 100 and 200 µg/ml of PRE then cell viability was measured at 24 and 48 hours. (The picture represents 3 independent experiments)

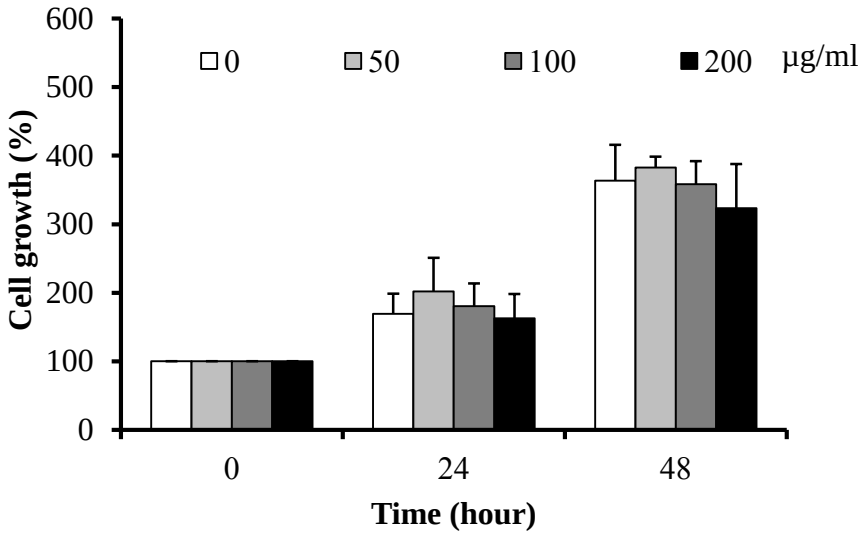

Figure 3.12 Effect of PRE on the growth of LNCaP. LNCaP cells were grown in RPMI media containing 50, 100 and 200 µg/ml of PRE then the growth of cell was measured at 0, 24 and 48 hours. (The picture represents 3 independent experiments)

3.5 Effect of PRE on expression of 5α-reductase in DU145

Because 5α-reductase is the key enzyme for the production of dihydrotestosterone (DHT) which important for prostate progression via AR, the expression of 5α-reductase mRNA was evaluated in DU145 cells by RT-PCR as show in Figure 3.13. PRE did not notably change the mRNA level of 5α-reductase in DU145 cells in all doses.

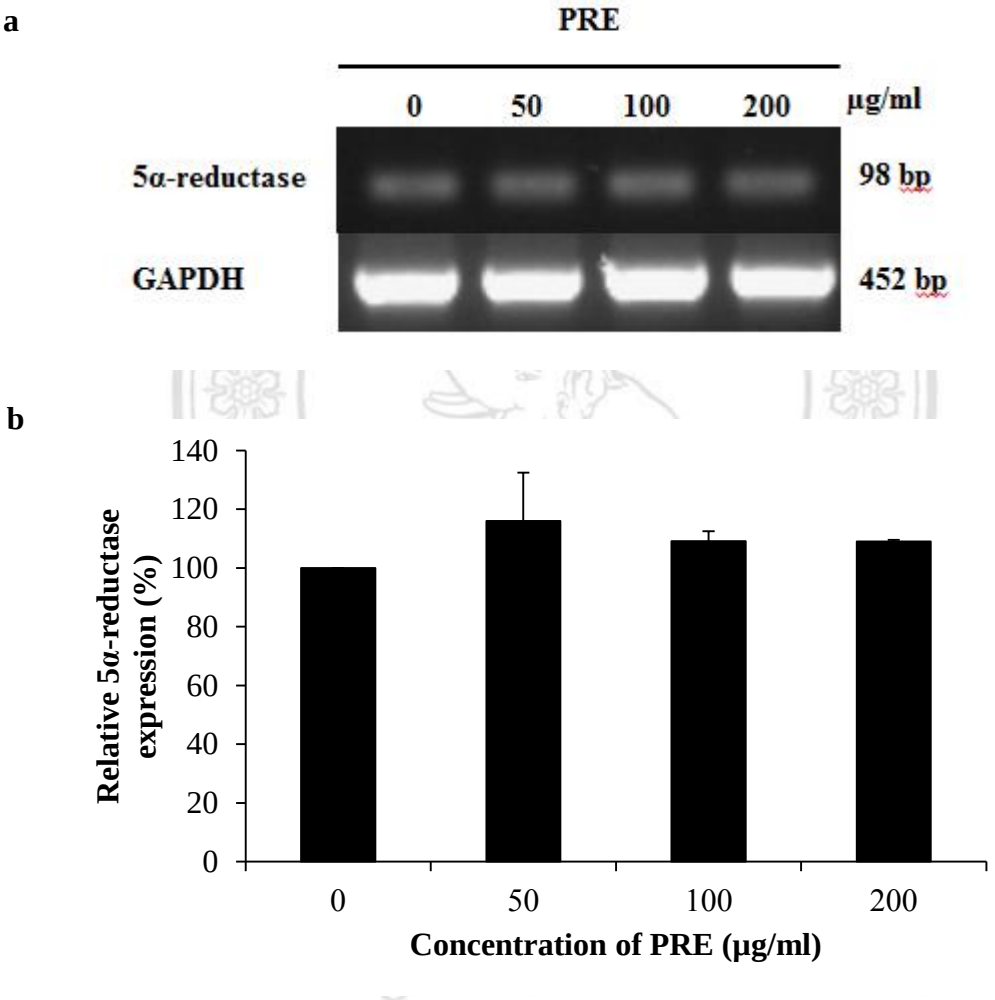

Figure 3.13 Effect of PRE on the expression of 5α-reductase in DU145. The mRNA levels of 5α-reductase were detected in DU145 cells treated with PRE for 24 h by RT-PCR and normalized by β-actin. The relative level of 5α-reductase expression is calculated and draws in histogram.

3.6 Effect of PRE on expression of androgen receptor in LNCaP

Because AR is an essential mediator of the androgen signaling for growth and differentiation, the effect of PRE on AR expression was determined in both mRNA and protein level. The levels of AR mRNA at 24 hours were slightly decreased when treated with PRE at 100 and 200 μg/ml by 3.8% and 21.1% respectively compared to no treatment group (Figure 3.14 and 3.15, respectively). Similar result was obtained in protein levels under the same experiment condition. The PRE treatment at dose 50, 100 and 200 μg/ml decreased the protein level by 9.3%, 22.2% and 57.0% respectively. These results suggested that PRE could suppress the AR expression in LNCaP in both mRNA and protein level.

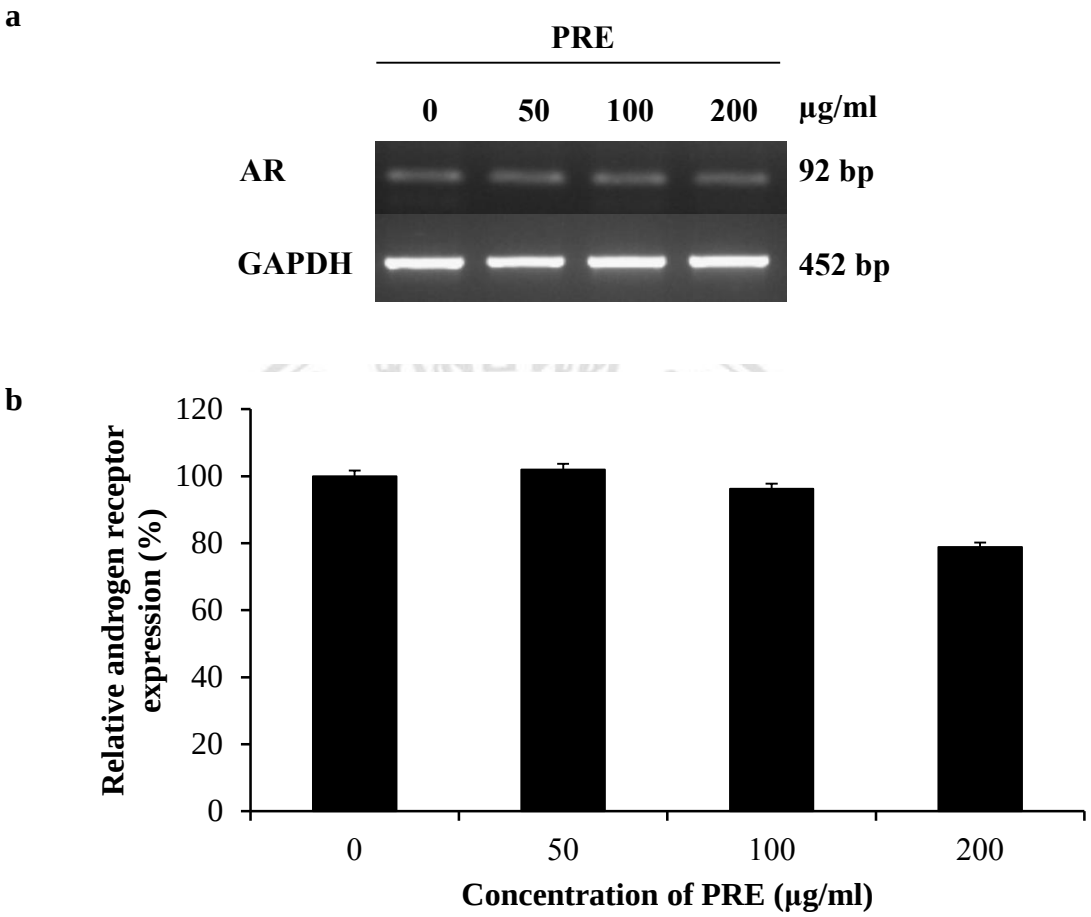

Figure 3.14 Effect of PRE on the expression of androgen receptor mRNA in LNCaP cells. The mRNA levels of androgen receptor were measured in LNCaP treated with PRE for 24 h by RT-PCR and normalized by β-actin and drew in histogram. (The picture represents from 3 independent experiments)

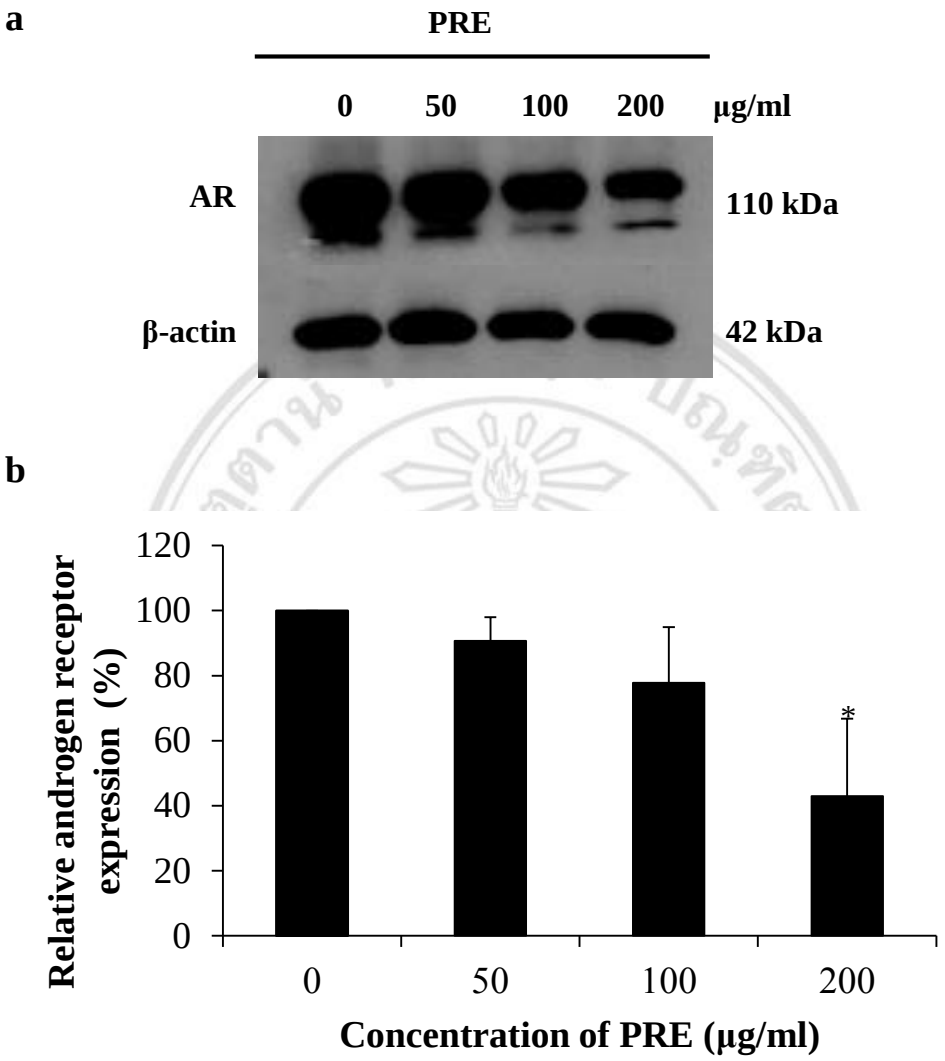

Figure 3.15 PRE suppressed AR expression in LNCaP cells. AR protein level was detected in LNCaP cells treated with PRE for 24 hours by Western blot analysis and normalized by β-actin. The relative levels of AR expression are calculated and draw in histogram. Mean values were calculated from three independent experiments; *P < 0.05 compared between PRE treatment and control.

3.7 Effect of PRE on expression of androgen regulated gene (PSA)

Prostate-specific antigen (PSA) is mainly regulated by androgens via interaction of the AR with androgen responsive elements. The effect of PRE on the expression and secretion of PSA was determined by western blot analysis and RayBio® Human PSA-total ELISA kit. Because the expression of PSA was quite low, the clearly change of PSA expression by treatment with PRE was not observed by Western blot analysis (Figure 3.16). However, the band intensity showed slightly decreases in PRE treatment at 200 μg/ml. On the other hand, the amount of PSA secreted to culture supernatant after 24 hours treatment was measured to confirm the production of PSA. The level of PSA secreted in culture supernatant of LNCaP treated with PRE 50 to 200 μg/ml were significantly decreased about 31 to 55 % respectively (Figure 3.17). Therefore, PRE could modulate the expression and secretion of PSA which correlated with the expression of AR in 3.6.

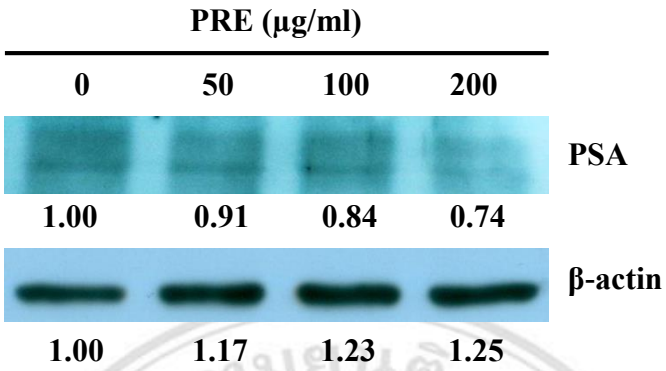

Figure 3.16 Effect of PRE on the expression of PSA of LNCaP. Western blot analysis was performed to quantified PSA protein and normalized by β-actin.
(The picture represents 3 independent experiments)

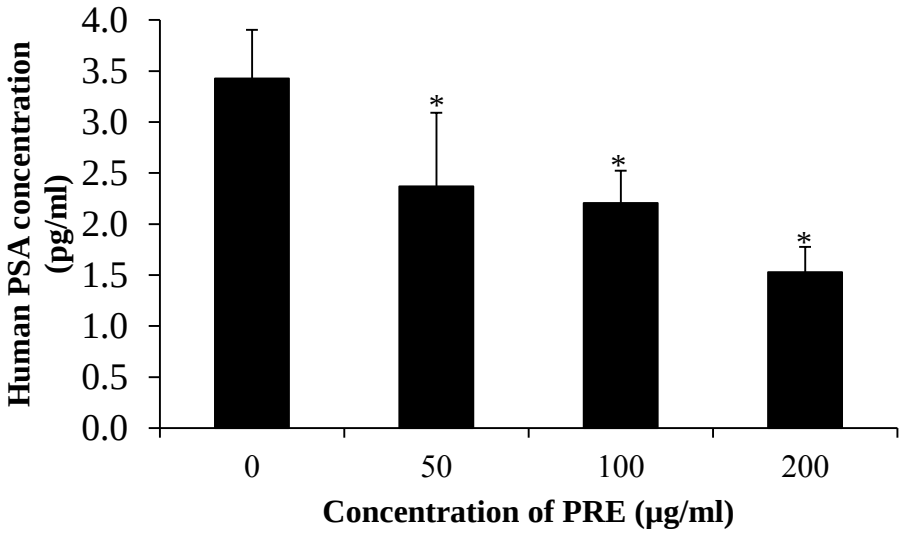

Figure 3.17 Secretory level of PSA from LNCaP determined by ELISA.
Mean values were calculated from three independent experiments;
*P < 0.05 compared between PRE treatment and control.